Monitoring of liver grafts with microdialysis catheters

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List of papers

1. Haugaa H, Thorgersen EB, Pharo A, Boberg KM, Foss A, Line PD, Sanengen T, Almaas R, Grindheim G, Pischke SE, Mollnes TE, Tønnessen TI: **Early bedside detection of ischemia and rejection in liver transplants by microdialysis**. Liver Transpl. 2012; 18: 839-49

2. Haugaa H, Thorgersen EB, Pharo A, Boberg KM, Foss A, Line PD, Sanengen T, Almaas R, Grindheim G, Waelgaard L, Pischke SE, Mollnes TE, Tønnessen TI: **Inflammatory markers sampled by microdialysis catheters distinguish rejection from ischemia in liver grafts.** Liver Transpl. 2012; DOI: 10.1002/lt.23503

3. Haugaa H, Almaas R, Thorgersen EB, Foss A, Line PD, Sanengen T, Bergmann GB, Ohlin P, Grindheim G, Wælgaard L, Pischke SE, Mollnes TE, Tønnessen TI. **Clinical Experience with Microdialysis Catheters in pediatric Liver Transplants.** Liver Transpl. 2012; DOI: 10.1002/lt23578

Abbreviations

ACR: Acute cellular rejection

ALT: alanine transaminase

AST: aspartate transaminase

ADP: adenosine diphosphate

ATP: adenosine triphosphate

AUC: Area under curve

C5a: complement activation product 5a

CE : Conformité Européenne

CRP: C-reactive protein

CXCL: C-X-C motif chemokine

CT: computed tomography

ELISA: enzyme-linked immunosorbent assay

ERC: endoscopic retrograde cholangiography

FDA: Food and Drug Administration

HMBG1: high mobility group box 1

HCV: hepatitis C virus

ICU: Intensive Care Unit

IL: interleukin

IP: Interferon-inducible protein

LDH: lactate dehydrogenase

MARS : Molecular adsorbent recirculating system

MELD: Model for end stage liver disease

MIP: macrophage inflammatory protein

MMF: mycophenolate mofetil

NAD^+: nicotinamide adenine dinucleotide (oxidized form)

NADH: nicotinamide adenine dinucleotide (reduced form)

PAMP: pathogen associated molecular pattern

ra: receptor antagonist

RAI: Rejection activity index

ROC: Receiver-operating characteristics

U/L: units per liter

Introduction

Evolution of liver transplantation

Organ transplantation is a treatment modality typically offered to patients with severe organ failure. As such, end stage liver disease is not consistent with life, and in the absence of other treatment alternatives liver transplantation saves thousands of lives yearly worldwide. In Norway 90-100 liver transplantations are now performed each year.

The first attempt of human liver transplantation was performed by Thomas Starzl et al in Denver, Colorado, in 1963.[1] Several attempts were undertaken before the first short term success with one year survival was performed in 1967. Despite improvements in surgical techniques, the procedure remained experimental throughout the 1970s with one year survival rates of about 25 %.

Except monozygotic twins, every human and animal is immunologically unique implying that organs from other bodies will be recognized as foreign and attempted rejected. As in other solid organ transplants, rejection was a major problem during the first years of liver transplantation. Patient and graft and patient survival were often restricted by rejection itself, or by severe infections as a result of high doses of glucocorticosteroids administered to prevent and treat rejections. As such, the introduction of the calcineurin inhibitor cyclosporine by Sir Robert Calne in 1978[2] was a milestone in the field of transplantation medicine. Cyclosporine is a very effective drug in preventing rejection and concurrently with growing use the graft survival rates increased rapidly. After a consensus conference in 1983 liver transplantation was no longer considered an experimental procedure,[3] but endorsed as an established treatment option for patients with end-stage liver disease. Cyclosporine has later widely been replaced by tacrolimus.

Advantages of tacrolimus are better efficacy enabling administration of corticosteroids at lower doses, and lower risk of developing hypertension and renal failure.[4;5] Both act as inhibitors of calcineurin, a calcium dependent protein phosphatase, and thereby inhibit the formation of interleukin (IL) 2 by T-lymphocytes.[6;7] Mycophenolate mofetil (MMF), a selective inhibitor of B- and T-lymphocyte proliferation, was introduced in the same period as tacrolimus.[8] One of the major advantages with MMF compared to the calcineurin inhibitors is that MMF is not nephrotoxic.

Along with the development and release of improved immunosuppressive agents, continuous improvements in surgical techniques contributed to higher graft- and patient survival rates throughout the 1980s. Introduction of venovenous bypass in the early 1990s at the University of Pittsburgh, Pennsylvania, represented a major improvement.[9;10] However, after introducing the piggyback technique enabling surgery without clamping of the inferior caval vein,[11] venovenous bypass is now considered superfluous in most transplantation centers. Today, a number of liver transplantations are performed without transfusion of red blood cells, but is still considered one of the most risky procedures performed in hospitals. [12]

Still, end stage liver disease where conventional treatment options have failed is the most common indication for liver transplantation. However, and because of the four decade long story of medical success, the specter of indications have grown. Most important is that patients certainly ending up with an end stage liver disease are transplanted at an earlier time point, but of importance is also that patients with e.g. malignancy[13] and metabolic disorders like oxalosis[14] are considered transplant candidates today. Earlier, the transplantation procedure was considered too risky for patients who are not at immediate risk of death. An overview of the primary diagnoses in 48,218 patients transplanted in Europe from 1999 to 2009 is showed in Table 1.[15]

Table 1. Primary Indications for Liver Transplantation Europe 1999-2009		
Indications for Liver Transplantation	Percentage of total	Percentage of disease
Acute hepatic failure	7	
Virus		19
Drug		21
Toxic		5
Postoperative/traumatic		3
Other/unknown		52
Cholestatic disease	10	
Primary biliary cirrhosis		41
Primary sclerosing cholangitis		46
Others		13
Congenital biliary disease	4	
Extrahepatic biliary atresia		77
Alagille syndrome		7
Others		16
Cirrhosis	53	
Virus related		43
Alcoholic		38
Combined virus and alcohol		6
Cryptogenic		7
Autoimmune		4
Others		2
Primary liver tumors	16	
Hepatocellular carcinoma, with cirrhosis		90
Hepatocellular carcinoma, without cirrhosis		3
Others		7
Secondary liver tumors	1	
Neuroendocrine		60
Colorectal		7
Others		43
Metabolic diseases	6	
Budd-Chiari	1	
Polycystic liver disease	1	
Others	1	
Note: Data is reprinted from Adam et al.[15] with permission from Elsevier.		

Surgical procedure in 2012

Liver transplantation is typically performed as orthotopic liver transplantation (OLT). Orthotopic means that the old liver is removed and the new liver is positioned at the same site. Although the hepatectomy may be challenging with risk of severe bleeding due to presence of (venous) portosystemic shunts and perihepatic tissue adherences, it is usually performed without venovenous bypass in an inferior caval vein saving procedure. The new liver, which may be a

whole liver or a split segment, is then transplanted. Children typically receive a split segment due to limited space in the abdominal cavity, and in such cases the largest split segment can be transplanted into an adult in a separate procedure performed simultaneously. Most often the piggyback technique is used, i.e. the liver graft with its hepatic veins is sutured to the recipient's inferior caval vein. Thereafter the portal veins from graft and recipient are coupled by an end to end anastomosis and the liver is perfused with portal vein blood. The time point of portal vein reperfusion represents the end of graft ischemia which starts with clamping of the donor's aortic artery. Severe systematic hemodynamic instability is frequently seen during the first minutes after coupling the graft to the recipient's circulation, and is usually termed reperfusion syndrome.[16] Thereafter, the arterial anastomosis is carried out. Frequently, two and sometimes three hepatic arteries must be sutured and in particular in pediatric liver transplantation this may be challenging due to small anatomical sizes. However, reperfusion syndrome is rarely seen after hepatic artery reperfusion. The biliary anastomosis is made either as choledochocholedochostomy or, in case of preexisting biliary disease, as choledochojejunostomy or choledochoduodenostomy. Finally, 1-2 abdominal drains are established before closure of the abdominal wall.

