Optimising outcomes after liver transplantation

Pathophysiology of biliary injury and the application of machine perfusion
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Pathophysiology of biliary injury and the application of machine perfusion

Dissertation

To obtain the degree of Doctor of Philosophy from The University of Queensland and the degree of Doctor at Maastricht University, on the authority of Rector Magnificus Prof. dr. Rianne M. Letschert, according to the decision of the Board of Deans to be defended in public on Wednesday, April 26, 2017 at 12.00 hours

by

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Chapter 1

General introduction and Aims
History of Liver transplantation

The history of liver transplantation dates back to the mid-20th century. The first orthotopic liver transplant in a canine model was performed by Vittorio Staudacher in Milan in 1952. Unfortunately at that time, his breakthrough was not recognized by the greater scientific community and not Staudacher but Stuart Welch and Jack Cannon were credited with performing the first heterotopic and orthotopic liver transplantation in large animal models in 1955 and 1956 respectively. Based on the extensive experience in dogs, the first liver transplant in humans was performed by Thomas Starzl in 1963. Unfortunately, none of his four patients described in his initial report survived past the 1-month mark and it would take him another 4 years to perfect the technique. Between July 23, 1967 and March 17, 1968 Starzl successfully transplanted seven consecutive patients with biliary atresia or hepatoblastoma, all of which were children. At the time of publication, the longest surviving recipient was still alive 9 months post transplantation. Key to his success were measures implemented to reduce the degree of ischemic insult to the liver. Cardiac massage and ventilatory support were initiated post mortem, the liver was rapidly cooled in situ using balanced electrolyte solution, and an ex situ oxygenated hypothermic organ perfusion device was used to bridge the period between donation and implantation.

Soon after the successful report by Starzl, Calne and Moore reported their first experience with human liver transplantation in Europe in a joint venture between the Addenbrooke’s hospital in Cambridge and King’s College Hospital, London. In their initial series of five patients published in 1968, they presented the first case of heterotopic liver transplantation in humans (the liver was placed in the splenic bed). Furthermore, they performed a transplant technique currently still used in clinical practice where the recipient inferior vena cava (IVC) was preserved and the donor liver was ‘piggybacked’ on the recipient IVC.

In the 10 years that followed, patient survival substantially improved. The acceptance of brain death criteria in 1968 and the introduction of cyclosporine by Calne in 1979 lead to higher quality organs and substantially improved graft survival.

In 1983, the National Institutes of Health in the USA hosted a consensus conference on liver transplantation. Up until that point, 540 human liver transplants had been performed in four medical centres in the US and Western Europe. During the meeting, the outcomes of these procedures were compared with patients who did not undergo liver transplantation. The transplant centre in Pittsburgh, led by Starzl, had performed by far the most liver transplants up until that point. Since the start of the program in 1963, 296 patients had undergone liver transplantation. The introduction of cyclosporine had a major impact on patient survival with 1-year survival rates of
32.9% before 1980 and 70% between 1980 and 1982. Calne and colleagues in Cambridge and King’s College performed 138 liver transplants between 1968 and 1983, predominantly for hepatic malignancy. Their survival rates were inferior to those reported by Starzl with reported rates of 30-40% on average. Pichlmayr and colleagues in Hannover, Germany, reported outcomes of 90 orthotopic transplants performed at their centre since 1979. Only 20% of patients that underwent transplantation for hepatic malignancy survived longer than 1 year. Cirrhotic patients that were transplanted in an elective setting however had the best chances of survival with 80% survival past the 1-year mark. The last series of 26 consecutive liver transplantations considered during the consensus conference were performed in Groningen, the Netherlands by Slooff and colleagues. Although this transplant program was by far the smallest, careful patient selection and the technique of biliary anastomosis had resulted in 1 and 2 year survival rates of 60%. Ultimately, the panel decided that survival advantages of patients undergoing this procedure had clearly been demonstrated and liver transplantation should no longer be considered experimental. Following on from this meeting, the pioneering centres started training other transplant teams and new liver transplant units were established around the globe.

Despite fierce opposition from both the medical and the political world, Professor Russell Strong and his team at the Princess Alexandra hospital carried out the first adult orthotopic liver transplant in Australia on the 29th of January 1985. This was successful and he went on to perform the first paediatric transplant 6 weeks later. During his short visit to Pittsburgh prior to establishing a transplant centre in Brisbane, Strong realised that a shortage of paediatric donors would remain a limiting factor in paediatric transplantation. This led him initially to develop a strategy by which an adult liver could be “cut down” to a size suitable for a child - this technique is currently still referred to as the Brisbane technique. Subsequently, he was the first in the world to successfully develop a technique where parents could be a living donor for their child. In July 1989, a segment 2/3 graft from a mother was successfully transplanted into her 17-month old son who suffered from biliary atresia.

Liver transplantation in the 21st century

In the current era of liver transplantation, the shortage of suitable donors is a pressing problem. Annually, over 500 patients with end stage liver disease are placed on the transplant waiting list throughout Australia. However only 50% will receive their lifesaving liver transplantation within a year of listing. As many as 10% of patients on the waiting list died or their disease progresses to a point where liver transplantation
is not considered a suitable treatment option anymore. To expand the number of available organs, extended criteria donors are being considered for transplantation. An important group of such organs are those donated after circulatory death (DCD). DCD organs are retrieved from donors allowed to progress to circulatory death after withdrawal of ventilatory and circulatory support and are therefore exposed to a period of warm ischaemia prior to cold perfusion. This in turn makes DCD livers more susceptible to cold preservation injury and recipients of these grafts frequently develop complications. Although short-term graft survival rates have been reported to be similar to that of livers from brain dead donors (DBD), long-term graft survival is significantly reduced. The analysis of a DCD liver recipient cohort from the Netherlands and Belgium, followed for up to 10 years, showed that 5-year graft survival of DCD livers was 54% compared to 66% of DBD controls (p=0.02). Patient survival was similar between the two categories.

Part I: Biliary stricture formation following DCD liver transplantation

One of the most important complications following transplantation of a DCD liver grafts is the development of biliary strictures, more specifically ischaemic type biliary strictures (ITBS) which are also referred to as non-anastomotic strictures (NAS). ITBS develops in between 10 and 33% of DCD recipients and the severity of disease is much greater compared to DBD recipients that develop ITBS. These strictures are mainly localised around or below the bifurcation of the common bile duct and occur within the first year of transplantation in over half the cases. Treatment options for ITBS are limited and consist mainly of endoscopic dilatation and stent placement. Ultimately, re-transplantation is required in a large proportion of ITBS sufferers. The pathophysiology of ITBS still remains largely unknown. Currently known risk factors mainly originate from case cohort studies however little is known about the true mechanistic processes involved. However studies assessing the pathophysiology of other diseases such as primary sclerosing cholangitis could provide helpful clues as to which processes could be responsible for the development of biliary stricture formation following transplantation of a DCD liver graft.

Mdr2 mice lack the canalicular phospholipid flippase transporter and as a result, no phospholipid is being excreted in bile. These animals develop severe biliary injury ultimately resulting in biliary stricture formation closely resembling the appearance of primary sclerosing cholangitis. Fickert at al. studied the sequences of events leading to the development of these strictures and found that the disruption of the blood-biliary-barrier (BBB) played a critical role in disease progression. The BBB consists of
tight junctions between adjacent hepatocytes or cholangiocytes, and serves as a natural barrier separating blood from bile.\textsuperscript{27} In the \textit{mdr2}\textsuperscript{-/-} mice, disruption of this barrier lead to leakage of toxic bile salts into the portal tract area.\textsuperscript{26} This in turn caused portal tract inflammation which ultimately induced periductal fibrosis and epithelial cell death. Primary sclerosing cholangitis (PSC) shows significant similarities with ITBS with regards to radiographic appearance. In fact, PSC recurrence after transplant is indistinguishable from the development of ITBS based on imaging. This lead us to hypothesise that dysfunction of the BBB could potentially play a role in ITBS formation, especially since increased bile toxicity early after transplantation has previously been associated with the development of ITBS.\textsuperscript{28}

Most insights into the modulation of this BBB result from cell culture experiments. Oxidative stress a well as pro-inflammatory cytokines like Tnf-\(\alpha\) and Ifn-\(\gamma\) were found to increase permeability of the BBB.\textsuperscript{29,30} Furthermore, lipopolysaccharides (LPS), the outer capsule of gram negative bacteria, induced tight junction disruption and increased para-cellular permeability in a time and dose dependent fashion.\textsuperscript{31} This effect was mediated by Toll-Like Receptor 4 (TLR-4) and LPS binding protein signalling pathways.

Currently, little is known about the integrity of the blood-biliary barrier following liver transplantation. A study examining common bile duct samples from DBD livers at time of retrieval, end of cold storage and just before the biliary anastomosis was made showed a marked destruction of tight junction architecture in patients with histologically severe bile duct damage.\textsuperscript{32} These patients were more likely to go on and develop biliary complications and graft loss. Although formal evidence is lacking, gut ischaemia during the DCD organ retrieval process is likely to occur which could ultimately result in the appearance of endotoxins in portal blood being flushed through the liver. We therefore hypothesised that exposure to endotoxins, in addition to ischaemia, would affect the BBB and, in turn, induce biliary stricture formation.

\textbf{Part II: Application of machine perfusion}

The application of machine perfusion to preserve organs \textit{ex vivo} was first described by Alexis Carrel and Charles Lindberg in 1935.\textsuperscript{33} They built a normothermic perfusion apparatus, which allowed them to study whole organs such as heart, kidneys and ovaries for several days \textit{ex vivo}. Several decades later, Starzl used a normothermic perfusion machine when he performed the first successful liver transplant.\textsuperscript{5} In the 1980’s and 90’s, the shift towards the use of brain death donors as well as the development of University of Wisconsin solution meant that organ quality significantly
increased and the complex and comprehensive technique of machine perfusion was largely abandoned. As currently more DCD livers are considered for transplantation, alternative organ preservation methods such as machine perfusion are explored yet again.

Compared to static cold storage, machine perfusion holds the potential to better preserve organ function, it allows for prolonged preservation thereby facilitating long distance transport and it allows for viability assessment of the graft prior to implantation. Currently, normothermic machine perfusion (NMP) is the only technique that allows for viability assessment as the metabolic functions of liver grafts are preserved at 37°C. Successful use of NMP has been described in different animal models.\textsuperscript{34-37} Furthermore, a pilot study using declined human donor livers has shown that NMP is technically feasible and allows assessment of graft viability.\textsuperscript{38} Besides the ability to assess metabolic processes in the liver, NMP offers a unique opportunity to study bile duct biology in a near physiological setting.
Aims of the thesis

The overarching aim of this thesis was to characterise the sequence of events associated with the development of ITBS following transplantation of DCD livers. To achieve this, we used both a partial hepatic ischaemia reperfusion model in rats as well as *ex vivo* normothermic machine perfusion of human donor livers. We hypothesised that increased permeability of the BBB, in combination with bile toxicity, was associated with the development of ITBS following transplantation of DCD liver grafts.

In Chapter 2 we determined the impact of DCD donation on the number of livers available for transplantation in Australia over the last decade. We furthermore assessed the risk factors associated with liver non-use.

Aims Part I: the development of biliary injury

The aim of part I of this thesis was to develop an animal model for biliary injury. As liver transplantation in rodent models is rather difficult, and requires extensive micro-surgical training, we choose a much simpler and more reproducible model of partial hepatic ischaemia-reperfusion. We furthermore aimed to test the attributive role of cofactors such as endotoxins in the development of biliary injury in this model. Lastly we aimed to develop a method to assess the permeability of the BBB *in vivo*.

In Chapter 3 we tested the role of endotoxins, in the form of LPS, in the development of biliary injury in a partial liver ischaemia-reperfusion model in rats. In this chapter, we further describe the use of horseradish peroxidase to assess the permeability of the BBB *in vivo*.

Chapter 4 explores the role of Kupffer cells in the development of LPS-induced biliary injury. Clodronate liposomes were used to deplete Kupffer cells from the liver and animals were subsequently exposed to LPS or a combination of warm hepatic ischaemia and LPS.

As it remained unclear if endotoxaemia occurred in DCD donors, we assessed the presence of inflammatory mediators in portal venous blood of both DBD and DCD donors during organ procurement in Chapter 5. We hypothesised that unlike organs donated after brain death, portal blood of DCD donors has an enhanced propensity to trigger inflammatory responses.
Aims Part II: machine perfusion

The aim of part II of this thesis was to develop and optimise a working mechanical model for *ex vivo* NMP of human donor livers currently declined for transplantation. We also used this perfusion technique as a platform to study the bile ducts and to assess whether a biomarker could be found to predict the development of stricture formation.

As machine perfusion of human livers is only used in limited centres around the world, we systematically reviewed the current perfusion protocols in chapter 6 and assessed their safety and applicability.

In our centre we developed an NMP protocol, which is described in chapter 7. To date, we perfused 10 livers deemed unsuitable for transplantation. Both DBD and DDB livers were perfused and the results are discussed in Chapter 8. In this chapter we also explored the impact of machine perfusion on the development of biliary injury. As biliary injury and stricture formation is a major concern when using DCD donors, we aimed to assess if NMP would modulate the degree of injury.

In the final chapter, chapter 9, a summary of the results of this thesis is provided and their contribution to the current literature is discussed.
References

Introduction and outline of the thesis


Chapter 2

Increased liver non-use: The implications of the shift towards Donation after Circulatory Death in Australia

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KR Bridle
LJ Britton
N Santrampurwala
DHG Crawford
CHC Dejong
J Fawcett

Submitted
Abstract

Background
In recent years, more livers are being declined for transplantation in Australia. The aim of this study was to evaluate the impact of donation after cardiac death (DCD) and other factors associated with organ quality on liver utilisation rates in Australia.

Method
Data on organ donors who donated at least one organ between 2005 and 2014 were obtained from the ANZOD Registry. Temporal changes in donor characteristics were assessed and a logistical regression analysis was performed to evaluate their association with liver non-use.

Key findings
The number of organ donors increased from 163 in 2005 to 332 in 2014 with 19% being DCD donors ($p<0.001$). The percentage of livers deemed unsuitable for transplantation increased from 26% in 2005 to 43% in 2014 ($p<0.001$). DCD was identified as the most important risk factor for non-use with an OR of 24.06 (95%CI: 17.47–33.14, $p<0.001$) followed by donor age, obesity and diabetes.

Conclusions
This study shows a significant decrease in liver utilization rates in Australia over a 10-year period with DCD donation as the most important independent risk factor. Novel approaches, such as machine perfusion, require further evaluation in order to be used to increase the number of livers available for transplantation.
Introduction

A shortage of available donors remains a major limiting factor in the field of liver transplantation. Currently in Australia, only 50% of patients with end stage liver disease on the waiting list receive a life-saving transplant each year. At the same time, 10% of patients die or their condition deteriorates to the point that they are no longer deemed suitable for transplant. In 2008, the Australian Federal Government announced a reform program to increase the number of organ donors. As a result, organ donor rates increased from 12 to 16.1 per million population (PMP). Unfortunately, the number of organ donors who donated their liver has failed to increase at the same rate, suggesting that more livers are deemed unsuitable for transplantation.

As well as encouraging the use of extended criteria livers (e.g. older donors and steatotic livers) and a drive to maximise splitting of suitable donor livers, one of the main strategies adopted has been the promotion of controlled donation after circulatory death (DCD, Maastricht classification type III). Currently, 28% of organ donors in Australia are DCD donors, which is low compared to the United Kingdom and the Netherlands, where 40% and 45% of organ donors are DCD respectively. DCD lung and kidney programs have been very successful with graft and patient survival rates comparable to conventional Donation after Brain Death (DBD) organs. Unfortunately the experience has not been the same for livers. The use of DCD livers has been associated with inferior graft quality and the development of non-anastomotic strictures (NAS) in 9-31% of recipients. As a result, the percentage of liver non-use attributable to DCD has increased in the US from 9% in 2004 to 28% in 2010. It is currently unclear what effect DCD donation has on liver non-use in Australia.

DCD livers, severely steatotic livers and livers from older donors are more susceptible to ischaemia-reperfusion injury. Alternative preservation methods such as ex-vivo machine perfusion have therefore been proposed in order to better preserve organ quality. Hypothermic machine perfusion at 4-10°C as well as normothermic machine perfusion using a blood based perfusate have proven to be safe and technically feasible in small clinical series and results are pending from the first randomised controlled trials. If extended criteria donor livers could be safely used following machine perfusion without increasing recipient morbidity, this could consequentially increase the number of donor livers available for transplantation. The aim of this study was to evaluate the impact of DCD and other donor characteristics associated with more marginal donors on liver non-use in Australia over the past 10 years. The study also aimed to determine the number of DCD livers that would be suitable candidates for machine perfusion if this technique could be implemented.
Chapter 2

Methods

Study population

All adult organ donors in Australia who donated at least one organ between 1st January 2005 and 1st of January 2015 were included in this study. As these donors had no absolute contraindication for the organ donation process, unsuitability for transplantation was therefore most likely the result of liver specific reasons. A liver donor was defined as an organ donor from whom the liver was retrieved and subsequently successfully transplanted into a recipient. Donors of split liver grafts (n=134) were excluded as they represent a specific subset of high quality donors and their characteristics differ from whole liver donors. De-identified donor data was obtained from the Australia and New Zealand Organ Donor (ANZOD) registry. In one Australian jurisdiction, the state of Queensland, approval was obtained from the human research ethics committee of the Princess Alexandra Hospital as well as the University of Queensland to access confidential donor data. This allowed access to donor files when data was incomplete or not available from the organ donor registry.

Data collection

Donor demographics associated with marginal grafts such as donor age, body mass index (BMI), donation type (DCD or DBD), hepatitis B (hepatitis B surface antigen positive) or hepatitis C infection (hepatitis C (HCV) antibody positive and presence of HCV RNA by nucleic acid testing), hypertension and diabetes mellitus (DM) were collected. As donor age was not normally distributed, the following age categories were used: <40, 40-49, 50-59 and >60 years. BMI was classified as underweight (<18.5 kg/m²), normal weight (18.5-24.99 kg/m²), overweight (25-29.99 kg/m²) and obese (>30 kg/m²). Liver biopsy results were not included from any of the donors, as it is not routine practice to perform a biopsy and the inclusion of biopsy results of selected donors would bias the results. Due to incomplete data in the organ donor registry during this period, it was not possible to include liver function test results as a parameter in the main analyses. Instead, a sub analysis was performed of the Queensland state donors (n=432) where alanine aminotransferase (ALT) was categorised as <100 versus >100 U/L, bilirubin as <20 versus >20 µmol/L and γ-glutamyl transferase as <40 versus >40 U/L.

Additional factors that potentially influence the decision to use a liver for donation such as gender, blood group, presence of hepatitis B core antibody, year of organ retrieval, state of organ retrieval, cause of death and smoking status were also recorded. If a donor was identified as a DCD donor, warm ischaemia time (WIT) prior to the start of cold perfusion was recorded. The start of this period was marked by a systolic blood pressure lower than 50mmHg or oxygen saturation lower than 50%,
whichever occurred first. In Australia, livers from DCD donors are generally considered for transplantation if the warm ischaemic time is limited to 30 minutes and they do not have any other extended criteria beyond DCD.\textsuperscript{17} From every donor, data on the organs retrieved and whether they were used or discarded was recorded (kidneys, liver, lung, heart, and pancreas). Lastly, from all donors in the State of Queensland, the distance between donor and recipient hospital was recorded. The state area comprises 1,727,000 km\textsuperscript{2}, with the liver transplantation centre located in the far south-east corner about 1700 km from the furthest donor hospital. For the purpose of this study travel distance was defined as metropolitan (<100 km) and non-metropolitan (>100 km). Overall each variable of interest was available from >95\% of organ donors unless stated otherwise.

**Impact of machine perfusion in the state of Queensland**

To determine likely candidates for machine perfusion, those that would fulfil current criteria for transplantation were identified from the United Kingdom and the Netherlands, as both these countries have very successful DCD liver transplant programs.\textsuperscript{8,9} Following review of current guidelines and recently published papers on the use of DCD donors in these countries, the DCD donor cohort was stratified by age, warm ischaemic time and BMI (as a surrogate marker for steatosis grade).\textsuperscript{18,19} Length of stay in the intensive care unit and cold ischaemic time are additional components that define marginality in the United Kingdom, however these parameters were not available in the present cohort. Instead, the distance between donor and recipient hospital was recorded and was used as an indicator of expected cold ischaemic time. Because of completeness of data, the focus was on organ donors in one jurisdiction, the state of Queensland.

**Statistical analysis**

All continuous variables are expressed as median (interquartile range) and all categorical variables as frequency (percentage). A chi-squared test was performed to compare categorical variables whereas a Mann-Whitney U test was used to compare all continuous variables. A multivariable logistical regression analysis was performed to identify donor characteristics associated with liver non-use controlled for all factors with a p-value <0.1 in univariable analysis.

A sub-analysis was performed on all Queensland donors to assess the association between donor hospital distance, abnormal liver function tests and liver non-use. The statistical analysis was performed using IBM SPSS Statistics for Macintosh, Version 23.0 (IBM Corp. IMB SPSS statistics, Armonk, NY) and a p-value of <0.05 was considered significant.
Results

Donor characteristics

Between 2005 and 2014 a total of 2413 organ donors in Australia were identified who have donated at least a single organ (Table 2.1). Of these, the majority of organ donors were Caucasian (93%) and 55% were male. In addition, 401 (16%) were older than 60 years old, 468 (19%) were DCD, and 900 (37%) and 607 (25%) were overweight or obese respectively. Only a very small proportion of organ donors tested positive for hepatitis B or hepatitis C (0.3% and 1% respectively), 8% were diabetic and 28% had a history of hypertension. Stroke was the most common cause of death (54%) followed by hypoxia. The median number of organs transplanted per donor was three. The majority of organ donors donated one or both kidneys (95%) followed by liver (63%), lung (42%), pancreas (31%) and heart (25%).

Table 2.1  Donor characteristics between 2005-2014.

