MACHINE PERFUSION OF HUMAN DONOR LIVERS WITH A FOCUS ON THE BILIARY TREE

Alix Matton

Machine Perfusion of Human Donor Livers with a Focus on the Biliary Tree

Alix Petra Margarita Matton

Different parts of this PhD project were financially supported by:

University Medical Center Groningen
Groningen University Institute of Drug Exploration (GUIDE)
Innovatief Actieprogramma Groningen (IAG-3)
Jan Kornelis de Cock Stichting
Tekke Huizingafonds
HBO_2 Therapeutics

The printing of this thesis was financially supported by:

University Medical Center Groningen
Research Institute GUIDE
Nederlandse Transplantatie Vereniging
Nederlandse Vereniging voor Hepatologie

Author: Alix Petra Margarita Matton
Cover: enviromantic
Printed by: Ridderprint B.V.
ISBN: 978-94-034-2215-2

rijksuniversiteit groningen

Machine Perfusion of Human Donor Livers with a Focus on the Biliary Tree

Proefschrift

ter verkrijging van de graad van doctor aan de
Rijksuniversiteit Groningen
op gezag van de
rector magnificus prof. dr. C. Wijmenga
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

woensdag 11 december 2019 om 11.00 uur

door

Alix Petra Margarita Matton

geboren op 28 november 1991
te Leuven, België

Promotores

Prof. dr. R.J. Porte
Prof. dr. J.A. Lisman

Beoordelingscommissie

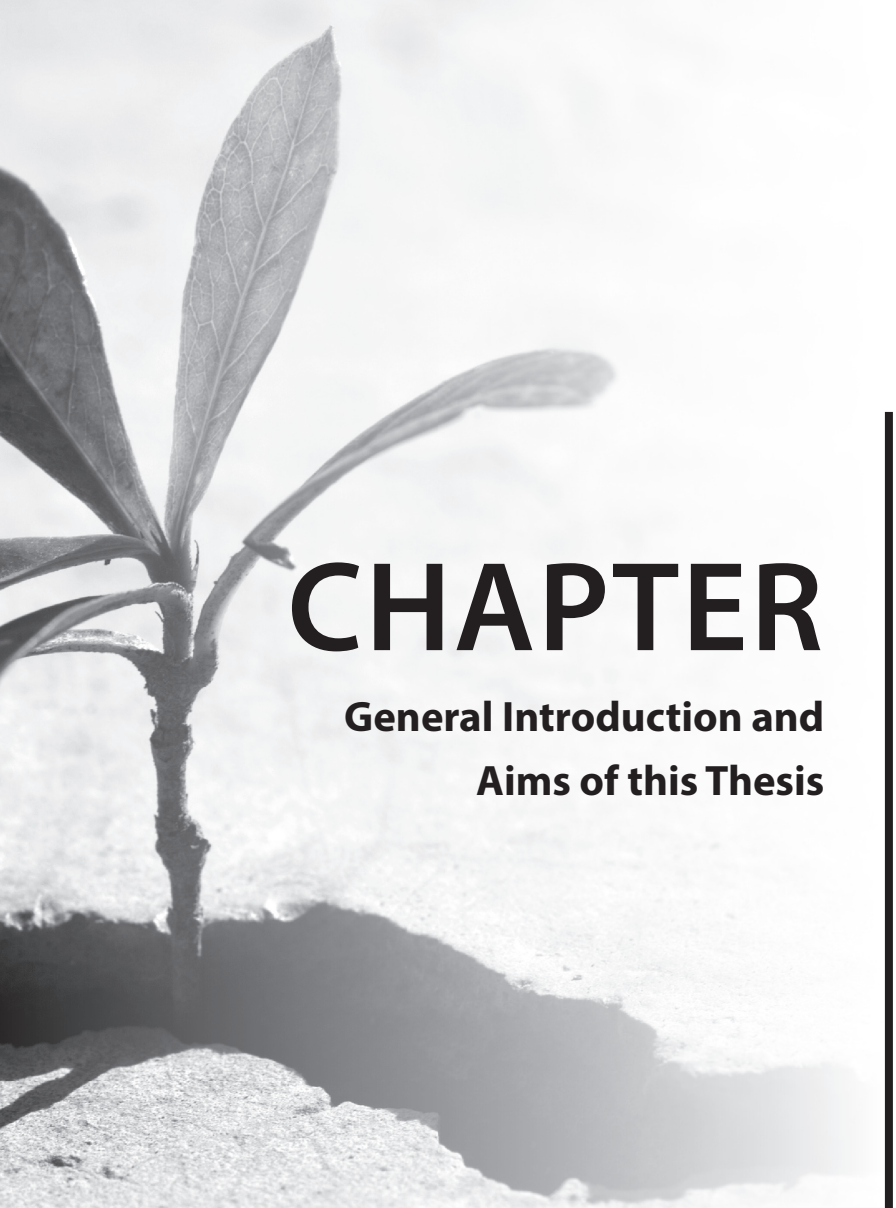
Prof. dr. U.H. Beuers
Prof. dr. H.G.D. Leuvenink
Prof. dr. J.M. Klaase

Paranimfen

Dr. L.C. Burlage

Drs. S. Karangwa

Table of Contents



CHAPTER 1

General Introduction and Aims of this Thesis

Orthotopic liver transplantation is the only curative treatment for patients with end-stage liver disease. Unfortunately, globally an immense gap exists between the demand and availability of donor livers for transplantation. This has led to the establishment of strict recipient criteria for transplantation, though still, many transplant candidates die awaiting a donor liver or are removed from the waiting list as they become too ill for transplantation. Of all patients listed for a liver transplant in the Eurotransplant region in 2017, 16% of patients deceased, nearly 4% became unfit to transplant and only 63% were actually transplanted.[1] For this reason, efforts to expand the donor liver pool are crucial. Aside from public campaigns aimed at increasing the number of registered donors, it is important to optimize the use of organs within the existing pool of donor livers. The present thesis focuses on the latter.

By loosening the criteria for liver donation, many more donor livers have become available for transplantation. Such livers are referred to as extended criteria donor (ECD) livers and include livers that are donated after circulatory death (DCD). Compared to donation after brain death (DBD), DCD livers inherently undergo a period of warm ischemia in which the organs are metabolically active but without circulation and provision of oxygen. This causes additional injury to the liver and results in higher complication rates after transplantation.[2-4] Nevertheless, the number of DCD liver transplantations is rising, with 40% of deceased donor liver transplants coming from DCD donors in the Netherlands in 2017.[1]

The most feared complication after DCD liver transplantation is the development of non-anastomotic strictures (NAS) of the biliary tree, also referred to as ischemic-type biliary lesions (ITBL). These strictures occur in 13 - 35% of DCD livers, compared to only 1 – 24% in DBD livers.[2,4-8] The development of NAS is multi-factorial, with the main causes including ischemia-related injury, immune-mediated injury, bile-salt toxicity and a lack of regenerative capacity of the biliary tree.[9-11]

Classically, surgeons could only base their decision to transplant a liver on donor characteristics, imaging results, macroscopic appearance of the liver and in some countries, also frozen histology sections of the liver. Machine perfusion, a technique that was re-introduced relatively recently after its initial publication in 1960s and 70s, is a technique in which donor livers are perfused *ex situ*, offering the possibility to objectively assess organ viability, a window for organ resuscitation and protection against ischemia and reperfusion injury. **Chapter 2** explains the various ways in which the donor liver pool can be expanded using ECD livers and machine perfusion.[12]

At 37°C, normothermic machine perfusion (NMP) renders the liver metabolically active, providing the possibility to objectively assess organ viability and allowing for the selection of livers that are suitable for transplantation.[13] NMP of human livers was first performed using a perfusion solution based on human blood products, including packed red blood cells (RBC) and fresh frozen plasma (FFP). The aim of **chapter 3** is to describe the procedure of NMP using the Liver Assist

(Organ Assist), which is further illustrated in the online video with this article, and share hepatocellular function and injury data that was obtained during NMP of human donor livers that were declined for transplantation.[14]

Due to the scarcity, logistical challenges and potential risk of transmitting infections, the aim of **chapter 4** was to find an alternative for the use of human blood products during NMP. In this chapter, we tested the feasibility and compared the efficacy of first replacing RBCs with an acellular hemoglobin-based oxygen carrier (HBOC-201, product name Hemopure), and as a second step replacing FFPs with gelofusine, a widely used gelatin-based colloid volume expander, and other nutrients.[15]

Hypothermic machine perfusion (HMP), performed at 10-12°C, has been shown to resuscitate mitochondria, replete adenosine triphosphate (ATP) levels and ameliorate ischemia-reperfusion injury after transplantation.[16] Especially in the case of DCD livers, oxygenated HMP prior to transplantation has been hypothesized to reduce the development of NAS. HMP can be performed using single perfusion through the portal vein, or dual through both the portal vein and hepatic artery. In particular dual hypothermic oxygenated machine perfusion (DHOPE) is expected to be most efficient as bile duct epithelial cells (cholangiocytes) are mostly dependent on arterial oxygenation.

A very important property of HBOC-201, compared to RBCs, is that it can be used at different temperatures. This allows for the possibility to first perfuse at lower temperatures using DHOPE, followed by a period of controlled oxygenated rewarming (COR) and lastly by NMP for viability assessment. The aim of the next study was therefore to test the feasibility and safety of performing machine perfusion at different temperatures using a HBOC-201-based perfusion solution. **Chapter 5** describes the results of a study on the first 7 human donor livers that were declined for transplantation nationally, of which 5, after being deemed viable in the NMP phase of the DHOPE-COR-NMP protocol, were transplanted.[17]

Several viability criteria have been established to assess hepatocellular injury of livers during NMP. These criteria include bile production, lactate clearance, pH buffering capacity, glucose metabolism, flows and macroscopic appearance of the liver. Criteria regarding bile duct viability, however, were lacking despite evidence that histological biliary injury prior to transplantation is a strong predictor for the later development of NAS. Therefore, the aim of **chapter 6** was to establish biomarkers of biliary viability during NMP by determining the value of biliary biomarkers in predicting the presence of histological biliary injury.[18] The assessed biomarkers were based on both cholangiocyte injury and function and included biliary pH, bicarbonate (secreted by cholangiocytes), glucose and the perfusate/bile glucose ratio (glucose is resorbed from bile by cholangiocytes), as well as biliary lactate dehydrogenase (LDH), reflecting biliary injury.

In order to establish other useful criteria during machine perfusion, we examined the release of microRNAs in NMP. MicroRNAs are small, non-coding RNAs that have emerged as sensitive, specific and stable markers for cell

function, stress and injury. The aim of **chapter 7** was to determine the value of hepatocyte-derived miRNA-122 and cholangiocyte-derived miRNA-222 in both perfusate and bile in predicting conventional hepato-cholangiocellular injury and function parameters during NMP.

Despite the emergence of machine perfusion as an alternative preservation technique, static cold storage (SCS), in which donor livers are transported on melting ice, remains the gold standard for liver preservation. The two most frequently used preservation solutions for static cold storage in clinical practice are University of Wisconsin (UW) solution and Histidine-tryptophan-ketoglutarate (HTK) solution. In the literature, conflicting results have been published regarding these preservation solutions' ability to preserve the liver and biliary tree. Furthermore, polyethylene glycols (PEGs) are non-immunogenic, non-toxic, water soluble and FDA-approved compounds with high flexibility, hydrophilicity, protein-rejecting properties and a greater hydrodynamic volume. PEGs have been shown to protect against ischemia reperfusion injury of different organs and we hypothesized that they would also play a significant role in protecting the biliary tree. Therefore, in **chapter 8**, human extrahepatic bile duct segments were preserved in various preservation solutions with added PEGs in order to determine the optimal preservation solution for protecting the bile ducts.

Lastly, machine perfusion has the potential to resuscitate donor livers. Injury to the peribiliary glands (PBG), which are niches of cholangiocyte progenitor cells embedded in the bile duct wall from which cholangiocytes regenerate, plays a role in the development of NAS. PBG, however, have only recently gained interest and have not been well described. Therefore, our aim was to develop a human *ex vivo* model to study human PBG in depth. **Chapter 9** describes the establishment of a novel technique, called precision-cut bile duct slices (PCBDS), and shows that progenitor cells in the PBG differentiate into mature cholangiocytes after severe biliary injury.[19] This technique involves the *in vitro* culturing of human bile duct slices and circumvents the use of laboratory animals.

In **chapter 10**, the chapters of the present thesis are summarized and discussed, followed by future perspectives to build on the present research. Lastly, this thesis concludes with a Dutch summary in **chapter 11**.

REFERENCES

1. Eurotransplant International Foundation. Annual Report 2017. 2017; Available at: https://www.eurotransplant.org/cms/mediaobject.php?file=Annual+Report+2017+HR10.pdf. Accessed 10 November, 2018.
2. de Vries Y, von Meijenfeldt FA, Porte RJ. Post-transplant cholangiopathy: Classification, pathogenesis, and preventive strategies. *Biochim Biophys Acta.* 2018;1864:1507-1515.
3. Blok JJ, Detry O, Putter H, et al. Longterm results of liver transplantation from donation after circulatory death. *Liver Transpl.* 2016;22:1107-1114.
4. Meurisse N, Vanden Bussche S, Jochmans I, et al. Outcomes of liver transplantations using donations after circulatory death: a single-center experience. *Transplant Proc.* 2012;44:2868-2873.
5. Jay CL, Lyuksemburg V, Ladner DP, et al. Ischemic cholangiopathy after controlled donation after cardiac death liver transplantation: a meta-analysis. *Ann Surg.* 2011;253:259-264.
6. Dubbeld J, Hoekstra H, Farid W, et al. Similar liver transplantation survival with selected cardiac death donors and brain death donors. *Br J Surg.* 2010;97:744-753.
7. Pine JK, Aldouri A, Young AL, et al. Liver transplantation following donation after cardiac death: an analysis using matched pairs. *Liver Transpl.* 2009;15:1072-1082.
8. Chan EY, Olson LC, Kisthard JA, et al. Ischemic cholangiopathy following liver transplantation from donation after cardiac death donors. *Liver Transpl.* 2008;14:604-610.
9. Op den Dries, Sanna Westerkamp, Andrie Karimian, Negin Gouw, Annette S H Bruinsma, Bote Markmann, James Lisman, Ton Yeh, Heidi Uygun, Korkut Martins,Paulo Porte, Robert. Injury to peribiliary glands and vascular plexus before liver transplantation predicts formation of non-anastomotic biliary strictures. *J Hepatol.* 2014;60:1172-1179.
10. Op den Dries S, Sutton ME, Lisman T, Porte RJ. Protection of bile ducts in liver transplantation: looking beyond ischemia. *Transplantation.* 2011;92:373-379.
11. Karimian, Negin Westerkamp,Andrie Porte, Robert. Biliary complications after orthotopic liver transplantation. *Current opinion in organ transplantation.* 2014;19:209-216.
12. Matton AP, Porte RJ. Opportunities for scientific expansion of the deceased donor pool. *Liver Transpl.* 2014;20 Suppl 2:S5.
13. op den Dries S, Karimian N, Sutton ME, et al. Ex vivo normothermic machine perfusion and viability testing of discarded human donor livers. *Am J Transplant.* 2013;13:1327-1335.

14. Karimian N, Matton AP, Westerkamp AC, et al. Ex Situ Normothermic Machine Perfusion of Donor Livers. *J Vis Exp.* 2015;(99):e52688. doi:e52688.

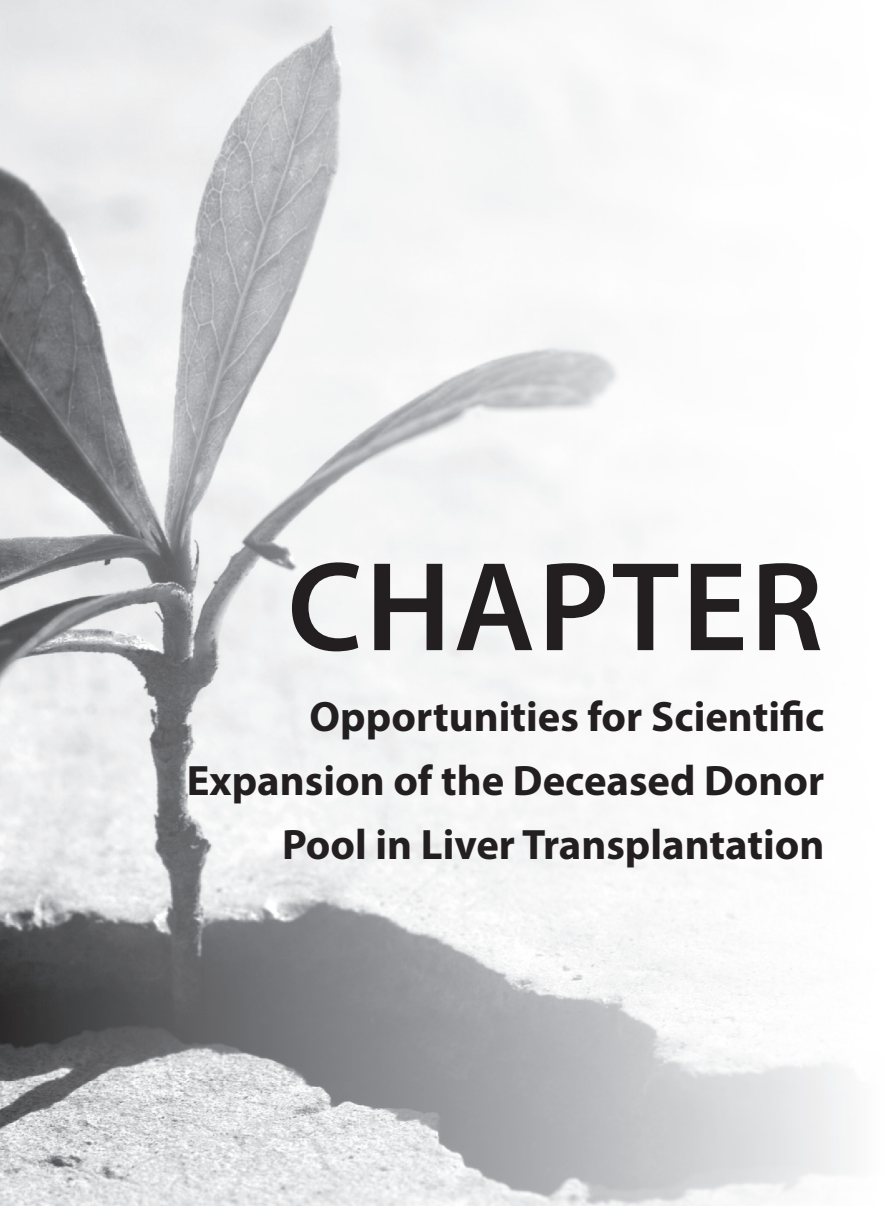
15. Matton APM, Burlage LC, van Rijn R, et al. Normothermic machine perfusion of donor livers without the need for human blood products. *Liver Transpl.* 2018;24:528-538.

16. Schlegel A, Kron P, Dutkowski P. Hypothermic Oxygenated Liver Perfusion: Basic Mechanisms and Clinical Application. *Curr Transplant Rep.* 2015;2:52-62.

17. de Vries Y, Matton APM, Nijsten MWN, et al. Pretransplant sequential hypo- and normothermic machine perfusion of suboptimal livers donated after circulatory death using a hemoglobin-based oxygen carrier perfusion solution. *Am J Transplant.* 2018.

18. Matton APM, de Vries Y, Burlage LC, et al. Biliary Bicarbonate, pH and Glucose Are Suitable Biomarkers of Biliary Viability During Ex Situ Normothermic Machine Perfusion of Human Donor Livers. *Transplantation.* 2018.

19. de Jong IEM, Matton APM, van Praagh JB, et al. Peribiliary glands are key in regeneration of the human biliary epithelium after severe bile duct injury. *Hepatology.* 2018.



CHAPTER 2

Opportunities for Scientific Expansion of the Deceased Donor Pool in Liver Transplantation

Alix P.M. Matton
Robert J. Porte

Liver Transplantation. 2014; 20 Suppl 2:S5.

ABSTRACT

The shortage of suitable donor livers in combination with the growing demand of liver transplants has led to the transplantation of increasing numbers of suboptimal livers from extended criteria donors (ECD). These livers have suffered more injury, resulting in significantly higher rates of graft failure and biliary complications. Further expansion of the pool of donor livers from deceased donors can only be obtained by a more effective and successful utilization of ECD livers such as livers obtained from donation after circulatory death (DCD). In most countries, the number of livers after donation after brain death (DBD) has been stable or even declining during recent years. Although DCD donation is increasingly considered in several countries, the percentage of DCD livers that are declined for transplantation is also increasing as the risk of early graft failure or graft-related complications is often too high. The current method of cold preservation and static cold storage of donor organs, which has been successful in low risk and optimal donor livers in the past, is insufficient for ECD or DCD donor livers. Those livers require more sophisticated methods of organ preservation to avoid or minimize any additional injury. To this end, machine perfusion of donor livers is receiving increasing attention as an alternative for graft preservation.

Various methods of machine perfusion have been and are being explored in experimental studies and the first clinical trials have been reported. The preliminary results are very promising and machine perfusion technology is going through a rapid development. Current data suggest that machine perfusion will provide an important new tool to optimize the utilization of ECD livers, such as livers obtained from DCD donors.