Immunosuppression in 2012

Immunosuppression is most often achieved administering a combination of a glucocorticoid, a calcineurin inhibitor, and mycophenolate mofetil. In cases of severe risk of rejection, such as redo transplantations and patients with primary sclerosing cholangitis (PSC), or preexisting impairment of renal function, an induction treatment with a specific anti IL-2 receptor antibody as basiliximab (Simulect®) may be preferred.[17]

Complications following liver transplantation

General considerations

Following a major procedure like liver transplantation with subsequent administration of potentially harmful immunosuppressive agents, the patients are at risk of a wide range of postoperative complications. Despite considerable therapeutic improvements during the last decades, up to 20 % of the transplanted liver grafts are lost within the first year. Importantly, most grafts are lost within the first week(s).[18] As a consequence, attempts to improve overall graft survival rates should imply early detection and adequate treatment of complications, such as acute rejection[19] and vascular occlusion with subsequent ischemia.[20]

Rejection

Most of the acute rejections are cellular (involving T-lymphocytes) rather than antibody mediated and thereby termed acute cellular rejection (ACR).[19;21] The reported incidence of ACR in the literature is 30-60 %.[19]

The condition is suspected when the activity of circulating liver transaminases and/or concentration of bilirubin increase, but a biopsy of the graft is required to confirm or refute the suspicion.[22] However, re-biopsies are frequently required because particularly early biopsies may be inconclusive.[23] Due to contraindications like coagulopathy or anti-coagulant treatment,[22;24] and that children in particular are in need of general anesthesia during the procedure, a number of patients are given high doses of corticosteroids based on clinical suspicion of cell mediated inflammation, rather than a definite diagnosis.[25] Most often, rejections are successfully treated with intravenously administered pulses of methylprednisolone. However, and more common in cases of antibody mediated humoral rejection, plasma exchange may be considered.[26]

Ischemia

Vascular complications like hepatic artery thrombosis usually occur during the first week(s) after transplantation and represent the most frequent cause for urgent re-transplantation. [27-29] Clinical signs are often absent since the graft is denervated and cessation of hepatic arterial blood flow does not usually affects systemic hemodynamics.

Ultrasound Doppler is an excellent diagnostic tool detecting vascular complications with high levels of sensitivity and specificity when performed by trained radiologists,[30;31] but continuous monitoring is currently not possible. In the future microprobes may gain clinical application.[32] In cases were traditional ultrasound Doppler is inconclusive a micro bubble contrast medium may be useful, but this application requires specially trained radiologists.[33] Accordingly, transportation of the patient to the radiologic department for a computed tomography (CT) angiography examination is often necessary to make the definite diagnosis.[34]

Other

Among a series of possible complications, infections and biliary complications are relatively frequent. In the literature the reported incidence of infection is approximately 30 % [35] and biliary complications occur in approximately 15 %.[36] Biliary leakage is usually caused by rupture of the biliary anastomosis and may thus be accompanied by peritonitis. Hence, biliary leakage and infection often go hand in hand. Bile leakage may be observed in the abdominal drain fluid and circulating bilirubin may be increased due to reabsorption. Increased concentrations of circulating bilirubin may also indicate presence of cholestasis. Macroscopic evident cholestasis may be verified by ultrasound examination, or by endoscopic retrograde cholangiography (ERC). ERC also enables therapeutic intervention.[37]

Energy release in glucose metabolism

Through several steps including the glycolysis, the Krebs cycle, and the electron transport chain, energy, water, and carbon dioxide is released from glucose ($C_6H_{12}O_6$) as adenosine triphosphate (ATP). ATP is the cells most important immediately available source of energy.

Each molecule of glucose broken down to pyruvate in the glycolysis implies net release of two molecules of ATP. Pyruvate is to some extent reduced to lactate by lactate dehydrogenase (LDH), maintaining a stable lactate/pyruvate ratio, even under normal aerobic conditions, but the gross amount of pyruvate undergoes several steps of decarboxylation in the Krebs cycle. These steps imply reduction of nicotinamide adenine dinucleotide (NAD^+) to NADH. Assumed that oxygen is available, NADH serves as an electron donor in the oxidative phosphorylation processes of adenosine diphosphate (ADP) to ATP in the electron transport chain located in the inner membrane of the mitochondria. Thereby, additionally approximately 36 ATP molecules are released by breakdown of each glucose molecule.

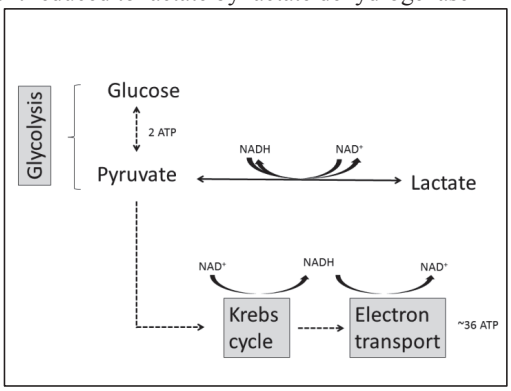

Figure 1. Overview of how energy is released as ATP in the breakdown processes of glucose.

Rejection

The energy demand in resting leukocytes is very low, but once activated they need huge amounts of energy. Unlike e.g. hepatocytes and epithelial biliary cells, proliferative cells like T-lymphocytes cannot fully utilize the theoretical achievable adenosine triphosphate (ATP) production in the inner mitochondria. Thus they are first of all dependent on increasing the less effective glycolysis.[21] Inflammatory processes such as rejection are accompanied by hyperemia

and we have a state of increased aerobic glycolysis. The lymphocytes increase their glucose uptake rate, glucose is broken down at a higher speed, and large amounts of pyruvate are produced. Since the supply of glucose is not negatively affected, similar amounts of lactate as pyruvate are produced. Potential benefits of monitoring these important intermediate metabolites of the glycolysis in detecting rejection are unresolved.

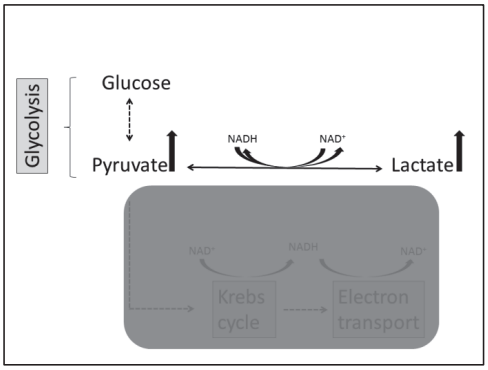

Figure 2. Overview of the glucose metabolism during activated lymphocytes during rejection. The shadowed area indicated that the lymphocytes cannot fully utilize the theoretical possibility for energy production in the inner mitochondria.

Ischemia

Ischemia is a state with restricted supply of blood to the tissues. Until the glucose stores are depleted, the affected cells can produce ATP by increasing the glycolysis, albeit only 2 mmol ATP per mmol of glucose. Since the supplies of glucose and oxygen are reduced, we have now a state of anaerobic glycolysis. Under anaerobic conditions the NADH produced in the Krebs cycle cannot be oxidized to NAD^+ in the electron transport chain since this requires oxygen. The toxic NADH accumulates and its role as cofactor in the reduction of pyruvate to lactate becomes more apparent. Accordingly, pyruvate is increasingly converted to lactate and the balance between pyruvate and lactate is moved towards lactate.[38]

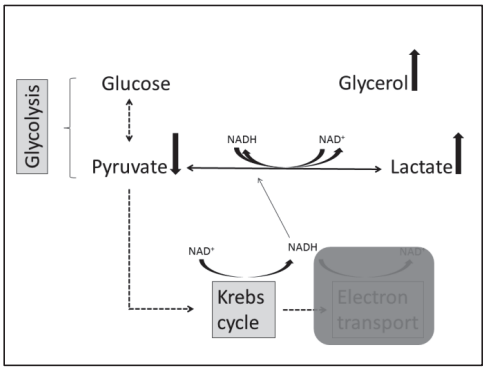

Figure 3. Overview of the anaerobic metabolism in ischemia. The electron transport chain is shadowed since the oxidation of NADH to NAD^+ is oxygen dependent.

