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Hepatic Ischemia-Reperfusion Injury

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To my Mum and Dad

ABSTRACT

Hepatic ischemia-reperfusion (I/R) injury is a complex phenomenon occuring in response to interruption of the liver's blood and oxygen supply and the subsequent restoration of blood flow and tissue oxygenation. Techniques to reduce blood loss and other intra-operative manoeuvers during liver resection can cause hepatic I/R injury. I/R injury to the liver is also unavoidable during the transplantation procedure. This directly impacts liver viability with consequences ranging from mild organ dysfunction to hepatic failure. Hepatic I/R injury has been extensively studied but there is still much to be understood.

Paper I studied the effect of portal triad clamping (PTC) on hepatic metabolism in patients undergoing liver resection using intrahepatic microdialysis to monitor glucose, lactate and pyruvate as markers of ischemia and glycerol as a marker of cell membrane damage. The lactate/pyruvate ratio (L/Pr) was also calculated. PTC induced considerable alterations, with anaerobic metabolism and increased glycogenolysis manifested by increased levels of glucose, lactate and L/Pr and cell membrane damage evidenced by increased levels of glycerol

Papers II and III were methodological studies of hepatic microdialysis in pig models. We could show that microdialysis catheters with membrane cut-off of 20 and 100 kDa could be used equally in hepatic microdialysis for monitoring the products of glucose metabolism and glycerol. However, microdialysis performed using a catheter placed directly in the middle hepatic vein was not equivalent to direct intrahepatic monitoring of the same metabolites.

Paper IV investigated the effects of warm I/R injury induced by PTC on hepatic morphology at the ultrastructural level and on the expression of the thioredoxin and glutaredoxin redox systems. On electron microscopy, a significant loss of the liver sinusoidal endothelial cell (LSEC) lining was observed and a decrease of hepatocyte microvilli. Hepatocellular morphology was well preserved apart from the appearance of crystalline mitochondrial inclusions. After reperfusion the LSEC lining showed signs of reactivation. No significant changes were observed in the TRX and GRX redox systems.

Paper V explored the value of L/Pr measured by microdialysis as a marker for ischemic complications in 45 patients undergoing liver transplantation (LT). Raised L/Pr defined according to protocol were identified in 24 patients but none were predictive of clinically significant ischemic complications. L/Pr is thus not a reliable marker of clinically significant ischemic events after LT.

Paper VI evaluated microdialysis as a postoperative monitoring tool for detection of acute cellular rejection (ACR) in patients undergoing LT. ACR was diagnosed in 33 of 71 transplanted patients. Results revealed metabolic patterns indicating a possible relation between the severity of primary I/R injury and the development of ACR.

In conclusion, warm ischemia induced by PTC causes significant alterations in hepatic metabolism and ultrastructure. L/Pr measured by microdialysis is not a reliable marker for detecting clinically significant ischemic complications early after LT. Primary I/R injury experienced by the organ during the LT procedure may be associated with the development of ACR. It may be possible to monitor larger molecules using microdialysis with 100kDa catheters without affecting the monitoring of small molecules. To get reliable results when monitoring hepatic metabolism by microdialysis the catheter should be placed intrahepatically.

LIST OF SCIENTIFIC PAPERS

I.   Isaksson B, D'souza MA, Jersenius U, Ungerstedt J, Lundell L, Permert J, Björnstedt M, Nowak G.
Continuous assessment of intrahepatic metabolism by microdialysis during and after portal triad clamping.
J Surg Res. 2011 Aug;169(2):214-9.

II.   D'souza MA, Ravn A, Jorns C, Nowak G, Isaksson B.
Membrane cut-off does not influence results regarding the measurement of small molecules - a comparative study between 20- and 100-kDa catheters in hepatic microdialysis.
Clin Physiol Funct Imaging. 2014 Mar;34(2):109-13.

III.   D'Souza MA, von Platen A, Rooyackers O, Nowak G.
Hepatic vein microdialysis is not equivalent to intrahepatic microdialysis monitoring for the detection of metabolic changes induced by arterial ischemia in a pig liver model.
Manuscript submitted

IV.   Jawad R*, D'souza M*, Selenius LA, Lundgren MW, Danielsson O, Nowak G, Björnstedt M, Isaksson B.
Morphological alterations and redox changes associated with hepatic warm ischemia-reperfusion injury.
World J Hepatol. 2017 Dec 8;9(34):1261-1269.

V.   von Platen A, D'Souza MA, Rooyackers O, Nowak G.
Evaluation of intrahepatic lactate/pyruvate ratio as a marker for ischemic complications early after liver transplantation - a clinical study.
Transplantation Direct 5(12):e505, December 2019

VI.   von Platen A*, D'Souza MA*, Rooyackers O, Nowak G.
Intrahepatic microdialysis for monitoring of metabolic markers to detect rejection early after liver transplantation.
Manuscript submitted

        * Authors contributed equally

LIST OF ABBREVIATIONS

Acetyl CoA	Acetyl coenzyme A
ACR	Acute cellular rejection
ALAT	Alanine aminotransferase
ANOVA	Analysis of Variance
ARE	Antioxidant-Response Element
ASAT	Aspartate aminotransferase
ATP	Adenosine triphosphate
AUC	Area under the curve
cDNA	Complementary DNA
CI	Confidence interval
CR	Chronic rejection
CT	Computerized tomography
CUSA	Cavitron Ultrasonic Surgical Aspirator
CV	Coefficient of variation
DAMP	Damage-associated molecular patterns
EM	Electron microscopy
ETC	Electron transport chain
FADH2	Flavin adenine dinucleotide hydroquinone
GCLC	Glutamate-cysteine ligase
GLRX	Glutaredoxin
GLUT2	Glucose transporter-2
GRX	Glutaredoxin system
GSH	Glutathione
GSR	Glutathione S-reductase
HAT	Hepatic artery thrombosis
HOCl	Hypochlorous acid
H_2O_2	Hydrogen peroxide
ICAM-1	Intercellular adhesion molecule-1
IFNγ	Interferon gamma
IL	Interleukin
I/R injury	Ischemia-reperfusion injury
KC	Kupffer cells
kDa	kDalton
L/Pr	Lactate/pyruvate ratio
LSEC	Liver sinusoidal endothelial cells

LT	Liver transplantation
mM	Millimole
MRI	Magnetic resonance imaging
NAD	Nicotinamide Adenine Dinucleotide
NO	Nitric oxide
NOS	Nitric oxide synthase
NRF2	Nuclear factor (erythroid-derived 2)-like 2
ONOO-	Peroxynitrite
$\cdot OH$	Hydroxyl radical
$\cdot O_2^-$	Superoxide anion
PEAS	PolyArylEtherSulfone
PHLF	Post-hepatectomy liver failure
PT-INR	Prothrombin time/international normalized ratio
PTC	Portal triad clamping
PVT	Portal vein thrombosis
qPCR	Qualitative polymerase chain reaction
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SEM	Standard error of the mean
SHVE	Selective hepatic vascular exclusion
SOD	Superoxide dismutase
TCA	Tricarboxylic acid
THVE	Total hepatic vascular exclusion
TNF-α	Tumor necrosis factor alpha
TRX	Thioredoxin system
TXN	Thioredoxin
TXNRD	Thioredoxin reductase
UDP	Uridine diphosphate
VCAM-1	Vascular adhesion molecule-1
xCT	Cysteine/glutamate antiporter
XDH	Xanthine dehydrogenase
μg	Microgram
μkat	Microkatal
μL	Microlitre
μM	Micromole
$^1O_2$	Singlet oxygen

CONTENTS

1 INTRODUCTION

Ischemia occurs when an organ or tissue is deprived of its blood supply and with it a lack of oxygen and nutrients. The cellular metabolic machinery is disrupted and when the blood supply is restored a compounding reperfusion injury occurs. This biphasic phenomenon is referred to as ischemia-reperfusion (I/R) injury [1]. Operative manoeuvres employed during liver resection, including those used to reduce blood loss can lead to hepatic I/R injury of the liver remnant. On the other hand, I/R injury is inherent to the process of liver transplantation (LT) and occurs during organ procurement, storage and the transplantation surgery. I/R injury in the liver is one of the most significant causes of morbidity and mortality after liver resection surgery and transplantation [2-5].

Studying the effects of I/R injury on liver metabolism could be of immense value with clinical implications for both liver resection surgery and transplantation. This information can be useful to understand the effect of various operative manoeuvres on hepatic metabolism and to establish the safety of various clamping manoeuvres during liver surgery. It may help to correlate metabolic alterations with clinical outcomes after LT especially in situations with extended ischemia times or marginal liver grafts. It may further help to modify intraoperative strategies and possibly postoperative management to attenuate the effects of hepatic I/R injury.

Hepatic I/R injury involves a complex cascade of cellular and humoral interactions eventually leading to cellular injury [6-8]. Hepatic I/R injury has been extensively studied and there is a vast literature on the topic but there is still much to be understood partly due to the complexity of the metabolic milieu of the liver and the cellular and molecular mechanisms involved [9, 10]. The hepatic response to ischemia and reperfusion may not necessarily be similar to the mechanisms seen in other organ systems.

The aim of this doctoral thesis is to contribute to the better understanding of the phenomenon of hepatic I/R injury. To this end, both clinical and experimental studies using techniques like microdialysis, electron microscopy (EM) and gene expression analysis of redox proteins in human subjects and animals have been performed.

2 BACKGROUND

2.1 LIVER ANATOMY

Since antiquity, the liver has held a central position in human awareness and was often considered to be the seat of emotions. The legend of Prometheus from Greek mythology reveals the ancient understanding that the liver has the unique capacity to regenerate itself [11]. It is this remarkable regenerative potential of the liver that has made possible the range and scope of liver surgery and transplantation.
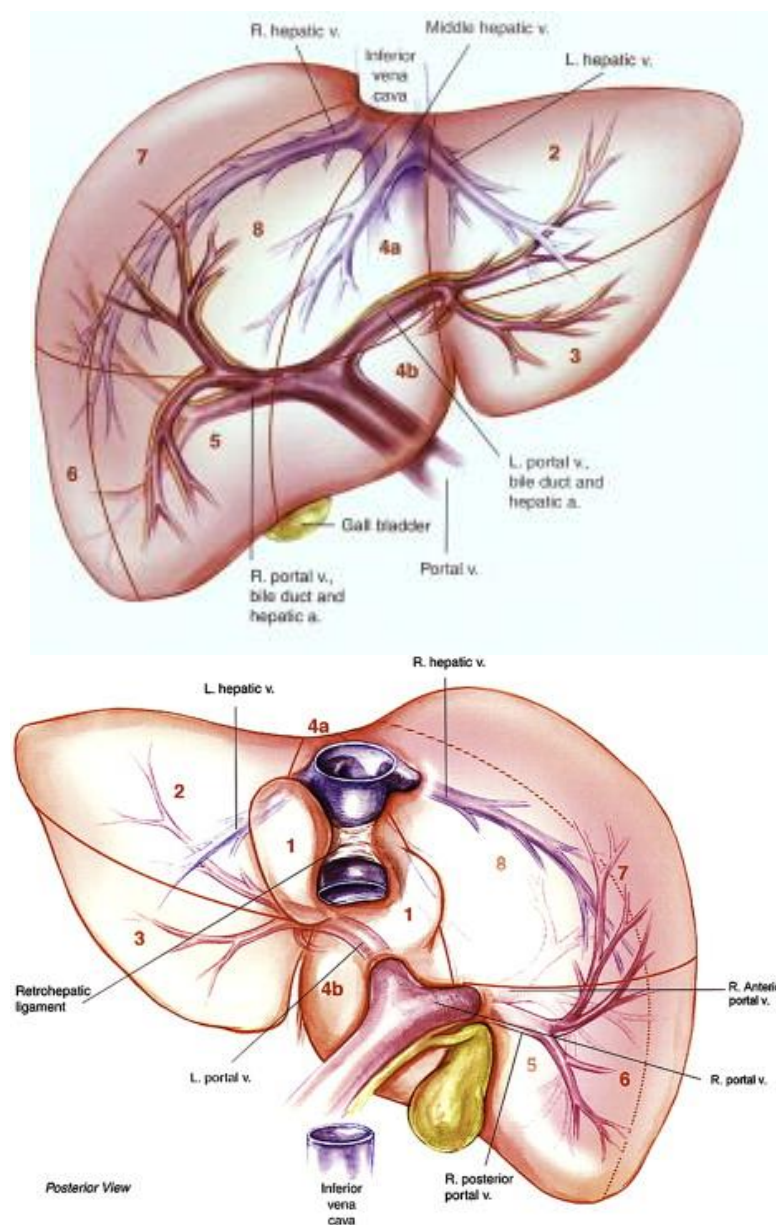

Figure 1. Segmental and vascular anatomy of the liver.

From: Abdel-Misih SR, Bloomston M. Liver anatomy. Surg Clin North Am. 2010 Aug;90(4):643-53. doi: 10.1016/j.suc.2010.04.017.
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Weighing approximately 2-3% of the total body weight in a normal human adult, the liver is the largest solid organ in the body. Suspended by peritoneal reflections or ligaments, the liver lies beneath the diaphragm to the right of the upper abdomen. The gallbladder lies on its inferior surface and drains via the cystic duct into the bile duct. The liver's dual blood supply comprising of the portal vein and the hepatic artery enters the organ through the 'porta hepatis' at the liver hilum [12]. The portal vein supplies 75% of the blood flow but the hepatic artery delivers half of the liver's oxygen supply.

Historically, the liver was anatomically divided into right and left lobes divided by the falciform ligament and the ligamentum teres as it enters the liver. This classical division however has little relation with the functional anatomy of the liver first described by the French surgeon and anatomist Claude Couinaud in the 1950s [13]. Based on third-order distribution of the portal pedicles, the liver was divided into 4 sectors and 8 segments (Figure 1). Each segment has its own inflow, both arterial and portal, as well as bile drainage. The Cantlie line passing from the middle hepatic vein to the gallbladder divides the right and left lobes of the liver [14]. The caudate lobe (segment 1) straddles the retrohepatic vena cava behind the porta hepatis.
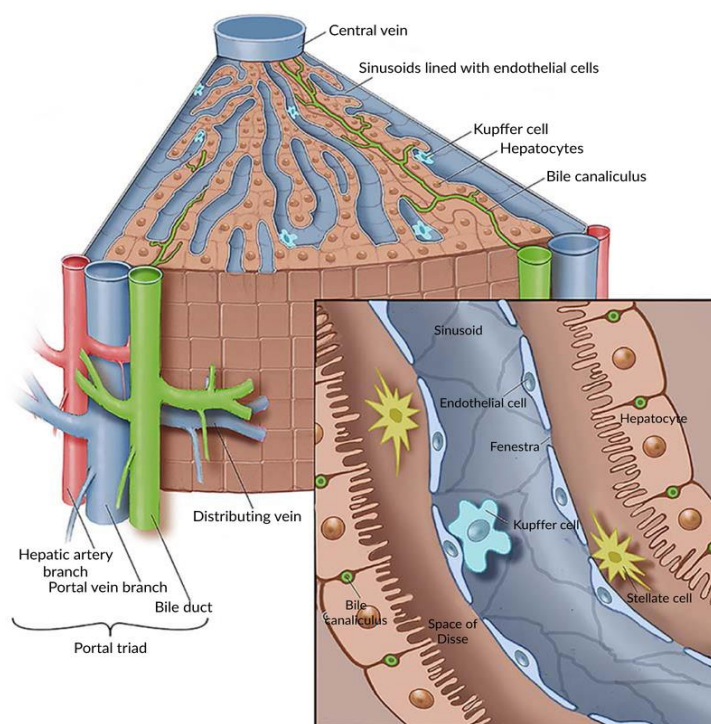

Figure 2. Microscopic anatomy of the liver depicting the hepatocytes, portal tracts and the sinusoidal spaces.

The 'portal tracts' comprising portal vein and hepatic artery divisions, bile ducts, lymphatic vessels and nerve fibers radiate from the hilum and course through the liver. Within the liver the portal and arterial blood mix and are distributed though the fenestrated sinusoids. The sinusoidal spaces are lined by hepatocytes (liver cells) arranged in cords, one or two cells thick and are lined by endothelial cells and Kupffer cells (KC), the latter belonging to the macrophage-phagocytic system. The liver sinusoidal endothelial cells (LSEC) form a fenestrated lining allowing easy exchange of molecules between blood and the hepatocytes (Figure 2). This exchange takes place through the 'space of Disse', which is a compartment between the LSEC and hepatocytes. Nutrients in the blood coming from the intestine diffuse through this space and equilibrate with the extracellular fluid [15]. Liver-associated lymphocytes or pit cells also line the sinusoids [16]. The sinusoidal blood then courses through hepatic venules finally joining to form the 3 main hepatic veins, which leave the liver to carry blood into the inferior vena cava and on to the heart.

