Linköping University Medical Dissertations No. 1418

Methods to Reduce Liver Ischemia/Reperfusion Injury

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Linköping 2014

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Printed in Sweden by LiU-Tryck, Linköping, Sweden, 2014

ISSN 0345-0082

ISBN 978-91-7519-245-1

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To:
Gunna, Hilda and Viktor

”Education is what survives when what has been learned has been forgotten”

Burrhus Frederic Skinner (1904-1990)

Cover photograph:

Snæfellsjökull, Iceland

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Abstract

Introduction: During the last two decades, liver surgery has expanded enormously, partly due to improved surgical equipment and techniques as well as new and more powerful chemotherapy agents. As the liver is a very well-vascularized organ, there is an inherent risk of bleeding during liver resection. One of the most popular methods employed to reduce this risk is to close the vascular inflow to the liver using the Pringle's maneuver (PM). However, this procedure has been recognized to cause ischemia/reperfusion injury (IRI) to the future liver remnant (FLR). In cases of extensive resection where the FLR is small and in cases when the liver suffers from chronic diseases, such as cirrhosis, IRI can greatly increase the risk of post-operative liver failure (POLF). Ischemic preconditioning (IPC) and, more recently, remote ischemic preconditioning (R-IPC) are methods that have been employed to reduce IRI.

Aim: 1) To compare the effects of IPC and R-IPC in a rat model; 2) to investigate the clinical effect of IPC during modern liver surgery; 3) to investigate the role of the nitric oxide (NO) system in IRI, IPC and R-IPC; and 4) to explore the possible protective effects of nitrite administration before IRI.

Methods: A rat model of segmental ischemia followed by 4 hours of reperfusion including microdialysis (μ D) was developed from earlier models. The effects of IPC and R-IPC were compared using transaminases and histology as well as continuous μ D sampling for glucose, pyruvate, lactate and glycerol. The role of the NO system was examined by serum and μ D measurements of NOx as well as tissue measurements of iNOS mRNA and IL-1R mRNA. In study II, patients were randomized to IPC or no IPC prior to liver resection, where intermittent PM was used to decrease bleeding.

Results: IPC was more effective in protecting the liver against IRI than R-IPC, as indicated by the levels of transaminases. Lower lactate levels were detected in patients treated with IPC before major liver resections than in controls. IPC reduced iNOS mRNA transcription during reperfusion; this result may be related to the early but not sustained increases in IL-1R transcription observed in the IPC group. Nitrite administered before ischemia reduced AST and ALT levels in the level after 4 hours of reperfusion; in addition, necrosis and glycerol release from the ischemic liver were reduced as well.

Conclusion: IPC is more effective than R-IPC in animal models; however, this effect is unlikely to be of clinical importance. NO_x decreases in the ischemic liver and the administration of nitrite before ischemia reduces IRI in rats. This may have clinical implications in the future.

Abbreviations

ALPPS = Associating liver partition and portal vein ligation for staged hepatectomy
ALT = Alanine aminotransferase
ANOVA = Analysis of variance
AST = Aspartate aminotransferase
ATP = Adenosine triphosphate
cGMP = Cyclic guanosine monophosphate
cPM = Continuous Pringle's maneuver
C-PTIO = 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide potassium salt
CRLM = Colorectal liver metastasis
CUSA = Cavitron Ultrasonic Surgical Aspirator
CVP = Central venous pressure
DAN = 2,3-diaminonaphthalene
ELISA = Enzyme-linked immunosorbent assay
eNOS = Endothelial nitric oxide synthase
FLR = Future liver remnant
GAPDH = Glyceraldehyde-3-phosphate-dehydrogenase
HCC = Hepatocellular carcinoma
HO-1 = Heme oxygenase 1
ICG-15 = 15 minute indocyanine green clearance test
ICAM = Intercellular adhesion molecule
ICG = Indocyanine green
IFN- γ = Interferon γ
IL = Interleukin
IL-1R = Interleukin 1 receptor
iNOS = Inducible nitric oxide synthase
INR = International normalized ratio
IPC = Ischemic preconditioning
iPM = intermittent Pringle's maneuver
IR = Ischemia/reperfusion
IRI = Ischemia/reperfusion Injury

L-NAME = N^Gnitro-L-arginine methyl ester

MAC-1 = Macrophage 1 antigen

μD = Microdialysis

NADPH = Nicotinamide adenine dinucleotide phosphate

NF-κB = Nuclear factor κB

nNOS = Neuronal nitric oxide synthase

NO = Nitric oxide

NO_x = The sum of nitrite and nitrate

ODQ = 1H-(1,2,3)oxadiazole(4,3-a)quinoxalin-1-one

PM = Pringle's maneuver

PMN = Polymorphonuclear neutrophil

POLF = Post-operative liver failure

PVE = Portal vein embolization

R-IPC = Remote ischemic preconditioning

RNS = Reactive nitrogen species

ROS = Reactive oxygen species

SD = Standard deviation

SEM = Standard error of the mean

sGC = Soluble guanylyl cyclase

SHVE = Selective hepatic vascular exclusion

THVE = Total hepatic vascular exclusion

TLR4 = Toll-like receptor 4

TNF-α = Tumor necrosis factor α

WBC = White blood cells

List of original papers

The thesis is based on the following original articles, which are referenced in-text by the respective roman numerals:

- I. **B. Björnsson**, A. Winbladh, L. Bojmar, L. Trulsson, H. Olsson, T. Sundqvist, P. Gullstrand, P. Sandström.
Remote or Conventional Ischemic Preconditioning - Local Liver Metabolism in Rats Studied with Microdialysis
Journal of surgical research 2012;176(1):55-62

- II. A. Winbladh, **B. Björnsson**, L. Trulsson, K. Offenbartl, P. Gullstrand, P. Sandström
Ischemic preconditioning prior to intermittent Pringle maneuver in liver resections
Journal of Hepatobiliary Pancreatic Sci 2012;19(2):159-170

- III. **B. Björnsson**, A. Winbladh, L. Bojmar, T. Sundqvist, P. Gullstrand, P. Sandström
Conventional, but not remote ischemic preconditioning, reduces iNOS transcription in liver ischemia/reperfusion
World J Gastroenterol 2014; 20(28):9506-9512

- IV. **B. Björnsson**, L. Bojmar, H. Olsson, T. Sundqvist, P. Sandström
Nitrite, a novel method to decrease Ischemia/Reperfusion Injury in the rat liver
Provisionally accepted for publication in World J Gastroenterol

1. History of liver surgery for neoplasia

Homer wrote in *The Iliad*:

“Achilles stabbed with his sword at the liver, the liver was torn from its place, and from it the dark blood drenched the fold of his tunic and Troy’s eyes were shrouded in darkness, and the light went out” (1).

This description from approximately 750 B.C. may be one of the earliest descriptions of liver trauma and certainly addresses one of the main risks of liver surgery, bleeding. The words attributed to Sir William Osler, *“if there wasn’t bleeding everybody would do surgery”*, are particularly applicable to liver surgery.

Before the introduction of the endotracheal tube in 1878 by William Macewen, elective abdominal surgery was almost unknown (2). The liver was only operated on in cases of trauma, and those operations were mostly or exclusively performed through wounds already present from the trauma. The first documented successful liver operation for trauma was performed by Wilhelm Fabry (Fabricius Hildanus), known as “the father of German surgery”, in the 17th century and consisted of removal of a portion of the liver present in a trauma wound (3).

Despite Lord Thurlow’s statement at the parliamentary debate on the establishment of Royal College of Surgeons in 1811, *“There is no more science in surgery than in butchering”*, some documentation can be found concerning the early days (until the mid-20th century) of planned liver surgery. In 1873, the British surgeon Sir John Eric Erichsen announced: *“The abdomen, the chest, and the brain will be forever shut from the intrusion of the wise and humane surgeon.”* (4). Despite Dr. Erichsen’s pessimism, the first documented planned hepatectomy was performed in 1886 by Dr. A Lius in Italy. The result was a deadly complication that later served as the cornerstone of liver surgery development: the patient bled to death 6 hours after surgery (5, 6). However, the die was rolled, and in 1888, the German surgeon Carl Johann August Langenbuch, who performed the first cholecystectomy in 1882, performed the first successful liver resection for a tumor. A portion of the left liver lobe was resected after first ligating the vascular pedicles (7). Although the patient survived, a reoperation for bleeding was required; unfortunately, the pathological examination revealed no malignancy. Concurrently, in the USA, liver surgery was also evolving, and in 1890, Louis McLane Tiffany, a professor of surgery at the University of Maryland in Baltimore,

performed the first liver resection for a tumor at Johns Hopkins Hospital (8). This achievement was closely followed by the first reported liver resection for a malignant tumor by another German surgeon, Lucke, in 1891 (9).

Sir James Cantlie published a significant contribution to the understanding of functional liver anatomy in 1897 by describing the true line separating the right and left liver lobes; however, this discovery was only first applied to clinical use a few decades later (10). In 1899, when Keen reported the first anatomical left lateral segmentectomy (segments II and III), he also updated his previous two reviews and noted that a total of 76 liver resections had been reported with a mortality rate of only 14.9% (11). Whether this represents publication bias or true progress in the field remains unclear, although Dr. Keen addresses over 20 cases that he excludes from the analysis as not being liver resections.

In 1903, Dr. W Anschutz described the technique called the “finger fracture”, which was later made popular by Lin (12, 13). Seven years earlier (1896), a technique based on the same principles, wherein sutures were passed through the liver tissue to create pressure before dividing the parenchyma, was described by Kousnetzoff and Pensky (14). The finger fracture technique was later further improved by Lin through the use of clamps along with the fingers (15).

In 1908, trauma surgeons made a substantial contribution addressing bleeding. While working at the Glasgow Royal Infirmary in 1908, Dr. James Hogarth Pringle described a novel method to minimize bleeding resulting from liver trauma; he developed his method using an animal model and tested it on two patients (16). Although neither of the patients survived, Pringle’s maneuver (PM), which involves temporary closure of the hepatoduodenal ligament along with the finger fracture, is likely the oldest technique in liver surgery that remains in use. This method of bleeding control was previously proposed by Clementi in 1890 (17).

The first major hepatectomy was performed by Wendel in 1911 and in 1920. While the patient was still alive, Wendel published the case of hepatocellular carcinoma (HCC) (18). In the Western world, liver surgery for malignant diseases is dominated by the resection of metastases, primarily from colorectal carcinoma (19, 20). The first reported hepatic metastasectomy for colorectal carcinoma was performed at the Lahey Clinic in Burlington, Massachusetts in 1940 by Dr. Richard B. Cattell (21). The first well-defined formal right hepatectomy was performed at the Beaujon Hospital in Paris by JL Lortat-Jacob in 1952 (22-24). This surgery marked the beginning of French involvement in liver surgery, which

increased during the latter half of the 20th century both in the fields of anatomy and nomenclature as well as in the introduction of two-stage liver resections (25-27). A year later, in 1953, Julian K. Quattlebaum, working in Savannah, Georgia, USA, reported his series of three major hepatectomies, placing the USA on the map of anatomical liver surgery (28).

The French surgeon and anatomist Claude Couinaud published the book *Le foie; etudes anatomiques et chirurgicales* (The liver: Anatomical studies and surgical studies) in 1957. This 530-page book describes in detail the segmental anatomy of the liver and is the foundation of the nomenclature used in Europe and Japan within liver surgery (26).

A review of the history of liver surgery cannot exclude liver transplantation. The first liver transplantation was performed in Denver, Colorado, USA in 1963 by a team led by Dr. Thomas Starzl (29). The first one-year survival was achieved in 1968 (for an operation that occurred in 1967). With the introduction of cyclosporin, liver transplantation became a viable clinical option in the 1980s. This step opened the door for more complex resections of the liver, ranging from in situ in vivo (with vascular exclusion of the organ and hypothermia by perfusion) as described by Fortner (Memorial Sloan-Kettering) in 1974 to ex situ ex vivo (where the liver is temporary removed from the body and operated on a back table) as performed by Pichlmayr (Hannover) in 1990 (30, 31).

One of the more significant advances in the diagnosis of liver neoplasia was the introduction of the ultrasound technique in the 1970s (32). Ultrasound spread fast in clinical practice, and its use during operations was introduced in Japan in 1983 and in France in 1984 (33, 34).

In his work published in 1987, Sir James Cantlie's stated:

"I believe that if, in the hands of future observers, the statements I have made receive closer investigation, the surgery of the liver will be advanced a step" (10).

His anatomical observations involving the hypertrophy of one liver lobe, the atrophy of the other, and the separate portal systems of the right and left liver lobes set the scene for the advance arriving from Japan in 1984 when Makuuchi described the effects of portal venous embolization (PVE) in human clinical settings (35).

Other more recent advances in liver surgery include the ligation of the portal vein during liver resections in one lobe followed by later hemi-hepatectomy and the application of laparoscopy to liver resections (25, 36). Finally, a new concept that combines liver

parenchymal transection with portal venous ligation, associating liver partition and portal vein ligation for staged hepatectomy (ALPPS), has emerged and has been demonstrated to stimulate rapid hypertrophy of the future liver remnant (FLR) (37).

2. Surgery for colorectal liver metastasis (CRLM)

Malignant tumors in the liver can be classified as primary malignancies (mainly hepatocellular carcinoma, cholangiocarcinoma and gallbladder carcinoma) and secondary malignancies (metastases). Metastases from cancers of the colon and rectum are historically most relevant to liver surgery, but during recent years, metastases from other solid have been recognized as an indication for liver surgery (38). Currently, surgery is the only treatment that offers a reasonable chance of cure for malignant liver tumors.

In Sweden, CRLM is by far the most common indication for liver resection. Over 14 years, the number of liver resections in Sweden has evolved from approximately 150 to approximately 800 (figure 6). A similar trend is noted in other countries (39-41).

Figure 1. Number of liver resections (JJBxx) in Sweden in 1998-2012. The number of liver resections performed in Sweden each year has increased approximately 500% over a 14-year period. Data from the Swedish National Inpatient Register (www.socialstyrelsen.se).

Several explanations for this dramatic increase are available. Before the introduction of active chemotherapy and surgery for CRLM, the prognosis of patients with the disease was dismal, with a 3-year survival rate below 5% (42). The high operative risk in the 1970s and early 1980s likely contributed to the strict criteria for metastatic liver surgery (43-45).

In addition, the high risk of relapse after surgery also contributed to the reluctance to perform major liver surgery (45). With the progress of the oncological treatment, the number of patients converted from a disease state not amenable for surgery to a state possible to treat with surgery has increased. Furthermore, the risk for relapse has also been reduced with better patient selection (46-48). In addition, technical advances in both surgery and anesthesiology have increased the safety of liver surgery (49).

Today, the perioperative mortality in liver surgery is as low as 1%, although the criteria for surgical resections have expanded substantially (50). In addition, the 5-year survival of patients treated surgically and oncologically for CRLM has increased to 55% in large centers (51).

3. Liver anatomy and nomenclature

The liver is the largest solid organ of the human body. A normal adult human liver weighs approximately 2-3% of the total body weight and lies beneath the diaphragm in the upper portion of the abdomen (52).

The liver possesses ligaments that attach to its surroundings (figure 2). In the midline, the teres ligament contains the remnants of the umbilical vein and continues as the falciform ligament, which attaches between segments IVa and IVb and segments II and III, respectively. Further back along the superior midline, the falciform ligament spreads out and becomes the coronary ligament (figure 2). On the right side, the coronary ligament continues to the bare area of the liver (area nuda hepatis), and lateral to the bare area the right triangular ligament is found (figure 3). On the left side, the coronary ligament continues towards the left triangular ligament laterally.