In contrast to muscle cells which can use produce ATP using creatine phosphate and thereby survive for 4 – 12 hours under ischemic conditions, hepatocytes cannot survive ischemia for more than approximately 30 – 60 minutes.[39] The subsequent cell death implies release of transaminases to the blood stream, but circulating transaminases increase at a time point when graft damage is already evident and in most cases irreversible.[40]

Glycerol serves, among other functions such as being an important energy reserve, as backbone for the fatty acids in forming the hydrophilic bilayer of phospholipids in the cell membranes.[41] Cell death therefore implies release of large amounts of glycerol to the surrounding tissue caused by phospholipases splitting glycerol from the fatty acids in the phospholipids in the cell membrane (Figure 3).

Inflammation

Immunological mechanisms are in one way or another involved in almost all pathological conditions, and play a major role in rejection and ischemia. Inflammatory mediators are traditionally linked to exogenous stimuli like bacteria, virus or donor tissue, termed pathogen associated molecular patterns (PAMP). At present, it is well known that the immune system can also become activated by recognition of endogenous molecules, termed alarmins. Alarmins, such as high mobility group box 1 (HMBG1) are released upon cell injury due to e.g. ischemia-reperfusion injury [42-47]. Both PAMPs and alarmins lead to a distinct and complex inflammatory response, including complement activation with release of the very potent C5a fragment, production of cytokines responsible for intercellular signaling, and chemokines inducing chemotaxis.

Microdialysis

Microdialysis is a technique which enables monitoring of the tissues and organs of interest. Depending on the membrane's pore size, metabolic substances (lactate, pyruvate, glucose and glycerol) and/or mediators of inflammation (cytokines, chemokines and complement) are sampled in a feasible way. [48] So far, the method's ability to detect brain ischemia is the best validated application. [49;50] In the United States clinical application is so far restricted to neurointensive care units as only the brain catheter (CMA 70, CMA Microdialysis AB, Stockholm, Sweden) is approved by the Food and Drug Administration for clinical use. However, there are more than 2000 clinical reports on microdialysis catheters and in Europe the catheters are Conformité Européenne (CE) marked for a wider range of indications. Accordingly, we consider the microdialysis method safe although bleeding may occur by insertion and theoretically also by removal of the catheters. Like other foreign bodies the catheters may get contaminated and thereby be source for bacterial and fungal infections, but this is not considered a major problem. The catheters have mainly been used to monitor tissues which are at risk of ischemia, [49;51-53] including liver grafts.[54-56] A pilot study from Wælgaard in our group suggested that also rejection of liver grafts may be detected.[56]

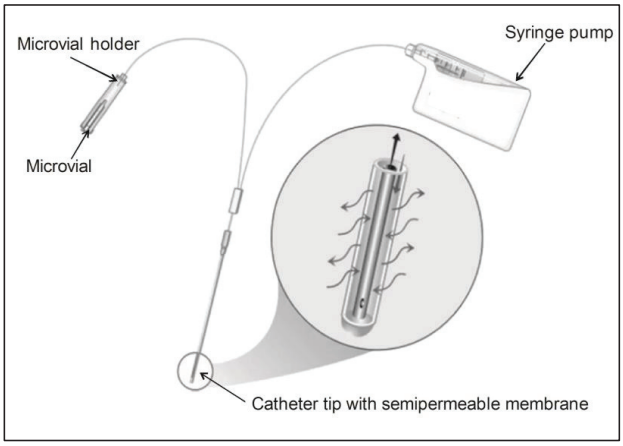

Figure 4. The figure is reprinted from Paper I with permission from M Dialysis AB.

Aims of the studies

General aim of the work

The overall goal of the presented thesis was to investigate the potential clinical utility of monitoring liver transplants by microdialysis catheters.

Study I

The primary objective of this study was to explore whether monitoring substances of glucose metabolism in the liver graft by microdialysis catheters detected ischemia and rejection. Subsequently, we aimed to explore if this method enabled earlier detection than current clinical monitoring practices.

Study II

The main objective of this study was to explore the ability of microdialysis catheters to sample inflammatory mediators occurring during rejection in liver grafts. Secondary we aimed to investigate if the inflammatory response in rejection can be distinguished from the inflammation associated with ischemia.

Study III

The objective of this report was to investigate microdialysis' ability to detect ischemia and rejection in pediatric liver transplants using the optimal cutoff values determined in Study I. As the presence of microdialysis catheters theoretically could interfere with mobilization of the patients we also wanted to explore how the catheters were tolerated by the children.

Materials and Methods

Ethical approval

The study protocol was approved by the Regional Ethical Committee (REK Sør-Øst C). Detailed oral and written information was given and a written, informed consent was obtained prior to inclusion. Children aged above 10 years and adolescents were given individually adjusted oral and written information.

Definition of clinical endpoints

Rejection

Grafts with biopsy confirmed acute cellular rejection.

Ischemia

Grafts with vascular occlusion/stenosis or infarction confirmed by ultrasound Doppler and/or liver computed tomography (CT).

Infection and cholestasis

Since leukocyte activation may imply lactate production, cases of infection, defined as elevation of C-reactive protein, concomitant antimicrobial therapy, and focus in or close to the graft and occurring while the microdialysis catheter was in the liver were analyzed separately. Cases with increments of circulating bilirubin and/or transaminases and histologically confirmed cholestasis in the graft were also analyzed separately as the biochemical picture of cholestasis may be similar to rejection.

Study population

Study I

During one year from October 2008 69 patients receiving 73 liver grafts were included. Each transplanted graft was defined as one study subject. Seven patients were given anti-rejection treatment without histological confirmation of the diagnosis and were excluded from analyses as the diagnosis was regarded uncertain in such cases. Thus, in this study we report on 66 liver transplants.

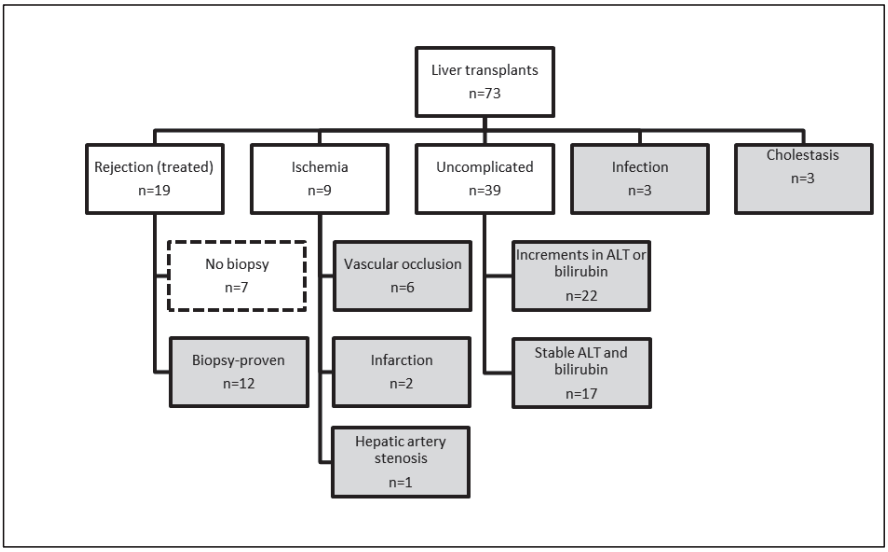

Figure 5. Overview of the study population in Study I. Seven patients were treated for rejection without biopsy confirming the diagnosis and were thus excluded. The darkened boxes represent the included grafts.

Study II

Due to immense costs and high work load related to the laboratory analyses, selected grafts from the population included in study I were used in this study. According to the specific aims, all grafts with biopsy proven ACR (n=12) and four grafts with vascular occlusion (ischemia) were included. In two cases of occluded hepatic artery the grafts were revascularized before we were able to collect samples for analyses of inflammatory mediators. Grafts with other cause of ischemia than total vascular occlusion (n=3) were not analyzed. The total cohort of grafts in which circulating ALT or bilirubin did not increase at any time point while monitored with microdialysis were included as references/controls (n=17).