KEY POINTS

1. The shortage of suitable donor livers in combination with the growing demand for liver transplants has led to the transplantation of increasing numbers of suboptimal livers from extended criteria donors (ECD).
2. Further expansion of the pool of livers from deceased donors can be obtained only with a more effective and successful utilization of ECD livers, such as livers obtained from donation after circulatory death (DCD).
3. Although DCD donation is increasing in several countries, the percentage of DCD livers that are declined for transplantation is also increasing because the risk of early graft failure or graft-related complications is often too high.
4. The current method of cold preservation and static cold storage of donor organs is insufficient for ECD or DCD livers. These livers require more sophisticated methods of organ preservation to avoid or minimize any additional injury.
5. Various methods of machine perfusion have been and are being explored in experimental studies, and the first clinical trials have been reported. The preliminary results are very promising, and machine perfusion technology is undergoing rapid development. Current data suggest that machine perfusion will provide an important new tool to optimize the utilization of ECD livers, such as livers obtained from DCD donors.

INTRODUCTION

Over the past decades, liver transplantation has become a successful treatment for patients with end-stage liver disease. A considerable number of patients awaiting a liver transplantation, however, die on the waiting list due to the significant global discrepancy between the demand and availability of suitable donor livers. In an attempt to expand the number of liver transplantations, physicians are currently pushing the limits by performing split and live liver donations, as well as accepting livers from extended criteria donors (ECD).[1,2] In the Western hemisphere, the vast majority of livers used for transplantation, however, remain livers from deceased donors. Livers can be either donated after brain death (DBD) or circulatory death (DCD). While in most Western countries the number of DBD donations has remained steady or even declined over the last decade, the number of DCD donations has been increasing.[3] The proportion of liver transplantations performed using DCD livers increased from 1.1% in 1995 to 11.2% in 2010 in the United States.[4] In the United Kingdom, the percentage of DCD livers was 18% in 2012, while in the Netherlands it had increased to 38% in 2013.[5,6] Simultaneously, however, the number of unused DCD livers has also been increasing over the past decade as a result of too many concomitant risk factors for graft dysfunction, such as older donor age, high BMI, and diabetes mellitus in the donor.[4] It is not likely that expansion of the deceased donor pool will come from more DBD livers. The largest gain in the number of suitable deceased donor livers could potentially be obtained by maximizing the usage of DCD livers.

Other types of ECD livers that carry an increased risk of graft failure include steatotic livers, and livers from elderly donors.[7] A common characteristic of DCD and other types of ECD livers is that they are at greater risk of developing significant ischemia/reperfusion injury, leading to parenchymal, endothelial and/or biliary injury and subsequent dysfunction (**Table 1**).[8]

Table 1. The risk of donor livers from extended criteria donors.

Parenchymal injury
Higher rate of primary non-function
Higher rate of Initial poor function
Endothelial injury
Higher rate of early hepatic artery thrombosis
Microvascular/sinusoidal thrombosis
Biliary injury
Higher rate of ischemic cholangiopathy (non-anastomotic biliary strictures)

Biliary injury, in particular, is a significant problem in the transplantation of DCD livers. Bile duct injury can result in leakage and fibrosis of the larger bile ducts, leading to so called non-anastomotic biliary strictures (NAS; also known as ischemic-type biliary lesions or ischemic cholangiopathy).[9] The development of NAS has been reported in up to 30% of DCD livers, of which 50% of patients die or require re-transplantation.[9,10] The pathophysiology of NAS is not yet fully understood, however ischemia-related injury, immune-mediated injury, bile salt toxicity and a lack of regenerative capacity of the bile ducts are thought to be responsible for the development of NAS.[11] Ischemia-related injury plays the largest role as biliary epithelial cells are very susceptible to ischemia and are mainly dependent on the oxygen supply through the hepatic artery.[11] As a result of the increased rates of graft failure and biliary complications, the costs of DCD transplantations are about 30% higher compared to DBD transplantations.[12,13]

It has become evident that the current method of organ preservation, which is based on cooling, is not good enough to protect suboptimal donor livers such as those from ECD and DCD donors. The current standard method of organ preservation is static cold storage (SCS), in which the organ is flushed with ice-cold preservation fluid and stored at low temperature (0-4°C) in a box with melting ice during transportation from the donor hospital to the transplant center. The advantages of preserving livers using SCS are that it is easily executable, transportable and cheap. However, SCS also causes damage to the organ, frequently resulting in an unacceptably low quality liver graft in suboptimal ECD livers (**Figure 1**). During SCS livers are not oxygenated, resulting in adenosine triphophosphate (ATP) depletion, and cold-induced damage occurs. Furthermore, there is no means of assessing the functionality and

viability of the organ short before implantation. Therefore, optimization of the utilization of ECD livers should come from novel organ preservation methods. To this end, machine perfusion is the most promising technique.

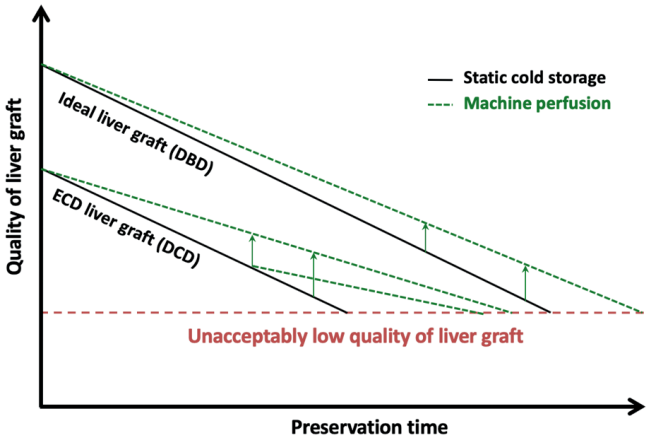


Figure 1. Schematic presentation of the decline in liver graft quality and viability during static cold storage (SCS) versus machine perfusion. In extended criteria donor (ECD) liver grafts, SCS results in a rapid decline in organ quality below a level at which it can still be transplanted with acceptable outcome. Machine perfusion has the potential to slow down the rate at which this decline in quality occurs, resulting in better organ viability after a given time period of preservation and potentially allowing for prolongation of the preservation time. In addition, machine perfusion may potentially allow for the resuscitation of liver grafts. *Abbreviations: DBD: donation after brain death; DCD: donation after circulatory death.*

MACHINE PERFUSION AS AN ALTERNATIVE PRESERVATION METHOD OF DONOR LIVERS

Experimental research has indicated that machine perfusion is superior to SCS in the preservation of donor livers. Machine perfusion leads to less ischemia / reperfusion injury[14], allows for prolonged preservation of the organs[15], and has the potential to restore and/or stimulate regeneration of damaged tissue. Moreover, machine perfusion also allows for the *ex vivo* assessment of graft viability [1,16] and provides the potential of (pharmacological) preconditioning.[17,18] In such a way, machine perfusion has the potential to increase the number and quality of donor organs. Disadvantages of machine perfusion, however, are that it is more complex and expensive to perform than SCS (**Table 2**).

Table 2. Advantages and disadvantages of static cold storage versus machine perfusion of donor livers.

Static Cold Storage	**Machine Perfusion**
Advantages	Advantages
Easy to execute	Reduced ischemia / reperfusion injury
Easy transportation	Prolonged preservation times
Low costs	Better *ex vivo* assessment of graft viability
	Potential for (pharmacological) preconditioning
	Potential to restore / regenerate damaged tissue
	Increase in numbers and quality of donor organs
Disadvantages	Disadvantages
No functional assessment	More complex
No oxygenation	More expensive than static cold storage
Cold induced injury	
Not good enough for ECD livers	

Abbreviations: ECD, extended criteria donor.

The technique of machine preservation and perfusion is still evolving and several questions remain unanswered (**Table 3**). It remains to be determined what is the optimal temperature at which organs should be perfused, whether or not an oxygen carrier should be added to the perfusion fluid, how long and at what pressure livers should be perfused, and finally what is the optimal timing of machine perfusion in the time period between procurement and transplantation. Furthermore, reliable criteria for the viability assessment of donor livers have yet to be confirmed in the clinical setting. With respect to the timing, machine perfusion can be performed in the donor (normothermic regional perfusion)[19], immediately after procurement, and/or during or after the storage and transportation of the organ (**Figure 2**).

Table 3. The various temperatures and timing of liver machine perfusion.

Technique	***Temperature***
Hypothermic perfusion	0 – 15°C
Subnormothermic perfusion	15 – 35°C
Normothermic perfusion	37°C
Timing	
In the donor (normothermic regional perfusion)	
Immediately after procurement	
During or after storage and transportation	

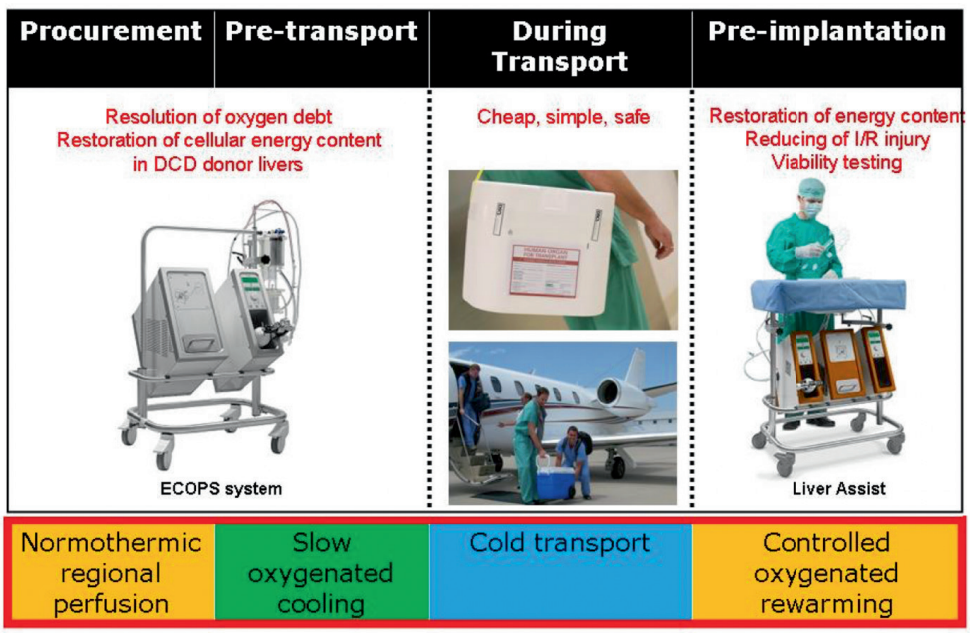


Figure 2. Schematic overview of the various combinations and types of liver machine perfusion that have been described. The optimal combination of different machine perfusion techniques remains to be determined and may very per type of donor livers.

A large number of animal experiments have been performed to explore the feasibility and potential benefits of machine perfusion. In one study, hypothermic oxygenated machine perfusion of porcine DCD livers has been shown to prevent arteriolonecrosis of the peribiliary vascular plexus, potentially reducing posttransplant biliary ischemia and leading to faster and more efficient regeneration of the biliary epithelium.[20] Another study recently suggested that

normothermic machine perfusion also improves biliary epithelial regeneration in a pig model of DCD livers.[21] Moreover, there is evidence from an experimental study that gradual warming up of DCD liver grafts is superior to SCS and hypothermic machine perfusion.[22]

The first clinical application of liver machine perfusion was reported by Guarerra *et al.* in 2010.[23] This study in 20 patients involved dual (portal vein and hepatic artery) non-oxygenated hypothermic machine perfusion of the donor liver prior to transplantation. This method resulted in lower cellular damage markers and less ischemia/reperfusion injury after transplantation.[24,25] A second clinical trial has been reported by Dutkowski *et al.* in 2014.[26] These investigators have reported on the feasibility and safety of hypothermic oxygenated machine perfusion through the portal vein in DCD livers and reported excellent early outcome after transplantation in eight patients. Our group has recently initiated a pilot study on hypothermic oxygenated machine perfusion using dual perfusion of both the portal vein and hepatic artery in DCD livers (Netherlands Trial Registry, NTR4493; www.trialregister.nl). This trial is still ongoing, but the initial results are encouraging.

More clinical trials will be needed to elucidate whether the different methods of machine perfusion are beneficial in the prevention of graft failure and biliary complications after transplantation, especially in DCD liver grafts. A multi-center randomized controlled clinical trial will soon be initiated by our group to compare hypothermic dual oxygenated machine perfusion with SCS in DCD liver grafts. Primary endpoint in this trial will be the development of NAS. Another randomized controlled clinical trial has been initiated to evaluate the effects of hypothermic oxygenated perfusion through the portal vein alone in DBD livers (ClinicalTrials.gov, ID: NCT01317342). In addition, a randomized controlled clinical trial on normothermic machine perfusion (Controlled-Trials.com, ID: ISRCTN39731134) will soon be launched, and a pilot study of normothermic regional perfusion in DCD organ donors was recently completed.[19]

SUMMARY, FUTURE PERSPECTIVE AND CHALLENGES

The largest potential gain to be obtained in expanding the deceased donor pool lies in the utilization of ECD livers, as there is an increasing number of unused DCD livers compared to a stable or even declining number of DBD livers. It is a crucial that measures are taken to improve the quality of ECD donor livers, especially of livers that are obtained from DCD donors. DCD livers form already a substantial proportion of all liver transplantations performed in countries such as the United Kingdom and the Netherlands. Increased utilization of DCD livers may contribute significantly to the number of available deceased donor livers in other countries as well. Moreover, improving the quality of DCD livers could lead to a substantial reduction in the rate of early graft failure after transplantation. Assessing the viability of livers, in particular suboptimal ECD livers, prior to transplantation would also lead to a more careful selection of transplantable livers. This would theoretically not only result in better outcomes after

transplantation, but also to the expansion of the number of available donor livers. A common characteristic of DCD and other types of ECD livers is that these livers have suffered a higher degree of injury prior to transplantation, explaining the higher risk of early graft failure after transplantation. It has become evident that the current method of organ preservation, which is based on cooling and static cold storage, is not sufficient to adequately preserve these preinjured ECD and DCD livers. If we want to improve the numbers and success rate of transplantation of livers from DCD and ECD donors, we have to introduce more sophisticated methods of organ preservation. Machine perfusion is receiving increasing attention as an alternative preservation method (**Figure 1**). Experimental studies have indicated that machine perfusion provides better protection of DCD livers and the first clinical trials have been initiated and reported. The potential role of machine perfusion in expanding the deceased donor pool is two-fold. Firstly, machine perfusion can be used for the resuscitation of liver grafts prior to transplantation, thereby not only improving the quality of DCD transplants but also increasing the number of transplantable ECD livers. Secondly, machine perfusion can be used to assess the function and viability of liver grafts prior to transplantation, thereby allowing for the careful selection of transplantable livers out of a pool of currently discarded ECD livers. Various protocols of machine perfusion have been described, but it remains to be established which method provides the best protection of DCD livers (**Figure 2**). The optimal and most cost-effective strategy of liver preservation based on machine perfusion technology may be a combination of different techniques for the different phases of organ preservation and transportation (**Figure 3**). An important outcome parameter to determine the efficacy of machine perfusion will be the degree of biliary injury and the rate of biliary complications (i.e. NAS) after DCD livers transplantation.

Procurement	Pre-transport	During Transport	Pre-implantation
Cold flush out	Cold storage	Cold transport	Cold
Cold flush out	Cold storage	Cold transport	Hypothermic (oxygenated) perfusion
Cold flush out	Cold storage	Cold transport	Controlled oxygenated rewarming perfusion
Cold flush out	Oxygenated perfusion (1-2 hr)	Cold transport	Controlled oxygenated rewarming perfusion
Cold flush out	(Sub) Normothermic perfusion	(Sub) Normothermic perfusion	(Sub) Normothermic perfusion
Normothermic regional perfusion	Slow oxygenated cooling perfusion	Cold transport	Controlled oxygenated rewarming perfusion

Figure 3. The optimal and most cost-effective strategy of liver preservation based on machine perfusion technology may be a combination of different techniques for the different phases of organ preservation and transportation, as depicted in this figure.

REFERENCES

1. Op den Dries S, Karimian N, Sutton ME, Westerkamp AC, Nijsten MW, Gouw AS, et al. Ex vivo normothermic machine perfusion and viability testing of discarded human donor livers. Am J Transplant 2013;13:1327-1335.
2. Barshes NR, Horwitz IB, Franzini L, Vierling JM, Goss JA. Waitlist mortality decreases with increased use of extended criteria donor liver grafts at adult liver transplant centers. Am J Transplant 2007;7:1265-1270.
3. Dominguez-Gil B, Haase-Kromwijk B, Van Leiden H, Neuberger J, Coene L, Morel P, et al. Current situation of donation after circulatory death in European countries. Transpl Int 2011;24:676-686.
4. Orman ES, Barritt AS,4th, Wheeler SB, Hayashi PH. Declining liver utilization for transplantation in the United States and the impact of donation after cardiac death. Liver Transpl 2013;19:59-68.
5. Johnson RJ, Bradbury LL, Martin K, Neuberger J, UK Transplant Registry. Organ donation and transplantation in the UK-the last decade: a report from the UK national transplant registry. Transplantation 2014;97 Suppl 1:S1-S27.
6. Nederlands Transplantatie Stichting Jaarverslag 2013 (Annual Report Dutch Transplantation Foundation). www.transplantatiestichting.nl
7. Merion RM, Goodrich NP, Feng S. How can we define expanded criteria for liver donors? J Hepatol 2006;45:484-488.
8. Monbaliu D, Pirenne J, Talbot D. Liver transplantation using Donation after Cardiac Death donors. J Hepatol 2012;56:474-485.
9. Op den Dries S, Sutton ME, Lisman T, Porte RJ. Protection of bile ducts in liver transplantation: looking beyond ischemia. Transplantation 2011;27;92:373-379.
10. Dubbeld J, Hoekstra H, Farid W, Ringers J, Porte RJ, Metselaar HJ, et al. Similar liver transplantation survival with selected cardiac death donors and brain death donors. Br J Surg 2010;97:744-753.
11. Karimian N, Westerkamp AC, Porte RJ. Biliary complications after orthotopic liver transplantation. Curr Opin Organ Transplant 2014 Jun;19(3):209-216.
12. Jay CL, Lyuksemburg V, Kang R, Preczewski L, Stroupe K, Holl JL, et al. The increased costs of donation after cardiac death liver transplantation: caveat emptor. Ann Surg 2010;251:743-748.
13. van der Hilst CS, Ijtsma AJ, Bottema JT, van Hoek B, Dubbeld J, Metselaar HJ, et al. The price of donation after cardiac death in liver transplantation: a prospective cost-effectiveness study. Transpl Int 2013;26:411-418.
14. Schlegel A, Rougemont O, Graf R, Clavien PA, Dutkowski P. Protective mechanisms of end-ischemic cold machine perfusion in DCD liver grafts. J Hepatol 2013;58:278-286.

15. St Peter SD, Imber CJ, Lopez I, Hughes D, Friend PJ. Extended preservation of non-heart-beating donor livers with normothermic machine perfusion. Br J Surg 2002;89:609-616.

16. Sutton ME, op den Dries S, Karimian N, Weeder PD, de Boer MT, Wiersema-Buist J, et al. Criteria for viability assessment of discarded human donor livers during ex vivo normothermic machine perfusion. Plos One 2014;9:e110642.

17. Van Raemdonck D, Neyrinck A, Rega F, Devos T, Pirenne J. Machine perfusion in organ transplantation: a tool for ex-vivo graft conditioning with mesenchymal stem cells? Curr Opin Organ Transplant 2013;18:24-33.

18. Wang B, Zhang Q, Zhu B, Cui Z, Zhou J. Protective effect of gadolinium chloride on early warm ischemia/reperfusion injury in rat bile duct during liver transplantation. PLoS One 2013;8:e52743.

19. Butler AJ, Randle LV, Watson CJ. Normothermic regional perfusion for donation after circulatory death without prior heparinization. Transplantation 2014;97:1272-1278.

20. Op den Dries S, Sutton ME, Karimian N, de Boer MT, Wiersema-Buist J, Gouw AS, et al. Hypothermic oxygenated machine perfusion prevents arteriolonecrosis of the peribiliary plexus in pig livers donated after circulatory death. PLoS One 2014;9:e88521.

21. Liu Q, Nassar A, Farias K, Buccini L, Baldwin W, Mangino M, et al. Sanguineous normothermic machine perfusion improves hemodynamics and biliary epithelial regeneration in donation after cardiac death porcine livers. Liver Transpl 2014;20:987-999.

22. Minor T, Efferz P, Fox M, Wohlschlaeger J, Luer B. Controlled oxygenated rewarming of cold stored liver grafts by thermally graduated machine perfusion prior to reperfusion. Am J Transplant 2013;13:1450-1460.

23. Guarrera JV, Henry SD, Samstein B, Odeh-Ramadan R, Kinkhabwala M, Goldstein MJ, et al. Hypothermic machine preservation in human liver transplantation: the first clinical series. Am J Transplant 2010;10:372-381.

24. Tulipan JE, Stone J, Samstein B, Kato T, Emond JC, Henry SD, et al. Molecular expression of acute phase mediators is attenuated by machine preservation in human liver transplantation: preliminary analysis of effluent, serum, and liver biopsies. Surgery 2011;150:352-360.

25. Guarrera JV, Henry SD, Chen SW, Brown T, Nachber E, Arrington B, et al. Hypothermic machine preservation attenuates ischemia/reperfusion markers after liver transplantation: preliminary results. J Surg Res 2011;167:e365-73.

26. Dutkowski P, Schlegel A, de Oliveira M, Mullhaupt B, Neff F, Clavien PA. HOPE for human liver grafts obtained from donors after cardiac death. J Hepatol 2014;60:765-772.

3

CHAPTER

Ex Situ Normothermic Machine Perfusion of Donor Livers

(Video Article)

Negin Karimian
Alix P.M. Matton
Andrie C. Westerkamp
Laura C. Burlage
Sanna op den Dries
Henri G.D. Leuvenink
Ton Lisman
Korkut Uygun
James F. Markmann
Robert J. Porte

Journal of Visualized Experiments. 2015; 26:e52688.