Figure 2. *Surface anatomy of the liver, anterior view.*

The inferior caval vein runs behind the liver (in a groove or sulcus), and the liver veins join the inferior caval vein before the vein enters the thoracic cavity through the diaphragm. The structures of the hepatoduodenal ligament (the portal vein, the common hepatic duct and the liver arteries) run into the liver hilum.

Figure 3. *Surface anatomy of the liver, inferior (visceral) view.*

On the liver surface, there are few landmarks that can reveal the liver's internal anatomy. Among these landmarks is the Rouviere's sulcus, named after Henri Rouviere, a professor of anatomy and embryology at the University of Paris (53). This cleft in the liver tissue runs to the right of the liver hilum and corresponds to the plane of the right portal pedicle within the liver (figure 3). Another landmark (that typically cannot be seen) on the liver surface is Cantlie's line, which runs from the fundus of the gallbladder and upwards to the center of the caval vein (10). This line (or plane as it follows the caval vein posteriorly) divides the liver anatomically into the right and left lobe (figure 4). Another important landmark on the liver surface is the attachment of the falciform ligament and the fissure to create the "ligamentum venosum" that runs on the inferior surface of the liver. Corresponding to this fissure, the portal pedicle to the anatomical left liver lobe can be found inside the liver.

Despite early studies on internal liver anatomy and the distribution of bile ducts, no accepted classifications systems used to describe the liver were formalized in the first half of the 20th century (54, 55). The most widely used systems, dating back to the 1950s, are those of Couinaud, with modifications by Bismuth; Healey and Schroy; and Goldsmith and Woodburne, referred to as the Anglo-Saxon system (26, 27, 56, 57). According to Couinaud, the liver is divided into 8 segments based on the third-order distribution of the portal vein branches. Goldsmith and Woodburne suggested a similar division into 4 segments (each with 2 sub-segments) based on the second-order bile duct and liver artery branches (figure 4, table 1).

Both classification systems utilize the distribution of hepatic veins. Each segment has its own inflow, both arterial and portal, as well as bile drainage (figure 4). The three hepatic veins drain most of the blood to the inferior caval vein. The right hepatic vein runs between segments VI and VII and segments V and VIII, respectively; the middle hepatic vein runs between segments V and VIII and segments IVa and IVb, respectively; and the left hepatic vein runs between segments IVa and IVb and segments II and III, respectively (figure 4). In addition, a variable number of short liver veins drain directly into the inferior caval vein. Segment 1 (caudate lobe) is drained by these short veins.

Figure 4. Internal anatomy of the liver. Roman numerals indicate segments.

Part	Healey and Schroy			Goldsmith and Woodburne			Couinaud		Bismuth	
	Segment	Subsegment	Segment	Subsegment	Sector	Segment	Sector	Segment		
Dorsal	Caudate	Right	Caudate lobe		Caudate lobe	I	Caudate	Posterior, I		
		Left								
Left	Lateral	Superior	Lateral	Superior	Lateral	II	Posterior	II		
		Inferior		Inferior					III	
	Medial	Superior	Medial	Superior	Paramedian	IV	Anterior	IVa, IVb		
		Inferior		Inferior						
Right	Anterior	Inferior	Anterior	Inferior	Paramedian	V	Anteromedial	Anterior, V		
		Superior		Superior					VIII	Posterior, VIII
	Posterior	Inferior	Posterior	Inferior	Lateral	VI	Posteromedial	Anterior, VI		
		Superior		Superior					VII	Posterior VII

Table 1. Comparison of the classification systems used to describe the internal anatomy of the liver. The system of Healey and Schroy is almost identical to that of Goldsmith and Woodburne, and the system described by Bismuth is similar to the one described by Couinaud.

Although the basic division of the liver into segments is generally agreed upon, the nomenclature used to describe various types of anatomical liver resections is not uniform (58). Two systems are the most widely used: in Europe and Japan, resections are primarily described according to Couinaud's system, and in the USA, the Anglo-Saxon system is used. The use of different systems to describe surgical procedures may lead to confusion and misinterpretation. At a meeting in Brisbane in 2000, the leading liver surgeons of Europe and the USA attempted to synchronize the nomenclature to avoid confusion. This is particularly important, as some of the names involved sound very similar (section and sector). This unification resulted in a system based on the course of the hepatic artery and the bile ducts that at least provides a translation for those terms. Although some confusion remains, the Brisbane 2000 terminology is gaining acceptance among liver surgeons (59-61). The terminology divides the liver into right and left (hemi-liver, first order). The right hemi-liver is divided into anterior and posterior sections, whereas the left hemi-liver is divided into medial and lateral sections. Each section consists of 2 segments, wherein segments II and III comprise the lateral left section, segments IVa and IVb comprise the medial left section, segments V and VIII comprise the anterior right section, and segments VI and VII comprise the posterior right section. This division forms the basis for the description of resections.

4. Techniques of anatomical liver resections

The article by Professor Henri Bismuth, “Surgical anatomy and anatomical surgery of the liver”, described the relevance of earlier anatomical studies of the liver. The internal anatomy of the liver was described from a surgical standpoint, including how individual segments or a group of segments (2 or more) can be resected without significantly interfering with vascular structures (27).

During the century since the beginning of liver surgery, much has changed in liver transection techniques. Despite these changes, the “finger fracture” technique with some modifications (mainly the use of clamps instead of fingers, referred to as the Kelly clamp crushing technique or Kelly-clasia) remains widely used (62). The Cavitron Ultrasonic Surgical Aspiration device (CUSA, Tycho Healthcare, Mansfield, MA) is another widely used method.

The CUSA is an ultrasound generator that also incorporates a suction device. The CUSA was first reported in the context of liver surgery in 1980 and has since gained considerable popularity (63). The vibrations at the tip of the CUSA generated by the ultrasound destroy the liver cells, leaving vessels and bile ducts intact. The destroyed cells are sucked up simultaneously, and the vessels and bile ducts are closed by the same methods used for these structures during the crush technique (suture, ligature, clips and diathermia). Numerous reports have been written comparing methods for liver resection with diverging results (64).

Figure 5. *Cavitron Ultrasonic Surgical Aspirator (CUSA).*

Unfortunately, none of these studies included a sufficient number of patients to draw firm conclusions regarding the superior technique (62, 64-67). In one study, the crushing method was accompanied by pedicle clamping, whereas the other methods (CUSA, radiofrequency, water jet) were not, making comparisons even more complicated (66).

In addition, there has been an explosion of other methods. These methods include the Ultracision harmonic scalpel (Ethicon), LigaSure (ValleyLab), TissueLink (Dover, NH), Habib sealer and other radiofrequency techniques, such as Cool-tip (Raduibucs, Tyco Healthcare), Hydro-Jet (Hydro-Jet, Erbe, Tubingen) and various stapling instruments. Although none of these methods has gained as much popularity as the CUSA and the crushing technique, all are used to some degree (62).

The ultrasonic scalpel (Ultracision Harmonic Scalpel, Ethicon Endo-Surgery) was introduced in the early 1990s. The ultrasonic technology is used to cut tissues and simultaneously seal the cut edge. The technique has gained increasing popularity with the introduction of laparoscopic liver surgery, but the advantage of this transection technique remains unclear (68, 69).

LigaSure was first reported in liver surgery in 2001 (70). LigaSures are bipolar diathermy forceps that claims to effectively seal vessels up to 7 mm. A comparison with other methods of transection provided diverging results (71, 72). However, this device has become increasingly popular with the increase in laparoscopic liver surgery (73).

TissueLink, Habib sealer and Cool-tip use radiofrequency energy to generate coagulation. The electrodes are inserted in the transection plane serially, and energy is applied to create coagulation. Subsequently, the tissue can be divided. Although it is claimed to be highly effective in achieving hemostasis, this result has not been proven (74-77).

Hydro-jet is a water propulsion dissector that uses a water jet to fragment the liver parenchyma and expose vessels and bile ducts. This method reduced bleeding compared with CUSA and the crush technique in a retrospective non-randomized study, but these findings were not confirmed in a prospective randomized trial (66, 78).

Regardless of what instrument is used to divide the liver parenchyma, anatomic liver resections are those in which the parenchymal division follows the functional anatomical lines of the liver. For hemi-hepatectomies (removal of the right or left hemi-liver), the vascular inflow to the part of the liver to be removed can be divided before parenchymal transection. The division of the portal pedicle can be achieved in an extra-hepatic manner, where the

corresponding hepatic artery and portal vein are isolated and ligated in a method described by Lortat-Jacob (22). Another approach for liver resection is intrahepatic ligation (Glissonian approach); for right hemi-hepatectomy, an opening is created in the liver capsule (Glisson's capsule) in the gallbladder fossa (segments IVb/V), and another opening is made to the right and posterior to the right portal branch. A vascular clamp is passed between the openings, and upon closure, the demarcation confirms its placement around the right portal pedicle. Subsequently, a stapling device is used to seal and divide the right portal pedicle. Another variant of the Glissonian approach involves dissecting the hilar plate and identifying the right anterior and the right posterior portal pedicles that then can be ligated and divided separately close to the liver. Damage to structures supplying the left hemi-liver can be avoided by following this procedure.

For left hemi-hepatectomy, the corresponding procedure involves opening the liver capsule at the umbilical fissure above the hilar plate and on the posterior aspect of segment II. Passing a vascular clamp between the openings will isolate the left portal pedicle, which can then be divided. Regardless of the method used to close the inflow to the portion of the liver to be resected, a demarcation appears along the line of anatomical division between the right and the left hemi-liver. Dividing the liver at this line will result in an anatomically correct hemi-hepatectomy.

In a previous study, the Glissonian approach did not result in more complications than the classic extra-hepatic approach in the settings of right hemi-hepatectomy (79). Although that study included a substantial number of patients, the study was not randomized. In addition, a substantial patient selection towards smaller tumors not close to the liver hilum was noted in the group with intraparenchymal division of vessels. The PM length was also significantly longer in that group. Retrospective studies have demonstrated that patients with intrahepatic division exhibit significantly reduced blood loss, fewer complications and less mortality as well as an increased frequency of R0 resections (resections where the histological examination reveals that the whole tumor has been removed) (80, 81). However, in these studies, significantly more liver resections (wedge and further segmentectomies) were performed in patients with extra-hepatic vessel control, and extended hepatectomies also frequently occurred in this patient group. Therefore, it might be prudent to adapt the method to the proximity of the tumor to the hilum given that radical removal of the tumors is of the utmost importance.

When individual segments or sections (2 adjacent segments) are to be removed, the inflow is located within the liver tissue; thus, the parenchyma must be divided somewhat before the inflow to the part to be resected is selectively divided. On the left side, the portal pedicle is identified in the fissure of the ligamentum venosum, and the branches to segments IVb and III can often be reached without significant parenchymal dissection, whereas the branches to segments II and IVa are observed in a more cephalic direction, necessitating further parenchymal dissection before these branches can be approached. When dealing with the medial section of the left hemi-liver (segments IVa and IVb), it should also be kept in mind that variable contributions from the right hemi-liver can exist.

The branches to individual sections (anterior and posterior) and segments in the right hemi-liver can typically be reached through dissection of the liver hilum (lowering of the hilar plate) and can thus be divided before parenchymal transection.

When liver resections are performed, another alternative is the so-called atypical liver resection. The main difference between anatomical and atypical resection is that the latter does not follow the anatomical lines of the liver. Atypical resection makes it possible to spare the liver parenchyma that is not affected by the tumor. The goal of atypical resection is to resect all tumors with sufficient margin of tumor-free tissue and to leave as much liver parenchyma as possible. Specific vascular inflow control is not achieved before parenchymal dissection, but the vessels and bile ducts are closed during the division of the parenchyma. Methods of vascular occlusion may be applied in a manner similar to those employed for anatomical resections. The necessary margin has been a matter of research; although a wide margin (> 10 mm), as recommended in the early days of modern liver surgery, may remain desirable, later studies have reported that even resections with smaller margins are sufficient in patients with colorectal liver metastasis (CRLM) (82-85). In the setting of HCC, greater surgical margins are proposed, as these tumors spread within the liver and as micrometastases that are typically present within 2 cm of the primary tumor are common (86). Altogether, 2-cm surgical margins should be pursued at a minimum, and theoretically, anatomical resection involving the affected segment may be superior to atypical resections (86). This suggestion has indeed been demonstrated in a randomized controlled trial comparing 1-cm and 2-cm surgical margins (87).

Anatomical and atypical resections for CRLM have been compared, and the results have varied between better results for anatomical resection and no difference between the two approaches (88, 89). The liver surgeon should be able to choose the type of resection that

offers the best overall results for the patient. This resection may indeed often necessitate the application of both anatomical and atypical resections.

5. Methods to control bleeding during liver surgery

In the early days of modern liver surgery, the perioperative mortality of liver resections ranged from 10 to 25% (43). A significant proportion of this mortality could be attributed to the inherent risk of bleeding. Currently, the perioperative mortality is less than 4% for liver resections performed for primary hepatobiliary disease and approximately 1% for other resections performed in centers performing more than 15 resections per year (50, 90). This reduction, despite more complex operations in older patients with more advanced diseases, can be largely attributed to a more systemic approach to the subject of hemostasis (90, 91). Apart from the obvious risk of exsanguination and death during surgery, transfusions have been related to increased morbidity, mortality and length of hospital stay in a dose-dependent manner (92).

The methods to decrease bleeding during liver surgery can be categorized as surgical methods and anesthesiological methods (or non-surgical methods).

As with all other wounds, much of the bleeding from the liver can be temporarily stopped or at least decreased by direct pressure and thereafter treated with sutures or other general methods of hemostasis. However, this approach is occasionally inadequate to control bleeding from larger vessels within the liver.

The first method specifically developed for liver surgery was the method described by Pringle (16). PM includes the isolation of the hepatoduodenal ligament, which is achieved by entering the foramen of Winslow (behind and to the right of the ligament) and the lesser omentum (on the left side of the ligament), thus giving the surgeon access to the ligament from all sides. The second step is to apply pressure to the ligament (either with a vascular clamp or a band), thus occluding the common hepatic artery, the portal vein and the common bile duct. Although this approach occludes both parts of the double circulation to the liver, it does not greatly affect bleeding from the liver veins.

Figure 6. *Pringle's maneuver. A cotton band or vascular clamp is placed around the hepatoduodenal ligament to close the inflow (hepatic artery and portal vein) to the liver.*

Total hepatic vascular exclusion (THVE) is another surgical method for reducing bleeding that is unique to liver surgery. In addition to the isolation of the hepatoduodenal ligament, the caval vein is isolated both inferiorly and superiorly to the liver. The accessory arterial supply to the left liver lobe from the left gastric artery is isolated. The inferior caval vein is clamped followed by clamping of the hepatoduodenal ligament and the accessory left liver artery; finally, the inferior caval vein is clamped superior to the liver. The clamping of the inferior caval vein inferior to the liver veins is either performed above the right adrenal vein, or the right adrenal vein is ligated. When applied for prolonged periods, hypothermic perfusion may decrease the adverse effects on the liver (93). This method has been compared

with PM, and both techniques reduce bleeding compared with no vascular occlusion. THVE, however, carries a considerably increased risk for hemodynamic instability, and its routine use is not advocated (94-96).

Selective hepatic vascular exclusion (SHVE) is another technique that has the advantages of total hepatic exclusion without the hemodynamic consequences. Instead of clamping the caval vein inferiorly and superiorly to the liver, the liver veins are clamped selectively (97). Thus, the flow in the inferior caval vein is not disrupted, and the risk of hemodynamic instability is reduced (98). With regard to reducing blood loss, SHVE is equally effective as THVE and is more effective than PM (98, 99). This method resembles a technique widely used for hemi-hepatectomies in which both the vascular inflow and the involved liver vein are divided before parenchymal transection (see previous chapter); however, SHVE also involves occlusion of the other liver vein(s) and the entire hepatoduodenal ligament. This approach may be indicated when tumors are located in close proximity to the liver veins as well as in patients with raised central venous pressure (CVP).