2.2 GLUCOSE METABOLISM IN THE LIVER

The liver plays a central role in glucose metabolism. The various pathways of glucose metabolism, including glycogenesis, glycogenolysis, glycolysis and gluconeogenesis are central to this vital function of the liver in glucose homeostasis. Carbohydrates are broken down by digestive processes in the gut into simple sugars, the monosaccharides glucose, galactose and fructose and reach the liver via the portal vein. Glucose is the main product of this breakdown and along with glucose from the systemic circulation via the hepatic artery reaches finally to the fenestrated sinusoids in the liver. Here, a passive equilibration occurs with the hepatic parenchymal interstitial fluid and glucose enters passively into the hepatocytes along the concentration gradient via the bidirectional glucose transporter-2 or GLUT2 [17]. Once in the hepatocyte, free glucose is then phosphorylated to glucose 6-phosphate by the enzyme glucokinase. Glucose 6-phosphate may be metabolized in 3 ways, isomerization into glucose 1-phosphate and uridine diphosphate-glucose (UDP-glucose), conversion to fructose 6-phosphate or oxidation to initiate the pentose phosphate pathway (Figure 3) [18].

UDP-glucose is the direct glucose donor for glycogen synthesis or the process of glycogenesis, which is the most important pathway of glucose utilization in the liver. After a meal, insulin is released from the pancreas and most glucose entering the liver cells is converted to and stored as glycogen to create a hepatic reserve that can be used during fasting. Glycogenesis may also proceed utilizing glucose derived from gluconeogenesis and

this represents the indirect pathway. The adult human liver can store up to 100-120 grams of glycogen. During fasting, when the insulin levels decrease, and glucagon levels rise, glycogen is broken down by the process called glycogenolysis. The enzymes glycogen phosphorylase and glycogen debranching enzyme are those responsible for glycogenolysis. Glucose molecules are sequentially removed from the glycogen branches and exit the hepatocytes by passive diffusion via the GLUT2 channels [18].
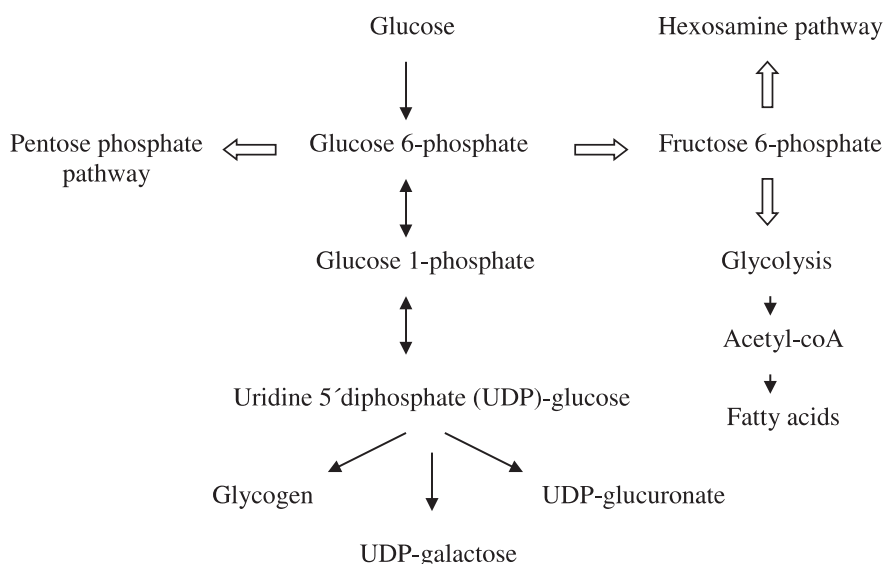
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                    Glucose                    Hexosamine pathway

                       |                              ⇑
                       v
Pentose phosphate ⇐  Glucose 6-phosphate   ⇒  Fructose 6-phosphate
    pathway
                       ↕                              ⇓

                 Glucose 1-phosphate              Glycolysis

                       ↕                              ↓
                                                  Acetyl-coA
      Uridine 5´diphosphate (UDP)-glucose            ↓
                                                  Fatty acids
              ↙        ↓        ↘
      Glycogen                  UDP-glucuronate

                UDP-galactose
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Figure 3. Glucose pathways in the liver.

The liver has the unique ability to release glucose into the blood stream when needed. This is achieved by the process of glycogenolysis as described above but also by de novo synthesis, referred to as gluconeogenesis. Glycogenolysis is responsible for the initial glucose production in times of need but when glycogen stores are depleted gluconeogenesis takes over as the source of hepatic glucose output [19]. In the liver, the gluconeogenesis pathway is used to synthesize glucose from fructose, glycerol, lactate and alanine.

Glucose is also oxidized in the liver to yield energy through the process of glycolysis that takes place in the hepatocyte cytosol. The tricarboxylic acid (TCA) or Kreb's cycle and the respiratory chain that follows next, occur in the mitochondria. Glycolysis does not need oxygen to proceed and it produces two molecules of adenosine triphosphate (ATP) and Nicotinamide Adenine Dinucleotide (NADH) per molecule of glucose. The end product of

glycolysis is pyruvate (two molecules), which in the presence of oxygen moves into the inner mitochondrial matrix to be decarboxylated by the enzyme pyruvate dehydrogenase [20]. Acetyl CoA formed in this reaction enters the aerobic TCA cycle. During the TCA cycle, high-energy molecules, including ATP, NADH, and flavin adenine dinucleotide hydroquinone (FADH2), are produced. Enzymatic reactions occurring in the inner mitochondrial membrane process NADH and $FADH_2$ to generate ATP. This electron transport chain (ETC) yields a net total of 36 ATPs for every glucose molecule [21].

A small percentage of cytosolic pyruvate is metabolized to lactate to yield two ATP molecules per glucose molecule. Pyruvate is converted into lactate by the enzyme lactate dehydrogenase. Lactate produced in this way in other tissues like muscle can only be metabolized in the liver where it is converted back to glucose via the Cori cycle [22]. Here, six molecules of ATP are utilized per glucose molecule generated. The glucose which is released into the blood by the liver can then be used by organs like the brain, muscle and the red blood cells for energy metabolism.
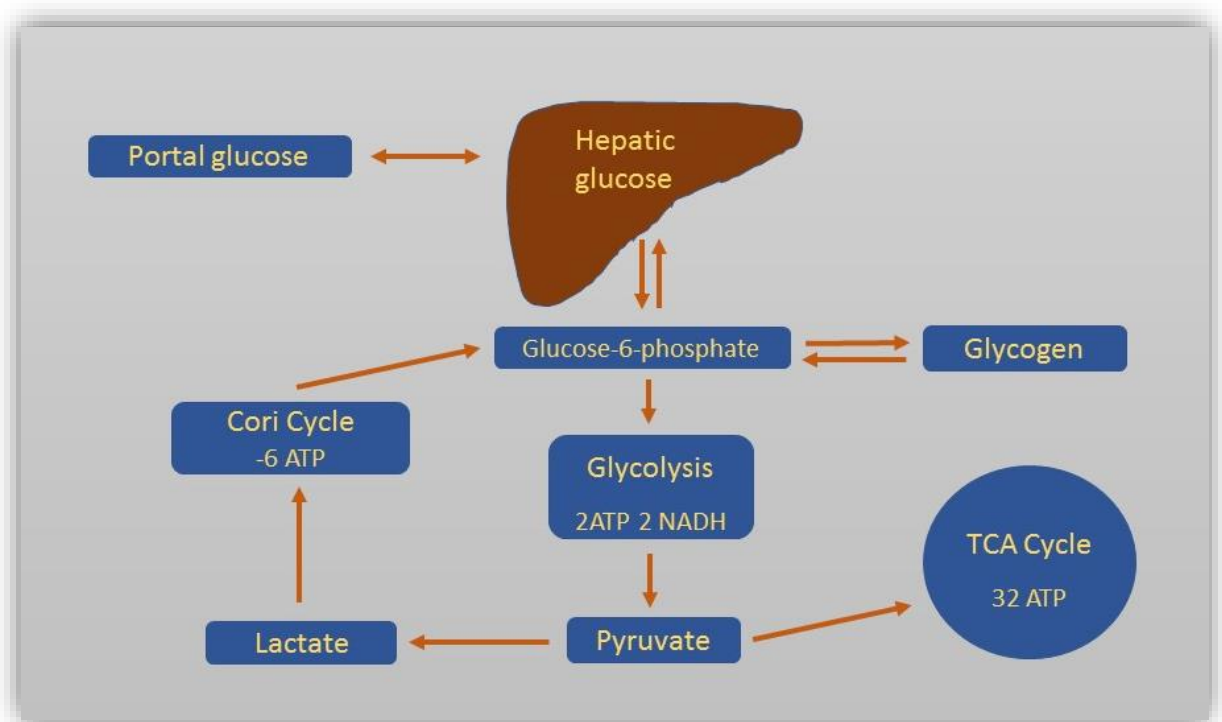

Figure 4. Schematic representation of glucose metabolism in the liver.

2.3 LIVER RESECTION AND TRANSPLANTATION

2.3.1 Liver resection surgery

Since Couinaud's description of the internal anatomy of the liver in the 1950s, the realization that the liver could be safely and successfully resected began to be established. Up until the 1980s it was still considered a dangerous operation with high morbidity and mortality and it took several decades for liver surgery to come into its own [23, 24]. The relative anatomic inaccessibility of the liver, it's concealed vascular anatomy and its immense vascularity has contributed to the complexity associated with liver surgery. A better understanding of the liver's anatomy, improved surgical techniques and advancements in perioperative management, have led to an exponential increase in the number and complexity of liver resections being performed for a variety of indications including primary and secondary hepatic malignancies [25-28]. With the modern multidisciplinary approach including effective oncologic treatment strategies, short- and long-term outcomes after liver resection for malignancy have improved [29]. More patients nowadays undergo complex and extended resections on livers exposed to chemotherapy than ever before.

The prerequisite for performing a liver resection is to preserve adequate volume of liver parenchyma to maintain function, preserve vascular inflow (portal venous and hepatic arterial) and outflow from the liver remnant to the vena cava and maintain a functioning biliary drainage. Liver function is of particular concern when operating on patients with hepatic fibrosis, steatosis, cirrhosis or livers exposed to chemotherapy [30-33]. Unfortunately, the estimation of preoperative liver function is an unexacting science and it is still difficult to accurately prognosticate the consequences of liver resection in a patient with compromised liver function [34]. Furthermore, the percentage of remnant liver volume correlates poorly with remnant liver function.

2.3.2 Bleeding during liver resection and clamping manoeuvres

Intraoperative bleeding is one of the major risks of liver surgery. Apart from being a cause of mortality, significant blood loss and transfusion has been related to increased morbidity and even tumor recurrence after liver resection [35, 36]. A number of technical operative refinements like the use of specialized instruments such as Cavitron Ultrasonic Surgical Aspirator (CUSA), Water Jet, LigaSure, TissueLink and intraoperative anaesthesiologic management like low central venous pressure (CVP) during parenchymal transection has been shown to reduce bleeding [28, 37]. In addition to the above, several techniques of inflow

occlusion can be used to reduce hepatic bleeding. The most commonly used in clinical practice is the classical 'Pringle manoeuvre' or 'Portal triad clamping' (PTC) first described in 1908 by James Hogarth Pringle [38, 39]. PTC is most commonly performed by placing a vascular clamp or a soft cotton band to occlude the hepatoduodenal ligament and thus the vascular inflow of the liver (Figure 5). PTC is usually applied for 15 minute-periods during parenchymal transection and released for 5 minutes.
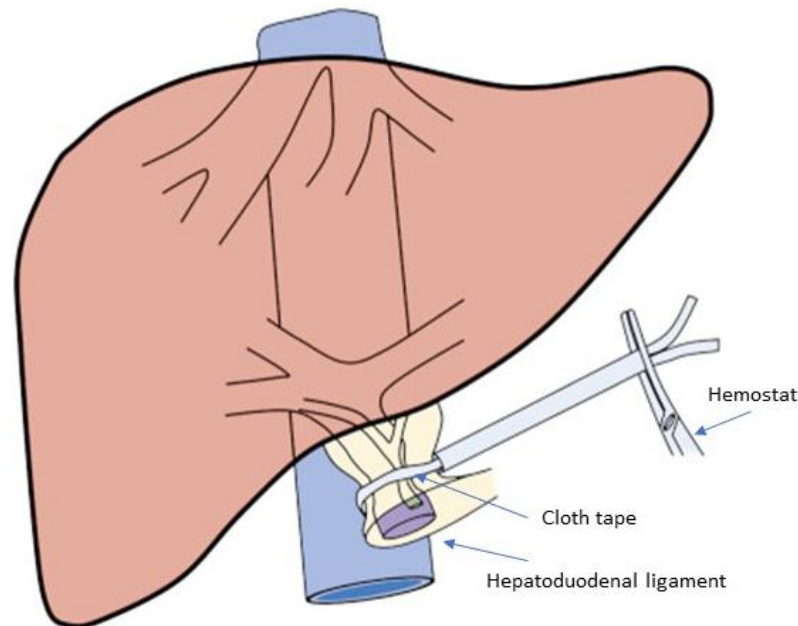

Figure 5. The Pringle manoeuvre.

From: François Cauchy, Olivier Scatton, Jacques Belghiti, Olivier Soubrane. Vascular isolation techniques in hepatic resection. Blumgart's Surgery of the Liver, Biliary Tract and Pancreas, 2-Volume Set
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Total hepatic vascular exclusion (THVE) is an additional method used to control bleeding during liver surgery and involves complete vascular inflow and outflow control. However, when applied for a prolonged time it can be associated with severe hemodynamic instability [39, 40]. As opposed to THVE, selective hepatic vascular exclusion (SHVE) is another technique wherein the vena cava is not clamped but the hepatic veins are clamped selectively thereby avoiding the hemodynamic consequences of THVE [41]. THVE and SHVE are however not used routinely in clinical practice. While these clamping manoeuvres may be effective in reducing blood loss they cause hepatic ischemia and subsequent reperfusion injury, I/R injury to the remnant liver. Depending on factors like the duration of clamping,

underlying quality of the hepatic parenchyma, volume of blood loss and other factors, the consequences of this injury may range from mild organ dysfunction to fulminant post-hepatectomy liver failure (PHLF) [3, 6, 42, 43]. Although PTC has been widely used to reduce blood loss during liver resection, concerns about I/R injury in patients with compromised liver function have led to its selective use [44, 45]. The newer technical tools used for liver parenchymal transection have probably decreased bleeding and as a consequence reduced the need for PTC, but it is still an effective method to reduce blood loss in difficult or urgent surgical situations.

2.3.3 Liver transplantation

A team led by Dr. Thomas Starzl performed the first human LT in Denver, Colorado, USA in 1963 [46]. Since then LT has become the gold standard for treatment of end stage liver disease of most etiologies, several metabolic disorders, some hepatic malignancies and uncommonly fulminant acute liver failure [47, 48]. As a clinical practice, LT is a science of multidisciplinary surgery and medicine and central to achieving optimal outcomes after LT, is the effective management of immunosuppression [49]. The quality of the donor graft can be assessed by several indexes. Age, liver fat percent, graft volume and ischemia time are some of the parameters used and donor grafts of borderline quality are referred to as "marginal" or "extended criteria" donors [50, 51]. The transplantation procedure involves donor hepatectomy, perfusion, transport, graft preparation and in the recipient, total hepatectomy and graft implantation with reconstruction of the liver vessels and biliary drainage. A veno-venous bypass is not routinely used during LT nowadays; instead, the 'piggyback technique' is preferred. I/R injury is thus unavoidable in the context of the LT procedure.

