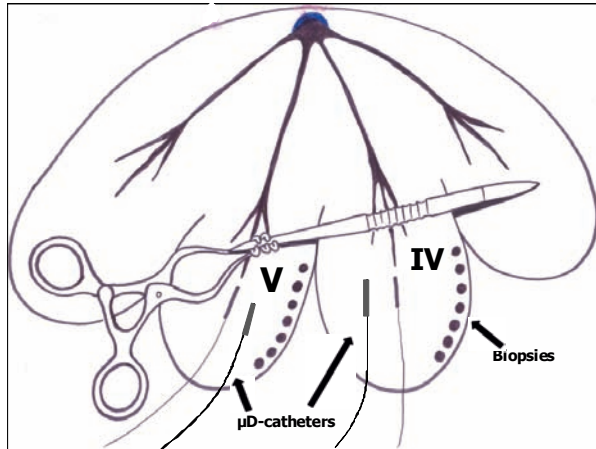


Microdialysis in Liver Ischemia and Reperfusion injury



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Cover Painting: Die Strafe. Christian Griepenkerl (1839-1916). The titan Prometheus was chained to a rock by the Greek deity Zeus because Prometheus had given the secret of the fire to the humans. Every day a vulture came to eat his liver, which then regenerated during the night. The process continued for 40 years until Prometheus finally was rescued by Hercules.

The vulture represents the essence of the liver surgeons today; meticulous dissection, fast patient recovery, maximum regeneration and high case load.

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ABSTRACT

Introduction: New chemotherapy regimens and improvements in surgical technique have increased the number of patients with liver tumours eligible for curative liver resection. There is a significant risk of bleeding during liver surgery, but this risk can be reduced if the portal inflow is temporarily closed; *i.e.* the Pringles maneuver (PM). When the PM is used, the liver will suffer from ischemia and reperfusion injury (IRI). If the liver remnant is too small or if the patient has chronic liver disease, the IRI may inhibit the regeneration of the liver remnant. The patient may then die from postoperative liver failure. Several strategies have been tried to protect the liver from IRI. For instance can the PM be applied in short intervals or reactive oxygen species can be scavenged by antioxidants. There are no sensitive methods available for studying IRI in patients and little is known how IRI affects the metabolism in the liver. Microdialysis is a technique that allows for continuous sampling of interstitial fluid in the organ of interest

Aim: To investigate the effects of ischemia and reperfusion on glucose metabolism in the liver using microdialysis technique.

Method: A porcine model of segmental ischemia and reperfusion was developed. The hepatic perfusion and glucose metabolism was followed for 6-8 hours by placing microdialysis catheters in the liver parenchyma (studies I-III). In study IV, 16 patients were randomized to have 10 minutes of ischemic preconditioning prior to the liver resection, which was performed with 15 minutes of ischemia and 5 minutes of reperfusion repetitively until the tumour(s) were resected.

Results: During ischemia the glucose metabolism was anaerobic in the ischemic segment, while the perfused segment had normal glucose metabolism. Urea was added in the perfusate of the microdialysis catheters and was found to be a reliable marker of liver perfusion. The antioxidant N-Acetylcystein (NAC) improved the hepatic aerobic glucose metabolism in the pig during the reperfusion, shown as reduced levels of lactate and improved glycogenesis in the hepatocytes. This can be explained by the scavenging of nitric oxide by NAC as nitric oxide otherwise would inhibit mitochondrial respiration. Also IP improved aerobic glucose metabolism resulting in lower hepatic lactate levels in patients having major liver resections.

Conclusion: Microdialysis can monitor the glucose metabolism both in animal experimental models and in patients during and after hepatectomy. Both NAC and IP improves aerobic glucose metabolism, which can be of value in patients with compromised liver function postoperatively.

ABBREVIATIONS

ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
au	arbitrary units
CcO	Cytochrome c Oxidase (Complex IV in the mitochondrial respiratory chain)
CVP	Central Venous Pressure
CRCM	Colorectal liver metastases
CUSA	Cavitron Ultrasonic Surgical Aspirator
Da	Dalton (molecular weight, gram/mol)
ATP	Adenosine triphosphate
dw	dry weight
GSH	The reduced form of glutathione
GSSG	The oxidized form of glutathione
G-6-P	Glucose-6-Phosphate
IL	Interleukin
IP	Ischemic preconditioning
IR	Ischemia and Reperfusion
IRI	Ischemia and Reperfusion Injury
i.v.	intravenously
kDa	kiloDalton (see Da above)
LDF	Laser Doppler flowmetry
LDH	Lactate dehydrogenase
LOS	Length of stay
L/P	Lactate-to-Pyruvate ratio
M	Molar (mol/liter)
MAP	Mean Arterial Pressure
NAD⁺	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)
NAC	N-Acetylcysteine

NF-κB	Nuclear Factor Kappa-B
NO	Nitric oxide
NO_x	The sum of nitrite and nitrate
pCO₂	partial pressure of carbon dioxide
PDH	Pyruvate dehydrogenase
PM	Pringles maneuver
PMN	Polymorph nuclear leukocytes
POD	Postoperative day
PT-INR	Prothrombin time – International Normalized Ratio
RCT	Randomized clinical trial
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SD	Standard deviation
SEM	Standard error of the mean
SNP	Sodium nitroprusside
SpO₂%	Peripheral oxygen saturation
SOS	Sinusoidal Obstruction Syndrome
TNF-α	Tumour necrosis factor – alpha
WBC	White blood cell count

DEFINITIONS

Cut off	The pore size of the microdialysis membrane described as the molecular weight (Daltons) able to pass the semi-permeable membrane.
(micro)Dialysate	The fluid that has equilibrated over the semi-permeable membrane with the interstitial fluid and is recovered and collected in the microvial.
Equilibrium	Equilibrium is achieved when the concentrations of the diffusing substance in the interstitial fluid and the perfusate are equal. In the case of microdialysis this occurs when the recovery is 100 %.
Ischemia	Reduced blood flow resulting in a subnormal oxygen tension.
IRI	The inflammation and oxidative/nitrosative damage that occurs in a tissue that has been reperfused after an episode of ischemia.
Microvial	A 60 µL plastic flask for collection of microdialysate.
Perfusate	The fluid that perfuses the microdialysis catheter. The fluid is propelled by a low flow micropump.
Recovery	The dialysate : interstitial concentration ratio for a particular substance expressed as a percentage.
Reperfusion	The return of blood supply in a tissue that has sustained an ischemic event.
15/5	Intermittent occlusion of the portal pedicle in the manner of 15 minutes of ischemia and 5 minutes of reperfusion repetitively.
10/10	Ischemic preconditioning in the manner of 10 minutes of ischemia and 10 minutes of reperfusion.

LIST OF ORIGINAL PAPERS

This thesis is based on the following papers which will be referred to by their roman numerals:

- I. A. Winblad**h, P. Sandström, H. Olsson, J. Svanvik and P. Gullstrand

Segmental Ischemia of the Liver – Microdialysis in a Novel Porcine Model

European Surgical Research 2009;43(3):276-85

- II.** S. Farnebo, **A. Winblad**h, E.K. Zettersten, P. Sandström, P. Gullstrand, A. Samuelsson, E. Theodorsson and F Sjöberg.

Urea clearance – a new technique based on microdialysis to assess liver blood flow studied in a pig model of ischemia/reperfusion

European surgical Research 2010;45(2):105-112

- III. A. Winblad**h, B. Björnsson, L. Trulsson, L. Bojmar, T. Sundqvist, P. Gullstrand and P. Sandström

N-Acetylcysteine improves glycogenesis after segmental liver ischemia and reperfusion injury in pigs

Submitted to the Scandinavian Journal of Gastroenterology

- IV.** **A. Winblad**h, B. Björnsson, L. Trulsson, K Offenbartl, P. Gullstrand and P. Sandström

Ischemic preconditioning prior to intermittent Pringles maneuver in liver resections

Accepted for publication in the Journal of Hepato-Pancreatico-Biliary Sciences

INTRODUCTION

The vascular anatomy of the liver

The liver is a central organ in vertebrates and death will occur if the liver is removed or seriously injured. The liver has a dual blood supply, the majority of which is being supplied by the portal vein. The hepatic artery contributes only about 25% of the blood flow, but delivers half the oxygen extracted by the liver parenchyma. Within the liver, the arterial and the portal blood mix in the fenestrated sinusoids (1). Nutrients from the gut diffuse through the space of Disse and equilibrate with the parenchymal extracellular fluid. The sinusoidal blood drains through venules to the liver veins and finally into the inferior caval vein (**figure 1**).

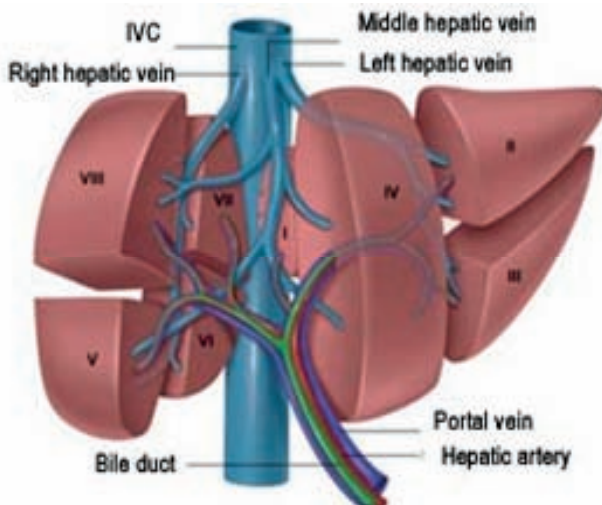


Figure 1. The segmentation of the liver according to Couinaud (1957) is based on the distribution of the portal structures. Each segment is supplied by one arterial and one portal venous branch. Each segment has its own biliary ductule draining the bile to the hepatic duct. The right, middle and left hepatic vein drains several segments each; except for segment 1, which drains directly into the caval vein.

The hepatocytes are the most common cells in the liver and they are responsible for most of the specific functions of the liver. Bile secretion, protein synthesis and conjugation of lipophilic substances are some of their functions together with having a central role in energy metabolism. Other cells are macrophages (i.e. the Kupffer cells), lymphocytes, sinusoidal endothelial cells, Ito cells and biliary epithelial cells.

The liver and the metabolism of glucose

Claude Bernard, a French laboratory assistant, was convinced that the liver was too large to produce nothing but bile. In 1843 he perfused livers with water and he found that he could extract glucose from the water that had perfused the liver, but after a while the glucose disappeared. He rested the perfusion briefly and after he restarted it, the glucose could again be found in the perfusate. From this he concluded that the liver was a storage organ for glucose and coined the term glycogen (2).

During physiological homeostasis, glucose is taken up from the intestines and transported to the portal vein and finally to the fenestrated sinusoids of the liver. Here the portal glucose passively equilibrates with the interstitial fluid of the liver parenchyma. Through the bidirectional GLUT-2 (3) membrane transporter, glucose is passively taken up and secreted by the hepatocyte driven by concentration gradients. In the hepatocyte, glucose is converted to glucose-6-phosphate (G-6-P) and if the insulin levels are high, glucokinase will be activated to synthesize glycogen from G-6-P. When the insulin levels go down and the glucagon levels increase (e.g. during fasting) the glycogen will be degraded (glycogenolysis). In the liver, G-6-P can, unlike in the muscles, be converted to glucose again and released into the circulation to supply vital organs (primarily the brain) with energy. G-6-P will also be metabolized in the liver to yield energy (glycolysis). The net effect of the glycolysis is 2 ATP and 2 NADH molecules per glucose molecule. The end product of the glycolysis, pyruvate, is transported into the mitochondria and there irreversibly decarboxylated by pyruvate dehydrogenase (4). Acetyl CoA is formed in the reaction and enters the citric acid cycle to yield 32 ATP per glucose molecule. In the citric acid cycle, NADH is converted back to NAD^+ , which is needed in the glycolysis (5).

Hepatic glucose metabolism:

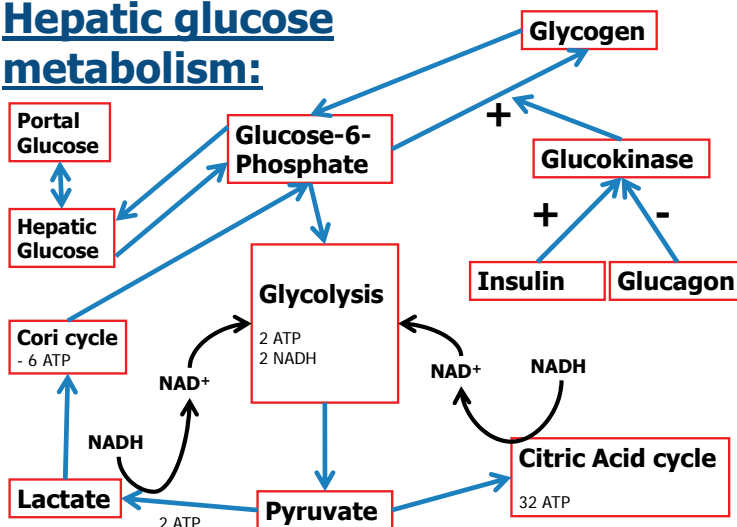


Figure 2. Schematic illustration of the glucose metabolism in the liver focused on the reactions central to this thesis.

About 4% of the cytosolic pyruvate is metabolized to lactate by lactate dehydrogenase to yield 2 ATP per glucose molecule. The lactate cannot be further metabolized in organs other than the liver. Accordingly, the lactate is transported to the liver and there metabolized in the Cori cycle (5, 6) to give glucose again, at the cost of 6 ATP per glucose molecule generated. The glucose can then be utilized by organs, like the brain and the red blood cells, which depend on glucose for energy metabolism (**figure 2**).

Liver surgery

Twenty years ago, very few patients survived more than a year after they had been diagnosed with a cancer in the liver (7). A malignant tumour in the liver can be primary (hepatocellular or cholangiocarcinoma) or secondary (i.e. metastases). In Sweden, the most common form of malignant liver tumour is colorectal cancer metastases (CRCM). Historically, Patients with hepatic CRCM had a 3-year survival rate less than 5% (7). If resections of the metastases were undertaken during the 70's, the perioperative mortality rate was 13% and as many as 20 % of these deaths were due to excessive intraoperative bleeding (8). If the patients survived the liver resection, they still faced a high risk of recurrent disease. Recent developments have led to reduced intraoperative bleeding (9) and perioperative mortality rates as low as 1 % (10). These improvements justify a more aggressive approach in order to achieve radical tumour resections.

The factors contributing to the decreased bleeding are:

- The recognition of the internal segmental anatomy of the liver was made in 1957 (11). Ideally, segmental resections are performed in a plane of dissection that does not damage the vascular contributory structures. This was fully appreciated first in the late 1980's, thereby fewer vessels need to be divided with less bleeding as a consequence (12).
- The perioperative reduction of the central venous pressure by fluid restriction and nitroglycerin infusion. This results in less bleeding from the hepatic veins (13).
- New instruments (CUSA, water jet and the Habib) have been developed, and all shown to reduce bleeding during the transection. These instruments destroy the parenchyma but spare the vessels, which then can be safely divided (14).

Along with these perioperative advances, the introduction of new effective adjuvant and neoadjuvant chemotherapy regimens has improved the long-term survival of hepatic CRCM patients. Today, combined oncological and surgical therapy has led to 5-year survival rates of between 30 and 50 % in selected patient materials (15-18). As a consequence of the improved multidisciplinary care, the number of liver resections performed has increased dramatically in Sweden (**Figure 3**) and worldwide (19-21).

Number of Liver resections, Sweden

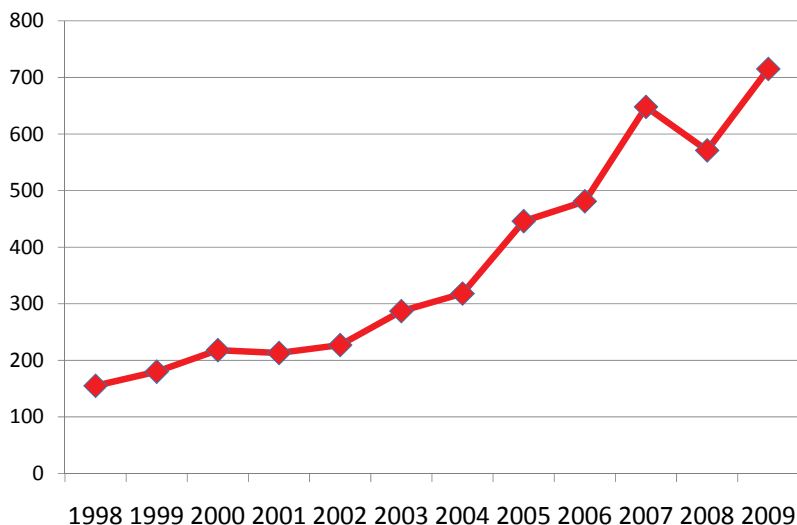


Figure 3. Data from the official inpatient register, The Swedish National Board of Health and Welfare. Search term: Liver resections (JJBxx), 19th of April 2011.