<table>
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<tr>
<th>Characteristic</th>
<th>2005 (N=163)</th>
<th>2014 (N=332)</th>
<th>Overall (N=2413)</th>
<th>p-value</th>
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<tr>
<td>Age (years)</td>
<td>48 (37 - 57)</td>
<td>52 (41 - 60)</td>
<td>50 (37 - 57)</td>
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<td>Gender (male)</td>
<td>85 (52%)</td>
<td>183 (55%)</td>
<td>1314 (55%)</td>
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<td>Race (Caucasian)</td>
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<td>396 (89%)</td>
<td>2337 (93%)</td>
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<td>Body mass index (kg/m²)</td>
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<td>27 (24-30)</td>
<td>26 (24-30)</td>
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<td>Donor type (DBD)</td>
<td>155 (95%)</td>
<td>237 (71%)</td>
<td>1945 (81%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Cause of Death</td>
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<td></td>
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<tr>
<td>Stroke</td>
<td>100 (61%)</td>
<td>171 (52%)</td>
<td>1310 (54%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>17 (10%)</td>
<td>94 (28%)</td>
<td>502 (21%)</td>
<td></td>
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<tr>
<td>Accident</td>
<td>25 (15%)</td>
<td>46 (14%)</td>
<td>368 (15%)</td>
<td></td>
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<tr>
<td>Other</td>
<td>21 (13%)</td>
<td>21 (6%)</td>
<td>233 (10%)</td>
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<td>Diabetes mellitus</td>
<td>12 (8%)</td>
<td>21 (6%)</td>
<td>199 (8%)</td>
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<td>Hypertension</td>
<td>39 (24%)</td>
<td>96 (29%)</td>
<td>669 (28%)</td>
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<td>Current</td>
<td>57 (35%)</td>
<td>145 (44%)</td>
<td>969 (40%)</td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>74 (45%)</td>
<td>105 (32%)</td>
<td>855 (35%)</td>
<td></td>
</tr>
<tr>
<td>Former</td>
<td>32 (20%)</td>
<td>82 (25%)</td>
<td>583 (24%)</td>
<td></td>
</tr>
<tr>
<td>Hepatitis B core antibody</td>
<td>11 (7%)</td>
<td>18 (5%)</td>
<td>128 (5%)</td>
<td>0.02</td>
</tr>
<tr>
<td>Hepatitis B surface antigen</td>
<td>0 (0%)</td>
<td>5 (2%)</td>
<td>7 (0.3%)</td>
<td>0.2</td>
</tr>
<tr>
<td>Hepatitis C antibody + HCV RNA</td>
<td>3 (2%)</td>
<td>2 (1%)</td>
<td>21 (1%)</td>
<td>0.2</td>
</tr>
<tr>
<td>WIT (min, 400/468)</td>
<td>Data not available</td>
<td>23 (20-28)</td>
<td>22 (18-27)</td>
<td></td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>28 (16 - 85)</td>
<td>42 (23 - 68)</td>
<td>38 (21 - 71)</td>
<td>0.5</td>
</tr>
<tr>
<td>Bilirubin (µmol/L)</td>
<td>13 (7-16)</td>
<td>13 (9 - 18)</td>
<td>12 (8 - 17)</td>
<td>0.4</td>
</tr>
<tr>
<td>Gamma-GT (U/L)</td>
<td>41(22-75)</td>
<td>32 (19 - 59)</td>
<td>43 (23 - 71)</td>
<td>0.4</td>
</tr>
<tr>
<td>Distance donor hospital (Km)</td>
<td>37 (0-870)</td>
<td>67 (8 - 619)</td>
<td>37 (8 - 122)</td>
<td>0.4</td>
</tr>
<tr>
<td>Nr organs retrieved and transplanted</td>
<td>4 (3 - 5)</td>
<td>3 (2 - 4)</td>
<td>3 (2 - 4)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Note: Because of rounding not all percentages add up to 100%. # The data presented here is from the Queensland state donor subgroup only. N=432. HCV: hepatitis C virus.
Changes in donor characteristics over time

Over the 10-year study period, the number of organ donors per year more than doubled from 163 in 2005 to 332 in 2014 (Figure 2.1). Since 2008 there has been a steady increase in the number of DCD organ donors with 29% of all organs donated being from a DCD donor in 2014. In 2005 the percentage of organ donors younger than 40 years old was 29%, which decreased to 22% in 2014. In contrast, the proportion of organ donors older than 60 increased by 11%. During the study period, the proportion of overweight and obese donors remained stable with a median BMI of 26 (24–30) kg/m².

More donors were current or former smokers, or died from hypoxic injury in 2014 compared with 2005 (Table 2.1). Overall the median number of organs retrieved per donor decreased from 4 (3–5) in 2005 to 3 (2–4) in 2014 (p<0.001). Among Queensland state organ donors, the median ALT, bilirubin and γ-glutamyl transferase values did not change over the 10-year study period with 71 (16%) organ donors having an ALT >100 U/L.

Temporal trends in liver non-use

Overall, a liver was retrieved and subsequently transplanted from 1509 (63%) organ donors. Over time, the proportion of livers deemed unsuitable for transplantation from DBD donors remained stable between 20-30% (Figure 2.2). Since 2005, only 62 (13%) DCD livers were used for transplantation. Over the 10-year study period, the
percentages of DCD livers rejected for transplantation was between 80% and 100%. As the proportion of DCD donors has increased over time, the overall number of livers not used for transplantation increased from 42 (26%) in 2005 to 141 (43%) in 2014 ($p<0.001$).

![Figure 2.2 Changing organ donor characteristics between 2005 and 2014. A) Increase in the number of organ donors and organ donors donating their liver (liver donors) between 2005 and 2014. B) Number of livers donated after circulatory death (DCD) and donated after brain death (DBD) between 2005 and 2014. C) Age of organ donors between 2005 and 2014. D) Body mass index (BMI) of organ donors between 2005 and 2014.](image)

Risk factors for liver non-use

In univariable analysis, liver non-use was strongly associated with DCD donor type ($p<0.001$, Table 2.2). Furthermore, livers from older donors and overweight or obese donors were less likely to be used. Organs from donors who died from hypoxia, compared to those who suffered a stroke, were more likely to be declined for transplant. In addition, liver non-use was associated with donors who were former smokers, compared to those who did not smoke. The presence of hypertension and diabetes in the donor population were also associated with liver non-use. In the subgroup of 468 DCD donors, a WIT over 20 minutes was associated with liver non-use (OR 3.10 (95% CI: 1.70–5.61), $p<0.001$). In univariable analysis of Queensland state organ donors, ALT concentration over 100 U/L and $\gamma$-glutamyl transferase greater than 40 U/L were associated with liver non-use (OR 3.04 (95% CI: 1.80–5.14) $p=0.029$ and OR 12.20 (95% CI: 7.290–20.42) $p<0.001$ respectively).
In multivariable analysis, DCD donor type was identified as the most significant independent risk factor for liver non-use with an odds ratio of 24.06 (95% CI: 17.47-33.14) \( p<0.001 \), Table 2.2). Furthermore, donor age over 40 years was associated with liver non-use with livers from donors over 60 years of age being the least likely to be used (OR 3.10 (95% CI: 2.21-4.34), \( p<0.001 \)). Donor obesity was also associated with higher odds for non-use (\( p<0.001 \)) as was diabetes. In the Queensland state organ donor subset, ALT >100 U/L and Gamma-GT >40 U/L were strongly associated with non-use (OR 4.43 (95% CI: 1.98-9.93) \( p<0.001 \) and OR 11.90 (95% CI: 5.97-23.76) \( p<0.001 \) respectively).

### Characteristics of DCD non-liver donors in the state of Queensland

Since 2008, there were 90 DCD organ donors in the state of Queensland but only 9 livers retrieved from these donors were used for transplantation. The non-liver donors were categorised by duration of warm ischaemia, age and BMI category to identify livers that could have been transplanted had some effective method, such as normothermic machine perfusion been available to better assess their suitability. Furthermore the distance between the donor hospital and the liver transplant centre was assessed. Compared to DBD donors, DCD donors were more often in the
metropolitan area (86% versus 66%, p<0.001). As can be seen in Table 2.3, the majority of DCD non-liver donors (85%) had a WIT of less than 30 minutes and 27 (33%) had recorded WIT less than 20 minutes. Only seven (9%) donors fulfilled all three optimal donor criteria (BMI <25 kg/m², WIT <20 minutes, age <50 years). All these donors were from hospitals in the metropolitan area. An additional 18 (22%) donors had one adverse parameter (age 50-60 years, BMI 25-30 kg/m² or WIT 20-30 minutes) three of which were non-metropolitan donors. A further eight (10%) donors had two adverse parameters and were within a 100 km from the liver transplant centre.

Table 2.3 Number of DCD non-liver donors in Queensland by age category, body mass index and warm ischaemic time.

<table>
<thead>
<tr>
<th>DCD donors N = 81</th>
<th>Body mass index</th>
<th>Donor age (years)</th>
<th>&lt; 40</th>
<th>40-50</th>
<th>50-60</th>
<th>&gt; 60</th>
</tr>
</thead>
<tbody>
<tr>
<td>WIT&lt;20 minutes</td>
<td>&lt; 25 kg/m²</td>
<td>5 (6 %)</td>
<td>2 (2 %)</td>
<td>3 (4 %)</td>
<td>1 (1 %)</td>
<td></td>
</tr>
<tr>
<td>N=27 (33 %)</td>
<td>25-30 kg/m²</td>
<td>2 (2 %)</td>
<td>4 (5 %)</td>
<td>2 (2 %)</td>
<td>2 (2 %)</td>
<td></td>
</tr>
<tr>
<td>&gt; 30 kg/m²</td>
<td>2 (2 %)</td>
<td>1 (0 %)</td>
<td>2 (2 %)</td>
<td>1 (1 %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WIT 20-30 minutes</td>
<td>&lt; 25 kg/m²</td>
<td>5 (6 %)</td>
<td>4 (5 %)</td>
<td>3 (4 %)</td>
<td>3 (4 %)</td>
<td></td>
</tr>
<tr>
<td>N=41 (51 %)</td>
<td>25-30 kg/m²</td>
<td>3 (4 %)</td>
<td>0 (0 %)</td>
<td>9 (11 %)</td>
<td>5 (6 %)</td>
<td></td>
</tr>
<tr>
<td>&gt; 30 kg/m²</td>
<td>1 (1 %)</td>
<td>1 (1 %)</td>
<td>4 (5 %)</td>
<td>3 (4 %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WIT&gt;30 minutes</td>
<td>&lt; 25 kg/m²</td>
<td>0 (0 %)</td>
<td>4 (5 %)</td>
<td>1 (1 %)</td>
<td>1 (1 %)</td>
<td></td>
</tr>
<tr>
<td>N=13 (16 %)</td>
<td>25-30 kg/m²</td>
<td>0 (0 %)</td>
<td>1 (1 %)</td>
<td>1 (1 %)</td>
<td>1 (1 %)</td>
<td></td>
</tr>
<tr>
<td>&gt; 30 kg/m²</td>
<td>0 (0 %)</td>
<td>1 (1 %)</td>
<td>3 (4 %)</td>
<td>0 (0 %)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

WIT: warm ischaemic time

Discussion

In recent years, DCD donors have been identified as a potential source to increase donor numbers. In the United Kingdom and the Netherlands, over one third of all organ donors are DCD donors and these countries have successfully implemented the use of DCD livers with 1-year graft and patient survival similar to conventional DBD donors.8,9 Unfortunately the Australian experience is rather different. Despite the fact that 468 (19%) organ donors in the present cohort were DCD, only 62 livers were retrieved and subsequently transplanted. This contributed to the significant rise in liver non-use rates from 26% in 2005 to 43% in 2014.

The decreasing rate of liver utilisation is a pressing problem around the globe. In the United Kingdom, the proportion of livers used for transplantation has dropped from 601 (79%) in 2005-06 to 812 (63%) in 2014-15.5,20 Furthermore, Orman et al.10 found that in the US, liver non-use increased from 15% in 2004 to 21% in 2010 with DCD as the most important risk factor (OR 21.31 (95%CI: 18.30-24.81)). The present study indicates that liver non-use is an even bigger problem in Australia, as the proportion of DCD donors in the total cohort is higher (19% versus 9%). Australian centres have
had a conservative approach to the use of DCD livers mainly due to a small increased risk of primary non-function that has been observed in our country (Jan 2006- Dec 2015: DBD; 20 (0.9%), DCD; 5 (5.1%), p<0.01, unpublished data, courtesy of Glenda Balderson, ANTLR) as well as NAS formation (21). The low population density (3.1 people/km²)22 and the large distances between the five transplant centres (up to 3600 Km) significantly reduce the chances of finding a new liver in case of primary non-function of the graft. Therefore, livers from DCD donors are rarely considered for transplantation if they are aged over 40-45, in an attempt to select more superior grafts.17

One of the most important questions remains is if some DCD donors, if given the time, would in fact progress to brain death.24 If so, this would mean that a proportion of DBD organ donors, with expected good organ quality, were converted into the less favourable DCD type, which the present study shows is associated with high odds of liver non-use. Reasons for this conversion could be that the DCD donation process is easier to comprehend by donor families and they prefer to accompany their family member until cardiac arrest has occurred.24 Furthermore, bed capacity in the intensive care unit (ICU) could put pressure on the time-consuming neurological observations required to medically and legally determine brain death. A recent study assessing transplant rates in 82 countries showed that between 2000 and 2010, countries with high DCD rates had declining or static DBD rates.23 Furthermore, this study by Bendorf et al. showed that the increase in the number of DBD donors, and not DCD, was the basis of higher sustained donation rates >20 PMP in countries such as Spain, France and the USA. Taken together, this might indicate that the focus needs to shift back to increasing the number of DBD donors.

To facilitate the use of high-risk donors such as DCD, modifiable risk factors such as WIT, cold ischaemic time (CIT) and distance travelled need to be modified. Currently in Australia, withdrawal of life support takes place in the ICU. Following cessation of circulation, a legally obligated 5-minute stand down period is in place to make sure that auto-resuscitation does not occur. The organ donor is then brought into the operating room, which can take 3-4 minutes. Withdrawal of life support in the operating room would significantly expedite the process and reduce WIT. CIT in Australia is largely dependent on travel time due to the large distances. Although actual flying times to distant parts of the state may only take 3-4 hours, ground transport between hospitals and airport has to be taken into account. Additional staff at the donor hospital (e.g. donation specialist nurse) to care for the donor once organ retrieval is finalised could facilitate a quicker departure. This approach has been implemented in the larger non-metropolitan hospitals throughout Australia. Furthermore, police escorts to and from the airport could significantly reduce travel time, especially during peak hour. Unfortunately this is not possible in most states. By far the most effective way to reduce travel time is to focus on metropolitan donors only. This approach has facilitated the use of more high-risk donors in other countries
such as Canada.25 Nevertheless, the biggest challenge to date is to build the confidence amongst transplant surgeons and physicians that the use of DCD donor livers can be safe.

One possible way to safely increase the number of livers suitable for transplantation could be the use of alternative preservation methods such as machine perfusion.16 Machine perfusion reduces the preservation injury and therefore holds the potential to better preserve organ quality and possibly even rescue marginal donors who are outside current criteria (e.g. DCD donor organs with WIT >30 minutes and macro-vesicular steatosis >30 %).26-28 Machine perfusion furthermore opens up the possibility for organ sharing and utilisation of marginal donors between geographically distant centres as preservation periods could be extended. Lastly, normothermic machine perfusion allows for assessment of viability, an important tool to avoid transplanting a liver that would in fact fail to function.29

The unit in the state of Queensland rarely uses DCD livers and has therefore explored the use of normothermic machine perfusion.30 When analysing the present cohort, 33 DCD donors (41%) were identified who either had nil, one or two marginal donor characteristics. When implementing machine perfusion, the use of these grafts would be the most logical first step. Obviously recipient selection to minimise the risk of adverse graft outcome is crucial.9,31

As the application of machine perfusion is still an upcoming new technique, it is hard to predict what the impact would be on the overall number of livers available for transplantation. Sutton et al. estimated that 50% of the discarded extended criteria livers (10 DCD, 2 DBD) they perfused showed signs of good organ function.32 Furthermore, based on results of a pilot series, Mergental et al. estimated a potential increase in the number of liver grafts in the United Kingdom by 15% if 70% of currently declined extended criteria livers could be used.33

The limitations of the study arise mainly from its retrospective nature. The reasons for non-use of each organ are registered in the database, but in the majority of cases this was not well defined (e.g. ‘medical unsuitability’ or ‘DCD’). Furthermore, no histological data were available to determine the impact of hepatic steatosis. Obesity and DM were however included in the cohort and these factors are strongly associated with hepatic steatosis.34 Finally it was only possible to assess the impact of abnormal liver function tests and donor hospital location in the Queensland state donor cohort. As this cohort only comprised 18% of all Australian organ donors, it might not represent the entire study cohort.

Despite these limitations, this study confirms and reiterates previous reports of a decrease in liver utilisation with DCD donation as the most important independent risk factor. Despite comparable graft and patient outcomes to DBD donors in selected centres, DCD livers are reluctantly used in Australia. Machine perfusion, by way of better preservation and viability testing, will likely play an important role in efforts to expand the donor pool and decrease waitlist mortality.
References

1. ANZLTR. 24th annual report Australia and New Zealand liver transplant registry. 2014.

31


Chapter 3

Low dose lipopolysaccharide causes biliary injury by blood biliary barrier impairment in a rat hepatic ischaemia-reperfusion model

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KR Bridle
M Gijbels
FG Schaap
L Jaskowski
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CM Campbell
SWM Olde Damink
DHG Crawford
CHC Dejong
J Fawcett

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Abstract

Background
This study explored whether bacterial endotoxins, in the form of lipopolysaccharides (LPS), could have an injurious effect on the biliary tract in conjunction with ischemia.

Method
Sixty-four rats were randomly assigned to four groups: sham operation (sham), 1 mg/kg LPS i.p. (LPS), hepatic ischemia reperfusion (IR), and IR combined with LPS (IR+LPS). Following 1 or 6 hours of reperfusion, serum liver tests, bile duct histology, immunofluorescence microscopy (zonula occludens-1), bile composition (bile salts, phospholipids, lactate dehydrogenase), hepatic gene expression (bile salt transporters and inflammatory mediators) as well as serum and biliary cytokine concentrations were quantified and compared between the study groups. In addition, the integrity of the blood biliary barrier (BBB) was assayed in vivo using horseradish peroxidase (HRP).

Key findings
LPS administration induced severe small bile duct injury following 6 hours of reperfusion. Furthermore, total bile salts and bilirubin concentrations in serum were increased in the LPS groups compared to sham controls (LPS: +3.3 fold and +1.9 fold, IR+LPS: +3.8 fold and +1.7 fold respectively). The BBB was impaired in the LPS groups as evidenced by elevated levels of HRP in bile (+4.9 fold), decreased expression of claudin-1 (-6.7 fold) and claudin-3 (-3.6 fold).

Conclusions
LPS was found to be a potent inducer of small bile duct injury following hepatic ischemia and 6 hours of reperfusion. This injury was associated with increased permeability of the BBB and impaired hepatic bile salt clearance.
Introduction

In the current era of organ shortage, an increasing number of extended criteria donors are considered for transplantation, such as those who donate after cardiac death (DCD). Although short-term graft survival rates similar to that of recipients of organs donated after brain death (DBD) have been reported, the use of these DCD livers has been hampered by the high incidence of ischemic type biliary strictures (ITBS).4 Systemic endotoxemia has previously been reported to occur during both organ retrieval and implantation of the liver graft.2,3 Furthermore, the underlying cause of death frequently causes increased endotoxin levels in organ donors.4 DCD organs in situ are exposed to a period of hypoperfusion and hypoxia prior to the start of cold perfusion. All of the intra-abdominal organs become ischemic, including the gut, and this could potentially result in the release of endotoxins into the portal circulation which might then be flushed into the liver during cold perfusion.5 Endotoxemia aggravates organ preservation injury and has been associated with increased mortality following transplantation in animal studies.6,7 It can substantially exacerbate hepatic ischemia-reperfusion injury (IRI) as it aggravates the inflammatory response by activating liver-resident macrophages and by enhancement of leukocyte recruitment.8 If endotoxemia is directly injurious to bile ducts, or augments the effects of ischemia, then potentially it might contribute to ITBS and it would be relevant to determine the acute effect of endotoxin on the biliary tract. The blood-biliary-barrier (BBB) is formed by tight junctions between cholangiocytes and keeps bile separated from the blood stream.9 Several in vitro studies using cultured cholangiocytes have shown that the integrity of these tight junctions is affected by endotoxins, cytokines and ER stress.10,11 Moreover, BBB dysfunction has been implicated in the pathophysiology of biliary stricture formation in Mdr2-/ mice, an animal model for primary sclerosing cholangitis (PSC).12 In Mdr2-/ mice, tight junction dysfunction was associated with leakage of bile in the portal tract area, with subsequent inflammation, periductal fibrosis and stricture formation. Bile toxicity has already been implicated in the pathophysiology of ITBS and disturbed tight junction architecture was observed in human common bile duct sections following cold preservation and reperfusion.13,14 However the relationship between BBB dysfunction and biliary complications was not assessed in this human study. The aim of the current study was to assess the effect of endotoxins, in the form of lipopolysaccharides (LPS) on the development of biliary injury in a model of warm hepatic-ischemia reperfusion.
Methods

Animals

Male Sprague-Dawley rats weighing between 250-350 grams were obtained from the Animal Resource Centre (ARC, Perth, Australia) and housed in a temperature and light-controlled facility. The Animal Ethics Committee of the University of Queensland (MED/PAH/472/13/PAH) approved the study protocol in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes.

Surgical procedure and experimental groups

Sixty-four animals were equally divided over 4 study groups: a sham group (Sham) underwent midline laparotomy only; a LPS group (LPS) underwent midline laparotomy and intra-peritoneal LPS administration; a hepatic ischemia-reperfusion group (IR) underwent 30 minutes of warm hepatic ischemia; and a combined group (IR+LPS) simultaneously received LPS into the peritoneal cavity and warm hepatic ischemia. Briefly, laparotomy was performed under isoflurane anaesthesia and an atraumatic vascular clamp (BH030R, BBraun, Bethlehem, PA, USA) was placed across the vasculo-biliary pedicle of the median and left lateral lobe inducing ischemia to 70% of the liver avoiding splanchnic congestion (IR groups). Animals also received either vehicle (0.9% sterile NaCl) or 1mg/kg LPS (dissolved in 0.9% NaCl) from Escherichia coli serotype 0111:B4 (L3012, Sigma-Aldrich, St. Louis, MO, USA) into the peritoneal cavity. After thirty minutes of ischemia, the clamp was removed and the abdomen was closed. Administered LPS was not rinsed from the peritoneal cavity prior to closure of the abdomen.

Following 1 or 6 hours of reperfusion, re-laparotomy was performed and the common bile duct cannulated to collect bile. Blood and tissue samples from the liver (ischemic and non-ischemic lobes) and common bile duct were collected for further analysis.

Bile composition analysis

After establishment of a steady bile flow during 10 minutes, two bile samples were collected. Lactate dehydrogenase (LDH) was measured via colorimetric assay (#5604-01, Bioo Scientific Corporation, Austin, TX, USA). Phospholipids were measured spectrophotometrically using an enzymatic assay (433-36201, Wako, Osaka, Japan). For quantitative determination of bile salts, a Nexera X2 Ultra High Performance Liquid Chromatography system (Shimadzu, Kyoto, Japan) was used. Further details are provided in the Supplementary Data. The individual bile salt concentrations were used to calculate the hydrophobicity index (HI).
Assessment of blood-biliary barrier permeability

The permeability of the BBB was assessed in vivo using a medium sized protein, viz. horseradish peroxidase (HRP, P8250, Sigma-Aldrich, St. Louis, MO, USA) as previously described by Takakuwa et al. Sterile water containing 1000 U of HRP was injected in the inferior vena cava 30 minutes after bile duct cannulation. Bile was collected for a further 10 minutes and HRP activity assayed (Amplex red Assay kit, #A22188, Thermo Fisher Scientific, Waltham, MA, USA).

Biochemical analysis of serum

Serum alanine transaminase (ALT), aspartate transaminase (AST), γ-glutamyl transferase, alkaline phosphatase and total bilirubin were measured using commercially available kits (Bioo Scientific Corporation, Austin, TX, USA). Total bile salt concentration in serum was measured spectrophotometrically using a 3α hydroxysteroid dehydrogenase enzymatic assay (#80460, Crystal Chem, Inc., Chicago, IL, USA).