2.3.4 Graft dysfunction after liver transplantation

The presentation of graft dysfunction post-LT is a challenging entity, difficult to detect clinically and by laboratory and radiological investigations. Blood liver biochemistry is the mainstay for detection and monitoring of complications in the post-transplant period and is often the first indication that the liver graft is not functioning optimally. However, these are very non-specific and can lead to a delay in detection and institution of appropriate treatment. The main causes of graft dysfunction are summarized in Figure 6 and the spectrum of complications can range from a mild biochemical derangement to life threatening liver failure

and graft loss [52]. For the purposes of this thesis, ischemic vascular complications and acute cellular rejection (ACR) will be discussed individually.
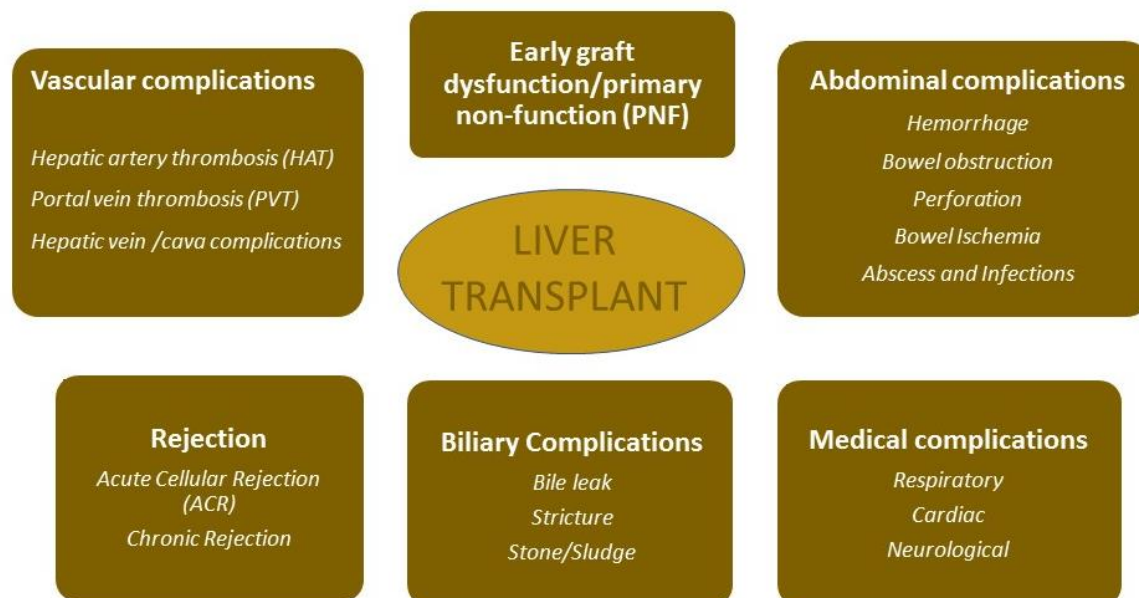

Figure 6. Complications and causes of graft dysfunction after liver transplantation

2.3.5 Vascular complications after liver transplantation

Hepatic artery thrombosis (HAT) and portal vein thrombosis (PVT) are the most important of the vascular complications occurring after LT [52]. HAT is the most common and the most dangerous of these and occurs in around 2-9% of LT patients [53]. It remains one of the foremost causes of early graft loss. The biliary tree is entirely dependent on hepatic arterial blood flow and HAT affects not only the graft globally but the bile ducts in particular. Even if HAT resolves, long term biliary complications may lead to secondary biliary cirrhosis and graft failure [54]. HAT can be differentiated into early and late. Early HAT usually presents within the first week post-LT and may present with massive liver enzyme elevations, coagulopathy, bile leak and signs of acute liver failure but early HAT can also be biochemically and clinically silent [55]. Late HAT on the other hand may present years after LT with gradually worsening liver function and recurrent cholangitis. Timely detection of HAT is thus of paramount importance and usually starts with Doppler ultrasound examinations on biochemical and clinical suspicion. This is usually followed up with computed tomography (CT) or magnetic resonance imaging (MRI). The management options include surgical or interventional radiological revascularization, retransplant, or conservative

treatment [55, 56]. PVT has a reported incidence of up to 7% of grafts. PVT however has a more indolent course but may present with portal hypertension or acutely with graft failure and significant hepatic necrosis [52].

2.3.6 Acute Cellular Rejection

The liver is said to be immunologically privileged; however ACR is relatively common and affects approximately 20 to 60% of LT patients and is usually detected within the first 6 weeks after LT [57]. The clinical and biochemical manifestations of ACR are non-specific and percutaneous liver biopsy is often required to verify the diagnosis [58]. Apart from the risks associated with the biopsy this may by itself delay diagnosis and treatment. ACR is usually treated with steroid boluses and higher doses of basic immunosuppression. ACR is a known risk factor for the development of chronic rejection (CR), which in turn may lead to a progressive immune-mediated damage to the bile ducts resulting in a cholestatic graft dysfunction and graft loss needing retransplantation [59]. Timely detection of ACR is thus of paramount importance for preserving graft function and improving outcomes after LT.

2.4 HEPATIC ISCHEMIA-REPERFUSION INJURY

Hepatic I/R injury occurs when the liver or part of it is deprived of blood. The initial ischemic insult (ischemia) is compounded by the inflammatory burst on restoration of the blood supply (reperfusion). There is a vast literature on the topic of I/R injury in general, but the mechanisms involved in hepatic I/R injury are still poorly understood. It is a biphasic phenomenon comprising a complex interaction of cellular and humoral events finally leading to cellular damage. The 2 distinct phases are the initial stage of '**Ischemic injury**', characterized by a mainly local metabolic derangement due to lack of oxygen supply, resulting in glycogen consumption, ATP depletion and cell death. The next phase of **'Reperfusion injury'** combines not only a metabolic derangement but also a profound immune mediated inflammatory response, which generates damaging free radicals or reactive oxygen species (ROS), which are directly toxic [60].

Hepatic ischemia is also classified as **'warm'** or **'cold'**. Warm ischemia occurs 'in situ' in the setting of liver resection, transplantation, trauma, and shock when the blood supply to the liver is interrupted. On the other hand cold ischemia occurs typically 'ex vivo' during organ preservation after flushing the liver with cold preservation solutions and transportation before LT [61]. While both types of injury share common mechanisms there

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are some fundamental differences between them. For example, the existing evidence is that warm I/R injury mainly affects hepatocytes, whereas cold I/R injury is initiated by damage to the LSEC and microcirculation [9]. However, both hepatocyte and LSEC injury occur in warm and cold ischemia. Indeed, both types of I/R injury have in common the activation of neutrophils and KC, generation of cytokines and chemokines, production of ROS, upregulation of adhesion molecules and lymphocyte/monocyte infiltration [60, 62].
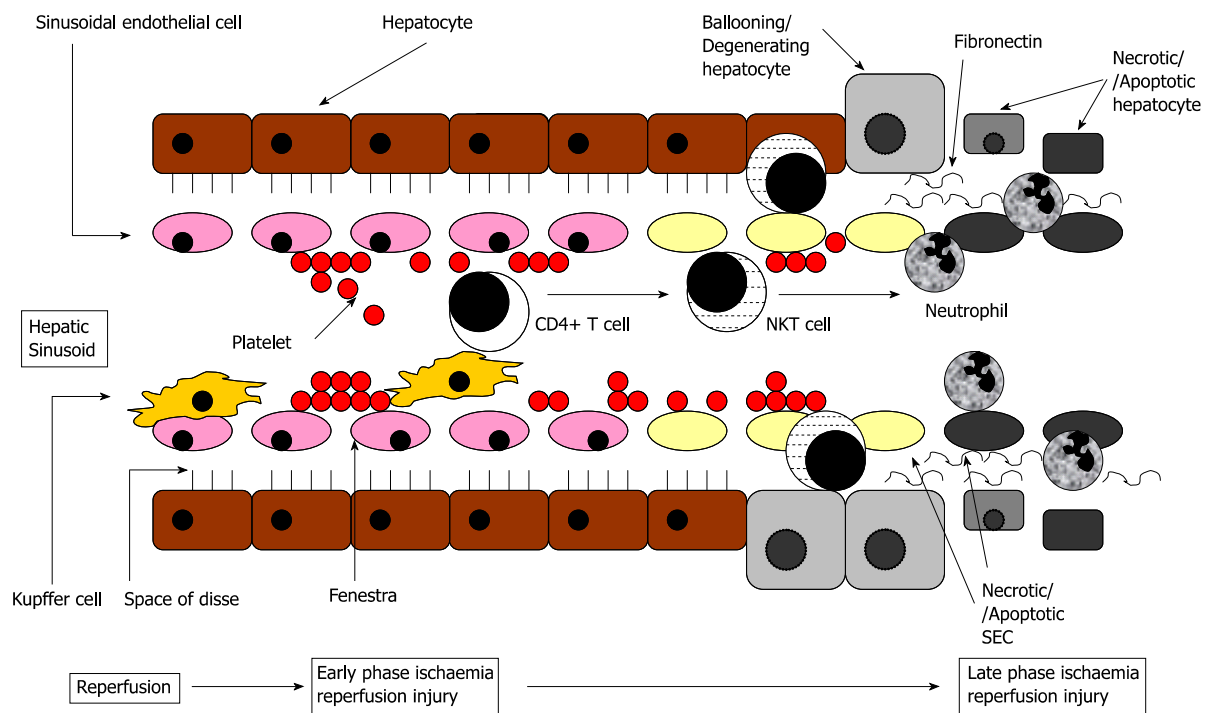
Figure 7. Schematic representation of events in hepatic ischemia-reperfusion injury

With the onset of ischemia, the oxygen tension in the liver tissue decreases and the ensuing mitochondrial dysfunction causes metabolism to shift from aerobic to anaerobic. The microenvironment becomes acidic and the production of high energy ATP decreases, becoming insufficient to meet the energy needs of cellular metabolism [63]. This ATP deficiency causes dysfunction of the ion pumps of the cell membrane. Sodium and calcium ions flood into the cells and their intracellular concentration increases. The cells swell, and the increased intracellular calcium activates phospholipases, which degrade the cellular

membrane phospholipids [63-65]. This results in a disruption of the cell membrane and intracellular contents leak out into the interstitial space including cell membrane glycerol. The cell death that occurs at this stage is mainly by apoptosis but if ischemia is prolonged necrosis will occur. Indeed, the acidic environment serves to delay necrosis and it is the onset of reperfusion injury that initiates the cellular necrosis [66, 67]. The cell swelling in combination with the imbalance between nitric oxide (NO) and endothelin production (relative excess of endothelin), leads to the narrowing of lumen of the sinusoidal spaces resulting in a microcirculatory dysfunction. This reduction in sinusoidal diameter and blood flow contributes to platelet and neutrophil accumulation and further compromise of the blood flow on reperfusion [9].

2.4.1 Cellular interactions in hepatic I/R injury

Hepatocytes, LSEC, KC, platelets and neutrophils are the major cellular components involved in the initiation and progression of hepatic I/R injury. The hepatocytes largely bear the effects of I/R injury as described in the early ischemic phase, but they also play a role in progression by releasing Interleukin-12 (IL-12). This interleukin may activate inflammatory responses, including release of the cytokines, tumor necrosis factor alpha (TNF-α) and Interferon gamma (IFNγ) [68]. Furthermore, hepatocytes may likely be responsible for the complement-driven activation of KC seen in I/R injury [69].

LSEC just like their hepatocyte-counterparts are largely the targets of I/R injury and similar to the hepatocytes in the initial phase of ischemia and ATP depletion, swell up and increase in volume [70]. As a result of the ensuing microcirculatory disturbance described above, platelets and neutrophils extravasate out into the sinusoidal spaces. The selectin family of adhesion molecules, P-, E- and L-selectin are expressed early during reperfusion by LSEC. These mediate the rolling adhesion of platelets and neutrophils that propagate the reperfusion injury. However, there is not much information available on the global physiological consequences of LSEC deregulation during hepatic I/R injury [71].

Parallel to hepatocyte and LSEC damage the KC are also subject to intense activation by damage-associated molecular patterns (DAMPs) released from the neighboring hepatocytes [72]. KC are also stimulated by complement and it has been reported that inhibition of the complement system can attenuate the severity of I/R injury. KC play a central role in the early phase of reperfusion and are thought to be the main source of ROS and pro-inflammatory cytokines generated early during I/R injury [72]. The activated KC release TNF-α and the Interleukins -1, 6 and 12 (IL-1, IL-6 and IL-12). Although TNF-α and IL-1

are proinflammatory, IL-6 may have an anti-inflammatory function [72-74]. The ROS and inflammatory cytokines released by KC attract lymphocytes to the liver, which in their turn further activate cytokine secretion by the KC [75].

The migrating neutrophils accumulate and adhere in the sinusoidal spaces in the liver attracted there by selectins and trapped by the mechanical disruption of the LSEC lining giving direct access to the hepatocytes [71]. Here the neutrophils adhere to the intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) expressed on the hepatocytes and get primed, partly under the influence of platelets and produce ROS by NADPH oxidase activity. The ROS diffuse into the hepatocytes and trigger mitochondrial dysfunction [71, 76]. Neutrophils also release proteases and may cause hepatocyte death but the implication of this mechanism is unclear [77].

2.4.2 Reactive oxygen species (ROS) and Reactive nitrogen species (RNS)

ROS is a term used for molecules having unpaired valence electrons or unstable bonds and includes radical as well as non-radical agents depending on the presence or absence of an unpaired electron. The most biologically significant radicals are superoxide anion ($\cdot O_2^-$), hydroxyl radical ($\cdot OH$) and nitric oxide (NO) whereas the non-radical species include hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hypochlorous acid (HOCl).

Xanthine dehydrogenase (XDH) is an enzyme involved in purine metabolism and in aerobic conditions, converts the ATP metabolite, hypoxanthine to xanthine and finally to uric acid using NAD^+ [78]. With prolonged ischemia XDH is converted to the ROS-forming xanthine oxidase (XO) [79]. The ATP depletion occurring due to the anaerobic shift as a result of ischemia, results in the accumulation of the products of its degradation including adenosine, hypoxanthine and xanthine. On reperfusion, initially the increase in oxygen delivery exceeds the rate at which cellular metabolism returns to aerobic pathways, which generates free radicals that damage cellular structures. Oxygen delivered to the ischemic tissue reacts with hypoxanthine and XO and forms superoxide and other ROS [80].

The most widely implicated ROS in hepatic I/R injury include the superoxide, hydroxyl radical and hydrogen peroxide [81]. These ROS are released by the KC and recruited neutrophils and through DNA modifications, lipid and protein peroxidation cause hepatocellular damage [82, 83]. ROS also increase expression of the proinflammatory cytokines TNF-α, IL-1β and IL-8 and are known to block mitochondrial respiratory

enzymes [84]. Several protective enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase, are present in mitochondria and help to detoxify ROS [85, 86].

Nitric oxide (NO) and peroxynitrite (ONOO$^-$) are the biologically most important reactive nitrogen species (RNS) and are produced endogenously from the amino acid L-arginine and oxygen by nitric oxide synthase (NOS), specifically the endothelial isoform, eNOS expressed in LSEC and hepatocytes [87, 88]. The inducible isoform, iNOS is upregulated in hepatocytes, KC and neutrophils during I/R injury and is a significant source of NO [89]. RNS bind to lipids, proteins and DNA and damage structural and functional components of the cell and the highly cytotoxic, peroxynitrite in addition inactivates several mitochondrial enzymes. NO produced by eNOS in the LSEC relax and dilate the sinusoids and thus control sinusoidal perfusion. This action is counteracted by endothelin. The eNOS isoform is dependent on oxygen for NO synthesis thus during hepatic ischemia there is sinusoidal contraction. This leads to decreased blood flow and trapping of neutrophils and platelets in the sinusoids [9, 90]. While it is accepted that RNS plays an important role in hepatic I/R injury, the exact mechanisms remain yet to be elucidated.

2.4.3 Glucose metabolism in ischemia and reperfusion

As described earlier, under aerobic conditions, the decarboxylation of pyruvate in the mitochondria yields acetyl CoA, which enters the TCA cycle [20]. Ischemia inhibits the further metabolism of acetyl CoA and it accumulates in the mitochondria. The accumulated acetyl CoA in the mitochondria is metabolized to less effective, energy substrates such as ketone bodies, acetoacetate and β-hydroxybutyrate [91]. The accumulation of acetyl CoA further inhibits pyruvate dehydrogenase leading to pyruvate accumulation in the cytosol which is then metabolized to lactate by the enzyme lactate dehydrogenase. This involves the oxidation of NADH to NAD$^+$, and glycolysis continues. This anaerobic shift yields 2 ATP molecules per glucose and additionally serves to keep the pyruvate concentration low. The result is an increase in lactate levels accompanied by a corresponding decrease in pyruvate levels resulting in an increased lactate/pyruvate ratio (L/Pr) [92]. While in most other tissues, glucose levels decrease during ischemia, in the liver, the hepatocytes respond to the ischemic stimulus by glycogenolysis, thereby resulting in increased intrahepatic glucose levels [93, 94]. This continues as long as glycogen reserves are present in the ischemic hepatocytes

2.5 REDOX REGULATORY ENZYME SYSTEMS

The cells line of defense against ROS mediated injury includes non-enzymatic and enzymatic antioxidants. These include the vitamins E, A and C and enzymes like SOD and the H_2O_2 reduction agents, catalase and Peroxiredoxin [85, 86, 95]. The thioredoxin (TRX) and glutaredoxin (GRX) systems also play a major role in maintaining redox balance. These enzyme systems are expressed in all cell types [96, 97]. Many of the antioxidant proteins involved in ROS scavenging are regulated at the transcriptional level by binding of nuclear factor (erythroid-derived 2)-like 2 (NRF2), to the Antioxidant-Response Element (ARE) which is located upstream of the promotor of these genes and initiates the response against ROS [78]. NRF2 also regulates glutamate-cysteine ligase (GCLC) and cysteine/glutamate antiporter (xCT), which are crucial for glutathione (GSH) synthesis. GSH is one of the most important cellular antioxidants [98, 99]. The genes coding for xCT and NRF-2 are SLC7A11 and NFE2L2 respectively.