Limitations in liver surgery

The liver is the only organ, other than the spleen and the skin, which can regenerate after a resection. The resected liver will regain most of its size and function within 3-4 weeks postoperatively (22). The surgeon must, however, make sure that the liver has an intact vascular inflow *i.e.* a patent portal vein and hepatic artery, as well as sufficient outflow through the liver veins. If any of these vessels are injured or thrombosized, that part of the liver will not regenerate and the patient can die from liver failure. For patient survival, it is therefore crucial to preserve the vascular supply and drainage.

Even if the vascular in- and outflow is preserved, a patient with too little functional parenchyma left after the resection may suffer from acute postoperative liver failure. Patients with liver diseases like fibrosis, cirrhosis or steatosis are particularly vulnerable to hepatic surgery as their parenchymal function is already compromised. These patients have to be recognized and staged preoperatively, and the resected volume held to a minimum to avoid lethal liver failure (23). As a rule of thumb: even in patients with healthy livers at least 2 segments (about 20-25% of the total liver volume) must be left or the risk of postoperative death increases significantly. Unfortunately, the instruments available for preoperative liver function assessment are not well developed. Especially for patients with slightly decreased liver function, it is difficult to prognosticate the consequences of major hepatic resections.

Sometimes the metastases are deemed irresectable if the tumours are close to the major vessels or biliary structures, which may make the resection too hazardous. Down-sizing

neoadjuvant chemotherapy has proven to make some of the patients resectable (24-26). When resectability is achieved by chemotherapy, these patients receive the same prognostic benefit as those who were initially resectable (25, 27, 28). During the last few years many patients with metastases in the liver have received neoadjuvant chemotherapy. Unfortunately, the chemotherapy damages the normal parenchyma as well. Oxaliplatin may cause sinusoidal obstructive syndrome (SOS) and irinotecan can cause steatohepatitis (29, 30). SOS is associated with an increased risk of intraoperative bleeding (31). Patients with steatohepatitis had a 15 % 90-day postoperative mortality rate compared to those without steatohepatitis that had mortality rate of 2 % (32). Beside the effects of chemotherapy, other chronic hepatic diseases have an increased risk of complications. Patients with steatosis have a higher risk of infectious complications (33, 34). Those with cirrhosis have an increased risk of bleeding and postoperative liver failure and careful patient selection is essential before considering surgical intervention (23).

Bleeding during liver surgery

Liver resections may cause severe intraoperative bleeding and the amount of bleeding has been correlated to patient mortality (35). To control bleeding is therefore the single most important factor to improve patient survival after liver surgery. The classical way to divide the liver parenchyma is the crush and clamp technique. The surgeon uses his/her fingers or a Kelly clamp to gently fracture the soft parenchyma, whereas the tougher structures, *i.e.* the vessels and the biliary ducts, are spared. These can then be clamped and ligated. It is difficult to control bleeding from the minor vessels with this technique as they are torn during the finger fracturing. New instruments (CUSA, water jet and the Habib) have been developed and all shown to reduce the bleeding during the transection (14). The common denominator for these instruments is that they divide the softer parenchyma (hepatocytes) while preserving vessel structures. The vessels can then be clipped, ligated or coagulated with electrocautery. Lowering the central venous pressure (CVP) has been shown to reduce retrograde bleeding from the liver veins. A low CVP can be achieved by perioperative fluid restriction and the infusion of nitroglycerin intravenously (13). Despite these developments, some patients, especially those with fibrosis or steatosis, can bleed extensively during the transection (23).

There are various inflow occlusion techniques that can be used to reduce the bleeding. The most common is the classic 'Pringles Maneuver', first described by James Hogarth Pringle in 1908 (36), hereafter abbreviated PM. It can be quickly performed by placing a vascular clamp over the hepatoduodenal ligament. Today a soft cotton, rubber or silicon band is used in elective surgery to minimize the trauma to the structures of the ligament. The use of PM, however, can compromise the residual liver parenchymal function due to ischemia and reperfusion injury (IRI). This is not considered clinically important if the parenchyma is otherwise healthy (37), but concerns have been raised in patients with cirrhosis (38) and severe steatosis (39) which both have increased risks of complications. In mice with inoculated hepatic metastases, IRI have been shown to accelerate growth of the metastasis (40), but this thesis has not been substantiated clinically in patients having vascular occlusion during the

hepatic resection. In a clinical retrospective study incorporating 355 patients, there was no increased risk of CRCM local recurrence rate or any change in the disease free survival compared to those that were operated without vascular occlusion (41).

In addition to the ischemic insult, excessive amounts of reactive oxygen species (ROS) are formed during the reperfusion and may damage cellular structures and functions by reacting with lipids, DNA, RNA and proteins (peroxidation). The harmful effects of IRI should be avoided (42, 43), but even so the PM was shown to be used by 93% of the surgeons in a Japanese survey 2002 (44). Since then, the introduction of the new technical devices of liver transection has probably diminished the need for PM. Despite its reduced use, PM remains a powerful adjunct that can be utilized in difficult resections and, indeed, IRI still remains a problem for those patients with marginal postoperative liver function.

Ischemia and reperfusion injury

Hepatic ischemia and reperfusion comprise a complex set of cellular and humoral events that eventually lead to cellular injury. These events are multifactorial, interact at multiple levels and change over time.

During ischemia, the oxygen tension decreases, metabolism goes anaerobic, the ATP levels decrease and the environment becomes acidic (45). This leads to dysfunction of the ATP-demanding ion pumps of the cellular membranes. The inability to maintain membrane ion potentials results in sodium and calcium leaking into the cells (46).

ATP degradation and generation of ROS:

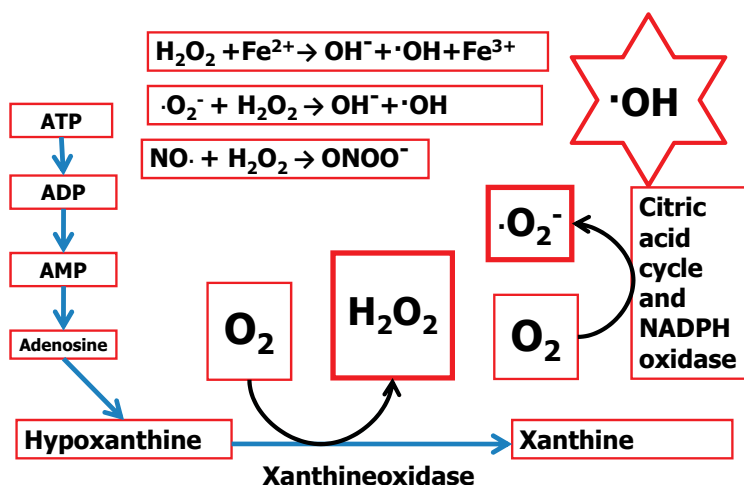


Figure 4. Degradation of ATP generates adenosine which likely functions as signal to the cells that the energy supplies are decreasing. Adenosine is in turn further degraded to yield hydrogen peroxide due to the action of xanthineoxidase. Hydrogen peroxide is highly reactive and may form the hydroxyl radical if it reacts with transition metals or superoxide. If hydrogen peroxide reacts with NO, peroxynitrite is generated. Note: The chemical reactions are not balanced.

The cells swell and the increased cytosolic calcium levels activate phospholipases, which degrade the phospholipids in the cellular membrane. The membrane disrupts and intracellular components (including phosphoglycerol of the membrane, see microdialysis below) leak to the interstitium (47). During this phase, the dominant mode of cell death is by apoptosis, but the longer the ischemia, the more necrosis will develop (48).

When ATP is degraded, it yields energy in steps until the end products adenosine and hypoxanthine are formed (**figure 4**). The earlier molecule acts as a signal that energy is depleted, which initiates mechanisms of protection from further ischemia (see ischemic preconditioning below).

The ischemia, possibly mediated by the complement factors 1 and 5a (49, 50), activates the Kupffer cells (51), which release ROS (52, 53). The Kupffer cells induce a complex network of cytokine signalling, including release of tumour necrosis factor alpha (TNF- α) and interleukin-1 β (IL-1 β) (54, 55). CD4+ lymphocytes are attracted by these and they release IFN- γ , which stimulates the Kupffer cells to produce more TNF- α and IL-1 β , and the hepatocytes to produce chemokines (56, 57). The CD4+ T-lymphocytes also produce the chemotactic agent IL-17, which stimulates PMN accumulation (58). These cytokines participate in the sinusoidal accumulation of PMN leukocytes and microcirculatory dysfunction (59, 60).

TNF- α stimulates both the expression of L-selectin and β 2-integrins on the cell surface of the PMNs and the intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule (VCAM-1) on the post-sinusoidal endothelial cells and the hepatocytes (55, 61). The PMNs now adhere to the endothelium and become primed by the translocation of their NADPH oxidase to the cell surface. Swelling and sinusoidal contraction also physically trap the PMNs in the sinusoids. Chemokines cause the trapped PMNs to migrate into the interstitial space (chemotaxis) where they adhere to the ICAM-1 and the VCAM-1 expressed on the hepatocytes. This triggers the primed PMNs to release ROS by NADPH oxidase activity, and to degranulate their cytoplasmic vesicles that contain various proteases (**Figure 5**). This degrades the extracellular matrix and the dead hepatocytes, but also damages viable hepatocytes (62).

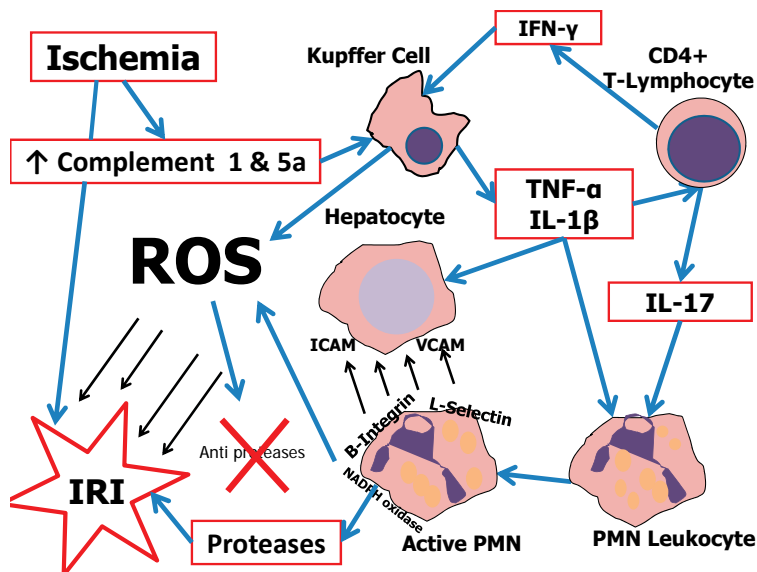


Figure 5. The pathways of ischemia and reperfusion injury (IRI) in the liver (simplified). Ischemia activates the Kupffer cells, which release ROS and secrete TNF- α and IL-1 β . This activates the CD4+ T-Lymphocytes, which secrete IFN- γ that further stimulates the Kupffer cells to release more TNF- α and IL-1 β . Hepatocytes and sinusoidal endothelial cells express adhesion molecules and the trapped PMNs migrate into the parenchyma and adhere to these. PMN secretes both ROS and proteases. The ROS are believed to inhibit circulating anti-proteases. These events lead to IRI.

Both the Kupffer cells and the recruited PMN leukocytes release ROS that cause significant damage to the parenchymal cells. However, a higher concentration of ROS than that seen in IRI is needed to cause enough peroxidation to injure the hepatocytes. It is believed that the initial burst of ROS inhibits circulating anti-proteases, thus making the proteases of the PMNs more effective in parenchymal degradation (63). It has been shown that the inhibition of Kupffer cells significantly reduces the IRI (64). ROS modulates the activity of NF- κ B. NF- κ B stimulates the transcription of both protective and the inflammatory cytokines, of course adding complexity to the understanding of IRI (54).

Reactive oxygen species (ROS)

ROS and reactive nitrogen species (RNS) are formed during IRI, and damage cellular structures. ROS and RNS can be divided into radicals and non radicals. The biologically most important radicals are the superoxide radical ($\cdot\text{O}_2^-$), hydroxyl radical ($\cdot\text{OH}$) and nitric oxide ($\text{NO}\cdot$). Radicals have an unpaired electron, which make them highly reactive. Hydrogen peroxide (H_2O_2) and peroxynitrite (ONOO^-) are not radicals, but can readily react to form radicals (Table 1).

The highly cytotoxic hydroxyl radical is formed when $\cdot\text{O}_2^-$ reacts with H_2O_2 (Haber-Weiss reaction) or when $\cdot\text{O}_2^-$ interacts with certain transition metals (e.g. iron or copper). This reaction is called the Fenton reaction, see **figure 4**. The hydroxyl radical can split covalent bonds thereby damaging lipids in the membranes, RNA, DNA and proteins.

Table 1.

Free Radicals	Nonradicals
<p>Reactive oxygen species (ROS)</p> <ul style="list-style-type: none"> Superoxide ($\cdot\text{O}_2^-$) Hydroxyl radical ($\cdot\text{HO}$) <p>Reactive nitrogen species (RNS)</p> <ul style="list-style-type: none"> Nitric oxide ($\text{NO}\cdot$) 	<p>Reactive oxygen species (ROS)</p> <ul style="list-style-type: none"> Hydrogen peroxide (H_2O_2) <p>Reactive nitrogen species (RNS)</p> <ul style="list-style-type: none"> Peroxynitrite (ONOO^-)

Table 1. Common Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS)

First and foremost, however, radicals are important mediators of cellular metabolism and signal transduction (65-67). Oxygen is not only vital in the life sustaining ATP production, but is also the main source of ROS production. Under physiological circumstances, 1-3 % of the oxygen is converted in the mitochondrial respiratory chain to become the superoxide radical, $\cdot\text{O}_2^-$ (68). The antioxidative systems (see below) can neutralize the physiological formation of ROS and RNS. When these protective systems become insufficient, e.g. during the reperfusion, excessive concentrations of ROS and RNS can cause oxidative and nitrosative stress, respectively. ROS increase the expression of the proinflammatory cytokines TNF- α , IL-1 β and IL-8, but also cause vasodilation from carbon monoxide release and stimulate the expression of the protective enzyme Heme Oxygenase 1. ROS and RNS are known to block the mitochondrial respiratory enzymes, thereby impairing the ATP production which is crucial for cell survival (69, 70).

Oxidative stress is known to induce apoptosis in hepatocyte cell lines (71). At reperfusion, the partial oxygen pressure increases and oxygen reacts with hypoxanthine forming ROS and xanthine (**figure 4**).

The main intracellular mechanisms for producing ROS during IR are the xanthine oxidase pathway, the mitochondrial respiratory chain, and NADPH oxidase systems (72-74).

Reactive nitrogen species (RNS)

The biologically most important RNSs are nitric oxide and peroxynitrite, see **table 1**. The latter is produced when NO reacts with the superoxide anion and molecular oxygen (75). RNS are highly reactive and bind to lipids, proteins and DNA; especially to amino acids containing thiol residues, thereby damaging structural and functional cell components. Mitochondrial respiratory complexes, ATP synthase, adenine nucleotide translocase and mitochondrial creatine kinase are inactivated by peroxynitrite.

It is known that also NO inhibits the mitochondrial respiratory chain (76-78). It is believed that the inhibition of these mitochondrial respiratory complexes reduces the harmful oxidative burst of ROS after reperfusion (79). This seems to be the case when complex IV (cytochrome c oxidase, CcO) is in its reduced form. During physiological cellular respiration, however, CcO is in the oxidized form and NO binds to the Cu_B centre, where it is converted to nitrite. The reduction of CcO increases when the mitochondrial oxygen concentration decreases, *i.e.* during ischemia. When CcO is reduced, NO competes with oxygen to bind reversibly at the a₃ site of CcO instead (80). As NO has a higher affinity for CcO than oxygen, the inhibition of the respiratory chain may be near complete (80).