In addition to the surgical methods, various pharmacological agents can be used to achieve hemostasis. However, these aspects of liver surgery are beyond the scope of this book. In addition, some of the methods used to divide the liver parenchyma have claimed to reduce bleeding (see previous chapter).

The main anesthesiological method to reduce bleeding during liver resections is low CVP anesthesia, which is targeted towards bleeding from the liver veins. The rationale behind this approach is that the liver veins drain directly into the caval vein, and thus, the pressure in the liver veins is roughly the same as in the caval vein. This concept became popular in the 1990s, and early reports suggested significantly reduced bleeding with low CVP anesthesia compared with anesthesia without specific measures to reduce the CVP (49). To achieve the goal of low CVP, between 2 and 5 mm Hg fluid restriction is applied before and during the surgery (until the transection of the liver is complete). In cases where this approach alone is insufficient, intravenous nitroglycerine is used to further reduce the CVP. A central venous line is necessary to monitor the patient. This approach minimizes the distention of hepatic veins and sinusoids, thus reducing “back bleeding”. Towards the end of the 1990s, this strategy was shown to be effective in non-randomized studies (100, 101). Later, small randomized studies summarized in a meta-analysis confirmed this finding (102). This approach is expected to reduce bleeding by up to 50% (49, 102).

No treatment is without risks or side effects, and low CVP, the “controlled hypovolemic state”, is no exception. This hypovolemia can cause inadequate perfusion, and in the event of sudden profuse bleeding, the volume reserve can be minimal. The kidneys are the organ system that is most likely to be affected and the most accessible. However, in one of the early studies, the incidence of renal failure was not increased with low CVP anesthesia (101).

THVE (see above) carries some anesthesiological implications given that it typically cannot be applied to a patient with (controlled) hypovolemia. For THVE, the CVP generally must be in the upper range, typically above 15 mmHg. This CVP allows the clamping of the inferior caval vein, which can decrease venous return and cause a sudden decrease in cardiac output as well as increased afterload. By maintaining a high CVP, clamping can be performed without jeopardizing adequate blood pressure and circulation in most cases. In some cases, vasoactive agents are needed in combination with the volume load to maintain the perfusion, and it should be kept in mind that if adequate volume load and the use of vasoactive agents fail to provide acceptable perfusion, veno-venous bypass may be required.

Autologous blood donation, hemodilution and hypoventilation are other non-surgical methods for reducing blood loss in liver surgery. However, none of these methods are regularly use in the clinic; therefore, these techniques are not included in this review.

6. Obstacles in liver surgery

Given that numerous obstacles, such as general anesthesia, the understanding of liver anatomy, the identification of methods for transecting liver tissue and bleeding control during liver surgery, have been solved, one might imagine that all obstacles to liver surgery had been removed. Unfortunately, further work is needed. In the 1980s and early 1990s, the criteria for resecting metastases from colorectal cancer in the liver included metachronous detection of the liver disease, no more than three metastases restricted to one of the liver lobes, no metastases greater than 5 cm, a possible resection margin of 1 cm and no signs of spreading outside the liver (103). These criteria were largely due to the prognosis of the malignant disease but also to the operative risk associated with more extensive operations.

With the implementation of modern chemotherapy, the prognosis for CRLM improved, and the indications for liver resections have broadened ever since. Today, extra-hepatic disease is not an absolute contraindication to liver surgery as long as the disease is treatable (104). The number and the distribution of liver lesions are not seen as a contraindication *per se*, but it is generally agreed that liver tissue corresponding to 20% of the liver must remain intact and circulated, provided that the patient has not received chemotherapy (105). This is one of the obstacles in liver surgery not likely to be overcome in the near future given that liver failure will result from more extensive resections.

Another challenge is that patients who initially possess unresectable liver metastases exhibit approximately the same prognosis as those who present with resectable disease if unresectable patients are successfully treated with chemotherapy before surgery and the disease is “down-sized” to a resectable situation (47). This challenge leads to a growing population of patients scheduled for liver surgery who have been heavily treated with chemotherapy before operation, which may decrease the quality of the FLR and increase the risk for complications (106). For patients undergoing operations after chemotherapy, a FLR of 30% has been suggested as a threshold due to the reduced functional reserve of the liver (107). In the setting of liver fibrosis or cirrhosis, which is common in HCC and may be present in CRLM as well, a 40% margin has been proposed (107).

To further complicate this situation, the operations need to achieve R0 or at least R1 status, are often technically demanding and might increase the usage of PM, thereby subjecting the FLR to IRI. In addition, the population of patients to be treated with

chemotherapy and liver surgery is growing older. Although chronological age *per se* is not considered a contraindication for surgery, the physiological reserve of the elderly with stage IV cancer (cancer with distant metastases) can be expected to be further reduced (91).

Operations for recurrent liver metastases from colorectal cancer exhibit a similar survival rate as first-time operations (108, 109). However, this approach will increase the intraoperative bleeding and will be demanding, both for the surgical team as well as the patient (108-110). Furthermore, additional obstacles might develop in a special situation wherein a two-stage operation is planned after initially unresectable metastases have responded to chemotherapy.

7. Ischemia/reperfusion injury (IRI)

Given that the surgical methods used to reduce bleeding in liver surgery potentially involve closure of the circulation to the liver, some degree of ischemia cannot be avoided. When the circulation is restored, reperfusion will occur. This combination and its consequences have been referred to as ischemia/reperfusion injury (IRI). Although concerns about hepatic inflow stasis were raised in the literature in 1963, the first publications regarding hepatic IRI date only to the late 1970s and early 1980s, although the topic has recently gained increasing interest (111-114).

IRI is an ill-defined injury after a period of ischemia followed by reperfusion with oxygenized blood. In the liver, IRI has historically been defined by the tissue damage observed after prolonged ischemia and reperfusion. The markers classically used to describe the severity of IRI include the liver enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), as well as histological signs of tissue damage. Given the multifactorial nature and complexity of IRI, these markers may be regarded as insufficient methods. Currently, IRI is recognized as a complex cascade of events initiated by the ischemic insult. The reperfusion phase starts with the return of oxygenized blood and can be divided into early (< 2 hours) and late (6 to 48 hours) reperfusion; however, the division is somewhat arbitrary and leaves a window of 4 important hours (115, 116). The injury leads ultimately to both necrosis and apoptosis in the liver.

Upon ischemia, the oxygen tension in the tissue will decrease, and the metabolism will change from physiological aerobic metabolism to anaerobic metabolism. As a consequence, the production of phosphorylated high-energy compounds (adenosine triphosphate, ATP) decreases, ultimately becoming insufficient for cellular metabolism of hepatocytes, sinusoidal endothelial cells and Kupffer cells (117). The ATP deficiency causes a loss of cellular membrane ion pump function, resulting in increased intracellular concentrations of sodium and calcium ions. This loss of function in turn causes cells to swell, and the increased concentration of intracellular calcium activates phospholipases, which degrade the membrane phospholipids (118). Simultaneously, the tissue becomes acidic, which further increases cellular dysfunction (118, 119). With the disruption of cellular membranes, cell contents, such as AST and ALT, begin to leak into the interstitium. The disruption of the cellular membrane and the leakage of cellular phospholipids (phosphoglycerol) forms the rationale for one of the microdialysis measurements further described in chapter 11 (120).

The main cells involved in IRI include hepatocytes, Kupffer cells (macrophages in the liver), sinusoid endothelial cells and polymorphonuclear neutrophils (PMNs).

Figure 7. The major cells and mediators involved in liver IRI.

ATP = Adenosine triphosphate

TNF- α = Tumor necrosis factor α

IL-1/IL-17 = Interleukin 1/interleukin 17

IFN- γ = Interferon γ

ROS = Reactive oxygen species

The main reactive oxygen species (ROS) involved in liver IRI include the superoxide radical ($\cdot\text{O}_2^-$), hydroxyl radical ($\cdot\text{OH}$) and hydrogen peroxide (H_2O_2). ROS are categorized as radicals and non-radicals depending on the presence (radicals) or absence (non-radicals) of an unpaired electron. Hydrogen peroxide alone is a non-radical but can react to form highly active radicals. The three systems that produce ROS during liver IRI are the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system, the mitochondrial respiratory chain and xanthine oxidase (115, 121).

Xanthine oxidase has been the focus of research during the last decades. Xanthine oxidase is an intracellular enzyme that exists as xanthine dehydrogenase under physiological conditions. Upon prolonged ischemia, xanthine dehydrogenase is converted to the ROS-forming oxidase (122). Furthermore, the substrates xanthine and hypoxanthine are metabolized relatively quickly and flushed out during reperfusion, whereas accumulation occurs during ischemia (123). Although the inhibition of xanthine oxidase with allopurinol has some effect on preventing IRI, it seems clear that the enzyme does not play a key role in the cascade (124).

Mitochondria-generated superoxide is another ROS that might be involved in the process of IRI. Again, prolonged ischemia appears to be required for the process to become important in oxidative stress (125). In the mitochondria, a number of protective enzymes, such as superoxide dismutase and glutathione peroxidase, are also present that detoxify ROS to some degree (126, 127).

NADPH oxidase is found in both Kupffer cells and PMNs that generate superoxide (128). This superoxide formation has been observed in the settings of hepatic IRI and has been suggested as a viable option in the treatment of IRI (129, 130). The inhibition of NADPH oxidase protects against hepatic IRI in mice (131). However, this result has not been observed in clinical studies.

Although hepatocytes are largely considered victims of IRI, they also contribute to the cascade by releasing IL-12. This interleukin may activate inflammatory responses, including TNF- α and IFN γ release, in livers subjected to IRI (132). As hepatocytes provide 80-90% of the complement factors found in plasma, these cells are likely to be responsible for the complement-derived activation of Kupffer cells (see below) observed in hepatic IRI (133).

Kupffer cells play an important role in the early phase of reperfusion and are the main source of ROS generated during that phase (134). The swollen and activated Kupffer cells also release TNF- α and IL-1 early. After a 2-hour delay (reperfusion), increased release of IL-6 is observed as well. TNF- α secretion appears to stimulate Kupffer cells to further secrete TNF- α (positive feedback), and inhibition of the IL-1 receptor in Kupffer cells reduces TNF- α production (135, 136).

Although TNF- α and IL-1 are cytokines with systemic proinflammatory properties, IL-6 is to some extent anti-inflammatory. IL-6 moderates the inflammatory response and reduces TNF- α expression as well as IRI (137-139).

Kupffer cells are also stimulated by complement. Complement-depleted rats respond to IRI with a reduced ALT elevation and less PMN infiltration than rats with intact complement; furthermore, this response appears to occur during the activation of Kupffer cells (140). Some studies have reported that inhibition of the complement system can reduce hepatic IRI (141-144). ROS, TNF- α and IL-6 secretion from activated Kupffer cells rapidly (within an hour of reperfusion) attracts lymphocytes to the liver (145, 146). The lymphocytes in turn further activate the Kupffer cells to secrete TNF- α with IFN- γ release (145). In addition, the secretion of IL-17 by lymphocytes appears to regulate the recruitment of neutrophils (147). In addition to the attraction of lymphocytes and the ROS production that is directly toxic, Kupffer cell secretion influences neutrophil recruitment and activation as well as sinusoid endothelial cells (see below).

The effect of PMNs is noticed later than the effect of Kupffer cells in hepatic IRI and appears to be initiated by the secretions described above (134, 148). TNF- α (released by Kupffer cells) has been shown to both activate neutrophils and facilitate their accumulation in the liver via the up-regulation of adhesion molecules (in hepatic post-sinusoidal venules), such as intercellular adhesion molecule-1 (ICAM-1) and P-selectin (137, 149-151). Although these mechanisms are potentially important in the post-sinusoidal venules, the importance of adhesion molecules in the liver sinusoids has been questioned (152). Regardless of the mechanism by which the PMNs accumulate and migrate into the liver (see below), these cells play an important role in the IRI. PMNs are a cell type that have the ability to form ROS. The main source of superoxide formation by PMNs is NADPH oxidase (153). Upon arrival to the liver, PMNs are primed, and the oxidant stress appears after 6-24 hours of reperfusion (148, 154). The PMNs adhere to damaged hepatocytes (similar to the interaction with endothelial cells) via interactions between the beta2-integrin MAC-1 and ICAM-1, the expression of which is stimulated by TNF- α (155). When activated, PMNs produce ROS that diffuse into the hepatocytes and trigger mitochondrial dysfunction. Intracellular oxidant stress might ultimately lead to hepatocyte death (156). The release of proteases (elastase, cathepsin G) is another mechanism by which PMNs kill hepatocytes, but the significance of this mechanism in vivo remains unclear (157, 158).

Similar to hepatocytes, sinusoid endothelial cells are largely a target of IRI. However, the role of these cells in the pathogenesis of IRI, which is mediated through interactions with other cells that are attracted to the liver, is important. The sinusoids have been identified as the main site for PMN extravasation into the liver, which occurs without the involvement of

ICAM-1 or P-selectin (152, 155, 159). It is reasonable to assume that another IRI-related mechanism is responsible for the accumulation and extravasation of PMNs. The initial ATP depletion associated with liver ischemia causes a volume increase in the endothelial cells in a manner similar to that observed in Kupffer cells and hepatocytes (160).

The accumulation of platelets in the sinusoids correlates with reduced sinusoidal perfusion. Furthermore, platelets adherent to the sinusoid endothelial cells (activated by Kupffer cells but independent of P-selectin) induce PMN accumulation in the sinusoids (161, 162). In addition to sinusoidal endothelial cell swelling and platelet adherence, the microcirculation is further impaired by the change in nitric oxide (NO) production (see chapter 8) with respect to endothelin production (figure 8) (163).

Figure 8. Sinusoidal swelling in liver IRI.

SEC = Sinusoid endothelial cell

NO = Nitric oxide

PMN = Polymorphonuclear neutrophil

In summary, liver ischemia/reperfusion (IR) leads to the activation of hepatocytes, Kupffer cells and sinusoid endothelial cells. The Kupffer cells produce ROS but also activate lymphocytes and neutrophils. These cells accumulate in the liver via extravasation in the liver sinusoids and are responsible for the later phase of the IRI cascade in which sinusoidal cells and hepatocytes are injured. Figure 9 summarizes the pathways involved in liver IRI.

Figure 9. Summary of liver IRI.
 SEC = Sinusoid endothelial cell
 PMN = Polymorphonuclear neutrophil
 IL-1/IL-6/IL-17 = Interleukin 1/6/17
 TNF-α = Tumor necrosis factor α
 ROS = Reactive oxygen species
 IFN-γ = Interferon γ

In addition to the detrimental effect of IRI on the FLR, liver ischemia might stimulate the malignant disease that served as the original cause of the surgery. Animal studies have indicated that ischemia stimulates tumor growth, possibly in a dose-dependent manner (164, 165). Furthermore, it has been suggested that selectively clamping the portal vein instead of performing PM might reduce this risk (166). However, to date, no human studies have demonstrated this effect in a clinical setting, possibly due to the multifactorial nature of both the malignant disease and the ischemia applied during liver surgery.

8. Reactive nitrogen species (RNS)

The main reactive nitrogen species (RNS) include nitric oxide ($\cdot\text{NO}$) and peroxynitrite (ONOO^-). Given that it possesses an unpaired electron, NO is a highly reactive free radical. The discovery of its signaling properties in the cardiovascular system earned three distinguished researchers the Nobel Prize in Physiology or Medicine in 1998. Since then, the role of NO in liver IRI has been studied vigorously, but much remains unclear within the field.