2.5.1 The Thioredoxin (TRX) and Glutaredoxin (GRX) systems

The TRX system consists of Thioredoxin (TXN), thioredoxin reductase (TXNRD), and NADPH. TXN has 2 isoforms, a cytoplasmic TXN1 and a mitochondrial TXN2 [100]. TXNRD also exists in cytoplasmic and mitochondrial forms, TXNRD1 and 2 respectively. These thioredoxins play a role in regulation of DNA synthesis and the inhibition of apoptosis by inhibiting enzymes involved in cell death [101, 102].

The GRX system includes Glutaredoxin (GLRX), GSH tripeptide (γ-glutamyl-cysteinyl-glycine), glutathione S-reductase (GSR) and NADPH [96, 103]. The isoforms include GLRX1, 2, 3 and 5 differing in their localization in cytosol, nucleus and mitochondria [104]. The GRX systems regulate differentiation, modulation of transcription factors, and apoptotic pathways and like TRX plays a role in DNA synthesis [105]. The exact role and involvement of the TRX and GRX redox systems in hepatic I/R injury is yet unknown.

2.6 MICRODIALYSIS

In both animals and humans, the effects of hepatic I/R injury have been usually studied using methodologies based on biopsies and blood investigations. These methods need to be repetitive to give a clear idea of the underlying pathophysiology and not just a momentary snapshot. Since its introduction in the 1950s and refinement in the following years, microdialysis has been developed as a tool for real-time and continuous monitoring of tissue metabolism, making it an ideal method to study hepatic I/R injury [106-109]. In physiological terms, microdialysis can be compared to repeated venous blood sampling from a specific area in a specific tissue to identify local metabolic events. Additionally, microdialysis avoids systemic dilution since it is performed directly in the tissue of interest.

In principle, microdialysis mimics the passive function of a capillary blood vessel. A microdialysis catheter has a semi-permeable dialysis membrane at its tip and is introduced into the tissue of interest and simulates the equilibrium between capillaries and interstitial fluid. This equilibrium exists due to the semi-permeable nature of the capillary wall. A passive diffusion of small molecules occurs through this wall along the concentration gradient of the substance and modulated by the oncotic pressure generated by larger molecules incapable of passing through the wall [110, 111]. The microdialysis catheter is a tube with a concentric double-lumen construction and with a semi-permeable membrane which is composed of PolyArylEtherSulfone (PEAS) at the tip. An isotonic solution (perfusate) is pumped at a steady flow rate through the inner lumen, and at the tip, this solution comes in contact with the semi-permeable membrane. The perfusate must be isotonic with the fluid surrounding the tip to prevent large shifts of water across the membrane. The semi-permeable membrane can have a pore size or cut-off ranging from 20 to 100 kDalton (kDa) that can be selected based on what substance is to be measured. The perfusate at the tip equilibrates across the membrane with the interstitial fluid by passive diffusion and the fluid returning through the outer lumen of the catheter is collected in a 60 μL microvial. The solution collected in the microvial is called the dialysate and can then be analysed for the molecules in question. Figure 8 illustrates the principle of microdialysis. Glucose, lactate and pyruvate are commonly analysed as markers of glucose metabolism and give us information of the oxidative status of the tissue whereas the L/Pr is used an indicator of the redox status of the tissue in anaerobic states [92, 112-114]. As a marker of cell membrane injury, glycerol has been measured by microdialysis [112-114].
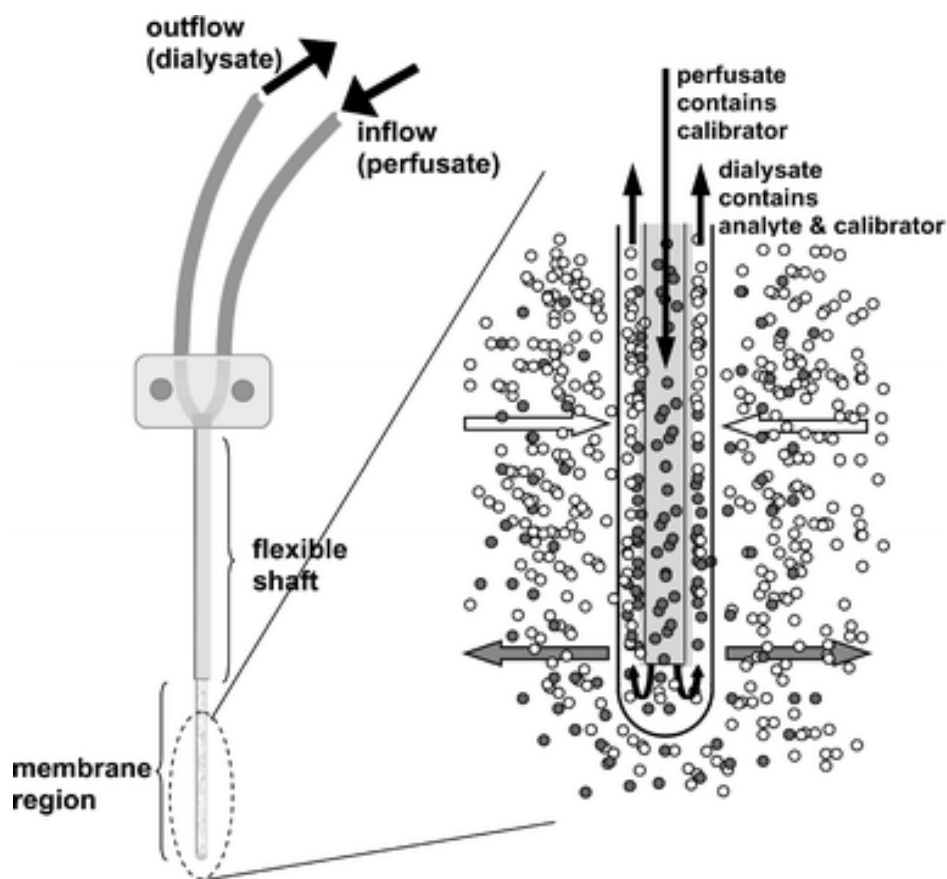
Figure 8. Schematic representation of the principle of Microdialysis.

From: Chaurasia CS, Müller M, Bashaw ED, Benfeldt E, Bolinder J, Bullock R, Bungay PM, DeLange EC, Derendorf H, Elmquist WF, Hammarlund-Udenaes M, Joukhadar C, Kellogg DL Jr, Lunte CE, Nordstrom CH, Rollema H, Sawchuk RJ, Cheung BW, Shah VP, Stahle L, Ungerstedt U, Welty DF, Yeo H. AAPS-FDA workshop white paper: microdialysis principles, application and regulatory perspectives. Pharm Res. 2007 May;24(5):1014-25.
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2.6.1. Technical aspects of microdialysis

"Recovery" reflects the dialysate concentration of the molecule being measured in relation to its true concentration in the tissue. The exchange of substances across the membrane and thus the recovery of metabolites is dependent on the rate at which the perfusate is pumped through the system and is also dependent on other factors including concentration gradient, molecular size, membrane surface area and pore size, temperature and probably other factors not well understood yet. Thus, standardization of the technique is essential for accurate results. A reliable pump system is a prerequisite and the perfusate injector needs to maintain a steady flow rate. A high flow rate can force fluid out into the interstitium and thus results in a lower recovery. Perfusion rates of 0.3, 0.5, 1, 2 and 5 μl/minute have been used with slower

velocities used to achieve highest possible recovery [115, 116]. As mentioned earlier the membrane pore size is selected depending of the metabolite being studied and molecules smaller than the pore size diffuse through. However, larger molecules even with a smaller size than the pores have a lower velocity and diffuse less easily across the membrane [117, 118]. To achieve high recovery, a long membrane with the right pore size and a low perfusion rate is needed. Temperature also influences the diffusion rate and recovery increases by up to 1-2% per degree Celsius increase in temperature [119, 120].

When the microdialysis catheter is introduced into a tissue, there is trauma to the tissue with an ensuing inflammatory response. This response 'normalizes by around 30-60 minutes and is the time needed for equilibration. This duration has been shown to vary depending on the metabolite being analyzed and longer equilibration times have been reported [121-123].

3 AIMS

The general aim of the thesis was to increase the understanding of the phenomenon of hepatic I/R injury at a metabolic and cellular level. Furthermore, we aimed to investigate the use of monitoring methods like microdialysis in both clinical and experimental settings of hepatic I/R injury.

The specific aims of this thesis were:

1. To study the metabolic effects of portal triad clamping in human subjects undergoing liver resection using microdialysis as a monitoring tool.

2. To study whether microdialysis catheters with a cut-off of 20 and 100 kDa can be used equally in the measurement of the small molecules, glucose, glycerol, lactate and pyruvate in a pig liver model.

3. To investigate whether intravascular microdialysis using a catheter placed by a transjugular approach in the middle hepatic vein is comparable to direct intrahepatic microdialysis for metabolic monitoring of arterial ischemia in a pig liver model.

4. To investigate the effect of warm I/R injury induced by PTC on hepatic cellular ultrastructure and on the expression of the thioredoxin (TRX) and glutaredoxin (GRX) systems in human subjects.

5. To evaluate whether monitoring the L/Pr ratio by intrahepatic microdialysis can be used clinically as a marker for the detection of ischemic complications early after LT.

6. To study if monitoring of glucose, lactate, pyruvate and glycerol by intrahepatic microdialysis can be used clinically to predict ACR early after LT.

4 METHODS

4.1 PAPER I

4.1.1 Study design and patients

This study was a feasibility study to establish the technique of microdialysis in human subjects undergoing liver resection and to characterize changes associated with warm ischemia induced by PTC. Eleven consecutive patients undergoing liver resection were included in the study and a prerequisite was that none had clinical or biochemical signs of chronic liver disease. Ten of these patients had metastases of colorectal cancer of which seven had received chemotherapy prior to surgery.

4.1.2 Study protocol and microdialysis

After laparotomy, the microdialysis catheter was inserted into segment IV of the liver using a plastic guider and steel cannula, taking special care not to penetrate tumor tissue. The CMA 70 microdialysis catheter, 0.9mm with a membrane cut-off of 20 kDa (CMA Microdialysis AB, Stockholm, Sweden) was inserted and a 5-0-prolene suture used to secure the catheter and fix it to the falciform ligament. The catheter was then connected to a syringe with perfusion fluid, which was a Ringer acetate-like solution, T1 (CMA Microdialysis AB) and then placed in a microinfusion pump. A flow rate of 1 μl/minute was set after which 30-60 minutes of perfusion was required for equilibration. PTC was then performed using a standard technique where a soft cloth tape was passed around the porta hepatis over which a rubber tubing was then slid. Using a hemostat, the rubber tubing was used to constrict the vessels in the hepatoduodenal ligament. Microvials were changed every 10 minutes and sampling was continued during equilibration, 20 minutes of portal triad clamping, and 10 minutes after reperfusion. The liver was not manipulated, and infusions of glucose and vasopressor drugs were withheld during the experimental period.

4.1.3 Statistical analysis

Statistical analyses were performed using Statistica 8.0 software. The data were presented as mean ± SE and comparison of more than two means were performed using Friedman ANOVA with Wilcoxon matched pair test. Correlations between microdialysis and reference variables were done using linear regression analyses. Tests with p values <0.05 were considered statistically significant.

4.2 PAPER II

4.2.1 Animals and anaesthesia

A total of six female pigs approximately 30-35 kg in weight were used in the study. All animals were fasted for 24 hours with free access to water prior to the experiments. After premedication, midazolam was administered intravenously and the animal intubated. Anaesthesia was maintained by halothane and complemented with fentanyl. Ringer acetate was infused intravenously at 37°C. Blood gas analysis, electrocardiogram, body temperature and urine production were monitored throughout the experiment. Body temperature was maintained at 38°C to 39°C.

4.2.2 Surgical procedure and microdialysis study protocol

A midline laparotomy was performed, and four microdialysis catheters were inserted into different segments of the liver of each pig using a steel cannula with a split catheter and then sutured to the liver parenchyma using a method described earlier [92]. Two of the catheters had the membrane cut-off of 20 kDa and two of 100 kDa (CMA 70 and CMA 71). After insertion the inlet of the tubings was connected to the CMA 106 microinfusion pump and perfusion started. Ringer acetate-like perfusion fluid T1 was pumped through the 20-kDa catheters (referred to as 20R and 20R1) and one of the 100-kDa catheters (100R) at a flow rate of 0.3 µl/minute. The other 100 kDa was pumped with hydroxyethyl starch (100V) (Voluven, Fresenius Kabi, Sweden) at the same flow rate. Equilibration was carried out for 60 minutes after catheter implantation and before the start of collection of dialysate samples. Microdialysate samples were collected at 40-minute intervals. The duration of the experiment was 240 minutes. The liver was not manipulated during the experimental period. The samples were analyzed using colorimetric methods with a CMA 600 microdialysis analyzer for glucose, glycerol, lactate and pyruvate using enzymatic reagents and colorimetric measurements.

4.2.3 Statistical analysis

Statistical analyses were performed using Statistica 8.0 software. Data were presented as mean ± SEM and coefficients of variation (CV) for dialysate concentrations of the given molecules in the different catheters. Data were analyzed using ANOVA with Scheffe's post hoc test and tests with p values <0.05 were considered statistically significant.

4.3 PAPER III

4.3.1 Animals and anaesthesia

A total of eight female littermate pigs, with a body weight of 30-35 kg, were used for the experiments. Before the operation, all animals were fasted for 24 hours with free access to water. The anaesthesia procedure was identical to study II.

4.3.2 Surgical procedure and microdialysis study protocol

Before the abdominal part of the experiment, a CMA 60 microdialysis catheter with a 20-mm shaft and a 30-mm membrane was inserted just under the skin over the left pectoral area as a subcutaneous reference catheter. A midline laparotomy was then performed and the intrahepatic microdialysis catheter was inserted into the middle lobe of the liver using a similar method as used for study II. The CMA 70 microdialysis catheter with a 60-mm shaft and a 30-mm membrane was used in the liver. In the next step the right internal jugular vein was isolated and the 67 IV microdialysis catheter (M Dialysis AB) with a 130-mm shaft and a 30-mm membrane was introduced into it. The tip of the catheter was placed in the middle hepatic vein and advanced till it stopped in the liver. Then, the inlets of the tubings were connected to microinfusion pumps (CMA 106) and perfusion started using T1 solution at a flow rate of 0.3 µl/min in the intrahepatic and subcutaneous catheters and 1 µl/minute in the hepatic vein catheter. A 2-hour period was needed for equilibration and a steady state was achieved after one hour of catheter placement. The hepatic arteries were dissected, isolated, ligated and divided one hour later. This "clamping" was carried on for a period of four hours. Dialysate samples were collected at 15-minute intervals from the intrahepatic and intravenous catheters and at 30-minute intervals from the subcutaneous catheter during the experimental period. The liver was not manipulated during the monitoring period. Dialysates were analyzed for glucose, glycerol, lactate and pyruvate and the lactate/pyruvate ratio (L/Pr) was calculated. The samples were analyzed using colorimetric methods with a CMA 600 microdialysis analyzer. At the end of the experiment the pigs were sacrificed by an overdose of anaesthesia.

4.3.3 Statistical analysis

Statistical analyses were performed using Statistica 13.2 software program. Data were presented as mean ± 95% confidence intervals (CI) for each metabolite (Glucose, Lactate, Pyruvate, Glycerol and the L/Pr) and for each catheter (Intrahepatic, Hepatic vein and Subcutaneous). Results were compared using ANOVA for repeated measurements. In case of

significant differences, analyses were complemented with the Bonferroni post-hoc test. Tests with p values <0.05 were considered statistically significant.

4.4 PAPER IV

4.4.1 Study design and patients

Eleven patients undergoing liver resection for differing indications were included in the study. Seven of these patients had colorectal liver metastases and had received preoperative chemotherapy. None of the patients had clinical or biochemical signs of chronic liver disease,

4.4.2 Study protocol and biopsy acquisition

After laparotomy and division of the falciform ligament, the hepatoduodenal ligament was isolated, and a PTC then performed as described for study I for a period of 20 minutes. Biopsies (one wedge and two needle) were taken at three time-points; baseline (just before the application of PTC), post-ischemia (after 20 minutes of PTC) and post-reperfusion (after 20 minutes of reperfusion). The needle biopsies were immediately transferred to the buffers and then stored at 4℃ for further analyses. The wedge biopsies were transferred to vials that were immediately frozen in liquid nitrogen and stored at -70℃ until analysis. The liver was not manipulated during the experimental period. The liver resection then proceeded as planned.

4.4.3 Transmission electron microscopy

The needle biopsies were fixed, washed and dehydrated with appropriate reagents and according to standard protocol. The embedded biopsies were sectioned using an ultramicrotome (Leica EM UC 6). A transmission electron microscope (Tecnai 12 Spirit Bio TWIN) was used to examination the sections and a Veleta® camera used for capturing digital images. The EM images were evaluated for cellular architecture, hepatocyte morphology, sinusoids and bile canaliculi.