The video component of this article can be found at
http://www.jove.com/video/52688/

ABSTRACT

In contrast to conventional static cold preservation (0-4 °C), *ex situ* machine perfusion may provide better preservation of donor livers. Continuous perfusion of organs provides the opportunity to improve organ quality and allows *ex situ* viability assessment of donor livers prior to transplantation. This video article provides a step by step protocol for *ex situ* normothermic machine perfusion (37 °C) of human donor livers using a device that provides a pressure and temperature controlled pulsatile perfusion of the hepatic artery and continuous perfusion of the portal vein. The perfusion fluid is oxygenated by two hollow fiber membrane oxygenators and the temperature can be regulated between 10 °C and 37 °C. During perfusion, the metabolic activity of the liver as well as the degree of injury can be assessed by biochemical analysis of samples taken from the perfusion fluid. Machine perfusion is a very promising tool to increase the number of livers that are suitable for transplantation.

INTRODUCTION

The current method of organ preservation in liver transplantation is flush out with and subsequent storage of donor livers in cold (0-4 °C) preservation fluid (such as University of Wisconsin solution or Histidine-Tryptophan-Ketoglutarate solution). This method is referred to as static cold storage (SCS). Although the metabolic rate of livers at 0-4 °C is very low, there is still demand for 0.27 µmol oxygen/min/g liver tissue, which cannot be provided during SCS.[1] The conventional method of SCS, therefore, results in some degree of (additional) injury of donor livers. While this amount of preservation injury is not a problem in donor livers of good quality, it can become a critical and limiting factor in suboptimal livers that have already suffered some degree of injury in the donor. For this reason, livers with suboptimal quality or so-called extended criteria donor (ECD) livers are frequently rejected for transplantation as the risk of early graft failure is considered to be too high. High rates of delayed graft function, primary non-function, and non-anastomotic biliary strictures (NAS) have been described in recipients of livers from donation after circulatory death (DCD), older donors or recipients of steatotic grafts.[2] NAS are a major cause of morbidity and mortality after liver transplantation. NAS may occur in both extra- and intrahepatic donor bile ducts and can be accompanied by intraductal biliary sludge and cast formation.[3,4] Although the etiology of NAS is thought to be multifactorial, ischemia/reperfusion injury of the bile ducts during graft preservation and transplantation has been identified as a major underlying mechanism.[2,5] Transplantation of a DCD graft has been identified as one of the strongest risk factors for the development of NAS. The combination of a period of warm ischemia in a DCD donor, cold ischemia during organ preservation, and subsequent reperfusion injury in the recipient is thought to be responsible for irreversible injury of the bile ducts, which, in combination with a poor regenerative capacity of the bile ducts, results in fibrotic scarring and narrowing of the bile ducts after liver transplantation.[2,5] NAS have been reported in up to 30% of patients receiving a DCD liver.[6-8] It has become clear that the current method of SCS of liver grafts for transplantation is insufficient for pre-injured ECD livers such as those from DCD donors. Alternative methods are needed to increase and optimize the use of ECD livers for transplantation.

Machine perfusion (MP) is a method of organ preservation that may provide better preservation of donor organs, compared to SCS. MP could be especially relevant for the preservation of ECD grafts. An important advantage of MP is the possibility to provide oxygen to the graft during the preservation period. MP can be performed at various temperatures, which have been classified as hypothermic (0-10 °C), subnormothermic (10-36 °C) and normothermic (36-37 °C) MP (NMP). Depending on the temperature used for MP, the type of perfusion fluid has to be adjusted and with increasing temperature more oxygen should be supplied. The first clinical application of MP in human liver transplantation was based on hypothermic perfusion without active oxygenation of the perfusion fluid.[9,10] In animal models, hypothermic oxygenated MP (0-10 °C) has been shown to have protective effects against

ischemia/reperfusion injury of liver grafts[11] and to provide better preservation of the peribiliary vascular plexus of the bile ducts.[12] Subnormothermic oxygenated MP at 20 °C or 30 °C has also been studied in animal models and was shown to provide earlier recovery of graft function of DCD livers, compared to SCS.[13,14] The feasibility of subnormothermic oxygenated MP of human livers was recently reported in a series of seven discarded human donor livers.[15] NMP (37 °C) allows for the assessment of graft viability and functionality prior to transplantation.[16,17] Additionally, MP allows for gradual rewarming of the liver graft before transplantation, which has been demonstrated to facilitate recovery and resuscitation of the graft.[18]

The perfusion device used in the current protocol for hepatic machine perfusion enables dual perfusion (via the portal vein and the hepatic artery) using two centrifugal pumps, that provide a continuous portal flow and a pulsatile arterial flow. The system is pressure-controlled, allowing auto-regulation of the flow through the liver, depending on the intrahepatic resistance. Two hollow fiber membrane oxygenators allow for the oxygenation of the liver graft, as well as for the removal of CO_2. The temperature can be set based on the intended type of MP (minimum temperature of 10 °C). Flow, pressure and temperature are displayed on the device in real-time allowing a continuous control of the perfusion process. A new sterile disposable set of tubing, reservoir and oxygenators is available for the perfusion of each graft (**Figure 1**).

The aim of this video article is to provide a step by step protocol for *ex situ* normothermic machine perfusion of human donor livers using this newly developed liver perfusion machine.

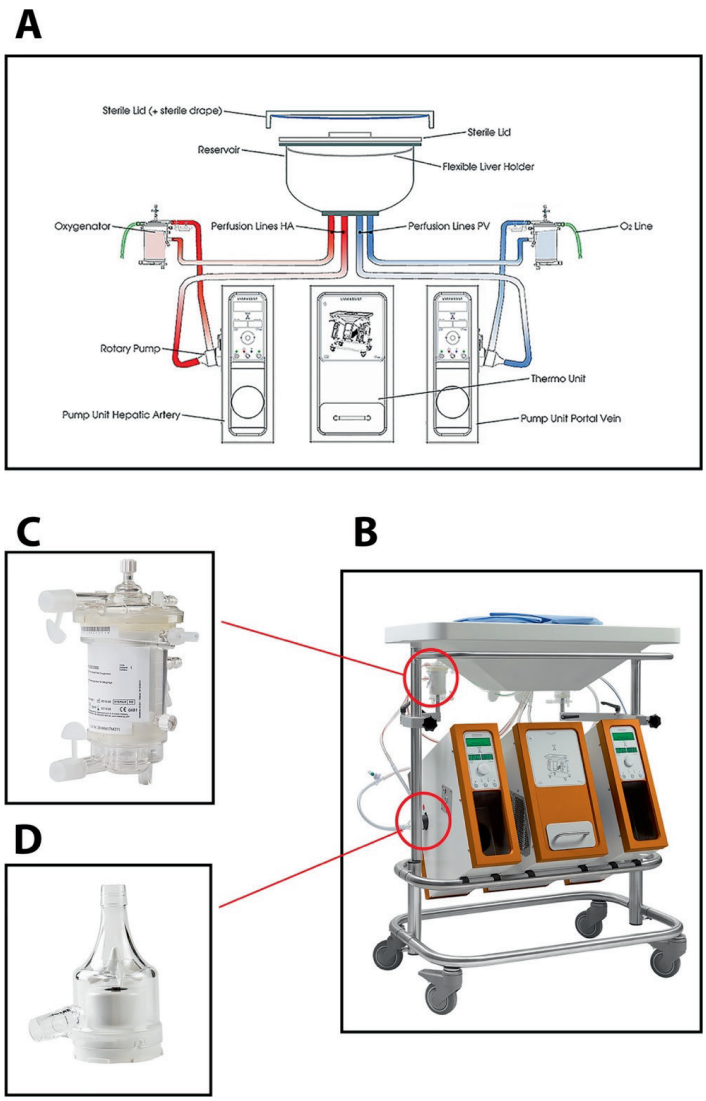


Figure 1: (**A**) A schematic drawing, (**B**) a photo of the perfusion machine, (**C**) a closer view of the oxygenator, and (**D**) centrifugal pump used for normothermic perfusion of human donor livers.

PROTOCOL

This protocol has been approved by the Medical Ethical Committee (Medisch Ethische Toetsingscommissie) of the University Medical Center Groningen, the Netherlands.

1. Preparation of the Perfusion Fluid

Note: The total volume of the perfusion fluid prepared for normothermic machine perfusion according to this protocol is 2,233 ml and the targeted osmolarity of the perfusion fluid is 302 mOsmol/L.

a. From the components of the perfusion fluid described in **Table 1**, keep the human packed red blood cells, fresh frozen plasma and human albumin separated. Mix the rest of the components in a sterile manner and store the solution in a sterile bag for transportation to the operating room (OR). Do this in a sterile environment (ideally a Good Manufacturing Practice facility) or in a laminar flow cabinet in a culture room.
b. Transfer human packed red blood cells (840 ml), fresh frozen plasma (930 ml), human albumin 200 g/L (100 ml) and the solution prepared in step 1.1 to the OR to be administered to the perfusion device.

Table 1: Components of the perfusion fluid.[16]

Components	Quantity
Packed red blood cell (Hematocrit 60%)	840 ml
Fresh frozen plasma	930 ml
Human albumin 200 g/L (Albuman, Sanquin)	100 ml
Modified parenteral nutrition (Clinimix N17G35E, Baxter International Inc.)	7.35 ml
Multivitamins for infusion (Cernevit, Baxter international Inc.)	7 µl
Concentrated trace elements for infusion (Nutritrace , B. Braun Melsungen AG)	7.35 ml
Metronidazol for i.v. administration (5 mg/ml) (Flagyl, Sanofi-Aventis)	40 ml
Cefazolin 1,000 mg flask 5 ml powder for i.v. administration (Servazolin,	2 ml
Fast-acting insulin (100 IU/ml) (Actrapid®, Novo Nordisk)	20 ml
Calcium glubionate, intravenous solution 10%, 137.5 mg/ml (Sandoz)	40 ml
Sterile H_2O	51.3 ml
NaCl 0.9% solution	160 ml
Sodium bicarbonate 8.4% solution	31 ml
Heparin 5,000 IE/ml for i.v. administration	4 ml
Total	2,233 ml

2. Priming of the Perfusion Device

a. Add the components of the perfusion fluid, including the human packed red blood cells, fresh frozen plasma, human albumin and the solution prepared in step 1.1 to the machine via the connector on top of the oxygenators and remove all the air bubbles from the tubing.
b. Switch on the venous pump and follow the manufacturer's instructions on the screen. Then turn on the arterial pump and follow the manufacturer's instructions on the screen.
c. Null the pressure meters against atmospheric pressure by following the instructions on the screen. This ensures that the pressure measured during the perfusion is the real pressure at the level of the portal vein and the hepatic artery.
d. Start the oxygenation using carbogen (95% O_2 + 5% CO_2) at a flow rate of 4 L/min. The air flow will be divided among the two oxygenators (2 L/min per oxygenator) and this should result in a $pO_2$of around 60 kPa (or 450 mmHg) in the perfusion fluid. For longer perfusions, it is advisable to use separate sources of oxygen and carbon dioxide. This allows for small adjustments in the O_2/CO_2ratio, which can be used to adjust the pH and pCO_2 of the perfusion fluid.
e. Take a perfusion sample for blood gas measurement 15-20 min after the device has been primed and monitor the pH and electrolytes accordingly. NOTE: Be sure to discard about 3 ml of perfusion fluid before taking the samples, as this fluid is in the peripheral tubing and does not represent the perfusion fluid in the system. Add an 8.4% sodium bicarbonate solution for buffering capacity, aiming for a physiological pH (7.35-7.45). For example, add 25-35 ml of an 8.4% sodium bicarbonate solution and check the pH and bicarbonate levels in the perfusion fluid by taking samples for blood gas measurement at regular intervals.

3. Procurement and Preparation of Donor Livers

Note: Procure the organ using the standard technique of *in situ* cooling and flush out with cold preservation fluid (0-4 °C)[19]. To facilitate cannulation of the artery, leave a segment of the supratruncal aorta attached to the hepatic artery (**Figure 2A**).

a. Flush out the bile ducts with the preservation fluid (*i.e.,* University of Wisconsin solution). Ligate the cystic duct with a surgical suture.
b. Pack and store the organ in a standard sterile donor organ bag and box with crushed ice for subsequent transportation to the MP center.
c. Start the back table procedure immediately upon arrival of the donor liver in the operating room.
    1. Take a sample of at least 10 ml of the preservation fluid for microbiological testing.

2. Remove the diaphragmatic attachments to the bare area of the liver as well as any remaining cardiac muscle from the upper cuff of the vena cava with surgical scissors.
3. Dissect the artery and portal vein using dissecting scissors and ligate side branches using surgical sutures or hemoclips.
4. Close the distal end of the supratruncal aorta segment using a non-absorbable monofilament suture (*e.g.,* 3-0 Prolene). Insert the arterial cannula into the proximal end of the supratruncal aorta and secure with sutures (**Figure 2A**). Use the cannula provided in the disposable package as supplied by the manufacturer of the perfusion device.
5. Insert the venous cannula in the portal vein and secure with sutures. Use the cannula provided in the disposable package. The hepatic vein remains uncannulated.
6. Flush out the bile duct with the preservation solution. Insert a silicon catheter into the bile duct and secure with sutures. NOTE: Do not insert the catheter too deeply into the bile duct as this may cause injury to the biliary epithelium.
7. Flush out the liver with 0.9% NaCl solution via the portal vein cannula as follows:
    a. If the graft has been preserved in University of Wisconsin solution as the preservation solution, flush out the liver with 2,000 ml of cold (0-4 °C) 0.9% NaCl solution followed by 500 ml of warm (37 °C) 0.9% NaCl solution.
    b. If the graft has been preserved in Histidine-Tryptophan-Ketoglutarate solution as the preservation solution, flush out the liver with 1,000 ml of cold (0-4 °C) 0.9% NaCl solution followed by 500 ml of warm (37 °C) 0.9% NaCl solution. The purpose of the warm flush is to prevent a significant drop in the temperature of the perfusion fluid.
    c. Perform the warm flush immediately before connecting the liver to the perfusion device. NOTE: Always keep the duration between warm flush and start of NMP less than 1-2 min.

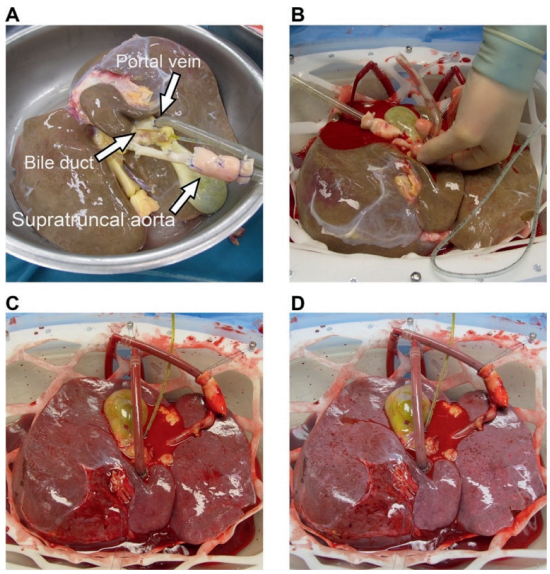


Figure 2: (**A**) Pictures of a human donor graft that has been prepared on the back table and (**B**-**D**) was subsequently perfused normothermically. (**A**) The arterial cannula is inserted into the surpratruncal aorta and the venous cannula is inserted into the portal vein. The bile duct is cannulated with a silicon biliary catheter. (**B**) The liver is positioned in the organ chamber with its anterior surface facing downwards and cannulas are connected to the tubings of the perfusion device. (**C**) 30 min after the start of normothermic machine perfusion. (**D**) 6 h after the start of normothermic machine perfusion. During operation the organ chamber is covered by a transparent cover to maintain a sterile moist environment for the liver (not shown in these pictures).

4. Normothermic Machine Perfusion

a. Position the liver in the organ chamber with the anterior surface facing downward. Immediately connect the liver to the primed perfusion device by connecting the portal vein cannula to the portal inflow tube of the perfusion device and the arterial cannula to the arterial inflow tube of the device.
b. Start perfusion on both portal and arterial side by following the manufacturer's instructions on the screen. Set the mean arterial pressure at 70 mmHg and the mean portal venous pressure at 11 mmHg.
c. Take perfusion fluid samples every 30 min for immediate analysis of blood gas parameters (pO_2, pCO_2, sO_2, HCO_2^- and pH) and biochemical parameters (glucose, calcium, lactate, potassium and sodium) using a conventional blood gas analyzer. Be sure to discard about 3 ml of perfusion fluid before taking the samples, as this fluid is in the peripheral tubing and does not represent the perfusion fluid in the system.
    a. To take these samples aspirate the perfusion fluid using a 1 ml syringe from the sampling connectors that are part of the disposable tubing set of the perfusion device. For each sample use a new syringe and

immediately remove any air bubbles from the syringe upon aspiration of perfusion fluid. Then insert the syringe in the blood gas analyzer and follow the manufacturer's instructions provided in the manual of the analyzer.

d. Collect plasma from the perfusion fluid, freeze and store at -80 °C for determination of alkaline phosphatase (AlkP), gamma-glutamyl transferase (gamma-GT), alanine aminotransferase (ALT), urea and total bilirubin. Collect plasma after 5 min of centrifugation of the perfusion fluid at 1,500 x g and 4 °C.

REPRESENTATIVE RESULTS

12 human livers that were declined for transplantation due to various reasons were used after obtaining informed consent for research from donor families. Donor characteristics are described in **Table 2**. The human donor livers were perfused normothermically for 6 h by using the protocol described in this paper. The quality of the liver grafts were evaluated by monitoring the macroscopic homogeneity of liver perfusion (**Figure 2A**-**D**). The hemodynamics of the livers were assessed by monitoring the changes in the arterial and portal flows. An initial increase in hepatic artery and portal vein flows and subsequent stabilization of the flows were observed, resulting in a mean arterial flow of 256 ± 16 ml/min (mean ± SEM) and a mean portal vein flow of 748 ± 34 ml/min (mean ± SEM) at 6 h, indicating stable hemodynamics of livers during perfusion (**Figure 3A**). Blood gas analysis of the perfusate samples collected from arterial perfusion fluid was used to monitor the status of oxygenation in the perfusion fluid. Oxygenation with carbogen (95% O_2 and 5% CO_2) at a flow of 4 L/min resulted in a continuous O_2 saturation of 100%. **Figure 3B** displays the oxygenation of the perfusion fluid and subsequent extraction of carbon dioxide in our experience.

Table 2: Donor characteristics.

Donor characteristics (N = 12)	**Number (%) or Median (IQR)**
Age (years)	61 (50-64)
Gender (male)	8 (67%)
Type of donor	
DCD, Maastricht type III	10 (83%)
DBD	2 (17%)
Body mass index (BMI)	27 (25-35)
Reason for rejection	
DCD+ age >60 years	5 (41%)
DCD+ high BMI	3 (25%)
DCD+ various reasons*	2 (17%)
Severe steatosis	2 (17%)
Preservation solution	
UW solution	6 (50%)
HTK solution	6 (50%)
Donor warm ischemia time in DCD (min)	14 (17 - 20)
Cold ischemia time (min)	389 (458-585)
Donor risk index (DRI)	2.35 (2.01-2.54)

* donor history of intravenous drug abuse for one graft and prolonged donor sO_2 <30% after withdrawal of life support for another graft. *Abbreviations: DCD, donation after circulatory death; DBD, donation after brain death; UW, University of Wisconsin; HTK, Histidine-tryptophan-ketoglutarate*

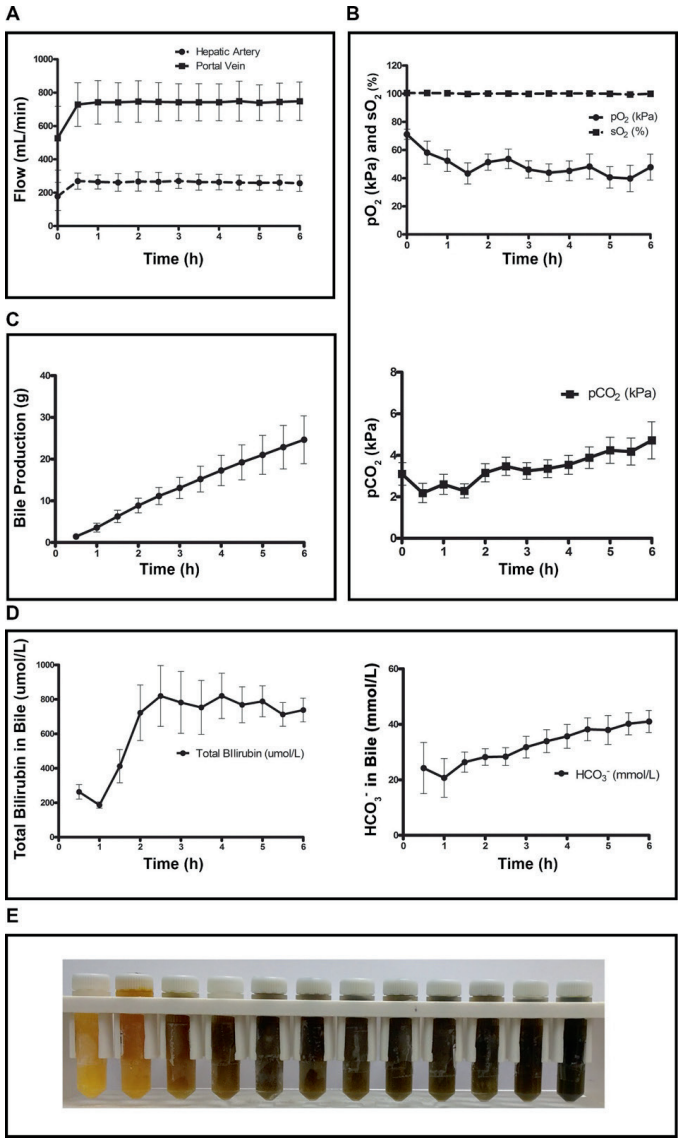


Figure 3: Graphical presentation of perfusion parameters and biochemical analyses of both the perfusion fluid and bile during 6 h of normothermic machine perfusion of 12 human livers. (**A**) Changes in arterial and portal flow. (**B**) Evolution of oxygenation characteristics and pCO_2 during 6 h of normothermic perfusion. (**C**) Cumulative bile production during perfusion. (**D**) Increasing concentrations of bilirubin and bicarbonate in bile samples taken during machine perfusion. (**E**) Microcentrifuge tubes containing bile from a representative graft, demonstrating a gradual darkening shade of the bile color over time. Data are expressed as mean ± SEM.