The main source of NO in the human body is endogenously produced by nitric oxide synthase (NOS). NOS has three isoforms: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS). nNOS is almost exclusively found in neural tissue and is therefore outside the scope of this book. eNOS is a calcium-/calmodulin-dependent enzyme that catalyzes the production of NO from the amino acid L-arginine and oxygen (figure 10). eNOS is expressed in liver endothelial cells and hepatocytes (167, 168).

Figure 10. Nitric oxide metabolism. Under physiological conditions, nitric oxide synthase (NOS) produces NO from L-arginine and oxygen. NO is degraded by oxidation to nitrite (NO_2^-) and nitrate (NO_3^-) but can also react with superoxide (O_2^-) to form peroxynitrite (ONOO^-). NOx formed from NO and from dietary sources can be reduced to NO.

The third isoform is iNOS, which is bound to calmodulin irrespective of calcium concentrations (calcium-independent). This enzyme is not expressed under physiological

conditions but is up-regulated in many cell types during IRI, including hepatocytes, Kupffer cells and neutrophils (169-171). In the setting of liver IRI, the main source of NO after the up-regulation of iNOS appears to be hepatocytes (169, 172, 173).

Although it is widely recognized that NO plays an important role in IRI, much of the details involved remain unclear. NO relaxes and dilates the liver sinusoids, thus making the perfusion of the liver sinusoids NO-dependent, at least to some degree. This action is opposed by endothelin, and studies have demonstrated that endothelin inhibition can improve the microcirculation in the liver during IRI (174, 175). On the other hand, the inhibition of NO production decreases liver circulation (176). Furthermore, it has been suggested that this effect is predominantly exerted through effects on the arterial circulation of the liver given that IRI is not increased when NO production is blocked in the context of common hepatic artery occlusions with intact portal circulation (176). However, NO clearly exhibits some positive effects on the IRI observed with total occlusion of the liver blood supply, as inhibition of NO production by the administration of N^G-nitro-L-arginine methyl ester (L-NAME) increases IRI in a rat model of total hepatic ischemia (177). These results potentially reflect the effect of blocking eNOS and thus inhibiting the physiological production of NO. This explanation is further supported by results demonstrating that eNOS-knockout mice experience more severe IRI than wild type mice (172). Furthermore eNOS overexpression decreases IRI in a mouse model (178). The administration of the NO substrate L-arginine immediately prior to partial (hepatic artery only) liver ischemia increases liver blood flow during reperfusion and reduces IRI (179). The source of NO in this treatment is likely to be eNOS. In addition, NO protects endothelial cells during IRI and improves the hepatic microcirculation (172, 180-184).

Given that eNOS produces relatively low amounts of NO compared with iNOS, the effects of high NO concentrations have been attributed to iNOS activation when studied in the setting of prolonged (greater than 60 minutes) ischemia. Substantial data from studies assessing iNOS inhibition support the detrimental effect of iNOS activation in both porcine and rat models of hepatic IRI (171, 185-188).

NO reacts with superoxide to form the potent oxidant and highly cytotoxic compound peroxynitrite, which might explain the protective effect on IRI observed with iNOS blockade (186, 189). In addition, excessive amounts of NO increase leucocyte activation and trafficking into the liver (188).

In the setting of short ischemic insults (less than 60 minutes), the effect of iNOS is more controversial. In a study on knockout mice, eNOS was protective in the setting of 1-hour partial hepatic ischemia, whereas iNOS increases IRI (190). A similar study reported increased signs of IRI in eNOS-deficient mice but no such increase in iNOS-deficient mice (191). The length of reperfusion has also been suggested to play an important role in the effects of iNOS as the effect of iNOS varies depending on the length of reperfusion. Three-hour reperfusion after 45-minute ischemia resulted in more severe IRI in iNOS knockout mice than wild type mice. However, this result was not observed after 1 or 6 hours of reperfusion (192).

Inhaled NO has been tested in the setting of human liver transplantation and was found to reduce the increase in transaminases associated with the procedure as well as the number of complications (193, 194). As the half-life of NO is only a few seconds, the protection observed with NO inhalation in this study may be related to nitrite (195). Nitrite is a relatively stable compound in the human body, thus making nitrite a pool for later NO production by reduction catalyzed by heme-containing proteins (196).

At the cellular level, NO affects the production of ROS and energy in the mitochondria (197). NO inhibits the mitochondrial respiratory chain, thus reducing the release/formation of ROS during reperfusion (196, 198-200). Under physiological conditions with a normal supply of oxygen, pyruvate enters the mitochondrial respiratory chain, and ATP is generated. In the process, NO is oxidized to nitrite by cytochrome c oxidase. When oxygen is lacking (ischemia), NO blocks cytochrome c oxidase, resulting in the accumulation of acetyl CoA and the reduction of pyruvate to lactate. Similarly, the amount of NO present during reperfusion might modulate the mitochondria to reduce the burst of ROS as well as ATP production (197).

9. Ischemic preconditioning (IPC)

The recommendation of short-acting ischemia followed by reperfusion before a longer-lasting ischemic insult as a means to protect an organ originated from studies on dog myocardium in the 1980s (201). In the mid-1990s, this concept started to spread to liver surgery and has been a topic of intense research during recent years (202). Multiple studies in rodents have demonstrated that ischemic preconditioning (IPC) is effective in reducing IRI in healthy animals as well as in obese animals and those with fatty liver (183, 203-213).

Of special interest are studies that report different effects of IPC on aged animals compared with younger animals (117, 214). This finding may be increasingly important given that aging increases IRI and as liver resections are more frequently performed on older patients than before (215).

The general hypothesis of the mechanisms underlying IPC is that the induction of low-grade oxidative stress prepares the cells for the insult of longer-lasting ischemia followed by reperfusion (216).

More specific mechanisms have suggested the induction of antioxidant survival genes, such as heme oxygenase-1 (HO-1) (217). Sinusoidal endothelial cell death decreases with IPC, and this effect appears to be mediated through the adenosine A(2) receptor pathway (218). Furthermore, it has been suggested that adenosine exerts its effect through the stimulation of NO production (219). In the setting of prolonged (75 minutes) total hepatic ischemia, IPC reduces caspase 3 activity and apoptosis (209). Other studies have suggested that additional apoptosis-regulating genes, such as c-jun, are involved in the protective effect of IPC and that IPC reduces transcription and inhibits apoptosis (203, 205, 210, 220). IL-6 is another possible mediator of the IPC effect; however, studies have reported diverging results, including reports of hepatoprotection when IL-6 is administered as well as reports of reduced levels of IL-6 after IPC compared with I/R alone (206, 207). IPC results in lower TNF- α levels than I/R alone, but depleting TNF- α mice of TNF- α ameliorates the protective effect of IPC (206, 207, 213). Thus, it appears that a low dose of TNF- α is required for the protective effect of IPC, as previously suggested (221). In addition to these protective mechanisms, IPC induces a regenerative response in the liver that might be mediated by NF- κ B activation (204, 211).

NO is a vasodilator and thus facilitates hepatic blood flow during reperfusion (222, 223). The positive effect of IPC on restoring hepatic microcirculation observed after 2 hours of reperfusion was demonstrated to not only disappear but even worsen when L-NAME, a competitive NOS inhibitor, was administered to rats before IPC+I/R (183). Moreover, NO has been implicated in the damping effect of IPC on TNF- α release (213). The effect of preconditioning on eNOS expression has been studied, and eNOS expression increases with IPC relative to I/R alone after 45 minutes of ischemia followed by 2 hours of reperfusion (168). In addition, the increased expression of eNOS was associated with higher plasma NOx. However, this study did not observe iNOS expression in IPC or the I/R group. On the other hand, iNOS gene expression is increased with IPC in living donor liver transplantation when the reperfusion period (before biopsies were obtained) is short (224).

The clinical effect of IPC has been studied in different settings. Studies on IPC before I/R during liver resections can be categorized based on the type of ischemia applied. A number of studies focused on the effect of IPC in the setting of inflow occlusion; thus, studies can be further subdivided into those assessing continuous inflow occlusion and those studying intermittent inflow occlusion. However, only one study has been published on the effect of IPC before intermittent inflow obstruction (225). The other available studies compare continuous inflow obstruction with IPC + continuous inflow obstruction or IPC + continuous inflow obstruction with intermittent inflow obstruction alone (226-233). In addition, studies have assessed the effect of IPC combined with hepatic vascular exclusion that can be either total or selective and continuous or intermittent (234-237). Table 2 summarizes the published clinical studies involving IPC and liver resections.

Study	Vascular occlusion	Main results of IPC treatment
Clavien et al. (226)	cPM	Reduced AST and ALT
Clavien et al. (227)	cPM	Reduced AST and ALT
Nuzzo et al. (230)	cPM	Reduced AST and ALT*
Li et al. (233)	cPM	Reduced AST, ALT and bilirubin
Chouker et al. (228)	cPM	Reduced PMN, AST and ALT
Azoulay et a. (234)	SHVE	No difference
Petrowsky et al. (231)	cPM + IPC vs. iPM - IPC	Equally effective
Smyrniotis et al. (235)	iSHVE vs. IPC+cSHVE	Equal for < 40 min occl., increased AST and caspase for > 40 min occl.
Heizmann et al. (229)	cPM	Reduced bleeding, fewer compl.
Arkadopoulos et al. (236)	SHVE +/- IPC	Reduced AST and apoptosis
Scatton et al. (225)	iPM +/- IPC	No difference
Hahn et al. (232)	iPM vs IPC+cPM	Reduced AST and ALT**
Jeon et al. (237)	THVE +/- IPC	No difference

Table 2. Clinical studies on the effectiveness of IPC. Only one of the studies reported a clinically significant difference when IPC was applied.

cPM = Continuous Pringle's maneuver

SHVE = Selective hepatic vascular exclusion

IPC = Ischemic preconditioning

iPM = Intermittent Pringle's maneuver

iSHVE = Intermittent selective hepatic vascular exclusion

cSHVE = Continuous selective hepatic vascular exclusion

occl = Occlusion

compl. = Complications

THVE = Total hepatic vascular exclusion

** Transection was performed during the IPC.*

*** Effect more pronounced in patients with cirrhosis.*

Although some of the studies presented in table 2 report differences in laboratory values that suggest the protective effect of IPC compared with continuous inflow obstruction alone, the clinical effectiveness of this approach appears to be at best questionable, as indicated in a Cochrane review conducted in 2009 (238). The only study able to demonstrate a clinical difference reported significantly more bleeding and blood transfusions in the non-IPC group, making the findings somewhat difficult to interpret (229). In addition, intermittent vascular obstruction compares favorably with continuous vascular obstruction with and without IPC.

However, a notable gap in the literature regarding the possible protective effect of IPC when applied before intermittent vascular obstruction is evident (225).

10. Remote ischemic preconditioning (R-IPC)

Hepatoduodenal ligament clamping to initiate IPC carries a small but significant risk of injury to the structures within the ligament. In addition, IPC is a time-consuming component of major liver resection, an operation that is often already long. Therefore, the idea of remote ischemic preconditioning (R-IPC), wherein another body part, such as a lower extremity, is rendered ischemic for a short period of time before the operation, is an appealing clinical concept. This concept was first published in the 1990s and has been primarily investigated in the heart (239, 240).

The first studies to investigate the possibility that R-IPC imparts protective effects in the setting of liver IRI were published in 2006 (241-243). With 3 cycles of 10 minutes of leg ischemia followed by 10 minutes of reperfusion before 25 minutes of total hepatic ischemia in rabbits, AST and ALT levels were significantly reduced at 2 hours of reperfusion compared with I/R alone (241). In addition, hepatic vein NOx was significantly increased in R-IPC-treated animals compared with those subjected to I/R alone. Hepatic blood flow at the end of reperfusion was also increased in R-IPC-treated animals. Taken together, the results indicated that R-IPC has a protective effect against hepatic IRI and that NO is involved in the early phase of reperfusion.

In a model involving 45 minutes of partial hepatic ischemia in rats preceded by three cycles of hind-limb preconditioning (10 minutes of ischemia and 10 minutes of reperfusion), serum ALT levels were significantly increased at 4 hours of reperfusion in the group subjected to I/R alone (242). The main possible mechanism identified in the study was the induction of HO-1, as observed in hepatocytes after R-IPC.

R-IPC was applied as a single 10-minute occlusion of the femoral artery in rats followed by 15 minutes of reperfusion before either closure of the hepatic artery (branch) or total inflow occlusion of 70% of the liver (243). In this model, R-IPC reduced the IRI when applied before partial (artery only) hepatic ischemia but not when total circulation to the liver was occluded.

The role of R-IPC was further established in 2009 when a study involving 45 minutes of 70% hepatic ischemia followed by 3 hours of reperfusion reported that R-IPC (applied as 4

cycles of 5 minutes of ischemia and 5 minutes of reperfusion to the hind limb) increased red blood cell velocity and sinusoidal flow as well as decreased PMN adhesion (244).

In a mouse model, R-IPC applied as 10 minutes of ischemia followed by 10 minutes of reperfusion decreased IRI compared with I/R alone. Given that this effect was not observed in toll-like receptor 4 (TLR4)-mutant mice, it was concluded that functional TLR4 is required for R-IPC (245).

In a mouse model where R-IPC was administered as 6 cycles of 4 minutes of ischemia in the hind limb and 4 minutes of reperfusion before 40 minutes of 70% hepatic ischemia followed by 2 hours of reperfusion, R-IPC reduced the level of aminotransferases in plasma and preserved hepatic microcirculation (246). In the same model, R-IPC (with and without I/R) elevated NO_x in the plasma. Furthermore, when the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-*o*-yl-3-oxide potassium salt (C-PTIO) was administered before R-IPC, the protective effect disappeared (195). Further studies using this mouse model demonstrated that eNOS^{-/-} mice are not protected by R-IPC and that eNOS protein expression is not increased with R-IPC compared with I/R alone in eNOS-wild type mice; thus, the protection is likely to be due to activation of the enzyme (247). To further evaluate the downstream mechanisms involved in the protective effects of NO in R-IPC, mice were administered 1H-(1,2,3)oxadiazole(4,3-*a*)quinoxalin-1-one (ODQ), an inhibitor of soluble guanylyl cyclase (sGC) (248). This inhibition resulted in levels of transaminases that did not significantly differ from those in I/R animals, whereas R-IPC without ODQ treatment offered the expected protection against IRI. Taken together, this series of studies provided evidence for the role of eNOS activation and subsequent NO production in the protective effects on the hepatic microcirculation via the cyclic guanosine monophosphate (cGMP) pathway during ischemia and early reperfusion.

The effects of R-IPC on the later phase of reperfusion have been less studied; however, 4 cycles of 5+5 minutes (ischemia + reperfusion) of R-IPC to the hindlimb was shown in a rat model to result in greater sinusoidal diameter and reduced hepatocellular injury (measured as AST and ALT levels as well as signs of cell death upon histological examination) (249).

In one study, the effect of R-IPC on regeneration was assessed, and increased liver proliferation was shown 24 hours after R-IPC+R/I compared with I/R alone. Furthermore, IL-6 mRNA was increased, whereas TNF- α expression was decreased, in the R-IPC group (250).

To date, no clinical studies exploring the usefulness of R-IPC in human liver surgery have been published. However, the preliminary results of 16 patients enrolled in a randomized study (www.clinicaltrials.gov NCT00796588) assessing the effects of R-IPC (3 cycles of 10 minutes of ischemia and 10 minutes of reperfusion to the lower limb) during major hepatectomy were presented at The Annual Meeting of the Society of Academic and Research Surgeons in January 2010 (251). In this report, the AST and ALT levels immediately after resection and 24 hours later were significantly reduced in the R-IPC group compared with the control group. Furthermore, the indocyanine green test (ICG-15) revealed enhanced liver function in the R-IPC group compared with controls immediately after resection (251).