4.4.4 Morphometric image analysis

Quantification analysis of the sinusoids in the EM images was performed using NIS Elements Basic Research software. Pixel length measurements were applied on the sinusoidal endothelial lining and the number of pixels was determined on one representative sinusoid for each patient and time point. The length of the endothelial lining was correlated to the length

of the entire sinusoid and the retrieved pixel value was related to actual μm for comparison between different images.

4.4.5 Immunoelectron microscopy

Needle biopsies were prepared and sectioned and primary antibodies of TRX1 and GRX1 were applied on the sections overnight in a humidified chamber at room temperature [124]. The sections were prepared further including rinse with gold at a dilution of 1:100. Then a transmission electron microscope (Tecnai G2 Bio TWIN) was used for examination and images captured by the Veleta® camera. Quantification of the staining was performed on five hepatocytes in close proximity to vessels for each tissue section. The number of gold particles in the cytosol and the nuclei were documented.

4.4.6 RNA purification, cDNA synthesis and qPCR

The fresh frozen wedge biopsies were processed according to standard protocol and RNA purification carried out. Assessment of RNA quality revealed that 6 out of 11 patients had good quality of samples from all time points. For cDNA synthesis, 2 μg RNA was subjected to reverse transcription and then subjected to qPCR. The genes of interest were isoforms of thioredoxins (TXN) and glutaredoxins (GLRX) and the gene of xCT and NRF-2, SLC7A11 and NFE2L2 respectively.

4.4.7 Statistical analysis

The statistical analysis was performed using GraphPad Prism 6.0 software. The non-parametric Friedman test followed by Dunn's post-hoc test was used for the analysis of endothelial lining, gene expression data, and immunogold staining data. Tests with p values <0.05 were considered to be statistically significant.

4.5 PAPER V

4.5.1 Patients, transplant procedure and postoperative blood glucose management

Forty-five patients undergoing LT for various indications at the Karolinska University Hospital, Stockholm, Sweden, were included prospectively in the study. The piggyback technique was the standard and in selected cases veno-venous bypass was used. Steroids and Tacrolimus were used as basic immunosuppression and high molecular weight Dextran for thrombosis prophylaxis. Ultrasound was the clinical routine to evaluate the liver circulation

within 24 hours after LT. Postoperatively blood glucose was measured every hour by arterial blood gas and patients were administered 5% glucose infusions at 30 ml/kg/day on day 1 and 2. Total parenteral nutrition and a full diet were successively introduced. Initially a target blood glucose value of 4–8 mmol/L was achieved with insulin.

4.5.2 Microdialysis and study protocol

At the end of surgery, but before abdominal closure, the CMA 61 microdialysis catheter with a 60 mm shaft (0.9 mm diameter), a 30 mm membrane (0.6 mm diameter) and a molecular cut-off of 20 kDa was inserted into segment IV of the liver graft. Another CMA 60 catheter reflecting systemic changes was placed subcutaneously in the right pectoral area as a reference catheter. Perfusion fluid T1 was perfused using CMA 106 microinfusion pumps at a flow rate of 0.3 μl/minute. Dialysate samples were collected once every hour and patients were monitored for up to six days postoperatively. Analyses were performed in the CMA 600 Microdialysis Analyzer and dialysates were analyzed for glucose, lactate, and pyruvate concentrations, and the L/Pr was calculated for both catheters. An ischemic complication was defined as vascular occlusion or graft infarction confirmed by radiology. Clinical or laboratory suspicion of such vascular complications, including raised L/Pr beyond cut-offs decided in the protocol were investigated by contrast ultrasound of the liver and a 4-phase liver CT scan if needed. According to the protocol an episode with three consecutive samples with increasing L/Pr where the increase was at least 30% in total was considered for further investigation. The 30% cut-off was based on our earlier studies and assuming that this level would be clinically relevant [92, 125].

4.5.3 Statistical analysis

Statistical analysis was performed using the Statistica 13.2 software. Episodes with increased intrahepatic L/Pr above the cut-off decided in the protocol were identified. The clinical outcome was compared for patients with and without such episodes. Episodes with systemic glucose increase (defined as three consecutive samples with increasing glucose and a minimum increase of 30% in total) measured in the reference catheter were identified. These episodes were studied for correlation in time to intrahepatic L/Pr increase. Also, the data were analyzed with respect to cut-off values for lactate and L/Pr at 3 mmol/L and 20, respectively, based on the study by Haugaa et al. [126]. Data were presented as mean ± SE.

4.6 PAPER VI

4.6.1 Patients, transplant procedure and postoperative management

Seventy-one consecutive patients undergoing LT at the Karolinska University Hospital, Stockholm for differing indications were included in the study. Aspects of LT were as described for study V (see before). A "time-zero biopsy" was obtained from the graft after complete revascularization. Blood work including transaminases, bilirubin and PT-INR were collected according to the standard clinical routine postoperatively. ACR within one month was diagnosed by either a sudden and marked increase in transaminases only or by increased transaminase levels confirmed by liver biopsy.

4.6.2 Microdialysis and study protocol

Intrahepatic microdialysis using the CMA 61 catheter was performed in a similar fashion as described in study V. Samples were collected at 1-hour intervals for up to six days post-operatively. Dialysates were analyzed with respect to glucose, glycerol, lactate and pyruvate concentrations and the L/Pr was calculated. Time-zero biopsies were graded for I/R injury using the modified Suzuki score [127].

4.6.3 Statistical analysis

Statistical analyses were performed using Statistica 13.2 software and SPSS 25 program. All patients with more than 24 hours of microdialysis data were included in the analyses. Patients were divided into 2 groups, those who experienced rejection and those without. Area under the curve (AUC) was calculated for 12-hour intervals for glucose, lactate, pyruvate, glycerol and the L/Pr for all patients. The two groups were compared with respect to these parameters, standard blood work (transaminases, bilirubin, PT-INR) and the "time-zero"- biopsy using ANOVA, t-tests and U Mann-Whitney or Kruskal-Wallis' test for nonparametric or non-normal distributed data as appropriate. A forward stepwise logistic regression analysis was performed to determine whether the changes detected were predictive for rejection or not. Data were presented as mean $\pm$ standard error and mean $\pm$ 95% CI where appropriate. The level of statistical significance for each test was set at $p < 0.05$.

4.7 Ethics

Human studies (Studies I, IV, V and VI): The study protocols of all the human studies conformed to the ethical guidelines of the 1975 Declaration of Helsinki and were approved by the Ethics Committee for Human Studies at the Karolinska Institute and by the Regional Ethics Committee for Human Studies, Stockholm, Sweden. Informed and written consent was obtained from all patients participating in the studies.

Animal studies (Study II and III): The animal studies were approved by the Regional Ethics Committee for Animal Experimentation, Stockholm, Sweden. All animals involved in the experiments received care in accordance with Swedish regulations.

5   RESULTS

5.1   PAPER I

There were no intra- or post-operative complications attributable to the microdialysis procedure. During PTC, there were significant increases in intrahepatic glucose, lactate, and glycerol levels. Levels increased from 9.1 ± 2.2 to 14.5 ± 2.4mM, from 2.2 ± 0.3 to 5.8 ± 0.5mM, and from 63 ± 14 to 142 ± 28 µM, for glucose, lactate and glycerol respectively (p=0.007, 0.008 and 0.012 respectively). Pyruvate levels during PTC however remained unchanged, resulting in an increased L/Pr from 39 ± 10 to 104 ± 32 (p=0.012). During reperfusion, glucose and pyruvate increased, while lactate remained stable, resulting in the normalization of the L/Pr (p=0.012). Glycerol continued to increase to 163 ± 42 µM during initial reperfusion.
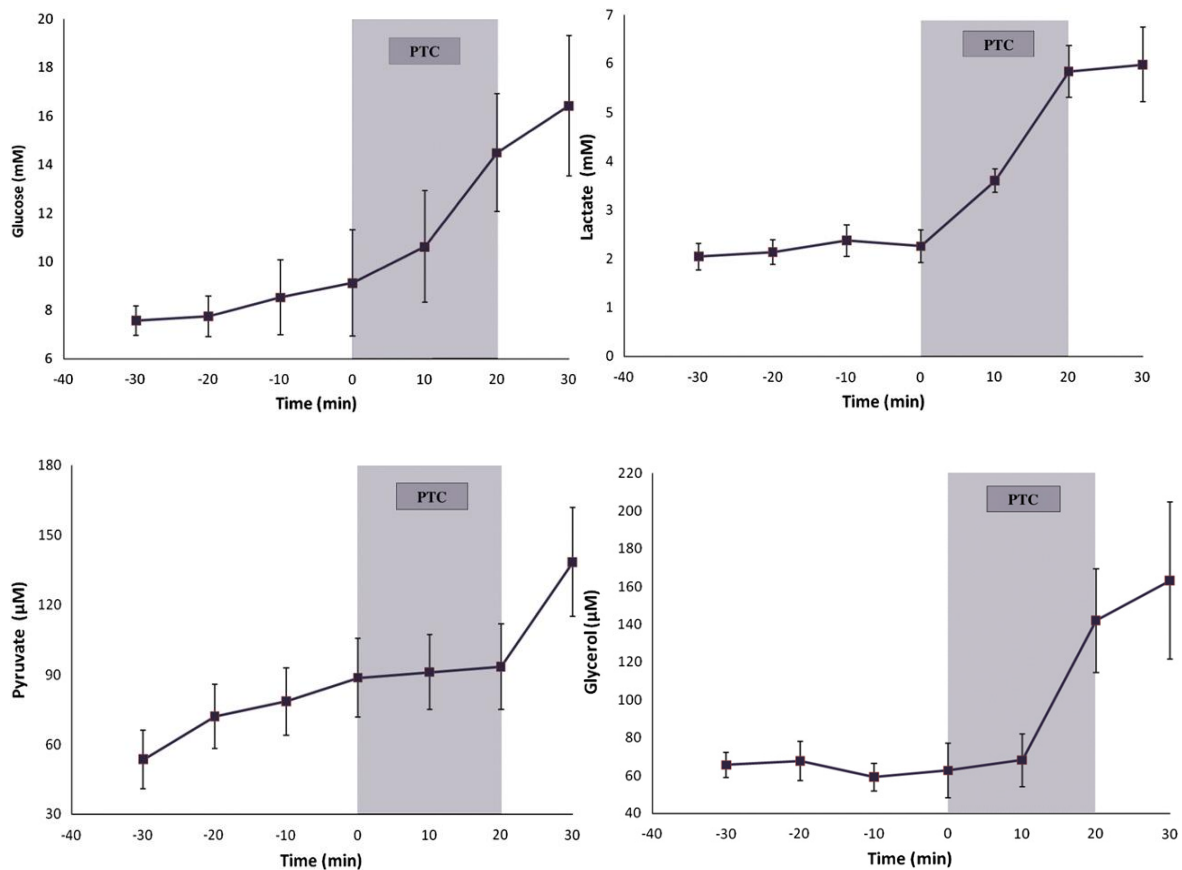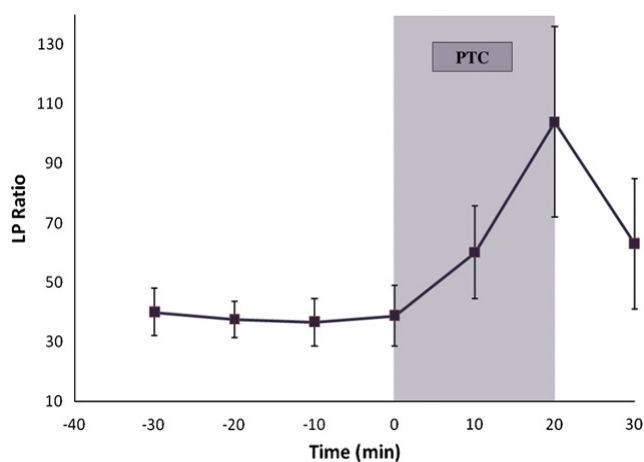
Figure 9. Glucose, lactate, pyruvate, glycerol levels and L/Pr measured by intrahepatic microdialysis before, during 20 minutes of PTC and after 10 minutes of reperfusion.

5.2 PAPER II

The average glucose concentrations measured by the catheters 20R, 20R1, 100R and 100V were 5.8 ± 0.5, 6.9 ± 0.7 mM, 7.0 ± 0.8 and 6.5 ± 0.7 mM (CVs 0.58, 0.65, 0.72 and 0.64), respectively, thus comparable and showing no difference.

Similarly, glycerol levels measured showed no significant difference between the four catheters. Levels measured by 20R, 20R1, 100R and 100V catheters were 10.9 ± 1.2, 9.9 ± 1.9, 13.6 ± 2.0 and 9.7 ± 2.0 µM (CVs 0.70, 1.19, 0.93 and 1.12) respectively.

Dialysate lactate concentrations measured by the 20R, 20R1, 100R and 100V catheters were 2.5 ± 0.1, 2.4 ± 0.1, 3.1 ± 0.2 and 2.7 ± 0.2 mM (CVs 0.44, 0.48, 0.42 and 0.46), respectively, showing no significant differences.

Dialysate concentrations of pyruvate measured by the 20R, 20R1, 100R and 100V catheters were 107.0 ± 7.6, 86.0 ± 7.3 µM), 128.1 ± 6.4 and 115.6 ± 8.2 µM (CVs 0.44, 0.52, 0.30 and 0.43) respectively. Pyruvate measurements by the 100R catheter were higher as compared to the 20R1 catheter ($p<0.01$).

The L/Pr for the catheters 20R, 20R1, 100R and 100V showed no difference and were 25.7 ± 1.6, 24.8 ± 1.2, 29.6 ± 1.5 and 24.2 ± 0.8 (CVs 0.38, 0.30, 0.32 and 0.22) respectively.
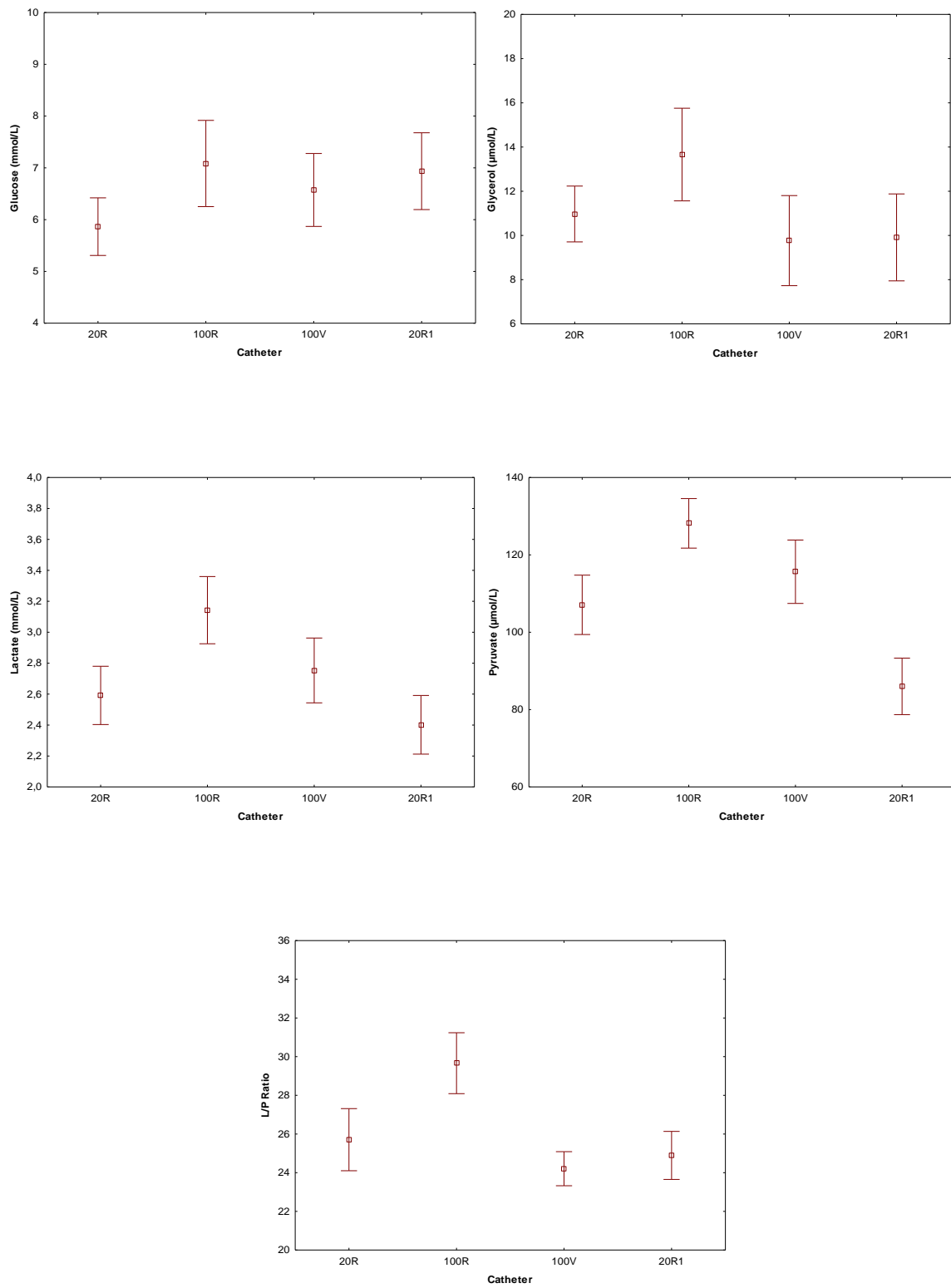
Figure 10. Mean (± SEM in whiskers) dialysate concentrations for glucose, glycerol, lactate, pyruvate and the calculated L/Pr for the catheters 20R, 100R, 100V and 20R1.