Assessment of small and large bile duct injury

Hilar and peripheral liver tissue as well as common bile duct were paraffin embedded and 4 µm thick sections were cut and stained with hematoxylin and eosin. Small and large intrahepatic bile ducts were identified based on their size (small ≤15 µm diameter, large >15 µm17) and semi-quantitative bile duct injury scoring was performed. The Small Bile Duct Injury Severity Score (SBDISS) encompassed 2 components: bile duct disruption (adopted from the Banff criteria for acute rejection18) and ductular proliferation as previously described by Cheng et al.19 Both items were scored using the following grades: 0: absent; 1: mild; 2: moderate and 3: severe. The Large Bile Duct Injury Severity Score (LBDISS) comprised 3 components: ductal disruption and the intra-epithelial and hilar infiltration of inflammatory cells. These components were scored using the same grading from 0-3 as the small bile ducts. Two independent expert pathologists (MG, CC) who were unaware of group assignments performed the scoring.

Immunofluorescent staining of tight junctions

Hepatic cryosections (4 µm thick) were incubated with polyclonal rabbit anti-ZO-1 (1:100, 61-7300 Thermo Fisher Scientific, Waltham, MA, USA) and mouse anti-Cytokeratin 19 (1:100, NCL-CK19, Novocastra) overnight. A donkey anti-rabbit IgG and donkey anti-mouse IgG were used as secondary antibodies (1:2000, A21207 and A21202, Thermo Fisher Scientific, Waltham, MA, USA). DAPI (D9542 Sigma-Aldrich, St.
Louis, MO, USA) staining was performed using a 1:500 dilution in 5% donkey serum and 2% BSA.

RNA extraction and RT-qPCR

Total RNA was extracted from ischemic and non-ischemic liver lobes and reverse transcribed into complementary DNA as previously described.20 Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using the ViiA 7 real-time PCR machine (Invitrogen, Carlsbad, CA, USA) and gene expressions were normalized to the geometric mean expression of 3 reference genes: glyceraldehyde 3-phosphate dehydrogenase, β2-microglobulin and basic transcription factor 3. Primer sequences can be found in Suppl. Table S3.1.

Cytokine array

The concentrations of Intercellular adhesion molecule-1 (Icam-1), Interferon γ (Ifnγ), Interleukin-1β (IL-1β), Interleukin-10 (IL-10), Interleukin-6 (IL-6), Leptin, L-Selectin, Monocyte chemoattractant protein-1 (Mcp-1), Tissue inhibitor metalloproteinase-1 (Timp-1) and Tumor necrosis factor-α (Tnf-α) were assessed using a Quantibody® multiplex ELISA array (QAR-CYT-2; RayBiotech, Norcross, GA, USA). The array was performed according to the manufacturer’s instructions on serum (1:2 dilution) and bile (1:4 dilution) samples. The fluorescence intensity was quantified and data were analyzed using the Q-analyzer® (RayBiotech, Norcross, GA, USA).

Statistical analysis

In each group, eight animals were studied unless stated otherwise. Non-parametric tests were used to evaluate differences between groups, and data are reported as median (inter quartile range). A Kruskal-Wallis test with Dunn’s correction for multiple comparisons was performed to compare intervention groups. For categorical data such as histological scoring, a Fisher’s exact test was performed. A cut-off p-value of 0.05 was used to determine significance. The statistical analysis was performed using GraphPad Prism 7 software (GraphPad, San Diego, CA, USA).

Results

Ischemia-reperfusion but not LPS induces significant hepatocellular injury

The intra-peritoneal administration of 1 mg/kg LPS alone had no effect on serum ALT compared to sham operated animals with a median value of 35.5 (31.1-40.3) U/L after
1 hour and 50.9 (43.7-56.9) U/L after 6 hours of reperfusion, respectively (P > 0.99 for both time points) (Figure 3.1). Liver ischemia and reperfusion, however, did induce hepatocellular injury as evidenced by increased serum ALT levels in the IR group at both time points (1h: +3.1 fold, \( p=0.007 \); 6h: +1.9 fold, \( p=0.046 \)). There was no additional effect of LPS on serum ALT in the ischemic group at either time point. This observation was further supported by serum AST elevation in the ischemic groups at the 6-hour time point (Suppl. Table S3.2).

Figure 3.1  Ischaemia-reperfusion results in hepatocellular injury following 1 and 6 hours of reperfusion. Serum alanine transaminase (ALT, U/L) was determined in groups of rats (n=7-8) that were sham-operated, exposed to 1 mg/kg lipopolysaccharides (LPS) or 70% hepatic inflow obstruction during 30 minutes (IR), or a combination of the latter two (IR+LPS) followed by reperfusion for 1 and 6 hours. Data are depicted as box and whisker plots showing median, minimum and maximum values. Statistical significance was tested using a Kruskal-Wallis test with Dunn’s correction for multiple comparisons, *\( p<0.05 \), **\( p<0.01 \), ***\( p<0.001 \).

LPS and not ischemia-reperfusion induces significant small bile duct injury following 6 hours of reperfusion

The intra-hepatic large bile ducts, including the peribiliary plexus, as well as the common bile duct showed no histological signs of injury in any of the study groups (Suppl. Table 3). In contrast, small bile ducts were affected and representative images of each group are shown in Figure 3.2A. Severe small bile duct injury (score \( \geq 4 \)) occurred in none of the sham animals and only in a single animal in the IR group (14%). LPS administration on the other hand caused severe biliary injury in 5 (63%) animals in both the LPS and IR+LPS group (\( p=0.004 \)). In addition to the histological scoring, LDH leakage into bile was measured as a biomarker for biliary injury.\(^{21}\) Hepatic IR induced a transient rise in LDH in bile following 1 hour of reperfusion,
which resolved at the later time point (Suppl. Figure S3.1). Following 6 hours of reperfusion, the administration of LPS, with or without IR, induced a marked increase in biliary LDH compared to sham operated animals (sham: 32.2 (29.5-35.8) U/L, LPS 91.1 (64.3-146.8) U/L, IR+LPS: 147.2 (84.6-234.0) U/L, p 0.01 and p<0.001 respectively) (Figure 3.2B). Only data from the six-hour time point will be presented below. All results from the one-hour time point can be found in the supplementary data.

**Figure 3.2** Severe small bile duct injury in LPS and IR + LPS groups following 6 hours of reperfusion. Groups of rats (n=7-8) were sham-operated, exposed to lipopolysaccharides (LPS) or 70% hepatic inflow obstruction during 30 minutes (IR), or a combination of the latter two (IR+LPS) followed by reperfusion for 6 hours. A) Representative images displaying portal tract histology (magnification 200x) with arrows indicating bile ducts. #: hepatic artery, PV: portal vein. B) Lactate dehydrogenase (LDH) activity in bile (U/L) depicted as box and whisker plot showing median, minimum and maximum values. Statistical significance was tested using a Kruskal-Wallis test with Dunn’s correction for multiple comparisons, *p<0.05, **p<0.01, ***p<0.001.

LPS impairs handling of biliary constituents following 6 hours of reperfusion

Bile flow was not significantly different between the intervention groups (Figure 3.3A). LPS administration, with or without IR, did result in a significant increase in the serum bile salt concentration. The mean concentration of total bile salt in serum was 45.7 (33.1-71.2) μmol/L and 52.0 (39.9-61.6) μmol/L in the LPS and IR+LPS group, respectively, compared to 13.7 (10.2-16.5) μmol/L in the sham-operated group.
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(p=0.001, Figure 3.3B). On a transcriptional level, expression of the main bile salt importer Na+/Taurocholate cotransporting polypeptide (Ntcp) was reduced (Figure 3.3C, p=0.02 and p=0.04 respectively). Furthermore, LPS administration led to a significant increase in serum bilirubin (figure 3.3D). The bilirubin concentration in the LPS and IR+LPS was 2.1 (1.9-2.5) and 1.9 (1.7-2.4) mg/dL compared to 1.1 (1.0-1.3) and 1.1 (1.0-1.5) mg/dL in the sham and IR groups respectively. This was accompanied by a reduction in mRNA expression of multidrug resistance protein 2 (Mrp2), the ATP-binding cassette protein responsible for secretion of glucuronidated bilirubin into the bile canaliculi, in the LPS group (Suppl. Figure S3.2). Note that, gene expression levels of the non-ischemic lobes will not be discussed in this manuscript but can be found in the Suppl. Figures S3.3 and S3.4.

Figure 3.3 Impaired hepatic bile salt clearance following 6 hours of reperfusion. A) Bile flow (µL/min). B) Concentration of total bile salts in serum (µmol/L) C) Hepatic Ntcp mRNA expression assessed using real-time quantitative polymerase chain reaction D) Serum bilirubin concentration (mg/dL). Groups of rats (n=7-8) were sham-operated, exposed to lipopolysaccharides (LPS) or 70% hepatic inflow obstruction during 30 minutes (IR), or a combination of the latter two (IR+LPS) followed by reperfusion for 6 hours. Data are depicted as box and whisker plots showing median, minimum and maximum values. Statistical significance was tested using a Kruskal-Wallis test with Dunn’s correction for multiple comparisons, *p<0.05, **p<0.01.
LPS increased the permeability of the blood-biliary-barrier following 6 hours of reperfusion

BBB permeability was measured in vivo using HRP to investigate whether the development of biliary injury in our model was accompanied by BBB dysfunction. IR did not cause an increase in biliary HRP above the background levels observed in the controls, indicating an intact barrier (Figure 3.4A). In the LPS group, however, the BBB was impaired as evidenced by significant leakage of HRP from blood to bile (+4.9 fold, \( p<0.001 \)), with a trend apparent in the IR+LPS group (+3.7 fold, \( p=0.06 \)). At the transcriptional level, the expression of tight junction proteins Claudin-1 and Claudin-3 was markedly lower in the LPS and IR+LPS group compared to the sham and IR groups (Figure 3.4B and 3.4C). Conversely, expression of tight junction protein Zonula occludens 1 (Zo-1) was 2-fold and 1.8-fold higher in the LPS and IR+LPS group compared to sham operated animals (\( p<0.01 \), Figure 3.4D).

![Figure 3.4](image_url)  
**Figure 3.4** Increased permeability of the blood biliary barrier following 6 hours of reperfusion. Groups of rats (n = 7-8) were sham-operated, exposed to lipopolysaccharides (LPS) or 70% hepatic inflow obstruction during 30 minutes (IR), or a combination of the latter two (IR+LPS) followed by reperfusion for 6 hours. Horseradish peroxidase (HRP) output in bile (A) following intravenous injection of 1000IU HRP and hepatic expression of genes encoding tight junction proteins Claudin-1 (B) Claudin 3 (C) and Zo-1 (D) was determined. Data are depicted as box and whisker plots showing median, minimum and maximum values. Statistical significance was tested using a Kruskal-Wallis test with Dunn’s correction for multiple comparisons, *\( p<0.05 \), ** \( p<0.01 \), *** \( p<0.001 \)
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Immunofluorescence co-staining for cytokeratin 19 and ZO-1 showed that ZO-1 was present at the junctions of neighbouring cholangiocytes in animals that did not develop biliary injury (Figure 3.5 A-D). Animals that developed biliary injury, such as those in the IR+LPS group, showed markedly altered tight junction morphology with an irregular ZO-1 staining pattern not confined to junctions of neighbouring cholangiocytes (Figure 3.5 E-H).

Bile composition following 6 hours of reperfusion was not toxic

There were no changes in total bile salt output following LPS administration in the IR+LPS group (Figure 3.6A). Biliary phospholipid output in bile was reduced following LPS administration combined with IR (IR+LPS), compared to IR alone or sham operated animals (sham: 390 (333-490) µg/10 minutes IR: 437 (343-528) µg/10 minutes, IR+LPS: 324.4 (209.6-323.7) µg/10 minutes, p=0.03 and p=0.008, Figure 3.6B). At the transcriptional level, the expression of the bile salt export pump (Bsep) was not affected in the intervention groups whereas the expression of the canalicular phospholipid flippase (Mdr2) was reduced following LPS administration (Suppl. Figure S3.2). Taken together, the biliary bile salt/phospholipid ratio was unaltered as both...
parameters were affected in a similar fashion (Figure 3.6C). Although the biliary bile salt/phospholipid ratio did not change across the intervention groups, we assessed whether the biliary bile salt pool itself was more hydrophobic and therefore potentially more toxic. Analysis of biliary bile salt composition revealed that glycine and taurine conjugates of cholic acid were most abundant in all groups (Suppl. Table S3.4). The hydrophobicity index (HI) of the biliary bile salt pool was calculated as a surrogate measure of pool toxicity (Figure 3.6D). LPS induced a significant reduction in the HI, suggesting a more hydrophilic and less toxic composition of the biliary bile salt pool ($p=0.02$).

![Figure 3.6 Bile composition following 6 hours of reperfusion. A) Total bile salt output (μmol) in bile. B) Phospholipid output (μg) in bile C) Ratio of bile salt/phospholipids (μmol/mg) D) Hydrophobicity index based on the concentration of individual bile salts. Groups of rats ($n=7-8$) were sham-operated, exposed to lipopolysaccharides (LPS) or 70% hepatic inflow obstruction during 30 minutes (IR), or a combination of the latter two (IR+LPS) followed by reperfusion for 6 hours. Data are depicted as box and whisker plots showing median, minimum and maximum values. Statistical significance was tested using a Kruskal-Wallis test with Dunn’s correction for multiple comparisons, *$p<0.05$, ** $p<0.01$](image)

Expression levels of genes coding for key enzymes of both the classical (e.g. Cyp7a1) and alternative (e.g. Cyp7b1) bile salt synthesis pathways were assessed (Figure 3.7). LPS induced a shift from the classical pathway to the alternative (or acidic) bile salt
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In the LPS group, gene expression of Cyp7a1 was 5.8 fold lower compared to sham animals \( (p=0.005, \text{Figure 3.7A}) \) whereas Cyp7b1 and Cyp27a1 expression were 6.4 and 2.6 fold higher, respectively \( (p=0.001 \text{ and } p=0.02, \text{Figure 3.7B and 3.7C}) \). A similar pattern was seen in the IR+LPS groups \( (\text{Cyp7b1} +10 \text{ fold, Cyp27a1} +2.9 \text{ fold}, \ p<0.001 \text{ and } p=0.003) \). Cyp8b1, coding for sterol 12 \( \alpha \)-hydroxylase, an enzyme determining the hydrophilicity/hydrophobicity balance of newly synthesized bile salts, was significantly lower in the LPS groups \( (6.7 \text{ fold and 7.8 fold, } p=0.02 \text{ and } p= 0.003 \text{ Figure 3.7D}) \).

**Figure 3.7** Hepatic expression of genes engaged in bile salt synthesis. mRNA levels of Cyp7a1 (A), Cyp7b1 (B), Cyp27a1 (C), and Cyp8b1 (D) were determined by RT-qPCR. Median and left lateral liver segments were assessed in rats \( (n=7-8 \text{ per group}) \) that were sham-operated, exposed to lipopolysaccharides (LPS) or 70\% hepatic inflow obstruction during 30 minutes (IR), or a combination of the latter two (IR+LPS) followed by reperfusion for 6 hours. Data are depicted using box and whisker plots showing median, minimum and maximum values. Statistical significance was tested using a Kruskal-Wallis test with Dunn’s correction for multiple comparisons, \( *p<0.05, ** p<0.01 \text{ ***, } p<0.001 \).

Induction of inflammatory response following 6 hours of reperfusion

To determine the inflammatory response of the liver and bile ducts to LPS administration, cytokines in liver tissue, serum and bile were examined (Figure 3.8, Suppl. Figure S3.5).
Figure 3.8  Hepatic expression of genes related to inflammation, and cytokine concentrations in serum and bile. Hepatic mRNA expression of Il-6, Icam-1, Tnf-α, and Mcp-1 in the median and left lateral liver segments was assessed using real-time quantitative polymerase chain reaction. Cytokine concentrations of Il-6, Icam-1, Tnf-α and Mcp-1 in serum and bile (pg/ml). Groups of rats (n=7-8) were sham-operated, exposed to lipopolysaccharides (LPS) or 70% hepatic inflow obstruction during 30 minutes (IR), or a combination of the latter two (IR+LPS) followed by reperfusion for 6 hours. Data are depicted using box and whisker plots showing median, minimum and maximum values. Statistical significance was tested using a Kruskal-Wallis test with Dunn’s correction for multiple comparisons, *p<0.05, ** p<0.01, *** p<0.001.
Following IP administration of LPS, serum concentrations of Icam-1, Tnf-α and Mcp-1 were significantly increased. Serum IL-6 levels were not significantly different between groups. In the liver, IR did not induce an inflammatory response whereas in the LPS groups (LPS and IR+LPS) IL-6, Icam-1, Tnf-α and Mcp-1 gene expression was significantly higher compared to the sham and IR groups. As cholangiocytes themselves are capable of producing cytokines such as Mcp-1, IL-6 and Tnf-α, their levels in bile were analyzed. Biliary Mcp-1 concentrations were significantly higher in the LPS and IR+LPS groups compared to sham (LPS +8.3 fold, IR+LPS +10.3 fold, p=0.03 and p<0.001). Biliary Icam-1 and IL-6 showed an inverse pattern compared to serum and mRNA expression levels with significantly decreased concentrations in the LPS and LPS+IR group. It however needs to be noted that IL-6 levels were overall very low in bile. The concentrations of Timp-1 protein in bile was found to be significantly increased in the LPS groups, matching hepatic gene expression levels (Suppl. Figure S3.5).

Discussion

The present study demonstrates that endotoxins, in the form of intra-peritoneal administered LPS, are capable of inducing small bile duct injury within 6 hours of exposure in rats. This occurred independently of hepatocellular injury. LPS administration furthermore disturbed the function of the BBB and impaired bile salt clearance in the groups that developed biliary injury.

In the current study, no evidence of biliary injury was found in the IR group following six hours of reperfusion, although increased levels of ALT were observed at that time point. This is in conflict with existing literature reporting that cholangiocytes are more vulnerable to reperfusion injury than hepatocytes. Furthermore, prolonged warm ischemia is a known risk factor for the development of ITBS. The hepatocellular injury in the current study however might have been transient as the serum half-life of ALT is 4.5 to 8 hours. This was further supported by the decrease in serum ALT levels between the one and six hour time point. Utilizing a longer duration of ischemia for this experiment may have resulted in biliary injury but the clamp time used was chosen to align with clinical DCD organ procurement. However, rats respond differently to IR injury than humans as they do not display any evidence of biliary injury on histology following exposure to 30 minutes of warm and three hours of cold ischemia and in future experiments, varying clamp times may need to be included. Since endotoxic shock can be a result of systemic exposure to LPS, it might be argued that the biliary damage seen in the LPS treated groups is the result of ischemic biliary injury due to hypotension (25) although we intended to explore a direct effect of LPS on cholangiocytes. For this reason the dose of endotoxin used in our experiments was...
an order of magnitude lower than the commonly used intra-peritoneal dose of 10-30 mg/kg that induces shock.\textsuperscript{25} Caraceni et al. have shown previously that 1 mg/kg LPS alone did not induce a significant drop in systolic blood pressure or liver perfusion.\textsuperscript{8} It was also considered that a lower dose might be more clinically relevant. In the present study, HRP was used to assess the permeability of the BBB in vivo. HRP appears in bile via two distinct routes, trans- and para-cellular, the latter being the fastest across tight junctions (26). More HRP appeared in bile in the LPS groups suggesting a loss of function of the tight junction barrier. This was further supported by almost undetectable expression of Claudin-1 and Claudin-3, two important components of tight junctions. ZO-1 staining showed marked disruption of tight junction morphology with an irregular staining pattern in animals that developed severe biliary injury. Endotoxins were found to disrupt tight junctions in cell culture experiments.\textsuperscript{10} However to the best of our knowledge, the present study is the first report of BBB disruption in an animal model of endotoxemia.

When the BBB is impaired, bile can leak back into the portal area resulting in clinical signs of cholestasis.\textsuperscript{27} Even though bile flow was not significantly impaired in this model, LPS was found to increase the circulating levels of bilirubin and bile salts and to lower the hepatic expression of Ntcp and Mrp2. The impaired tight junction function could be an explanation for elevated serum bile salts. Alternatively, the latter may be due to LPS-mediated repression of bile salt uptake via Ntcp.\textsuperscript{28} This study did not reveal an alteration in biliary bile salt/phospholipid ratio or an increased proportion of hydrophobic/toxic bile acids. This could partially be explained by the use of rats in our experiments since they have less toxic bile compared to humans.\textsuperscript{18} Additionally, our injury model was not extreme, with a short duration of IR, a low dose of LPS and a short follow up duration.

The exact mechanisms by which LPS induces biliary injury in our model remain to be determined. Kupffer cells have previously been shown to play a pivotal role in the development of LPS-enhanced preservation injury and endotoxemia-induced cholestasis, as their depletion from the liver was found to reduce the injury.\textsuperscript{7,28} Cholangiocytes themselves however also participate in the innate and adaptive immune response. They express toll like receptor 4 (TLR-4) and they produce cytokines such as IL-6 and TNF-\alpha in response to LPS.\textsuperscript{29} This TLR mediated immune response not only aids in the defence against pathogens, but it has also been implicated in the pathophysiology of PSC, primary biliary cholangitis and cystic fibrosis-associated cholangiopathies.\textsuperscript{29,32} In the present study, cytokines were detected in bile and their levels were altered by LPS administration. This suggests that the local inflammatory milieu contributes to the development of biliary injury.

Previous studies have linked immunological factors with the development of ITBS, especially when occurring in the periphery of the liver.\textsuperscript{13,33} Furthermore, a study by Friedrich et al. identified that CD14/TLR-4 signaling, a critical element in the innate immune response to factors such as LPS, played a pivotal role in ITBS formation.\textsuperscript{34} In
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the current study we found that, after 6 hours of reperfusion, the biliary Mcp-1 protein concentration was significantly increased upon LPS administration and this chemokine has previously been linked to myofibroblastic transformation and differentiation of portal myofibroblasts.\(^{35}\) In addition, the concentrations of Timp-1 protein in bile and hepatic gene expression were significantly elevated in the LPS groups, which could suggest the initiation of a fibrotic response.\(^{36}\) As with any experimental animal study, though, there are potential design limitations that could affect the interpretation of results and a single set of experiments cannot address all the relevant questions. The difficulty of replicating human ischemic injury conditions in rodents has been alluded to above, as has the choice of endotoxin dose. In the present study only short-term effects were examined and a more complex study will be needed to determine the occurrence of stricture formation over time.

This study has indicated that endotoxins, even at a low concentration of 1 mg/kg, are potent inducers of biliary injury. It might be the case that DCD organ procurement exposes donor livers to higher levels of endotoxins compared to DBD donors due to the period of shock prior to the start of cold perfusion, although human data supporting this is lacking.\(^{37}\) Our studies, albeit only using a short duration experimental protocol, indicate that it may be worthwhile exploring endotoxin exposure during human organ retrieval and its effect on the development of ITBS. If endotoxin is implicated in ITBS, then it might be possible to limit endotoxin exposure during organ procurement by ligating or venting the portal vein.
References

20. The Laboratory Rat. Mark A. Suckow SHW, Craig L. Franklin, editor: Elsevier Inc; 2006.
# Supplementary material

## Table S3.1 Prime sequences used for real time RT-PCR.

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Table S3.3 Large Bile Duct Injury.