11. Microdialysis

Bitto introduced microdialysis (μ D) in 1966 as a method to investigate the concentration of electrolytes and amino acids in dog brain and subcutaneous tissue (252). In 1974, Urban Ungerstedt further developed the concept by applying a microdialysis membrane to a catheter, thus creating the first version of modern μ D catheters (253).

The technique simulates the equilibrium between capillaries and interstitial fluid, which is based on the semi-permeable properties of the capillary wall. Small molecules can diffuse passively through this wall in a process that is driven by the concentration gradient of the substance and the oncotic pressure generated by larger molecules that do not pass through the wall.

The μ D catheter is a double-lumen tube with a semi-permeable membrane at the tip. Through the outer lumen, an isotonic solution (perfusate) is continuously pumped, and at the tip, the solution comes into contact with the semi-permeable membrane that resembles capillary walls. The membrane has a pore size of 20 to 100 kDalton (kDa) that can be selected based on what substance is measured. At the tip, the substance diffuses passively into the solution, which thereafter flows through the inner lumen of the catheter and is collected in a microvial (figure 11). The solution collected in the microvial (dialysate) can then be analyzed for the substances in question.

When presenting the results from μ D studies, it must be kept in mind that the concentration in the fluid does not generally represent the actual concentration in the tissue but is a product of the recovery of the substance in the specific setting used during the sampling. When the recovery approaches 100%, the concentration of the substance in the dialysate is close to the true concentration in the interstitial fluid. Given this limitation, μ D studies are best performed as a comparison of concentrations from the same tissue/organ subjected to different situations or temporal change within the same organ.

Figure 11. *The principles of microdialysis. Perfusion fluid pumped through the catheter equilibrates with the interstitial fluid by diffusion over a semi-permeable membrane. The fluid is then sampled for analysis.*

Many factors influence the recovery rate of substances within a microdialysis system. The membrane of the μ D catheter is one of the most important factors that impacts recovery. Both the length of the membrane and the pore size determine the recovery rate. Thus, it is of great importance to choose catheters that allow for the diffusion of the substance to be analyzed. Another factor that can be controlled and standardized is the perfusion rate. At higher perfusion rates, the absolute recovery increases, but the dialysate becomes diluted, resulting in a net decrease in the recovery. Thus, lower perfusion velocities (typically 0.1-0.3 μ l/min) are desirable to increase the recovery rate. This necessity becomes somewhat problematic when short sampling intervals are needed to follow concentration changes over time because the low velocity combined with short sampling times make the recovered volumes small and difficult to analyze. Another factor that can be relatively easily controlled is the level of the microvial used to sample the dialysate in relation to the pump. If the microvial is positioned below the semi-permeable membrane, hydrostatic pressure at the tip of the catheter is increased, thus imposing the risk of excess water loss to the tissue from the perfusate. This loss increases the osmolality of the dialysate and yields a falsely elevated concentration of the substances to be analyzed. The sizes of the molecules in question are

important given that larger molecules have less kinetic energy than smaller ones, resulting in less diffusion through the membrane. The kinetic energy is also affected by temperature; higher temperatures increase the kinetic energy, thus increasing the recovery. It has been estimated that a 1 degree Celsius increase in temperature increases the recovery by 1-2% (254).

In addition to the above- mentioned factors, it must be kept in mind that the introduction of microdialysis catheters is a traumatic process. Therefore, time must be allowed to pass before any reliable measurements can be performed. The time to reach equilibrium or “steady state” is typically set to 30 or 60 minutes; however, this time may be longer for some substances (255-257).

The microdialysis technique was introduced to clinical neurosurgery in the early 1990s and has also been used in the setting of liver transplantation (258-261). In addition, the technique has been tested in the setting of liver resection (262). One of the advantages of this technique is that analysis can be performed “bedside” immediately after sampling, thus providing direct information about the organ in question.

In experimental settings, μ D with an analysis of glucose, pyruvate, lactate and glycerol has been used in porcine models (263). An increase in glucose and lactate accompanied by decreased pyruvate during ischemia represents anaerobic metabolism, whereas increased glycerol is indicative of cell membrane disruption and thus tissue damage (120).

12. Liver IRI study methodologies

IRI is poorly defined and numerous obstacles are encountered when liver IRI is studied. First, the ethical aspects of performing such studies in human subjects must be considered. Although liver surgery has evolved dramatically from the high complication rates of the early days, there remains a large risk involved in this type of surgery. Thus, any unnecessary ischemic events are unethical, as is the prolongation of already long operations for research. These ethical concerns establish the boundary for clinical studies on liver IRI. To date, the trials published have largely relied on a combination of liver biopsies and blood sampling.

To gather more information on the nature of IRI, many animal models have been developed and used in various studies. The animal strains used ranges from small rodents (mice and rats) to larger mammals (dogs and pigs). Due to legislation, the use of dogs in clinical experiments has stopped in the Western world. The main motivation for the use of mammals, such as pigs, is that the anatomy and biology of these animals more closely resembles humans than rodents. On the other hand, rodents are more widely available for clinical research, and more commercially available reagents and kits can be used in the experiments, thus making the results more reliable.

Irrespective of what species is used, the study of IRI mechanisms is complicated as the cascade is multifactorial and interactions between different signaling molecules and cytokines may change over time. The early models of total liver ischemia posed the problem of blood stagnation in the bowel. This problem was overcome by non-physiological shunts that were cumbersome and might have affected the results (264). Today, most animal models of liver IRI rely on partial or segmental liver ischemia, circumventing this problem. However, this approach makes it difficult to know how much ischemia is applied, and no reliable method is available to measure or grade the ischemia. A possible method for investigating the different effects of various grades of ischemia in addition to the time factor is the use of selective occlusion of the hepatic artery compared with PM (243).

The sampling has largely been based on repeated biopsies from the liver and organ harvesting as well as blood sampling. Thus, the chain of events and temporal changes have been difficult to follow in one or a few animals, and many animals have been sacrificed to reveal causal relationships. This experimental design, however, is complicated by the fact that IRI is poorly defined and by the fact that various methods are used to measure the

phenomenon. The undisputed basic biochemical markers include the liver transaminases and ultimately the histological signs of tissue injury. Although the assessment of histology can be considered a subjective art, some objective guidelines can be used. In addition to the factors included in the score used in our studies, the infiltration of PMNs is commonly used to describe histological signs of IRI. The dimensions of the score used in the present studies (sinusoidal congestion, cytoplasmic vacuolation, liver necrosis) are the same as those used in another widely used system, although the grading is somewhat different (265). Apoptosis is an important component of IRI that is difficult to assess with basic pathology staining, and the signs of necrosis evolve over time; therefore, the length of the experiment influences the findings. The appearance of PMNs is also a time-dependent (see chapter 7) sign of inflammation that is not captured with the scoring system that is commonly employed.

In this thesis, a known model of rat liver IRI was modified with the addition of μ D to gather more information about the local metabolism and temporal changes in the liver (266). In study II, μ D was added to clinical praxis along with a short period of IPC.

13. Aims of the thesis

The **overall aim** of this thesis was to investigate methods to reduce IRI in the liver.

Specific aims for studies I-IV:

- I To compare the effectiveness of IPC and R-IPC before IRI in a rat model and to investigate whether commercially available kits for microdialysis could be used to monitor liver IRI.
- II To investigate whether IPC before the interrupted PM exhibits clinical benefits compared with the interrupted PM alone in a randomized controlled trial.
- III To compare the involvement of NO and iNOS in the effects of IPC and R-IPC.
- IV To investigate whether nitrite administered before ischemia reduces IRI and the activation of iNOS transcription.

14. Material and methods

Ethical

The study protocols for study I (2-09), study III (2-09) and study IV (56-12) were approved by the regional ethics committee for animal experiments, Linköping, Sweden. The study protocol for study II (M100-06) was approved by the regional ethics board, Linköping, Sweden.

Animals (study I, III and IV)

Male Sprague-Dawley rats (258-444 g) were used in studies I, III and IV. Before the experiments began, the animals were acclimatized for one week in the laboratory pet house. The rats had free access to standard rat food pellets and tap water in a 12-hour light/dark environment at 21°C.

Patients (study II)

Patients with planned resections of at least two liver segments due to metastatic liver disease or the suspicion of gallbladder cancer (T1-2, tumors confined to the gallbladder wall) were asked to participate in the study. Patients with intended portal venous ligation, multi-organ resection or suspected or proven chronic liver disease were not included in the study.

If no differences were observed in 16 patients with IPC, any difference missed due to low power would be of minimal clinical interest.

The patients were stratified according to the size of the intended resection and randomized to either preconditioning or no preconditioning prior to the PM (figure 12).

Figure 12. Randomization and stratification (study II). Patients were stratified according to the number of segments resected and randomized within each stratum to IPC or no IPC prior to the PM.

Anesthesia and surgery

Animals

The rats (studies I, III and IV) were anesthetized with isoflurane, and 0.05 mg/kg buprenorphine was administered s.c. for pain relief. The animals were then intubated with a 16-G intravenous catheter and ventilated throughout the experiment. During the experiment, the animals were monitored, and body temperature was maintained within 38-39°C. Laparotomy was performed via a midline incision, and the ligament attachments of the liver were divided. Directly after intubation and every hour throughout the experiment, the animals received 5-ml warm Ringer's acetate s.c.

Patients

All patients (study II) received anesthesia according to the low CVP concept with a target CVP ≤ 5 mmHg. Fluid restriction and nitroglycerine were used according to clinical practice. The operations were performed through a right subcostal incision angled and extended to the sternum.

After exploration without findings contraindicating resection, patients were stratified (2-3 or > 3 segments) and then randomized to either IPC or no IPC. PM was applied with cotton tape around the hepatoduodenal ligament. The intermittent PM was performed with 15-minute closure of the hepatoduodenal ligament and 5-minute open circulation for 1 to 3 cycles.

The IPC group underwent 10-minute closure of the ligament followed by 10-minute open circulation (IPC) administered prior to the intermittent PM.

Parenchymal transection was achieved with the CUSA, and liver biopsies were obtained from microscopically healthy portions of the liver at the end of the operation.

IPC, R-IPC and liver ischemia (studies I, III and IV)

In the IPC group, a clamp was applied over the vascular pedicle to the left lateral lobe and then removed after 10 minutes, allowing for 10-minute reperfusion before the ischemic assault.

Remote IPC was induced by placing a tight tourniquet around the right hind leg for 10 minutes followed by 10 minutes of reperfusion.

The ischemia lasted 60 minutes (study I and III) and was followed by 4 hours of reperfusion. In study IV, no preconditioning was applied, and the ischemia was reduced to 45 minutes while maintaining 4 hours of reperfusion.

Administration of nitrite (study IV)

Two minutes before the application of ischemia, 480 nM sodium nitrite dissolved in NaCl (0,24 ml) was administered directly into the caval vein using a 30-G needle.

Local metabolism (microdialysis)

Animals

The model of segmental ischemia in the left lateral lobe has been previously established (266). In studies I, III and IV, microdialysis was added to this model. A CMA 20 Elite microdialysis probe was inserted in both the left lateral and right lateral (control) liver lobes and perfused at a rate of 1.0 $\mu\text{l}/\text{min}$ with perfusion fluid T1 (NaCl 147 mmol/L, KCl 4 mmol/L CaCl_2 2.3 mmol/L). Before insertion, the probes were pre-perfused. After probe insertion, 35 minutes were allowed to pass to achieve steady state, and 20 minutes of baseline sampling of the microdialysate was performed before ischemia was induced. From the beginning of ischemia and throughout the reperfusion period, the microdialysate was sampled. Glucose, glycerol, lactate and pyruvate were instantly analyzed with the clinical bedside analyzer ISCUS (CMA Microdialysis).

Patients

In study II, 63 microdialysis CMA catheters were used. Two catheters were inserted into the FLR and attached to the Glisson capsule using 4/0 absorbable sutures. The μD catheters were fastened to the skin with tape and connected to a 107- μD CMA pump that the patients kept in a waist belt during the post-operative period (figure 13).

Figure 13. *Microdialysis in a clinical setting. Perfusion fluid is pumped through the catheters and sampled in the vials.*

The catheters were perfused with Ringers acetate at a rate of 2.0 µl/min during surgery at the surgical recovery department. Initially, 20 minutes were allowed to achieve a steady state; subsequently, samples were collected every ½ hour during surgery and every hour at the recovery department.

When the patient was transferred to the surgical ward, the perfusion velocity was changed to 0.3 µl/min, and sampling was performed every 4th hour until the evening of post-operative day (POD) 4 when the catheters were removed.

The dialysate from one of the sampled microvials was immediately analyzed using the bedside analyzing equipment ISCUS, whereas the other microvial was frozen at -20°C for later analysis of NOx.

General

In all of the studies, the automatic bedside analysis machine ISCUS was used for immediate analysis of glucose, pyruvate, lactate and glycerol. The ISCUS machine uses a single-beam filter photometer to detect light emitted at a wavelength of 530 nm. All of the enzymatically catalyzed reactions lead to the formation of quinoneimine at a rate that is proportional to the substance to be measured (information from CMA Microdialysis).

Glucose concentrations are measured as follows:

Detection range: 0.1-25 mM, deviation < 5%.

Pyruvate concentration is measured by:

TOOS = N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine

Detection range: 10-1500 µM, deviation < 5%.

Lactate is measured by:

Detection range: 0.1-12 mM, deviation < 5%.

Glycerol concentration is obtained by:

DCHBS = 3,5-dichloro-2-hydroxy-benzene sulfonic acid

Detection range: 0.01-1.5 mM, deviation < 5%.

Blood sampling and analysis

Animals

In studies I and III, blood was sampled in a 2-ml syringe both at the beginning and the end of the experiment from the exposed external jugular vein. In study IV, blood was only sampled immediately before the animals were exsanguinated, as previous studies have demonstrated only small variations at baseline. The blood was centrifuged at 3000 rpm for 7 minutes in LH tubes.

AST and ALT were analyzed according to laboratory standards (Department of Clinical Chemistry, University Hospital, Linköping, Sweden). Other analyses are described below.

Patients

Blood sampling in study II for Hb, WBC, albumin, AST, ALT, bilirubin, INR and lactate was performed preoperatively, directly after PM, at the end of surgery, at 8 pm on the day of surgery and on POD 1-4 at 7 am. All measurements were performed according to laboratory standards at the Department of Clinical Chemistry, University Hospital, Linköping, Sweden. In addition, capillary glucose levels were measured every 4th hour.

Tissue sampling

Animals

Directly after the final blood sampling, the liver was harvested. For study I, the tissue was preserved in formalin for histological analysis; for study III, the tissue was preserved in liquid nitrogen. In study IV, both methods were used. In study IV, liver biopsies were also obtained after 15 minutes of ischemia and after 40 minutes of ischemia; this tissue was stored immediately in liquid nitrogen.

The tissue sampled for histology was fixed in formalin (4%) overnight and embedded in Technovit 8100-plastic. Liver sections (2 μ m) were hematoxylin-eosin (H&E) stained and coded before examination by a pathologist blind to the experimental design (studies I and IV). In study I, samples from 3 randomly chosen animals in each group were examined, whereas samples from all the animals were examined in study IV.

The degree of liver injury was estimated using the scoring system described by Calabrese et al. (267). The signs noted are as follows: sinusoidal congestion, cytoplasmic vacuolation, and liver necrosis. Sinusoidal congestion was scored as 0-4, where 0 = none, 1 = less than 10% hepatic tissue, 2 = 10-40%, 3 = 40-70% and 4 = greater than 70%. Cytoplasmic vacuolation was scored as 0-4: 0=none, 1= less than 10%, 2=10-40%, 3=40-70% and 4= more than 70% of hepatic tissue. Liver necrosis was scored as 0-2, where 0 = none, 1 = less than half of the hepatocytes and 2 = more than half of the hepatocytes.