Bile production was used as an indicator of liver function. Metabolically functioning livers produced bile during NMP, resulting in a mean total bile production of 24.6 ± 6 g after 6 h of NMP (**Figure 3C**). An increase in the concentration of total bilirubin and bicarbonate in the bile represented an improvement in the quality of the bile produced during NMP (**Figure 3D, E**). Liver tissue ATP content as an indicator of mitochondrial function increased during NMP, resulting in mean ATP of 30 ± 5 µmol/g protein (mean ± SEM) after 6 h of NMP (**Figure 4**). Biochemical analysis of hepatic injury markers in the perfusion fluid, such as ALT, AlkP, gamma-GT and potassium, was used to assess the amount of graft injury. Stable concentrations of hepatic injury markers reflected minimal injury of the grafts during perfusion (**Figure 5A**). Lactate and glucose levels in the perfusion fluid as well as oxygen consumption have been described previously[17]. Furthermore, histological examination of H&E stained biopsies collected from liver tissue and the distal end of the extrahepatic bile duct, as illustrated in **Figure 5B, C** did not reveal any additional injury to the grafts during normothermic machine perfusion.

Microbiological testing of the perfusion fluid did not reveal any bacterial contamination during NMP. In one case a positive culture for *S. epidermidis* was obtained from the sample collected immediately after cold preservation. However, culture of the perfusion fluid after 6 h of NMP was negative for any bacteria, showing the efficacy of the antibiotics used in the perfusion fluid.

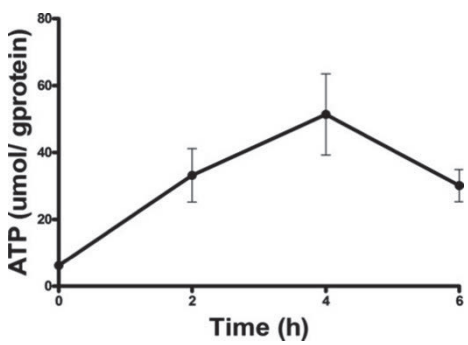


Figure 4: Changes in the level of liver tissue ATP content during NMP. Increased liver tissue ATP content during NMP showed improvement of mitochondrial function. Data are represented as mean ± SEM.

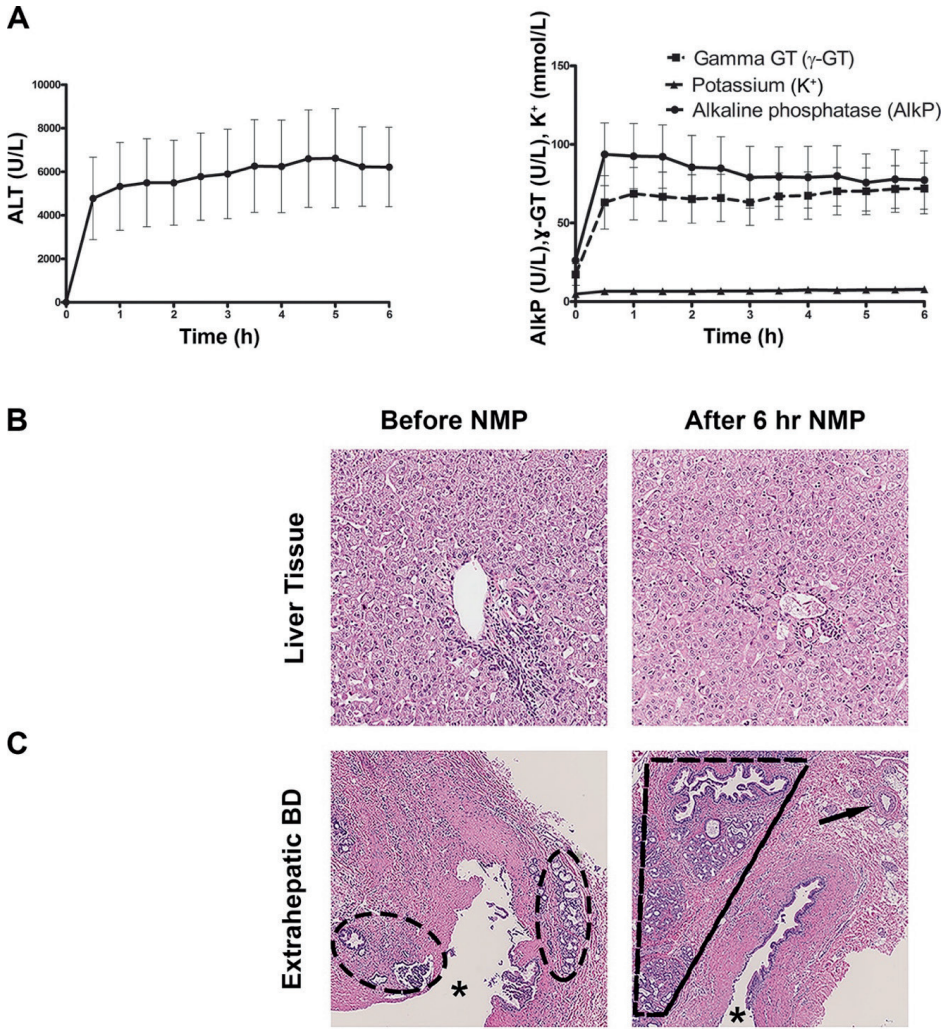


Figure 5: (**A**) Markers of hepatobiliary injury and (**B**) staining of liver parenchyma and (**C**) the extrahepatic bile duct taken from a representative graft before (0 h) and after (6 h) machine perfusion. (**A**) Stable concentrations of injury markers in the perfusion fluid indicated minimal injury of grafts during machine perfusion. (**B**) Well-preserved microscopic architecture of a representative liver graft. (**C**) Histology of the extrahepatic bile duct (lumen marked by an asterisk) of a representative graft. Moderate biliary epithelial injury indicated by partial loss of the luminal epithelial layer was observed at baseline and this did not worsen during 6 h of MP. A similar degree of biliary injury has been described in a series of human livers before transplantation.[20] Peribiliary vasculature (arrow) and peribiliary glands (area within dashed lines) displayed no worsening of injury after normothermic machine perfusion.

DISCUSSION

This video provides a step by step protocol for normothermic machine perfusion of human donor livers using a device that enables pressure controlled dual perfusion through the hepatic artery and portal vein. While following this protocol, technical failures of the perfusion machine did not occur and all grafts were well perfused and well oxygenated. The *ex situ* perfused livers had stable hemodynamics and were metabolically active, as defined by the production of bile.[16,17]

This is a well-established protocol for machine perfusion of human donor livers. This technique has several potential advantages over the conventional method of SCS[21]. Machine perfusion provides the opportunity to preserve donor liver grafts at different temperatures depending on the intended endpoint of organ preservation. Hypothermic oxygenated machine perfusion provides better perfusion and wash-out of the microvasculture and may help to restore intracellular energy contents by stimulating adenosine triphosphate (ATP) regeneration. However, full assessment of graft viability requires perfusion at a more physiological temperature (subnormothermic or normothermic). With increasing perfusion temperatures, the liver will become metabolically more active and start to produce bile. A recent study has suggested that bile production as an indicator of liver function might be an asset during *ex situ* NMP to evaluate graft viability prior to transplantation. This study showed that bile production correlated with the liver tissue ATP level and histological and biochemical markers of liver injury.[17] These findings remain to be confirmed by clinical trials. Although bile production is a suitable potential marker of liver parenchyma viability, markers of bile duct viability that can be assessed during *ex situ* NMP are still lacking. Therefore, it is currently still not possible to predict whether a liver assessed during NMP will develop NAS after transplantation or not. However, using this protocol, *ex situ* NMP did not reveal any worsening of bile duct injury during 6 hours of NMP. Moreover, this technique has the potential to allow for preconditioning of the graft before transplantation, resulting in reduced post-transplant injuries or recurrence of underlying diseases.[22]

The optimal fluid for *ex situ* oxygenated machine perfusion of donor livers is dependent on the temperature used. The solubility of oxygen in water is temperature-dependent and the amount of oxygen that can be dissolved in a watery fluid decreases with increasing temperature.[23] When using low temperatures for MP, the amount of oxygen dissolved in the perfusion fluid can be sufficient. However, at 37 °C an oxygen carrier should be added to the perfusion fluid to provide enough oxygen to the graft. For hypothermic MP, a preservation solution such as Belzer Machine Perfusion Solution can be sufficient.[11] For subnormothermic or normothermic MP, more complex perfusion fluids that also contain nutrients and an oxygen carrier have been used in different studies.[15,16] In our studies on normothermic MP, we have used ABO- and Rhesus matched packed red blood cells from the local blood bank as an

oxygen carrier.[16] It remains to be established whether similar results can be obtained with artificial hemoglobin-based oxygen carriers such as Hemopure or Hemarina.

The most critical technical aspects for successful perfusion of human livers are: to correctly secure the cannulas in the portal vein and supratruncal aorta segment, to ligate all small side braches to avoid any leakage of perfusion fluid which could disturb the pressure and flow regulations of the machine, to maintain a physiological environment for the liver especially by adjusting the pH and electrolyte concentrations of the perfusion fluid, and to maintain sterility of the perfusion environment.

Due to technical constraints, the perfusion device used in the described protocol cannot lower the temperature of the perfusion fluid below 10 °C. Although this can be considered a limitation, it does not provide a real problem concerning ischemia. The reason is that more than sufficient amounts of oxygen can be supplied to the perfusion fluid by the two membrane oxygenators regardless of the temperature. An advantage is that the temperature can be easily adjusted during the perfusion period, which allows gradual rewarming of the donor liver. A recent study in porcine livers has shown important advantages of gradual rewarming prior to normothermic reperfusion using the same device as described here.[18]

The ability to perfuse donor livers at different temperatures and the opportunity of adding extra agents to the perfusion fluid during organ perfusion offer the potential to assess and improve organ quality prior to transplantation. Therefore, this method can considerably increase the number of available organs for transplantation.

ACKNOWLEDGEMENTS

This research work was financially supported by grants provided by Innovatief Actieprogramma Groningen (IAG-3), Jan Kornelis de Cock Stichting and Tekke Huizingafonds, all in the Netherlands. We are appreciative to all the Dutch transplantation coordinators for identifying the potential discarded livers and obtaining informed consent.

REFERENCES

1. Plaats A, et al. Hypothermic machine preservation in liver transplantation revisited: Concepts and criteria in the new millennium. Ann. Biomed. Eng. 2004;32(4):623–631.
2. op den Dries S, Sutton ME, Lisman Y, Porte RJ. Protection of bile ducts in liver transplantation: Looking beyond ischemia. Transplantation. 2011;92(4):373–379.
3. Seehofer D, Eurich D, Veltzke-Schlieker W, Neuhaus P. Biliary complications after liver transplantation: Old problems and new challenges. Am. J. Transplant. 2013;13(2):253–265.
4. Buis CI, et al. Nonanastomotic biliary strictures after liver transplantation, part 1: Radiological features and risk factors for early vs. late presentation. Liver. Transpl. 2007;13(5):708–718.
5. Karimian N, op den Dries S, Porte RJ. The origin of biliary strictures after liver transplantation: Is it the amount of epithelial injury or insufficient regeneration that counts. J. Hepatol. 2013;58(6):1065–1067.
6. Gastaca M. Biliary complications after orthotopic liver transplantation: A review of incidence and risk factors. Transplant. Proc. 2012;44(6):1545–1549.
7. Sanchez-Urdazpal L, et al. Ischemic-type biliary complications after orthotopic liver transplantation. Hepatology. 1992;16(1):49–53.
8. Dubbeld J, et al. Similar liver transplantation survival with selected cardiac death donors and brain death donors. Br. J. Surg. 2010;97(5):744–753.
9. Guarrera JV, et al. Hypothermic machine preservation in human liver transplantation: The first clinical series. Am. J. Transplant. 2010;10(2):372–381.
10. Henry SD, et al. Hypothermic machine preservation reduces molecular markers of ischemia/reperfusion injury in human liver transplantation. Am. J. Transplant. 2012;12(9):2477–2486.
11. Schlegel A, Rougemont O, Graf R, Clavien PA, Dutkowski P. Protective mechanisms of end-ischemic cold machine perfusion in DCD liver grafts. J. Hepatol. 2013;58(2):278–286.
12. op den Dries S, et al. Hypothermic oxygenated machine perfusion prevents arteriolonecrosis of the peribiliary plexus in pig livers donated after circulatory death. PLoS. One. 2014;9(2):e88521.
13. Tolboom H, et al. Subnormothermic machine perfusion at both 20 degrees C and 30 degrees C recovers ischemic rat livers for successful transplantation. J. Surg. Res. 2012;175(1):149–156.
14. Gringeri E, et al. Subnormothermic machine perfusion for non-heart-beating donor liver grafts preservation in a swine model: A new strategy to increase the donor pool? Transplant. Proc. 2012;44(7):2026–2028.

15. Bruinsma BG, et al. Subnormothermic machine perfusion for ex vivo preservation and recovery of the human liver for transplantation. Am. J. Transplant. 2014;14(6):1400–1409.

16. op den Dries S, et al. Ex vivo normothermic machine perfusion and viability testing of discarded human donor livers. Am. J. Transplant. 2013;13(5):1327–1335.

17. Sutton ME, et al. Criteria for viability assessment of discarded human donor livers during ex vivo normothermic machine perfusion. PLoS. One. 2014;9(11).

18. Minor T, Efferz P, Fox M, Wohlschlaeger J, Luer B. Controlled oxygenated rewarming of cold stored liver grafts by thermally graduated machine perfusion prior to reperfusion. Am. J. Transplant. 2013;13(6):1450–1460.

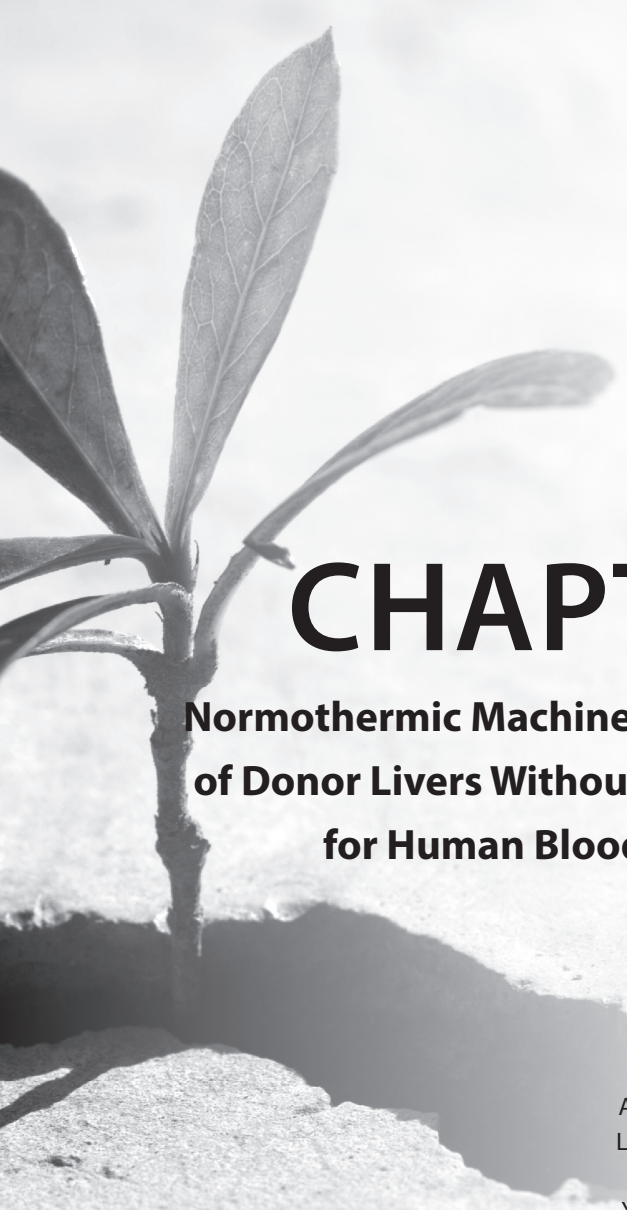
19. Makowka L, et al. Surgical technique of orthotopic liver transplantation. Gastroenterol. Clin. North. Am. 1988;17(1):33–51.

20. Hansen T, et al. Histological examination and evaluation of donor bile ducts received during orthotopic liver transplantation--a morphological clue to ischemic-type biliary lesion? Virchows Arch. 2012;461(1):41–48.

21. Monbaliu D, Brassil J. Machine perfusion of the liver: Past, present and future. Curr. Opin. Organ. Transplant. 2010;15(2):160–166.

22. Oldani G, et al. Pre-retrieval reperfusion decreases cancer recurrence after rat ischemic liver graft transplantation. J. Hepatol. 2014;61(2):278–285.

23. Tromans D. Temperature and pressure dependent solubility of oxygen in water: A thermodynamic analysis. Hydrometallurgy. 1998;48(3):327–342.



4

CHAPTER

Normothermic Machine Perfusion of Donor Livers Without the Need for Human Blood Products

Alix P.M. Matton
Laura C. Burlage
Rianne van Rijn
Yvonne de Vries
Shanice A. Karangwa
Maarten W. Nijsten
Annette S. H. Gouw
Janneke Wiersema-Buist
Jelle Adelmeijer
Andrie C. Westerkamp
Ton Lisman
Robert J. Porte[2]

Liver Transplantation. 2018; 24:528-538.

ABSTRACT

Background: Normothermic machine perfusion (NMP) enables viability assessment of donor livers prior to transplantation. NMP is frequently performed using human blood products including red blood cells (RBC) and fresh frozen plasma (FFP). Our aim was to examine the efficacy of a novel machine perfusion solution based on polymerized bovine hemoglobin HBOC-201.

Methods: Twenty-four livers declined for transplantation were transported using static cold storage. Upon arrival, livers underwent NMP for 6 hours using pressure-controlled portal and arterial perfusion. Twelve livers were perfused using a solution based on RBCs and FFPs (historical cohort), 6 livers with HBOC-201 and FFPs, and another 6 livers with HBOC-201 and Gelofusine, a gelatin-based colloid solution.

Results: Compared to RBC + FFP perfused livers, livers perfused with HBOC-201 had significantly higher hepatic ATP content, cumulative bile production and portal and arterial flows. Biliary secretion of bicarbonate, bilirubin, bile salts, and phospholipids was similar in all three groups. The ALT concentration in perfusate was lower in HBOC-201 perfused groups.

Conclusion: NMP of human donor livers can be performed effectively using HBOC-201 and Gelofusine, eliminating the need for human blood products. Perfusing livers with HBOC-201 is at least similar to perfusion with RBCs and FFPs. Some of the biomarkers of liver function and injury even suggest a possible superiority of an HBOC-201 based perfusion solution and opens a perspective for further optimization of machine perfusion techniques.

INTRODUCTION

Liver transplantation is the only curative treatment option for end-stage liver disease. Unfortunately, a global discrepancy exists between the availability and need for human donor livers, resulting in substantial waiting list mortality.[1] Over the past decades, machine perfusion has been gaining interest as a promising tool for expanding the human donor liver pool.[2]

Normothermic machine perfusion (NMP) is a technique whereby human donor livers are perfused *ex situ* at 37°C. This technique can be used for the entire period of preservation, as is currently being evaluated in a clinical trial by Friend et al. in Oxford[3], and for viability assessment of the organ prior to transplantation.[4-6] In this manner, only well-functioning organs are transplanted, including those that initially may have been declined for transplantation. Furthermore, NMP has the potential to allow for the resuscitation of donor livers.

NMP is generally performed using a perfusion solution based on packed red blood cells.[3,7-9] The NMP perfusion solution requires an adequate oxygen carrier to deliver oxygen throughout the organ, as well as physiological osmolarity and oncotic pressure. Previous NMP perfusions at our center were performed using matched packed red blood cells (RBCs) and fresh frozen plasma (FFP) obtained from the blood bank, with the addition of nutrients and antibiotics.[7] Other centers have performed NMP with RBCs and Gelofusine [3,8] or Steen solution[9], and one previous study has also performed NMP using HBOC-201 and Gelofusine.[10]

The use of human blood products is expensive and logistically challenging due to their short preservation time and need for matching. Furthermore, human blood products are scarce, and carry the risk of transmitting blood borne infections. For these ethical, financial, and logistical reasons it would be favorable to avoid the use of RBCs and FFPs for NMP. Consequently, the aim of the current study was to design a perfusion solution for NMP that circumvents the use of human blood products. We did this by replacing RBCs with HBOC-201 (Hemopure®, HbO_2 Therapeutics LCC), a bovine-derived free hemoglobin oxygen carrier, and FFPs with Gelofusine, a widely used commercially available colloid solution.

MATERIALS AND METHODS

Organ Procurement

The present study was performed at the University Medical Center Groningen, the Netherlands and was approved by the Medical Ethical Committee of the institute. Between July 2012 and July 2015, twenty-four human donor livers that were declined for transplantation were included after consent for research had been obtained from relatives. All donor livers were procured using the standard technique of *in situ* cooling and flush out with ice-cold preservation solution (University of Wisconsin [UW] or histidine-tryptophan-ketoglutarate [HTK]

solution, in line with the national organ procurement protocol), as has previously been described.[11] Livers were packed in ice-cold preservation solution (UW or HTK), stored on ice and transported to our center. Upon arrival, an experienced liver surgeon performed the back table preparation and cannulated the portal vein, supratruncal aorta and bile duct for machine perfusion. Meanwhile, the machine perfusion device was set up and primed and machine perfusion was commenced as soon as possible.