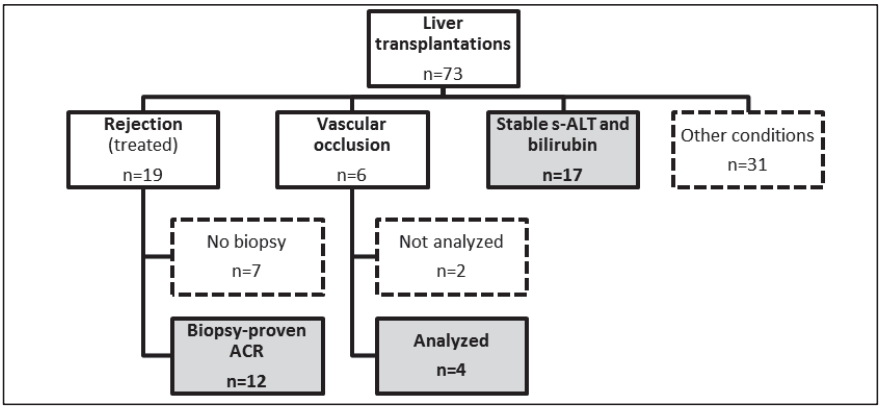

Figure 6: Overview of grafts included in Study II. The darkened boxes are the included grafts; the stapled boxes represent excluded grafts. Reprinted from Paper II with permission from John Wiley and Sons.

Study III

Between October 2008 and May 2011 16 patients (11 girls, 5 boys) aged median (range) 1.9 (0.4 – 15.7) years undergoing totally 20 liver transplantations were included. Eighteen of the

transplantations were performed as split liver transplantations. Ten of the grafts (eight recipients) were also reported in Study I.

Microdialysis

Catheter insertion (Study I, II and III)

The microdialysis system consists of a small battery driven syringe pump coupled to a double tubular catheter with a semi permeable membrane at the tip (Figure 4). The catheters used in the present study have an outer diameter of 0.6 mm. Their membranes have 100 kDa pore size, and a length of 30 mm. A secure thread is positioned 60 mm from the tip (CMA 65, M Dialysis AB, Stockholm, Sweden). At the end of surgery the left abdominal wall was punctured from the inside by a hypodermic needle (Sterican® 14G X 3 1/8 “, B. Braun AG, Melsungen, Germany) through which the catheters were led. Each catheter entered the abdomen through a separate hole and the left side of the abdominal wall was chosen to avoid the access for postoperative ultrasound examinations. The caudal part of each liver lobe was punctured 1-3 cm lateral from the falciform ligament in cranial-lateral direction using a splitable introducer (1 mm Ø). Only one catheter was used in split transplants. If bleeding was present after removal of the splitable introducer, hemostasis was achieved by manual compression and, if necessary, by a clot inducing material (Surgicel®, Ethicon, Cornelia GA, USA). The catheters were secured in the falciform ligament or liver capsule by a 6.0 vicryl suture at which the catheter’s secure thread was fixated. Finally, a skin secure was performed using a 4.0 thread. The catheters were perfused with solution containing dextran and electrolytes (Plasmodex®, Meda AB, Stockholm, Sweden) at a velocity of 1 µl per minute by microinjection pumps (CMA 107, M Dialysis AB, Stockholm, Sweden). The perfusion of the catheters was started prior to insertion in the liver tissue. The first

microvials were discarded after approximately 30 minutes of liver perfusion to avoid analyzing fluid that had not ben perfused through the liver.

Analyses of metabolic variables (Study I and III)

Metabolic variables were analyzed bedside every one to two hours after arrival in the Intensive Care Unit (ICU). A clinical chemistry analyzer using substrate specific reagents for colorimetric measurements of glucose, lactate, pyruvate, and glycerol was used (Iscus, M Dialysis AB, Stockholm, Sweden). Lactate/pyruvate ratio was automatically calculated (lactate (mM)/pyruvate (μM) x 1000). The catheters were kept in situ for as long as they were able to sample microdialysate and then removed transcutaneously.

Analyses of inflammatory variables (Study II)

After the patients´ arrival at the ICU, samples were collected and immediately, without analyzing metabolic mediators, frozen to -70˚C at following time points: 0, 4, 8, 12 and 24 hours. Thereafter, samples were collected and frozen twice daily. Freezing samples at an exact time point after graft reperfusion was not feasible, and all reported time points relate to the time point of reperfusion of the portal vein. In the cases where two catheters were used, samples from the catheter which sampled microdialysate for the longest period were chosen for analyses. The complement activation product C5a was analyzed en bloc by Human C5a ELISA Kit II (BD Biosciences, San Jose, CA). Following pilot analyses of three patients with rejection using the Bio-Plex Human Cytokine 27-Plex Assay (Bio-Rad Laboratories Inc., Hercules, CA), the following six mediators were analyzed en bloc: Interleukin-1 receptor antagonist (IL-1ra), C-X-C motif chemokine (CXCL) 10, CXCL-8, IL-6, IL-10, and macrophage inflammatory protein (MIP)-1β.

Statistical analyses

Statistical analyses were performed with PASW 18.0 (IBM®, Chicago, IL, USA) in all studies. Due to the non-normal distribution nature of several of the data, non-parametric statistical methods were used when needed. The presented *P*-values are 2-tailed and *P*-values < 0.05 were considered statistically significant.

Linear mixed models (Study I)

In order to verify particularly our rejection hypothesis in a mathematical, researcher independent way, the initial exploration of the metabolic data were performed by linear mixed models. Since the residuals for all investigated variables were normally distributed the data were not logarithmically transformed prior to analyses. Residuals are estimates of the statistical errors, and in contrast to the classical statistical error using the average of an entire population the residual is calculated using the mean of the investigated population. [57]

Except for ischemic events which could clearly be defined by radiological examinations, data from other grafts were coded according to the endpoints of the study, i.e. rejection, infection and cholestasis. Thus, the time point of occurrence of e.g. rejection was not regarded at this stage in the data analyses. We searched for potential group differences, time dependency and possible effects of donor, recipient or graft characteristics. The measured metabolic mediators were dependent variables. A random effects model was used and model selection was done for each variable by choosing the model achieving the lowest Information Criteria. The random effects of patient and groups were included in the model.

Receiver operating characteristics curves (Study I and II)

Receiver operating characteristics (ROC)-curves were constructed to explore the ability of the investigated mediators to discriminate ischemia and rejection from uneventful conditions (Study I and II) and from other pathological conditions (Study II). Area under curve (AUC) was calculated and the null hypothesis was AUC = 0.5. The optimal cutoff value for each variable was defined as the value closest to the top left corner.

Mann Whitney *U*-test (Study I, II and III)

Mann Whitney *U*-tests were performed to compare data between groups.

Wilcoxon Signed Rank test (Study I, II and III)

Wilcoxon Signed Rank tests were used for repeated measurements.

Contingency table analyses (Study I and III)

Values for sensitivity and specificity (Study I and III) were calculated using standard formulas for analyses of contingency tables.

Post-hoc correction of *P*-values

For post hoc correction of *P*-values from tests involving several groups the Bonferroni method was used. This is considered a conservative method and implied that in analyses involving more than two groups each *P*-value was divided by the number of tests used to analyze the data.

Summary of main results

Study I

Study population

Nine liver transplant recipients were diagnosed with *ischemia* and 12 experienced episodes of rejection confirmed by biopsy. In 39 recipients, no major events occurred during the period of sampling of metabolic substances (*reference group).* During the observation period, we identified three patients with *infection* in or close to the graft and three developed *cholestasis* confirmed by graft-biopsy. Two of all investigated patients died; one patient died due to complications related to ischemia the first postoperative day and one patient in the reference group died due to pulmonary aspergillosis 16 days post transplantation. This patient was included in the reference group since her liver function tests were normal for the whole postoperative period.

Of the nine cases of *ischemia*, five had hepatic artery thrombosis, one graft had thrombosis of both the portal vein and hepatic artery, one had stenosis of the right hepatic artery, and in two grafts infarction was detected. In 11 of the 12 cases of *rejection*, circulating ALT increased and in nine cases bilirubin increased. All rejections were successfully treated with pulses of methylprednisolone.