NO, nitrite and nitrate

Nitric oxide synthase (NOS) is the enzyme regulating the production of NO, which is synthesized from arginine and oxygen. Citrulline is formed in the process. (**figure 6**). There are three isoforms of NOS (eNOS, nNOS and iNOS) and the endothelial NOS (eNOS) is particularly important in the liver (1, 81). NO is the only factor that can relax and dilate the sinusoids and the perfusion of the sinusoids is therefore dependent on NO produced by eNOS. The dilating action is opposed by endothelin. Unfortunately, eNOS needs oxygen for NO synthesis and therefore the sinusoids contract during ischemia. The blood flow decreases and PMNs and platelets are trapped in the sinusoids (82). Experimental animal liver IRI studies have shown that endothelin blocking agents can improve the microcirculation and reduce IRI (83-85).

The sum of nitrate and nitrite can be measured together, hereafter called NO_x. The levels of nitrate and nitrite are in balance with each other and the levels of NO. In deoxygenated and acidic environments the reduction of nitrite to NO increases (79), and as the nitric oxide synthases (NOS) need oxygen to produce NO, nitrite is the major source of NO during anaerobic conditions. If NO is consumed, nitrite and nitrate levels are reduced to maintain the levels of NO. When NO is produced in excess quantities (*i.e.* the induction of iNOS), the nitrite levels are increased as are the nitrate levels. NO has an affinity for transition metals and the sulfhydryl groups of the amino acid cysteine (**Figure 6**). This is the proposed therapeutic effect of endogenous scavengers like glutathione, which have a high affinity for both ROS and RNS, see below.

Vascular occlusion techniques

The PM is used to reduce bleeding during liver transection (36, 86). The risk with inflow occlusion is that it causes IRI to the liver parenchyma (43, 87, 88). To overcome the postoperative morbidity and mortality risks in resectional surgery, the PM is used for the shortest possible time. There are some protective strategies that can reduce the IRI.

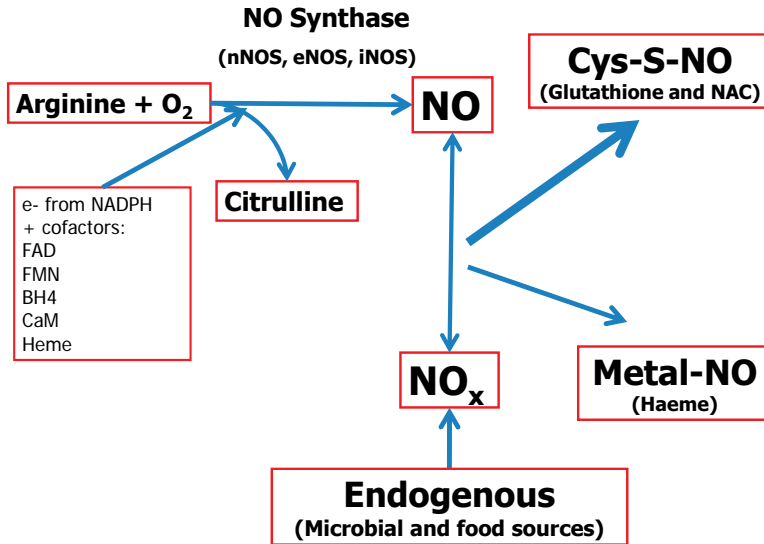


Figure 6. The nitrogen oxide (NO) metabolism. Oxygen is needed as a substrate when NO is synthesized from the amino acid arginine by the enzyme NO synthase (NOS). Sinusoidal dilation is dependent on eNOS activity. During ischemia the sinusoids are constricted because the action of endothelin is unopposed. The NO levels are low, but some NO is derived from the reduction of nitrite and nitrate (NO_x). NO have a T_{1/2} of only a few seconds and bind reversibly to sulfhydryl groups of the amino acid cysteine. NO is also scavenged by iron which exists abundantly in the body (e.g. haemoglobin and myoglobin).

Intermittent clamping lets the parenchyma recover from the ischemia intermittently during a few minutes and this was first described in rats. Intermittent clamping has experimentally shown better survival and less reduction in ATP levels (89, 90) and lower ALT levels (91) compared to continuous clamping. There is only one RCT that compares intermittent clamping (15 minutes of ischemia and 5 minutes of reperfusion repetitively (15/5)) with continuous clamping. The 15/5 group lost more blood than the controls, but the duration of the ischemia correlated stronger to the levels of the aminotransferases in the control group. Moreover, those with steatosis and fibrosis tended to tolerate the continuous clamping less, as seen in the liver function tests (92). Retrospective analyses of patients having intermittent PM for more than 2 hours, indicate that this method of vascular occlusion is well tolerated (93, 94).

Another way to reduce the IRI is to expose the liver to a brief episode of ischemia before the subsequent continuous ischemic insult, so-called ischemic preconditioning (IP). This was first tested in 1986, and it was shown that IP reduced ATP depletion in the heart during the subsequent ischemic insult (95). IP also protects the liver from IRI if it is employed before continuous PM (96-99). IP has been shown to reduce the release of TNF-α from Kupffer cells (100), reduce the levels of endothelin (82), reduce oxidation and the aminotransferases lev-

els (101). The mechanisms mediating the effect of hepatic IP remain obscured, but the inhibition of adenosine, the inactivation of adenosine (A_2) receptors or the inhibition of NO synthesis abolish the protective effect of IP. Administration of NO-donors can give similar protection as IP against IRI, even when adenosine is inhibited (102, 103). These interesting findings were concluded when the levels of ALT, AST and LDH were used as end points of IRI. However, adenosine alone could reduce IRI when lactate accumulation was used as the marker of IRI. Adenosine can activate adenosine monophosphate-activated protein kinase (AMPK), which in turn reduces the lactate accumulation in rat livers (104). This occurs irrespective of the blocking or administration of NO.

IP has also been tried in humans and found to protect against IRI. This is evident by the resulting lower levels of AST and ALT and also less apoptosis, possibly due to inhibition of caspase-3 activity (86, 105, 106). The latest RCT (107) studied the effects of 10/15 IP (n=41) when liver resections were performed under selective vascular exclusion. This study showed reduced apoptosis (TUNEL stain) and oxidative stress (malondialdehyde) when IP was performed prior to liver transection. Moreover, AST, IL-6 and IL-8 levels were lower in the IP group during the first 24 hours compared to the controls (n=43), but later, in the postoperative course no differences were seen.

There are only two randomized controlled trials that compare the effects of IP prior to continuous PM with intermittent PM during liver resections. In 2006, Petrowsky performed 10/10 IP (n=36) and could not show any advantage regarding AST and ALT levels compared to 15/5 intermittent PM (n=37). There was, however, less bleeding in the IP group, and he concluded that the methods are equally efficient with regard to IRI (108). In the same year, Smyrniotis published an RCT involving 27 patients who had 15/5 intermittent PM and 27 who had 10/10 IP followed by continuous PM. No differences were seen when the ischemic time was less than 40 minutes, but prolonged ischemia resulted in higher AST levels and more apoptosis in the IP group (109). In this study, the changes were transient and the result risks being *ex-post facto* fallacy.

No RCT has studied the effect of IP in the liver when this is used together with 15/5 intermittent vascular clamping.

Glucose metabolism in ischemia and reperfusion

As mentioned above, pyruvate is irreversibly decarboxylated by pyruvate dehydrogenase in the mitochondria to yield acetyl CoA, which enters the citric acid cycle (4). A small amount of the cytosolic pyruvate is metabolized to lactate, which can become glucose again in the Cori cycle (5), see **Figure 2**. The lack of oxygen during ischemia inhibits further metabolism of acetyl CoA in the citric acid cycle and acetyl CoA accumulates in the mitochondria.

Accumulation of acetyl CoA inhibits pyruvate dehydrogenase competitively and pyruvate therefore accumulates in the cytosol and is metabolised to lactate for energy. In the mitochondrion, Acetyl CoA is metabolised to other, less effective, energy substrates such as ketone bodies, acetoacetate and β -hydroxybutyrate (110). The Cori cycle is energy demand-

ing and costs more ATP than is synthesized in the glycolysis. The accumulation of lactate persists. When complex IV (Cytochrome c Oxidase, CcO) in the mitochondrial respiratory chain is in its oxidised form, NO is converted to NO_2^- at the Cu_B centre of CcO. During ischemia CcO is reduced and NO binds the a_3 site instead. NO has a higher affinity than oxygen for the a_3 site and hence, the mitochondrial respiration is almost completely blocked by NO (80), see (Figure 7).

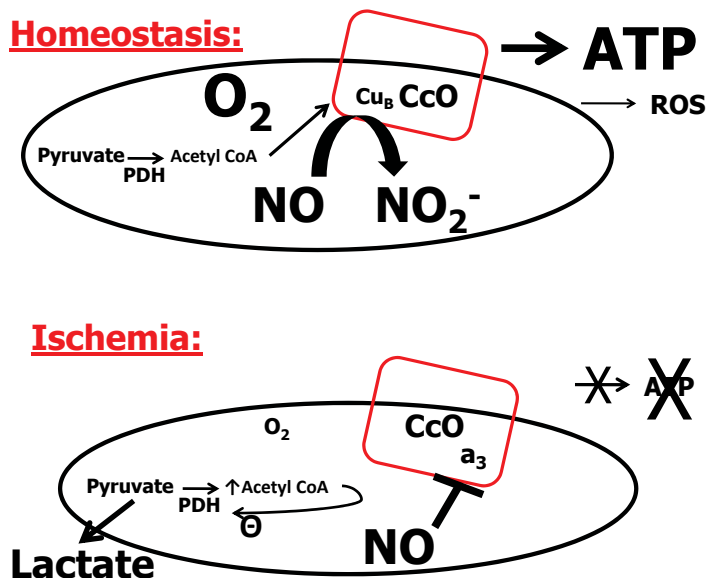


Figure 7. Simplified schematic illustration of the hepatic mitochondrion during homeostasis and ischemia. During normal partial oxygen pressure pyruvate enters the respiratory chain in the mitochondria and generates ATP. A small amount (1-3%) of the oxygen becomes reactive oxygen species (ROS) and NO is converted to nitrite by Cytochrome c oxidase (CcO). During ischemia NO blocks CcO and no ATP or ROS is generated. Acetyl CoA accumulates and pyruvate dehydrogenase is inhibited. Pyruvate is reduced to lactate, generating NAD^+ and 2 ATP. Lactate accumulates in the cytosol.

During the reperfusion the concentration of oxygen increases, pyruvate enters the citric acid cycle and ATP is again synthesized. Excess formation of $\cdot\text{O}_2^-$ occur in the mitochondrial respiratory chain. Nitrite is generated from NO at the Cu_B centre of CcO. If excess concentrations of NO have been present during the ischemia, the a_3 site will still be blocked with less ATP synthesis as the consequence. The oxidative burst and the generation of $\cdot\text{O}_2^-$ will also be attenuated (80), see **figure 8**.

Ischemia causes ATP breakdown to ADP, AMP and finally adenosine. Several experimental studies indicate that it is these two latter metabolites that mediate the protective effect of IP (111-114). AMP activates adenosine monophosphate kinase (AMPK). It is possible that AMPK activation conserves energy by preserving ATP, resulting in less lactate accumulation

(104). Adenosine activates its G-protein coupled A_2 -receptor. This eventually increases the intracellular cAMP concentration, which improves the glucose uptake and activates mitochondrial ATP-dependent K^+ -channels, thereby improving mitochondrial respiration and reducing ATP depletion (115). Coherent with this hypothesis, IP has been shown to reduce the ATP degradation in both the ischemic rat liver (114) as well as in humans (105). In the rat, the accumulation of lactate was also reduced at the same time (114). It is also known that the preservation of ATP and glycogen improves graft survival after liver transplantation (116, 117).

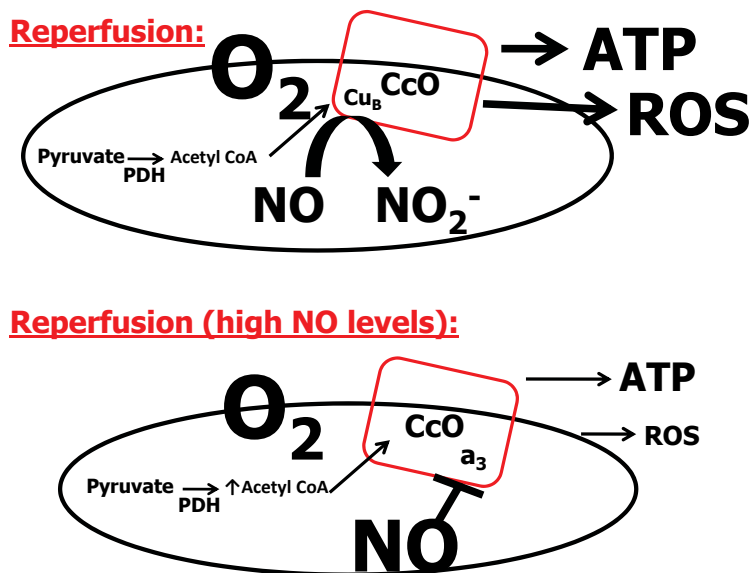


Figure 8. Simplified schematic illustration of the mitochondrion during reperfusion. The mitochondrial respiration generates both ATP and large amounts of ROS. The lower illustration shows the mitochondrion when high concentrations of NO have blocked CcO and consequently only small amounts of ATP and ROS are generated.

Methodology of liver ischemia and reperfusion studies

The effects of ischemia and reperfusion are difficult to study as the multifactorial nature of the cytokine interactions, vary with the setting and may change over time. Isolated molecular interactions can be studied *in vitro*, but when the findings in the cell cultures are applied in murine and rodent models of IRI, the results are difficult to reproduce due to the complex interactions of the cytokines *in vivo*. There are, however, animal models of liver IRI that clearly can show that interventions directed at specific IR phenomena can reduce the injury. The results from these studies are usually based on blood samples, liver biopsies or organ harvesting. Repetitive sampling of blood can be used to study dynamic changes in metabolism or cytokine signalling. Unfortunately, small but significant changes in the molecular concentrations occurring in the organ of interest can be lost due to dilution in the systemic circulation. Moreover, sampling of blood in small animals make them hypovolemic and

anaemic just after a few samples. Biopsies and harvested organs provide a more accurate assessment of the parenchymal concentrations of the molecules studied, although it is not possible to follow a chain of events in the same animal without the biopsies causing serious injury. As a rule, huge amounts of animals need to be sacrificed in order to show a chain of events during IRI.

Clinical IRI has historically been defined as increasing serum levels of aminotransferases. The non specific AST also exists in muscle and heart tissue, but the cytosolic ALT is a specific hepatic enzyme (118, 119). It should be remembered that the IRI hits all cells in the liver parenchyma, but only the hepatocytes contain aminotransferases. The fate of the other cells remains obscure in the blood sample analyses. The exclusive role of the transaminases as indicators of IRI can therefore be questioned.

In the clinical trials described above (92, 97, 105, 106), the methodology depends on liver biopsies which are taken up to one hour after reperfusion. After closure of the abdomen, the methodology changes to blood samples and we do not know the fate of the parameters studied with the biopsies. Optimally, all parameters of IRI should be studied continuously, with the same method in the organ of interest, and with a minimum of discomfort for the subjects. The microdialysis (μ D) technique fulfils all these criteria, but is limited by the need to be inserted intrahepatically during an operative procedure. It is also crucial that the investigator is aware of the relativity of the recovered concentrations. This is in practice a negligible problem for small molecules, but interpretation must be cautioned when the recovery is low.

Microdialysis

Glucose metabolism can be continuously monitored in different anatomic locations simultaneously using microdialysis (120-126). Commonly, glucose, lactate and pyruvate are analyzed. During ischemia there is only little oxygen available and the mitochondrial respiration will not work. Pyruvate will not enter the citric acid cycle and almost all the pyruvate produced during anaerobic glycolysis is consumed for lactate production. The lactate concentration increases and the anaerobic condition can be seen in the microdialysate as an increased lactate-to-pyruvate (L/P) ratio (127).