Tissue preserved in liquid nitrogen was analyzed for iNOS and IL-1 receptor mRNA (study III) and iNOS mRNA (study IV) as described below.

Patients

The liver biopsies were immediately submerged in liquid nitrogen and later freeze-dried. Glycogen in the liver tissue was quantified with the BioVision Glycogen Assay kit (BioVision Research Products, Mountain View, CA, USA). Freeze-dried tissue was placed in ice-cold Eppendorf tubes, crushed with mortar and pestle and extracted into sterile water. Enzymes were then inactivated by boiling for 5 min. Subsequently, the tubes were centrifuged at 13,000 rpm for 5 min. The supernatants were added to 96-well plates. As recommended in the assay kit, the glucose levels in the extracts (corresponding to 500-1000 mg freeze-dried tissues) were measured with and without hydrolyzing the glycogen by glucoamylase. After the reaction with OxiRed, the absorbance was finally colorimetrically measured in an enzyme-linked immunoassay (ELISA) reader at 550 nm. The glycogen level was calculated

using the standard curve included in the kit, and the result is reported as μg glycogen per mg freeze-dried tissue.

NO_x (studies II, III and IV)

In the serum and microdialysate, the sum of NO_2^- and NO_3^- were analyzed following the instructions in the commercial “Nitrite/Nitrate Fluorometric Assay Kit”. Prior to analysis, the serum was ultrafiltrated through a 10-kDalton cut-off filter. The microdialysate was directly analyzed. Standard curves were plotted. For nitrate, 10 μL of sample was diluted in 70 μL assay buffer. Aliquots of 10 μL of enzyme cofactor and 10 μL of nitrate reductase mixture were added to the buffered sample. After 30 minutes of incubation at room temperature, 10 μL of DAN (2,3-diaminonaphthalene) was added and incubated for another 10 minutes. For nitrite, 10 μL of sample was diluted with 90 μL assay buffer, and 10 μL of DAN was added. Both nitrate and nitrite samples were then incubated for 10 minutes, and 20 μL of NaOH was added. All samples were read in triplicate with fluorometry using an excitation wavelength of 355 nm and emission wavelength of 430-460 nm.

iNOS mRNA in liver tissue (studies III and IV)

Approximately 10 mg of dry weight liver tissue was disrupted and homogenized in a Micro-Dismembrator at 2900 rpm for 30 sec. The RNA extraction was performed according to the manufacturer’s protocol (Qiagen, Valencia CA, USA) with the modification of eliminating β -mercaptoethanol. The RNA was eluted in 300 μL of RNase-free water, and the RNA concentration and purity (~ 2.0) were measured at 260 nm and 260/280 nm, respectively. RNA was stored at -70°C until assayed. Approximately 0.5 μg RNA was used in the reverse transcriptase cDNA conversion with High Capacity cDNA Reverse Transcription Kit in a total volume of 20 μL according to manufacturer’s protocol (Applied Biosystems, CA, USA). cDNA samples were stored at -20°C until assayed. For the real-time PCR reaction, 2 μL of sample containing 50 ng cDNA (0,025 $\mu\text{g}/\mu\text{L}$) was used in a total reaction volume of 20 μL . Samples were analyzed in triplicate with the Fast Master Mix and TaqMan Gene Expression Assay in a 7500 Fast Instrument. For results, the mean Ct values were normalized against the mean Ct for GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) and are presented as ΔCt .

IL-1R mRNA in liver tissue (study III)

Quantitative PCR for IL1R was performed with the Fast SYBR Green Master Mix. The final concentration of the primers was 100 nM. In the negative controls, cDNA was replaced by

distilled water. The final amount of cDNA in each reaction was comparable to 1 ng of RNA. The PCR program was set for 40 cycles of 95°C for 20 sec, 95°C for 1 sec and 60°C for 20 sec. At the end of the reaction, a dissociation curve analysis was performed. Specific primers for rat IL-1R were used. GAPDH and beta-actin (b-actin) were utilized as housekeeping genes. The primers were designed using Primer Express. All reactions, including no-template controls and endogenous control probes, were performed by the epMotion pipetting robot in duplicate. The results were analyzed using the ΔC_t method and are presented as relative gene expression. The 7900 Fast Real-Time PCR system with 7900 System SDS 2.3 Software was used according to the manufacturer's protocol.

Statistical methods

Animals

Data are presented as the means (SEMs) unless otherwise stated. A p -value < 0.05 was considered statistically significant for all statistical calculations. ANOVA followed by post-hoc analysis with Fisher's LSD method was used to compare multiple groups in study I. Temporal changes within the groups were analyzed using the t-test or the Mann-Whitney U test. Statistica 8.0 software (StatSoft Inc., Tulsa, Oklahoma, USA) was used for all statistical calculations.

Patients

Patients with IPC ($n = 16$) were compared with the controls ($n = 16$). The patients with IPC ($n = 8$) were then compared with the controls ($n = 8$) within the same stratum (2-3 segments and > 3 segments resected). The results are presented for these stratified groups unless otherwise indicated in the text.

A secondary comparison was made between minor (2–3 segments, $n = 16$) and major (>3 segments, $n = 16$) resections to control for confounding changes depending on resection size. The μD samples were grouped into the following 7 different phases: baseline (sample 1), preoperative (samples 2–7), post-operative on the day of surgery (samples 8–24), POD 1 (samples 25–30), POD 2 (samples 31–36), POD 3 (samples 37–42) and POD 4 (samples 43–46). Individuals were nested in the IPC and control groups and assigned as the random factor in an ANOVA. A Tukey post-hoc analysis was performed if significant differences were observed between the groups in the ANOVA. Non-parametric values are presented as medians (ranges) and were analyzed with the Mann–Whitney U test; parametric values are presented as the means (SEMs) and were analyzed using Student's t-test. STASTICA 8.0

software (StatSoft; Tulsa, OK, USA) was used for all statistical calculations. A p -value of < 0.05 was considered statistically significant.

15. Results

Study I

Blood analyses

Serum AST levels were increased in the IRI group (155 (20.9) IU/L) compared with the IPC group (71.5 (19.6) IU/L) or in the R-IPC group (96.6 (12.4) IU/L) at 4 hours of reperfusion. The differences were significant with p -values of 0.004 and 0.04, respectively. However, the difference between the IPC and R-IPC groups was not significant (figure 14).

After 4 hours of reperfusion, serum ALT levels were significantly increased in the IRI group (107.4 (15.5) IU/L) compared with the IPC group (41.6 (11.3) IU/L) ($p = 0.003$). The levels in the R-IPC group (68.1 (14.9) IU/L) did not differ significantly from the IRI group ($p = 0.064$) or the IPC group (figure 15). In the sham group, only a small increase in the AST and ALT levels was observed during the reperfusion.

Figure 14. Serum AST levels (study I). Mean $\pm$ SEM serum AST levels after 1-hour segmental liver ischemia followed by 4 hours of reperfusion in rats pre-treated with IPC or R-IPC compared with non-treated rats (IRI). The shaded area represents the ischemic phase.
* $p = 0.004$ (IRI vs. IPC) and $p = 0.04$ (IRI vs. R-IPC).

Figure 15. Serum ALT levels (study I). Mean $\pm$ SEM serum ALT levels after 1-hour segmental liver ischemia followed by 4-hour reperfusion in rats pre-treated with IPC or R-IPC compared with non-treated rats (IRI). The shaded area represents the ischemic phase.

* $p = 0.003$ (IRI vs. IPC).

Glucose

Glucose increased approximately 2-fold in the ischemic lobe in the IRI group compared with the control lobe during ischemia. In both the IPC and the R-IPC groups, similar patterns were noted, although the overall changes were of a reduced magnitude.

In the IPC group, glucose levels increased significantly to 10.2 (0.7) mM in the ischemic lobe after preconditioning ($t = 20$) compared with 7.2 (0.4) mM at baseline ($t = 0$, $p = 0.0002$) (figure 16). This early increase in parenchymal glucose was not observed in the IRI or the R-IPC groups. At $t = 20$, glucose levels were significantly increased in the ischemic lobes in the IPC group (10.2 (0.7) mM) compared with the corresponding lobes in the IRI group (7.5 (0.6) mM) ($p = 0.006$) and with the ischemic lobe in the R-IPC group (7.0 (0.6) mM) ($p = 0.001$). On the other hand, the rise in glucose during ischemia was less pronounced in the IPC (7.1 (1.2) mM) group than in the IRI (12.7 (1.6) mM) group. At $t = 110$, this difference achieved statistical significance ($p = 0.005$). In the R-IPC (10.3 (1.1) mM) group, a trend towards increased glucose levels was observed compared with the IPC group, although the difference was not statistically significant ($p = 0.091$).

Figure 16. μ D glucose (study I). Mean $\pm$ SEM μ D glucose in the ischemic liver segment after 1-hour segmental liver ischemia and 4-hour reperfusion in rats pre-treated with IPC or R-IPC compared with non-treated rats (IRI) and the combined results from the control (non-ischemic) segments (control). The shaded area represents the ischemic phase.

* $p = 0.006$ (IRI vs. IPC) and $p = 0.001$ (IPC vs. R-IPC).

** $p = 0.005$ (IRI vs. IPC).

Pyruvate

In the ischemic segment in the IRI group, pyruvate levels were reduced to approximately 20% of the level in the control segment at the end of ischemia. The corresponding values in the IPC and R-IPC groups were 65% and 40%, respectively. Given the large interindividual variations in the pyruvate levels, no significant differences were noted between the groups (figure 17).

In the sham group, no significant differences were noted between the two lobes in the microdialysate (data not shown).

Figure 17. μD pyruvate (study I). Mean $\pm$ SEM μD pyruvate in the ischemic liver segment during 1-hour segmental liver ischemia and 4-hour reperfusion in rats pre-treated with IPC or R-IPC compared with non-treated rats (IRI) and the combined results from the control (non-ischemic) segments (control). The shaded area represents the ischemic phase.

Lactate

In ischemic lobes in the IRI group, the lactate levels increased 3 fold during the ischemic phase compared with the control lobe. The same pattern was observed in the other groups. In the IPC group, increased lactate was observed in the ischemic segment during preconditioning ($t = 20$). This increase was 2.5-fold higher than the initial value ($t = 0$; from 0.92 (0.08) to 2.5 (0.2) mM) ($p < 0.001$). This change was not observed in the IRI or the R-IPC groups. The differences between the IPC group compared with the IRI and R-IPC groups (2.5 (0.2) mM vs. 1.3 (0.2) and (1.8 (0.4) mM) at $t = 20$ were statistically significant ($p = 0.002$ and $p = 0.045$, respectively). Otherwise, no differences were observed between the groups (Figure 18).

Figure 18. μ D lactate (study I). Mean $\pm$ SEM μ D lactate in the ischemic liver segment during 1-hour segmental liver ischemia and 4-hour reperfusion in rats pre-treated with IPC or R-IPC compared with non-treated rats (IRI) and the combined results from the control (non-ischemic) segments (control). The shaded area represents the ischemic phase.

* $p = 0.002$ (IRI vs. IPC) and $p = 0.045$ (IPC vs. R-IPC).

Glycerol

An approximately 7-fold increase in glycerol was observed in ischemic lobes compared with control lobes in the IRI group during the ischemic phase. Both the IPC and R-IPC groups exhibited similar patterns, but the increase in the IPC group was less pronounced than in the other two groups. At the end of ischemia ($t = 80$), glycerol was significantly lower in the IPC group (514 (70) μM) in the ischemic lobe than in the IRI group (731.8 (66.8) μM) and the R-IPC group (759 (84) μM) ($p = 0.046$ and $p = 0.022$, respectively). An early significant increase in glycerol was observed after preconditioning ($t = 20$) in the IPC group compared with $t = 0$ ($p = 0.001$). This effect was not observed in the IRI or R-IPC groups. Glycerol, in the ischemic lobe, returned to the level of the control lobe after approximately 90 minutes of reperfusion (figure 19).

Figure 19. μD glycerol (study I). Mean $\pm$ SEM μD glycerol in the ischemic liver segment during 1-hour segmental liver ischemia and 4-hour reperfusion in rats pre-treated with IPC or R-IPC compared with non-treated rats (IRI) and the combined results from the control (non-ischemic) segments (control). The shaded area represents the ischemic phase.

* $p = 0.001$ ($t = 0$ min vs. $t = 20$ min in IPC).

** $p = 0.046$ (IRI vs. IPC) and 0.022 (IPC vs. R-IPC).

Histology

The only abnormal histological finding in the control lobes (all groups and all stratas) was slight sinusoidal congestion of the liver parenchyma (scores 0-1).

In the ischemic lobes (all groups), sinusoidal congestion tended to be more obvious after 4 hours of reperfusion (scores 2-4) than in the early state (1-hour reperfusion). Histological signs of ischemia-reperfusion injury, identified as hepatocellular cytoplasmic vacuolation, sinusoidal congestion and hepatocyte necrosis, were more obviously observed in the IRI and the R-IPC groups than in the IPC group after 4-hour reperfusion. According to the grading system, the scores were higher in these 2 groups than in the IPC group. Figure 20 presents representative slides from the livers of the different groups after 4-hours reperfusion.

Figure 20. *Histology (study I). H&E-stained (x 40 magnification) specimens from the ischemic lobes of rat livers after 1-hour segmental ischemic followed by 4-hour reperfusion. IR only (A), IR after IPC (B), IR after R-IPC (C) and sham operated (D).*

Study II

Demographics

The IPC group (n = 16) and the control group were similar with respect to demographics (age, sex and chemotherapy before operation), operative data (bleeding, transfusion, durations of ischemia, resections volume and glycogen levels) and length of stay.

When stratified according to resection size, subjects undergoing major resection (> 3 segments) had larger liver volumes resected than those having three or less segments resected (700 (87) vs. 184 (26) ml, $p < 0.001$). The transectional area was also larger in major resections (122 (7) vs. 93 (9) cm², $p < 0.05$). The operation time was longer for the major resections group ((328 (24) vs. 251 (13) min, $p < 0.05$). The length of stay was longer for major resections compared with minor resections (10 vs. 9 days, $p < 0.05$). Table 3 presents the demographic and perioperative data.

	2 - 3 segments		> 3 segments	
	IPC (n = 8)	Control (n = 8)	IPC (n = 8)	Control (n = 8)
Sex, (% males)	4 (50)	5 (62)	4 (50)	5 (62)
Age (years)	67 (15)	62 (9)	60 (13)	66 (10)
Preop glycogen levels (µg/mg fdt)	40 (6.0)	42 (9.8)	41 (7.4)	37 (7.7)
Preop chemotherapy (n (%))	4 (50)	3 (38)	7 (88)	6 (75)
Units of insulin given (total # (range))	51 (0-47)	24 (0-20)	84 (0-60)	60 (0-60)
LOS (days)	7-13 (9)	6-11 (6)	8-13 (10)	7-51 (11)
Blood loss (mL)	100-1700 (359)	50-2400 (300)	100-1500 (625)	200-1800 (750)
Units of blood transfused (total # (range))	6 (0-4)	4 (0-2)	10 (0-5)	7 (0-3)
Units of plasma transfused (total # (range))	9 (0-7)	7 (0-5)	13 (0-6)	18 (0-7)
Resected volume (mL)	55-340 (190)	60-400 (165)	225-1150 (698)	300-1200 (550)
Transected area (cm ²)	91 (36)	95 (40)	132 (33)	113 (23)
Bleeding/cm ² (mL/cm ²)	1.0-11 (5.2)	0.5-15 (5.9)	0.9-25 (4.4)	2.3-21 (6.6)
Duration of ischemia (min)	44 (10)	44 (13)	35 (11)	44 (8.0)
Transection time (min)	64 (22)	77 (40)	62 (32)	60 (11)
Duration of Surgery (min)	242 (57)	261 (42)	292 (60)	359 (112)
Bleeding/min (transection) (mL/min)	1.8-19 (5.7)	0.9-20 (6.5)	2.6-36 (9.2)	3.9-42 (11.2)
Bleeding/min (total) (mL/min)	0.5-5.2 (1.8)	0.6-2.9 (1.1)	0.4-5.2 (2.0)	0.9-4.7 (3.6)
Complications (n (%))	3 (38)	2 (25)	4 (50)	3 (38)
Diagnosis:				
Metastases (n (%))	7 (88)	4 (50)	8 (100)	7 (88)
Benign (n (%))	1 (12)	3 (38)	0	0
Carcinoma (HCC?) (n (%))	0	0	0	1 (12)
Gall bladder cancer (n (%))	0	1 (12)	0	9
Steatosis (n (%))	1 (12)	0	0	3 (38)
Portal fibrosis (n (%))	1 (12)	0	0	0

Table 3. Demographic and perioperative data (study II). Patients underwent operations for resections of either 2-3 liver segments or > 3 liver segments with or without pre-treatment with IPC.