Concentrations of intrahepatic metabolic variables

Ischemia was detected as increased lactate and lactate/pyruvate-ratio. Rejection was detected as simultaneously increased lactate and pyruvate. Glycerol increased exclusively in cases of severe ischemia, whereas glucose was similar in all groups (data not shown). The concentrations during the different conditions are shown in Figure 7.

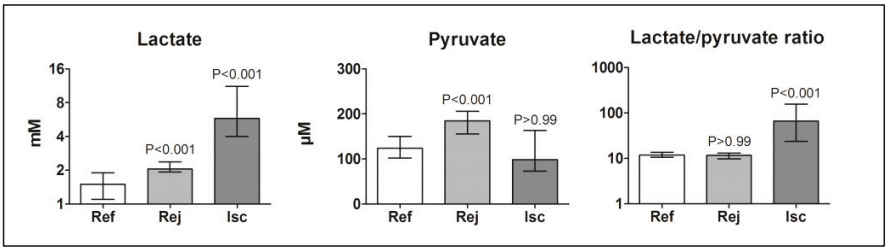

Figure 7: Median (interquartile range) concentrations of lactate and pyruvate and calculated lactate/pyruvate (LP-ratio) during episodes of acute cellular rejection (ACR, n=12) and ischemia (ISC, n=9) compared to the cohort of reference grafts (REF, n=39) with uncomplicated course. The groups were compared by the Mann Whitney U-test. The P-values are Bonferroni adjusted for comparison of three groups. Reprinted from Paper I with permission from John Wiley and Sons.

Time points of detection of rejection and ischemia

Lactate measured in arterial blood, increased in only three of the six cases of vascular occlusion; in the other three cases arterial lactate remained unchanged. In the patients where arterial lactate increased, these increments were observed 0.3, 6, and 57 hours later than by the microdialysis catheters. Accordingly, pathological intrahepatic metabolism was detected by microdialysis catheters prior to radiological confirmation of vascular occlusion in all cases. Rejection, defined as lactate >2mM and LP-ratio <20 lasting ≥6 hours, was detected median four days before ALT ($P = 0.02$) and bilirubin ($P = 0.04$) increased. This was median 4 (range = 0-10) days before biopsies were performed ($P = 0.01$).

Discrimination of ischemia and rejection

ROC-curves (Figure 8) revealed that lactate discriminated ischemia with an AUC of 1.00 (95 % CI, 1.00-1.00) and an optimal cutoff value of 3.0 mM. Likewise, lactate/pyruvate ratio discriminated ischemia with an AUC of 0.99 (95 % CI, 0.98-1.00) and an optimal cutoff value of 20. Lactate discriminated rejection with an AUC of 0.87 (95 % CI, 0.77-0.97) and an optimal cutoff value of 2.0 mM. Pyruvate discriminated rejection with an AUC of 0.88 (95 % CI, 0.78-0.97) and an optimal cutoff value of 170 µM. Using these criteria in contingency table analyses, two consecutive positive measurements detected ischemia with 100 % sensitivity and more than 90 % specificity. Consecutive positive measurements from both liver lobes over a period of at least six hours detected rejection with 89 % sensitivity and 83 % specificity.

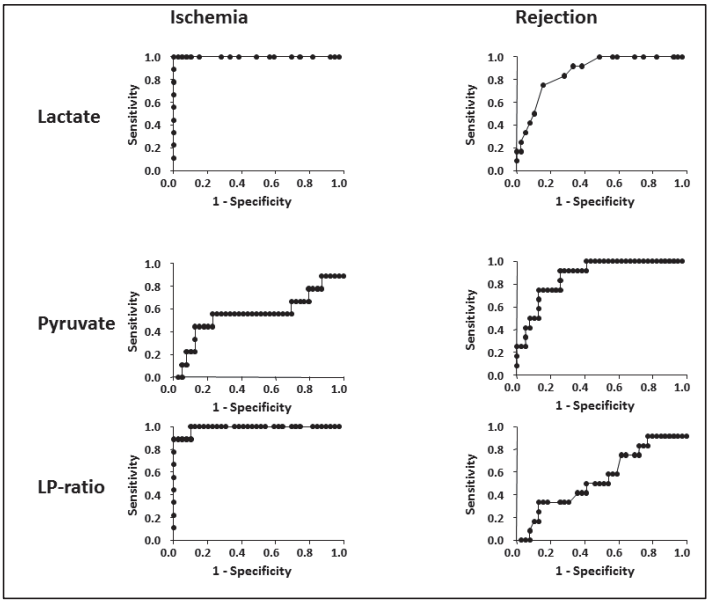

Figure 8. Receiver operating characteristics (ROC) curves showing how lactate, pyruvate and lactate/pyruvate (LP)-ratio discriminate ischemia (n=9) and rejection. Reprinted from Paper I with permission from John Wiley and Sons.

Study II

Initial concentrations of inflammatory variables

From initially relatively high concentrations, there were time dependent decrements of C5a, IL-1ra, IL-6, IL-10 and MIP-1β after graft reperfusion ($P < 0.001$ for all analyses), whereas CXCL-8 and CXCL-10 decreased non-significantly. No differences in any of the investigated periods were found between the reference group and the grafts that later developed rejection.

Rejection

The concentrations of CXCL-10 increased in both the rejection- and the reference group, but it increased significantly earlier and to higher values in the rejection group, reaching statistically significant differences at day 3 ($P = 0.05$) and at day 4 ($P = 0.02$), i.e. in samples collected before any patient had been given anti-rejection treatment. After the initial obligatory peak in ALT following graft reperfusion, increments of CXCL-10 were observed during a period of physiological decrease in transaminases. Increments of more than 100 % were observed after median 2.2 (range = 1.2-3.8) days. This was 1.7 days before any increment in ALT (P=0.02), and 5.2 days before bilirubin increased with ≥ 25 % ($P = 0.008$).

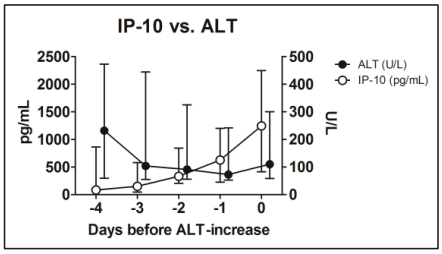

Figure 9. The course of intrahepatic CXCL-10 compared to circulating ALT in 12 grafts with acute cellular rejection

The peak concentrations of CXCL-10 were significantly higher in the rejection group as compared to both the reference group ($P = 0.008$) and the ischemia group ($P=0.004$). In the latter group, no increase in CXCL-10 was detected. The peak concentrations of IL-6 and CXCL-8 were

also higher in the rejection- as compared to the control cohort, but the differences were not statistically significant.

Ischemia

The peak concentrations of C5a, CXCL-8 and IL-6 were significantly higher in grafts with ischemia as compared to the controls. C5a was the only mediator that was significantly higher in the ischemia group than in the rejection group (P=0.008).

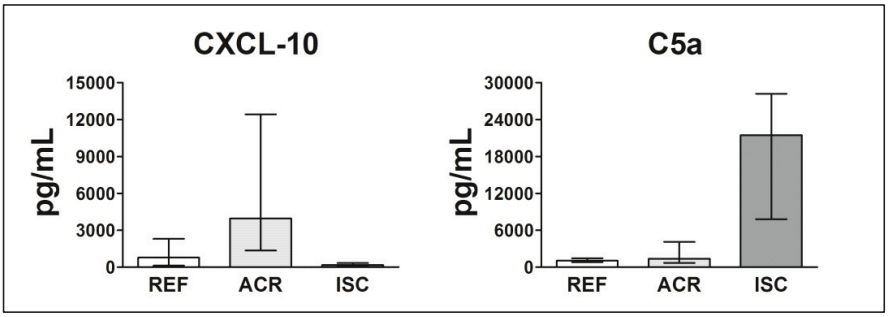

Figure 10: Peak concentrations of CXCL-10 and C5a in rejection (ACR), ischemia (ISC) and references (REF). Reprinted from Paper II with permission from John Wiley and Sons.

Discrimination abilities

C5a discriminated ischemia from controls with an AUC of 0.96 (95 % CI, 0.87-1.00) (P = 0.005) and from ACR with an AUC of 0.88 (95 % CI, 0.69-1.00) (P = 0.03). CXCL-8, IL-1ra and IL-6 also discriminated well between ischemia and controls, but not as well as C5a.