The methodological principle of microdialysis is based on the ability of capillaries to recover metabolites from the interstitium and then transport these molecules into the systemic circulation. Cells secrete metabolites to the extracellular interstitial space where the metabolites accumulate. Capillaries and sinusoids function as semi-permeable membranes and the smaller molecules diffuse passively over the vessel walls through tiny pores. The whole process is driven by concentration gradients and modulated by oncotic pressure. Equilibrium develops between the interstitial fluid and the lumen of the vessel, whereas, the larger molecules remain in their specific compartment and maintain the oncotic pressure.

Microdialysis was first introduced in 1966 by Bito (128), who investigated the concentrations of amino acids and electrolytes in the brain and subcutaneous tissue by implanting dialysis bags in these tissues. Ungerstedt used a dialysis membrane together with a hollow

fibre in 1974 and created the concept of the microdialysis catheters used today (129). Microdialysis was first reported in humans in 1987 by Lönnroth, who reported a glucose recovery rate of 28% in adipose tissue when he used 3 kDa membranes and a perfusion velocity of 2.5 $\mu\text{L}/\text{minute}$ (130). Experiments incorporating the microdialysis technique have now been performed in several different organs, including the brain, skin, muscle and recently also in the liver. The first papers reported the use of hepatic microdialysis to monitor metabolism in rodent transplant models (131-133).

The microdialysis catheter has a concentric double lumen construction, and the micro-pump continuously perfuses the outer channel with an isotonic solution (hereafter called “the perfusate”). At the distal end of the catheter, the perfusate comes into contact with the semi-permeable membrane. It has a pore size of between 20 and 100 kDalton (kDa), which mimics the passive diffusion occurring through the capillary walls. The molecules of the interstitial fluid diffuse across this membrane and equilibrate with the perfusate. At the very tip of the catheter, the perfusate enters the inner channel through a small hole. The recovered solution (hereafter called “the dialysate”) flows through the inner tubing and is collected in a 60 μL microvial (**figure 9**).

Microdialysis incorporates several advantages compared to sampling blood or tissue. It is performed in the organ of interest without systemic dilution and can be performed without additional needle punctures. The method is especially valuable when metabolic events are studied over time, as the sampling is performed continuously while the catheters remain in the study object. This reduces the number of experimental animals sacrificed or the discomfort for patients who thus do not need repetitive biopsies.

There are, however, several issues to remember when the results are to be interpreted. The recovery of metabolites from the interstitial space is rarely 100%. When the recovery approaches 100%, the concentration of the metabolite in the dialysate mirrors the true concentration in the interstitial fluid. The smaller molecules have a higher kinetic energy and thereby hit the membrane much more often than the larger slow moving molecules. Some larger metabolites may have recovery rates as low as a few percentages and then the recovery rate may have a larger impact on the result than the concentration variations in the target organ. The kinetic energy increases with higher temperatures, and a one Celsius degree change alters the recovery with 1-2 % (130, 134). This rarely has a major impact *in vivo*, but needs to be considered when *in vitro* recovery rates are compared to those recorded *in vivo*.

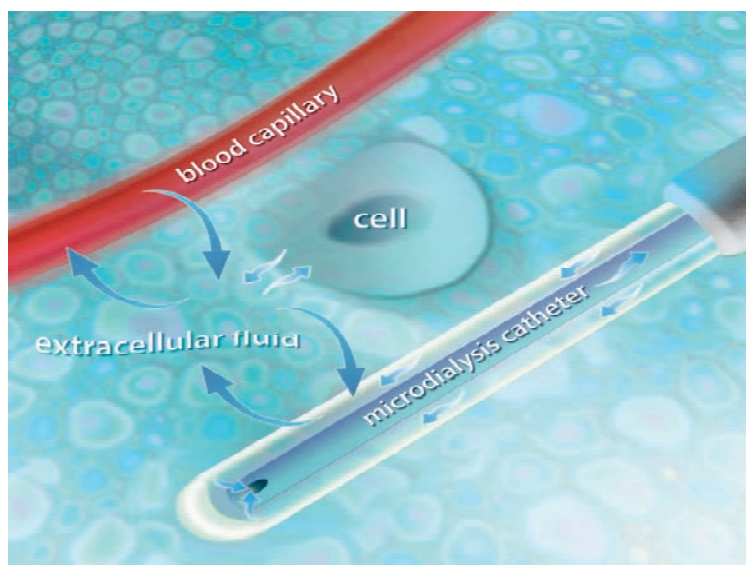


Figure 9. The microdialysis catheter mimics a capillary. The perfusate flows through the outer tube and equilibrates with the interstitial fluid over a semipermeable membrane (20 kDa) at the tip of the catheter. The dialysate is recovered through the inner tube. Published with the courtesy of CMA microdialysis.

It is therefore of utmost importance to standardize the sampling technique in every individual experiment in order to minimize variations in recovery. The area and pore size of the membrane are the most important factors, together with the velocity of the perfusion fluid. These factors are controlled for by using calibrated equipment. Increasing the velocity increases the absolute recovery of metabolites, but also renders a dilution of the sample, giving a low dialysate concentration of the metabolite. Decreasing the velocity improves equilibration and gives metabolite concentrations closer to the true tissue concentrations (135). Slow perfusion velocities (0.1-0.3 $\mu\text{l}/\text{minute}$) are therefore desirable to ensure the highest possible relative recovery rate (136). A problem occurs when slow perfusion is combined with short sampling intervals, as the recovered volumes will be too small for analysis.

Increased hydrostatic pressure can increase the amount of water lost to the tissue by osmosis (ultrafiltration) and thus yield a higher osmolality in the dialysate. It is therefore important to keep the pump and the microdialysis catheter at the same level as the membrane (catheter tip) throughout the study. In addition, a decreased oncotic pressure in the perfusate can increase the ultrafiltration of water, and this phenomenon becomes more important when large pore size membranes are used. Ultrafiltration can be controlled for by comparing the dialysate and perfusate volumes. There are mathematical models to estimate the true concentration in the interstitial fluid of a recovered metabolite (130, 137). Unfortunately, the calculations are time consuming and cumbersome, and therefore not practical in the clinical setting. The recovery rates of several metabolites have been established *in vitro* and in muscle and adipose tissue. Using 30 mm long membranes at physiological temperature and low

perfusion velocities increase the recovery rates, which then approximate the true tissue concentration.

As can be deduced from **table 2**, all factors mentioned above are necessary to optimize the relative recovery (136, 138). *In vitro* sampling with short membranes and high perfusion velocities render low recovery rates (Data provided by CMA microdialysis), **table 2**. Moreover, the insertion of the catheter induces a traumatic response that is said to have normalized in 30-60 minutes (139, 140), although one study states that the local inflammatory response in the tissue around the catheter tip may persist for up to 4 hours after insertion (141). The consequence of these aspects of microdialysis methodology is that the results are to be viewed as relative changes rather than absolute values even though the units are given as concentrations (Molars).

Table 2. Recovery rates.

Reference	CMA	Study III	138	138	136
Study design	<i>in vitro</i>	<i>in vitro</i>	<i>in vitro</i>	<i>in vivo</i>	<i>in vivo</i>
Membrane length	3 mm	30 mm	30 mm	30 mm	30 mm
Perfusion velocity (µl/min)	2.0	1.0	2.5	2.5	0.3
Recovery rates (%)					
Glucose	18		50	23	90-100
Lactate	34		73	34	90-100
Pyruvate	38				90-100
Glycerol			69	30	90-100
Urea			84	31	
NO _x		70			

Table 2. Recovery rates with 20 kDa membranes at various membrane lengths and at different perfusion velocities. A low perfusion velocity yields a recovery rate close to 100%.

In practice, microdialysis is best used when temporal changes in metabolite concentrations are to be studied or when groups are to be compared. Taking the microdialysis technique into the clinic requires it to be reliable, as well as simple to use. Today, there are commercially available equipment for quick bed-side analyses of microdialysate (**figure 10**), which facilitates the use of microdialysis. Having a sample analyzed takes 7 minutes and thus making bedside monitoring feasible. This makes microdialysis a promising tool for the clinical monitoring of patients who have had liver surgery. In fact, in some neurosurgical units it is already routine practice to monitor cerebral ischemia with an intracranial microdialysis catheter (142).

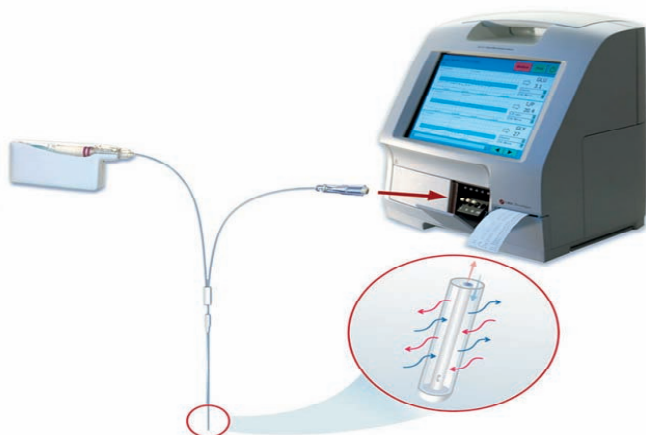


Figure 10. Bed-side analysis of the microdialysate. The microvial is collected and immediately analyzed in the ISCUS. The result is shown within seven minutes. Published with the courtesy of CMA microdialysis.

There have been a number of clinical publications describing hepatic microdialysis in liver transplant recipients (126, 143-149). One study describes the use of microdialysis after liver resections. This study showed increased levels of lactate, glucose and glycerol during the ischemia (150).

Liver microcirculation

Several studies have shown that IRI decreases the perfusion of the liver sinusoids (99, 151-153) and this is regarded as another of the detrimental effects of IRI. A reduction in the flow through the sinusoids will of course decrease the oxygen delivery to an already injured tissue.

Variations in the microperfusion will affect the recovery of the metabolites in microdialysis studies performed in various tissues (154) and the liver (155), see below. Fluctuations in the hepatic blood flow are a confounding factor that needs to be controlled when metabolism is to be experimentally studied with microdialysis. This can be done with parallel methods, *e.g.* laser Doppler flowmetry (LDF) or the use of radioisotopes. These methods are impractical, if not impossible, to use in clinical intraabdominal investigations. The microdialysis ethanol clearance technique was developed to make the assessment of tissue perfusion easier. Ethanol is added to the perfusate and diffuses through the semipermeable membrane to the interstitial fluid. The ethanol diffuses faster when the tissue perfusion is high and consequently less ethanol will be recovered in the dialysate. It has been shown that the ethanol concentration in the dialysate is inversely proportional to the blood flow in muscle (156-158). This method has been validated in muscle, but can be less reliable in the liver

as the enzyme alcohol dehydrogenase exists abundantly there. Recently, urea has successfully been validated to act as a flow marker in muscle tissue (159). Urea is a small inert molecule that is readily distributed in the interstitium and does not have the volatile and toxic characteristics of ethanol.

Endogenous antioxidants and N-acetylcysteine

Endogenous antioxidative molecules (e.g. glutathione and thioredoxin) contain sulfhydryl groups with high affinity for ROS/RNS and can therefore protect the cells from damage during the reperfusion (160). Glutathione is mainly synthesized by the hepatocytes and the intracellular concentrations in the liver are in mM concentrations compared to μM concentrations in the extracellular space. The peptide is secreted by the liver and remains active until it is lost through renal clearance. The rate limiting enzyme of synthesis is glutamate cysteine ligase and this enzyme is inhibited by high glutathione levels and depends on the availability of the rate limiting substrate cysteine. In the reduced form (GSH) the sulfhydryl group of the cysteine residue binds ROS and RNS with high affinity. The enzyme glutathione peroxidase or the ROSs can oxidize GSH (161). The oxidised form (GSSG) can be reduced by the enzyme glutathione reductase, again making it able to scavenge ROS (162). *In vitro* depletion of glutathione stores in the hepatocytes have been shown to correlate with hepatocyte death after paracetamol intoxication (163). High levels of GSH have been proposed to protect the hepatocytes from IRI (164), but some reports show contradictory results (165) and the results are difficult to interpret mainly due to variations in study designs. With this knowledge of glutathione metabolism it is theoretically attractive to counteract the IRI by exogenous administration of glutathione. Unfortunately, glutathione is not taken up by the cells, so other antioxidants have been investigated in the pursuit of reducing IRI.

The effects of exogenous antioxidant administration have been studied in various experimental ischemia and reperfusion (IR) models (166). N-Acetylcysteine (NAC) is one of these molecules, and it is primarily known as the only available treatment of acute liver failure secondary to paracetamol intoxication (167). Paracetamol intoxication generates high levels of ROS and NAC has been found *in vitro* to reduce apoptosis and almost completely inhibit the ROS formation generated by hepatocytes during the reperfusion. Also, NAC can prolong cell survival *in vitro* by replenishing the glutathione stores in hepatocytes (163). Most of the ROS were then derived from the mitochondria and partly from NADPH oxidase (71).

About 80% of the experimental animal liver IRI models show beneficial effects of NAC, usually having the transaminase levels as study endpoint. *In vivo*, NAC reduces the serum levels of transaminases and acute phase proteins (168-171), scavenges ROS/RNS and improves glutathione synthesis (170, 172). NAC scavenges NO (173, 174), and recently this was shown to reduce the formation of plasma S-nitrosothiols after liver IRI (69). Despite these mechanisms of cellular protection proposed, none of the 9 published clinical trials in which NAC was administered before IR showed any clinical benefits, as reviewed by Jegatheeswaran and Siriwarden (175). The trials included liver transplantation patients only and 3 of them could show reduced transaminase levels, whereas the others could not. Intravenous

administration of NAC increased the GSH levels in patients undergoing liver transplant (176). No published paper describes the use of NAC in resectional surgery. One study evaluated the use of NAC in patients having abdominal surgery, but could not see any advantages of the treatment (177). The mechanisms of protection, however, remain obscure as the effects likely are multiple and vary in the different phases of the IRI (175, 178). One interesting murine model could show that the beneficial effect of IP was abolished when NAC was administered before the ischemic preconditioning (179).

Another interesting effect of NAC has been shown in nutritional and toxicology studies, where NAC was found to improve aerobic glucose metabolism (180), favour glycogenesis (181, 182) and reduce insulin resistance (183). The mechanism behind the improved glycogenesis and its relation to the antioxidative property of NAC is not known and has not been studied in IR models.

Experimental animals

Porcine metabolism is closer than rodent metabolism to that of humans (184-186) and thus a porcine model with segmental liver IR was chosen for studying nitrosative stress and glucose metabolism. One of the goals with this thesis was to transfer the microdialysis technique to the clinic, and it was therefore important that the technique and the results from the experimental studies were possible to extrapolate to the clinical study protocol.

AIMS OF THE THESIS

General

To investigate the effects of ischemia and reperfusion on glucose metabolism in the liver using the microdialysis technique.

Specific aims – Study I-IV

- I. To develop a new porcine model of segmental liver ischemia and reperfusion and to investigate whether ischemia and reperfusion injury occurs in only the exposed segment in the newly developed model. A secondary aim was to see if microdialysis could be used to monitor and possibly quantify the ischemia and reperfusion injury *in vivo*.
- II. To find out if the microdialysis clearance technique is applicable for blood flow assessment in an established porcine hepatic ischemia and reperfusion model and to validate the microdialysis urea clearance technique against the established microdialysis ethanol technique in the assessment of blood flow at a higher perfusate flow rate (2μL/minute).
- III. To investigate whether NAC maintains the ATP levels and improves the glycogenesis in the liver after segmental ischemia in the pig liver. A secondary aim was to investigate whether NAC changes the levels of glutathione or the NO metabolites nitrite and nitrate.
- IV. To investigate whether IP (10/10) before 15/5 Pringles maneuver reduces the ischemia and reperfusion injury or changes the glucose metabolism compared to using 15/5 Pringles maneuver alone in surgical liver resections and for the first time follow patients for 5 days with hepatic microdialysis.

MATERIAL AND METHODS

Experimental animal model (studies I-III)

A novel porcine experimental model of segmental liver ischemia and reperfusion was developed in **study I**. The same model was then used in **studies II** and **III** to investigate liver perfusion and the metabolic effects of N-Acetylcysteine, respectively. The animals in **study II** (n=6) were part of the control group of **study III** (n=8). As discussed above, pigs were used because of their metabolic resemblance to humans. This improves the extrapolation of the results to clinical study protocols.