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* From some animals insufficient hilar liver tissue was available to perform the analysis. Groups of rats were sham-operated, exposed to lipopolysaccharides (LPS) or 70% hepatic inflow obstruction during 30 minutes (IR), or a combination of the latter two (IR+LPS) followed by reperfusion for 6 hours. Data is shown as number (%). Statistical significance was tested using a Fishers exact test. \( P < 0.05 \) was considered significant.

Figure S3.1 Lactate dehydrogenase in bile following 1 hour of reperfusion. Activity of lactate dehydrogenase in bile (U/L) was measured as biomarker for biliary injury. Groups of rats \((n=7-8)\) were sham-operated, exposed to lipopolysaccharides (LPS) or 70% hepatic inflow obstruction during 30 minutes (IR), or a combination of the latter two (IR+LPS) followed by reperfusion for 1 hour. Data are depicted as box and whisker plots showing median, minimum and maximum values. Statistical significance was tested using a Kruskal-Wallis test with Dunn’s correction for multiple comparisons, * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \).
Figure S3.2 mRNA expression of bile salt and organic acid transporters following 6 hours of reperfusion. Hepatic mRNA expression levels of \textit{Mdr2}, \textit{Mrp2} and \textit{Bsep} in the median and left lateral liver segments following 6 hours of reperfusion assessed using real-time quantitative polymerase chain reaction. Groups of rats (n=7-8) were sham-operated, exposed to lipopolysaccharides (LPS) or 70% hepatic inflow obstruction during 30 minutes (IR), or a combination of the latter two (IR+LPS) followed by reperfusion for 6 hours. Data are depicted as box and whisker plots showing median, minimum and maximum values. Statistical significance was tested using a Kruskal-Wallis test with Dunn's correction for multiple comparisons, *p<0.05, ** p<0.01 ***, p<0.001.
Low dose lipopolysaccharide causes biliary injury by blood biliary barrier impairment

Figure S3.3 Expression of inflammatory genes in the non-ischaemic lobes following 6 hours of reperfusion. Hepatic mRNA expression of Il-6, Tnf-α, Mcp-1, Icam-1, Il-1 beta and Il-10 in the median and left lateral liver segments as well as caudate lobe assessed using real-time quantitative polymerase chain reaction. Data are depicted as box and whisker plots showing median, minimum and maximum values. Groups of rats (n = 7-8) were sham-operated, exposed to lipopolysaccharides (LPS) or 70% hepatic inflow obstruction during 30 minutes (IR), or a combination of the latter two (IR+LPS) followed by reperfusion for 6 hours. From the IR and IR+LPS animals, tissue was collected from both the ischaemic and non-ischaemic lobes (n = 6). Statistical significance was tested using a Kruskal-Wallis test with Dunn’s correction for multiple comparisons comparing Sham with non-ischaemic lobe IR and LPS with non-ischaemic lobe IR+LPS, P < 0.05 was considered significant.
Figure 53.4 Expression of genes engaged in bile salt synthesis and transport, and those encoding for tight junction proteins in the non-ischaemic lobes following 6 hours of reperfusion. Hepatic mRNA expression of Cyp7a1, Cyp7b1, Cyp8b1, Cyp27a1, Ntcp, Mrp2, Bsep, Mdr2, Claudin 1, Claudin 3, and Zo-1 in the median and left lateral liver segments as well as caudate lobe assessed using real-time quantitative polymerase chain reaction. Groups of rats (n = 7-8) were sham-operated, exposed to lipopolysaccharides (LPS) or 70% hepatic inflow obstruction during 30 minutes (IR), or a combination of the latter two (IR+LPS) followed by reperfusion for 6 hours. From the IR and IR+LPS animals, tissue was collected from both the ischaemic and non-ischaemic lobes (n = 6). Data are depicted as box and whisker plots showing median, minimum and maximum values. Statistical significance was tested using a Kruskal-Wallis test with Dunn’s correction for multiple comparisons comparing Sham with non-ischaemic lobe IR and LPS with non-ischaemic lobe IR+LPS, P < 0.05 was considered significant.
Low dose lipopolysaccharide causes biliary injury by blood biliary barrier impairment

Table S3.4  Bile salt composition following 6 hours of reperfusion (μmol/L).

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>LPS</th>
<th>IR</th>
<th>IR+LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ursodeoxycholic acid</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Glycoursodeoxycholic acid</td>
<td>172 (160-232)</td>
<td>171.0 (131-270)</td>
<td>160.0 (109-176)</td>
<td>111.5 (104-171)</td>
</tr>
<tr>
<td>Tauroursodeoxycholic acid</td>
<td>120 (86-181)</td>
<td>95 (88-99)</td>
<td>117 (107-139)</td>
<td>6457 (92)</td>
</tr>
<tr>
<td>Cholic acid</td>
<td>656 (493– 759)</td>
<td>574 (289-769)</td>
<td>604.0 (332-778)</td>
<td>535.5 (418 – 820)</td>
</tr>
<tr>
<td>Glycocholic acid</td>
<td>4945 (4168-5380)</td>
<td>3890 (3060-5750)</td>
<td>4310 (3320-4920)</td>
<td>3020 (2665-3630)*</td>
</tr>
<tr>
<td>Taurocholic acid</td>
<td>2165 (1628 – 2520)</td>
<td>2390 (2200-3350)</td>
<td>3380 (2130-3740)</td>
<td>2275 (1358-3525)</td>
</tr>
<tr>
<td>Chenodeoxycholic acid</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Glycochenodeoxycholic acid</td>
<td>1060 (812.8–1373)</td>
<td>816 (540-945)</td>
<td>781 (611-1140)</td>
<td>485 (354-960)*</td>
</tr>
<tr>
<td>Taurochenodeoxycholic acid</td>
<td>535 (438-725)</td>
<td>464 (355-591)</td>
<td>644 (618-740)</td>
<td>322 (271-425)*</td>
</tr>
<tr>
<td>Deoxycholic acid</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Glycodeoxycholic acid</td>
<td>398(269-621)</td>
<td>241.0 (148-281)</td>
<td>463 (374-514)</td>
<td>229 (144-282)</td>
</tr>
<tr>
<td>Taurodeoxycholic acid</td>
<td>164 (118-336)</td>
<td>133 (82-218)</td>
<td>295 (181-512)</td>
<td>107 (89-285)</td>
</tr>
<tr>
<td>Lithocholic acid</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Glycolithocholic acid</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Taurolithocholic acid</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Alpha-Muricholic acid</td>
<td>102 (85-116)</td>
<td>109(83-185)</td>
<td>98 (58-173)</td>
<td>113 (81-193)</td>
</tr>
<tr>
<td>Beta-Muricholic acid</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Hyodeoxycholic acid</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Glycolithocholic acid</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Tauro-α-Muricholic acid</td>
<td>745 (543-1021)</td>
<td>823 (665-1020)</td>
<td>989 (850-1090)</td>
<td>579 (501-757)</td>
</tr>
<tr>
<td>Tauro-β-Muricholic acid</td>
<td>549 (379-1068)</td>
<td>862(707-1180)</td>
<td>626 (557-902)</td>
<td>728 (498-906)</td>
</tr>
</tbody>
</table>

Data is shown as median (interquartile range). Groups of rats (n=7-8) were sham-operated, exposed to lipopolysaccharides (LPS) or 70% hepatic inflow obstruction during 30 minutes (IR), or a combination of the latter two (IR+LPS) followed by reperfusion for 1 and 6 hours. Statistical significance was tested using a Kruskal-Wallis test with Dunn’s correction for multiple comparisons, p<0.05 was considered significant. α: p<0.05 compared to sham, β: p<0.05 compared to IR, N.D.: not detected. Taurohyodeoxycholic acid was not determined due to a technical issue.
Figure S3.5  Expression of genes related to inflammation, and cytokines concentrations in serum and bile following 6 hours of reperfusion. Hepatic mRNA expression of Timp-1, Il-1 beta and Il-10 in the median and left lateral liver segments assessed using real-time quantitative polymerase chain reaction. Serum concentrations of Il-1 beta, Il-10, L-selectin and Ifn-γ (pg/ml). Biliary concentrations of Timp-1, Il-10 and L-selectin (pg/ml). Biliary concentrations of TIMP1, Il-10 and L-selectin (pg/ml). Data are depicted as box and whisker plots showing median, minimum and maximum values. Groups of rats (n=7-8) were sham-operated, exposed to lipopolysaccharides (LPS) or 70% hepatic inflow obstruction during 30 minutes (IR), or a combination of the latter two (IR+LPS) followed by reperfusion for 1 and 6 hours. Statistical significance was tested using a Kruskal-Wallis test with Dunn’s correction for multiple comparisons, *p<0.05, **p<0.01 ***p<0.001.
Experimental protocol for the assessment of bile salt composition using an Ultra High Performance Liquid Chromatography system

Chemicals

*Sigma-Aldrich (St. Louis, MO)*

Cholic acid (CA), taurocholic acid (TCA), glycocholic acid (GCA), deoxycholic acid (DCA), taurodeoxycholic acid (TDCA), glycodeloxycholic acid (GDCA), chenodeoxycholic acid (CDCA), taurochenodeoxycholic acid (TCDCA), glycochenodeoxycholic acid (GCDCA), lithocholic acid (LCA), taurolithocholic acid (TLCA), ursodeoxycholic acid (UDCA) and glycoursoxycholic acid (GUDCA)

*Makaira Ltd (London, England)*

Glycolithocholic acid (GLCA)

*Merck Millipore (Billerica, MA)*

Tauroursodeoxycholic acid (TUDCA)

*Steraloids Inc (Newport, RI)*

Alpha-muricholic acid (α-MCA), beta-muricholic acid (β-MCA), omega-muricholic acid (ω-MCA), tauro alpha-muricholic acid (Tα-MCA), tauro beta-muricholic acid (Tβ-MCA), hyodeoxycholic acid (HDCA), taurohyodeoxycholic acid (THDCA) and glycohyodeoxycholic acid (GHDCa)

*CDN Isotopes (Pointe-Claire, Quebec, Canada)*

D4-cholic acid (D4-CA), D4-chenodeoxycholic acid (D4-CDCA), D4-glycochenodeoxycholic acid (D4-GCDCA) and D4-glycocholic acid (D4-GCA)

*Medical Isotopes (Pelham, NH)*

D4-taurochenodeoxycholic acid (D4-TCDCA) and D4-taurocholic acid (D4-TCA)

Sample preparation

Bile samples were diluted in ultrapure water (1:1000) and subsequently homogenized. One quality control standard plasma sample was included for every 10 tested samples. 25 µL diluted bile was transferred into a clean tube and 250 µL of internal standard solution was added. The sample was vortexed for 60 s and centrifuged at
15800 x g for 10 minutes. The supernatant was then poured into a clean glass tube and the fluid was evaporated under nitrogen at 40°C. If samples were not measured immediately, they were stored in this stage at -20°C.

Before measurement, samples were reconstituted in 200 µL 50 % methanol in water, vortexted for 60 s and centrifuged for 3 min at 1800 x g. The supernatant was subsequently transferred into a 0.2 µm spin-filter and centrifuged at 2000 x g for 10 min. After filtering, the samples were transferred into LC-MS vials and analysed (10 µL injection volume).

Instrumentation
For the quantitative determination of bile acids we used a Nexera X2 Ultra High Performance Liquid Chromatography system (SHIMADZU, Kyoto, Japan), coupled to a SCIEX QTRAP 4500 MD triple quadruple mass spectrometer (SCIEX, Framingham, MA, USA) (UHPLC-MS/MS). The LC-MS/MS system is controlled by Analyst MD 1.6.2 software.

Liquid chromatographic and mass spectrometric conditions:
Bile acids were separated with an ACQUITY UPLC BEH C18 Column (1.7 µm x 2.1 x 100 mm) equipped with an ACQUITY UPLC BEH C18 VanGuard Pre-Column (1.7 µm x 2.1 x 5 mm), (Waters, Milford, MA, USA). Separation was achieved in 28 minutes using 10 mM ammonium acetate in 20% acetonitrile (mobile phase A) and 10 mM ammonium acetate in 80% acetonitrile (mobile phase B), total flow rate: 0.4 ml/min.

The mass spectrometer (MS) parameters such as temperature, ion spray voltage, gas pressures, etc., were optimised by infusing the bile acids and internal standards (IS) in a 50% MeOH solution via the internal syringe pump of the mass spectrometer. Bile acids with the same molar mass were infused as a mixture. All bile acids and internal standards were detected in negative mode with the mass spectrometer settings shown in Table S3.6. The multiple reaction monitoring (MRM) transitions for each bile acid and internal standard, as well as their optimum MS parameters such as collision energy (CE), de-clustering potential (DP), entrance potential (EP) and collision cell exit potential (CXP) are shown in Table S3.7.
Low dose lipopolysaccharide causes biliary injury by blood biliary barrier impairment

Table S3.6  Mass spectrometer source settings API4500.

<table>
<thead>
<tr>
<th>Detection mode</th>
<th>MRM, negative mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution Q1</td>
<td>Unit (scan speed 10 Da/s)</td>
</tr>
<tr>
<td>Resolution Q3</td>
<td>Unit (scan speed 10 Da/s)</td>
</tr>
<tr>
<td>IonSpray Voltage (IS)</td>
<td>-4500</td>
</tr>
<tr>
<td>Temperature</td>
<td>500 °C</td>
</tr>
<tr>
<td>Curtain gas (CUR)</td>
<td>40</td>
</tr>
<tr>
<td>CAD gas (CAD)</td>
<td>-3</td>
</tr>
<tr>
<td>Gas 1 (GS1)</td>
<td>40</td>
</tr>
<tr>
<td>Gas 2 (GS2)</td>
<td>70</td>
</tr>
</tbody>
</table>

Table S3.7  MRM settings API4500.

<table>
<thead>
<tr>
<th>Component</th>
<th>Q1 mass (Da)</th>
<th>Q3 mass (Da)</th>
<th>Declustering potential (DP)</th>
<th>Collisions energy (CE)</th>
<th>Entrance potential (EP)</th>
<th>Collision cell exit potential (CXP)</th>
<th>Dwell (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDCA, HDCA, CDCA, DCA</td>
<td>391.3</td>
<td>391.3</td>
<td>-140.0</td>
<td>-12.0</td>
<td>-8.0</td>
<td>-10.0</td>
<td>50.0</td>
</tr>
<tr>
<td>CA, MCA’s</td>
<td>407.3</td>
<td>407.3</td>
<td>-120.0</td>
<td>-12.0</td>
<td>-8.0</td>
<td>-10.0</td>
<td>50.0</td>
</tr>
<tr>
<td>GUDCA, GHDC, GCDCA, GDCA</td>
<td>448.4</td>
<td>74.0</td>
<td>-120.0</td>
<td>-80.0</td>
<td>-8.0</td>
<td>-10.0</td>
<td>50.0</td>
</tr>
<tr>
<td>GCA</td>
<td>464.2</td>
<td>74.0</td>
<td>-120.0</td>
<td>-85.0</td>
<td>-8.0</td>
<td>-10.0</td>
<td>50.0</td>
</tr>
<tr>
<td>TUDCA, THDCA, TCDC, TDCA</td>
<td>498.3</td>
<td>80.0</td>
<td>-120.0</td>
<td>-110.0</td>
<td>-8.0</td>
<td>-10.0</td>
<td>50.0</td>
</tr>
<tr>
<td>TCA, T-MCA’s</td>
<td>514.3</td>
<td>79.9</td>
<td>-120.0</td>
<td>-115.0</td>
<td>-8.0</td>
<td>-10.0</td>
<td>50.0</td>
</tr>
<tr>
<td>LCA</td>
<td>375.2</td>
<td>375.2</td>
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<td>-25.0</td>
<td>-8.0</td>
<td>-10.0</td>
<td>50.0</td>
</tr>
<tr>
<td>GLCA</td>
<td>432.2</td>
<td>74.0</td>
<td>-120.0</td>
<td>-75.0</td>
<td>-8.0</td>
<td>-10.0</td>
<td>50.0</td>
</tr>
<tr>
<td>TLCA</td>
<td>482.2</td>
<td>79.9</td>
<td>-120.0</td>
<td>-110.0</td>
<td>-8.0</td>
<td>-10.0</td>
<td>50.0</td>
</tr>
<tr>
<td>D4-CA</td>
<td>411.3</td>
<td>411.3</td>
<td>-120.0</td>
<td>-12.0</td>
<td>-8.0</td>
<td>-10.0</td>
<td>50.0</td>
</tr>
<tr>
<td>D4-GCA</td>
<td>468.2</td>
<td>74.0</td>
<td>-120.0</td>
<td>-85.0</td>
<td>-8.0</td>
<td>-10.0</td>
<td>50.0</td>
</tr>
<tr>
<td>D4-TCA</td>
<td>518.3</td>
<td>79.9</td>
<td>-120.0</td>
<td>-115.0</td>
<td>-8.0</td>
<td>-10.0</td>
<td>50.0</td>
</tr>
<tr>
<td>D4-GCDCA</td>
<td>452.4</td>
<td>74.0</td>
<td>-120.0</td>
<td>-80.0</td>
<td>-8.0</td>
<td>-10.0</td>
<td>50.0</td>
</tr>
<tr>
<td>D4-TCDCA</td>
<td>502.3</td>
<td>80.0</td>
<td>-120.0</td>
<td>-110.0</td>
<td>-8.0</td>
<td>-10.0</td>
<td>50.0</td>
</tr>
<tr>
<td>D4-CDCA</td>
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<td>395.3</td>
<td>-120.0</td>
<td>-12.0</td>
<td>-8.0</td>
<td>-10.0</td>
<td>50.0</td>
</tr>
</tbody>
</table>
Chapter 4

The role of macrophages in the development of biliary injury in a lipopolysaccharide enhanced hepatic ischaemia-reperfusion model

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FG Schaap
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SWM Olde Damink
DHG Crawford
CHC Dejong
J Fawcett

Submitted
Abstract

Background
Endotoxins, in the form of lipopolysaccharides (LPS), are potent inducers of biliary injury. However, the mechanism by which this develops remains unclear. We hypothesised that macrophages are pivotal in the development of LPS-induced biliary injury and no injury would occur in their absence.

Method
Clodronate liposomes were used to deplete macrophages from the liver. Forty-eight rats were equally divided across six study groups: sham operation (sham), liposome treatment and sham operation (liposomes+sham), 1 mg/kg LPS i.p. (LPS), liposome treatment and LPS administration (liposomes+LPS), hepatic ischaemia-reperfusion injury with LPS administration (IRI+LPS) and liposome treatment followed by IRI+LPS (liposomes+IRI+LPS). Following six hours of reperfusion, blood, bile, and liver tissue was collected for further analysis. Small bile duct injury was assessed, serum liver tests were performed and bile composition was evaluated. The permeability of the blood biliary barrier (BBB) was assessed using horseradish peroxidase (HRP).

Key findings
The presence of hepatic macrophages was reduced by 90% in the liposome pre-treated LPS and IRI+LPS groups ($P < 0.001$). Despite macrophage depletion, severe small bile duct injury occurred in 4 (50%) animals of the liposomes+IRI+LPS group compared to 6 (75%) animals of the liposomes+LPS group. Furthermore, BBB impairment persisted with leakage of HRP in bile. LPS-induced elevation of the chemokine Mcp-1 in bile was unaffected by macrophage depletion.

Conclusions
The depletion of macrophages did not prevent the development of biliary injury following LPS or LPS-enhanced IRI. Cholangiocyte rather than macrophage activation may underlie this injury.
Introduction

Ischaemic-type biliary stricture (ITBS) formation remains one of the most troublesome complications following liver transplantation using livers donated after circulatory death (DCD).\(^1\),\(^2\) During the DCD retrieval process, ischaemia of intra-abdominal organs such as the gut occurs, and this may result in the release of endotoxins into the portal circulation.\(^3\) We previously showed that endotoxins, in the form of lipopolysaccharides (LPS), are potent inducers of biliary injury.\(^4\) Administration of LPS in a hepatic ischaemia-reperfusion injury (IRI) model resulted in severe bile duct injury, which was characterised by degenerative changes of cholangiocytes and ductular proliferation. The injury was associated with the development of the clinical features of cholestasis and increased leakage of macromolecules across the blood-biliary-barrier (BBB).\(^4\) In the present study, we further explored the mechanisms by which LPS induced biliary injury in this model by addressing the involvement of liver-resident macrophages.

Kupffer cells respond to LPS via activation of the Toll-like receptor-4 (TLR-4) pathway.\(^5\) Kupffer cell activation plays a pivotal role in the development of IRI.\(^6\) Upon activation by endogenous damage-associated and/or pathogen-associated molecular pattern (DAMP/PAMP) molecules, Kupffer cells produce reactive oxygen species and pro-inflammatory cytokines, which result in the recruitment of neutrophils and the development of tissue damage.\(^7\) Through enhanced Kupffer cell activation, endotoxaemia can substantially aggravate IRI, as was observed during organ retrieval and liver transplantation.\(^7\),\(^9\),\(^10\) Moreover, primary graft non-function following DCD liver transplantation was associated with a higher degree of Kupffer cell activation.\(^11\) In animal models, IRI and LPS-enhanced IRI was ameliorated by depleting Kupffer cells as evidenced by reduced hepatocellular damage, increased portal flow, and decreased recipient mortality.\(^8\),\(^12\) It is currently unclear whether macrophages have a role in the development of LPS-induced biliary injury.

The role of cholangiocytes in immune responses is increasingly recognised.\(^13\) They express various TLRs, and upon stimulation they release pro-inflammatory cytokines such as tumor necrosis factor-\(\alpha\) (Tnf-\(\alpha\)) and interleukin-6 (II-6) in the bloodstream and biliary compartment.\(^13\),\(^14\) Additionally, interferon-\(\gamma\) (IFNy) and Tnf-\(\alpha\) increase the expression of Tlr4 on cholangiocytes in vitro augmenting their response to LPS.\(^15\) Lastly, cholangiocytes can act as antigen-presenting cells as they express major histocompatibility complex II (MHC II) on their surface.\(^16\) Collectively, these data suggest that LPS-induced damage of the cholangiocytes might be a direct effect or mediated via Kupffer cell response.

The aim of the current study was to explore the role of macrophages in the development of biliary injury resulting from LPS administration in a hepatic IRI model.
We hypothesised that in the absence of macrophages, the BBB would remain intact and that biliary injury would be ameliorated.

Methods

Animals and experimental groups

Male Sprague-Dawley rats (250 g; Animal Recourses Centre, Perth, Australia) were used for this study. Procedures were in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes and approved by the Animal Ethics Committee of the University of Queensland.

For the purpose of the study, forty-eight animals were equally divided across six study groups:

1. Laparotomy without any additional intervention (sham);
2. Administration of 1 mg/kg LPS in the peritoneal cavity (LPS);
3. 30 minutes of liver ischaemia and simultaneous administration of LPS in the peritoneal cavity (IRI+LPS);
4. Pre-treatment with clodronate liposomes followed by laparotomy (liposomes+sham);
5. Pre-treatment with clodronate liposomes followed by administration of LPS in the peritoneal cavity (liposomes+LPS); and
6. Pre-treatment with clodronate liposomes followed by 30 minutes of liver ischaemia and simultaneous administration of LPS in the peritoneal cavity (liposomes+IRI+LPS).