CXCL-10 discriminated rejection from controls with an AUC of 0.81 (95 % CI, 0.65-0.97) (P = 0.005) and from ischemia with an AUC of 1.00 (95 % CI, 1.00-1.00) (P = 0.004).

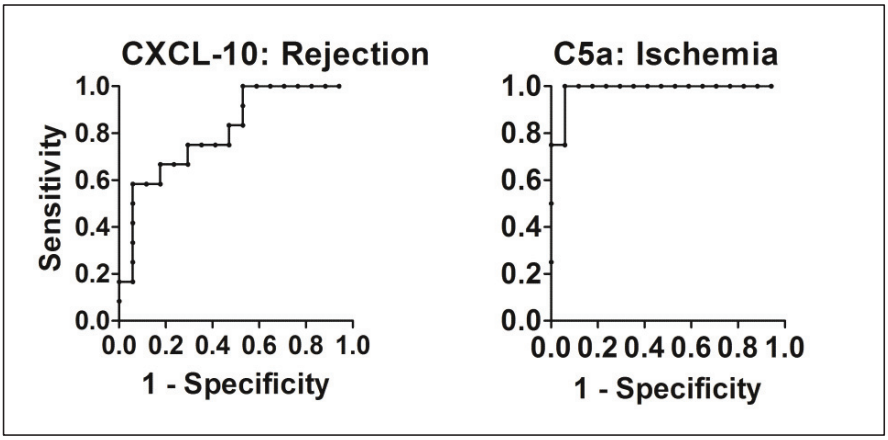

Figure 11: ROC curves depicting how CXCL-10 and C5a discriminate rejection and ischemia from reference grafts. Reprinted from Paper II with permission from John Wiley and Sons.

Study III

Study population

Of the 20 investigated liver grafts, ischemic vascular complications were detected in six, and rejection was proven by histology in eight. Complications other than ischemia and rejection occurred in three grafts; infection was detected in one, one had biopsy proven cholestasis, and one had primary non function (PNF). One of the sixteen recipients died due to ischemic graft complications.

Sensitivity and specificity in detecting rejection and ischemia

Using the criteria lactate > 3.0 mM and LP-ratio > 20 the intrahepatic microdialysis catheters detected ischemia with 100 % sensitivity. However, when requiring only single time point measurements the specificity was as low as 57 %. When requiring two or three consecutive positive measurements, only one graft with rejection and one with cholestasis (Figure 12) were misjudged as ischemia and the value for specificity was 86 %.

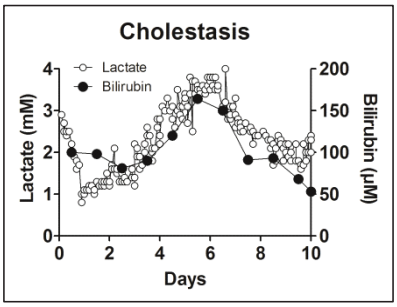

Figure 12. This patient had a typical rejection pattern of the lactate curve, but the biopsy revealed intracanalicular cholestasis. Reprinted from Paper III with permission from John Wiley and Sons.

Using the criteria lactate > 2.0 mM and LP-ratio < 20, single time point measurements detected rejection with 100 % sensitivity, but with 0 % specificity. Requiring consecutive positive measurements throughout a period of six hours, one graft which displayed rejection values lasting just above five hours was coded as rejection and the sensitivity was 88 %. Rejection was falsely detected in six grafts and the specificity was thus 45 %. However, in five of

these cases the clinical- picture and context ruled out rejection as a possible diagnosis. Two were in the period of metabolic normalization after already confirmed occluded hepatic artery, two had severe peritonitis and abdominal pain due to spontaneous perforation of the duodenum and leakage from the choledochojejunostomy respectively, and the last case was in a graft with primary non function (PNF) (Figure 13). When not considering rejection in these grafts, the specificity was 83 %. One episode of histologically proven intracanalicular cholestasis (RAI 1) (Figure 12) could not be ruled out as possible rejection and was thus classified "true false positive rejection". The results did not change when increasing the requirement for consecutive positive measurements to 12 hours, but further increment resulted in a decrease in sensitivity.

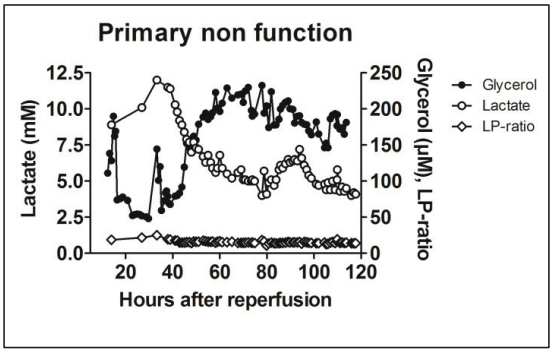

Figure 13. Graft with primary non function due to prolonged ischemia. Increased lactate with stable lactate/pyruvate-ratio (LP-ratio). Glycerol was increased as sign of ongoing necrosis. Reprinted from Paper III with permission from John Wiley and Sons.

Time point of detection of rejection and ischemia in pediatric transplants

In general, the results from Study I were confirmed in this smaller series of pediatric liver transplants. Ischemia was detected close to real time. In one case only intrahepatic, not arterial, lactate increased at the time point of thrombosis of the hepatic artery (Figure 14). In another case, microdialysis catheters correctly displayed normal metabolism whereas ultrasound Doppler falsely suggested an occluded hepatic artery. As in Study I, rejection was detected before circulating ALT or bilirubin increased, and before biopsy was performed.

Feasibility, complications and acceptance of microdialysis catheters

Minor bleeding occurred by insertion of two catheters and was easily handled by manual compression and Surgicel®. Significant bleeding was not registered at the time of removal of any catheter despite 15 of 20 cases receiving low molecular weight heparin (LMWH) and/or acetyl salicylic acid (ASA). No episodes of infection could be related to the catheters. Microdialysate from the liver was sampled for median 10 (range = 1 – 28) days and no differences were found between children younger and older than two years in terms of catheter durability. The parents of two patients asked for withdrawal of the catheter after 12 and 28 days respectively as they perceived the many measurements as a strain to the children who both were fully mobilized and doing well. All other patients tolerated and kept their catheters until they were removed because of malfunctioning (n=13), re-transplantation (n=4) or re-laparotomy (n=1). No patients were immobilized because of the catheters.

Discussion

General considerations

According to our knowledge, each of the present studies are the largest of its kind worldwide. In Study I, the largest of the three studies including 66 liver transplants, we showed that rejection and ischemia may be detected bedside several days before current standard methods. In Study II we found a potentially specific biomarker of rejection. Despite Study III being the smallest of the three studies, it was performed in the very important and vulnerable group of pediatric transplants and is likely to have most immediate clinical impact of the three studies.

Pathophysiology

Metabolic variables

Simplified overviews of the intermediate metabolism in normal conditions, rejection, and ischemia are shown in Figures 1 - 3. We consider the simultaneous measurements of lactate and pyruvate enabling distinction between aerobic and anaerobic glycolysis of immense importance. Contrary to lactate, pyruvate is a more unstable metabolite sensitive to storage time and conditions. Pyruvate is thus not a routine variable in a clinical setting. The bedside approach of the microdialysis system enables pyruvate measurements in a feasible way and is important for the accuracy of diagnostics.

With reduced oxygen supply as in ischemia the condition is dominated by anaerobic glycolysis; NADH, a cofactor to the enzyme LDH, is increased and the balance between pyruvate and lactate is shifted towards lactate.[38] Thus, the lactate/pyruvate-ratio increases in anaerobic glycolysis/ischemia. In the present material pyruvate decreased only in the most severe cases of ischemia as e.g. the case of combined hepatic artery- and portal vein thrombosis. Mix of blood

from intact vessel systems (hepatic artery or portal vein) with normal contents of glucose and oxygen is likely to explain this observation.