Diagnosis and complications

The most common diagnosis was liver metastases ($n = 26$) followed by benign lesions ($n = 4$), gallbladder cancer ($n = 1$) and hepatocellular cancer ($n = 1$).

Complications occurred in 12 (37.5%) patients, and no differences were noted between the groups (table 3).

Blood analyses

When serum and blood analyses were compared, no difference was observed between the IP group and controls. However, patients treated with IPC before major resections exhibited reduced levels of lactate (2.4 (0.1) vs. 4.4 (0.5) mM, $p < 0.05$) and INR (1.2 (0.04) vs. 1.5 (0.04) $p < 0.05$) immediately postoperatively compared with controls.

Microdialysis

Minor resections

The only difference noted between the treatment and control groups undergoing minor resections was that glucose was reduced in the treatment group on POD 2 compared with the control group.

Major resections

Immediately after surgery and up to POD 1, the levels of μ D-glucose, μ D-pyruvate and μ D-lactate were lower in the IPC group compared with the controls.

Glucose was reduced in the IPC-treated group compared with the control group (4.9 (0.1) vs. 6.6 (0.1) mM, $p < 0.001$) on the operation day (figure 21). In addition, on POD 2, glucose was lower (6.1 (0.1) vs. 8.3 (0.2) mM, $p < 0.05$) in patients treated with IPC before major resection compared with controls.

Figure 21. μ D glucose (study II). Mean $\pm$ SEM μ D glucose levels in patients undergoing major liver resection with IPC and without IPC (control) before intermittent PM. Tran... = Transection phase. *** $p < 0.001$. * $p < 0.05$.

Similarly, the pyruvate level was lower in the IPC-treated group than in controls (147 (3.2) vs. 198 (3.5) μM , $p < 0.001$) on the operation day (figure 22). From POD 1 onwards, no significant differences in the pyruvate levels were noted between the groups.

Figure 22. μD pyruvate (study II). Mean $\pm$ SEM μD pyruvate levels in patients undergoing major liver resection with IPC and without (control) IPC before intermittent PM. Tran... = Transection phase. *** = $p < 0.001$.

Lower lactate levels were observed during the transection phase in IP-treated patients undergoing major resections than in controls (4.25 (0.12) vs. 5.8 (0.13) mM, $p < 0.001$). This difference continued through the entire day of operation (2.6 (0.06) vs. 4.0 (0.08) mM, $p < 0.001$) and was also observed on POD 2 (1.8 (0.05) vs. 2.3 (0.06) mM $p < 0.05$) (figure 23).

Figure 23. μ D lactate (study II). Mean (SD) μ D lactate levels in patients undergoing major liver resection with and without IPC before intermittent PM.
 Tran... = Transection phase.
 *** = $p < 0.001$.
 * = $p < 0.05$.

Glycerol levels did not differ in μ D between the groups, and no difference was found between major and minor resections.

NO_x levels decreased continuously postoperatively in the IPC group from 31 (3) to 22 (3) μM ($p < 0.01$) up to POD 3, when the levels started to return to the levels seen before surgery. In the control group, a similar pattern was observed, and no difference was noted between the groups. The NO_x levels in the μ D were not affected by the volume of the resection.

Glycogen

No difference in glycogen levels was noted between the groups or between major and minor resections. Male patients exhibited increased glycogen levels compared with females (45.0 (0.5) vs. 36.7 (0.5) $\mu\text{g}/\text{mg}$ freeze-dried tissue, $p < 0.05$).

Study III

NOx in serum

In the R-IPC group, an initial rise (20.0 to 25.2 μM) during ischemia and 1-hour reperfusion followed by a decline (25.2 to 23.4 μM) during later parts of reperfusion was observed. In the IPC group, the rise (20.2 to 27.7 μM) was more pronounced early than in the R-IPC group (ns), but the decline (27.7 to 23.4 μM) started directly in the reperfusion phase (figure 24). In both preconditioning groups, the rise and decline in S-NOx was statistically significant (both groups $p < 0.001$), but no significant difference was noted between the two groups.

Figure 24. Serum NOx (study III). Mean $\pm$ SEM se-NOx in rats treated with IPC, R-IPC or no preconditioning before 1-hour segmental liver ischemia followed by 4-hour reperfusion. The shaded area represents ischemic phase.

NOx in microdialysate fluid

In both the IPC (11.8 to 6.4 μM) and R-IPC (12.3 to 4.7 μM) groups, significant decreases in NOx in the μD fluid were observed at the end of ischemia ($p = 0.007$ and $p = 0.002$, respectively) compared with the levels in early ischemia. In both groups, the levels quickly increased again during reperfusion (figure 25). In the control lobes, only small variations were observed, and no difference was noted between the groups (data not shown).

Figure 25. $\mu\text{D NOx}$ (study III). Mean $\pm$ SEM NOx in the microdialysate in rats treated with IPC, R-IPC or no preconditioning before 1-hour segmental liver ischemia followed by 4-hour reperfusion. The shaded area represents ischemic phase.

iNOS mRNA in liver tissue

iNOS transcription at the end of reperfusion was significantly increased in both the IPC (ΔCt 5.86) and R-IPC (ΔCt 3.44) groups compared with levels at the end of ischemia (ΔCt 10.78 and 11.49, respectively) ($p < 0.001$ in both groups) (figure 26). When the groups were compared, level of iNOS mRNA was significantly higher in the R-IPC group than in the IPC group at the end of reperfusion ($p = 0.03$).

Figure 26. *iNOS transcription (study III). Mean $\pm$ SEM iNOS mRNA in liver tissue from rats treated with IPC, R-IPC or no preconditioning before 1-hour segmental liver ischemia followed by 4-hour reperfusion.*

* = $p < 0.05$.

IL-1 receptor mRNA in liver tissue

IL-1 receptor expression was measured with semi-quantitative real-time PCR. In the IPC group, a trend towards reduced IL-1 transcription (ΔC_t 1.88 to 4.81) was noted during reperfusion, although this trend did not reach statistical significance ($p = 0.0636$). In the R-IPC group, this decrease was not observed given that the transcriptional activity increased slightly (ΔC_t 3.26 to 2.99) (ns) (figure 27).

When IPC and R-IPC were compared, the IPC group exhibited significantly increased IL-1 receptor mRNA levels at the end of ischemia ($p = 0.0034$). At the end of reperfusion, the R-IPC group exhibited a trend towards higher transcription of IL-1 receptor mRNA compared with the IPC group; however, this difference was not statistically significant ($p = 0.273$).

Figure 27. *IL-1R transcription (study III). Mean $\pm$ SEM IL-1R mRNA in liver tissue from rats treated with IPC, R-IPC or no preconditioning before 1-hour segmental liver ischemia followed by 4-hour reperfusion.*

* = $p < 0.05$ (IPC vs. R-IPC).

Study IV

Blood analyses

Liver transaminases

Higher serum AST levels (figure 28) were observed in the IRI group (40 (6.8) $\mu\text{kat/l}$) after 4 hours of reperfusion than in the nitrite-treated group (22 (2.6) $\mu\text{kat/l}$) ($p = 0.022$).

Figure 28. Serum AST (study IV). Mean $\pm$ SEM se-AST in rats subjected to 45-minute segmental (left lateral lobe) liver ischemia and 4-hour reperfusion with (Nitrite) or without (IRI) pre-treatment with 480 nmol nitrite intravenously. Animals treated with nitrite before ischemia and reperfusion exhibited significantly lower AST levels than untreated animals.

* $p = 0.022$.

ALT levels (figure 29) at 4 hours of reperfusion were significantly increased in the IRI group (34 (6) $\mu\text{kat/l}$) compared with the nitrite group (14 (1.5) $\mu\text{kat/l}$) ($p = 0.0045$). AST and ALT levels were approximately normal after reperfusion in the sham group.

Figure 29. Serum ALT (study IV). Mean $\pm$ SEM se-ALT in rats subjected to 45-minute segmental (left lateral lobe) liver ischemia and 4-hour reperfusion with (Nitrite) or without (IRI) pre-treatment with 480 nmol nitrite intravenously. Animals treated with nitrite before ischemia and reperfusion exhibited significantly lower AST levels than untreated animals. * $p = 0.0045$.

NO_x

The sum of nitrite and nitrate (NO_x) measured in the serum at the end of reperfusion did not significantly differ between the groups.

NO_x

NO_x in the liver tissue measured via microdialysis was significantly ($p = 0.031$) increased in the nitrite group ($10.1 \pm 2.9 \mu\text{M}$) compared with the IRI group ($3.2 \pm 0.9 \mu\text{M}$) after the administration of nitrite (figure 30). During ischemia, the levels of NO_x decreased in both groups and thereafter increased during reperfusion. After 4-hour reperfusion, a trend towards increased levels of NO_x in the IRI group compared with the nitrite group was noted ($p = 0.067$).

Figure 30. $\mu\text{D NO}_x$ (study IV). Parenchymal (microdialysis) NO_x (nitrite and nitrate) (mean $\pm$ SEM) in the ischemic liver lobe in rats subjected to 45-minute segmental (left lateral lobe) liver ischemia and 4-hour reperfusion with (Nitrite) or without (IRI) pre-treatment with 480 nmol nitrite intravenously. After the administration of nitrite, parenchymal NO_x was increased compared with animals not receiving nitrite ($p = 0.031$). During ischemia, the levels decreased in both groups and then increased again during reperfusion. After 4-hour reperfusion, a tendency towards increased levels in animals not treated with nitrite was noted ($p = 0.067$). The shaded area represents the ischemic phase.

Glucose

In the both groups, glucose increased approximately 2 fold (6 to 12 mM) during ischemia in the ischemic lobe compared with the control lobe as well as the pre-ischemic value in the ischemic lobe. However, no significant difference was observed between the groups (figure 31).

Figure 31. μD glucose in the ischemic lobe (study IV). Parenchymal (microdialysis) glucose (mean $\pm$ SEM) in the ischemic liver lobe in rats subjected to 45-minute segmental (left lateral lobe) liver ischemia and 4-hour reperfusion with (Nitrite) or without (IRI) pre-treatment with 480 nmol nitrite intravenously. During ischemia, parenchymal glucose increased in both groups, and no difference was noted between the groups. In the reperfusion phase, a steady decline in glucose levels was noted. The shaded area represents the ischemic phase.

In the control lobe in the nitrite group, a significant ($p < 0.001$) increase (6.1 to 7.7 mM) in glucose was observed after nitrite administration. At $t = 00:43$, during ischemia and early (30 minutes) reperfusion, the difference between the groups was significant ($p = 0.049$ and $p = 0.02$, respectively) (figure 32).

Figure 32. μ D glucose in the control lobe (study IV). Parenchymal (microdialysis) glucose (mean $\pm$ SEM) in the control (non-ischemic, right) liver lobe in rats subjected to 45-minute segmental (left lateral lobe) liver ischemia and 4-hour reperfusion with (Nitrite) or without (IRI) pre-treatment with 480 nmol nitrite intravenously. After the administration of nitrite, parenchymal glucose increased significantly ($p < 0.001$) and achieved levels higher than in the untreated group ($p = 0.02$).

* $p < 0.001$ ($t = 00:20$ vs. $t = 00:42$ in Nitrite) and $p = 0.02$ (IRI vs. Nitrite).

Pyruvate

In the IRI group, pyruvate in the ischemic segment was reduced to approximately 40% of the level in the control segment at the end of ischemia. In the nitrite group, the corresponding value was 15%. In the early phase of reperfusion, there was a transient increase in both groups. However, large interindividual variations were observed in the pyruvate levels and no significant differences were noted.

Lactate

The lactate levels increased more than four fold in the ischemic lobe (4 mM) compared with the control lobe (0.7 mM) in the IRI group during the ischemic phase. The nitrite group followed similar pattern (3.3 and 1 mM, respectively), although the change was less in magnitude.

During ischemia and during the first 30 min of reperfusion, lactate was significantly increased in the IRI group compared with the nitrite group ($p = 0.01$) (figure 33).

Figure 33. μD lactate in the ischemic lobe (study IV). Parenchymal (microdialysis) lactate (mean $\pm$ SEM) in the ischemic liver lobe in rats subjected to 45-minute segmental (left lateral lobe) liver ischemia and 4-hour reperfusion with (Nitrite) or without (IRI) pre-treatment with 480 nmol nitrite intravenously. During ischemia, parenchymal lactate increased in both groups, and during the ischemic phase and the first 30 minutes of reperfusion, the levels were increased in the untreated group than in the treated animals. The shaded area represents the ischemic phase.

* $p = 0.01$ (IRI vs. Nitrite).

Lactate increased from 0.9 to 1.2 mM ($p = 0.012$) in the control lobe in the nitrite group after the administration of nitrite; this effect that was not observed in the IRI group. Furthermore, the levels of lactate were significantly ($p = 0.01$) increased during the first 95 minutes of the experiment in the control lobe in the nitrite group compared with the IRI group (figure 34).

Figure 34. μ D lactate in the control lobe (study IV). Parenchymal (microdialysis) lactate (mean $\pm$ SEM) in the control (non-ischemic, right) liver lobe in rats subjected to 45-minute segmental (left lateral lobe) liver ischemia and 4-hour reperfusion with or without pre-treatment with 480 nmol nitrite intravenously. After the administration of nitrite, parenchymal lactate increased significantly ($p = 0.012$) and achieved levels higher than in the untreated group ($p = 0.01$).

* $p = 0.012$ ($t = 00:20$ vs. $t = 00:42$ Nitrite) and $p = 0.01$ (IRI vs. Nitrite).

Glycerol

An approximately 38-fold increase in glycerol in the ischemic lobe (18 to 686 μM) was noted in the IRI group compared with the control lobe. The nitrite group exhibited the same pattern, but the increase (25 to 602 μM) was less pronounced than that observed in the IRI group (24-fold).

During the ischemic phase and during the early 30 minutes of the reperfusion phase, glycerol levels were significantly higher in the IRI group than in the nitrite group ($p = 0.049$). In the ischemic lobe, the glycerol level returned to the level of the control lobe after approximately 60 minutes of reperfusion (figure 35).

Figure 35. μD glycerol (study IV). Parenchymal (microdialysis) glycerol (mean $\pm$ SEM) in the ischemic liver lobe in rats subjected to 45-minute segmental (left lateral lobe) liver ischemia and 4-hour reperfusion with or without pre-treatment with 480 nmol nitrite intravenously. During ischemia, parenchymal glycerol increased in both groups. During the ischemic phase and the first 30 minutes of reperfusion, the levels were increased in the non-treated group ($p = 0.049$). The shaded area represents the ischemic phase.

* $p = 0.049$ (IRI vs. Nitrite).