Macrophage depletion and surgical procedure

Clodronate encapsulated in liposomes (5 mg clodronate per milliliter, www.clodronateliposomes.org, Amsterdam, the Netherlands) was used to deplete macrophages. Animals in groups 4, 5 and 6 were injected with clodronate liposomes (0.5 ml/100 grams body weight) in the tail vein 48 hours prior to surgical intervention. No PBS or saline liposomes were administered to animals in group 1, 2 and 3 as they might disable the phagocytosis function of macrophages by saturation.

Midline laparotomy was performed under general anaesthesia with isoflurane and the common bile duct, hepatic artery and portal vein of the left lateral and medial lobes were identified. A vascular clamp (BH030R, B Braun, Bethlehem, PA, USA) was then placed across these structures to induce ischaemia to approximately 70% of the liver (IRI groups). Simultaneously, vehicle (0.9% sterile saline) or 1 mg/kg LPS from Escherichia coli serotype 0111:B4 (L3012, Sigma-Aldrich, St. Louis, MO, USA; in sterile saline) was administered in the peritoneal cavity. Following 30 minutes of ischaemia, the clamp was removed and the animals were allowed to recover.

Following six hours of reperfusion, re-laparotomy was performed and a cannula was inserted in the common bile duct to allow for bile collection. Animals were
The role of macrophages in the development of biliary injury

subsequently euthanised by exsanguination, and liver tissue from the left lateral and median lobes (subjected to ischaemia in the IRI groups) was collected for RNA extraction and (immuno) histological evaluation.

Immunohistochemical staining for mature Kupffer cells

Paraffin-embedded liver sections stained with a monoclonal antibody against CD163 (ED-2, 1:300, Serotec, Oxford, UK) were used to visualise the presence of mature macrophages. The average number of positive cells per field were counted in five non-overlapping fields at 200 x magnification using ImageJ image analysis software.19

Serum and bile analysis

Serum alanine transaminase (ALT), aspartate transaminase (AST), γ-glutamyl transferase (GGT), alkaline phosphatase (ALP), and bilirubin, as well as lactate dehydrogenase (LDH) in bile, were assessed using commercially available kits (Bioo Scientific Corporation, Austin, TX, USA). Total bile salt concentration in serum and bile, as well as phospholipid concentration in bile, were measured enzymatically using a kit according to the manufacturer's instructions (#80460, Crystal Chem, Inc., Chicago, IL, USA and #433-36201, Wako, Osaka, Japan).

Blood-biliary-barrier integrity assessment

Horseradish peroxidase (HRP, Peroxidase from horseradish type II, P8250, Sigma-Aldrich, St. Louis, MO, USA) was used to assess the permeability of the BBB in vivo as previously described.20 Two hundred microliters of HRP (5000 IU/mL in sterile water), was injected in the inferior vena cava of each animal. Bile was subsequently collected for 10 minutes to assess para-cellular transport across tight junctions.21 A commercially available kit (#A22188, Thermo Fisher Scientific, Waltham, MA, USA) was used to determine activity of HRP in bile.

RNA extraction and real-time polymerase chain reaction

Total RNA was extracted from liver tissue using Trisure and cDNA was synthesised using a kit (SensiFAST, Bioline, Taunton, MA, USA). Real-time quantitative polymerase chain reaction (RT-qPCR) was performed on a Viia7 Real-time PCR system (Invitrogen, Carlsbad, CA, USA) using Sybr Green chemistry (Bioline, Taunton, MA, USA). Normalisation was performed using the geometric mean of glyceraldehyde phosphate dehydrogenase, β2-microglobulin and basic transcription factor 3 mRNA expression. The primer nucleotide sequences and accession numbers can be found in Supplementary Table S4.1.
Histological evaluation

Paraffin-embedded liver sections, stained with haematoxylin and eosin, were used for the semi-quantitative assessment of small bile duct injury. Depending on the size of the ducts,\(^2\) a small bile duct injury severity score (SBDISS) was used as previously described by Cheng et al.\(^3\) The score was based on bile duct damage (adapted from the Banff criteria for acute rejection) and ductular proliferation, each of which scored from 0 (absent) to 3 (severe). An expert liver pathologist (CC) who was blinded to study group allocation performed the histological scoring.

Cytokine array on bile samples

Biliary concentrations of cytokines were determined to gain insight into the local inflammatory milieu surrounding cholangiocytes. Levels of intercellular adhesion molecule-1 (Icam-1), monocyte chemoattractant protein-1 (Mcp-1), tissue inhibitor metalloproteinase-1 (Timp-1), Il-6, interleukin–10 (Il-10) and L-selectin concentrations were determined using a multiplex ELISA array (QAR-CYT-2, RayBiotech, Norcross, GA, USA). Diluted bile samples were analysed according to the manufacturer’s instructions. Fluorescence intensity was determined and data analysis was performed using the Q-analyser (RayBiotech, Norcross, GA, USA).

Statistical analysis

GraphPad Prism 7 software (GraphPad, San Diego, CA, USA) was used for statistical analysis. Continuous variables are reported as median [interquartile range] and graphed using box plots. A Kruskal-Wallis test was performed to compare all experimental groups, and in case of significant outcome was followed by three pre-defined post-hoc comparisons with Dunn’s correction for multiple comparison to evaluate the effect of macrophage depletion: sham versus liposomes+sham, LPS versus liposomes+LPS and IRI+LPS versus liposomes+IRI+LPS. Categorical variables are reported as frequencies (%) and represented in tabular form. A Fisher’s exact test was performed to compare categorical data. Eight animals were studied per group unless stated otherwise and a \(p\)-value <0.05 was considered statistically significant.

Results

Effective depletion of Kupffer cells from the liver

Effectiveness of clodronate to deplete Kupffer cells was studied by CD163 immunohistochemistry (Figure 4.1). In the sham group there were 105 [90–128] CD163\(^+\) cells per field. In the LPS and IRI+LPS groups this increased to 158 [147–176]
The role of macrophages in the development of biliary injury

and 145 [130-170] per field. Following liposome pretreatment, the number of positive cells per field decreased with 84% (p=0.01), 90% p<0.001 and 89% (p<0.001), respectively. In addition, hepatic Tnf-α mRNA expression, induced by exposure to LPS or IRI+LPS, was normalised by macrophage depletion (Figure 4.2A). Mcp-1 expression, crucial for the recruitment of monocytes and macrophages to areas of inflammation and markedly induced following LPS administration, was comparable to controls when LPS groups were pretreated with clodronate liposomes (Figure 4.2B).

**Figure 4.1** Successful depletion of mature macrophages following clodronate liposome treatment. Groups of rats were pre-treated during 48 hours with vehicle or clodronate liposomes, followed by sham operation, administration of lipopolysaccharides (LPS), or a combination of LPS and 30 minutes warm hepatic ischaemia (IRI+LPS). Animals were sacrificed after 6 hours of reperfusion. Representative light microscopy images of liver sections (200x magnification) stained for CD163 are depicted for an animal receiving vehicle (A) or clodronate liposomes (B). Quantification of CD163+ cells per high-powered field (C). Data is graphed using box plots presenting median, minimum and maximum values. Statistical significance was evaluated using a Kruskal-Wallis test, and -if appropriate- three predefined post-hoc comparisons (sham versus liposomes+ sham, LPS versus liposomes+LPS and IRI+LPS versus liposomes+IRI+LPS). ** p<0.01, ****p<0.0001.
Figure 4.2  Decreased hepatic expression of genes related to inflammation following clodronate liposome treatment. Groups of rats were pre-treated during 48 hours with vehicle or clodronate liposomes, followed by sham operation, administration of lipopolysaccharides (LPS), or a combination of LPS and 30 minutes warm hepatic ischaemia (IRI+LPS). Animals were sacrificed after 6 hours of reperfusion, and hepatic mRNA expression was analysed (left lateral and median segments) using RTqPCR. Hepatic mRNA expression of Tnf-α (A) and Mcp-1 (B). Data is graphed using box plots presenting median, minimum and maximum values. Statistical significance was tested using Kruskal-Wallis multiple comparison test. Statistical significance was evaluated using a Kruskal-Wallis test, and -if appropriate- the three predefined post-hoc comparisons. ** p<0.01.

Macrophage depletion causes elevated liver tests

Serum ALT activity was not affected by macrophage depletion (Figure 4.3A). However, AST was significantly increased in the macrophage-depleted groups compared to their non-depleted controls (sham: p=0.001, LPS: p=0.03 and IRI+LPS p=0.003, Figure 4.3B). ALP and GGT were both significantly increased following macrophage depletion in the LPS-treated groups (Suppl. Data Figure S4.1). LPS-induced hyperbilirubinemia was ameliorated in the absence of macrophages (Figure 4.3C). On a transcriptional level, this was associated with a trend towards increased gene expression of ATP-binding cassette protein Mrp2, which facilitates the biliary secretion of glucuronidated bilirubin (Figure 4.3E). Following macrophage depletion, the serum elevation of bile salts tended to improve in the LPS group (p=0.06) but remained elevated in the IRI+LPS group (Figure 4.3D). The latter occurred despite a significant increase in all groups (p<0.01 for all comparisons, Figure 4.3F) of Na⁺-taurocholate co-transporting polypeptide (Ntcp), the transporter responsible for basolateral uptake of conjugated bile salts.
The role of macrophages in the development of biliary injury

Figure 4.3  Serum liver tests. Groups of rats were pre-treated during 48 hours with vehicle or clodronate liposomes, followed by sham operation, administration of lipopolysaccharides (LPS), or a combination of LPS and 30 minutes warm hepatic ischaemia (IRI+LPS). Animals were sacrificed after 6 hours of reperfusion and serum activity or level of A) alanine transaminase (ALT), B) aspartate transaminase (AST), C) bilirubin and D) bile salts (µmol/L) were determined. Furthermore, hepatic mRNA expression of Mrp2 (B) and Ntcp (C) was measured using real-time quantitative polymerase chain reaction. * p<0.05, **p<0.01.

Effective macrophage depletion did not result in reduced small bile duct injury

A small bile duct injury severity score (SBDISS) was calculated, and representative portal tract images are depicted in Figure 4.4A. None of the animals developed severe small bile duct injury in the sham groups. LPS administration induced severe small bile
duct injury (SBDISS>4) in 5 (62.5%) animals compared to 6 (75%) in the liposomes+LPS group. In the IRI+LPS group, severe small bile duct injury occurred in 5 (62.5%) animals compared to 4 (50%) in the liposomes+IRI+LPS group. Macrophage depletion did not alter LDH activity in bile, a biomarker for biliary injury, following LPS administration (Figure 4.4B). Although biliary LDH activity was slightly increased in the liposomes+sham group compared to sham controls, this did not reach significance ($p=0.09$).

![Image](image.png)

**Figure 4.4** Macrophage depletion did not ameliorate LPS induced biliary injury. A) Representative light microscopy images of portal tracts (magnification 200x) with arrow indicating bile ducts, PV: portal vein, $\heartsuit$ hepatic artery. B) Concentration of lactate dehydrogenase (LDH) in bile as a biomarker of biliary injury.

**Persistent impairment of the BBB following macrophage depletion**

In the sham group, macrophage depletion led to a significant increase in the output of HRP in bile suggesting increased BBB permeability (Figure 4.5A). Elevated biliary HRP activity was not affected by macrophage depletion in the LPS and IRI+LPS groups. We subsequently assessed gene expression levels of tight junction proteins *Claudin-1* and...
The role of macrophages in the development of biliary injury

Zonula occludens-1 (Zo-1) (Figure 4.5B and 4.5C). Claudin-1 expression remained low in the liposomes+LPS group but was significantly increased in the liposomes +IR+LPS group. The expression did not exceed those of the untreated sham controls (-1.4 fold versus Sham). Zo-1 expression was not altered in the absence of macrophages in any of the groups.

**Figure 4.5** Persistent impairment of the blood biliary barrier following depletion of macrophages. A) Units of Horseradish peroxidase (HRP) output in bile during a 10-minute collection period following intravenous injection of 1000 IU HRP. Hepatic mRNA expression of B) Claudin-1 and C) Zo-1 measured using RT-qPCR. * p<0.05

Macrophage depletion increased bile flow and total bile salt excretion

Bile flow was increased following macrophage depletion, reaching significance in the LPS group, and total bile salt and phospholipid output were increased in the LPS-treated groups (Figure 4.6A-C). This was accompanied by increased expression of the bile salt export pump (Bsep) in the LPS group (Figure 4.6D), but reduced levels of Mdr2, encoding the flippase that facilitates canalicular extraction of phospholipids, in both LPS-treated groups (Figure 4.6E).
Cytokine production in bile remains largely unaltered

The biliary concentration of several cytokines and chemokines were measured to determine the effect of macrophage depletion on the local inflammatory milieu surrounding cholangiocytes (Figure 4.7 and Supplementary Figure S4.2). Clodronate liposome treatment increased biliary Icam-1 protein in the LPS+IRI group to control levels (Figure 4.7A). Elevation of Mcp-1 in bile in the LPS groups, was not affected by macrophage depletion (Figure 4.7B). Timp-1 levels were elevated in the LPS-treated groups, and further increased upon macrophage depletion in the IRI+LPS group (Figure 4.7C).
The role of macrophages in the development of biliary injury

Discussion

In the current study, clodronate liposomes were used to deplete macrophages from the liver. The development of biliary injury, resulting from exposure to LPS or LPS-enhanced IRI, occurred independently of the presence of macrophages and impairment of the BBB persisted despite macrophage depletion. To our knowledge, this is the first study to explore the role of macrophages in the development of LPS-induced biliary injury.

In this study, CD163+ cells, a marker of mature macrophages, were largely depleted from the liver following treatment with liposomes. This observation was supported by a significant reduction in hepatic expression of pro-inflammatory cytokine Tnf-α and chemokine Mcp-1.

In the current study, serum elevations of AST, ALP and GGT were observed following clodronate liposome treatment. These findings differ from those previously reported.
Macrophage depletion from donor livers using gadolinium chloride was found to be beneficial for early graft function and increased survival in animal studies.\textsuperscript{12} It furthermore reduced hepatic LPS toxicity and endotoxin-aggravated IRI.\textsuperscript{8,25} A possible explanation for the increased serum liver enzymes in this study, could be impaired clearance of the enzymes, a role recently attributed to Kupffer cells.\textsuperscript{26} This was further supported by absence of histological evidence of hepatocellular injury in the liposomes+sham group (data not shown).

Despite strong evidence that macrophages play an important role in the development of IRI and LPS-enhanced IRI, this study failed to show improvement in biliary injury following macrophage depletion. To determine the role of cholangiocytes in injury development we measured the biliary concentrations of several cytokines to assess the local inflammatory milieu surrounding cholangiocytes. Biliary Mcp-1 and Timp-1 protein levels were most strongly increased in the LPS and IRI+LPS groups. The presence of Mcp-1 in bile was not affected by macrophage depletion, whereas Timp-1 was more abundant in the clodronate liposome-treated groups. These findings suggest that macrophages only have a small effect on local biliary inflammatory milieu, which could explain the persistence of biliary injury in our model. Especially since TLR-4 signalling and subsequent production of cytokines by cholangiocytes, has been implicated in the pathophysiology of biliary diseases such as primary sclerosing cholangitis.\textsuperscript{13}

Another possible explanation for the development of biliary injury in the absence of macrophages could be a direct toxic effect of LPS on cholangiocytes. Cells from the reticulo-endothelial system together with hepatocytes are responsible for clearance of agents such as LPS from the portal circulation, and result in secretion of LPS in bile.\textsuperscript{27,29} An accumulation of endotoxins has been observed in cholangiocytes of patients with primary biliary cholangitis and primary sclerosing cholangitis.\textsuperscript{30} Previous studies have shown that endotoxin clearance and secretion into bile can occur in the absence of macrophages, as hepatocytes are able to take up LPS.\textsuperscript{12,31} As cholangiocytes would remain exposed to LPS, this could explain why biliary injury developed in the macrophage-depleted groups.

The impairment of the BBB following exposure to LPS persisted in the absence of macrophages. Macrophages may play a role in maintaining the BBB as increased levels of HRP appeared in bile in the liposomes+sham group. Tight junctions are the main structural components of the BBB, and its permeability can be tested using macromolecules (e.g. HRP) that cross the barrier from the blood stream via the paracellular route.\textsuperscript{21} Endotoxins as well as individual (pro-inflammatory) cytokines have previously been found to impair BBB function.\textsuperscript{32,34} Even though serum cytokine concentrations are likely reduced following macrophage depletion, the production of cytokines by cholangiocytes was maintained, which could have affected the BBB function. Alternatively, the increased concentration of HRP in bile observed in the
liposomes+sham group could also be the result of reduced phagocytosis of HRP due to the absence of macrophages. Endotoxaemia can induce impaired bile salt clearance via suppression of canalicular bile salt export (via Bsep) and basolateral bile salt uptake (via Ntcp). In the present study, we found that LPS-induced Ntcp suppression was reversed by macrophage depletion. This supports previous reports that macrophages play a key role in regulating hepatocytic Ntcp expression and portal venous bile salt clearance. In the present study, we found that LPS‐induced Ntcp suppression was reversed by macrophage depletion. This supports previous reports that macrophages play a key role in regulating hepatocytic Ntcp expression and portal venous bile salt clearance. In conclusion, macrophage depletion did not prevent the development of biliary injury following LPS or LPS-enhanced IRI. Cholangiocytes can produce cytokines in a TLR4-dependent fashion, and results from this study suggest that these cells themselves, rather than macrophages, may be key players in LPS-induced biliary injury. Studies investigating agents such as TLR4 or TNF-alpha signalling inhibitors in preventing LPS-induced biliary injury could provide additional information regarding the aetiology of LPS-induced biliary injury.

The role of macrophages in the development of biliary injury
References


Supplementary material

Table S4.1  Primer sequences used for real time RT-PCR.

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Figure S4.1  Serum liver tests. A) Serum activity of alkaline phosphatase (U/L) and B) \(\gamma\)-glutamyl transferase (U/L). Groups of rats were pre-treated during 48 hours with vehicle or clodronate liposomes, followed by sham operation, administration of lipopolysaccharides (LPS), or a combination of LPS and 30 minutes warm hepatic ischaemia (IRI+LPS). Animals were sacrificed after 6 hours of reperfusion. Data is graphed using box plots presenting median, minimum and maximum values. Statistical significance was evaluated using a Kruskal-Wallis test, and -if appropriate- three predefined post-hoc comparisons (sham versus liposomes+ sham, LPS versus liposomes+LPS and IRI+LPS versus liposomes+IRI+LPS). * \(p<0.05\), ** \(p<0.01\).
Chapter 5

Comparable levels of inflammatory mediators in portal venous blood collected from organ donors donating after circulatory death and those donating after brain death

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Submitted
This chapter is embargoed at request
Chapter 7

Urea production during Normothermic Machine Perfusion- Price of Success?

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Abstract

Background
Normothermic machine perfusion (NMP) holds great promise as an ex-vivo organ maintenance system but many facets of liver physiology need to be considered in its establishment. We have successfully performed ex vivo normothermic oxygenated perfusion in 4 human donor livers rejected as unsuitable for transplantation. This manuscript describes one such perfusion where hyper osmolality in the perfusate from urea production and hyperglycaemia highlights the complexity of organ maintenance in NMP systems.

Method
Organ retrieval was performed in a standard fashion using University of Wisconsin solution. In addition, blood from the donor was collected and used as NMP perfusate. In the circuit, perfusate draining from the IVC was driven through a single centrifugal pump, then onwards through an oxygenator / heat exchanger before being split into a pressure-controlled hepatic artery supply and gravity fed portal venous supply via a reservoir. Throughout the perfusion period of twenty-four hours there was continuous monitoring of haemodynamic parameters and blood, bile, liver and bile duct tissue samples were collected.

Key findings
Acid-base homeostasis was restored quickly and, even though there was a substantial initial transaminase release, thereafter little ongoing enzyme flux was observed. Perfusate urea concentration steadily rose to a final concentration of 103 mmol/L. In conjunction with the electrolyte and glucose measurements, this contributed greatly to a steady increase in calculated serum osmolality from 342 mmol/L to 414 mmol/L over time.

Conclusions
We believe that it is important to maintain physiological parameters, including osmolality, in NMP systems as near normal as possible. Provision for dialysis of the perfusate during NMP may be necessary especially for long duration of perfusion.
To the editors

Normothermic oxygenated Machine Perfusion (NMP) has been proposed as a technique that may provide the means to preserve organ function but moreover accurately predict clinical outcome because graft function can be analysed pre-transplant. Promising results have been obtained in several animal models and a recent pilot study in discarded human donor livers showed that normothermic machine perfusion was feasible and graft viability could be assessed.1 We have successfully established an ex vivo normothermic oxygenated perfusion circuit using only a single centrifugal pump. We have perfused 4 human donor livers, currently deemed unsuitable for transplantation by local criteria. Interestingly, we observed high levels of calculated osmolality in the perfusate; a problem which we believe should be addressed in order to implement this new technique in clinical practice.

Methods

Organ retrieval

The human donor livers used in this report were deemed unsuitable for transplant based on current local criteria. Three livers were rejected due to their age as a donation after cardiac death (DCD) donor and one, retrieved from a brain dead (DBD) donor, due to the presence of steatohepatitis. Organ retrieval and cold perfusion were undertaken according to local protocol using heparinised saline followed by University of Wisconsin solution, with priority given at all times to organs destined for clinical transplantation. In accordance with local legislation, no heparin was administrated to DCD donors prior to organ retrieval. Donor blood was collected from the right atrium and later used to prime the perfusion circuit. Both the liver and the collected blood were stored on ice until the normothermic liver perfusion was commenced. The mean total period of cold ischaemia prior to perfusion was 5 hours and 42 minutes (± 2 hours and 45 minutes).

Approval was obtained from the Metro South Human Research Ethics Committee and the University of Queensland to perform the below described study protocol.

Perfusion circuit

The perfusion circuit was designed in our centre and consisted of a single centrifugal pump (ROTAFLOW; Maquet, Rastatt, Germany) to drive pressure controlled hepatic artery perfusion and maintain filling of a reservoir for a gravity fed portal venous supply (Figure 7.1). Other circuit components included an oxygenator (Dideco TM Kids
D101; Sorin Group, Mirandola, Italy) and a heat exchanger set at 37 °C. A scavenger system with a roller pump returned leaked perfusate back to the portal reservoir. The circuit was primed with 4% albumin solution and bubbles were removed. Blood was then slowly added and bicarbonate was administered to correct acidosis. When a citrate-based anticoagulation solution was used during donor blood retrieval, calcium gluconate was also added to normalise calcium levels. A minimum circuit volume of 1.5 L was used with a haematocrit of at least 20%.

![Schematic overview of the perfusion circuit](image)

Figure 7.1  **Schematic overview of the perfusion circuit.** The liver is placed in the organ chamber and connected to the centrifugal pump which directly supplies the hepatic artery. The portal vein is gravity fed via the reservoir. A roller pump circulates leaked perfusate back into the reservoir.