Study Groups

Twelve donor livers were perfused with RBCs and FFPs (RBC + FFP group). Subsequently, 6 livers were perfused with HBOC-201 and FFPs (HBOC-201 + FFP group) and thereafter, 6 livers were perfused with HBOC-201 and Gelofusine (HBOC-201 + Gelofusine group). Due to the scarcity of available donor livers at our research center, we used a cohort of livers that had already been perfused and previously published (RBC + FFP group).[11] Perfusions in the three study groups were not randomized but instead performed consecutively. All perfusions were performed in the presence of the principal investigator and, after having optimized our perfusion technique extensively before including any of the liver grafts of the present study, no changes were made in perfusion technique.

Oxygen Carrier HBOC-201

The HBOC-201 oxygen carrier solution contains polymerized hemoglobin, which is much smaller than a human erythrocyte, is less viscous than RBCs and has the ability to release oxygen more easily than human hemoglobin.[12] This gives it the ability to perfuse tissues more deeply and oxygenate more remote regions. [12] Due to the extraction and purification process, potential contaminants including plasma proteins, endotoxins, bacteria, viruses and the prions responsible for bovine spongiform encephalopathy and variant Creutzfeld-Jakob disease are removed, resulting in a sterile, pyrogen-free solution.[13] The *in vivo* half-life of HBOC-201 is about 20 hours .[13] A downside to the use of HBOC-201 is the potential formation of methemoglobin (metHb), however the small amount of HBOC-201 that would reach the recipient in a transplantation setting is minimal as the perfusion solution would be washed out prior to transplantation.[13] Lastly, HBOC-201 cannot be spun down and therefore renders the perfusate colored red, which may interfere with spectrophotometric analyses.[14]

Machine Perfusion Solution

The perfusion solutions of the three study groups were based on three main components: 1) an oxygen carrier, provided by either 3 units of RBCs or 4 units of HBOC-201 (Hemopure®, HbO_2 Therapeutics LCC, PA), both with a total of 120 g Hb, 2) a colloid solution, consisting of either 3 units of FFPs supplemented with 100mL 20% human albumin or 500 mL 4% Gelofusine® (B Braun, Melsungen,

Germany) supplemented with 250 mL 20% human albumin and 3) additional supplements containing nutrients, trace elements, antibiotics, vitamins, insulin and heparin as described previously.[7] The total volume of perfusion solution was similar in all three groups and around 2200 mL. All blood products were supplied by Sanquin, the Dutch blood bank, and were not expired. In each perfusion solution, the colloid oncotic pressure and osmolarity were targeted to reach physiological levels. Prior to connecting the liver, the pH of the perfusion fluid was optimized.

Normothermic Machine Perfusion

The Liver Assist (Organ Assist, Groningen, the Netherlands) machine perfusion device was used. It simulates the physiological environment by providing pressure-controlled pulsatile flow to the hepatic artery and continuous flow to the portal vein and gravitational outflow through the vena cava. The hepatic artery and portal vein perfusion circuits are each comprised of a rotary perfusion pump, a membrane oxygenator with integrated heat exchanger and flow and pressure sensors.

The perfusion solution was maintained at 37°C and NMP was performed for 6 hours. Pressures were set at a mean of 70 mmHg (systolic and diastolic pressures ±20%) on the arterial and 11 mmHg on the portal side. Perfusion fluid was oxygenated using a total of 4 L/min (95% oxygen and 5% carbon dioxide) through the two oxygenators. Before NMP and every 30 min during NMP, samples of the arterial and venous perfusion fluid, as well as bile samples, were taken for analysis of blood gas parameters (pH, pO_2, pCO_2. sO_2, HCO_3^-, lactate, glucose and metHb) using an ABL800 FLEX or ABL90 FLEX analyzer (Radiometer, Brønhøj, Denmark). If needed, sodium bicarbonate (8.4% solution) was added to maintain a pH within the physiological range of 7.35-7.45, as described previously.[7,15] Liver parenchyma wedge biopsies were taken before and every 2 hours during NMP, stored in formalin and embedded in paraffin or snap frozen in liquid nitrogen and stored at -80ºC. Bile produced by the liver was collected and measured every 30 min and stored at -80ºC. Perfusion fluid samples were collected every half hour and stored at -80ºC (after 5 min centrifugation at 2700 rpm at 4ºC).

Assessment of Hepatobiliary Function and Injury

Adenosine-5'- triphosphate (ATP) in liver parenchyma biopsies was determined as described previously.[4]

To calculate the peak oxygen extraction, the difference between arterial and venous oxygen content was calculated and corrected for the flow. The following formula was used to calculate the oxygen content:

*Oxygen content = (pO_2 * K) + (sO_2 * Hb * c), where pO_2 is the partial pressure of oxygen in kPa, K a constant (0.0225), sO_2 the oxygen saturation expressed as a fraction (where 1.00 is 100% saturation), Hb the hemoglobin concentration in g/dL and c the oxygen binding capacity of hemoglobin (1.39 for human Hb; 1.26 for HBOC-201).*

Total bilirubin concentration in bile was determined using a competitive ELISA kit (Human Total Bilirubin ELISA kit, #MBS756198, MyBioSource, Inc., San Diego, CA, USA) utilizing a monoclonal anti-TBB antibody and a TBB-HRP conjugate as indicated by the manufacturer. Samples were applied undiluted. Color intensity was measured spectrophotometrically at 450nm using VersaMax ELISA microplate reader and SoftMax Pro 5.4, and concentrations were calculated.

Total bile salt concentrations in bile were determined by adding 250 µL trisbuffer and 50 µL of the reagent 3α-hydroxysteroid dehydrogenase (H1506-50UN, Sigma-Aldrich) and resazurine (Acros Organics) to 10 µL (diluted 1:100) of each sample.[16] Fluorescence was measured using a Perkin Elmer Wallac 1420 Victor3 microplate reader and concentrations were calculated.

Phospholipid concentrations in bile were determined by adding 150 µL of reagent out of a commercially available Phospholipids kit (Refnr. 15741 9910 930, Diagnostic systems, GmbH, Holzheim, Germany) to 10 µL (diluted 1:9) of each sample. Color intensity was measured spectrophotometrically at a wavelength of 570 nm (VersaMax Molecular devices) in SoftMax Pro 5.4 and concentrations were calculated. In order to calculate the biliary secretion of bicarbonate, bilirubin, total bile salts, and phospholipids, their concentrations were multiplied by the volume of bile produced, corrected for the weight of the liver.

After centrifugation, perfusate samples were 10x diluted and analyzed for alanine aminotransferase (ALT) using routine diagnostic laboratory procedures. As HBOC-201 hemoglobin is freely suspended in solution and cannot be spun down, ALT concentrations in the HBOC-201 groups were corrected for the 20% hematocrit present in the RBC + FFP group by multiplying ALT values in the HBOC-201 groups by 1.25 (1 / 0.80 = 1.25).

Paraffin-embedded slides of liver biopsies were prepared for hematoxylin and eosin (H&E) staining and semi-quantitatively assessed using the Suzuki liver injury scoring system.[17] All liver slides were examined in a blinded fashion by an expert liver pathologist (ASHG).

Statistics

Continuous variables are presented as median with interquartile range (IQR); categorical variables as absolute numbers. Continuous variables were compared between groups by calculating the area under the curve (AUC) when indicated and the Kruskal-Wallis H or Mann-Whitney U test with Bonferroni correction. Categorical variables were compared with the Fisher's exact test. The level of significance was set at a p-value <0.05. All statistical analyses were performed

using SPSS software version 22.0 for Windows (IBM SPSS, Inc., Chicago, IL, USA) and Microsoft Excel 2010 for Windows.

RESULTS

Donor Liver Characteristics

Table 1 shows the donor liver characteristics in the three study groups. There were no significant differences in donor liver characteristics between the groups. Of note, the number of livers discarded due to expected steatosis (based on donor BMI, ultrasound and laboratory results) was 5 in the RBC + FFP group compared to none and one in the HBOC-201 + FFP and HBOC-201 + Gelofusine groups respectively. However, the level of actual microscopic steatosis, which was only known after the liver had been offered for research, was much lower. Only 2 livers (17%) in the HBOC-201 + FFP group, none in the HBOC-201 + FFP group and 1 (17%) in the HBOC-201 + Gelofusine group had a clinically relevant degree of microscopic steatosis (greater than 30%).

Normothermic Machine Perfusion

Figure 1 shows photographs of NMP using RBC + FFP (**Figure 1A**) and HBOC-201 + Gelofusine (**Figure 1B**). The color of HBOC-201 is darker than that of human blood. During one HBOC-201 + FFP perfusion, there was blood present in the bile and this liver was consequently excluded for biliary analyses, as this would result in the recording of falsely elevated bile production. The fraction of metHb during NMP reached maximally 0.02% in the RBC + FFP group, 0.22% in the HBOC-201 + FFP group and 0.28% in the HBOC-201 + Gelofusine group (healthy human adults range <1%).

Hemodynamics

As shown in **Figure 2A**, the portal vein flow increased during the first hour of NMP and thereafter remained stable in all three groups. In both HBOC-201 groups, the portal flow was significantly higher at each time point compared to the RBC + FFP group, reaching a median [IQR] of 848 [663 – 1393] mL/min/kg liver weight in the RBC + FFP group, 1890 [1530 – 2173] in the HBOC-201 + FFP, and 1830 [1713 – 2030] in the HBOC-201 + Gelofusine group at 6 hours of NMP.

Table 1. Donor Liver Characteristics.

	RBC + FFP (n=12)	**HBOC-201 + FFP** (n=6)	**HBOC-201 + Gelofusine** (n=6)	**p-value**
Age (years)	61 (53 - 63)	54 (39 -67)	65 (63 - 66)	0.22
Gender				0.43
Male	8	4	3	
Female	4	2	3	
BMI	27 (25 - 35)	19 (17 - 29)	25 (24 - 28)	0.19
Type of donor				1.00
DCD	9	5	5	
DBD	3	1	1	
Warm ischemia time[a] (min)	35 (24 - 39)	31 (25 - 37)	39 (28 - 45)	0.56
Cold ischemia time[b] (hrs)	9.1 (7.2 - 10.2)	7.6 (7.1 - 8.6)	8.0 (7.1 - 8.4)	0.38
Donor risk index[c]	2.8 (2.4 - 3.2)	2.7 (2.0 - 3.2)	3.0 (2.6 - 3.2)	0.86
Cause of death				0.19
Anoxia	5	4	2	
CVA	1	2	2	
Trauma	6	0	2	
Reason for discarding				0.17
Expected steatosis	5[d]	0	1	
DCD and age > 60	5	2	4	
High AST/ALT/GGT	1	3	0	
Other[e]	1	1	1	
Preservation solution				0.39
HTK	3	0	0	
UW	9	6	6	

Continuous variables are presented as median and interquartile range, categorical variables as absolute numbers. [a] Time between withdrawal of life support until the aortic cold flush in the donor (DCD only). [b] Time between the donor aortic cold flush until the start of normothermic machine perfusion. [c] Donor risk index was calculated according to Braat et al. 2012.[25] [d] Only 2 of these 5 livers turned out to have microscopic steatosis >30%. [e] RBC + FFP group: unknown; HBOC-201 + FFP group: DCD in combination with 26 min between cardiac

arrest and aortic cold flush; HBOC-201 + Gelofusine group: DCD age 57 in combination with out-of-hospital cardiac arrest. *Abbreviations: DCD: donation after circulatory death; DBD: donation after brain death; CVA: cerebrovascular accident; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyl transferase; HTK: histidine-tryptophan-ketoglutarate solution; UW: University of Wisconsin solution.*

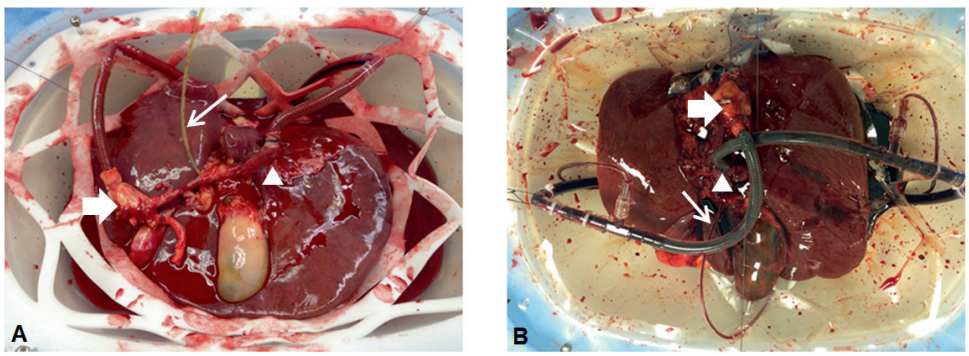


Figure 1. Photographs of donor livers during NMP. Panel A: NMP using a perfusion fluid based on RBC + FFP. Panel B: NMP using a perfusion fluid based on HBOC-201 + Gelofusine. The supratruncal hepatic artery (large arrow), portal vein (arrowhead) and bile duct (thin arrow) are cannulated. Note the darker color of the HBOC-201 perfusion solution.

The hepatic artery flow was higher after the first two hours of NMP in both HBOC-201 groups compared to the RBC + FFP group, reaching a median [IQR] of 273 [231 – 327] mL/min/kg liver weight in the RBC + FFP group, 742 [480 – 867] in the HBOC-201 + FFP, and 533 [187 – 741] in the HBOC-201 + Gelofusine group at 6 hours NMP. The arterial flow remained stable in the RBC + FFP group, continued to increase in the HBOC-201 + FFP group, and declined slightly after 3 hours of NMP for unknown reasons in the HBOC-201 + Gelofusine group (**Figure 2B**). The total flow (portal + arterial), however, remained stable in all three groups. This is in line with the fact that the portal vein and hepatic artery compete for blood flow (**Figure 2C**). There were no significant differences in either portal or arterial flow between the two HBOC-201 groups. Furthermore, there were no significant differences in resistance between the three groups (data not shown). The higher flow rates, despite equal pressures and resistance, in the HBOC-201 groups can be explained by the fact that the viscosity of the HBOC-201 perfusion fluid is lower than that of the RBC perfusion fluid.

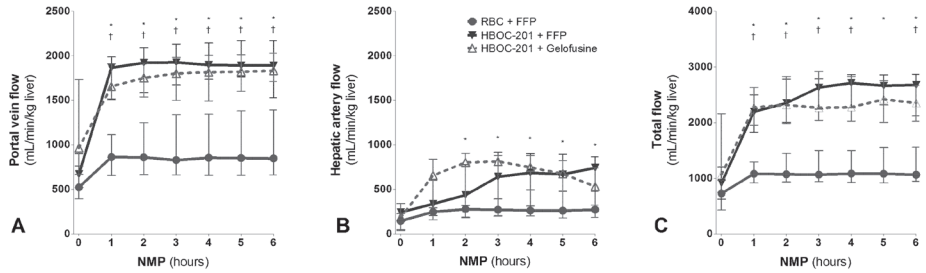


Figure 2. Portal vein, hepatic artery and total flow during NMP. Panel A: The portal vein flow during NMP was significantly higher at each time point after the first hour in both HBOC-201 groups compared to the RBC + FFP group. Panel B: The hepatic artery flow was significantly higher after the first two hours of NMP in the HBOC-201 + FFP group compared to the RBC + FFP group. Panel C: The total (portal vein + hepatic artery) flow during NMP remains significantly higher at nearly each time point after the first hour in both HBOC-201 groups compared to the RBC + FFP group. There were no significant differences in hepatic or portal vein flow in between the two HBOC-201 groups. * significant difference between RBC + FFP and HBOC-201 + FFP; † significant difference between RBC + FFP and HBOC-201 + Gelofusine. Median values and interquartile ranges are shown.

ATP Content in Liver Parenchyma

The median ATP content in liver parenchyma was higher in both HBOC-201 groups at each time point during NMP compared to the RBC + FFP group, reaching significance at two time points (**Figure 3A**). At 6 hours NMP, the median [IQR] ATP content was 24 [14 – 51] µmol/g protein in the RBC + FFP group, 50 [35 – 59] in the HBOC-201 + FFP group, and 79 [50 – 103] in the HBOC-201 + Gelofusine group. Furthermore, the ATP content in the HBOC-201 + Gelofusine group was higher at each time point compared to the HBOC-201 + FFP group, however this did not reach significance.

The normal value of ATP content in healthy livers using our assay is around 60 µmol/g protein, implying that physiological ATP levels were reached during NMP with HBOC-201.

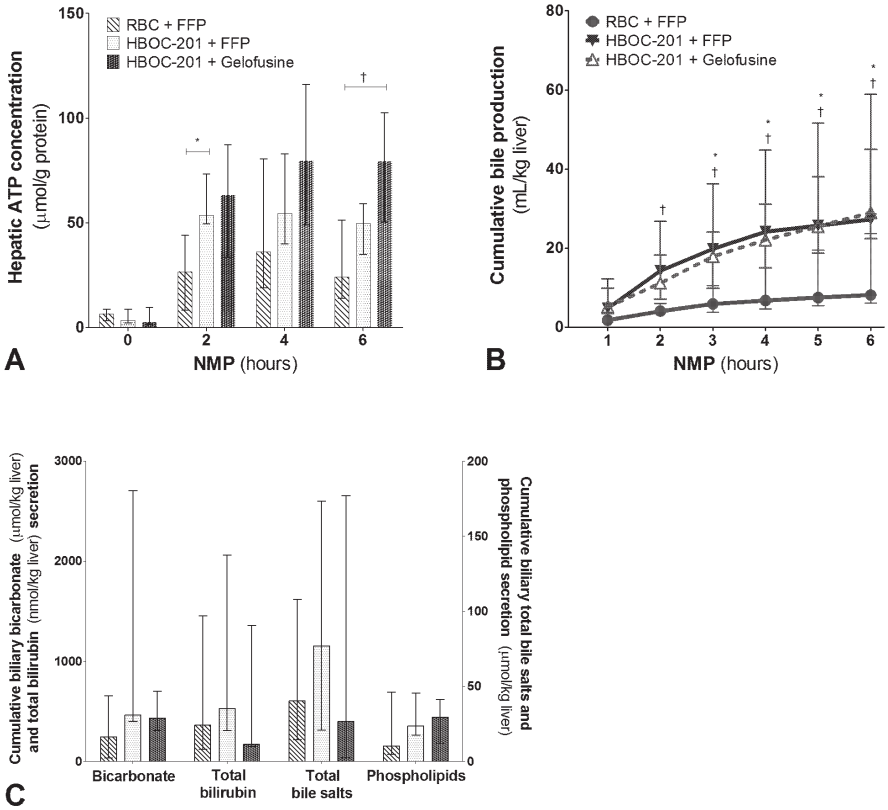


Figure 3. ATP content in liver parenchyma, cumulative bile production and cumulative biliary secretion of bicarbonate, bilirubin, bile salts and phospholipids during 6 hours of NMP. Panel A: The hepatic ATP content was highest in the HBOC-201 + Gelofusine group, followed by the HBOC-201 + FFP group, and lastly the RBC + FFP group at each time point. Panel B: Cumulative bile production during NMP was significantly higher at each time point in both HBOC-201 groups compared to the RBC + FFP group, after the second hour of NMP. Panel C: The cumulative secretion of bicarbonate, bilirubin, bile salts and phospholipids in bile during 6 hours of NMP were not significantly different between the three study groups. * significant difference between RBC + FFP and HBOC-201 + FFP; † significant difference between RBC + FFP and HBOC-201 + Gelofusine. Median values and interquartile ranges are shown.

Peak Oxygen Extraction

The peak oxygen extraction was higher in the HBOC-201 perfused groups, however this did not reach statistical significance. The median [IQR] peak oxygen extraction was 0.0014 [0.0010 – 0.0022] mL O2/min/g liver in the RBC + FFP group, 0.0023 [0.0020 – 0.0024] in the HBOC-201 + FFP group, and 0.0024 [0.0022 – 0.0033] in the HBOC-201 + Gelofusine group.

Bile Production

After the second hour of NMP, the cumulative bile production was significantly higher in the HBOC-201 groups compared to the RBC + FFP group, reaching a median [IQR] of 8.2 [6.1 – 17.7] mL/kg liver weight in the RBC + FFP group, 27.3 [26.6 – 31.2] in the HBOC-201 + FFP group, and 29.0 [25.6 – 39.4] in the HBOC-201 + Gelofusine group (p=0.04 and p=0.03 respectively) at 6 hours of NMP (**Figure 3B**). There were no significant differences between the two HBOC-201 groups.

Biliary Composition

The biliary secretion of bicarbonate (marker for cholangiocyte function), bile salts, phospholipids and bilirubin (markers for hepatic function) were not significantly different between the three groups (**Figure 3C**).

Lactate and Glucose in the Perfusion Fluid

As shown in **Figure 4A**, the lactate concentration during NMP declined more quickly in the HBOC-201 groups compared to the RBC + FFP group, with an approximately two-fold higher median lactate concentration at 2 hours NMP in the RBC + FFP group compared to the HBOC-201 perfused groups (median [IQR] of 6.7 [4.1 – 10.0] mmol/L in the RBC + FFP group, 3.6 [1.8 – 10.3] in the HBOC-201 + FFP and 2.6 [0.5 – 6.0] in the HBOC-201 + Gelofusine group at 2 hours NMP). Although the differences did not reach significance, these data could suggest that the HBOC-201 perfused livers have a more adequate aerobic metabolism than the RBC + FFP perfused livers. The glucose concentration also seemed to normalize more rapidly in the HBOC-201 perfused livers compared to the RBC + FFP perfused livers (**Figure 4B**), though this did not reach significance.