5.3 PAPER III

Glucose and glycerol levels measured by the three catheters showed no differences over the course of the experiment before and after arterial clamping.

After arterial clamping, between 60-120 minutes in the subcutaneous catheter and between 105-150 minutes in the intrahepatic catheter lactate levels were significantly higher as compared to before clamping. These differences were significant (p<0.01).

There were significant differences between pyruvate measured by the three catheters (p<0.001). Pyruvate levels increased subcutaneously between 60-120 minutes after arterial clamping (p<0.01) and decreased as measured in the intrahepatic catheters from 90 minutes onwards after clamping (p<0.001).

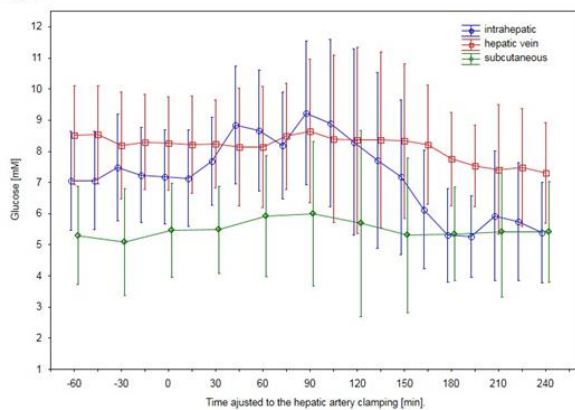L/Pr measured by the three catheters throughout the experiment showed no differences.

Figure 5.

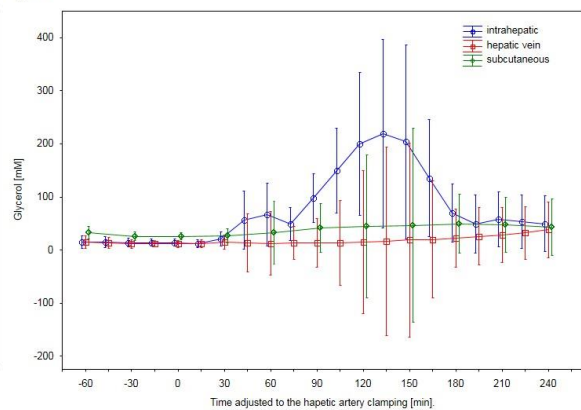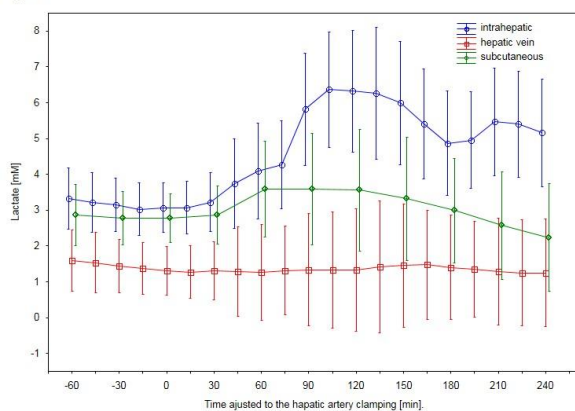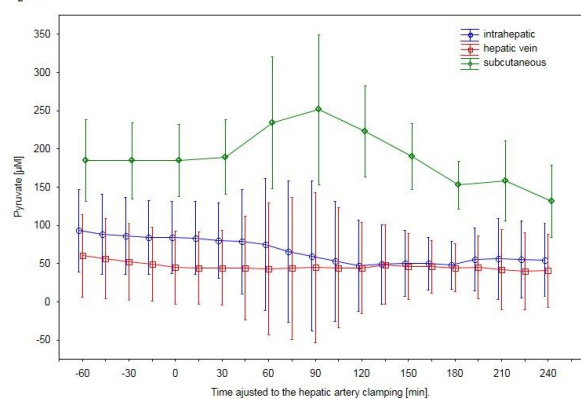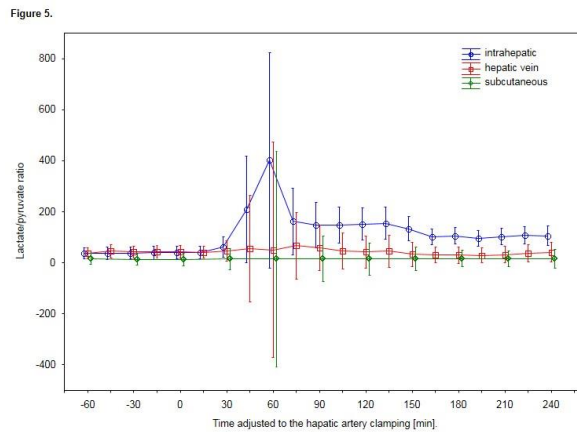**Figure 11. Glucose, glycerol, lactate and pyruvate levels and L/Pr (mean±95% CI) measured in the liver, hepatic vein and subcutaneous catheters before and after arterial clamping. Lactate and pyruvate levels showed significant differences.**

5.4 PAPER IV

5.4.1 Hepatic ultrastructure and morphometry

On EM examination at baseline, the biopsies showed normal hepatocyte and LSEC morphology. The hepatocytes had large numbers of mitochondria. The hepatocytes in the space of Disse showed intact microvilli extensions and normal fenestrated LSECs lined the sinusoids.

Post-ischemia the most prominent finding was a disruption of the LSEC lining in 10 out of 11 patients. The hepatocyte microvilli decreased in number and were undetectable in some cases. Apart from this the hepatocytes had a remarkably preserved morphology with intact membranes. The hepatocyte mitochondria were dilated and exhibited aggregates, so-called "crystalline inclusions" post-ischemia seen in 7 of 11 patients.

Post-reperfusion, there was an apparent reactivation of the LSECs with pseudopod-like extensions appearing from the LSEC surface. In some sections LSEC apoptosis and phagocytosis by KC was noticed. The hepatocyte morphology remained normal and microvilli returned to their normal state. The mitochondrial crystalline inclusions persisted. These findings are shown in Figures 12 and 13 and quantified in Table 1.
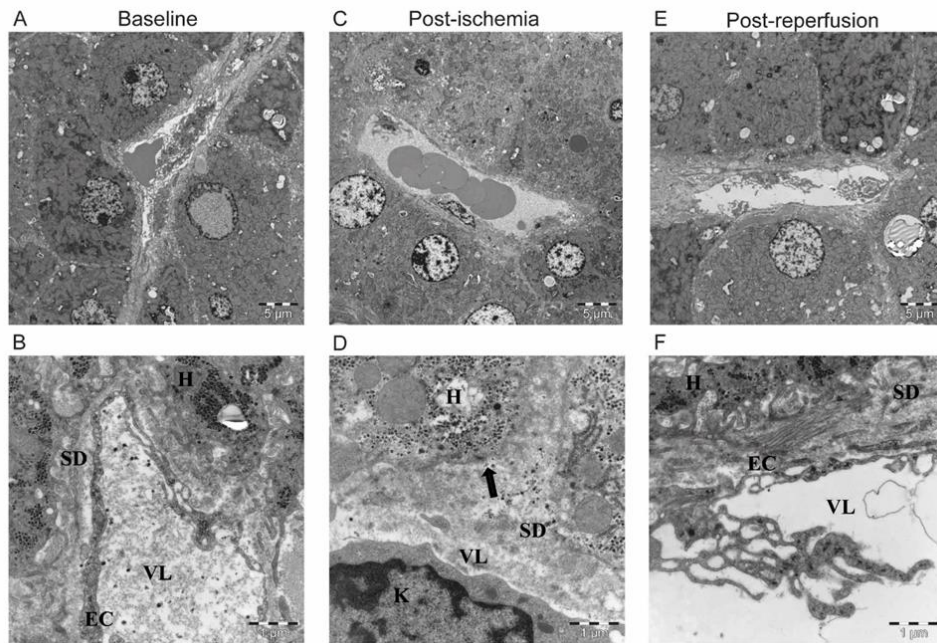
Figure 12. EM images at baseline, post-ischemia and post-reperfusion showing the salient findings. LSEC disruption, and loss of hepatocyte microvilli are seen post-ischemia and LSEC reactivation post-reperfusion.
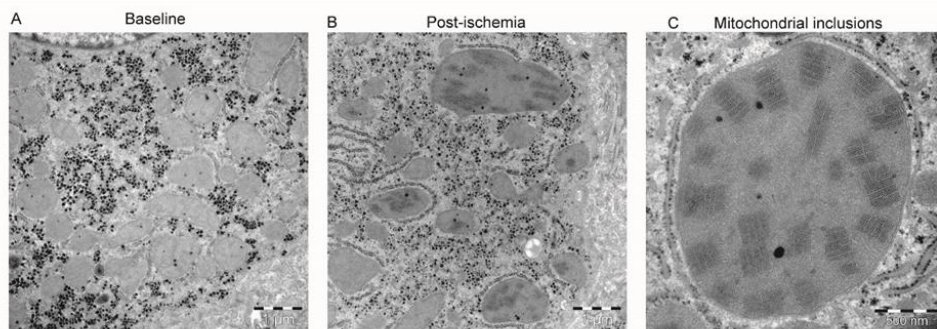
Figure 13. Crystalline mitochondrial inclusion and a few dilated mitochondria seen post-ischemia in the hepatocyte.

Table 1. Quantitative summary of ultrastructural changes. N observations/11 patients

	Baseline	Post-ischemia	Post-reperfusion
Disruption of endothelium	2/11	10/11	2/11
Endothelial activation	4/11	3/11	9/11
Mitochondrial inclusions in hepatocytes	1/11	6/11	7/11
Lipids, lipofucsin	10/11	10/11	10/11

Morphometric image analysis to evaluate the loss and reactivation of the LSEC lining was expressed as a percentage of intact LSEC lining of the total length of the hepatic sinusoid. This revealed a quantitatively significant reduction in the lining post-ischemia compared to baseline (p=0.0003). There was no difference between the baseline level and the post-reperfusion, indicating LSEC lining recovery (Figure 14).
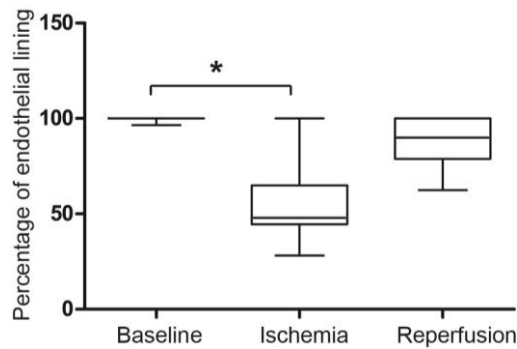
Figure 14. Morphometric analysis showing reduction in LSEC lining post-ischemia.

5.4.2 Immunoelectron microscopy
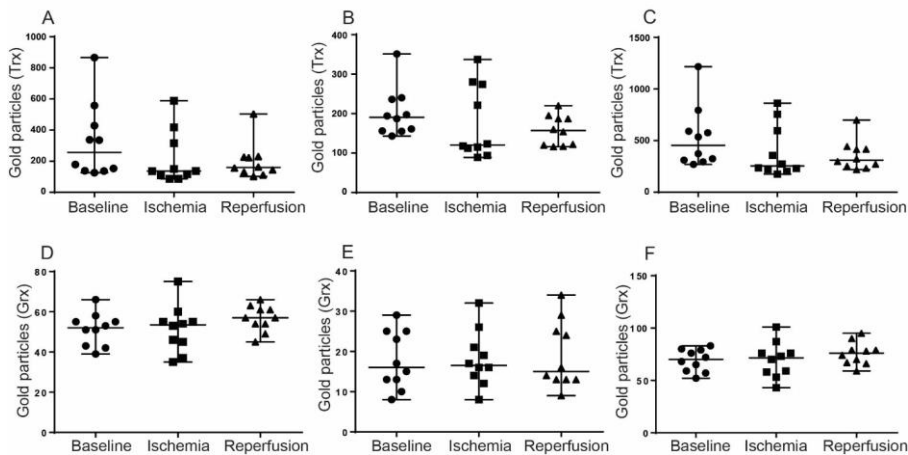
Figure 15. Quantification of immunogold staining. Number of gold particles of 5 hepatocytes for each time point and patient. A - C for TRX and D - F for GRX. A and D – Cytosol, B and E – Nuclei, C and F – Cytosol and nuclei

Immunogold staining for GRX1 and TRX1 showed no significant changes in the amount of TRX1 in the hepatocyte nuclei or cytosol during I/R injury. The total amount of TRX differed in the hepatocytes between the time points. During ischemia TRX level decreased in five,

remained unaffected in three, and increased in two patients (Table 2) while GRX1 levels did not change in any of the patients (Figure 15).

Table 2. Levels of TRX1, evaluated by immunogold staining, total number of gold particles present in five hepatocytes

Patient	Baseline	Post-ischemia	Post-reperfusion
P1	794	357	414
P2	310	202	323
P3	1217	235	219
P4	373	176	298
P5	324	231	232
P6	269	273	443
P7	575	569	252
P8	294	221	699
P9	535	863	417
P10	590	755	272

5.4.3 Gene expression analysis of redox regulating systems

Relative mRNA expression of NFE2L2 and SLC7A11, coding for the redox regulatory proteins NRF-2 and xCT, were investigated along with the TRX family of proteins. There were no differences post-ischemia or post-reperfusion compared with baseline (Table 3).

Table 3. Gene expression of redox proteins changes in mRNA expression compared to baseline, calculated using the 2-ΔΔCT method

	TNX1	*TNX2*	*TNXR1*	*TNXR2*	*GLRX1*	*GLRX2*	*GLRX3*	*GLRX5*	*NFE2L2*	*SLC7A11*
Ischemia	1.09±0.18	1.14±0.30	1.02 ± 0.28	1.05 ± 0.23	1.19 ± 0.41	1.24 ± 0.46	1.03 ± 0.16	1.20 ± 0.27	0.83 ± 0.15	1.27 ± 1.24
Reperfusion	1.12±0.25	1.31±0.57	0.97 ± 0.33	1.39 ± 0.79	1.12 ± 0.67	1.16 ± 0.43	1.16 ± 0.26	1.18 ± 0.63	1.00 ± 0.44	1.22 ± 0.78

5.5 PAPER V

5.5.1 Illustrative Case reports
Patient 1

A young male recipient underwent LT and 22 hours after LT the intrahepatic L/Pr, measured by microdialysis, increased by 70% over a period of two hours, reaching a level of 17.6. Lactate remained stable at around 1.2 mM and pyruvate levels decreased during this L/Pr increase. L/Pr soon returned to previous levels even though pyruvate and lactate levels continued to rise. On day 10, HAT was diagnosed and the patient reoperated and a thrombectomy performed. Unfortunately, the patient eventually developed cholestatic liver failure and had to be retransplanted. It is likely that HAT occurred during the episode with raised L/Pr but the rise was expected to be higher and more prolonged and did not result in any further radiologic investigation at the time.

A   B

Figure 16. Intrahepatic L/Pr and systemic glucose (A) and intrahepatic lactate and pyruvate levels (B) measured by microdialysis for patient case report 1.