The simultaneous increments of lactate and pyruvate with stable lactate/pyruvate ratio in rejection most likely reflect an increased aerobic glycolysis due to lymphocyte-activation.[21] Being aware that we did not measure intrahepatic concentrations of transaminases or bilirubin, this statement is supported by detection of significantly increased metabolism several days before the traditional markers of cell death or dysfunction increased in blood.

An increased energy demand due to activation of neutrophil granulocytes is likely to explain the observed hypermetabolism associated with infection, apparent as increased lactate and pyruvate. In infection, lactate is also released by the oxidative burst mechanism involved in bacterial killing,[58] and a somewhat higher lactate/pyruvate ratio would be expected with infection than in rejection. However, the restricted number of infectious cases in the present material does not allow any conclusion to be drawn regarding expected levels of lactate/pyruvate ratio in infection. Accordingly, infection should be considered a differential diagnosis to rejection in cases where the microdialysate reveals hypermetabolism. Interestingly, no intrahepatic changes were found in the case of severe peritonitis suggesting that the liver graft was not infected in this case. However, an additional intraperitoneal microdialysis catheter would probably have detected the case of peritonitis as reported by others.[59-61] Studies aiming to explore the clinical utility of intraperitoneal microdialysis positioned between the loops of the small intestine are ongoing and may provide valuable experience, in particular in monitoring vulnerable groups of postoperative immunosuppressed patients.

In one case of canalicular cholestasis (Figure 12) we observed an increased metabolism, similar to the hypermetabolism observed in rejection. Cholestasis, with intrahepatic bile duct pressure exceeding the capillary pressure, might explain the metabolic condition,[62] but absence of cholestasis by ultrasound investigation contradicts this explanation. Bilirubin's powerful uncoupling effect on oxidative phosphorylation may be an alternative explanation.[63-66] The patient was administered 10 mg/kg methylprednisolone intravenously and recovered spontaneously. We therefore consider immunological mechanisms possible although not revealed by the histo-pathological examination of the biopsy.

Inflammatory variables

Despite CXCL-10 being produced by a variety of cells like mononuclear cells, activated stellate cells, endothelial cells, and hepatocytes, various reports indicate that it possibly is a specific marker of rejection of liver grafts,[67-70] as well as of other solid organ transplants.[71-75] CXCL-10 expression is induced by γ-interferon and contributes via stimulation of its receptor CXCR3 to migration of T-lymphocytes into the inflamed tissue.[67-70;76] Thus, γ-interferon and CXCL-10 may be considered early mediators predominantly of the cell mediated, adaptive immune system. Our results support that CXCL-10 may be less dependent from the innate than the adaptive immune system; it did not increase in ischemia and seemed independent of the ischemia/reperfusion injury. A biological explanation to why CXCL-10 is increased in rejection, and not ischemia, may be that the precursor γ-interferon probably is independent from complement activation.[77;78] The complement system mainly represents the innate immune system and the biologically highly potent activation product C5a is induced by activation through all initial activation pathways. It has a number of effector functions and is e.g. a powerful chemo attractant causing accumulation of neutrophil granulocytes and an important inducer of

inflammation.[79;80] Our data support studies showing that the complement system plays a key role in the ischemia/reperfusion injury.[44;81] In contrast to C5a, IL-1ra has potent anti-inflammatory activities.[82] Interestingly, these two inflammatory mediators, which counteract each other, closely reflected ischemia/reperfusion, indicating that the inflammatory network functions to balance the detrimental effects in this condition. CXCL-8, a member of the CXC-chemokine family,[83] and IL-6,[84] a pro-inflammatory cytokine, increased with ischemia and a positive trend was detected also with rejection. This indicates that these mediators are expressed by a general activation of the immune system and not specifically by the adaptive (cell mediated) or innate immune system. Consequently, neither IL-6 nor CXCL-8 seems to be attractive candidates in the search for specific markers of rejection.

The current study (Study II) using a derived cohort of transplanted patients as "controls" (discussed in Limitations), does not formally answer whether the somewhat increased levels of CXCL-10 in the reference group represent cell mediated inflammation or constitute a part of the normal course after liver transplantation. Some degree of alloreactivity after liver transplantation is probably common and/or inevitable, and can be transient. Coinciding minor discharges of transaminases or bilirubin may not always be detected in peripheral blood samples, or the increases might not be high enough to justify a liver biopsy. Contrary to the study performed by Wælgaard et al, [56] C5a did not increase with rejection (Study II). This may be due to a more complicated course of the recipients in the former study; two were critically ill prior to transplantation and were treated with the molecular adsorbent recirculating system (MARS) and one of these had several bowel perforations needing surgical corrections. The third graft had, in addition to rejection and stenosis of the choledochus anastomosis, also evidence of vascular graft ischemia.

Clinical application

Metabolic variables

The results suggest that bedside sampling of metabolic mediators using microdialysis catheters may be used to detect episodes of ischemia and rejection in liver grafts and that these conditions may be identified earlier than by current standards of monitoring and care. The method's ability to detect ischemia and thus alert for possible vessel occlusion is particularly important as prolonged ischemia is detrimental for the graft. Consequently, an early detection by microdialysis may be graft saving and improve patient survival. The levels of sensitivity and specificity of which rejection were detected indicate that this method might also be a useful clinical tool in cases of suspected allograft rejection. Thus, this method might act as an alert to carry out a biopsy for verification, particularly in the early stage of rejection.

As indicated in this work and reported in the literature particularly vascular complications occur more frequently in the pediatric- as compared to the adult population.[85;86] As a consequence of the promising results in Study I and III microdialysis is now implemented as part of the standard monitoring and care of pediatric liver transplants in our hospital. Slightly modified optimal cutoff values (lactate >2.6 mM and LP-ratio > 20) detected in study I and explored in Study III are used as an alert to carry out Doppler ultrasound examination which is performed after two consecutive positive measurements, the second one analyzed 30 min after the first. As the specificity in detecting ischemia was as high as 86 % (Study I) and 83 % (Study III) we expect that the number of negative ultrasound Doppler examinations will be acceptable, but this will be a question for exploration of future data.

When considering using microdialysis in diagnosing rejection, the varying sensitivity and specificity depending on the number of repeated measurements should be considered; relying on single time point measurements in making decisions about initiating anti-rejection therapy would imply that a number of patients would unnecessarily be administered potentially harmful high doses of corticosteroids. By requiring consecutive positive measurements for six hours before initiating anti-rejection therapy, clinically acceptable levels of both sensitivity and specificity were achieved. However, as shown in Study III, the microdialysis data should be assessed together with the clinical context and patient condition before making decisions regarding performing biopsies, or initiating anti-rejection treatment in cases of contraindications to biopsies.[22] As stated earlier in this discussion, particularly infection should be considered an important differential diagnosis to rejection.

Inflammatory variables

Having detected CXCL-10 as a potentially specific marker of rejection in liver grafts is most likely the most important finding in Study II. If confirmed in larger studies, and at other centers than ours, CXCL-10 might turn out to be an important additional marker to the metabolic variables. The absence of C5a in in a microdialysate sample with high concentration of CXCL-10 (and lactate and pyruvate) would support the presence of rejection.

As opposed to metabolic mediators which are analyzed bedside relatively cheap and feasible, analyzing inflammatory variables is still time and cost consuming laboratory work. Thus, both further research and technical development of a fast track analysis kit is needed before e.g. CXCL-10 and C5a can be introduced in clinical practice.

Feasibility, complications of and acceptance of microdialysis catheters

More than 2000 clinical microdialysis studies, including gastrointestinal studies, have so far been published. The kits for analyzing the reported metabolic variables lactate, pyruvate, glucose, and glycerol are commercially available. The equipment used in the current studies is Conformité Européenne (CE)-marked, also for livers. Thus, the method may be implemented in clinical practice, including liver grafts, in most European countries. In the United States, however, only the brain catheter (CMA 71, M Dialysis AB, Stockholm, Sweden) is so far approved by the Food and Drug Administration for clinical use. Accordingly, monitoring liver transplants in the United States at this stage can only be done in a clinical research setting approved by the Institutional Review Board.