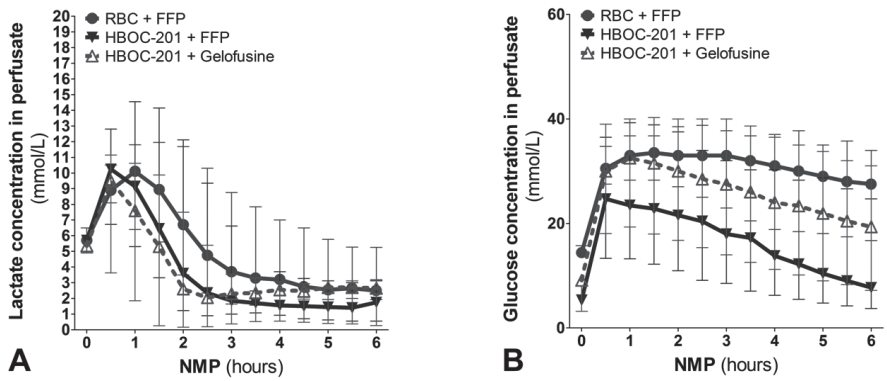


Figure 4. Lactate and glucose concentrations in perfusion fluid during NMP. Panel A: The perfusate lactate concentration declined more quickly in the HBOC-201 groups compared to the RBC + FFP group, with an approximately two-fold higher median lactate concentration at 2 hours NMP in the RBC + FFP group compared to the HBOC-201 perfused groups. There were, however, no significant differences in perfusate lactate concentrations between the three groups. Panel B: Although glucose concentration during NMP normalized more quickly in the HBOC-201 groups compared to the RBC + FFP group, this did not reach statistical significance. Median values and interquartile ranges are shown.

Buffering Capacity

The amount of bicarbonate that needed to be added to the perfusion system was not statistically different between the three groups. The median [IQR] volume of 8.4% sodium bicarbonate added during NMP was 20 [3 – 44] mL in the RBC + FFP group, 10 mL [10 – 10] in the HBOC-201 + FFP group, and 25 [10 – 40] in the HBOC-201 + Gelofusine group.

ALT Concentration in the Perfusion Fluid

The concentration of ALT in perfusate during NMP was higher in the RBC + FFP group compared to both HBOC-201 groups during NMP, nearly reaching significance at 4 hours of NMP (both p=0.07) and at 6 hours of NMP between the RBC + FFP and HBOC-201 + FFP group (p=0.06) (**Figure 5**). The median [IQR] ALT concentration at 6 hours NMP was 5817 [2957 – 14023] IU/L in the RBC + FFP group, 2550 [942 – 5562] in the HBOC-201 + FFP group, and 2418 [1968 – 3768] in the HBOC-201 + Gelofusine group.

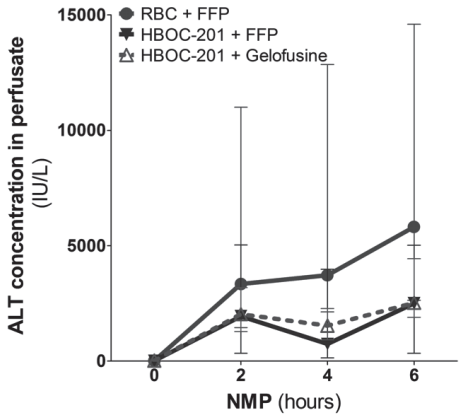


Figure 5. Alanine aminotransferase (ALT) concentration in perfusion fluid during NMP. The ALT concentration is higher in the RBC + FFP group compared to both HBOC-201 groups during NMP, nearly reaching significance at 4 hours of NMP (both p=0.07) and at 6 hours of NMP between the RBC + FFP and HBOC-201 + FFP group (p=0.06). Median values and interquartile ranges are shown.

Histological Analysis of Liver Injury

The amount of histological injury of liver parenchyma was not significantly different between the three groups before or after NMP. The median [IQR] total Suzuki injury score was 2.0 [1.0 – 3.0] before and 3.0 [2.0 – 4.3] after NMP in the RBC + FFP group; 1.0 [1.0 – 1.0] before and 2.0 [2.0 – 2.0] after NMP in the HBOC-201 + FFP group; and 1.5 [1.0 – 2.0] before and 2.5 [1.3 – 4.5] after NMP in the HBOC-201 + Gelofusine group. The main factor contributing to the total injury score was the degree of necrosis, with a median increase of 1.0 point in each group, as is shown in representative H&E stained liver sections in **Figure 6**.

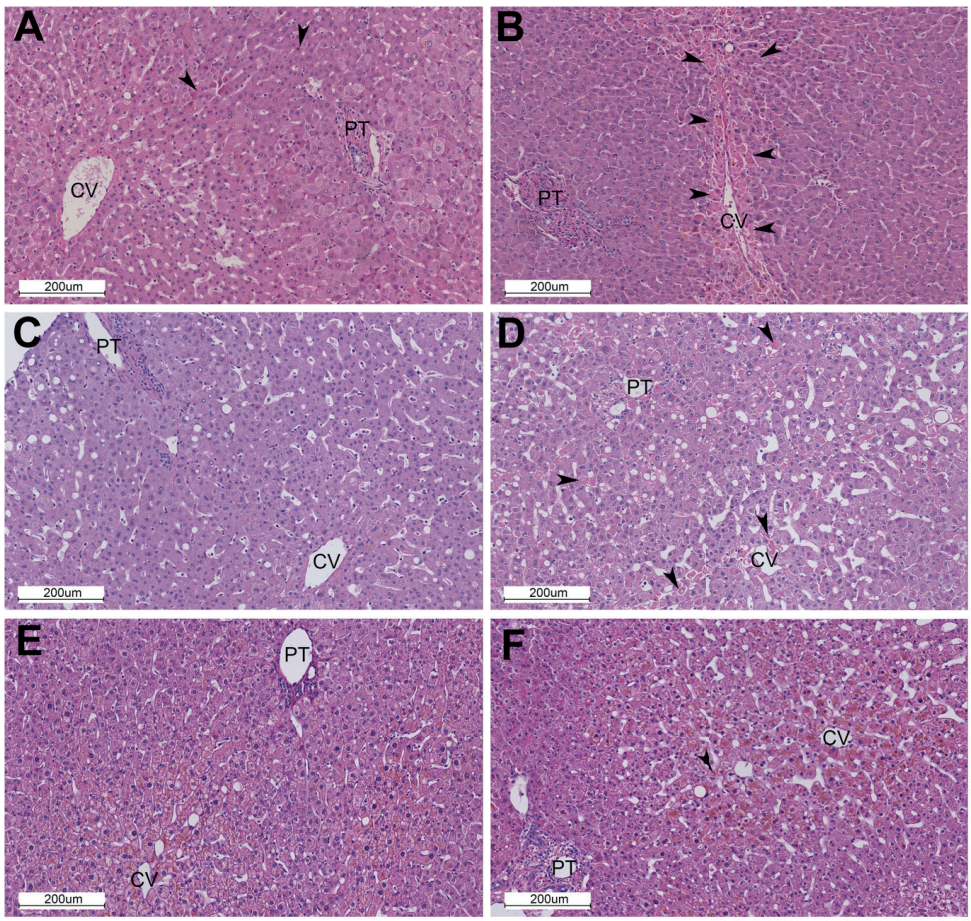


Figure 6. Histological liver injury. Representative H&E stainings of liver biopsies prior to and after 6 hours NMP in each study group. There were no significant differences in the degree of liver injury between the three study groups before or after NMP. Arrowheads indicate necrotic cells. Slide a: liver section of an RBC + FFP liver prior to NMP; slide b: liver section of the same RBC + FFP liver after 6 hours NMP; slide c: liver section of an HBOC-201 + FFP liver prior to NMP; slide d: liver section of the same HBOC-201 + FFP liver after 6 hours NMP; slide e: liver section of an HBOC-201 + Gelofusine liver prior to NMP; and slide f: liver section of the same HBOC-201 + Gelofusine liver after 6 hours NMP. *Abbreviations: CV: central vein; PT: portal triad.*

DISCUSSION

Machine perfusion is revolutionizing the field of organ transplantation and, as it is rapidly making its way into the clinic, is responsible for increases in the quality and quantity of liver transplants. Finding an alternative to using scarce, expensive and logistically complex human blood products for NMP is an important step in making NMP more widely applicable and accessible. In this study, we have shown that 1) NMP can be effectively performed without the use of human blood products by replacing RBCs with HBOC-201, a polymerized bovine hemoglobin, and FFPs by Gelofusine, a widely available colloid solution, and 2) that perfusion with HBOC-201 is at least as effective as with RBCs. Some end-points in our study indicate than an HBOC-201 based perfusion fluid may even be superior, as shown by the increased recovery of hepatic ATP content, bile production and improved glucose and lactate metabolism, as well as lower injury markers (ALT).

After having performed perfusions with RBCs and FFPs, we first replaced RBCs with HBOC-201 and kept FFPs, and subsequently also replaced FFPs with Gelofusine. The ATP content in liver parenchyma was continuously higher in both HBOC-201 groups compared to the RBC + FFP group. Previous research has shown that during static cold storage, hepatic ATP levels are depleted and these levels can be restored during machine perfusion.[11,18] Livers with higher ATP levels show significantly better outcomes after transplantation, as has been validated in several animal and clinical studies[19-21], holding great promise for future clinical perfusion with HBOC-201.

A possible explanation for the higher ATP content in liver parenchyma in the HBOC-201 perfused livers lies in the properties of HBOC-201. The HBOC-201 molecule has a lower affinity for oxygen than human hemoglobin with a dissociation curve that is shifted to the right, causing HBOC-201 to give off oxygen more readily.[12] In addition, HBOC-201 solution is less viscous and contains free hemoglobin, which is much smaller than erythrocytes, thereby allowing it to penetrate more deeply into the tissue.[12] The peak oxygen extraction also appeared higher in the HBOC-201 perfused groups than in the RBC + FFP group, although this did not reach significance.

Bile production is an ATP-dependent process. In line with this, the cumulative bile production was also significantly higher in both HBOC-201 groups compared to the RBC + FFP group. According to the “viability criteria” described by Sutton et al., 7 out of 12 livers in the RBC + FFP group, 4 out of 5 in the HBOC-201 + FFP group, and 6 out of 6 livers in the HBOC-201 + Gelofusine group would have potentially been transplantable.[4] Similarly, bile production is a transplantation criterion established in a clinically validated group of livers described by the Birmingham group.[22]

The amount of bicarbonate, bile salts, phospholipids, and bilirubin secreted into bile was, however, not significantly different between the three groups. Bile flow is mainly driven by the secretion of bile salts, but a significant part is also driven by bile salt-independent factors.[23] It could be possible that the secretion

of other molecules, such as HBOC-201 or derivatives thereof, are hypercholeretic and thereby cause higher bile flow with an altered bile composition.

Both the lactate and glucose concentrations in perfusion fluid declined more rapidly in the HBOC-201 perfused livers compared to the RBC + FFP perfused livers, though this did not reach significance. This may indicate that the HBOC-201 perfused livers were able to metabolize lactate and glucose at least equally well, or perhaps even better, as the RBC perfused livers, reflecting proper restoration of aerobic metabolism.

The HBOC-201 perfused livers consistently showed significantly higher flows through the portal vein compared to the RBC + FFP perfused livers. Flow through the hepatic artery was also consistently higher in the HBOC-201 perfused groups, reaching significance between the RBC + FFP and HBOC-201 + Gelofusine groups. The increased flow is likely a result of the aforementioned lower viscosity of HBOC-201, compared to human blood, and not caused by a difference in intrahepatic resistance between the groups. The size of HBOC-201 is 1 x 10^{-8} the size of an RBC. This makes an HBOC-201 based perfusion fluid much less viscous than an RBC based fluid, resulting in higher flows at a given intrahepatic resistance.

Interestingly, the concentration of the liver injury marker, ALT, in perfusion fluid was consistently lower in the HBOC-201 groups compared to the RBC + FFP group, despite no histological differences in the amount of liver parenchyma injury. There were no significant differences in donor parameters between the three groups; in fact, the DRI was even slightly higher in the HBOC-201 + Gelofusine group. The number of livers declined for transplantation due to expected steatosis was higher in the RBC + FFP group, however this did not translate into a higher number of livers with microscopically confirmed clinically relevant steatosis and is therefore unlikely to have played a major role in the results of the present study.

Two other studies have reported the use of HBOC-201 in a machine perfusion setting. In the first study, subnormothermic (21ºC) machine perfusion was compared to static cold storage using pig donor livers. The investigators noted significantly higher survival, superior graft function and bile production after liver transplantation in the machine perfused group, compared to static cold stored livers.[24] The second study compared NMP using RBCs with HBOC-201 and reported similar flows, lactate clearance and histological findings. They also reported significantly higher oxygen extraction in the HBOC-201 perfused group.[10] The results of these studies are in line with the results of our study and indicate that machine perfusion with HBOC-201 is equal or even superior for the function and quality of liver grafts.

Limitations of this study are relatively small samples sizes in the HBOC-201 study groups, lack of transplant validation and the fact that the livers in the different study groups were not randomized but instead perfusions of the three study groups were performed consecutively. We do not, however, believe that a

potential learning curve could have played a role in the current study as our research team had extensively optimized its NMP perfusion technique prior to the perfusion of any of the included liver grafts, after which no changes in perfusion technique were made.

In conclusion, NMP can be performed without the use of RBCs and FFPs by replacing them with HBOC-201 and Gelofusine, respectively. This reduces the costs and logistical complexity of NMP and avoids the use of scarce human blood products, which carry the potential to transmit blood-borne infections. The current study indicates that perfusing livers with HBOC-201 is at least similar to perfusion with human RBC. Some of the biomarkers of liver function and injury used in this study even suggest a possible superiority of an HBOC-based perfusion solution. Altogether, this suggests that NMP with HBOC-201 and Gelofusine is a favorable method and opens a perspective for further optimization of machine perfusion techniques. Future studies are needed to assess the safety of performing NMP with HBOC-201 and Gelofusine in a clinical transplantation setting. For this reason, a clinical trial has recently been initiated at our center (Dutch Trial Register www.trialregister.nl, nr. NTR5972).

ACKNOWLEDGEMENTS

The authors thank the Dutch transplantation coordinators for identifying potential donor livers and for the effort in achieving informed consent for research from the donor families. We would like to thank Zaf Zafirelis for making this research possible, and Greg Dubé, Jenny Kootstra-Ros and Jeroen van Leeuwen for their expertise in laboratory and chemical analyses.

REFERENCES

1. Barshes, N R Horwitz, I B Franzini, L Vierling, J M Goss,J A. Waitlist mortality decreases with increased use of extended criteria donor liver grafts at adult liver transplant centers. American journal of transplantation 2007;7(5):1265-1270.
2. Matton AP, Porte RJ. Opportunities for scientific expansion of the deceased donor pool. Liver Transpl 2014 Nov;20 Suppl 2:S5.
3. Ravikumar R, Jassem W, Mergental H, Heaton N, Mirza D, Perera MT, et al. Liver Transplantation After Ex Vivo Normothermic Machine Preservation: A Phase 1 (First-in-Man) Clinical Trial. Am J Transplant 2016 Jun;16(6):1779-1787.
4. Sutton ME, op den Dries S, Karimian N, Weeder PD, de Boer MT, Wiersema-Buist J, et al. Criteria for viability assessment of discarded human donor livers during ex vivo normothermic machine perfusion. PLoS One 2014 Nov 4;9(11):e110642.
5. Watson CJ, Kosmoliaptsis V, Randle LV, Russell NK, Griffiths WJ, Davies S, et al. Preimplant Normothermic Liver Perfusion of a Suboptimal Liver Donated After Circulatory Death. Am J Transplant 2016 Jan;16(1):353-357.
6. Mergental H, Perera MT, Laing RW, Muiesan P, Isaac JR, Smith A, et al. Transplantation of Declined Liver Allografts Following Normothermic Ex-Situ Evaluation. Am J Transplant 2016 Nov;16(11):3235-3245.
7. op den Dries S, Karimian N, Sutton ME, Westerkamp AC, Nijsten MW, Gouw AS, et al. Ex vivo normothermic machine perfusion and viability testing of discarded human donor livers. Am J Transplant 2013 May;13(5):1327-1335.
8. Bral M, Gala-Lopez B, Bigam D, Kneteman N, Malcolm A, Livingstone S, et al. Preliminary Single-Center Canadian Experience of Human Normothermic Ex Vivo Liver Perfusion: Results of a Clinical Trial. Am J Transplant 2017 Apr;17(4):1071-1080.
9. Selzner M, Goldaracena N, Echeverri J, Kaths JM, Linares I, Selzner N, et al. Normothermic ex vivo liver perfusion using steen solution as perfusate for human liver transplantation: First North American results. Liver Transpl 2016 Nov;22(11):1501-1508.
10. Laing RW, Bhogal RH, Wallace L, Boteon Y, Neil DAH, Smith A, et al. The use of an Acellular Oxygen Carrier in a Human Liver Model of Normothermic Machine Perfusion. Transplantation 2017 May 18.
11. Westerkamp AC, Karimian N, Matton AP, Mahboub P, van Rijn R, Wiersema-Buist J, et al. Oxygenated Hypothermic Machine Perfusion After Static Cold Storage Improves Hepatobiliary Function of Extended Criteria Donor Livers. Transplantation 2016 Apr;100(4):825-835.
12. Dube GP, Vranckx P, Greenburg AG. HBOC-201: the multi-purpose oxygen therapeutic. EuroIntervention 2008 May;4(1):161-165.

13. Anbari KK, Garino JP, Mackenzie CF. Hemoglobin substitutes. Eur Spine J 2004 Oct;13 Suppl 1:S76-82.

14. Chance JJ, Norris EJ, Kroll MH. Mechanism of interference of a polymerized hemoglobin blood substitute in an alkaline phosphatase method. Clin Chem 2000 Sep;46(9):1331-1337.

15. Karimian N, Matton AP, Westerkamp AC, Burlage LC, Op den Dries S, Leuvenink HG, et al. Ex Situ Normothermic Machine Perfusion of Donor Livers. J Vis Exp 2015 May 26;(99):e52688. doi(99):e52688.

16. Turley SD, Dietschy JM. Re-evaluation of the 3 alpha-hydroxysteroid dehydrogenase assay for total bile acids in bile. J Lipid Res 1978 Sep;19(7):924-928.

17. Suzuki S, Toledo-Pereyra LH, Rodriguez FJ, Cejalvo D. Neutrophil infiltration as an important factor in liver ischemia and reperfusion injury. Modulating effects of FK506 and cyclosporine. Transplantation 1993 Jun;55(6):1265-1272.

18. van Rijn R, Karimian N, Matton APM, Burlage LC, Westerkamp AC, van den Berg AP, et al. Dual hypothermic oxygenated machine perfusion in liver transplants donated after circulatory death. Br J Surg 2017 Jun;104(7):907-917.

19. Dutkowski P, Schlegel A, de Oliveira M, Mullhaupt B, Neff F, Clavien PA. HOPE for human liver grafts obtained from donors after cardiac death. J Hepatol 2014 Apr;60(4):765-772.

20. Guarrera JV, Henry SD, Samstein B, Reznik E, Musat C, Lukose TI, et al. Hypothermic machine preservation facilitates successful transplantation of "orphan" extended criteria donor livers. Am J Transplant 2015 Jan;15(1):161-169.

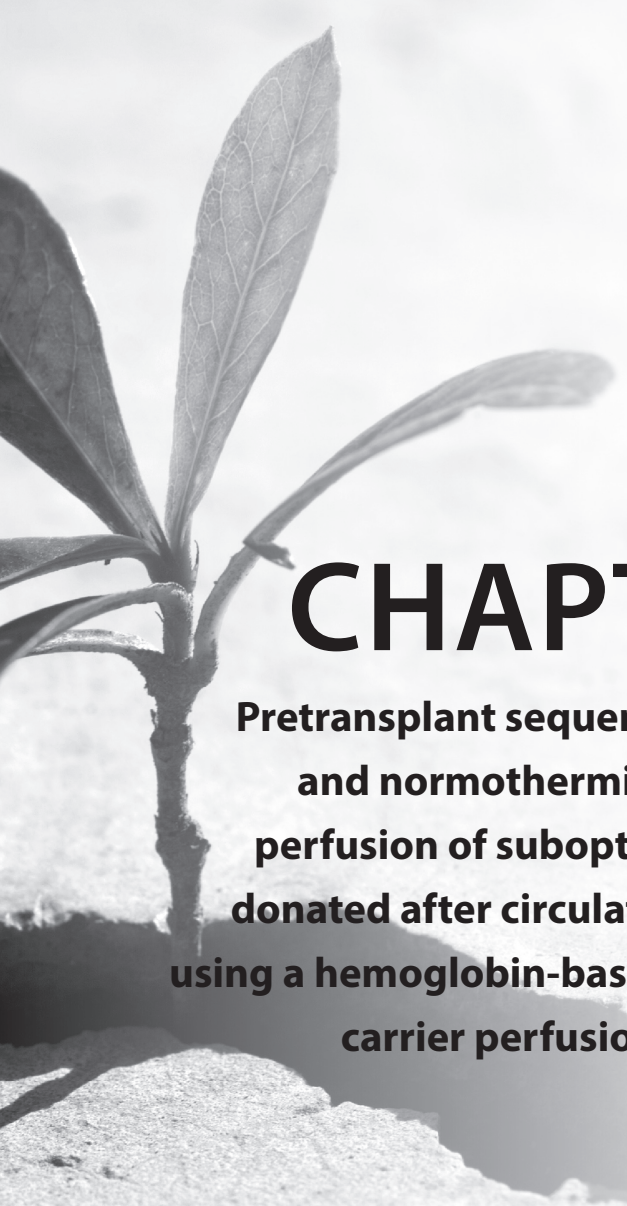
21. Dutkowski P, Furrer K, Tian Y, Graf R, Clavien PA. Novel short-term hypothermic oxygenated perfusion (HOPE) system prevents injury in rat liver graft from non-heart beating donor. Ann Surg 2006 Dec;244(6):968-76; discussion 976-7.