Patient 2

A middle-aged male underwent LT and 65 hours after LT, a 50 % increase in the L/Pr was seen over a 3-hour period, reaching a level of 10.8. HAT or PVT was suspected, and an ultrasound followed by a CT angiography was performed showing normal liver circulation. The patient had an uneventful postoperative course thereafter. Analysis showed that during the L/Pr rise pyruvate levels decreased while lactate levels remained stable. Also, the patient experienced an increase in systemic glucose 3 hours after the episode of elevated L/Pr due to a stop in the intravenous insulin infusion.
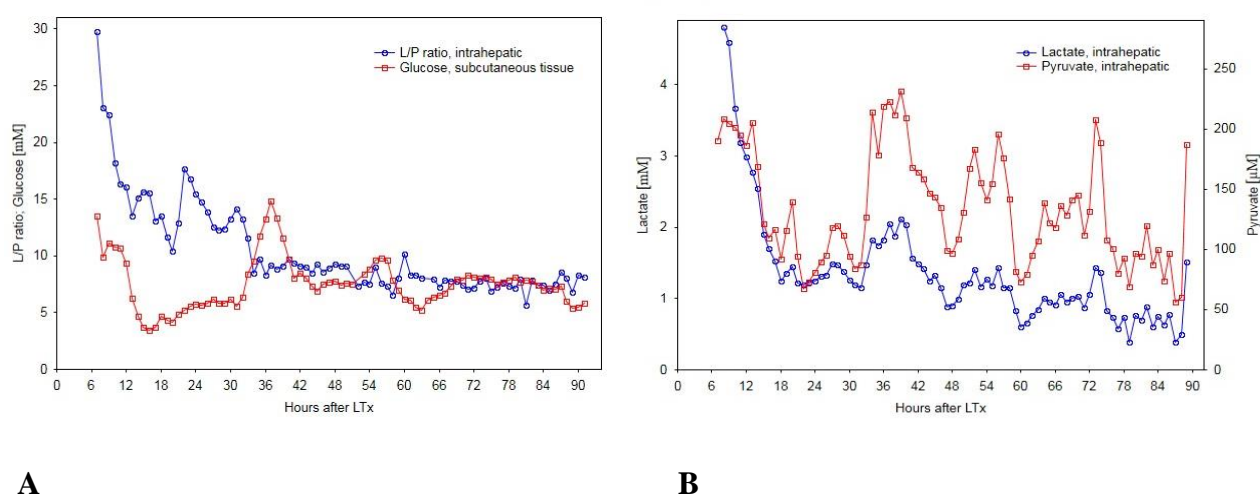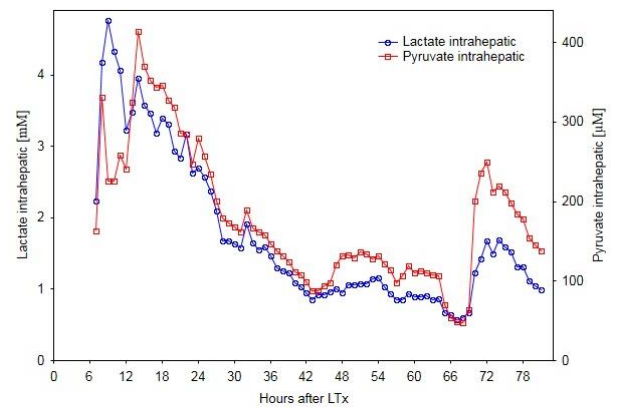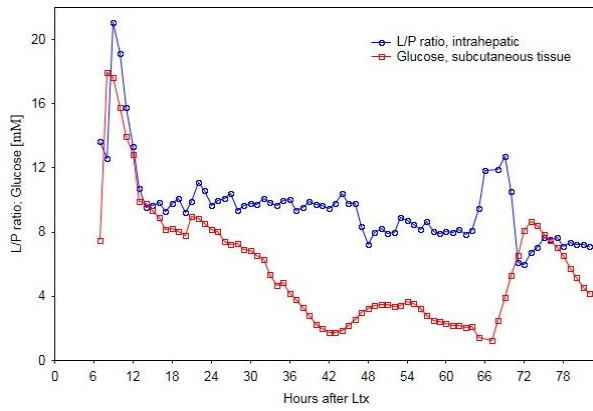
A   **B**

Figure 17. Intrahepatic L/Pr and systemic glucose (A) and intrahepatic lactate and pyruvate levels (B) measured by microdialysis for patient case report 2.

5.5.2 Overall results

No complications related to the microdialysis procedure were observed. Liver graft cold ischemia time ranged from 322-960 minutes (median=718 minutes).

Intrahepatic glucose, lactate, pyruvate and the L/Pr levels in the entire cohort are shown in Figure 18. There was no time correlation between episodes of increased L/Pr and episodes of increase in systemic glucose and no correlation was between intrahepatic glucose and lactate.
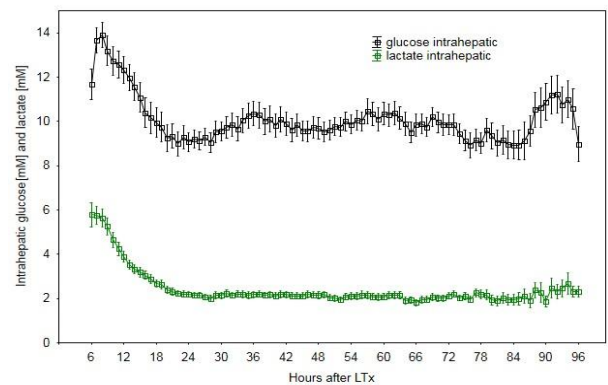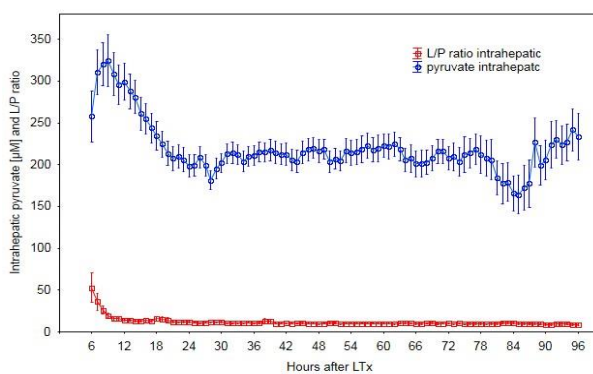
A   **B**

Figure 18. Intrahepatic L/Pr and pyruvate (A) and glucose and lactate (B) levels following LT for the entire cohort. Data presented as mean ± SE.

One patient of 45 in the cohort developed HAT (case report 1). The data revealed 44 events of increased L/Pr (as per study protocol) in 24 patients, none had any clinical, biochemical or radiological signs of ischemic complications. Further analysis of these episodes showed that in 26 of 44 cases the increase was with decreasing lactate and pyruvate levels. In 10 cases there was an increase in lactate and pyruvate levels and in 8 cases the increase in L/Pr was associated with an increase in lactate and decrease in pyruvate. Eight patients (17%) had at least one L/Pr value above the cut-off of 20 and a lactate value above the cut-off of 3mM but without any ischemic complications [126]. L/Pr and lactate values in the patient with HAT did not reach the protocol suggested cut-off values. The results are summarized in Tables 4 and 5.

Table 4. Metabolic events and ischemic complications

	1 patient with ischemic complication*	44 patients without ischemic complications
Events of increase in L/Pr	0	44 (in 24 patients)
Events of increase in systemic glucose	0	116 (in 39 patients)
Events of increase in systemic glucose with simultaneous increase in L/Pr	0	15
Number of simultaneous L/Pr and lactate values above cut-off	0	8

Table 5. Analysis of lactate and pyruvate levels in relation to increased L/Pr

	Lactate ↑	Lactate ↓
Pyruvate ↑	n = 10 (23%)	n = 0 (0%)
Pyruvate ↓	n = 8 (18%)	n = 26 (59%)

5.6 PAPER VI

There were no major adverse events related to the microdialysis procedure. Mean ischemia time was 563 minutes for patients who experienced rejection and 561 for those who did not. Of the 71 patients in the study, 33 (46%) developed ACR within the first month post-LT and was detected on an average 11 days (range 5-23) after transplantation. There was no difference between the groups regarding donor related parameters such as age, cold ischemia time and time-zero biopsy pathology or liver blood chemistry (Tables 6 and 7). However, ASAT was higher in patients with rejection group on day 7 post-LT.

Table 6. Donor age, ischemia time and time-zero biopsy evaluation.

	Mean (Non-rejection)	Mean (Rejection)	Standard Error (Non-rejection)	Standard Error (Rejection)	p-value
Donor age (years)	47.4	53.8	3.1	2.7	0.09
Ischemia time (min)	561	563	28.7	29.2	0.9
Graft steatosis[1]	1.2	1.3	0.07	0.1	0.4
Graft ischemia[2]	2.0	1.8	0.1	0.1	0.2

[1] Graft steatosis was graded on a scale of 1-3; 1 = <10% steatosis / 2 = 11-20% steatosis / 3 = 20-30% steatosis

[2] Graft ischemia was graded on a scale of 1-3; 1 mild ischemia / 2 moderate ischemia / 3 severe ischemia

Table 7. Standard liver blood tests during the first week post-transplantation.

42	ALAT (µkat/L)		ASAT (µkat/L)		Bilirubin (µmol/L)		PT-INR	
	Non-rejection	Rejection	Non-rejection	Rejection	Non-rejection	Rejection	Non-rejection	Rejection
Day 1	18.81±2.68	21.01±2.82	23.30±3.58	30.50±5.13	53.56±6.21	56.33±10.21	1.75±0.05	1.68±0.07
Day 2	17.35±2.33	21.17±3.50	15.30±2.10	22.86±5.49	46.28±5.78	47.33±6.40	1.58±0.07	1.53±0.07
Day 3	14.58±2.09	17.16±2.51	9.63±1.44	13.05±2.21	54.90±7.39	80.01±35.54	1.43±0.05	1.36±0.04
Day 4	10.32±1.45	12.49±1.63	4.81±0.58	6.95±0.99	71.77±9.10	50.78±5.08	1.32±0.03	1.30±0.03
Day 5	7.27±0.99	8.88±1.08	2.56±0.27	3.71±0.50	77.80±10.0	54.87±5.60	1.31±0.04	1.27±0.03
Day 6	5.11±0.62	6.75±0.72	1.59±0.16	2.30±0.22	73.54±9.96	64.60±6.20	1.31±0.03	1.30±0.03
Day 7	4.07±0.46	5.53±0.57	**1.13±0.12**	**1.75±0.22***	75.59±9.86	66.43±8.45	1.28±0.03	1.29±0.04

Data presented as mean±SEM for patients without vs. with rejection. Significant differences between groups are highlighted * ($p < 0.05$).

In the cohort only three patients remained with microdialysis monitoring up to day 6 or longer while the majority had the catheter removed around day 3. Only the first 72 hours of data was available for all patients and microdialysis results are presented in Figure 19.
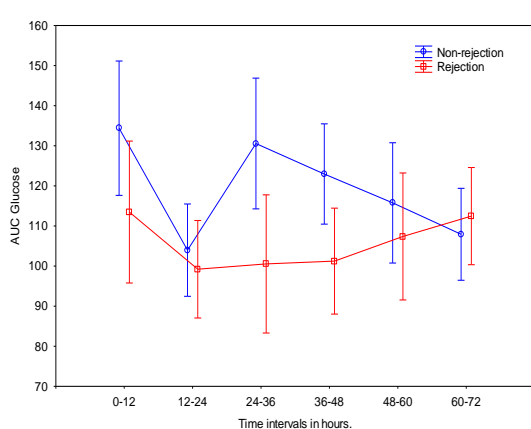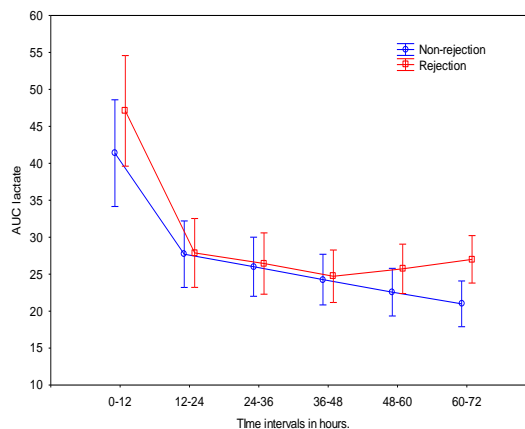
A   B
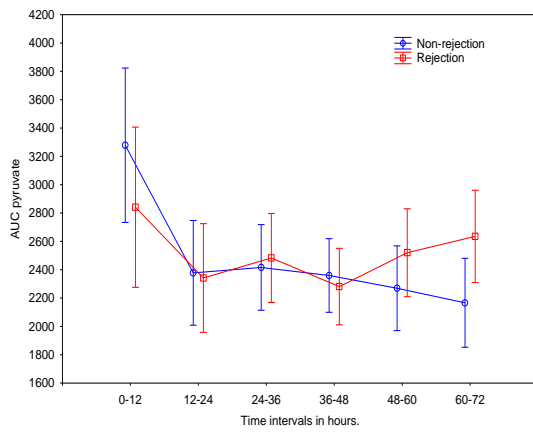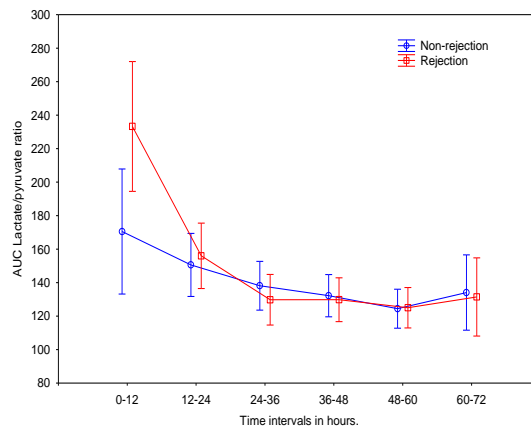
C   D

Figure 19. AUC values for glucose, lactate, pyruvate and L/Pr (A-D) for patients with and without rejection. Data presented as mean ± 95% confidence intervals.

The L/Pr was higher in the group with rejection at 1-12 hours after LT. Glucose was higher in the group without rejection at 24-48 hours. Lactate levels were higher at 48-72 hours and pyruvate levels were higher at 60-72 hours post-LT in the rejection group as compared to the non-rejection group. Glycerol levels showed no difference between the groups.

The forward stepwise logistic regression model showed that only L/Pr during the first 12 hours post-LT was an independent predictor of early post-transplant rejection (p=0.05).

6 DISCUSSION

6.1 PAPER I

At the time of publication, this study was one of the first to investigate microdialysis in human non-transplanted livers. The aim was to establish the feasibility of intraoperative microdialysis in liver resection patients and to study the effects of warm ischemia induced by PTC. We could show that microdialysis could be safely performed in these patients without any procedure-related complications.

The results of the study revealed substantial metabolic alterations associated with PTC with increasing glucose, lactate and glycerol levels measured by intrahepatic microdialysis. Pyruvate levels were stable and the L/Pr thus rose significantly. These findings were consistent with previously published animal studies on warm ischemia [92, 112]. The increase in glucose levels could be attributed to hypoxia induced glycogenolysis and possibly gluconeogenesis [128]. On reperfusion glucose levels continued to rise due to the reestablishment of the blood supply. Due to the shift to anaerobic metabolism as a result of PTC, ATP production is sustained by the reduction of pyruvate to lactate and the resulting raised L/Pr is a better indicator of ischemia than just the raised lactate alone [92, 129, 130]. Pyruvate levels did not decrease during PTC-induced warm ischemia but remained stable and could be due to the increased glucose levels, similar to previous reports [112, 114]. Pyruvate levels increased significantly on return to aerobic conditions during reperfusion and lactate levels remained stable during the initial phases reflecting a post-ischemic hypermetabolism.

Glycerol is a key constituent of the cell membrane. Damage to the cell wall as a result of ischemia causes glycerol to leak out into the interstitium, which can then be measured by microdialysis as a marker of cell membrane damage [112-114, 131, 132]. In our study glycerol levels rose during PTC and during reperfusion suggesting a persistent cell membrane damage probably a result of the free radical mediated damage to cellular structures caused by reperfusion injury [60].

This study showed that at the metabolic level there were significant changes induced by PTC-associated warm I/R injury and these could be measured by intrahepatic microdialysis. The findings of the study documented the deviations in glucose metabolism and the cell membrane damage occurring as a result of this injury. PTC or the Pringle manoeuvre has been proven to be an effective method to reduce blood loss during liver surgery and is sometimes an indispensable surgical tool but is still used selectively [44, 45]. The results of

our study suggest that in situations with compromised liver function the hepatic parenchymal injury induced by PTC must be taken into consideration when deciding on its use and duration.

6.2 PAPER II

Hepatic microdialysis is conventionally performed using catheters with a membrane cut-off of 20-kDa to measure the small metabolites glucose, lactate, pyruvate and glycerol. Larger molecules like cytokines, chemokines and complement play a significant role in I/R injury and studying these molecules entails the use of microdialysis catheters with a larger cut-off of 100 kDa [133, 134]. The aim of this study was to evaluate whether 100-kDa catheters could be used to measure small molecules such as glucose, lactate, pyruvate and glycerol with equivalent results as 20-kDa catheters in a pig liver model.

The study design was to use two catheters with 20 kDa cut-off and two with a 100 kDa cut-off. To circumvent the issue of volume loss when using 100-kDa catheters we used the colloid solution Voluven (hydroxyethyl starch) as perfusion fluid for one of the 100-kDa catheters in our study. In doing so, the aim was to counteract the osmotic effect of plasma proteins and thus help conserve volume inside the catheter [135, 136]. Ringer acetate-like T1 solution was used in the other catheters.