The swine (Swedish Pigham landrace, 29-35 kg, castrated males) were sedated and anaesthetized before they were orally intubated. Systemic variations in the blood flow may affect the concentrations of the metabolites to be studied in the dialysate, as variable oxygen delivery may alter the metabolism. It was also important that the recovery of urea and ethanol depended on the local blood flow rather than the systemic flow (**study II**). Physiological homeostasis was therefore rigorously monitored during all three studies. Buffered glucose, 25 mg/mL, was given i.v. at a rate of 240 (**studies II** and **III**) or 316 mL/h (**study I**) during the experiment (totally 50 g glucose in 2000 mL). Colloids were individually administered i.v. depending on the central venous pressure, to keep the pigs normovolemic.

Access to the abdomen was achieved through a right subcostal incision, and the round and the falciform ligaments were divided. The liver of the pig is lobulated and the peripheral segments easy to access surgically. In order to achieve segmental ischemia, the base of the peripheral segment 4 was clamped, but the vascular clamp crushed the parenchyma. The parenchyma, however, remained intact when an ordinary 25 cm forceps was placed “around” the base of segment 4 and with its tips approximated and secured with a towel clamp (**figure 11**).

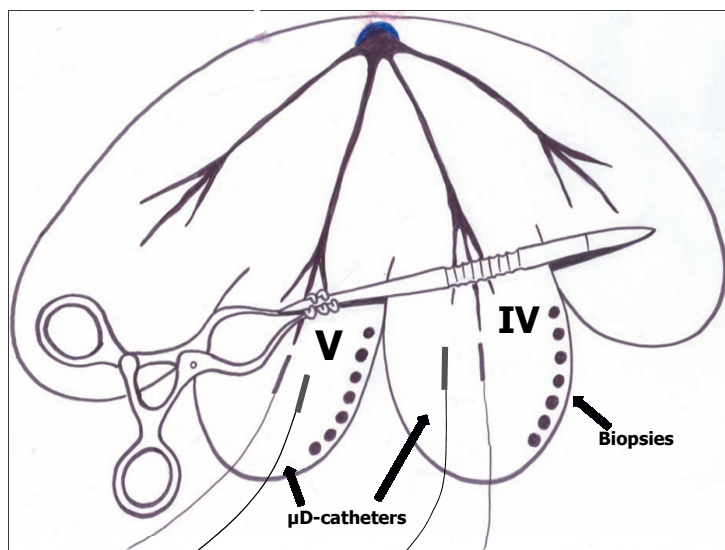


Figure 11. Schematic figure showing the pig liver in the ischemia and reperfusion experimental model used in study I-III. A microdialysis catheter is placed in segment IV and V (two in each segment in study III), respectively. After steady state, 80 minutes of ischemia was achieved by closing the towel clamp around the tips of the forceps at the base of segment IV. Releasing the clamp allows reperfusion. Puncture biopsies are shown as dark dots.

After 60 minutes, the pigs had liver segment IV clamped for 80 minutes and the reperfusion was followed for 4 (**studies I and II**) and 6 hours (**study III**), see **figure 12**.

Laser Doppler probes were placed on the surface of both segment 4 and of the adjacent non-ischemic segment 5. Measuring started 10 minutes before ischemia and continued after the animals were sacrificed. The main purpose of using laser Doppler flowmetry was to verify that there was no blood flow during ischemia. Due to practical reasons that concerned laboratory space, only half of the animals had laser Doppler probes placed.

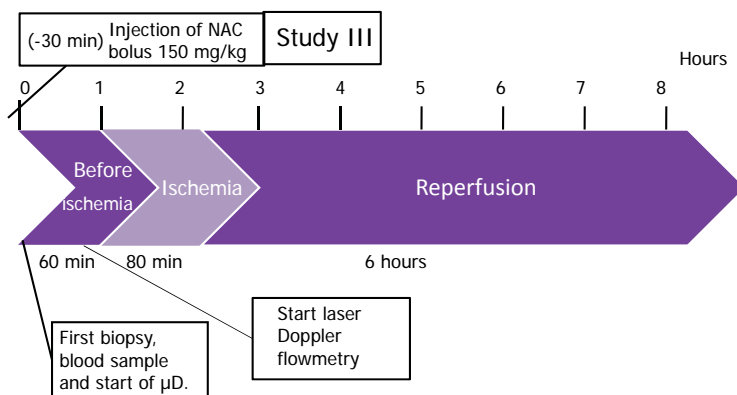


Figure 12. Treatment protocol of the pigs subjected to segmental liver IRI, “Time points” 0 through 8 are shown above the arrow. Biopsies and blood samples were taken hourly. The duration of each phase is shown below the arrow. In study III, 8 pigs were treated with a bolus injection of NAC of 150 mg/kg, followed by an infusion of 50 mg/kg/h. The bolus injection of NAC was given 30 minutes before μ D sampling started at time 0. Reperfusion was followed for 240 minutes (study I), 220 min (study II) and 360 min (study III).

Microdialysis catheters (CMA 63, CMA microdialysis, Solna, Sweden) were placed in segments 4 and 5 (**figure 11**). To check for possible systemic variations of the metabolites studied, a subcutaneous reference catheter (CMA 60, CMA microdialysis) was placed subcutaneously in the area of the left pectoral muscle. All catheters had 30 mm long membranes and a pore size of 20 kDalton. Perfusate flow velocity was set at 1.0 μ L/minute. After the microdialysis catheters were positioned, the animals rested for 60 minutes before the experiment started. The 60 minutes were used to achieve a steady state of the microdialysate (40 minutes) and base line sampling (20 minutes). Lactate, glucose, pyruvate and glycerol were immediately analyzed in the clinical bedside analyzer ISCUS (CMA microdialysis). In **study III** two catheters were placed in each of the segments 4 and 5. The microdialysate from the second catheter of each segment were sampled at 1-hour intervals and immediately frozen at -20° C.

Blood was sampled in 4 test tubes (4 x 4 mL) hourly throughout **studies I-III** and immediately analyzed for pH, base excess, bicarbonate, $p\text{CO}_2$, blood glucose, sodium, and potassium. In the evening after the experiment the PT-INR, the blood levels of haemoglobin and the serum levels of bilirubin, ALT, AST and LDH were analyzed. The fourth test tube was centrifuged and the serum was frozen before batch analyses of NO_x .

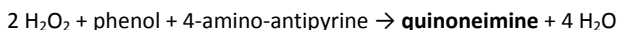
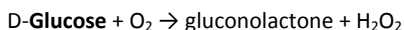
Biopsies were immediately submerged in liquid nitrogen and later in the same night freeze-dried. ATP, glycogen, apoptosis, and glutathione were analyzed in the freeze-dried

tissue biopsies. Apoptosis was measured with histone associated DNA-fragmentation. For further information, see the respective individual paper at the end of this book.

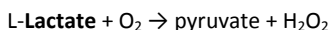
The microdialysate was frozen in -20° C and then NO_x was analyzed in batches.

Microdialysis analyzing equipment (studies I-IV)

In **studies I, III and IV** the microdialysate was analyzed immediately in the ISCUS bedside analysator. In **study II**, the microdialysate was analysed in batches using the CMA 600. Both instruments use the same wavelength (530 nm) spectrophotometer, and the chemical reactions used are:



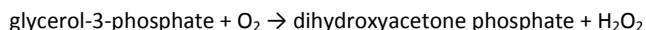
Detection ranges: 0.1-25 mM with a deviation of < 5 %.



Detection ranges: 0.1-12 mM with a deviation of < 5 %.



Detection ranges: 10-1500 μM with a deviation of < 5 %.



Detection ranges: 0.01-1.5 mM with a replicate deviation of < 5 %

Both analyzing instruments are calibrated every time they are started or when new reagents are placed in them. Under operation both are calibrated every 6th hour. If the ISCUS response rate deviates from the stated response factor the analyses are rerun.

Study I

The reperfusion was followed for 4 hours and there was no pharmacological intervention, otherwise the study protocol is identical to **figure 12**. The schematic illustration (**figure 11**) fits **study I**, apart from the fact that only one microdialysis catheter was placed per liver segment.

Study II

In this study, 6 pigs had an additional microdialysis catheter (CMA 70) placed in the ischemic segment 4. This catheter was perfused with Ringers acetate containing 20 mmol urea/l and 5 mmol ethanol/l at a perfusion velocity of 2.0 µl/minute. The ethanol concentration was measured by adding alcohol dehydrogenase and NAD⁺ to the sampled microdialysate according to method described by Bernt and Gutmann (187). The concentration of NADH produced in the enzymatic reaction is proportional to the concentration of ethanol. NADH was spectrophotomerically analyzed, whereas the levels of urea, glucose, lactate and pyruvate were measured in the CMA 600 analyser. Correlation analyses were made between the concentrations of ethanol (gold standard) and urea recovered. **Figure 11** corresponds with **study II**, except for the extra microdialysis catheter placed in segment 4. The reperfusion was followed for 220 minutes and no NAC was given.

Study III

At the start of the experiment, half of the pigs (n=8) received NAC (150 mg/kg) as a bolus injection and then as a continuous infusion (50 mg/kg/h) throughout the experiment. The Ischemia-Reperfusion (IR)-group (n=8) received an infusion of 0.9 % sodium chloride at the same volume and rate, see **figures 11** and **12**. Reperfusion was followed for 360 minutes.

Study IV (Clinical study)

The protocol of **study IV** was approved by the Regional Ethics Committee at the University of Linköping, University Hospital, Linköping, Sweden and the RCT was registered at the web site 'www.controlled-trials.com'.

This randomized single-blinded controlled trial was conducted between January 16th 2008 and June 8th 2009 at the Department of Surgery, University Hospital, Linköping, Sweden. Patients intended for resection of two or more segments of the liver due to metastatic liver disease or the suspicion of T1-2 gallbladder cancer were asked to participate in the study. No patients intended for multiorgan resection, portal ligature or with suspected chronic liver disease were included.

In this trial we introduced a novel method of assessing postoperative liver metabolism after IRI. No reliable calculations of power could thus be made; instead we looked at the clinical significance. IP takes 20 minutes to perform and the benefit obtained from this maneuver must justify the extra time spent in the operating theatre. It was therefore decided that if no differences were seen in 16 patients having IP (i.e. 5 hours of surgery), the results would be of no clinical interest.

Patients were stratified according to the size of the resection, and 16 closed envelopes were prepared for patients who had resections of 2-3 segments and another 16 envelopes for those who had >3 segments resected. Half of the patients in each of the strata were randomized to have IP prior to the PM, thus making a control group (n=8) within each stratum (**figure 13**). The patients were blinded to their group allocation whereas the surgeons were not.

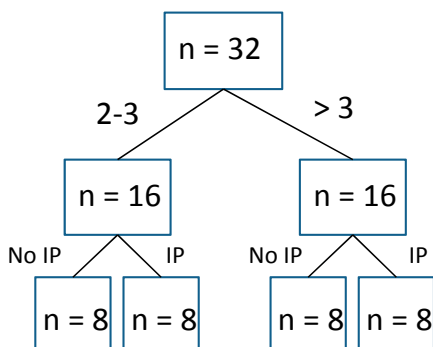


Figure 13. Schematic illustration of the randomization. The study population was set at 32 subjects. When the size of the resection was decided, an envelope of that stratum was opened to reveal IP or no IP.

All patients were operated through a right subcostal incision angled and extended to the sternum. After the patient was considered resectable, he or she was stratified (2-3 or >3 segments) and then randomized to either IP or no IP. A cotton tape around the hepatoduodenal ligament was used to induce ischemia to the liver. The PM was performed according to 15/5 and the IP group had 10/10 IP performed prior to that (**figure 14**).

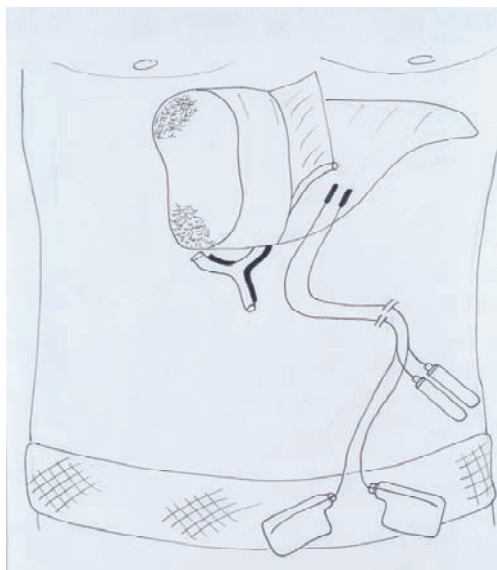


Figure 15. Schematic illustration showing the positioning of the catheter tips within the residual liver parenchyma. The micropumps are kept in a waist belt.

Pulse, blood pressure, respiratory frequency, temperature, urinary output and patient consciousness were postoperatively assessed every fourth hour and scored according to the modified early warning score (MEWS) (188). The most severe complication was registered for each patient according to the Dindo-Clavien classification (189).

Statistical methods

Non-parametric values are shown as ranges (median) and analysed with the Mann-Whitney U-test and parametric values given as mean (SD) or mean (SEM) as indicated in the text. Parametric data concerning two variables were analysed with Student t-test.

The microdialysate samples were retrieved at the time points specified in the respective studies. The samples were then grouped into phases (**studies II-IV**), except in **study I** where the individual time points were used in creating the statistical model. The phases (or the individual time points in the case of **study I**) were compared using ANOVA. The Individuals were assigned as the random factor and nested in their respective groups. If statistically significant differences were found in the ANOVA, a Sidak (**Studies I and III**) or a Tukey (**study IV**) post Hoc analysis was performed.

In **study II**, the temporal changes were analyzed with the Friedman's one-way analysis of variance with Kendall's coefficient of concordance. Correlations were made with Pearson's correlation coefficient analysis.

SPSS 15.0 software was used in **studies I and II** and STASTICA 8.0 software was used in **studies III and IV**. A p-value < 0.05 was considered statistically significant.

RESULTS

All animals in the **studies I – III** thrived throughout the respective studies and there were no events of haemodynamical instability in any of the pigs. There were no differences between the groups in **study II** regarding MAP, CVP, pH, base excess, bicarbonate, pCO₂, spO₂, sodium or potassium.

Study I

Compared to baseline, all animals peaked in their microdialysate levels of glucose (4-fold, $p<0.01$), lactate (3-fold, $p<0.001$) and glycerol (4-fold, $p<0.001$), whereas pyruvate fell to a tenth of the preischemic levels ($p<0.001$) in the ischemic segment during the ischemic phase. No such changes were seen in the perfused segment or in the subcutaneous reference catheter. The segmental ischemia did not elevate the levels of glucose in the blood (figure 16).

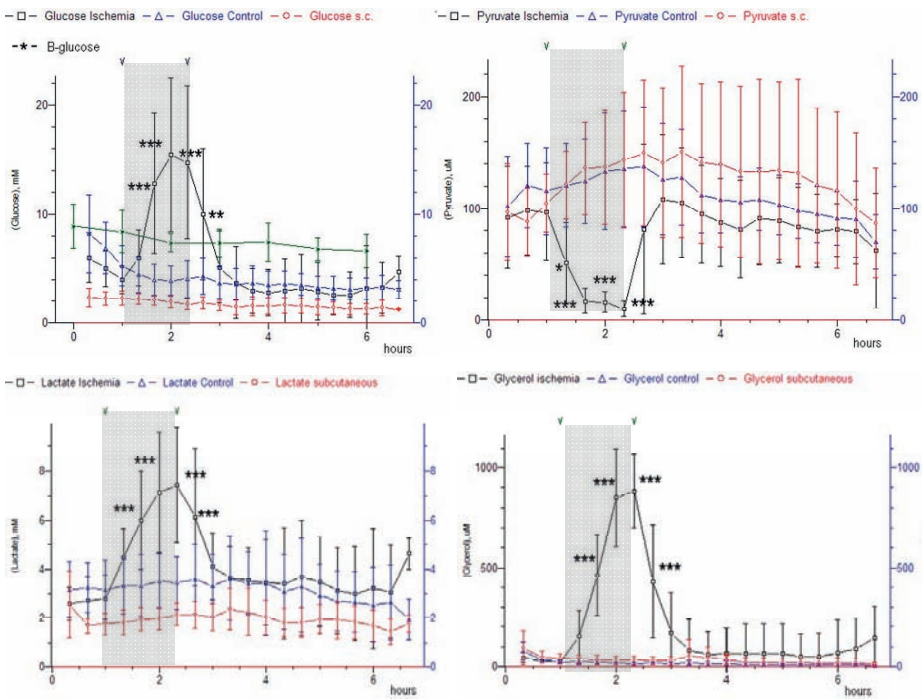


Figure 16. In **study I** the microdialysates from the subcutaneous tissue, the ischemic and perfused segments of the liver were analyzed. There were significant changes in the glucose, lactate, pyruvate and glycerol levels, but only so in the ischemic segment. No changes were seen in the blood levels of glucose. The shaded area indicates the ischemia. Mean (SD). *= $p<0.05$, **= $p<0.01$ and ***= $p<0.001$.