22. Perera T, Mergental H, Stephenson B, Roll GR, Cilliers H, Liang R, et al. First human liver transplantation using a marginal allograft resuscitated by normothermic machine perfusion. Liver Transpl 2016 Jan;22(1):120-124.

23. Boyer JL. Bile formation and secretion. Compr Physiol 2013 Jul;3(3):1035-1078.

24. Fontes P, Lopez R, van der Plaats A, Vodovotz Y, Minervini M, Scott V, et al. Liver preservation with machine perfusion and a newly developed cell-free oxygen carrier solution under subnormothermic conditions. Am J Transplant 2015 Feb;15(2):381-394.

25. Braat AE, Blok JJ, Putter H, Adam R, Burroughs AK, Rahmel AO, et al. The Eurotransplant donor risk index in liver transplantation: ET-DRI. Am J Transplant 2012 Oct;12(10):2789-2796.



5

CHAPTER

Pretransplant sequential hypo- and normothermic machine perfusion of suboptimal livers donated after circulatory death using a hemoglobin-based oxygen carrier perfusion solution

Yvonne de Vries
Alix P. M. Matton
Maarten W. N. Nijsten
Maureen J. M. Werner
Aad P. van den Berg
Marieke T. de Boer
Carlijn I. Buis
Masato Fujiyoshi
Ruben H. J. de Kleine
Otto B. van Leeuwen
Peter Meyer
Marius C. van den Heuvel
Vincent E. de Meijer
Robert J. Porte

American Journal of Transplantation
2019;19:1202-1211.

ABSTRACT

Ex situ dual hypothermic oxygenated machine perfusion (DHOPE) and normothermic machine perfusion (NMP) of donor livers may have a complementary effect when applied sequentially. While DHOPE resuscitates the mitochondria and increases hepatic ATP content, NMP enables hepatobiliary viability assessment prior to transplantation. In contrast to DHOPE, NMP requires a perfusion solution with an oxygen carrier, for which red blood cells (RBC) have been used in most series. RBC, however, have limitations and cannot be used cold. We, therefore, established a protocol of sequential DHOPE, controlled oxygenated rewarming (COR), and NMP using a new hemoglobin-based oxygen carrier (HBOC)-based perfusion fluid (DHOPE-COR-NMP trial, NTR5972).

Seven livers from donation after circulatory death (DCD) donors, which were initially declined for transplantation nationwide, underwent DHOPE-COR-NMP. Livers were considered transplantable if perfusate pH and lactate normalized, bile production was ≥ 10 ml and biliary pH >7.45 within 150 min of NMP. Based on these criteria five livers were transplanted. The primary endpoint, 3-month graft survival, was a 100%.

In conclusion, sequential DHOPE-COR-NMP using an HBOC-based perfusion fluid offers a novel method of liver machine perfusion for combined resuscitation and viability testing of suboptimal livers prior to transplantation.

INTRODUCTION

Ex situ machine perfusion is increasingly investigated as a tool to increase the number of donor livers for transplantation and to reduce post-transplant complications. Machine perfusion, was recently introduced in clinical practice using two different temperature protocols: hypothermic (4-12°C) or normothermic (37°C) machine perfusion.[1-5] (Dual) hypothermic oxygenated perfusion ((D)HOPE) can be applied to resuscitate the mitochondria and increase hepatic ATP content, resulting in less cell injury, including less cholangiocyte injury. Normothermic machine perfusion (NMP) allows for *ex situ* functional testing of (extended criteria) donor livers prior to transplantation and suboptimal donor livers have been successfully transplanted after NMP.[2,6-8] (D)HOPE and NMP may therefore have a complementary effect when applied sequentially.[9,10] In preclinical studies using human donor livers, it was previously shown that a short period of DHOPE prior to NMP results in increased ATP concentrations, less hepatobiliary injury and improved function during the NMP phase, compared to direct end-ischemic NMP.[9,10]

Different perfusion solutions have been used for (D)HOPE and NMP. A perfusion fluid based on human red blood cells (RBC) is frequently used for NMP.[11-13] The use of RBC, however, has several drawbacks. Firstly, RBC are a relatively scarce human blood product.[14] Secondly, RBC may induce an immune reaction or cause an infection.[14,15] Lastly, RBC cannot be used during (D)HOPE due to increased stiffness of the erythrocyte lipid membranes and hemolysis at low temperatures. These drawbacks press the need for an alternative oxygen carrier, especially when hypothermic and normothermic machine perfusion are combined. Hemoglobin-based oxygen carriers (HBOC) are a suitable alternative for the use of RBC in *ex situ* liver machine perfusion. The bovine derived HBOC-201 (Hemopure) has previously been used successfully in experimental and preclinical studies of liver machine perfusion.[14,16,17]

Based on the presumed complementary effect of (D)HOPE and NMP we have combined these two techniques in a clinical machine perfusion protocol using an HBOC-201-based solution. The use of this perfusion solution eliminates the need to change the perfusion fluid during different temperature phases. Donor livers that were initially declined for transplantation nationwide were subjected to a combined protocol of DHOPE, controlled oxygenated rewarming (COR), and subsequent viability testing during NMP (DHOPE – COR – NMP Trial). This report describes the first transplantations of initially nation-wide declined livers that underwent *ex situ* machine perfusion with the HBOC-201-based perfusion fluid.

METHODS

Study Protocol

Between August 2017 and April 2018, 20 livers were offered for inclusion in the DHOPE-COR-NMP study. All livers were declined for regular transplantation by the three liver transplant centers in the Netherlands. Thirteen livers were

secondarily declined because of logistical reasons, long agonal phase (in case of donation after circulatory death), or macroscopic fibrosis/cirrhosis (**Figure 1** and **Figure 1S**). Seven livers were accepted to undergo DHOPE-COR-NMP. All seven livers were initially declined for transplantation because of a combination of risk factors, as described in **Table 1**. The median donor risk index was 2.82 (IQR 2.52 – 2.97), reflecting the suboptimal quality of these livers.

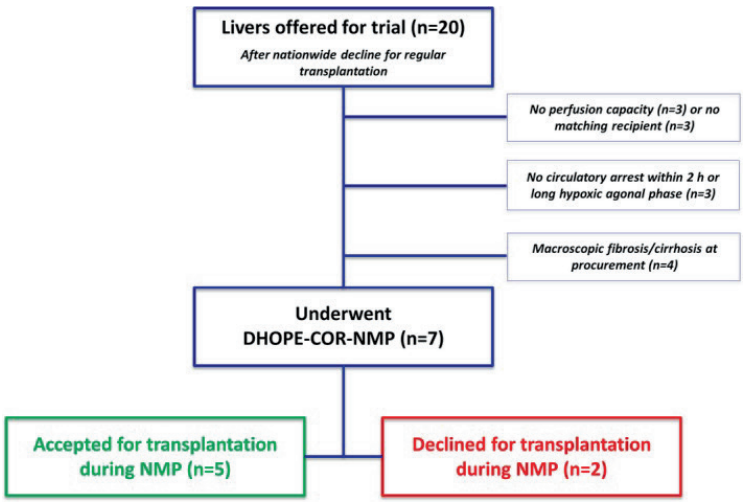
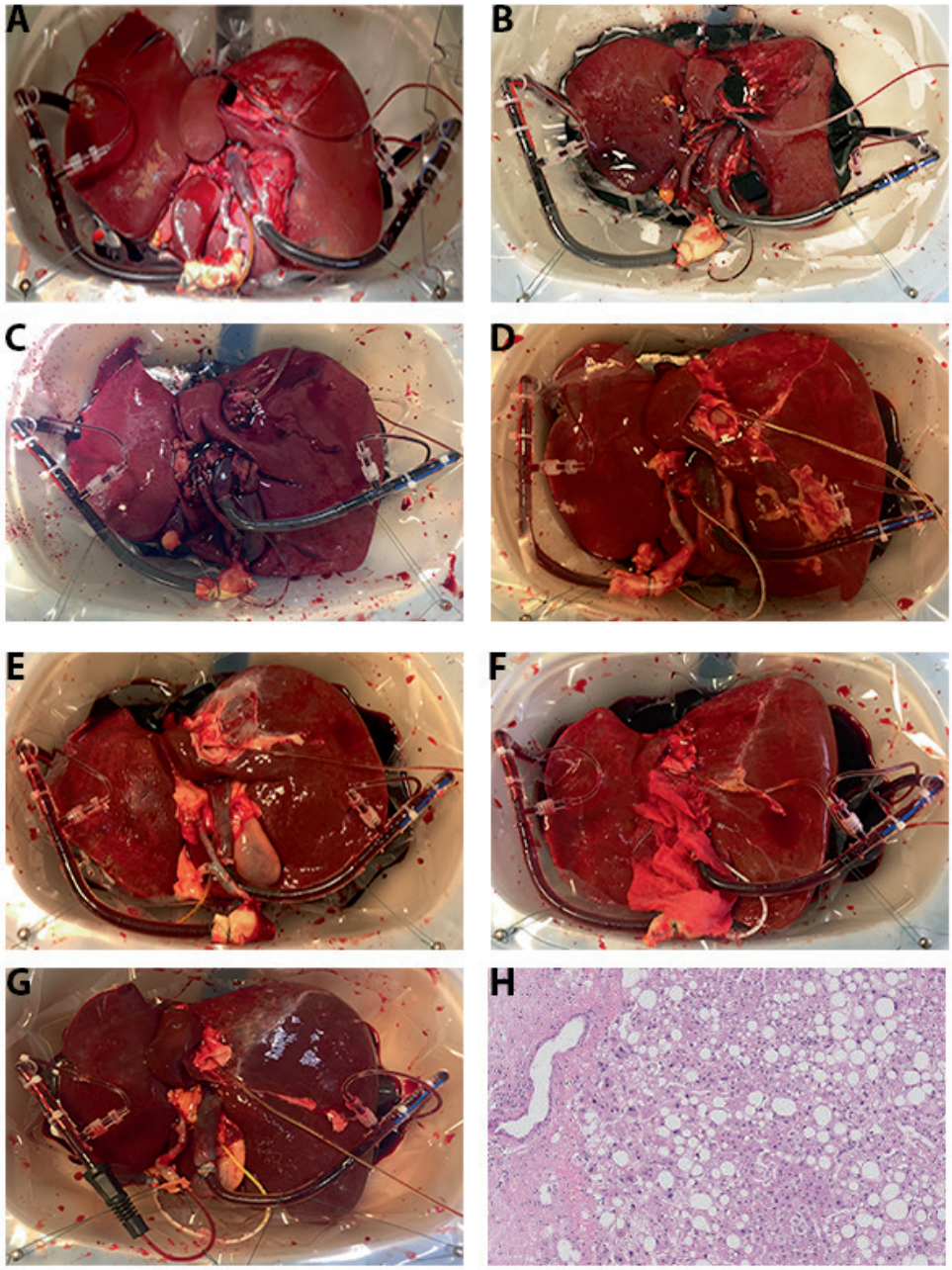


Figure 1. Flow chart of livers offered in the context of the DHOPE – COR – NMP Trial. After initial decline by all Dutch liver transplant centers a total number of 20 livers were offered for inclusion in this trial. Thirteen livers did not undergo machine perfusion due to logistical reasons, long agonal phase, or macroscopic findings. Seven livers underwent machine perfusion for resuscitation and viability assessment.

The study protocol was approved by the medical ethical review committee of our center (METc2016.281) and published in the national registry of clinical trials (www.trialregister.nl; NTR5972). The primary outcome parameter was 3-month graft survival. All recipients gave written informed consent.

Procurement of Donor Livers

All donor livers were procured in a standard manner by a dedicated procurement team. After withdrawal of life support, circulatory death was awaited, followed by a mandatory five minutes 'no touch' period before procurement surgery was started. Cold *in situ* flush was performed with UW cold storage solution with the addition of 50,000 IU of heparin. After procurement, the livers were transported to our center using static cold storage. Upon arrival, the livers were prepared for machine perfusion, as described previously.[11]



Supporting figure 1. Photos of all livers and histology of donor liver #3 with severe steatosis. Panels A-G: Photos of liver #1 to #7 on the perfusion machine in chronologic order. Panel H: Light microscopy of a parenchymal biopsy of liver #3 after static cold storage (before machine perfusion) revealed 60% macrovesicular steatosis.

Table 1. Donor characteristics of livers that were accepted to undergo machine perfusion.

	Liver 1	**Liver 2**	**Liver 3**	**Liver 4**	**Liver 5**	**Liver 6**	**Liver 7**
Age (years)	42	63	47	52	46	62	63
DBD/DCD donor	DCD	DCD	DCD	DCD	DCD	DCD	DCD
BMI / degree of steatosis	BMI 21	BMI 28	BMI 33, >60% histological steatosis	BMI 28	BMI 27	BMI 23	BMI 25
Notably increased laboratory values in the donor	Peak AST 1676 U/L, peak ALT 1375 U/L, peak γGT 166 U/L	-	-	Peak γGT 340 U/L	Peak AST 161 U/L, peak ALT 270 U/L, peak γGT 254 U/L	Peak AST 201 U/L, peak ALT 175 U/L	-
Intoxications	Binge drinking	-	-	Frequent alcohol consumption	Alcohol, heroin, speed cocaine, ecstasy	-	-
dWIT (min)*	29	23	30	33	27	35	25
CIT (min)#	289	306	525	294	256	278	221
Donor hepatectomy time (min)^	59	82	96	28	11	44	36
DRI[30]	2.53	2.82	2.46	2.92	2.50	3.75	3.03
ET-DRI[31]	2.65	2.92	2.47	3.31	2.85	2.88	2.87

* dWIT is defined as the time from withdrawal of life support until the start of *in situ* cold perfusion. # CIT is defined as the time from *in situ* cold perfusion until the start of machine perfusion. ^Donor hepatectomy time is defined as the time from *in situ* cold perfusion until the time of hepatectomy. *Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body-mass index; CIT, cold ischemia time; DBD, donation after brain*

death; DCD, donation after circulatory death; DRI, donor risk index; dWIT, donor warm ischemia time; ET-DRI, Eurotransplant donor risk index; γGT, γ-glutamyltransferase.

Machine Perfusion Settings

A combined machine perfusion protocol of one hour of DHOPE (resuscitation phase), one hour of COR, and subsequent NMP (viability testing phase) was established (**Figure 2**). For machine perfusion at different temperatures the Liver Assist (Organ Assist, Groningen, the Netherlands) perfusion device was used. DHOPE was performed at 10 °C. During COR the temperature was gradually increased about 1°C per 2 minutes, to 37°C at the start of NMP. Portal vein and hepatic artery pressures were set at 5 and 11 mmHg during DHOPE, and gradually increased during COR to 11 and 70 mmHg at the start of NMP, respectively.

During DHOPE, the perfusion fluid was oxygenated with 1 L/min 100% O_2, resulting in a perfusate pO_2 >80 kPa, as described previously[2]. During NMP an air/oxygen mixture was used aimed to reach an arterial perfusate pO_2 of 10.0 – 13.3 kPa and a venous oxygen saturation of 55-75%. To obtain these targets, FiO_2 was varied between 21 and 40%.

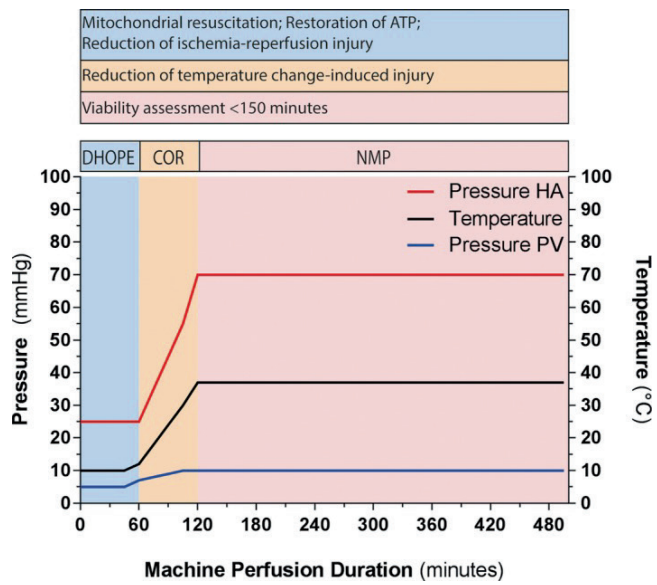


Figure 2. Overview of the machine perfusion protocol. Panel A: The machine perfusion protocol included one hour of DHOPE, one hour of COR, and subsequent NMP for at least 150 min. Each phase of machine perfusion served a different purpose as described in the upper part of the figure. Machine perfusion settings were adjusted according to the perfusion temperature. The temperature was kept at 10°C during DHOPE and was gradually increased to 37°C during the COR phase, after which the liver was functionally tested during NMP. Portal vein (PV) and mean hepatic artery (HA) pressure were set at 5 and 25 mmHg, respectively, during DHOPE and were gradually increased during COR to 10 and 70 mmHg, respectively, at the start of NMP.

Arterial perfusate samples were collected every half hour and analyzed using the ABL 90 Flex analyzer (Radiometer, Brønhøj, Denmark). In addition, venous perfusate samples were collected and analyzed every hour, to determine oxygen consumption. Oxygen consumption was calculated based on the difference between arterial and venous oxygen content. The following equation was used, ([{ApO_2-VpO_2} x K /760] x total flow) + ([{AsO_2 - VsO_2} x Hb x c x 0,0001] x total flow) / Liver weight x 100.[18] Where pO_2 was in mmHg, sO_2 in %, Hb in g/dL, total flow (sum of arterial and portal flow) in mL/min and liver weight in g. K was a constant (0.0225) and c the oxygen binding capacity of HBOC (1.26).

Bile was collected from COR-NMP onwards and its quantity measured. Additionally, every half hour bile was collected under mineral oil to determine biliary pH and HCO_3^-, as described previously.[8,19] Mineral oil prevented exposure of bile to ambient air, thus preventing the exchange of CO_2 molecules, which influences biliary pH via HCO_3^-.

Perfusion Fluid

To facilitate perfusion at different temperatures, an acellular perfusion solution based on a bovine-derived HBOC (HBOC-201, HBO_2 Therapeutics, Souderton, PA) was used. In addition to HBOC-201, the perfusion fluid contained gelofusine, albumin, metronidazole, cefazolin, nutrients, glutathione, insulin, heparin, and $NaHCO_3$. Details of the perfusion solution composition are provided in **supplementary Table 1S**. As of liver #6, taurocholate was added to the perfusion fluid (50 mg at baseline, followed by a continuous infusion of 7.7 mg/h during the NMP phase.[20] Taurocholate was produced according to GMP by our hospital pharmacy.

Table 1S. Composition of the HBOC-201-based machine perfusion solution

Component	Manufacturer/Distributor	Volume (mL)
HBOC-201 (Hemopure)	HbO_2 Therapeutics, Shouderton, PA, USA	1250
Gelofusine 4%	B Braun, Melsungen, Germany	300
Albumin 20%	Sanquin, Amsterdam, Netherlands	250
Total parenteral nutrition (TPN	Hospital pharmacy	20
Addamel (trace elements)	Fresenius Kabi, Netherlands	10
Metronidazol (Flagyl) 5 mg/ml	Baxter BV, Utrecht, Netherlands	44
Sterile water	B Braun, Melsungen, Germany	335
Insulin (NovoRapid) 100 IU/ml	Novo Nordisk BV, Alphen aan den Rijn, Netherlands	1
Cernevit (Multi vitamins)	Baxter BV, Utrecht, Netherlands	2
Heparin (5000 IU/ml)	Leo Pharma, Amsterdam, Netherlands	2

Cefazolin 1g /5mL	Baxter BV, Utrecht, Netherlands	2
Taurocholic acid sodium salt 0.1% (1mg/mL)	Hospital pharmacy	7,7
Sodium bicarbonate 8.4%	B Braun, Melsungen, Germany	35
KCl 1mmol/mL	B Braun, Melsungen, Germany	2
Glutathion (Tationil) 600mg/4mL	Teofarma, Pavia, Italy	14
Total volume		**2274,7**

Viability Testing – Assessment of Hepatobiliary Function

Liver viability and function were assessed during the NMP phase using predefined viability criteria, including sufficient bile production with a biliary pH of >7.45, and normalization of perfusate pH and lactate (**Table 2**).[5,7,8,21,22] The liver was considered acceptable for transplantation, if all criteria were met within the first 150 min of NMP. If the liver did not meet the predefined viability criteria, machine perfusion was terminated. If the liver did meet the predefined viability criteria, machine perfusion was continued and the recipient was brought to the operating room. When the hepatectomy of the native liver was almost complete, machine perfusion was terminated and the donor liver flushed out with 2 L of cold UW cold storage solution to remove the HBOC-based machine perfusion fluid. As routinely performed in our center, the first 400 ml of venous blood from the liver was drained and discarded to avoid spill of UW Cold Storage Solution into the recipient circulation.

Table 2. Viability criteria for donor liver assessment during NMP phase.

Viability Criteria
Cumulative bile production of ≥10 mL at 150 min of NMP and ≥4 mL in the preceding hour.[8]
Lactate concentration in perfusate is within 'normal' values [0.5 – 1.7 mmol/L] within 150 min of NMP.[5,7]
Perfusion fluid pH is within normal values [7.35 – 7.45] within 150 min of NMP, without the need for repeated addition of $NaHCO_3$.[5,7]
Biliary pH of >7.45 within 150 minutes of NMP.[21,22]

All predefined viability criteria had to be met in order to consider a liver transplantable.
Abbreviations: NMP: normothermic machine perfusion.