Microdialysis catheters were inserted in 83 liver grafts in the present studies. We had no severe complications related to the catheters. By introducing the splitable needle, some episodes of minor bleeding occurred that were easily handled by manual compression and a common hemostatic agent. Accordingly, this work suggests that microdialysis safely can be used in liver transplants.

Significance of early detection of complications

Rejection

Although there is no general agreement regarding the significance of initiating anti-rejection treatment at an early time point[19;87] we argue that early treatment of rejection is important. It is well known that hepatocytes are able to regenerate.[88] However, hepatocytes are not the main targets in the course of rejection. Sinusoidal endothelial cells, bile ducts and small intrahepatic arteries are more affected and these do not have the same ability as hepatocytes to

regenerate.[87] As the blood flow in the portal vein is diminished with rejection, the risk for portal vein occlusion is increased.[89]Importantly, specific patient populations undergoing liver transplantation have distinct postoperative profiles of complications. Patients with primary sclerosing cholangitis (PSC) have a higher incidence of both acute and chronic rejection. In these patients, acute rejection is associated with higher incidence of chronic rejection and relapse of PSC in the graft which again is associated with decreased long term outcome.[90] In the large population of patients transplanted for hepatitis C virus (HCV) cirrhosis, anti-rejection treatment with intravenous corticosteroids have been reported to cause massive viral replication and thus recurrence of the disease.[91] As a consequence, it is theoretically conceivable that early detection of rejection followed by tailored treatment regimens guided by continuous microdialysis monitoring to establish the lowest effective dose of immunosuppression would be beneficial in these patients. Individualized regimens could theoretically also be advantageous in patients infected with Epstein Barr virus (EBV) and in patients already diagnosed with malignancy. In these patients, aggressive anti-rejection treatment is associated with higher incidence of post-transplant lymphoproliferative disease [92] and immunosuppressive medication may trigger growth of malignant cells. [93]Accordingly, patients with PSC, HCV-cirrhosis, EBV-infection, and malignancy may all represent subgroups of patients in which close intrahepatic monitoring might be particularly useful.

Ischemia

Study I and II show that early detection of potentially reversible ischemic conditions may be detected at a very early time point with the microdialysis method. Compared to rejection, it is generally agreed upon that early detection of vascular occlusions is of immense importance to be able to intervene before irreversible graft failure has occurred to possibly save the graft.[94]

Importantly, we have not shown that the patients actually benefit from having their ischemic complications detected at an early time point and this will be a question for future studies.

Infection

Infectious complications are severe and common following liver transplantation. [35] It is shown that early detection and treatment of severe infections is important to improve the survival rates in a general population.[95] We consider it conceivable that the vulnerable group of liver transplanted patients may benefit from early detection and subsequent treatment of infectious complications, and we consider exploring the role of microdialysis for this indication of particular importance.

Limitations of the work

The design in all studies can be characterized as open labeled, prospective, observational and non-interventional. The hypotheses were known by the staff treating and nursing the patients and by the patients. The potential benefit of early detection of ischemic vascular complications would make a blinding ethically difficult. We had no instructions regarding observed hypermetabolism and suspected rejection, but strongly assume that the microdialysis data were taken into consideration when making decisions regarding performing biopsies or initiating treatment without biopsies. In fact, seven patients were given anti-rejection treatment without biopsy confirming or refuting the suspicion and thus forcing us to exclude these grafts from data analyses. This may have influenced the reported values for sensitivity and specificity and was the reason for not reporting positive and negative predictive values. Implementing biopsies in the study protocol would most likely have secured a better validation of the data, but protocol

biopsies of liver grafts are controversial and abandoned in most centers. [96] As children are in need of general anesthesia to enable biopsy, implementing biopsies in the study protocol would imply an unfortunate higher level of invasiveness of the study. We assume that in particular the number of included children would have been substantially reduced. Reducing the fraction of investigated liver grafts in certain diagnostic groups would certainly have impact on the values for sensitivity and specificity.

All studies were performed without a formal control group. The presented groups including the reference group(s) are better described as derived cohorts rather than true controls. An imaginable control group would have been a group of patients undergoing liver surgery other than transplantation, e.g. liver resection. This would undoubtedly be a group of patients without any possible risk of rejection and thereby be interesting subjects for comparison of the role of CXCL-10 after transplantation. On the other hand, implementing a group of patients in an invasive study solely for the purpose of serving as controls would raise ethical concerns. From our point of view, such a study design is appealing, but probably more suitable and applicable in a laboratory setting.

The studies were not primarily designed to neither detect, nor elucidate, the clinical course of infection and cholestasis. Besides vascular complications and rejection, these complications are however frequent and potentially severe complications after liver transplantation. In Study II, such complications were not at all taken into consideration. In the literature, the reported incidence of infection is approximately 30 %[35] and biliary complications occur in approximately 15 %.[36] We have only described cases of infection and cholestasis occurring while the microdialysis catheters were in the liver grafts. Accordingly, several patients had infections at other sites, as well as suffering from infections after the catheters were removed. In order to

present as validated data as possible, we chose to only present data from grafts with cholestasis which were proven by biopsy. Accordingly, there may certainly have been cases of subclinical cholestasis in the reference group. Hence, the results regarding infection and cholestasis should be interpreted with caution.

In general, the number of grafts with complications is restricted, and care should be taken drawing any strict conclusions. In Study II the number of grafts with ischemia was particularly restricted, and in Study III, the number of investigated subjects is very small.

According to the aim of Study II, pilot analyses with the 27-plex kit were exclusively performed in grafts with rejection. Thus, mediators likely to be important in ischemia and ischemia/reperfusion injury, e.g. tumor necrosis factor-α were not investigated.

The microdialysis catheters' life span is time-limited as the membranes become clotted by e.g. cellular debris. There is no possibility to predict the time-point of catheter malfunction.[97] As shown by Wælgaard et al, the capability to recover larger proteins is restricted.[98] This phenomenon most likely limited our possibility to investigate the effect of treatment of rejection other than showing the effect of treatment in only 4/12 grafts receiving anti-rejection treatment in Study I.

Main conclusions

Study I

- Bedside measurements of lactate and pyruvate detected ischemia and rejection with clinically highly acceptable levels of sensitivity and specificity.
- Episodes of vessel occlusion were detected with intrahepatic microdialysis catheters while arterial lactate remained unchanged.
- Rejection was detected several days before significant changes were observed in routine blood samples.

Study II

- CXCL-10 increased selectively in liver grafts with rejection.
- Intrahepatic CXCL-10 increased before circulating ALT or bilirubin increased.
- C5a was elevated selectively in ischemic grafts and was thus able to discriminate rejection from ischemia.

Study III

- The results from Study I were confirmed and ischemic vascular complications and rejections were detected in close to real time with clinically satisfactory levels of sensitivity.
- The restricted specificity in detecting rejection urges the need for additional intrahepatic markers.
- The catheters were well tolerated by the children and the method is feasible and safe also for this patient group.

Further perspectives

As a consequence of the present studies and clinical need we have implemented microdialysis catheters as part of the standard postoperative monitoring of pediatric liver transplants. This is the patient group which is most likely to benefit from close intra organ monitoring because of high complication rates. Also due to somewhat high costs for the equipment needed it is reasonable to use it in the patients having the highest potential for developing resolvable complications.

The ability for the microdialysis method to detect postoperative infections will be explored in ongoing and planned studies in both transplanted and not transplanted patients. Postoperative infections are common, and they have negative impact on survival rates, duration of hospital stay, as well as costs for hospitals and communities. With that in mind, the microdialysis method may turn out to be cost-effective.

Microdialysis should be considered a semi continuous monitoring tool, and human intervention is required at a regular basis to carry out the analysis. An automatic system would be of benefit, but so far struggles to develop such a system including the important metabolite pyruvate have not succeeded.

The relative high costs of the microdialysis system and also the technical limitations ask for easier and cheaper tools for intra organ monitoring. One of the main interests of research in our group is developing a sensor for continuous monitoring of tissue PCO_2 as increasing levels of PCO_2 measured in organs is a sensitive and specific variable to detect hypoperfusion and ischemia [99-105]. Our hope is to develop a sensor which can do continuous readings of e.g.

intrahepatic PCO_2 for several days. The sensor is fully automatic and does not require human intervention.

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