Liver preparation and commencement of normothermic perfusion

A routine back table procedure was performed and the bile duct was cannulated with an infant feeding catheter for bile collection. The top end of the vena cava was closed and a 34F cannula (Medtronic; Minneapolis, MN, USA) was inserted in the inferior vena cava.
The common hepatic artery was cannulated with a 16F cannula (Medtronic; Minneapolis, MN, USA) and a portal venous cannula (Maquet; Rastatt, Germany), inserted during organ retrieval, was left in situ. Before connecting the liver to the circuit, it was flushed with 2 L cold saline to wash out residual UW solution. The pump was started at 1.4 L/minute and adjusted to limit the hepatic artery pressure to 70 mmHg and flows were confirmed using dedicated Doppler probes (Medistim; Oslo, Norway) on the hepatic artery (300-375 mL/minute) and portal vein (550 - 750 mL/minute). Pressures were measured in the hepatic artery and portal vein with a dual pressure monitoring kit (Edwards Lifesciences; Irvine, CA, USA). Throughout the perfusion period there was continuous monitoring of hemodynamic parameters and blood, bile, liver and bile duct tissue samples were collected.

**Perfusate additives**

Taurocholic acid (7 mL/hour), prostacyclin (8 mcg/hour) and insulin (50-200 IU/hour) were continuously infused and heparin was administered as a bolus according to the activated clotting time. In addition, amino acids (20 mL/hour) were continuously infused during the latter two perfusions as has been reported in other systems.1

**Results**

The four livers included in this study were perfused for between 3 and 24 hours. Serial perfusate samples were taken and used for biochemical analysis (Figure 7.2A). During the perfusion the livers showed an initial large release of transaminases followed by steady decline over time. Lactate levels in the circuit rapidly normalised in all four cases to less than 1.5 mmol/L following a mean peak of 7.12 ± 1.76 mmol/L (data not shown). All four livers produced bile during the perfusion period (data not shown). Perfusate urea concentration steadily increased over the perfusion period; in the fourth NMP (NMP4), perfused for 24 hours because graft performance was very good based on appearance, metabolic parameters and bile production, the final concentration exceeded 100 mmol/L (Figure 7.2B). Glucose concentration was also noted to be high during perfusion, which was unresponsive to the administration of large doses of insulin (>50 units/hour) into the circuit (Figure 7.2C). In conjunction with the electrolyte measurements, this meant that, in all four liver perfusions, the calculated serum osmolality was over the upper limit of normal (normal range 275-295 mmol/L); in the case of NMP4 this was quite markedly so (Figure 7.2D).
Figure 7.2  Functional parameters liver perfusion up to 24 hours. A) Alanine Transaminase (U/L), B) Urea (mmol/L), C) Glucose (mmol/L) and D) Osmolality (mmol/L). The dotted lines reflect the normal range of serum osmolality 275-295 mmol/L.

Discussion

The results described represent an early experience in NMP of the human liver but already an important lesson has emerged. The functions of the liver are many and complex and NMP is a sophisticated in vitro organ maintenance system. There may be many facets of physiology to consider in determining the effectiveness of in vitro organ support. We found that it is straightforward to establish perfusion with a single pump system (using a portal venous reservoir at a specified height to achieve pressure-regulated flow) and that acid-base homeostasis can be restored quickly. This single pump circuit, similar to that developed in Oxford, is simpler and less expensive than a 2 pump setup but still able to provide the physiological dual blood supply at different pressures (although the portal venous perfusion is non-physiological to the
extent that it is oxygenated). It was straightforward to set up the centrifugal pump to deliver the arterial perfusate at a fixed pressure with the “extra” perfusate going into the portal venous supply reservoir, which functions as a volume buffer. After starting at 1.4 L/min, no more than 1 or 2 pump flow adjustments were required and the circuit never ran dry. Centrifugal pumps are less injurious to red blood cells than roller pumps which may be relevant for the development of prolonged perfusion protocols. While there was substantial transaminase release into the perfusion circuit initially, thereafter we observed no further rise. This suggests that ongoing hepatocyte damage is limited. Likewise, bile production was observed. However, the steadily rising urea levels in the circuit, which can only result from the continuation of urea cycle activity in the hepatocytes, and can therefore be considered a parameter of a functional NMP system, contribute substantially to a steady rise in osmolality. This was most strikingly seen in NMP4, successfully perfused for 24 hours. Contributing to hyper-osmolality, very high levels of perfusate glucose were observed in all four liver perfusions which were persistent for 6 – 12 hours despite the administration of large doses of insulin: this phenomenon has also been observed in pig livers perfused by the Oxford group in their NMP system (Friend, personal communication). Human tissues are sensitive to osmolar stress; high serum sodium levels in organ donors are linked with adverse transplant outcomes and may be a surrogate for osmolar stress.

Although we have not yet directly measured osmolality in the perfusion circuit, a priori it seems unlikely that it could be lower because the calculated osmolality formula, described by Bhagat et al., used by our laboratory accounts for urea and glucose concentrations and the principle electrolytes. Additional to an osmotic effect, it has been shown that a high concentration of urea (100 mmol/L) negatively affects hepatic metabolism via proteolysis inhibition.

The mechanisms underlying high levels of both glucose and urea production remain to be fully elucidated. It is possible that the NMP system stimulates high glycogenolytic activity resistant to insulin and urea production may be driven by the infusion of amino acids in the circuit. We and others have made the assumption that liver homeostasis in NMP may be supported by amino acids. While yet unproven, there are data to show that L-arginine, for example, reduces preservation injury by stimulation of nitric oxide production. Further work is needed to determine whether the benefit of amino acid infusion outweighs any deleterious effects. If it does, high levels of urea production may be unavoidable and would need to be addressed in another way. One strategy would be to incorporate a dialyser in the circuit - this could clear both urea and glucose. This would be straightforward in our static setup, and indeed we intend to explore this next, but it would not be feasible in a portable system. If dialysis does prove to be a requisite component of perfusion systems for organ storage beyond a few hours then portable devices in development will need the provision of a connection to dialysis before and/or after transport.
Finally, one of the main problems in expanding the use of DCD livers for transplantation has been the intractable problem of ischemic cholangiopathy. It would be plausible to consider that osmotic stress in biliary epithelium could be another injury to the biliary tract that might result from long periods of NMP for organ preservation and assessment.

We believe that it would be sensible to maintain physiological osmolality in NMP systems. This could be achieved with the provision of dialysis of the perfusate during NMP, especially for the successful development of perfusion systems beyond 6 hours.
Urea production during Normothermic Machine Perfusion: Price of Success?

References

Chapter 8

Does normothermic machine perfusion preserve bile ducts as well as it preserves hepatocytes? A study of human donor livers

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Submitted
Chapter 9

General discussion
General discussion

Liver transplantation is the preferred treatment option for patients with end stage liver disease. Unfortunately, an insufficient number of donor livers are available to transplant every patient on the waiting list; annually up to 10% of patients are delisted or die due to disease progression while waiting for a lifesaving liver transplant. More marginal organ donors, such as those donated after circulatory death (DCD) are being considered for transplantation in order to close the gap between supply and demand. Unfortunately, their use has been hampered by inferior graft performance and the development of biliary strictures in a significant proportion of recipients. This thesis aimed to identify factors responsible for the development of biliary injury using an animal model of partial hepatic ischaemia-reperfusion. Furthermore, the technique of normothermic machine perfusion of human donor livers was established and used as a platform to study the potential items involved in the pathophysiology of bile ducts injury in DCD livers.

In this chapter, the main findings of this thesis will be discussed and the implications for clinical practice assessed. Lastly, the conclusions of this thesis will be provided as well as future research opportunities.

DCD donors are an underutilised resource in Australia

Over the last several years there has been an initiative by the Australian Commonwealth Government to increase the number of organs available for transplant. In order to achieve this goal, the use of DCD organ donors was encouraged. In our experience, DCD donors are however often unsuitable liver donors. In chapter 2 we reviewed the characteristics of all Australian liver donors over a 10-year period to determine the impact of DCD organ donation on liver transplantation rates.

Although the number of organ donors and the number of livers donors substantially increased over the study period, a greater proportion of livers was deemed unsuitable for transplantation. Use rates of DBD donor livers remained stable around 20-25%. DCD donor livers on the other hand were only rarely used for transplantation and as the number of DCD donors increased, the overall liver utilisation rates declined from 74% in 2005 to 57% in 2014.

The likely reasons for the high non-utilisation rates of DCD donor livers are the inferior long-term graft survival rates, as well as the increased risk of ischaemic type biliary strictures (ITBS) compared to livers from DBD donors. To facilitate the use of these high-risk donors in Australia, a better understanding is required of the pathophysiology of ITBS. This was addressed in Part I of this thesis. Another way to safely use DCD livers may be by applying normothermic machine perfusion (NMP) as a
preservation method. This technique also allows for organ function assessment prior to transplantation, thereby avoiding the transplantation of non-viable liver grafts. The application of this technique was further addressed in part II of this thesis.

Part I. biliary injury

The development of an animal model of biliary injury and stricture formation

So far, much of what is known about the pathophysiology of biliary strictures originates from patient cohort studies. Prolonged ischaemia, as well as immunological factors and bile toxicity have been implicated in disease development. In order to study the sequence of events responsible for the development of biliary injury and stricture formation we used an animal model of partial hepatic ischaemia-reperfusion (Chapters 3 and 4). This model was considered less technically challenging than a liver transplantation model and we expected it to provide more reproducible results within a short timeframe. Within this model we tested the role of ischaemia as well as lipopolysaccharides (LPS) in the development of biliary injury. Animals were exposed to warm hepatic ischaemia and reperfusion (IRI), administration of lipopolysaccharides or a combination of both IRI and LPS and bile liver and bile duct tissue was collected.

As the study described in Chapter 3 only presents short-term effects of both IRI and LPS, further studies need to be conducted to confirm stricture formation does occur over time. Filling of the biliary tree with a methacrylate polymer mixture to obtain a bile duct cast or cholangiography could be used to confirm the presence of biliary strictures.

The role of ischaemia in the development of biliary injury and stricture formation

Cholangiocytes are more sensitive to IRI than hepatocytes. Upon reperfusion, they produce more reactive oxygen species, which combined with a lower basal level of anti-oxidants lead to a higher rate of cell death. Although these experiments described by Noack et al. were conducted using cultured cells, there is convincing evidence from clinical scenarios and studies to confirm these findings. Firstly, the blood supply to the biliary tree is mainly arterial and patients with hepatic artery thrombosis following transplantation develop biliary necrosis and stricture formation. Furthermore, extended periods of warm ischaemia prior to organ retrieval were found to be associated with the development of ischaemic type biliary strictures following transplantation In addition, a warm ischaemic period
exceeding 25 minutes was associated with an inferior graft survival rate of DCD grafts.2

Although it seems clear that ischaemia is a risk factor, we did not find any evidence of biliary injury following 30 minutes of warm ischaemia in our rat model as described in Chapter 3. This may have been due to the short duration of blood flow interruption to the liver. Longer periods of warm ischaemia have previously been used in animal models of IRI and is well tolerated by rats.14 Our rationale for using the 30 minute cut off however was that this is currently the maximum duration of ischaemia acceptable for the use of DCD grafts.

Another explanation could be that rats respond in a different fashion to warm ischaemia than humans. Op den Dries et al. previously conducted an experiment where rat livers were exposed to 30 minutes of warm ischaemia and 3 hours of cold storage.15 Following 2 hours of reperfusion, they did not find any evidence of biliary injury using routine histological assessment. When transmission electron microscopy was performed however, the number of mitochondria in cholangiocytes was markedly reduced, microvilli were lost and contact between neighbouring cholangiocytes was reduced.

Although we have successfully established a rat model of biliary injury, the development of ischaemia-induced biliary injury could not be determined using routine histological assessment. As ischaemia is considered an important risk factor for the development of ITBS following transplantation, the use of a different species such as pigs should be considered for future animal studies.

Endotoxins as a mediator of biliary injury

Endotoxins, in the form of LPS, can substantially enhance hepatic IRI resulting in more severe necrosis compared to LPS or IRI alone.14 In a transplantation setting, the deleterious effects of endotoxin exposure in the donor can be transferred to the recipient resulting in inferior graft function.16,17 Zipfel et al. reported that livers of donors that had plasma LPS concentrations over 12 pg/ml failed in 75% of cases within the first two years of transplant compared to no graft failures in the group with LPS levels <12 pg/ml.18 As the above-mentioned studies only focussed on hepatocellular function and injury, the study described in Chapter 3. aimed to determine the effects of LPS on cholangiocytes. In this study we administered 1mg/kg of LPS. This is a relatively low dose compared to the 15-30mg/kg LPS used in sepsis studies.19 Despite the low dosage used, small cholangiocytes in the portal triads were severely damaged following six hours of exposure to LPS with most or all ducts infiltrated by inflammatory cells, severe degenerative changes and ductular proliferation. We however did not find any evidence of injury to the large intrahepatic or common bile duct. One possible explanation might be that 6 hours is too early for large bile duct damage to occur. Histological scoring of the large ducts did indicate
some early changes with atypical large bile duct cells, but not to the extent of the injury we saw in small bile ducts.

Although at this stage it remains unclear if LPS-mediated injury would progress to stricture formation, previous studies have linked immunological factors to the onset of ITBS in transplant recipients.\textsuperscript{5,6} Chemokine receptor polymorphisms resulting in loss of function, cytomegalovirus infection as well as transplantation across blood groups have all been implicated in late ITBS development, occurring more often in the periphery of the liver. In addition, Friedrich et al. recently identified the crucial role of CD14/TLR-4 signalling in ITBS formation.\textsuperscript{20} As this pathway becomes activated in response to LPS, it possibly implicates a role for LPS-mediated injury in ITBS formation.

The role of macrophages in the development of LPS-induced biliary injury

Kupffer cells, the resident macrophages of the liver, become activated upon stimulation with pathogen-associated molecular patterns (PAMP) such as LPS and release reactive oxygen species and cytokines in response.\textsuperscript{21} This leads to the recruitment of neutrophils, which can eventually result in cellular injury.\textsuperscript{14} Hepatic depletion of macrophages has previously been shown to have a protective effect on the development of IRI and endotoxin-induced hepatocellular injury.\textsuperscript{16,22} We therefore aimed to determine the role of macrophages in the development of LPS-induced biliary injury in Chapter 4. Macrophages were successfully depleted from the liver using clodronate liposomes and rats were exposed to LPS or IRI+LPS for 6 hours. Histological assessment of small bile duct injury revealed that severe biliary injury persisted in the absence of macrophages. These data suggest that cholangiocytes are directly affected by LPS. Cholangiocytes are exposed to endotoxins as they are excreted in bile following their removal from the circulation.\textsuperscript{23} As this process is not altered by macrophage depletion, it could well be the reason for the development of biliary injury in the absence of macrophages. Furthermore, cholangiocytes play an active role in innate immunity as they release cytokines upon stimulation.\textsuperscript{24} Assessment of biliary concentration of cytokines in our study revealed that the concentrations of monocyte chemo-attractant protein-1 (Mcp-1) and tissue inhibitor of metalloproteinase-1 (Timp-1) were most significantly increased following LPS administration. Macrophage depletion did not alter this local inflammatory environment, which could explain the persistent biliary injury. Ultimately, specific assessment of cholangiocytes, by means of cell sorting, is required to determine the exact mechanism by which LPS induces biliary injury.
Blood biliary barrier assessment and involvement in LPS-mediated biliary injury

Epithelial barrier function is often tested in vitro using non-metabolised sugars such as inulin or mannitol.\textsuperscript{25} Furthermore trans-epithelial electrical resistance is assessed to determine the permeability of the epithelial barrier.\textsuperscript{26} The blood-biliary barrier (BBB) has not frequently been assessed in vivo. Horseradish peroxidase (HRP) as well as fluorescence labelled UDCA were used in previous animal studies.\textsuperscript{27-29} As HRP is readily available, repeated measures can be performed and leakage across the barrier can be assessed without the need to obtain cryosections, we decided to use HRP in our experiments described in \textbf{Chapter 3 and 4}. Following intravenous injection, HRP appears in bile via two distinct routes, the para-cellular route (across tight junctions), and trans-cellular transport (30). The administration of LPS increased the amount of HRP that leaked across the BBB by the para-cellular route. Immunofluorescence staining for tight junction protein zonula occludens-1, showed a severely disturbed staining pattern in animals that developed biliary injury. Whether impaired BBB function leads to the development of a specific biliary injury (as bile is retained in the portal tract area) or if it is merely the result of severe systemic insult remains to be determined.

Comparative inflammatory propensity of portal blood obtained from DCD donors to that of DBD donors

As LPS was found to be a potent inducer of biliary injury in our rat model, we aimed to determine whether endotoxaemia occurs during organ retrieval of DCD donors in humans (\textbf{Chapter 5}). DCD organ donation takes place following the withdrawal of life support and cessation of circulation. Throughout this process, hypoxia and hypoperfusion of organs such as the gut occurs which could result in endotoxaemia.\textsuperscript{31} Previous studies had shown that blood collected during DBD donation contained increased concentrations of endotoxins, especially at the time of hilar dissection.\textsuperscript{32} However, no data were available on the occurrence of endotoxaemia during the DCD donation process. We therefore collected portal venous blood samples from DBD and DCD donors throughout the organ retrieval process and assessed the concentration of endotoxins both directly and indirectly. We performed the conventional Limulus amebocyte lysate (LAL) test on a subset of samples to determine the content of endotoxins. Additionally, we performed an NFκB-cell based assay, which measures the inflammatory response mediated by all pro-inflammatory factors present in the tested samples such as damage-associated molecular patterns and endotoxin. As opposed to what we anticipated, no differences were found in the concentration of endotoxins or the propensity of portal samples to induce an inflammatory response between DBD and DCD donors. A possible contributing factor could have been the
timing of sample collection in this study. It was often difficult to obtain a portal venous sample at the start of cold perfusion in DCD donors. At the time of sample collection, cold perfusate might have already washed out the inflammatory mediators from the portal system. It however needs to be noted that even portal samples collected directly following the initiation of cold perfusion failed to induce an inflammatory response.

Up until this point it has been unclear to what extent endotoxaemia contributed to the development of IRI in DCD transplantation. Although inflammatory mediators were not present in blood flushing through the liver, hepatic macrophage activation was enhanced in DCD donors at the time of organ retrieval. This increase in macrophage activation is likely the result of exposure to local damage-associated molecular patterns (DAMPs) released by the liver upon ischaemic injury.

In addition to the blood samples, bile and common bile duct (CBD) sections were collected to assess the degree of bile duct damage at the time of organ retrieval. When systematically assessing CBD sections of DCD donors, only mild injury was observed to all components apart from the peribiliary glands, which were severely damaged. In addition to CBD histology, lactate dehydrogenase (LDH) in bile was measured as a biomarker of biliary injury. This likely represents damage to the entire biliary tree and LDH levels were significantly higher in bile collected from DCD donors compared to DBD donors. Although we were not able to determine if the sustained biliary injury ultimately resulted in stricture formation, results from this study might indicate the need for early intervention in order to prevent the development of ITBS.

**Part II: Machine perfusion**

The second half of this thesis focussed on the application of machine perfusion to safely increase the use of DCD donor livers for transplantation. This setup was furthermore used as a platform to assess the bile ducts of DCD livers prior to transplantation. The safety and feasibility of different perfusion setups used around the world was reviewed and a normothermic perfusion protocol was subsequently developed at the Princess Alexandra Hospital in Brisbane.

**Systematic review of different perfusion modalities used across the globe**

A systematic review of the literature identified 22 manuscripts describing the use of machine perfusion techniques to perfuse liver grafts from marginal donors (Chapter 6). Of those we identified nine studies that described hypothermic perfusion (HMP, 4-10°C), four described mid-thermic perfusion (MMP, 13-24°C), and nine discussed normothermic protocols (NMP, 37°C). Each centre considered their
protocol technically feasible and no major technical complications were encountered that might led to graft loss or exclusion from the study. Compared to NMP, HMP is rather uncomplicated, as no oxygen carrier is needed at this low temperature. Furthermore, static cold storage is the default backup in case of pump failure. NMP on the other hand, requires extensive knowledge of multiple aspects of liver metabolism and pump failure immediately exposes the livers to a warm ischaemic insult. When it comes to biliary complications, oxygenated HMP of DCD donor livers significantly reduced the incidence of ITBS compared to non-perfused controls zero versus 11 (22%), \( p=0.015 \) \((34)\). NMP only just entered the clinical trials phase and no results are available yet on the influence on ITBS formation. It was however the only technique that allowed for viability testing prior to transplantation. Both lactate clearance as well as bile production have been proposed as indicators of graft viability however results from large randomised trials are awaited to determine if these factors truly predict graft function in short or long term.\(^{50,52}\) Nevertheless, we aimed to establish a NMP protocol at our centre as we believe that viability testing is one of the most important features of the application of machine perfusion.

The establishment of a normothermic machine perfusion protocol at the Princess Alexandra Hospital

Encouraged by the favourable results published by op den Dries et al., and later the Oxford group led by Professor Friend, we decided to develop a NMP protocol at our centre \((46,49)\). The circuit was custom made using locally available resources. A single centrifugal pump perfused the hepatic artery directly whereas the portal vein was gravity fed via a reservoir. During the perfusion period, taurocholic acid, amino acids, insulin and prostacyclin were continuously infused and blood collected from the donor was used as perfusate. Finally, a scavenger system was in place to pump leaked perfusion solution back into the closed circuit. In this thesis, the results of ten consecutive perfusions are discussed in Chapter 7 and 8. In our experience, it was relatively straightforward to establish perfusion and only minor adjustments had to be made to the pump settings throughout the perfusion period to maintain near physiological pressures and flows. As the metabolic functions of the liver are diverse and complex, we would like to discuss some different aspects separately.

Viability assessment during NMP

Lactate clearance has been proposed as a way to distinguish the “good livers” from the “bad”. In the landmark papers by Perera et al. and Watson et al. describing the first transplants of declined marginal DCD livers following NMP, lactate clearance was used as one of the major determinants of viability.\(^{50,53}\) In a later report by the Birmingham group, a more comprehensive assessment protocol was presented which
besides lactate clearance included two out of the three following factors: macroscopic appearance, pH and arterial and portal flow.\textsuperscript{55} Bile production was only used to determine viability if a graft failed to clear lactate. When we applied the lactate clearance cut-off to our cohort, seven livers were deemed viable and three were deemed non-viable. During the perfusions, other parameters associated with liver function such as potassium concentrations, γ-glutamyl transferase and AST correlated well with lactate clearance. At the end of perfusion, hepatocellular architecture was preserved and no evidence of necrosis was present upon histological assessment. We therefore concluded that lactate clearance was a good determinant of viability from a hepatocellular aspect.

**Glucose metabolism of DCD donor livers during NMP**

Throughout the perfusion period we observed a distinct pattern in the glucose levels of DCD compared to DBD livers. Within the first hour of perfusion of DCD grafts, glucose concentrations increased despite the administration of 100IU of insulin. During the subsequent hours of perfusion, glucose levels decreased to below baseline. These findings are consistent with the current literature (53, 56) and glycogenolysis is the most likely explanation.\textsuperscript{57} Especially as venous glucose concentrations during this period were higher than arterial glucose concentration. Ultimately, glycogen stores are restored at the end of NMP as was shown by Megenthal et al.\textsuperscript{55}

Glucose concentrations at the start of each perfusion were largely dependent on the type of anticoagulant (citrate-based anticoagulation solution or heparin) used to store donor blood. Furthermore, during the first two perfusions we did not use a haemofilter and a 5%-glucose solution was used to counteract the sodium increase resulting from the administration of sodium bicarbonate. This, in combination with the glycogenolysis of the DCD grafts resulted in peak glucose concentrations of 32.6 (20.9–34.5) mmol/L. As glucose is a major component of the calculated osmolality, this resulted in osmolality levels well above the physiological threshold of 295 mmol/L. The use of a dialysis machine prior to, or during, machine perfusion might therefore be considered.