In the sham group, no significant differences were noted between the two lobes in the microdialysate (data not shown).

iNOS mRNA

Fifteen minutes into the ischemic phase, no difference in iNOS mRNA was noted between the groups (IRI $\Delta\text{Ct} = 9.3 \pm 0.72$, ΔCt nitrite = 8.6 ± 0.24). In both groups, iNOS transcription increased at the end of the experiment to $\Delta\text{Ct} = 3.8 \pm 0.52$ in the IRI group and 3.4 ± 0.26 in the nitrite group ($p < 0.01$ in both groups). However, no significant difference was noted between the groups.

Histology

All sham animals exhibited only zero scores except for 1 animal that scored 1 (10 - 40% of hepatic tissue) with respect to sinusoidal congestion. In the IRI group, the mean score for sinusoidal congestion was 1.29 compared with 0.86 in the nitrite group. The mean score for cytoplasmic vacuolization was also higher in the IRI group (0.71) than in the nitrite group (0.43). Of the 14 animals in the IRI group, 4 (29%) exhibited signs of necrosis on histology, whereas no animals in the nitrite group exhibited necrosis (figure 36).

Figure 36. Histology (study IV). H&E-stained liver tissue from the ischemic liver lobes in rats subjected to 45-minute segmental (left lateral lobe) ischemia and 4-hour reperfusion. (A and D) Without pre-treatment, (B and E) with 480 nmol nitrite administered intravenously before ischemia and (C) sham operated. A – C 20 x magnification, D and E 40 x magnification.

16. Discussion

This thesis focuses on methods to prevent or reduce IRI in the liver. As a secondary focus, this thesis explores the use of microdialysis as a tool for continuously monitoring IRI.

In liver surgery, some degree of I/R is often inevitable, and it has become clear that IRI may have detrimental effects on clinical outcomes. As liver surgery continues to increase, both in terms of the number of patients undergoing operation as well as in the complexity of the procedures, this phenomenon has gained much interest in recent decades. This increasing interest has led to a large amount of research, both in the pursuit of increased understanding of the phenomenon and to identify methods to diminish its effect. Although this search for understanding has lasted over 20 years, much is still unknown about the mechanisms involved, and no reliable method to abolish the injury has been described. Hepatic IRI is known to be a multifactorial cascade involving many cell types and substances. As the process is complex and dynamic, results are often divergent and difficult to extrapolate to other slightly different scenarios. The methods used to investigate IRI in the past have ranged from tissue analysis to serum analysis of peripheral sampled blood or blood samples drawn in proximity to the liver. The lack of methods to investigate changes in the liver continuously inevitably leads to the sacrifice of a number of laboratory animals to make up for these shortcomings. In addition, clinical studies involving multiple biopsies from the liver are unethical and hazardous, and monitoring the changes using only blood tests is an inadequate method. Recent technical advances that may account for the shortcomings of the traditional methods described above include microdialysis, laser Doppler imaging, intravital microscopy and functional magnetic resonance imaging. Microdialysis offers a tool for investigating the changes that occur within the liver parenchyma in real time.

The method to counteract IRI when vascular occlusion is applied that has garnered the most support in the literature is the use of intermittent PM instead of continuous PM; however, the difference between these methods is small (94). Another available method that has been explored in the clinical setting is IPC, as listed in table 2. R-IPC is an exciting alternative to IPC that could reduce the inherent risk of the IPC procedure and shorten operation times. R-IPC has only been reported in one small clinical trial (251). Despite an increased interest in R-IPC and a number of experimental studies demonstrating a protective effect of R-IPC on IRI, R-IPC has not been compared directly to IPC. If preconditioning is to be used in the clinical setting, it is important to know how to apply it. Therefore, study I was

conducted to compare IPC and R-IPC. If μ D is to be used in the clinic, it is advantageous to be able to use commercially available kits for the analysis; therefore, study I also included the hypothesis that μ D could be performed exclusively with commercially available kits to monitor IRI in the liver.

IPC is more effective than R-IPC in reducing AST and ALT early (after 4 hour) in the reperfusion phase of IRI (figures 14 and 15, study I). Furthermore, animals treated with IPC exhibit fewer histological signs of IRI after IR than animals treated with R-IPC (figure 20). Similarly, less μ D glycerol is detected after IR when animals are pre-treated with IPC than when R-IPC is applied as a pre-treatment (figure 19). The substances measured with μ D in study I represent metabolic markers (glucose, pyruvate and lactate) and an injury marker (glycerol). The changes in these markers detected with μ D were those expected with anaerobic metabolism with sufficient availability of glycogen (increased glucose and lactate) and cell damage (increased glycerol).

The increase in glucose noted with microdialysis in the IPC group prior to ischemia (figure 16) likely resulted from the preconditioning. This increase may be due to anaerobic metabolism already occurring during the short ischemia produced by preconditioning. However, glycolysis, which provides additional energy, is also a possible explanation for the increase. In a study using a porcine model, it was shown that glycogen in the liver is depleted during ischemia, a finding that may support this explanation (268). On the other hand, the significant increase in lactate noted in the same segment (figure 18) supports the explanation that the preconditioning initiates anaerobic metabolism. The increase in glycerol noted at the same time further supports the notion that the preconditioning results in some degree of membrane disruption.

The protective effect of IPC in the clinical setting has largely been investigated with continuous PM. However, any difference between intermittent and continuous PM that might exist is to the advantage of intermittent PM. With this fact in mind and given the results indicating that a greater effect can be expected from IPC than R-IPC (study I), it seems reasonable to investigate the effect of IPC before intermittent PM in the clinical setting.

Although study II was somewhat underpowered, the study revealed no clinical advantages when IPC was added to intermittent PM. In the setting of minor liver resections (2-3 segments), the only difference between patients who received IPC and those who did not

was in μ D glucose on POD 2. In the subgroups undergoing major liver resections (> 3 segments), a potentially beneficial effect of IPC was observed in laboratory values; lactate was reduced during the early post-operative phase in patients receiving IPC than in patients not receiving IPC (table 3). This finding was further supported by reduced μ D-glucose (figure 21), μ D-pyruvate (figure 22) and μ D-lactate (figure 23) immediately post-operative in the IPC group compared with the control group.

Although intermittent PM is recommended over continuous PM, the effectiveness of intermittent PM in the clinical setting in reducing IRI appears to be limited (94). The addition of IPC to intermittent PM is an insufficient means to counteract hepatic IRI (study II). In addition, it is somewhat cumbersome to apply IPC to all patients when PM is not always needed during liver resections. Our unit has changed the application of intermittent PM from routine application in a 15 + 5 fashion to a more selective application for shorter periods of time when needed. This approach may decrease the total I/R. Furthermore, this approach is often applied in a manner that mimics IPC, making it important to pursue other means of protecting the liver against IRI when vascular occlusion is utilized during liver resections.

NO has been implicated in both IRI mechanisms and preconditioning and appears to be one of the key substances involved in these processes. iNOS rather than eNOS has previously been shown to be responsible for the majority of the NO production during acute inflammation. Therefore, iNOS may be implicated in IRI. Study III was designed to investigate the mechanisms involved in IPC and R-IPC with a special focus on NO involvement.

In study III, iNOS transcription was shown to be significantly increased in the R-IPC group compared with the IPC group (figure 26). Furthermore, the transcriptional activity in the R-IPC group was approximately identical to what was observed in the IRI group. This difference is likely a sign of greater inflammatory activity in the R-IPC group than in the IPC group. IL-1 receptor transcription was increased directly after ischemia in the IPC group compared with the R-IPC group (figure 27). The only difference in the treatment of the groups, and therefore the only plausible explanation for this difference, was how the preconditioning was applied. The initial ischemic insult to the liver (IPC) may have triggered the transcription of the IL-1 receptor, resulting in reduced IL-1 receptor transcription during reperfusion due to negative feedback. In study III, NOx was decreased during ischemia in both groups (figure 25). Until recently, NOx has been considered a product of the elimination

of NO and has thus been used as a surrogate marker for NO production. If this is the case, NO_x would be expected to remain stable in ischemic tissue without any circulation. A possible explanation for the decrease in μ D-NO_x that occurs during ischemia is the reduction of nitrite to NO (and nitrate to nitrite) (figure 10) that occurs primarily in acidic environments but also under physiological circumstances. A further explanation might be that NO can bind to other metabolic groups, such as thiols (269, 270). Despite the changes in parenchymal NO_x detected with microdialysis, only minor changes were observed in the serum, indicating that serum measurements of NO_x do not accurately reflect the situation in the liver parenchyma.

As NO_x concentrations decreased in the liver parenchyma during ischemia (study III), it is possible that the administration of nitrite before the ischemic insult would decrease the IRI. When IRI is measured by serum AST and ALT levels 4 hours after 45-min segmental liver ischemia, the administration of nitrite before IR reduces IRI (figures 28 and 29, study IV). These findings are further supported by pathology findings demonstrating fewer signs of IRI in animals pre-treated with nitrite before IR than in non- treated animals (figure 36). Furthermore, lower parenchymal (μ D) glycerol levels accompany ischemia and early reperfusion when nitrite is administered before IR compared with no pre-treatment (figure 35), indicating less cell damage. This field has limited data; however, it has been shown in a mouse model that administering nitrite before the onset of reperfusion (intraperitoneal injection) decreases both liver and heart IRI (271). It has been proposed that the protective effect of nitrite can be attributed to modifications of the mitochondrial electron transport chain that slow the ATP production as well as the production of ROS (197, 272). In study IV, μ D-glucose (figure 32) was found to increase in the control lobe directly after injection in the group treated with nitrite compared with untreated animals. At the same time, μ D-lactate (figure 34) was also increased. These changes, although subtle, might reflect the metabolic effects on the inhibition of the electron transport chain. Another possible explanation for the protective effect of nitrite administration is the inhibitory effect of nitrite on platelets (273). As demonstrated in figure 8, platelets play an active role in the swelling of the liver sinusoids that is central to the IRI. It is, however, worth noting that this effect also may increase the risk for bleeding, which represents the livery surgery complication that the use of PM aims to prevent, and IRI is the main drawback of PM. Thus, the development of a method to reduce IRI that increases the risk of bleeding might be challenging.

During liver resections, a low CVP is frequently maintained to reduce bleeding. One of the methods by which this effect is achieved is the administration of intravenous nitroglycerine. Nitroglycerine is a compound with a short half-life that is metabolized to nitrite in the liver (274). The studies performed on low CVP have largely focused on the effect of this method on bleeding and possible side effects related to systemic hypoperfusion. However, an additional effect might be buried within the administration of nitrite that can exert a protective effect against IRI. In a small randomized study where nitroglycerine was used to lower CVP, a tendency towards reduced ALT postoperatively among patients in the low CVP group was noted (275). These findings potentially reflect effects of nitroglycerine and nitrite that should be further explored in the clinical setting.

In summary, IPC as well as R-IPC exhibits some protective effects against liver IRI; however, the clinical effectiveness of these methods is questionable. Nitrite administered before I/R also exerts a protective effect in these settings, and this finding should be further investigated in the clinical setting. Microdialysis appears to be a useful method to investigate liver IRI in experimental and clinical settings.

17. Conclusions

The main conclusions of this thesis are as follows:

IPC offers more protection against IRI than R-IPC when applied for the same amount of time.

μ D with commercially available reagent kits can be used to monitor hepatic anaerobic metabolism and IRI in experimental and clinical settings.

The addition of IPC to intermittent PM does not offer clinically significant advantages compared with intermittent PM alone.

IPC decreases IRI-induced transcription of iNOS compared with R-IPC.

The administration of nitrite before liver ischemia reduces IRI.

18. Future perspectives

Currently, no secure means are available to avoid IRI when vascular occlusion is used to reduce bleeding during liver surgery. It appears unreasonable to believe that such a trauma to a well-vascularized organ can ever be completely avoided by any means. Therefore, the best method to avoid IRI should be to limit the use of vascular occlusion as much as possible. The use of the PM has changed from continuous to intermittent, and in our unit, intermittent PM is seldom applied for 15 minutes. With better anesthesiological methods as well as surgical instruments and techniques, the use of vascular occlusion can be minimized, thus decreasing the need for means to protect the liver against IRI. The more selective application of the PM also makes preconditioning less attractive given that the use of preconditioning could prove unnecessary in numerous cases.

From the technical aspect of the work presented in this thesis, it is clear that microdialysis can be applied in clinical and experimental liver surgeries, and this technique has great potential to reveal more about the mechanisms involved after liver resection. One interesting field of innovation that has become a clinical reality during the last two years is the ALPPS procedure. During this treatment, the patient is subjected to two liver operations over a period of 1-2 weeks. This timeframe with fast hypertrophy of the liver is an exciting field for research that could add to the general understanding of liver hypertrophy. Because of the short time span between the two procedures planned for this patient group, μ D may serve as an optimal tool to investigate the causes of the hypertrophy.

Given that many liver surgery units prefer low CVP during liver surgery, which is often accomplished by treatment with intravenous nitroglycerine (glyceryl trinitrate), the results from study IV might become clinically relevant. Nitroglycerine exerts its vasodilatory effects via breakdown to NO, and NO rapidly oxidizes to nitrite. Thus, some of the beneficial effects of low CVP might be attributed to the use of nitroglycerine to achieve the low CVP. Fluid restriction is often used as the first method to maintain low CVP; we could compare the “classical approach” in patients who achieve the target CVP with a novel approach that omits this step and liberally applies nitroglycerine. In these settings, μ D may be applied to assess the concentration of NOx in relation to nitroglycerine administration. The efficacy and safety (including bleeding) of nitrite should be further examined in animal studies and hopefully later in the clinical setting.

What really is needed though is the development of a surgical technique not requiring vascular occlusion. Inventing new equipment allowing transection of liver parenchyma without bleeding in spite of intact circulation to the liver could eliminate the risk of ischemic injury to the remnant liver parenchyma.

Acknowledgments

The research presented within this book is the work of many people in a research group and others to whom I owe my sincere gratitude. This list includes but is not limited to the following:

Per Sandström. Primary supervisor and good friend. For always finding time in your rather busy schedule to discuss research findings and future steps. You have provided me with the opportunity to combine the research with the clinic in a most rewarding way.

Per Gullstrand. Earlier primary supervisor, current co-supervisor and good friend. For your enthusiasm, encouragement and the ability to ask critical questions and never accept incomplete answers.

Tommy Sundqvist. Co-supervisor. For encouraging me to study the mechanisms involved in our results, for stimulating discussions and for the help you have provided in looking for explanations to our findings.

Anders Winblad. Co-supervisor. For taking me into the world of liver IRI research without knowing me and for explaining many of the important steps involved. You have the ability to encourage and inspire your co-workers whenever needed.

Lena Trulsson. Earlier co-supervisor. For teaching me the fundamentals in laboratory science.

Linda Bojmar. Research engineer and fellow PhD student. For your patience and understanding and excellent work with the small samples provided to you. Good luck with your studies!

Hans Olsson. Co-author. Your pathology skills and critical thinking in the interpretation of the results have helped me better understand the limitations of my work.

Rune Sjö Dahl. Professor of surgery, experienced clinical and academic surgeon. Your advice and ability to ask the right questions are always welcomed and can only increase the quality of the work.

The staff at the upper GI unit. Thank you for being supportive and generally being a pleasure to work with.

Other colleges at the department of surgery. You have put up with me on a daily basis in clinical work as well as during periods of more research-orientated endeavors. Your patience, understanding and encouragement mean a considerable amount to me.

The staff at the surgical ward and department of anesthesia and operation. Thanks for all of the help provided during the clinical study and for the positive collaboration in the clinical work.

The staff at the animal laboratory. You have been helpful and positive throughout the time I have spent at your workplace.

And last but certainly not least:

My family. Thank you for all your love, support, encouragement and understanding. You made this journey worthwhile!

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