The results of our study showed stable values for the parameters measured during the monitoring time, given that no manipulations of the liver or vascular clamping manoeuvres were carried out. The average levels measured by the 100kDa catheter perfused with Ringer like solution (100R) for glucose, glycerol and lactate tended to be higher as compared to the other catheters, although this difference was not statistically significant. Pyruvate measurements were however significantly higher in the 100R catheter when compared to the 20R1 catheters (P<001). This could be because of volume loss in or higher recovery of the 100R catheter. The 100V catheter however measured the four molecules comparably to the 20-kDa catheters perfused with Ringer acetate-like T1 solution. It can thus be concluded that when using microdialysis catheters with 100 kDa cut-off for the measurement of small molecules in hepatic microdialysis, a high osmolality solution (Voluven) should be used as perfusate.

6.3   PAPER III

Hepatic microdialysis has been used extensively in the setting of both liver resection surgery and transplantation [113, 114, 126, 134, 137]. Although an invasive method, its safety has been established and it has a very low complication rate. There are a few problems associated with the method which have prevented its widespread clinical application for postoperative monitoring. Most patients find the catheter to be uncomfortable and are disturbed by the hourly sampling especially during night time.

We hypothesized that placing an intravascular microdialysis catheter with the tip in the hepatic vein would be theoretically as close to the hepatic interstitial compartment as possible. This could be done relatively non-invasively using the transjugular approach and advancing the microdialysis catheter as far as it would go into the middle hepatic vein using techniques used for placement of TIPS (Transjugular Intrahepatic Portosystemic Shunt) creation and HVPG (Hepatic venous pressure gradient) measurement [138, 139]. This would circumvent some of the issues discussed above and also the catheter could be replaced easily if needed. Conceptually, the microdialysis catheter placed in the hepatic vein, would sample blood directly leaving the liver, which in terms of composition should be as close to the hepatic interstitium that an intrahepatic microdialysis catheter samples. Several studies have proven the technical feasibility of intravascular microdialysis and results have been validated [140-142]. We devised an animal model of hepatic arterial ischemia comparing hepatic vein microdialysis and intrahepatic microdialysis for the monitoring of the metabolites, glucose, lactate, pyruvate and glycerol.

Analysis of the microdialysis data from the intrahepatic catheters showed that glucose, lactate, glycerol levels and the L/Pr after an initial rise, continued to decline despite persistent clamping similar to findings of a study previously published by us [125]. This can be explained by a possible increase in oxygen extraction from portal venous blood by the hepatocytes. An interesting finding was that pyruvate levels in the subcutaneous catheter increased significantly during clamping but declined in the liver suggesting a possible systemic hypermetabolism as a result of hepatic ischemia.

Glucose, glycerol, lactate and pyruvate and the calculated L/Pr levels remained stable throughout the experiment in the hepatic vein catheter. None of the changes in response to hepatic arterial clamping seen in the intrahepatic and subcutaneous catheters were registered in the hepatic vein catheter. It could be that in fact the tip of the hepatic vein catheter was too far from the interstitium resulting in a "dilution effect" or that the composition of the hepatic

venous blood is not comparable to that of the interstitium as hypothesized. Also, the recovery of metabolites could be reduced due the hepatic venous system being a "high flow" one. The recovery could be improved by increasing membrane length or adjusting the perfusion flow rate [115, 143, 144]. Thus, although hepatic vein microdialysis as a concept was an interesting idea, it could not in this study be used with equivalent results as standard intrahepatic microdialysis. Technical refinements could make it a feasible concept in the future.

6.4   PAPER IV

In this study we used PTC for a fixed period of time to establish controlled experimental conditions to study the effects of warm I/R injury in the human liver. The scope of the study was broad in that we studied ultrastructural changes using EM and in addition even redox enzyme systems with purported roles in I/R injury. Given that PTC is routinely performed usually for not more than 20 minutes, ethical considerations did not permit longer ischemia times which would have been desirable. The limitations notwithstanding, this study gives us a valuable information to further the understanding of hepatic I/R injury.

The most striking finding of this study was the disruption of the LSEC post-ischemia seen on EM. This loss was quantitatively significant as seen on morphometric analysis. This goes against the conventional understanding that hepatocytes are more susceptible to warm I/R injury and LSECs to cold [9, 71]. However, LSEC death preceding that of hepatocytes has been reported in warm I/R injury too [145]. Apart from the fact that LSECs bore the major impact of the ischemic insult, another remarkable finding was the formation of pseudopod-like projections from the LSEC surface on reperfusion. This was interpreted as a reactivation or reattachment of the LSECs. This finding was seen early and in 9 of 11 patients suggesting a reversibility of LSEC damage when the magnitude of ischemia is limited. Similar findings had been reported in cold ischemia [146]. Due to the short duration of the experiment and the fixed time-points of biopsy acquisition, the later phases of reperfusion injury could not be studied.

The hepatocyte morphology seemed however remarkably well preserved as a response to warm I/R injury in this study, consistent with previous reports of intermittent PTC [147]. There was however, a loss of microvilli in the space of Disse after PTC and some had condensed nuclear chromatin. Additionally, the hepatic mitochondria were dilated and

showed the presence of crystalline inclusions which persisted after reperfusion. The nature of these crystalline inclusions usually seen in early alcohol and other liver diseases and aspirin toxicity is still unknown [148-151]. These inclusions could be an evolutionarily preserved adaptive response to ischemia.

Gene expression analysis of TXN and GLRX isoforms and the associated redox proteins were not altered in this study. Immunogold results for TRX1 and GRX1 showed no changes in the hepatic GRX1 levels, but the levels of TRX1 present in the hepatocytes varied between the time points, suggesting a possible redistribution of the protein and thus a tentative role for the TRX family of proteins in warm I/R injury. This merits further investigation.

6.5   PAPER V

Only 1 of 45 (~2%) patients in our cohort developed HAT and thus describing the metabolic changes accompanying ischemic complications after LT was not feasible in this study. This patient however did not have L/Pr or lactate levels reaching the cut-offs defined by the study protocol to suggest ischemia. In case report 2 the patient reached cut-off levels but had no documented vascular complication. Analysis revealed that the rise in L/Pr in both patients was due to stable lactate levels and decreasing pyruvate and was followed by decreasing L/Pr as a result of increasing lactate and pyruvate levels suggestive of hypermetabolism.

We analyzed the events of protocol-defined L/Pr rise in our study. Of a total of 44 such events in 24 patients, 26 (59%) had decreasing lactate and pyruvate levels during the episodes with faster decrease in pyruvate. This suggests recovery of metabolism in the transplanted liver rather than ongoing ischemia with a faster rate of metabolism of pyruvate. This preferential metabolism of pyruvate may be due to the fact that it can regulate its own metabolism and that there are more pathways for its metabolism [152]. In 10 patients (23%) with raised L/Pr, both lactate and pyruvate levels rose. The rising pyruvate makes ischemia unlikely and indicates a higher rate of lactate accumulation than pyruvate suggestive of hypermetabolism. Eighteen percent of the events (8 of 44) with increased L/Pr were accompanied by an increase in lactate and a decrease in pyruvate. Furthermore 17% of patients had L/Pr and lactate above the cut-off levels suggested by Haugaa et al. [126]. None of these patients had any clinical or radiological signs of or diagnosed ischemic complications. The possibilities are that the raised L/Pr was due to reasons other than graft

ischemia or that there indeed occurred ischemic events, but they were of no clinical significance as the clinical course continued uneventfully.

Glucose metabolism in the liver is complex and differs fundamentally from other tissues. The liver enzymes involved in glucose metabolism are uniquely sensitive and the liver responds rapidly to ischemia and decreased glucose supply. The metabolic patterns detected by microdialysis in the post-LT phase probably reflect the effects of primary I/R injury on enzyme activity regulating glucose metabolism in the liver and the recovery of the metabolic machinery after LT. Intrahepatic L/Pr as an independent variable measured by microdialysis is a product of the complex metabolic interplay occurring in the liver and systemically in the early post-transplant phase. In our study using the suggested cut-off levels resulted in far too many false positives and L/Pr is thus not a reliable or specific marker of clinically significant ischemic complications post-LT.

6.6 PAPER VI

Rejection is not an instantaneous phenomenon and develops over time. Relative changes of metabolites measured by microdialysis over time more accurately reflect changes in the interstitium as compared to absolute values [144, 153]. In this study we have used AUC values for 12-hour intervals for the parameters glucose, lactate, pyruvate, glycerol and the L/Pr.

Analysis of the microdialysis data shows that in the first 12 hours post-LT, patients who developed ACR had higher levels of intrahepatic L/Pr as compared to those without rejection. Logistic regression showed L/Pr AUC to be the only parameter in the first 12 hours post-LT to be associated with rejection. This finding suggests that grafts that develop ACR suffered greater primary I/R injury during storage and transplantation which is inherent to the transplant procedure [5]. This injury is seen only in the metabolic parameters and not in the time-zero biopsy. It is known that L/Pr decreases in the early post-LT phase, and in this cohort similar patterns were noted indicating a recovery from ischemia [113]. Additionally, in the rejection group, intrahepatic glucose levels were lower on day 2 after transplantation indicating a slower recovery of the gluconeogenetic pathway consequent to ischemia. Primary I/R injury to the graft before and during the transplant is an independent predictor of poor outcome after LT and has been linked to the development of rejection [154-157]. Our data supports this association.

On day 3 after LT, intrahepatic lactate and pyruvate levels were higher in patients who developed ACR. This could be the consequence of post-ischemic-hypermetabolism or the result of increased aerobic glycolysis due to lymphocyte activation as suggested by Haugaa et al. [126, 158, 159]. It could also be that the glucose produced as a result of ischemia induced glycogenolysis in the hepatocytes cannot enter the citric acid cycle at the rate of production and is thus converted to pyruvate and lactate, leading to higher levels on day 3. Glycerol levels showed no increase as would be expected in the patients who developed ACR and it could be that if microdialysis had been carried out for a longer period this would have been observed, but this was not practically possible.

In the modern practice of LT, patients are discharged by postoperative day 8 to 10 and it is very hard to justify long periods of microdialysis monitoring. Consequently its use has not been introduced in the standard post-transplant monitoring protocol. Furthermore, it is evident that the interpretation of microdialysis results in the setting of LT is extremely challenging due to the complexity of the metabolic state of the post-transplant liver. Indeed, microdialysis monitoring following LT may detect some of the metabolic events that precede ACR but maybe not rejection itself. Devising strategies to attenuate the duration and severity of primary I/R injury to the liver graft may help to reduce the impact of ACR.

7 CONCLUSIONS

This thesis allows the following conclusions to be made:

Microdialysis allows monitoring of intrahepatic metabolism during liver resection surgery and is easy to use in humans.

Warm ischemia-reperfusion injury induced by portal triad clamping is associated with considerable alterations with hepatic anaerobic metabolism, increased glycogenolysis, and cellular membrane damage.

Microdialysis catheters with membrane cut-off of 100 kDa can be used equally as 20 kDa catheters in intrahepatic microdialysis for the monitoring of glucose, glycerol, lactate and pyruvate, and lactate/pyruvate ratio if a high osmotic solution such as Voluven is used as the perfusion fluid. It may be possible to monitor larger molecules using microdialysis with 100kDa catheters without affecting the monitoring of small molecules.

Hepatic vein microdialysis cannot be used equally as compared to direct intrahepatic microdialysis for the monitoring of glucose, glycerol, lactate and pyruvate.

The major impact of hepatic warm I/R injury induced by portal triad clamping is borne by the sinusoidal endothelial cells with detachment and reactivation at ischemia and reperfusion, respectively. Hepatocyte morphology is well preserved apart from crystalline inclusions in the mitochondria. In situ protein observations suggest a tentative role for the thioredoxin family of proteins in liver ischemia-reperfusion injury

Using microdialysis as a monitoring tool, an increase in the intrahepatic lactate/pyruvate ratio is not necessarily indicative of ischemic complications and may be raised in the absence of clinically significant ischemia in the liver early after transplantation.

The metabolic patterns detected by microdialysis early after liver transplantation indicate a possible relation between primary ischemia-reperfusion injury and the development of acute cellular rejection.

8 FUTURE PERSPECTIVES

The full potential of microdialysis as a clinical monitoring tool in liver resection surgery and transplantation has not been fully realized. Whether it is due to the complexity of the liver's metabolic machinery or need for better refinement of the technique merits further research. Microdialysis can be used to study the hepatic response to not only I/R injury but also to investigate other conundrums like liver regeneration. To this end studies monitoring inflammatory markers, ROS, NO and gene products can add to the current knowledge with possible implications for clinical practice. Also, the use of large pore microdialysis catheters makes possible the study of bigger molecules.

In our study investigating the ultrastructural changes associated with warm I/R injury, two striking findings were the hepatocyte mitochondrial crystalline inclusions and the apparent LSEC reactivation on reperfusion. Studies to further investigate these findings and shed light on their significance would be very valuable. Indeed, the LSEC reactivation occurs very early during reperfusion, probably indicating a key role for this cell type in the liver's tolerance to ischemia and possibly other insults.

Complementary to the EM study on liver resection patients, a similar study in the transplant setting would be extremely interesting but a logistically difficult one to perform. Apart from the ultrastructural findings, we would even be better able to study the role of the redox enzyme systems in I/R injury. The longer duration of I/R injury during LT would make it feasible to study changes at the gene expression level.

The concept of hepatic vein microdialysis could be taken further by employing the TIPS technique to place the transjugular microdialysis catheter with the tip directly in the liver to get an intraparenchymal liver monitoring. However, the technicalities and risks with this approach need to be evaluated first.

Lastly, the findings of our study on rejection suggested a possible link between primary I/R injury and the development of ACR. This association merits further investigation due to its immense implications for the clinical practice of liver transplantation.

9 POPULÄRVETENSKAPLIG SAMMANFATTNING

Blödning är en av de vanligaste och allvarligaste komplikationerna i samband med leverkirurgi, då delar av levern tas bort. I samband med leveroperationer strävar man efter att minska blödningen så mycket som möjligt. Det vanligaste sättet att göra detta är att stänga av leverns blodflöde under tiden delningen av levervävnaden pågår. Dessa och andra intraoperativa tekniker vid leverkirurgi orsakar förändringar i leverns ämnesomsättning och vävnadskada i levern (s.k. ischemi-reperfusionsskada; I/R skada). Levertransplantation medför att organet är utan blod under en tidsperiod. När levern sedan opereras in i kroppen och blodflödet återställs kan ytterligare skada ske. I/R skada påverkar direkt leverns viabilitet och kan ge upphov till alltifrån organdysfunktion till livsfarlig leversvikt. Effekterna av I/R skada på leverns ämnesomsättning samt på mer subtila funktioner är ofullständigt studerade. Mikrodialys är en metod för att mäta olika ämnen direkt i vävnaden. Oftast mäts metaboliter i kolhydrat- och fettomsättning, såsom glukos, laktat, pyruvat och glycerol.

I vår första studie undersöktes metabola förändringar i lever med mikrodialys hos patienter som genomgick leverkirugi. Blodflödet till levern stängdes av i 20 minuter och mikrodialys genomfördes. Resultaten visar ökade nivåer glukos, laktat, glycerol och ökad laktat/pyruvat-kvot (L/Pr) i samband med blodflödesavstängningen, vilket tyder på betydande vävnadsskada.

Vår andra och tredje studie är experimentella grisstudier med mikrodialys. I ena studien jämfördes två mikrodialyskatetrar med olika membranstorlek (20 och 100kDa). Vi kunde påvisa att 100 kDa katetrar var likvärdiga som 20 kDa katetrar för mätning av små molekyler. I den andra studien undersöktes huruvida en mikrodialyskateter som placerats i levervenen gav likvärdig mätning som mikrodialyskateter som placerats i levervävnaden. Studien visar att intravenös mikrodialys inte ger samma resultat som direkt mikrodialys i levern.

I vår fjärde studie studerades förändringar i levervävnad med olika tekniker hos patienter som genomgick leverkirurgi. Leverbiopsier togs vid tre tidpunkter: före och efter blodflödesavstängningen samt efter att blodflödet återställts. Vävnadsproverna studerades med elektronmikroskopi samt analyser för protein- och genuttryck. Vi kunde observera morfologiska förändringar främst i celler som omger blodkärl runt levercellerna. Levercellerna klarade sig ganska bra utan större tecken till skada. Ingen påverkan på redoxenzymer kunde ses under studietiden.

Studie 5 och 6 utfördes på patienter som genomgick levertransplantation. De övervakades med mikrodialys postoperativt. Tidigare studier har visat att L/Pr är en känslig markör vid vävnadsischemi. Vi kunde inte påvisa något samband mellan ökad L/Pr och kärlkomplikationer efter transplantation. Vi studerade också sambandet mellan tidiga metabola förändringar i den transplanterade levern och senare utveckling av akut avstötningsreaktion. Hos patienter som utvecklade akut avstötning efter levertransplantation sågs högre L/Pr under de första 12 timmarna efter transplantationen. Detta tyder på ett samband mellan primär I/R skada och akut avstötning efter levertransplantation.

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