The infiltration of PMN leukocytes increased in both the ischemic (from 21 (7.9) to 142 (61), $p<0.001$) and the perfused segment (from 18 (5.9) to 72 (48), $p<0.01$) at 6 h compared to baseline. The density tended to be higher in the ischemic segment after 4 hours of reperfusion (142 (61) vs. 72 (48), $p=0.09$), see **figure 17**.

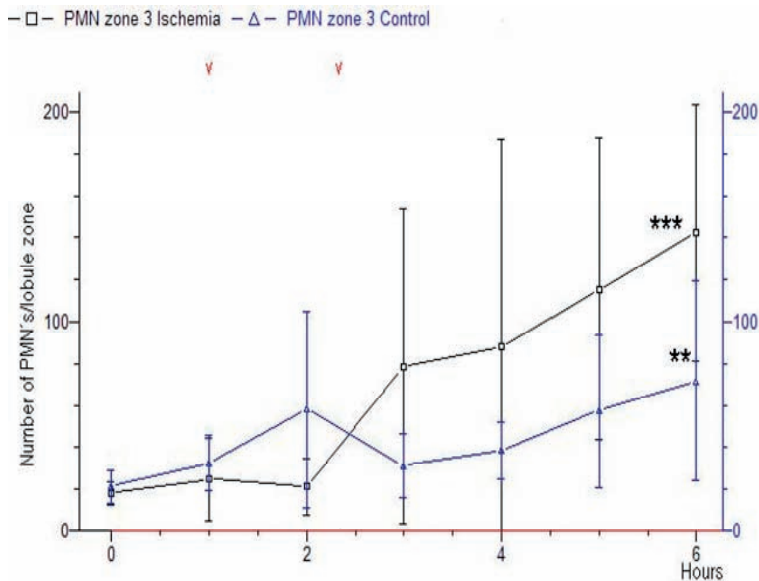


Figure 17. The density of PMN leukocytes in Rappaport zone 3 of the hepatocyte. The number of PMNs was counted in 2 zones on three separate sections. The shaded area indicates the ischemic phase. Mean (SD). **= $p<0.01$ and ***= $p<0.001$, compared to baseline.

The histological examination of the ischemic segment revealed vacuolization early in the reperfusion whereas necroses were detected later. No such changes were seen in the perfused segment 5 although PMN leukocyte infiltration increased significantly (**figure 18**).

The main result was that all animals were haemodynamically stable and had the same metabolic pattern in the microdialysate, indicating that this porcine experimental model is stable and reproducible.

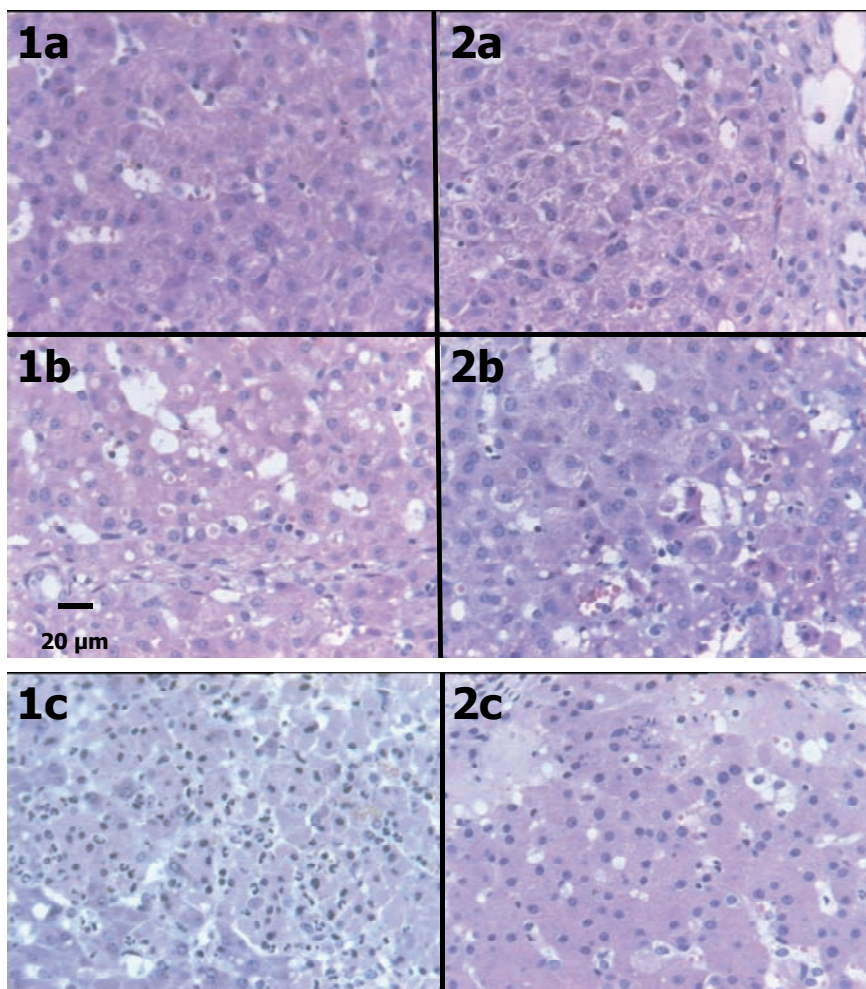


Figure 18. Micrographs of the perfused segment (1) and the ischemic segment (2), baseline (1a and 1b), after 1 hour of ischemia (1b and 2b), after 3 hours of reperfusion (1c and 2c). Increased leukocyte infiltration is illustrated in figure 1c and a necrotic area is shown in figure 2c, although there was no evidence of differences between the segments regarding such phenomena. H & E, magnification 40x.

Study II

During the ischemic phase, the blood flow was zero according to the laser Doppler flowmetry. Both urea and ethanol peaked during the ischemic phase (both $p<0.005$ compared to baseline), but as the segment was reperfused, the concentrations of both ethanol and urea returned to the pre-ischemic levels (**figure 19**).

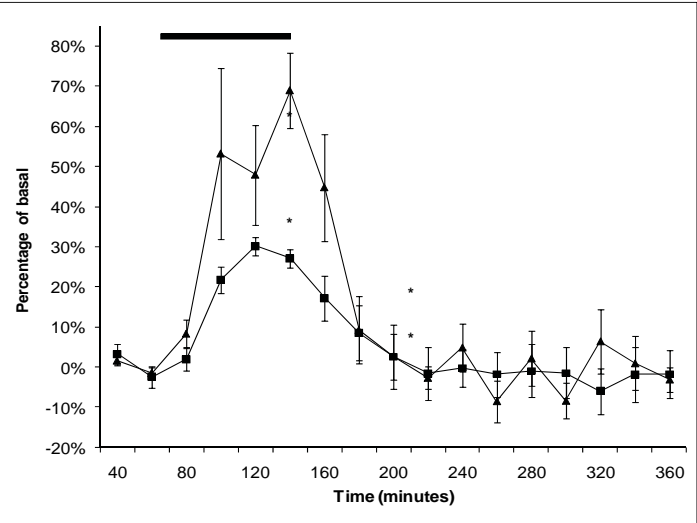


Figure 19. Changes in ethanol (triangles) and urea (squares) (% of basal). The ischemic phase is indicated by a black line. Mean (SEM). *. $p<0.05$

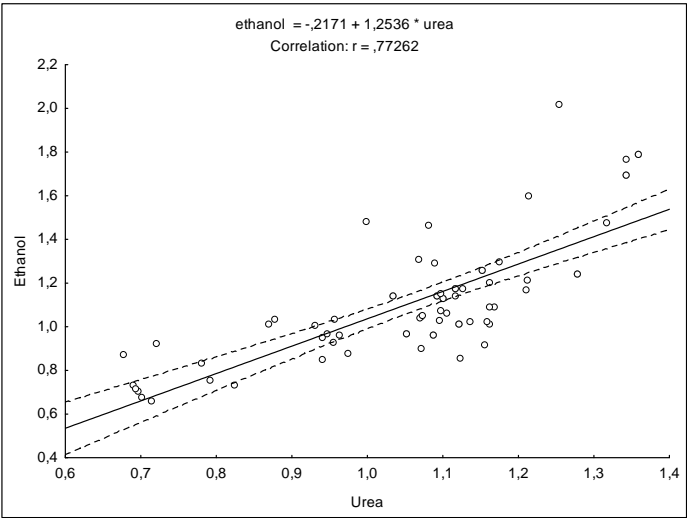


Figure 20. Simultaneously analyzed ethanol values (% of baseline) plotted as a function of urea values (% of baseline) during the whole experimental period. A regression line has been drawn ($r = 0.77$). Dotted lines show 95% confidence intervals.

There was a good correlation between the ethanol and urea concentrations in the microdialysate during all phases ($r=0.77$) (**figure 20**). Laser Doppler flowmetry during the reperfusion phase confirmed the restoration of the blood flow in segment 4.

Study III

During ischemia, the laser Doppler median flow in segment 4 of both the NAC and IR-groups were at the same level as after the animals had been sacrificed (**figure 21**). This confirmed that there had not been any blood flow in segment 4 during the ischemic phase.

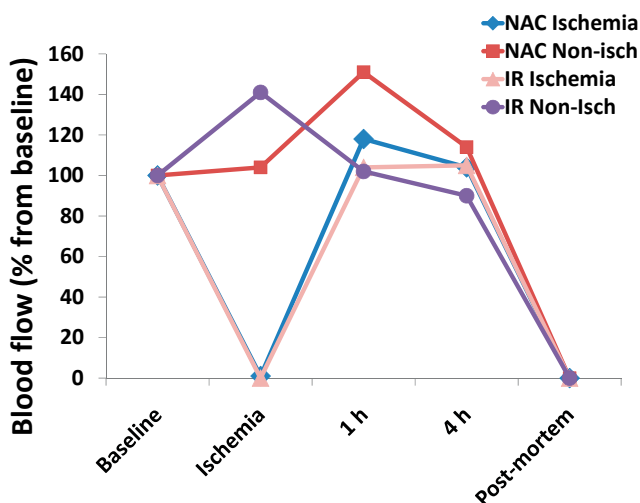


Figure 21. Laser Doppler flowmetry recordings from 4 pigs in each group. Baseline is set at 100%. Percentages of baseline flow are shown during ischemia, after 1 and 4 hours of reperfusion and post-mortem.

There were no differences between the groups regarding the blood levels of glucose, haemoglobin, PT-INR, ALT, LDH, or bilirubin levels at any time point. AST, however, increased from 49 (4.6) to 261 (72) IU/L in the IR-group at the end of the experiment ($p<0.001$). During the reperfusion phase, AST was significantly lower in the NAC-group, 176 (13) vs. 251 (25) IU/L (ANOVA, $p=0.0099$).

The number of PMNs in zone 3 of the IR-group increased continuously in the ischemic segment from 19 (3.1) to 135 (48), $p=0.021$, and in the non-ischemic segment from 24 (5.5) to 71 (13), $p=0.0038$. At the end of the reperfusion phase the number of PMNs were reduced in the non-ischemic segment of the NAC group (32 (4.0) vs. 71 (13), $p=0.0090$). There were signs of necrosis in the ischemic segment of both groups, and the densities of PMNs were similar, 135 (48) vs. 95 (18), $p=0.39$ (**figure 22**).

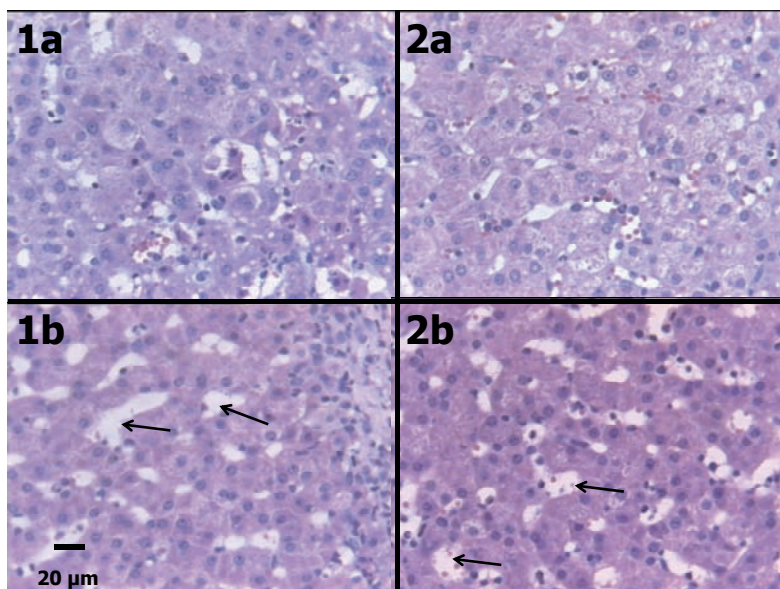


Figure 22. Micrographs of the ischemic segment in the control group (1) and the NAC-group (2), after 1 hour of ischemia (1a and 2a), after 5 hours of reperfusion (1b and 2b). The sinusoids are markedly dilated (arrows) in both groups at the end of the experiment (2a and 2b). H & E, magnification 40x.

Apoptosis, measured as histone associated DNA fragmentation, increased in the ischemic segment of the IR-group (0.43(0.05) to 1.04(0.17) au/mg dw tissue, $p=0.012$), but treatment with NAC did not reduce this mode of cell death.

The ATP levels in the IR-group decreased in the ischemic segment from 0.41(0.08) to 0.17(0.03) M/g dw tissue ($p=0.0180$) during ischemia, and were restored during reperfusion, 0.17(0.03) vs. 0.46(0.16), $p=0.0039$. The ATP levels tended to be higher in the NAC group, 0.66(0.10) vs. 0.46(0.16), $p=0.12$ (figure 23A). During the reperfusion in the non-ischemic segment (Figure 23B), the level of ATP was higher in the NAC-group than in the IR-group, (0.59(0.01) vs. 0.40(0.01), $p=0.0213$).

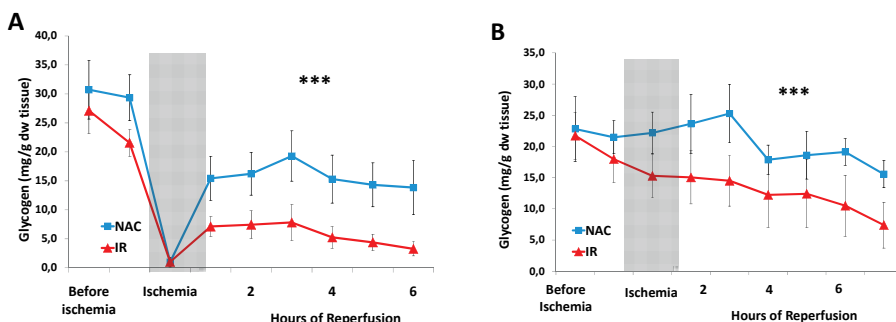


Figure 23. The ATP levels in the ischemic (A) and the non-ischemic (B) liver segment. Eight pigs were treated with NAC and 8 were controls (IR). ANOVA. Mean (SEM). * = $p<0.05$

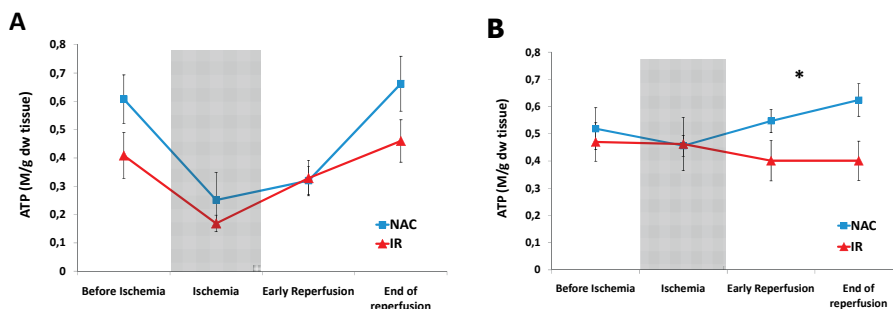


Figure 24. The glycogen levels in the ischemic (A) and the non-ischemic (B) liver segment. Eight pigs were treated with NAC and 8 were controls (IR). ANOVA. Mean (SEM). ***= $p < 0.001$.