**Urea production during NMP**

Although we initially regarded urea production as a good indicator of synthetic liver function, its effect on osmolality of the perfusate meant that urea built-up in the closed system might need to be avoided when prolonged perfusion is required. One possible way of doing so is the use of a dialysis system as was employed by Banan et al.\textsuperscript{58} A major disadvantage of the use of a dialysis system within the first 2 hours of perfusion is that it might mask the production of lactate, potassium and other small
proteins that indicate organ dysfunction. Although dialysis is unlikely to counteract the ongoing production of lactate by non-viable grafts, it might mean that the cut-off of 2 mmol/L becomes jeopardised.

Assessment of biliary viability during machine perfusion

To determine the effect of NMP on the bile duct integrity we collected samples of the CBD at the time of organ donation, end of cold storage and end of perfusion. Unfortunately, NMP was not able to prevent biliary injury and the majority of CBD sections showed evidence of severe injury, regardless of metabolic performance. In addition to the role of NMP, we determined if the degree of injury to the CBD correlated with the severity of injury to the intrahepatic ducts. In total, 15 livers deemed unsuitable for transplantation were included in the comparison discussed in Chapter 8. Compared to the CBD, significantly less biliary injury was observed at the level of the main intrahepatic ducts as well as segmental ducts. These findings are in stark contrast to what has previously been reported by Karimian et al. The livers included in their study were however not re-perfused and therefore the overall degree of injury was much less compared to what we observed in our cohort. When a sub-analysis was performed including those livers that did not undergo NMP in our cohort, the degree of injury to the CBD was much less in these unperfused grafts and correlated well with the degree of injury to main ducts or the segmental ducts. It might therefore not be possible to rely on the histological assessment of CBD injury to determine biliary viability, and a more reliable biomarker is needed.

We therefore measured the levels of LDH in bile as has previously been used as a biomarker of biliary injury. Immediately following re-perfusion of the graft, LDH levels in bile were significantly higher than those measured in the donor, correlating with a higher degree of injury observed upon histological assessment. At the end of the perfusion period however, LDH levels reduced by 1.6 fold although significant histological damage was present. This might indicate that by that time, no more LDH is released as the majority of cholangiocytes have died. Therefore caution has to be taken when interpreting LDH results without the support of histological evidence.

Assessment of the blood-biliary-barrier during NMP

As we previously identified impairment of the BBB as a possible contributor to the development of biliary injury in Chapter 3, we aimed to determine its integrity during NMP in Chapter 8. In line with the rat studies, we administered HRP to the circuit and measured the output of HRP in bile during the perfusion of two livers. We were able to measure the concentrations of HRP in bile however the interpretation of the results is complicated by several factors. The liver needs to produce sufficient amounts of bile
to allow for the assay to be conducted. Furthermore, as extension tubing or paediatric feeding tubes are used to facilitate bile collection outside the main sterile field, the concentration of bile collected might reflect what has been excreted several minutes earlier. Therefore trans-cytosis rather than para-cellular movements could accidentally be assessed. In future experiments, a different approach to assessment of the BBB should be considered.

Conclusion

This thesis assessed ways to improve the use and outcome of DCD liver transplantation. Different aspects of the pathophysiology of bile duct injury following IRI and transplantation were assessed and endotoxins were shown to be potent mediators of bile duct injury. This acute injury was not mediated by macrophages but evidence suggested that a local inflammatory milieu surrounding the bile ducts more likely played a role. Although endotoxins were identified as potent mediators of biliary injury, such factors were not observed during the DCD donation process.

Another way to facilitate the safe use of DCD donor livers is by machine perfusion. In our unit at the Princess Alexandra Hospital in Brisbane, we successfully established a normothermic perfusion protocol and were able to use this technique to assess suitability for transplantation. Viability of the bile ducts is however critical to reduce the incidence of ITBS and further work is needed to find biomarkers that predict viability.

Future perspectives

Macrophages were shown not to play a role in the development of LPS-mediated biliary injury. Future work should therefore focus on the direct effect LPS has on cholangiocytes. A proposed way to do this is to obtain tissue from the biliary tree at the end of the experiment and assess gene expression patterns of cholangiocytes using RTqPCR.

As biliary cytokine concentrations were still present following macrophage depletion, it would be interesting to test TLR-4 or Tnf-α receptor agonists as a therapeutic option. We have performed some preliminary experiments using TACE inhibitor TIMP-3. This protein prevents the cleavage of pro-TNF-alpha into its active soluble form and pre-treatment with TIMP-3 protected the animals from the LPS-induced biliary injury. As was highlighted at the beginning of this discussion, rats respond differently to ischaemic injury than humans and might therefore not be the best animals to assess biliary injury in. Alternatively, pig models should be considered for future...
experiments. Experiments in these animals have shown similar biliary injury patterns and due to the size of the animal, machine perfusion or transplant experiments could be considered.

Viability of the bile ducts is an important factor determining long-term graft survival of DCD donor livers. Future research should be directed to determine a suitable biomarker of biliary viability during NMP.

We have shown that NMP of human donor livers is feasible and that viability can be assessed. Ultimately, we would like move toward a setting where livers deemed viable by NMP are used for transplantation.
Chapter 5

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Summary
Summary

**Chapter 1** provides a general introduction to liver transplantation and the current clinical limitations as well as overview of the outline of this thesis. Currently more livers donated after circulatory death (DCD) are being considered for transplantation in order to increase the number of donor livers available for patients on the transplant waiting list. Unfortunately, the use of these grafts is often complicated by the formation of ischaemic type biliary strictures (ITBS). As a result, DCD liver recipients frequently undergo re-interventions and long-term graft survival is limited. The overarching aim of this thesis was to better understand the pathophysiology of biliary stricture formation in DCD donors. To achieve this we developed an animal model of biliary injury as well as an ex-vivo normothermic machine perfusion setup used to perfuse livers declined for transplantation.

In **Chapter 2** the characteristics of all adult organ donors over the last decade were reviewed to determine the impact of DCD donation on liver transplantation rates in Australia. Since the implementation of DCD donation in 2008, their contribution to the overall number of organ donors steadily increased to 29% in 2014. Unfortunately DCD grafts were used only in a small proportion of cases, which contributed to an increase in the number of livers declined for transplantation (42 (26%) in 2005 versus 141 (43%) in 2014). Furthermore, multivariable regression identified DCD donor type as the most important independent risk factor for liver non-use (OR 24.06 (95%CI 17.47-33.14)) followed by advanced donor age, obesity, and diabetes. The limited use of DCD donors is likely the result of the unacceptably high risk of ITBS formation. A better understanding of the pathophysiology of these strictures is required to ultimately facilitate the use of more DCD grafts. Another possible approach could be the use of machine perfusion as an alternative preservation method. Additionally, modifiable risk factors such as the period of warm and cold ischaemic time should be kept to a minimum. Characterisation of DCD grafts declined for transplantation in the state of Queensland identified 33 (41%) donors who had zero, one or two marginal donor criteria that could be likely candidates for the application of machine perfusion.

**Chapter 3** focussed on pathophysiology of biliary injury. In this study, the role of lipopolysaccharides (LPS) and ischaemia-reperfusion was assessed. Rats were exposed to 1mg/kg of LPS or 30 minutes of warm hepatic ischaemia to 70% the liver. The animals were subsequently allowed to recover for one or six hours after which bile, blood, liver and common hepatic bile duct tissue was collected. No evidence of biliary injury was observed following 30 minutes of warm ischaemia and six hours of reperfusion. However, severe small bile duct injury was present six hours after the administration of LPS, which was associated with impaired clearance of bile acids from...
the circulation. In this experiment we furthermore assessed the permeability of the blood-biliary-barrier (BBB) using horseradish peroxidase (HRP). Following intravenous injection, more HRP leaked across the BBB and appeared into bile in the LPS groups. These findings were associated with a significant decrease in Claudin-1 and claudin-3 gene expression, both genes encoding for important tight junction proteins. Furthermore, immunofluorescence staining for Zonula occludens-1, showed markedly deranged tight junction morphology with a staining pattern not confined to the junctions of neighbouring cholangiocytes. The findings of this study highlighted the potency of endotoxins such as LPS to induce biliary injury.

In chapter 4 we aimed to determine the role of Kupffer cells in the development of LPS-mediated biliary injury. Kupffer cells become activated upon stimulation with LPS and we therefore hypothesised that in their absence, LPS mediated biliary injury would not occur. Rats were pre-treated with clodronate liposomes and exposed to sham operation, 1mg/kg LPS administration or LPS administration combined with 30 minutes of warm hepatic ischaemia to 70% of the liver. Despite successful depletion of macrophages from the liver, no reduction in biliary injury was observed following six hours of reperfusion. In addition, increased BBB permeability persisted. As cholangiocytes themselves can play a role in innate immunity and respond to LPS in a toll-like receptor-4 dependant fashion, biliary concentrations of several cytokines and chemokines were assessed to gain insight into the local inflammatory milieu surrounding cholangiocytes. Monocyte chemoattractant protein-1 (Mcp-1) and tissue inhibitor of metalloproteinase-1 (Timp-1) were 8.3 and 10.3-fold increased following LPS administration or LPS combined with warm hepatic ischaemia. Macrophage depletion did not affect the levels of Timp-1 and Mcp-1. Results from this study show that macrophages do not play a pivotal role in the development of LPS-induced biliary injury. Instead, cholangiocytes themselves might be responsible for the induction of small bile duct injury however further studies are required to confirm this.

Results from the animal experiment prompted the study conducted in human organ donors as described in chapter 5. The concentration of endotoxins and other inflammatory mediators was assessed in portal blood from organ donors. We hypothesised that the concentration of inflammatory mediators was increased in portal blood collected from DCD donors due to a period of hypo-perfusion and hypoxia prior to organ retrieval. Based on the convincing results of the rat study we further hypothesised that these pro-inflammatory mediators such as endotoxins were responsible for the induction of biliary injury in DCD donors. In total, nine DCD donors and 21 brain death donors (DBD) were included in this study. No differences were found in the presence of pro-inflammatory mediators in portal blood between DCD and DBD donors, both by the direct measurement of endotoxin and by the use of a bioassay for inflammatory stimuli. Despite the absence of pro-inflammatory
mediators, Kupffer cell activation was enhanced and prolonged in DCD donors. In addition, biliary injury could already be detected in DCD donors at the time of organ retrieval.

The second section of this thesis was aimed at the development of an *ex vivo* normothermic machine perfusion (NMP) protocol at our hospital. This system served as a platform to further study bile ducts of DCD donors and was also used as an assessment tool to determine graft viability of livers previously deemed unsuitable for transplantation. In Chapter 6 the different machine perfusion setups used in preclinical and clinical settings around the globe were reviewed. The aim of this systematic review was to determine the safety and applicability of machine perfusion. In total, 22 manuscripts were identified and all but one were published within the last five years. The corresponding author of each manuscript was approached to provide information regarding the safety of their perfusion protocol and none had encountered major technical complications that resulted in the loss of a liver graft. Both hypothermic perfusion as well as normothermic perfusion has been used prior to transplantation in a small case series and the results are promising. Equal or superior graft function is observed and hypothermic machine perfusion convincingly reduced the incidence of ITBS following transplantation of DCD grafts. However as NMP is the only modality that allows for graft assessment prior to transplantation, we proceeded to establish this technique at our centre.

An NMP protocol was developed at the Princess Alexandra Hospital and the first results were described in Chapter 7. Perfusate was pumped from the inferior vena cava using a centrifugal pump and flushed through an oxygenator with an inbuilt heat exchanger. Oxygen rich perfusate was then supplied directly to the hepatic artery whereas the portal vein was gravity fed via a reservoir. During the perfusion, amino acids, insulin and bile acids were continuously infused and a scavenger system was in place to recirculate leaked perfusate back into the closed system. To date, ten livers, all declined for transplantation, have been perfused using our custom-made perfusion circuit as described in chapter 8. Based on the currently available literature, lactate clearance within two hours of perfusion was used to distinguish viable from non-viable livers. Ultimately, seven livers were deemed viable whereas three livers were deemed non-viable. Compared to non-viable livers, viable grafts had significantly lower concentrations of potassium, AST and γ-glutamyl transferase and increased urea concentrations in their perfusate. Furthermore at end of perfusion, liver morphology was preserved upon histological assessment.

As NMP was shown to have a protective effect on bile ducts in animal studies, we assessed the degree of injury to the common bile duct (CBD) at the time of organ donation, prior to the start of machine perfusion and at the end of perfusion in a
semi-quantitative fashion. Unfortunately, severe biliary injury was observed in CBD sections collected at the end of perfusion, regardless of metabolic function. The degree of injury to the CBD however was not found to correlate with the degree of injury to the main hepatic ducts or the segmental ducts. These findings underline the need for a biomarker of biliary injury or viability in order to determine which grafts are unsuitable for transplantation due to a high risk of ITBS formation. This will be the focus of future studies.

**Chapter 9** described the key findings of this thesis and discussed the results and implications for clinical practice. Furthermore, avenues for future research were described.
Samenvatting
Samenvatting

In Hoofdstuk 1 wordt in het een kort overzicht gegeven van de geschiedenis van de levertransplantatiechirurgie. Daarnaast worden de huidige klinische limitaties besproken en wordt het doel van dit proefschrift gepresenteerd. Momenteel zijn er niet genoeg donorlevers beschikbaar voor alle patiënten op de wachtlijst waardoor er wordt overwogen om meer levers afkomstig van non-heartbeating donoren te gebruiken voor transplantatie. Echter is het gebruik van deze levers geassocieerd met de ontwikkeling van galwegstructuren. Dit heeft tot gevolg dat patiënten die een dergelijke lever ontvangen frequenter re-interventies ondergaan en uiteindelijk is re-transplantatie in eenderde van de gevallen noodzakelijk. De voornaamste doelstelling van dit proefschrift was om de pathofysiologie van galwegstructuren na transplantatie van een non-heartbeating donorlever in kaart te brengen. Om dit doel te bereiken werd er gebruik gemaakt van zowel een diermodel voor galwegschade als een ex-vivo normotherm machineperfusiesysteem voor humane levers.

In hoofdstuk 2 worden de resultaten van een review van alle Australische orgaandonoren tussen 2005 en 2014 gepresenteerd. Sinds de implementatie in 2008 is het aantal non-heart-beating orgaandonoren gestaag toegenomen. Echter werden levers afkomstig van deze donoren maar zelden gebruikt voor transplantatie, wat ertoe leidde dat de proportie niet geaccepteerde levers toenam van 42 (26%) in 2005 naar 141 (43%) in 2014. Een multivariabele regressieanalyse liet tevens zien dat non-heart-beating donatie de grootste onafhankelijke risicofactor was om een lever niet te accepteren voor transplantatie, gevolgd door hoge leeftijd, obesitas en diabetes. Naast het verkrijgen van een beter inzicht in de ontwikkeling van galwegstructuren kan ook het gebruik van machineperfusion worden overwogen om meer levers afkomstig van non-heart-beating donoren te gebruiken voor transplantatie. In de Australische staat Queensland werden 33 (41%) non-heart-beating orgaandonoren geïdentificeerd die geschikte kandidaten zouden kunnen zijn voor machine perfusie.

In hoofdstuk 3 wordt de pathofysiologie van galwegschade verder bestudeerd. In de studie beschreven in dit hoofdstuk, werd gekeken naar de rol van lipopolysachariden (LPS) en ischemische-reperfusion op het ontstaan van galwegschade. Ratten werden blootgesteld aan 1mg/kg LPS, warme ischemie van de lever voor 30 minuten of een combinatie van beiden. Eén of zes uur na de ingreep werden vervolgens bloed, gal, lever en galwegweefsel verzameld. Leverischemie gedurende 30 minuten en reperfusie gedurende zes uur bleek geen nadelig effect te hebben op de galwegen. Echter er ontwikkelde zich wel ernstige galwegschade zes uur na blootstelling aan LPS of ischemie gecombineerd met LPS, welke gepaard ging met verminderde hepatische
klaring van galzouten uit de systemische circulatie. In dit experiment werd horseradish peroxi-
dase (HRP) gebruikt om de permeabiliteit van de bloed-gal-barrière te testen. Deze barrière bestaat uit naburige cholangiocyten die met elkaar verbonden zijn middels tight-junctions. Na intraveneuze injectie werd meer HRP in gal gevonden in de
groepen ratten die behandeld waren met LPS, dit duidt op verminderde functie van de
bloed-gal-barrière. Deze bevinden waren geassocieerd met een verlaagde genexpressie van claudin-1 en claudin-3, die beiden coderen voor tight-junction-
eiwitten. Tevens liet een immunofluorescente kleuring een sterk verstoord kleurs-
patroon zien van het tight-junctioneitwit zonula-occludens-1. Resultaten van deze
rattenstudie tonen aan dat endotoxines zoals LPS zeer potent zijn in het veroorzaken
van galwegschade.

In hoofdstuk 4 wordt de rol van Kupffercellen in de ontwikkeling van LPS gemedieerde
galwegschade bestudeerd. Bloodstelling aan LPS kan leiden tot activatie van
Kupffercellen. De hypothese was dat LPS-gemedieerde galwegschade niet zou
optraden in afwezigheid van Kupffercellen. Ratten werden voorbehandeld met
clodronaatbevattende liposomen waarna ze werden blootgesteld aan LPS of een
combinatie van LPS en warme ischemie van de lever. Ondanks het feit dat
Kupffercellen succesvol werden verwijderd uit de lever, ontstond nog steeds ernstige
galwegschade. Tevens bleef de functie van de bloed-gal-barrière verminderd. Om een
beter inzicht te krijgen in het inflammatoire milieu rondom cholangiocyten, werd de
concentratie van verschillende cytokines in gal gemeten. De hoeveelheid monocyte-
chemoattractant protein-1 (Mcp-1) en tissue inhibitor of metalloproteinase-1 (Timp-1)
waren 8.3x en 10.3x verhoogd na blootstelling aan LPS en LPS gecombineerd met
warme ischemie. Kupfferceldepletie had geen effect op de concentratie Timp-1 en
Mcp-1. Op basis van deze studie kan worden geconcludeerd dat Kupffercellen geen
prominente rol spelen in de ontwikkeling van LPS-gemedieerde galwegschade. Echter
lijken cholangiocyten zelf mogelijk een rol te spelen.

De bevindingen in de rattenstudies leidden tot de initiatie van een studie met humane
orgaandonoren welke wordt beschreven in hoofdstuk 5. De concentratie van endotoxines en andere pro-inflammatoire factoren werden bepaald in portaal bloed
van orgaandonoren. De hypothese was dat portoveneus bloed van non-heart-beating
donoren een hogere concentratie pro-inflammatoire factoren zou bevatten als gevolg
van hypoxie en hypoperfusie van de darm, gedurende orgaandonatie. Tevens werd
verondersteld dat pro-inflammatoire factoren zoals endotoxines verantwoordelijk
waren voor de inductie van galwegschade in non-heart-beating donorlevers. In totaal
werden negen non-heart-beating en 21 heart-beating orgaandonoren geïncludeerd in
de studie. Er werd geen verschil gevonden in de hoeveelheid pro-inflammatoire
factoren tussen deze twee typen orgaandonoren. Desondanks, werd er een
verhoogde activatie van macrofagen in de lever vastgesteld; en galwegbiopten van
non-heart-beating donoren vertoonden meer galwegschade in vergelijking met die van heart-beating donoren.

Het tweede gedeelte van dit proefschrift is met name gericht op het gebruik van normotherme machine perfusie (NMP) van humane marginale levers. Dit systeem werd niet alleen gebruikt om te differentiëren tussen levers die geschikt zijn voor transplantatie en levers die uiteindelijk onvoldoende functioneren, maar ook om de galwegen van non-heart-beating donoren verder te bestuderen. Allereerst werd systematisch de beschikbare literatuur bestudeerd in hoofdstuk 6 met als doel de veiligheid en bruikbaarheid van machine perfusie protocollen in pre-klinisch en klinisch onderzoek in kaart te brengen. In totaal werden 22 publicaties geïdentificeerd die hypotherme, midtherme of normotherme machineperfusie-protocollen beschreven welke elke werden gezien als veilig en bruikbaar. Een klein aantal hypotherm en normotherm geperfuseerde marginale levers zijn inmiddels gebruikt voor transplantatie met goede resultaten. Tevens ontwikkelden patiënten die hypotherm geperfuseerde non-heart-beating donorlevers ontvingen significant minder galwegstructuren in vergelijking met patiënten die een niet geperfuseerde non-heartbeating donorlever ontvingen. Echter aangezien NMP als enige methode de mogelijkheid biedt om leverfunctie te testen alvorens de lever wordt getransplanteerd, werd besloten om een protocol op te zetten in ons centrum in Brisbane (Australië).

Er werd een NMP protocol ontwikkeld in het Princess Alexandra Ziekenhuis, in Brisbane (Australië) en de eerste resultaten worden besproken in hoofdstuk 7. Het perfusiesysteem werkt als volgt: perfusievloeistof (bloed van de orgaandonor) werd uit de vena cava inferior gepompt met behulp van een centrifugaal pomp, waarna de perfusievloeistof door een oxygenator stroomde welke tevens de perfusievloeistof opwarmde tot 37 graden Celcius. Zuurstofrijk bloed werd vervolgens direct naar de a. hepatica geleid terwijl de vena porta werd gevoerd door middel van een reservoir. Tijdens de perfusie werden constant aminozuren, insuline en galzouten toegediend en werd er elke vier uur een bolus heparine gegeven om embolieën te voorkomen. Over de afgelopen twee en een half jaar werden tien levers, voorheen bestempeld als ongeschikt voor transplantatie, middels dit systeem geperfuseerd (hoofdstuk 8). Lactaatsclaring gedurende de eerste twee uur op de perfusiemachine werd gebruikt om te differentiëren tussen levers die geschikt zijn voor transplantatie en levers die uiteindelijk onvoldoende functioneren. Op basis van dit criterium zouden zeven normotherm geperfuseerde levers mogelijk bruikbaar zijn geweest voor transplantatie. Vergeleken met levers die onbruikbaar werden geacht hadden bruikbare levers een lagere concentratie kalium, AST en γ-glytamyl transferase en een hogere concentratie ureum in de perfusievloeistof. Aan het einde van de
perfusieperiode van gemiddeld zes uur werden geen histologische tekenen van leverschade gevonden. Eerdere dierstudies hebben aangetoond dat NMP schade aan de galwegen kan verminderen. Om de invloed van NMP op humane galwegen te beoordelen werden er biopten genomen van de ductus choledochus tijdens orgaandonatie en aan het begin en eind van NMP. Helaas was schade aan de ductus choledochus onvermijdelijk, ongeacht de metabole functie van de lever. Echter, de hoeveelheid schade aan de ductus bleek niet te correleren aan de hoeveelheid schade aan de grote intrahepatische galwegen en de segmentale galwegen. Toekomstige studies dienen zich daarom te focussen op het ontwikkelen van een biomarker voor galwegschade, om zo veilig non-heartbeating donorlevers te kunnen gebruiken voor transplantatie.