Glycogen was almost depleted in the ischemic segment during ischemia, but the stores partly refilled after reperfusion (**figure 24A**). In the non-ischemic segment, NAC treatment prevented the gradual decrease in the glycogen stores seen in the IR-group (**figure 24B**). During the reperfusion phase the glycogen levels in the NAC-group were higher than for those in the IR-group in both the ischemic (15.8(0.23) vs. 5.88(0.11), $p = 0.0001$) and perfused (20.0 (0.25) vs. 12.0(0.23), $p = 0.0001$) segments.

NAC did not change the levels of GSH, GSSG or the ratio of GSH/GSSG at any time point.

In the non-ischemic segment and the subcutaneous tissue there were no changes in the microdialysate over time or between the groups. In the IR-group, the levels of lactate (**figure 25A**) peaked in the ischemic segment during ischemia (3.4(1.3) to 8.9(1.5) mM) compared to the levels at the baseline and in the non-ischemic segment, both $p < 0.001$. The lactate levels during the reperfusion phase were lower in the ischemic segment of the NAC-group compared to the IR-group, 2.8(0.2) vs. 3.8(0.2) ($p < 0.001$), respectively.

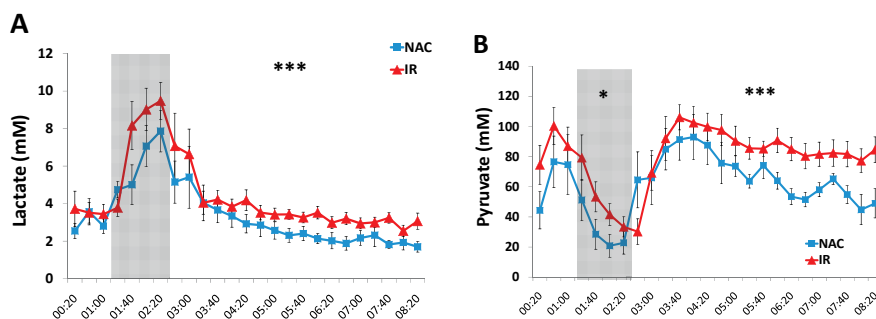


Figure 25. Recovered levels of lactate (A) and pyruvate (B) in the microdialysate. Shading represents the ischemic phase. Analysis every 20 minutes, baseline (time point 3), ischemic phase (time points 4-7), reperfusion phase (8-25). ANOVA. Mean (SEM). *= $p < 0.05$, ***= $p < 0.001$.

The difference during the ischemic phase, 6.3(0.4) vs. 7.5(0.6) mM, approached statistical significance ($p=0.0736$). The NAC-group had lower levels of pyruvate (**figure 25B**) than the IR-group in both the ischemic phase (31(4.9) vs. 52(5.5), $p=0.0250$) and the reperfusion phase (68(2.5) vs. 85(2.3), $p<0.001$). The glucose and glycerol levels peaked during ischemia as expected, but there were no differences between the groups.

The levels of NO_x in the serum or in the microdialysate did not change over time within the groups. The NO_x levels in the NAC-group were lower than in the IR-group both in the serum (112 (12) vs. 159 (15) μM , ($p<0.05$) and in the microdialysate. The difference was seen in both the ischemic (82(8.1) vs. 164 (25) μM , $p<0.01$) and the non-ischemic segment (76(8.5) vs. 167(25) μM , $p<0.01$) (**figure 26**).

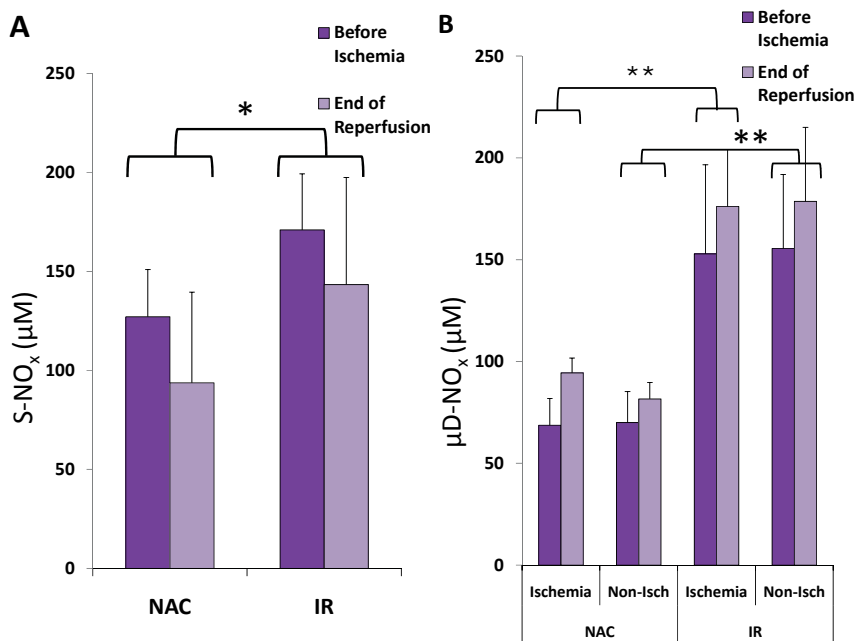


Figure 26. The nitrite/nitrate (NO_x) levels in the serum (**A**) and the microdialysate (μD) (**B**) in pigs subjected to segmental liver IRI. Eight pigs were treated with NAC and 8 were controls (IR), μD results are shown for both the ischemic and non-ischemic segments. Mean (SEM). * = $p<0.05$, ** = $p<0.01$.

These results indicated that NAC scavenged NO and thereby decreased the levels of NO_x . We therefore followed up this theory with an *in vitro* experiment. Dose-dependent decreasing levels of NO were seen when increasing concentrations of NAC (0.02, 1 and 10 mM) were added to 1 mM of the NO-donor SNP.

Study IV

All randomized patients completed the study according to intention to treat and there was no mortality within the study.

There were no differences between the whole IP group (n=16) and the controls (n=16) or between the stratified groups with regard to demographics, preoperative glycogen levels, intraoperative data, insulin administration, LOS or postoperative diagnosis. Complications occurred in 12 patients (37.5 %) and these were scored according to Dindo-Clavien (189). No differences were seen between the groups.

No differences were seen between the IP group (n=16) and the controls (n=16) when serum and blood analyses were compared, neither were any such differences seen between the groups when the patients who had minor resections were compared. The patients that had IP prior to a major resection had lower levels of lactate and PT-INR immediately postoperatively (2.4(0.4) vs. 4.4 (1.4), $p=0.003$ and 1.2(0.1) vs. 1.5 (0.1), $p=0.041$, respectively).

After surgery and up to POD 1 there were lower levels of μ D-glucose, μ D-pyruvate and μ D-lactate in the IP group (n=16) compared to the controls (n=16). These differences disappeared in the minor resections subgroups (2-3 segments, IP (n=8) vs. controls, n=8), apart from a lower level of glucose on POD 2 in the IP treated patients. Analyses of the patients who had IP treatment before a major resection, however, showed lower lactate levels already during the transection phase (4.25 (0.34) vs .5.8 (0.37), $p<0.001$). This decrease in the lactate levels continued throughout the rest of the day of the operation (2.6 (0.17) vs .4.0 (0.22), $p<0.001$) and also included lower levels of glucose (4.9 (0.32) vs .6.6 (0.35), $p<0.001$) and pyruvate (147 (9.1) vs .198 (9.8), $p<0.001$) in addition. On the POD 2 the levels of glucose (6.1 (0.38) vs .8.3 (0.52), $p=0.022$) and lactate (1.8 (0.13) vs. 2.3 (0.16), $p=0.047$) were lower in the IP treated patients (**Figure 27 a-c**).

No differences whatsoever were seen in the μ D-glycerol levels. We also compared all patients having major resections (n=16) with those having minor ones (2-3 segments, n=16), but could not detect any differences in the μ D analyses.

The NO_x levels decreased postoperatively in the IP group from 31 (11) to 22 (11) μM ($p=0.0018$) up to POD 3, where they started to return to the original levels. The control group behaved similarly, and there were no differences between the groups. The volume of the resection did not affect the NO_x levels in the μ D.

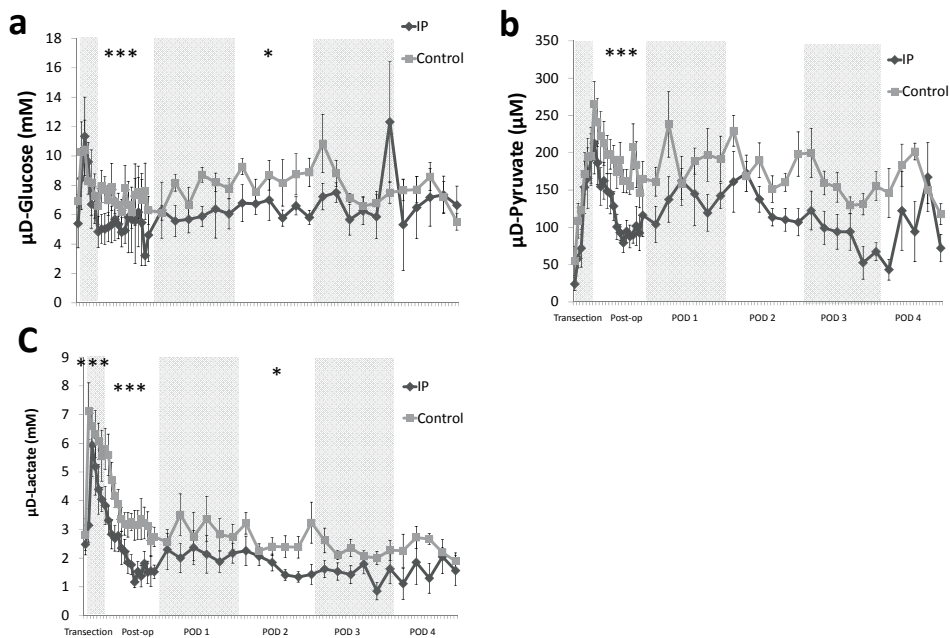


Figure 27. Glucose (a), pyruvate (b) and lactate (c) levels in the microdialysate (μ D). The IP group ($n=16$) and the control group ($n=16$). Shaded fields separate the phases used in the ANOVA and the significant differences found in the Tukey post hoc analysis are noted: * = $p < 0.05$ and *** = $p < 0.001$. POD = Post Operative Day.

Case presentation

One patient not involved in **study IV** was followed with microdialysis postoperatively. She had hepatitis and Child B cirrhosis complicated with a hepatocellular carcinoma too large for radiofrequency ablation. After having the tumour resected, she suffered from liver failure until she succumbed 14 days postoperatively. Her lactate levels were extremely elevated even during the transection of the liver and the elevated lactate levels persisted throughout the time spent in the ICU (**figure 28**).

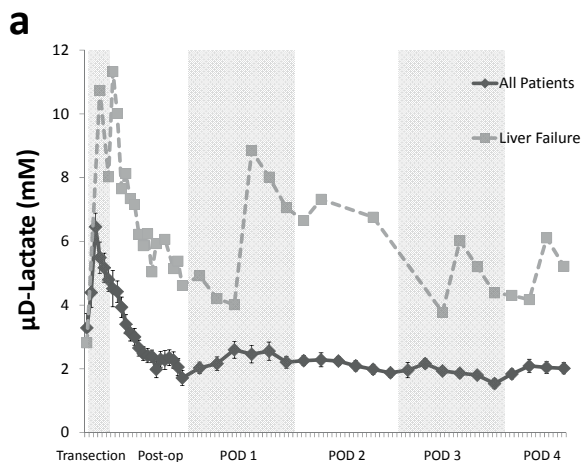


Figure 28. A patient suffering from acute postoperative liver failure presented with extremely elevated lactate levels in the microdialysate already during the transection. The patient was not part of study IV.

This tragic outcome points out the need for improved assessment of operability to avoid postoperative liver failure. Microdialysis can be a novel tool of liver function assessment in the perioperative situation.

DISCUSSION

The focus of this thesis is the glucose metabolism in the liver after hepatic ischemia and reperfusion injury.

Despite the enormous wealth of documented knowledge on the topic of hepatic IRI (4186 hits in PubMed), we are just beginning to understand the cellular and humoral mechanisms. The extent of IRI depends on the duration and magnitude of the ischemia, if it is continuous or intermittent, but also on the degree of oxidative/nitrosative stress during the reperfusion. The latter depends on the amount of ROS/RNS produced but also on the antioxidative capacity of the host, which may vary over time and between individuals. If just some of the experimental circumstances change, the results from a previous experiment may be impossible to repeat. The complexity of IRI has thus resulted in divergent findings, making extrapolation of the results to other species or to the clinic hazardous.

The hepatic IRI hits several different cells in the liver parenchyma and many different cellular functions are altered. The activity of the ATP-dependent membrane ion pumps can be inhibited, causing for example reduced bile secretion. The ATP synthesis can be impaired through the blocking of the mitochondrial respiratory chain. Various enzymes alter configuration when ROS/RNS binds to their amino acid residues, thereby modifying the enzymatic activity. One example of that is the enzyme glucokinase, which is under the influence of insulin and glucagon. Glucokinase incorporates the G-6-P molecule into the glycogen macromolecule and is thus necessary for the synthesis of glycogen. If glucokinase is exposed to ROS/RNS it will change its configuration and become inactive (190). This means that less G-6-P will become glycogen during the reperfusion and hence, the G-6-P will be metabolized in the glycolysis or return to the systemic circulation. The reduced glycogen levels after ischemia are evident in **figure 24** and it is possible that the scavenging of ROS/RNS by NAC protects the function of glucokinase. This may explain the increased restoration of the glycogen stores seen in the NAC treated animals. In **figure 23A** the ATP levels are low during the ischemia and we know that ATP is necessary to uphold the energy demanding membrane ion potential (46). A failure will result in sodium and calcium influx and cellular death. Depletion of reduced glutathione has likewise been linked to hepatocyte death (163). A functioning glucose metabolism, ATP production and an antioxidative defence system are crucial for hepatocyte viability. It is therefore appealing to consider the antioxidative capacity or the ATP levels in the hepatocytes as more relevant markers of cellular viability after hepatic IRI than mere enzyme leakage. The susceptibility to oxidation and short half lives make these substances difficult to study clinically.

Until recently, also the dynamics of hepatocyte energy metabolism *in vivo* has been elusive for the scientists, as the metabolites produced in the injured liver are diluted in the systemic circulation. Repetitive biopsies or the sacrifice of huge numbers of laboratory animals have been impractical, but has remained the method of choice to follow energy metabolism

over time. Whatever and how good the experimental study design, the results will always need to be extrapolated if they are to be applied in the clinic. It is, however, the belief of this author that efforts should be made to further develop the technology of experimental studies in the clinical settings. New techniques for *in vivo* analyses are beginning to emerge, of which microdialysis is but one. Other interesting techniques are for instance functional magnetic resonance imaging, contrast enhanced ultra sound, laser Doppler perfusion imaging and intravital microscopy.

The paradigm of quantifying clinical hepatic IRI with transaminase leakage only, is therefore theoretically opposed to the increasing understanding of the many aspects of hepatic IRI. To challenge the paradigm of the aminotransferases it is necessary to correlate new markers of IRI to hepatocyte death. If it can be shown that increasing anaerobic metabolism correlates to hepatocyte apoptosis or necrosis, it will be clinically relevant to consider the hepatic glucose metabolism as at least a complementary marker of IRI.

Study I showed that the glucose metabolism in a perfused segment was unaffected by the IR occurring in the ischemic segment. This finding justifies future studies using the perfused part of the liver as an internal control to the ischemic segment, thereby reducing the number of experimental animals needed. The recruitment of PMN leukocytes is not just a phenomenon localized to the injured part of the liver, as seen in the increasing density of PMN leukocytes in both segments. The hepatic expression of leukocyte adhesive molecules and chemotactic agents is therefore at least partly mediated by systemic signalling secondary to a localized ischemic insult. This novel porcine model of segmental liver ischemia is reproducible, as all pigs showed the same metabolic changes in the parameters studied.

We continued to explore our experimental model and in **study II** we found that the metabolic changes during ischemia paralleled the changes in liver perfusion. Both markers of flow in the perfusate (urea and ethanol) paralleled the metabolic markers of ischemia (lactate and glucose). These findings confirmed that the changes in the lactate and the glucose levels are dependent on the tissue perfusion. There were no differences when we compared the two markers of flow, but we can conclude that urea is superior to ethanol, since urea is easier to handle and is metabolically inert. The urea clearance technique contributes to the diagnostic arsenal of microdialysis, especially when the perfusion is to be studied *per se*. Adding urea to the perfusate can also be helpful in situations when markers of metabolism are unreliable, e.g. in starvation or states of hypermetabolism. Previous methods of monitoring the blood flow in the liver (like LDF or scintigraphic methods) have been unreliable and impractical clinically, but the urea clearance technique is easy to use and easily transferable to the clinical setting. A possible confounder for interpretation of the ethanol recovery is the existence of alcohol dehydrogenase in the liver tissue. The enzyme did not, however, diminish the reliability of ethanol as a marker of flow during 4 hours of reperfusion. The enzymatic activity of alcohol dehydrogenase still has to be recognized if longer times of observation are expected.

Moreover, urea can be analyzed in the same reagent kit as lactate, glucose and pyruvate in the ISCUS analyzer. If urea is added to the perfusate and then analyzed together with lactate, glucose and pyruvate, the changes in the markers of ischemic metabolism can immediately be compared to the recovered level of urea. If urea is elevated at the same time as the metabolism indicates ischemia, the ischemia is due to low perfusion. On the other hand, when the urea levels are stable, changes in metabolism are not due to low perfusion.

The histological evaluation of 6 independent zones of Rappaport in both segments in each animal showed infiltration of PMN leukocytes that increased throughout the study period. An effort was made to compare qualitative variables (sinusoidal congestion, necrosis, vacuolization and hepatocyte swelling), but when the slides were examined the histopathology was morphologically very divergent within the same section. The qualitative approach was therefore abandoned due to this regional heterogeneity within the same section. It seemed that this regional variation of IRI varied with the degree of sinusoidal dilation. This could mean that in areas where the sinusoidal endothelial cells are viable, there is also eNOS activity keeping the sinusoids open by means of the relaxing effect of NO. This theory cannot be further explored within this thesis, but eNOS activity and cell viability in the context of the histological findings is an interesting venue for future experiments.

The effects of the antioxidant NAC have been divergent and difficult to interpret when NAC has been studied in liver IRI models (175). A reason for this may be that the outcome parameters previously studied have been secondary to the direct effects of NAC. In **study III** we could show for the first time that NAC improves glycogenesis, decreases lactate levels and tends to improve ATP levels in the liver after IRI. This occurred without changes in the redox state of glutathione or reduction in apoptosis. The levels of pyruvate and lactate were lower in the microdialysate in the NAC-treated animals. This indicates a higher rate of mitochondrial oxidative metabolism as opposed to when the glucose is anaerobically metabolized to lactate. There were lower levels of NO_x in both the serum and the microdialysate, indirectly indicating that NAC scavenges NO. This finding is important in glucose metabolism as NO can inhibit the mitochondrial respiratory chain (76-78). It is believed that the inhibition of these complexes reduces the harmful oxidative burst of ROS after reperfusion (191). This seems true when complex IV (cytochrome c oxidase, CcO) is in its reduced form. During physiological cellular respiration, however, CcO is in the oxidized form and NO binds to the Cu_B centre, where it is converted to nitrite. The reduction of CcO increases when the mitochondrial oxygen concentration decreases, *i.e.* during ischemia. When CcO is reduced, NO competes with oxygen to bind reversibly at the a₃ site instead (80). As NO has a higher affinity for CcO than oxygen, the inhibition of the respiratory chain may be almost complete (80), see **figure 29a**.

Removing NO from the mitochondrion during ischemia may then facilitate continued electron transport and ATP production even when the bioavailability of oxygen is low. ATP is needed for phosphorylation of glucose to yield glucose-6-phosphate, in the glycogenesis and to reduce the lactate accumulation of lactate through the Cori cycle. So, NAC scavenges NO, and thereby some CcO activity is maintained, which prevents the accumulation acetyl CoA in the mitochondrion. In this way PDH activity is increased and pyruvate is allowed to flux

through the citric acid cycle (with what little oxygen is available), see **figure 29b**. In a mouse model, it could be established that NAC stimulated the activity of PDH (180). It is not impossible that NAC exerts a direct effect on the PDH activity, but it seems more likely that the activity of PDH is maintained when acetylCoA levels are low due to its metabolism in the citric acid cycle.

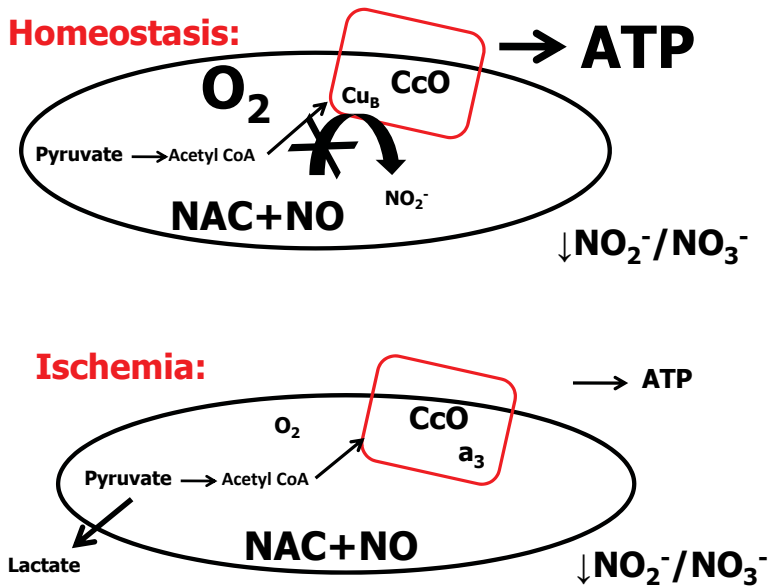


Figure 29. Simplified schematic illustration showing the effects of NO and NAC at the mitochondrial level. At normal oxygen tension NO is competing with O_2 at the Cu_B site of cytochrome c oxidase (CcO). During ischemia CcO is reduced and NO instead binds at the a_3 site where it inhibits CcO and hence blocks ATP production. During ischemia the inhibition of CcO causes acetyl CoA accumulation inhibits PDH, resulting in lactate production (**a**). When NAC scavenges NO there is less NO to be converted to nitrite and the nitrite concentration decreases. During ischemia the lower levels of NO diminishes the block of the a_3 site and therefore some ATP production can occur from the small amounts of available O_2 . CcO activity allows acetyl CoA to flux through the citric acid cycle resulting in less lactate production (**b**).

The clinical value of NAC is still debated (192), but the improvement of the hepatocyte energy metabolism has not been addressed in previous studies. Future studies involving NAC should incorporate variables of mitochondrial function and energy metabolism to justify its use in clinical practice.

One of our goals with the three experimental studies was to simplify the methodology of microdialysis to enable it to be transferred to the clinic. The RCT (**study IV**) reports some beneficial effects of IP before intermittent PM in patients who have had major resections, indicating that IP modulates glucose metabolism in livers in which the parenchymal mass is

significantly reduced. In analogy with the findings in **study III**, the lower levels of glucose lactate and pyruvate in the microdialysate can indicate a better mitochondrial function. ATP depletion during ischemia increases the adenosine levels (**figure 4**), which acts on the Adenosine-2 receptors. The activation of this receptor has been shown to improve the mitochondrial respiration and reduce the ATP depletion (114, 115). Hence, IP can reduce the lactate accumulation through improved mitochondrial function. In addition, IP causes a short burst of ROS and the positive effect of IP can be abolished if the oxidative stress of IP is inhibited by NAC (179). So, also the generation of ROS is important in regulating the cellular responses to IRI.

The results are of doubtful clinical value at this point as the study was underpowered with regard to clinical outcome parameters (10). The findings imply, however, that IP can improve the energy extraction from glucose and can thus be important in the postoperatively functionally marginalized liver. Importantly, **study IV** shows that microdialysis is simple and easy to use in the clinical setting.

Technical aspects of microdialysis

The insertions of the catheters (**studies I-IV**) were performed using split introducers, and could be done with minimal bleeding from the puncture site. The membranes were located centrally in the liver segment of interest, at least one cm below the capsule of Glisson. During the porcine experiments, there were some events of catheter dislocations, but the catheters could be reinserted with ease. Some catheters stopped functioning intermittently, but could always be restarted after temporary microflushing.

According to the protocol (**study IV**), the use of the μ D-catheters prolonged the operations by about 40-60 minutes, including the IP. Just placing a catheter and starting the sampling should take no more than 5 minutes. At the ward, sampling intervals were set at 4 hours to match the regular schedule of postoperative patient monitoring. We had no indications of any need to have shorter sampling intervals. Instead, the intervals could have been at least doubled and still provide high resolution monitoring of glucose metabolism. The IS-CUS analyser requires several μ l of the microdialysate to enable analysis. During the operation, we wanted to sample the microdialysate every half hour due to the short resolution of the ischemic event. Therefore the micropump flow velocity was higher (2.0 μ l/minute) during surgery and ICU than on the ward (0.3 μ l/minute) to ensure adequate volume recovery. When the patients were moved to the ward, the perfusion velocity was reduced, possibly enhancing recovery of metabolites from POD1 and onwards. This reduction was made at about the same time for all patients, and the possible changes in recovery therefore have no practical implication when the groups are compared. No catheter dislocations occurred and all catheters could be removed with a slight jerk and pull on POD 4 with no more than a slight discomfort for the patient. Two catheters stopped functioning on POD 3, possibly due to clotting of the semi-permeable membrane.

The findings in our μ D analyses have doubtful clinical implication at this point, but the study shows that postoperative sampling is feasible and the method can be used with ease by investigators and with little discomfort for patients. Academically, however, μ D has prom-

ising venues of postoperative metabolic surveillance in the clinical setting. For instances, pharmacologic interventions can be studied almost in real-time at the parenchymal level in postoperative patients. Studying the target organ without the systemic dilution seen in venous blood can for example help establishing optimal therapeutic concentrations of drugs.

Further, the use of microdialysis together with segmental ischemia challenges the concept of organ harvesting and laboratory animal sacrifices. From the findings in this thesis it is valid to use the perfused segment as a control, at least when hepatic metabolism is to be investigated. Both measures reduced the number of laboratory animals needed in our studies, but this approach must of course be validated if other experimental models are to be developed. In **study III**, for example, it would have been necessary with another group of sham operated animals if segmental ischemia had not been employed. If organ harvesting had been chosen instead of microdialysis, at least four times as many animals would have been sacrificed. The pigs could sustain the biopsies better than small animals, but there were some blood loss (and possibly also an inflammatory reaction) for each biopsy taken. Biopsies are not ideal in smaller animals and usually organ harvesting is necessary in rodent models. Study III included 16 pigs instead of (3 (groups) x 8 x 4) 96 smaller animals, had a sham group and organ harvesting been used.

In summary, the coherency seen in all the studies of this thesis indicates that the microdialysis technique is reliable for studying the hepatic glucose metabolism *in vivo*. The energy, and in particular, the glucose metabolism are important aspects of hepatic IRI. With bed-side analyzing equipment it is possible to have the results in near real-time, making bed-side monitoring possible. Microdialysis offers liver surgeons a new tool for understanding postoperative hepatic energy metabolism.

CONCLUSIONS

The main conclusions of this thesis are:

- Microdialysis can be used with ease to study glucose metabolism in the liver *in vivo*, both experimentally and clinically.
- The glucose metabolism in a perfused part of the liver is unaffected by ischemia in an adjacent ischemic segment, and can be held as a control in animal studies.
- The urea microdialysis clearance technique is a reliable method for assessing the perfusion of the liver.
- N-Acetylcysteine improves glycogenesis and decreases lactate levels in the liver after ischemia and reperfusion injury.
- Ischemic preconditioning decreases the postoperative levels of lactate in the liver after major liver resections performed with intermittent Pringles maneuver.
- During the reperfusion, both the perfused and ischemic parts of the liver are infiltrated by PMN leukocytes.

FUTURE PERSPECTIVES

The feasibility of clinical hepatic microdialysis makes it an interesting tool to observe temporal changes postoperatively.

After major liver resections some metabolic events trigger hepatic regeneration. The whole regenerative process as a whole is largely unknown, but microdialysis makes it possible to follow this process postoperatively. Interventions aiming at improving the regeneration can be studied. Moreover, with the microdialysis catheters in position it is possible to add a pharmacologic agent in the perfusate. The effects of the drug can then be observed on the parenchymal level rather than in the blood only. A receptor agonist or antagonist can be added in the perfusate and then the dialysate can be analyzed for an effector of this receptor activation/inactivation. This way, interactions at the molecular level can be investigated *in vivo* instead of *in vitro*, as previously has been the case.

Microdialysis has proven its value when glucose metabolism is to be studied, but temporal profiles of other small molecules, like amino acids or peptides, have been described previously. As long as the relative recovery is high, many aspects of hepatic metabolism can be investigated. To develop the microdialysis technique it is important to look for new applications and find new substances to analyze. For instance, the average size of the gene regulatory microRNAs is about 22 base pairs which correspond to about 5-15 kDa. Interventions directed at the microRNA regulation of gene expression could thus be studied *in vivo* over several days. The clinical importance of this can only be imagined.

SUMMARY IN SWEDISH

Bakgrund: Tack vare nya cellgifter och en förbättrad operationsteknik kan allt fler patienter opereras och botas från metastaser i levern. Fortfarande finns dock en avsevärd blödningsrisk vid ingrepp i levern. För att motverka blödning under operationen kan blodkärlen till levern tillfälligt stängas; så kallad Pringles manöver (PM). Om PM tillgrips, drabbas den del av levern som skall vara kvar efter operationen av syrebrist och återflödesskada (ischemi och reperfusions skada, IRI). Om patienten har en kronisk leversjukdom eller endast en mindre del av levern lämnas kvar, kan IRI medföra att levern aldrig tillväxer efter operationen. Patienten kan då dö av leversvikt. Flera tekniker har provats för att skydda levern från IRI. Bland annat kan man ge läkemedel som binder fria syre radikaler, vilka bildas vid reperfusionen eller så kan man utföra PM i korta intervaller. Det finns inga bra tekniker att studera IRI hos människa och det är föga känt hur leverns ämnesomsättning påverkas av IRI. Mikrodialys är en teknik som möjliggör kontinuerlig analys av vävnadsvätskan i levern.

Mål: Att undersöka effekterna av IRI på leverns glukos omsättning med hjälp av mikrodialys-teknik

Metod: En grismodell utvecklades där endast en del av leverns kärlförsörjning stängdes. Ämnesomsättningen och blodgenomströmningen i levern följdes under 4-6 timmar med hjälp av mikrodialyskatetrar i levern (studie I-III). I studie IV lottades hälften av 32 patienter att genomgå ischemiska prekonditionering (IP) i 10 minuter innan operationen i levern påbörjades. Samtliga patienter genomgick PM enligt protokoll med 15 minuter ischemi och 5 minuters reperfusion, vilket upprepades till tumören var bortopererad.

Resultat: Glukosets omsättning påverkades kraftigt i den del av levern som var ischemisk, medan den del som hade ordinarie genomblödning hade fortsatt normal ämnesomsättning. Urinämne (urea) tillsattes i mikrodialysvätskan och befanns vara en pålitlig markör för genomblödningen i levern. Antioxidanten N-acetylcystein (NAC) förbättrade glukosets omsättning i grisens lever under reperfusionen med bland annat minskade mjölksyrenivåer och bättre glykogeninlagring i levercellerna som följde. Sannolikt berodde detta på att NAC binder kväveoxid, vilket annars skulle blockera cellandningen i mitokondrierna. Även IP förbättrade glukosomsättningen med minskade laktatnivåer i levern hos de patienter som genomgått större leveroperationer.

Konklusion: Mikrodialys kan på ett tillförlitligt sätt mäta leverns glukosomsättning både i djurmodeller och hos patienter under och efter leveroperation. Både NAC och IP förbättrar glukosomsättningen, vilket skulle kunna vara avgörande för patienter med dålig leverfunktion efter leverkirurgi.

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