**In hoofdstuk 9** worden de belangrijkste bevinden van dit proefschrift beschreven en worden de implicaties voor de kliniek bediscussieerd. Tevens wordt de focus van toekomstige studies besproken.
Valorisation
Valorisation

This chapter will discuss how the research results presented in this thesis could be used in clinical practice. The implications of the study results to the wider community will be described. Lastly, the necessary steps to implement machine perfusion into clinical practice will be reviewed.

Implications of research results for the wider community

In Australia, there has been a steady increase in the number donor livers deemed unsuitable for transplantation over the last ten years. Despite the strong wishes of donor families to use the organs of their loved ones to save the lives of others, the number of liver declined for transplantation increased from 26% in 2005 to 43% of in 2014. On the other hand, annually about 10% of patients with end stage liver disease waiting to undergo life-saving liver transplantation became ineligible as their disease progressed beyond acceptance criteria for transplantation. The decreasing rate of liver utilisation is not only a pressing problem in Australia, but has been reported around the globe. In the United Kingdom, the proportion of livers used for transplantation has dropped by 16% between 2005-06 and 2014-15.\textsuperscript{1,2} Furthermore, Orman \textit{et al.} found that in the US, liver non-use increased from 15% in 2004 to 21% in 2010,\textsuperscript{3} which was accompanied by declining annual liver transplant rates.

If we could make better use of the organs currently available, it would mean that the wishes of more donor families could be honoured. Furthermore, as this could be achieved without an increase in the number of organ donors, it would be the most cost effective way to save the lives of patients that succumb to their disease while waiting to undergo liver transplantation.

Currently a large proportion of livers declined for transplantation are from organ donors who donate their organ after the cessation of circulation (donation after cardiac death, DCD). The use of these liver grafts is currently limited by the slightly increased risk of primary non-function, especially when transplanted into a high-risk recipient. More importantly, the development of ischaemic type biliary strictures (ITBS) in up to one in three recipients limits long-term graft survival.\textsuperscript{4,5} As a results, the cost per life year gained is far greater in DCD donors where € 112,376 was spent per annum compared to € 88,913 per year for those that receive a liver donated after brain death.\textsuperscript{6}

The aim of this thesis was to characterise the sequence of events associated with the development of ITBS, as this is currently the most limiting factor in the utilisation of DCD livers. Improved knowledge about the pathophysiology of ITBS may help find new therapies or preventing measures, and these may benefit the wider community. In
context, the use of normothermic machine perfusion was explored as a tool to assess graft function of livers currently declined for transplantation. Combined, these two avenues of research have an impact on the wider community as a whole as liver disease and organ donation touches the lives of many patients worldwide.

The implementation of normothermic machine perfusion

The application of normothermic machine perfusion (NMP) has several advantages over the use of conventional simple cold storage. First of all, the quality of marginal donor organs can be preserved which would allow for the use of more livers currently declined for transplantation. Furthermore, it possibly allows for extended periods of preservation, which in turn facilitates long distant transport but also improves transplant logistics as the transplant can take place during daytime hours. In addition, results from several animal studies have indicated that NMP is preventing biliary injury, which could reduce the incidence of ITBS. Equally importantly, graft function can be assessed during perfusion at 37°C, which could avoid transplanting a non-viable graft.

An NMP protocol was established at the Princess Alexandra Hospital and ten livers declined for transplantation were successfully perfused. Based on the viability criteria previously described by other centres, seven could potentially have been used for transplantation. Over the last 2 years, 146 DCD liver grafts were declined for transplantation in Australia. If 70% of these grafts could have been recovered using NMP, this would have increased the donor pool by 16%. A similar prediction of the effect of NMP on organ donor numbers was made by Mergenthal et al. Based on the number of livers declined for transplantation and the results of their pilot study, they estimated that a 15% increase in the total number of donor livers could be achieved in the United Kingdom.

Based on the experience with NMP described in this thesis and reports of other centres that have implemented the technique in clinical practice, the next aim is to start using those livers that are deemed viable during NMP for transplantation.

What is required to implement NMP in clinical practice?

Machine perfusion post static cold storage or in transit

Currently, NMP has been applied following a period of cold storage as well as during transit. Although in transit perfusion limits the period of cold preservation injury to about one hour, it comes with some logistical challenges. It would mean that a new
vehicle fitted with a main power supply needs to be purchased to facilitate the transport of this large piece of equipment. However air-transportation is often required in countries such as the United States, Canada and Australia and the use of NMP in a small airplane has not previously been performed. Furthermore, banked matched blood will have to be taken to the donor hospital to serve as perfusate during NMP although the blood may not be used if a DCD donor does not die within the currently accepted time frame. Lastly, in some instances the airplane is shared between the abdominal and cardiothoracic team when retrieving organs from donors outside the metropolitan area. As the maximum cold ischaemic time of a heart is already in jeopardy when flight time exceeds 2 hours, there may be time restrictions in establishing NMP for the donor liver prior to transportation. Therefore in Australia, NMP will probably only be initiated at the recipient hospital following a period of conventional cold storage.

Organ donor inclusion criteria

An important consideration is which liver grafts require NMP prior to transplantation. Some reported studies have included “within criteria” grafts in their perfusion studies (8). These livers are however already acceptable for transplantation using current empirical criteria and the application of NMP exposes them to the potentially catastrophic effects of pump failure. Furthermore it will not increase the number of grafts available for transplantation but it may increase the costs associated with using them. In fact, as the appropriate criteria to determine viability are still being established, perfusion of “standard criteria” donor livers could potentially lead to a reduction in the number of transplants. One group of patients that could benefit from machine perfusion of “standard criteria” grafts are those with a high model for end-stage liver disease (MELD) in the intensive care. Machine perfusion reduces the reperfusion injury and this could reduce the incidence of post-reperfusion syndrome during transplantation.\(^\text{14}\)

However as increasing the number of donor livers available for transplantation is the primary goal, livers currently declined for transplantation should be the target of NMP. Furthermore, the use of livers with evidence of structural damage or organ donors infected with hepatitis B, C or HIV should be avoided. Initially, warm ischaemic time of DCD donors will be limited to 30 minutes, which can be extended once we become more experienced.

Criteria for viability assessment

A standardised protocol will be established outlining the criteria used to determine viability. Based on the pre-clinical results described in this thesis, lactate clearance below 2 mmol/L within two hours will be the main component. Furthermore, the liver
needs to have a satisfactory macroscopic appearance and both the surgeons as well as
the anaesthetist involved in the care of the recipient need to agree on proceeding.
While the recipient is prepared for transplantation, machine perfusion will continue
until transplantation. This period is anticipated to be up to six hours.

Recipient selection

The selection of suitable recipients of these high-risk NMP livers is crucial in obtaining
favourable long-term outcomes. In countries such as Australia and the United
Kingdom, no nationwide liver transplant waiting list exists and organ allocation is
performed by individual transplant centres. Patients with hepatocellular carcinoma
could be suitable low-risk recipients to receive an NMP perfused liver. These patients
often do not have end stage liver disease with portal hypertension and ascites, which
makes the transplant operation more straightforward from a technical perspective.

Commercial machine versus custom-made setup

Currently, two commercial machines have been used for normothermic liver perfusion
in clinical studies in Europe and the United Kingdom however both are not approved
by the Therapeutic Goods Administration for use in Australia.8,35 These machines cost
up to AUD 260,000, which excludes the disposables (personal communication). These
are priced between AUD 9000 and AUD 18,000 per perfusion. The perfusion setup
described in this thesis, is comprised of components used for ECMO, and costs AUD
1500 per perfusion.

As experience was gained with our custom made setup and it is significantly cheaper
than the commercially available machines, we will continue to use the perfusion
machine described in this thesis.

Cost implications for transplant program

An increase in donor numbers will come at a price. Not only is machine perfusion
more expensive than conventional static cold storage, staff and facility costs needed
to run them have to be considered. Currently, the kidneys from DCD donors are often
the only abdominal organs retrieved from DCD donors and their retrieval is performed
by an urologist or general surgeon. If the livers of those donors are considered for
transplantation, this will mean that HPB surgeons will have to perform more retrievals
and an increase in surgical staff will need to be considered. Despite the increase in
cost in the short term, it will reduce the cost required for ongoing care of patients
with end stage liver disease. Furthermore, if the incidence of biliary complications can
be limited by the use of NMP, it will significantly reduce the costs of ongoing care for
liver transplant recipients.
In conclusion, machine perfusion will have a distinct impact on global transplantation practices in the near future as it will ultimately lead to a significant increase in the number of livers available for transplantation.
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Acknowledgements
Acknowledgements

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Dear Rich, I don’t know how you managed to put up with me for the last 2.5 years! All the trips and plans that had to be cancelled because I had to go and help out on a transplant. Or the times you had to pick me up from the hospital because I was too tired to drive (or had a broken finger). You were always there to help me, no questions asked. I furthermore have a great deal of respect for the way you combine a full-time job with studying while still being there to support me. It has been very hard at times but you have always managed to succeed. I am looking forward to finally show you the beautiful country I grew up in and introduce you to all my friends and family in the Netherlands. Rich, you make me feel like the luckiest girl in the world!
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Curriculum vitae
Curriculum vitae

Janske Reiling was born on October 31, 1987 in Maarheeze, the Netherlands. After obtaining her Atheneum degree at the Strabrecht College in Geldrop in 2005, she went on to study medicine at the University of Maastricht. During her fourth year she went to South Africa for her ophthalmology rotation and this sparked her passion for travel. In the following year she travelled to Brisbane, Australia, for her elective rotation as well as a research participation. Here she was introduced to the liver and kidney transplant department of the Princess Alexandra Hospital, a place that would play a vital role in her future career. She completed her research project on the role of statins in the onset of graft dysfunction following kidney transplantation under the supervision of Professor Darrell, professor David Johnson and associate Professor Peter Kruger. As the combination of living in Australia and a PhD in the transplant field seemed appealing, she decided to eventually come back to Brisbane. Upon her return to the Netherlands she completed her participation in Healthcare at the Atrium Medical Centre in Heerlen under the supervision of dr W.G. van Gemert and obtained her medical degree in December 2011. During the following year she worked as a surgical resident at Maastricht University Medical Centre, The Netherlands, while finalising the application process to start a PhD in Brisbane. In April 2013 the necessary paperwork was finalised and she started her PhD under the joint supervision of Professor Jonathan Fawcett and Professor Kees Dejong at the University of Queensland and the University of Maastricht. In October 2016 she will commence her medical career in Australia at Greenslopes Private Hospital in Brisbane.
Appendix

University of Queensland thesis preliminary pages
Appendix

Abstract

Liver transplantation is the preferred treatment option for end stage liver disease. The demand for donor livers greatly exceeds the supply and organs from more marginal donors are being considered for transplantation. An important group of such donor livers are those obtained by donation after circulatory death (DCD). These livers are more susceptible to cold preservation injury and their use has been associated with a slight increased incidence of primary non-function in Australia. More importantly, biliary complications such as ischaemic type biliary strictures (ITBS) are more prevalent in recipients receiving a DCD liver resulting in a higher rate of re-intervention, re-transplantation and inferior long-term graft survival. This thesis describes a series of studies examining potential factors responsible for the development of biliary injury. Furthermore, a normothermic hepatic machine perfusion protocol was established at our centre and used as a platform to study the development of biliary injury in human livers.

A review of all adult Australian organ donors between 2005 and 2014 revealed that there was a 17% increase in the number of livers deemed unsuitable for transplantation, mainly attributable to the rise in DCD donors. Over the study period, only 62 (13%) livers from DCD donors were used for transplantation. In addition, advanced donor age, obesity as well as diabetes were associated with liver non-use.

The pathophysiology of biliary stricture formation following transplantation remains largely unknown and better insights into the sequence of events leading to their development are urgently needed. The work in this thesis has shown that endotoxins, in the form of lipopolysaccharides (LPS), are potent inducers of small but not large bile duct injury. Following six hours of administration of 1mg/kg LPS, small bile ducts were severely affected with biliary epithelial cells showing significant degenerative changes, infiltration of inflammatory cells and ductular proliferation. This was further associated with an increased permeability of the blood-biliary-barrier and the development of clinical features of cholestasis.

Macrophages are activated by LPS and to determine their role in LPS-induced biliary injury, an additional group of animals was pre-treated with clodronate liposomes prior to LPS exposure. These macrophage depleted animals showed a similar degree of biliary injury in response to LPS, which suggests that macrophages don’t play a crucial role in the pathophysiology of LPS-induced injury.
These findings prompted the initiation of a prospective patient cohort study aimed to examine the levels of endotoxins and other pro-inflammatory mediators during DCD organ donation. Portal blood samples were collected from DCD donors and the inflammatory propensity of these samples was compared to samples collected from donors who donated after brain death (DBD). Using a cell-based assay as well as a Limulus Amebocyte Lysate quantification method, we were unable to show a difference in inflammatory mediators present in portal blood between DBD and DCD donors. We did however find increased levels of hepatic macrophage activation and evidence of more severe biliary injury in samples collected from DCD donors.

The second part of this thesis focuses on the application of machine perfusion techniques. Machine perfusion could facilitate the use of more marginal grafts and opens up opportunities to assess viability prior to transplantation. In this thesis, we systematically reviewed all machine perfusion protocols currently applied in pre-clinical or clinical transplantation settings. Hypothermic perfusion (HMP), mid-thermic perfusion (MMP) and normothermic machine perfusion (NMP) have been applied and each centre described the method as safe and feasible. Only small case series have been conducted so far. Nevertheless, oxygenated HMP of DCD livers prior to transplantation lead to a significant reduction in ITBS formation compared to non-perfused controls. In our centre, we developed a normothermic machine perfusion apparatus and perfused ten livers that were declined for transplantation. Using the suitability criteria of lactate clearance within the first two hours of perfusion, we estimated that 7/10 rejected livers could have been suitable for transplantation. When assessing biliary injury we found that degree of injury to the common bile duct did not correlate with the level of injury observed to the large intra-hepatic bile ducts.

In summary, this thesis has identified the gross underutilisation of DCD donor livers in Australia. It furthermore explored the role of endotoxins as a co-factor in the development of biliary injury, which is currently considered the Achilles heel of DCD liver transplantation. The application of machine perfusion was established and hepatic viability could be assessed under normothermic conditions. Future work focussing on the assessment of biliary viability is needed to facilitate the safe use of DCD donor livers.
Abstract (Dutch)

Levertransplantatie is vaak het laatste redmiddel voor patiënten met leverfalen. Helaas zijn er niet genoeg donorlevers beschikbaar voor alle patiënten op de wachtlijst, waardoor er wordt overwogen om marginaal lever te gebruiken voor transplantatie. Deze levers, afkomstig van oudere danwel non-heart-beating donoren of met hooggradige steatose, zijn gevoeliger voor reperfusie schade. Dit heeft tot gevolg dat deze levers soms niet werken na transplantatie. Daarnaast vormen galwegcomplicaties zoals galwegstructuren voor veel morbiditeit, wat resulteert in meer re-interventies, re-transplantaties en inferieure leverfunctie op de langere termijn. In dit proefschrift wordt een serie studies beschreven die zich focust op mogelijke factoren die een rol spelen in de pathofysiologie van deze galwegstructuren. Daarnaast wordt het gebruik van een leverperfusietechniek beschreven, welke wordt ingezet om de fysiologie van de humane galwegen te bestuderen.

Een review van alle Australische orgaandonoren tussen 2005 en 2014, liet zien dat er tijdens deze jaren een stijging van 17% was in het aantal levers dat niet werd geaccepteerd voor transplantatie. Non-heart-beating donoren waren de voornaamste oorzaak van deze stijging. Maar 62 (13%) levers afkomstig van non-heart-beating donoren werden gebruikt voor transplantatie gedurende de studieperiode. Tevens waren hogere leeftijd, obesitas en diabetes geassocieerd met het niet accepteren van levers voor transplantatie.

De pathofysiologie van galwegstructuren na levertransplantatie is grotendeels onbekend. Een studie, beschreven in dit proefschrift, heeft geconstateerd dat toediening van lipopolysacchariden (LPS) kan leiden tot ernstige schade aan kleine galwegen. Cholangiocyten vertoonden degeneratieve veranderingen, infiltratie van inflamatoire cellen en proliferatie, zes uur na de intra-peritoneale toediening van LPS. Deze veranderingen waren verder geassocieerd met een toegenomen permeabiliteit van de blood-gal-barrière en klinische tekenen van cholestase.

LPS kan leiden tot Kupffercel activatie en dit zou een rol hebben kunnen gespeeld in de ontwikkeling van deze LPS-gemedieerde galwegschade. Om dit verder te bestuderen, werd een extra groep ratten voorbehouden met liposomen die gevuld waren met clodronaat om zodoende Kupffercellen te verwijderen uit hun lever. Deze ratten vertoonden een gelijke hoeveelheid galwegschade na toediening van LPS, wat dus suggereert dat Kupffercellen geen rol spelen in de LPS gemedieerde galwegschade.
De bevindingen in de ratenstudies leidden tot de initiatie van een studie met humane orgaandonoren. In dit cohort werden de concentratie van endotoxines en andere inflammatoire factoren in portovenue bloed van heart-beating en non-heart-beating donoren met elkaar vergeleken. De hypothese was dat portovenue bloed van non-heart-beating donoren een hogere concentratie pro-inflammatoire factoren zou bevatten, als gevolg van hypoxie en hypoperfusie van de darm gedurende orgaandonatie. Zowel de Lumulus Amebocyte Lysate assay als een in vitro assay werden gebukt, echter werd er geen verschil gevonden in de hoeveelheid inflammatoire mediatoren tussen deze twee typen orgaandonoren. Desondanks, werd er een verhoogde activatie van macrophagen in de lever vastgesteld; en galwegbiopten van non-heart-beating donoren vertoonden meer galwegschade in vergelijking met heart-beating donoren.

Het tweede gedeelte van dit proefschrift richt zich op het gebruik van marginale donorlevers middels toepassing van machineperfusie (MP). Een van de belangrijkste voordelen van MP is dat het de mogelijkheid biedt om levers die geschikt zijn voor transplantatie te differentiëren van levers die uiteindelijk onvoldoende functioneren. In dit proefschrift wordt allereerst een systematic review beschreven omtrent het gebruik van MP-protocollen in pre-klinisch en klinisch onderzoek. Hypotherme perfusie, mid-therme perfusie en normotherme perfusie zijn elk gebruikt voor de preservatie van humane levers en deze technieken worden gezien als veilig en bruikbaar. Tevens ontwikkelden patiënten die een hypotherm geperfuseerde non-heart-beating donorlever ontvingen significant minder galwegstructuren in vergelijking met patiënten die een non-heart-beating donorlever ontvingen die niet werd geperfuseerd.

Er werd een normotherm MP-protocol ontwikkeld in het Princess Alexandra Ziekenhuis in Brisbane (Australië) en tien levers die als ongeschikt waren bestempeld voor transplantatie werden hierop geperfuseerd. Op basis van de lactaatklaring gedurende de perfusie, zouden zeven levers van deze groep mogelijk bruikbaar zijn geweest voor transplantatie. Schade aan de ductus choledochus was echter onvermijdelijk, ongeacht de metabole functie van de lever. De hoeveelheid schade aan de ductus correleerde echter niet met de hoeveelheid schade aan de intra-hepatische grote galwegen.

Samengevat, hebben de studies geïncludeerd in dit proefschrift aangetoond dat levers afkomstig van non-heart-beating orgaandonoren nu nauwelijks worden gebruikt voor transplantatie in Australië. Verder werden endotoxines geïdentificeerd als een mogelijk bijdragende factor aan de ontwikkeling van galwegschade, iets dat wordt gezien als de belangrijkste limiterende factor in het gebruik van non-heart-beating donorlevers voor transplantatie. Daarnaast werd een normotherm MP-protocol
ontwikkeld, dat kan worden gebruikt om de leverfunctie en bruikbaarheid voor transplantatie te testen. Toekomstige studies dienen zich te focussen op het ontwikkelen van een methode om galwegschade beter in kaart te brengen, om zo veilig non-heart-beating donorlevers te kunnen gebruiken voor transplantatie.
Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications included in this thesis


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Contributions by others to the thesis

Concept and design of the project
Dr Kim Bridle, Professor Darrell Crawford, Professor Kees Dejong and Professor Jonathan Fawcett contributed to the experimental design of each project.

Tissue processing and sectioning
Formalin fixed liver and bile duct sections from the rat experiments were processed and sectioned by the histotechnology facility at the QIMR Berghofer Medical Research Institute. They furthermore performed the Haematoxylin and eosin staining of these sections. The human liver and common bile duct sections were processed and stained by Envoi Pathology.

Histology scoring
Liver and bile duct histology scoring (Chapter 3, 4 and 8) was performed by Catherine Campbell (Envoi Pathology) and Marion Gijbels (Department of Pathology and Department of Molecular Genetics, Cardiovascular Research Institute Maastricht, the Netherlands), both specialist pathologists.

Serum analysis during normothermic machine perfusion
Hourly serum samples were collected during normothermic machine perfusion of discarded human donor livers (Chapter 7 and 8) and sent to Queensland Pathology for analysis.

Design of the perfusion circuit used for normothermic machine perfusion
The perfusion circuit used for the perfusions of the discarded human donor livers (Chapter 7 and 8) was co-designed by Dr Andrew Simpson and manufactured by Cellplex Pty, Ltd, Melbourne, Australia.

Liquid chromatography of bile samples
Bile samples (Chapter 3) were sent to the department of Laboratory Medicine at the University Medical Centre Groningen, The Netherlands, for assessment of bile salt composition using UHPLC-MS/MS.

Cytokine array serum and bile samples
The concentrations of several cytokines in bile and serum samples included in Chapter 3 and 4 were measured using multiplex ELISA arrays. The arrays was performed in our lab and sent to RayBiotech (Norcross, MA, USA) for assessment of fluorescence intensity. The obtained results were analysed in our lab using specific software.
Data collection of organ donors
Organ donor data was obtained from The Australian and New Zealand Organ registry (ANZOD), and used for Chapter 2. When data was not available in the registry, donor records held by DonateLife Queensland were reviewed with the help of Jade Carey, donation specialist.

Assistance with experiments
Dr Kim Bridle has assisted with the animal experiments (Chapter 3 and 4) and Lesley Jaskowski has performed several Immunohistochemical and Immunofluorescence stains, as well as qRT-PCR’s.

NF-κB-dependent cell-based assay and Limulus Amebocyte Lysate quantification method
Professor Matt Brown, and other members of his laboratory (Centre for Inflammation and Disease Research, Institute for Molecular Bioscience, University of Queensland), kindly assisted with the analysis of portal blood samples using a NF-κB-dependent cell-based assay (Chapter 5). Dr Ashok Raj furthermore assisted with the measurement of endotoxins in portal blood samples using the Limulus Amebocyte Lysate quantification method (Chapter 5).

Critical review of the thesis
Dr Kim Bridle, Professor Darrell Crawford, Professor Kees Dejong and Professor Jonathan Fawcett reviewed and edited each chapter of this thesis and approved the final version.
Statement of parts of the thesis submitted to qualify for the award of another degree

None