RESULTS

Machine Perfusion Characteristics

Five of the seven livers (liver #1, and #4 to #7) that underwent DHOPE-COR-NMP were identified as transplantable based on functional assessment during NMP (**Table 3**). Median cold ischemia time of all livers was 289 min (IQR 256 – 306 min). Machine perfusion times per liver are provided in **Table 4**. Median total duration of machine perfusion was shorter for the non-transplanted livers due to termination of machine perfusion after these livers did not meet all predefined viability criteria within 150 min of NMP.

Table 3. Viability criteria overview and transplantation decision per liver

	Liver 1	Liver 2	Liver 3	Liver 4	Liver 5	Liver 6	Liver 7
Perfusate pH	+	+	-	+	+	+	+
Perfusate Latate	+	+	-	+	+	+	+
Bile production	+	+	+	+	+	+	+
Biliary pH	+	-	-	+	+	+	+
Transplantation (yes/no)	Yes	No	No	Yes	Yes	Yes	Yes

+: The liver met this viability criterion. –: The liver did not meet this viability criterion.

Table 4. Machine perfusion times per liver.

	Liver 1	Liver 2	Liver 3	Liver 4	Liver 5	Liver 6	Liver 7
Total duration of NMP (min)	347	163	180	397	301	373	391
Duration of NMP from viability assessment onwards (min)	197	-	-	247	157	223	241
Total machine perfusion time (DHOPE – COR – NMP) (min)	467	283	300	517	421	493	511

Abbreviations: NMP, normothermic machine perfusion.

Both portal vein and hepatic artery flow remained low during DHOPE and increased during COR. At 150 min of NMP, median portal vein flow was 1680 ml/min (IQR 1460 – 1740 ml/min) (**Figure 3A**). Overall, resistance in the portal vein decreased towards NMP, but remained relatively high in liver #3 (**Figure 3B**). At 150 minutes of NMP, median hepatic artery flow was 547 ml/min (IQR 240 – 737 ml/min). Furthermore, hepatic artery flow was variable between the livers (**Figure 3C**). Resistance in the hepatic artery, generally remained <0.2

mmHg*min/L/g, except for resistance in liver #7 (**Figure 3D**). During COR, total flow increased as well, to a median of 2512 min (IQR 2133 - 2570 min) at 150 minutes of NMP (**Figure 3E**).

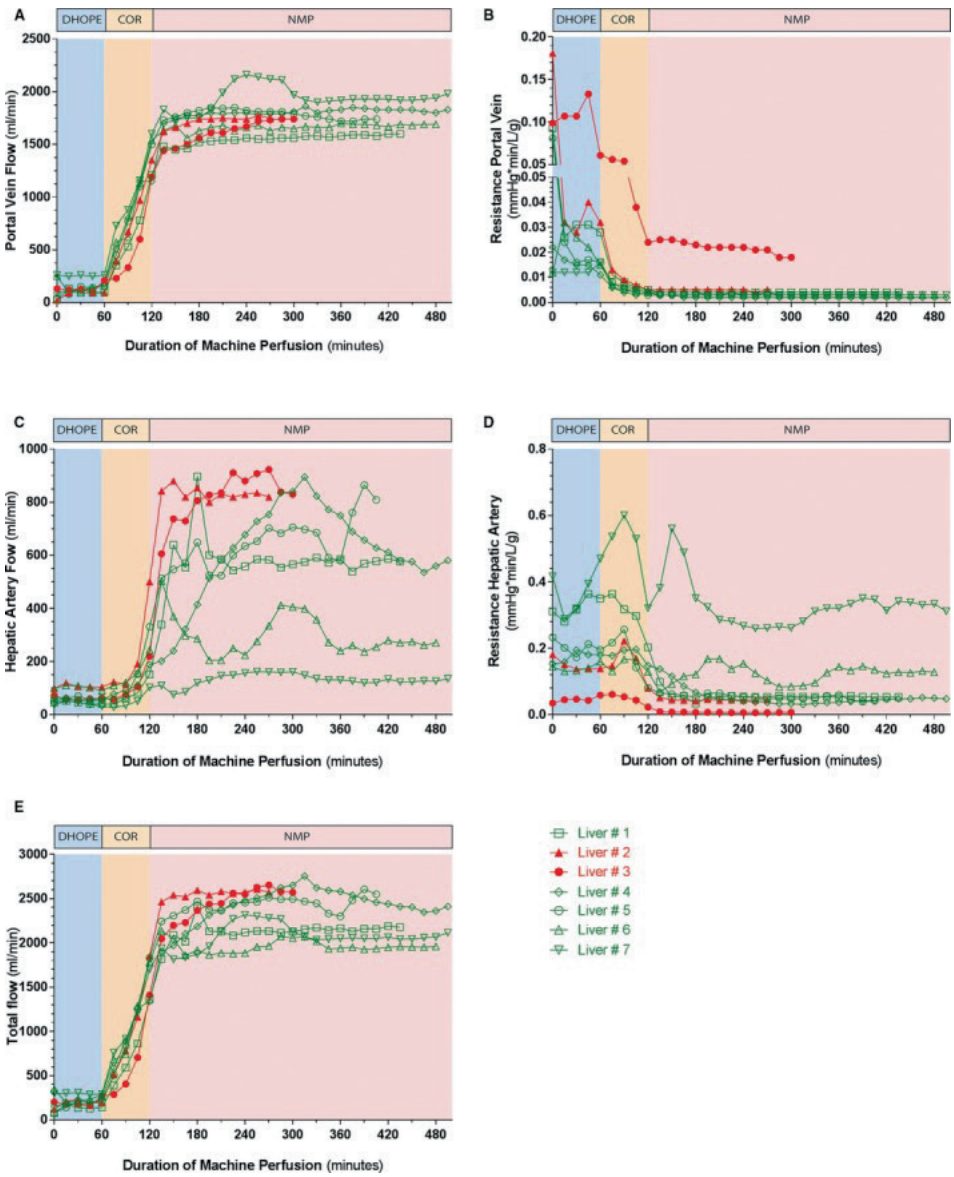


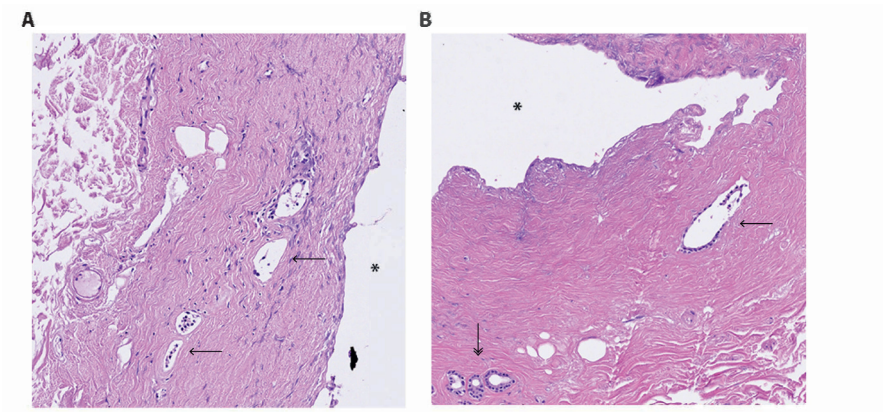
Figure 3. Flows and resistance during machine perfusion. Panel A: Portal vein (PV) flows were low during DHOPE. After 150 min of NMP, median portal vein flow was 1680 ml/min (IQR 1460 – 1740 ml/min). Panel B: Resistance in the portal vein was low, except for liver #3. Panel C: Hepatic artery (HA) flows were low during DHOPE and COR. During NMP hepatic artery flows varied between 100 and 900 ml/min. At 150 min of NMP, median hepatic artery flow was 547 mL/min (IQR 240 – 737 mL/min). Panel D: Resistance in the hepatic artery was <0.2

mmHg*min/L/g, except for liver #7. Panel E: Total flow increased to a median of 2512 min (IQR 2133 - 2570 min) at 150 minutes of NMP. The red lines represent the non-transplanted livers and the green lines represent the transplanted livers. *Abbreviations: Tx, transplantation.*

Hepatobiliary Function and Damage during Machine Perfusion

Perfusate pH normalized within 150 min of NMP of liver #1, #2 and #4-7, but not in liver #3, despite the addition of 25 mL 8.4% $NaHCO_3$ (**Figure 4A**). Perfusate lactate normalized within 150 min of NMP in all livers, except for liver #3 (**Figure 4B**). Furthermore, all livers produced sufficient amounts of bile. Bile production of liver #6 appeared lower, however, this was caused by a cannulation problem of the bile duct (**Figure 4C**). Alanine aminotransferase (ALT) concentrations in perfusate of the transplanted livers were <2,000 U/L. In the two non-transplanted livers ALT concentrations were >2,000 U/L, with a peak ALT concentration of 8,460 U/L in liver #3 (**Figure 4D**). Livers #1 and #4 to #7 produced bile with a pH >7.45, whereas livers #2 and #3 did not (Figure 4E). Bile duct biopsies of these two livers revealed signs of substantial histological injury (**Figure 2S**).

Median oxygen consumption was 0.14 mlO_2/min/100 g liver weight during DHOPE and increased during COR to a median peak value of 2.77 mlO_2/min/100g liver weight during NMP.



Supporting figure 2. Bile duct histology of secondarily declined donor livers #2 and #3. Panel A: Hematoxylin & eosin (H&E) stained extrahepatic bile duct (EHBD) of donor liver #2 revealed complete loss of the luminal epithelium. Stromal necrosis was mild, yet >50% of the epithelium of the intramural peribiliary glands (PBG) was lost. Furthermore 50% of the epithelium of the extramural PBG was lost. Panel B: H&E stained EHBD of liver #3 revealed severe stromal necrosis and arteriolonecrosis. Furthermore, >50% of the epithelium of the intra- and extramural PBG was lost. Magnification 20x. Asterisks indicate the bile duct lumen. A single arrowhead indicates the intramural PBG and a double arrowhead the extramural PBG.

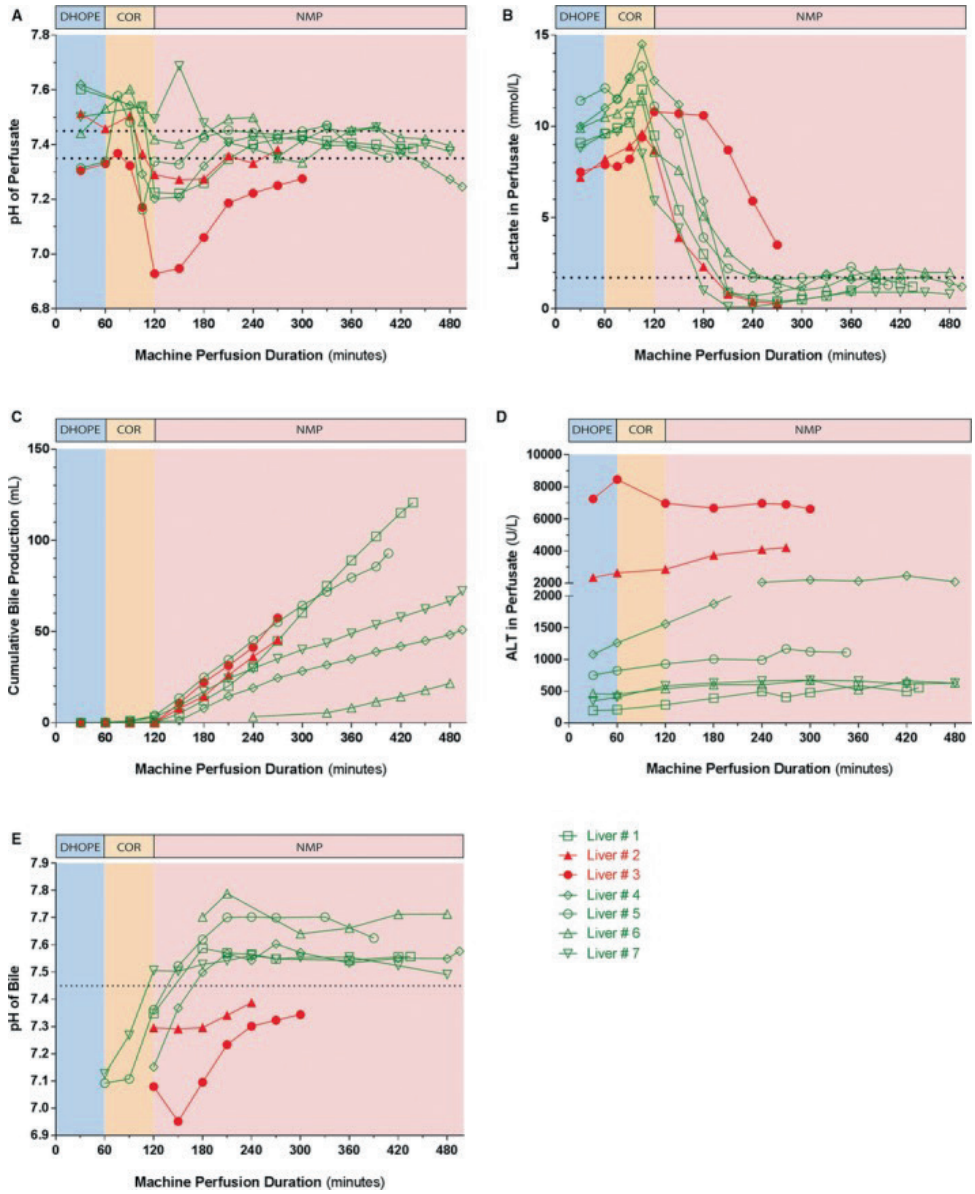


Figure 4. Machine perfusion fluid biochemistry. Panels A-B: Biochemical parameters used for viability assessment of the liver. In all but one liver, perfusate pH and lactate values normalized within 150 min after start of the NMP. Panel C: All livers produced sufficient amounts of bile. Liver #6 seemingly produced less bile due to a cannulation problem of the bile duct. Panel D: ALT perfusate levels were <2,000 U/L in the transplanted livers and >2,000 U/L in the non-transplanted livers. Panel E: Biliary pH, a marker of biliary epithelial viability, increased to >7.45 in all livers that were transplanted, whereas biliary pH remained <7.45 in the livers that were not transplanted livers. The red lines represent the non-transplanted livers and the green lines represent the transplanted livers.

Peri- and Postoperative Outcomes

Median follow up after transplantation was 197 days (IQR 152 – 307 days). Laboratory values of the recipients are shown in **Figure 5**. Serum ALT levels rapidly decreased during the first week after transplantation and values were (nearly) normal at 30 and 90 days after transplantation. The recipients of liver #1 and #7 had remarkably low peak serum ALT concentrations of 201 and 337 U/L, respectively (**Figure 5A**). Serum total bilirubin levels rapidly decreased during the first postoperative days in all recipients. However, a temporary peak in serum bilirubin was noted at the end of the first week in recipients of liver #4 and #6 (**Figure 5B**). None of the transplanted livers classified for early allograft dysfunction.[23]

Thus far we have observed a 100% patient- and graft survival. None of the recipients has developed clinically evident non-anastomotic strictures of the biliary tree during the median follow up of 197 days (6.5 months).

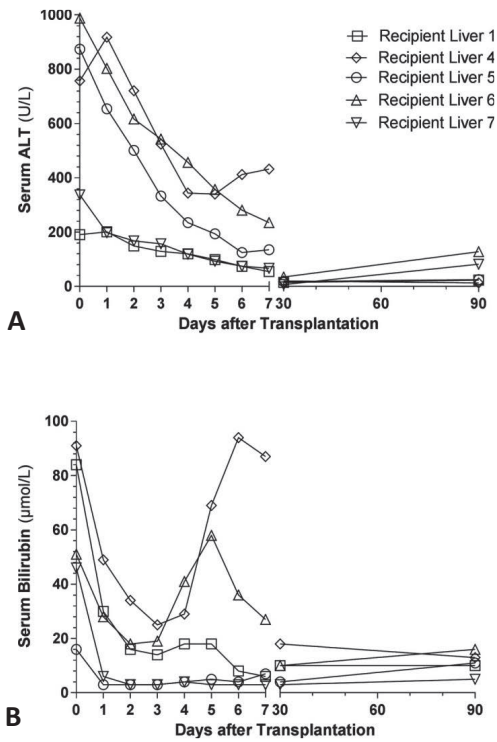


Figure 5: Post-transplantation serum alanine aminotransferase (ALT) and total bilirubin. Laboratory values were recorded at post-operative day 0 until 7, and at 1 and 3 months. Post-operative day 0 was defined as the time from reperfusion in the recipient until midnight of the same day. Panel A: Post-operative serum ALT concentrations rapidly decreased during the first week. The recipients of liver #1 and #7 had low peak serum ALT concentrations of 201 and 331 U/L, respectively. Panel B: Post-operative total bilirubin concentration likewise decreased during the first week, except for a transient increase in the recipients of livers #4

and #6 at the end of the first week. Bilirubin levels of these livers, however, normalized during the weeks thereafter.

DISCUSSION

The clinical series described in this report provides two novel findings that are relevant for the further development of machine perfusion technology in organ transplantation. First, we have successfully used a combination of hypothermic and normothermic machine perfusion to resuscitate and select initially declined suboptimal livers for transplantation. The 100% graft survival at 3 months, which was the primary end point, indicated the safety and feasibility of the procedure. Secondly, we have shown that an HBOC-based machine perfusion solution can be safely used in clinical liver transplantation.

Several groups have described the successful use of NMP to select suboptimal and initially declined donor livers.[7,12] Although the optimal parameters for viability assessment are still under debate, most groups have been using a combination of bile production, perfusate lactate levels, and pH as markers of hepatocellular function. We have previously suggested to use biliary pH and bicarbonate as markers of biliary epithelial (cholangiocellular) viability.[22] Biliary epithelium actively modifies bile composition by the secretion of bicarbonate, resulting in an alkalotic biliary environment. This alkalotic environment protects biliary epithelial cells against the detergent effects of hydrophobic bile salts, a phenomenon known as the “bicarbonate umbrella”.[24] In a clinical series of liver NMP, Watson et al. have recently confirmed the potential usefulness of biliary pH as marker of biliary viability.[12] We have, therefore, added biliary pH as a bile duct viability criterion to our protocol. Of the four predefined selection criteria, the criterion for biliary pH was the most frequent reason for secondary discard in these clinical series.

Although, favorable outcomes after NMP regarding graft and patient survival have been reported, it has not yet been demonstrated that NMP protects the cholangiocyte compartment.[7,12,25] (D)HOPE in DCD liver grafts has, on the other hand, been described to reduce histological signs of biliary ischemia-reperfusion injury and the incidence of post-transplant cholangiopathy.[2,3,26] Furthermore, DHOPE has been shown to reduce ischemia reperfusion injury via resuscitation of the mitochondria and the increase in hepatic ATP–content, thereby also protecting the hepatocytes.[2,3,6] The latter might explain why post-operative peak ALT was lower in our recipients than in the studies that compared SCS to end-ischemic NMP alone.[7,12] However, definitive conclusions on this topic cannot be drawn from the current study due to the lack of a control group.

In this study we combined the presumed benefits of DHOPE and NMP to resuscitate and select high-risk donor livers that can be safely transplanted, despite initial nation-wide decline. Besides a 100% graft survival at a median follow up of 197 days, none of the recipients has developed clinical signs of post-transplant cholangiopathy so far. The development of post-transplant cholangiopathy was, however, not the primary outcome of this study and was

based on clinical symptoms and laboratory findings, rather than on imaging studies. Therefore, subclinical cases of cholangiopathy may have been missed and final conclusions on the efficacy of combined DHOPE, COR and NMP in the prevention of post-transplant cholangiopathy require longer follow-up in a larger series.

We applied sequential DHOPE and NMP linked by a controlled rewarming phase, as sudden temperature shifts may contribute to cellular injury.[27,28] A previous clinical study has indicated that a short period of COR prior to implantation of donor livers results in less hepatocellular injury, compared to direct implantation of a cold stored donor liver, as evidenced by lower post-operative peak transaminases and a higher graft survival rate at 6 months after transplantation.[28] Based on the relative small number of livers included in the current study and the absence of a control group, we cannot draw conclusions on the value of the COR phase. Previous preclinical studies have shown that (D)HOPE and NMP can also be combined without a COR phase.[9,10]

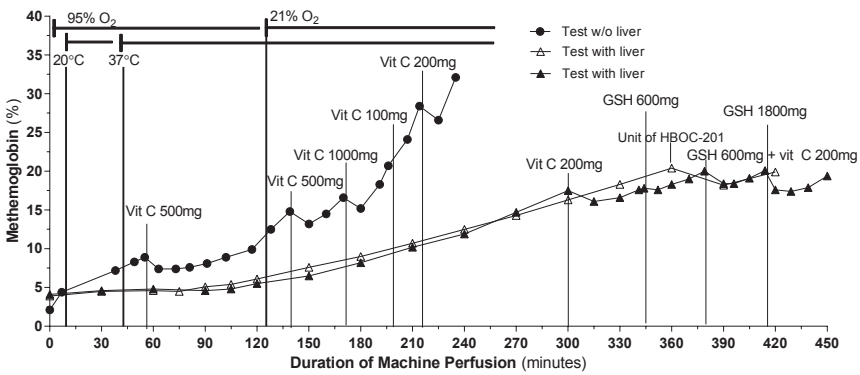
For the application of this combined machine perfusion protocol we have developed a perfusion fluid that can be used at various temperatures and eliminates the use of a third party human blood product. While during hypothermic machine perfusion, oxygen can be dissolved in the perfusion solution, during NMP an oxygen carrier is necessary. Perfusion solutions based on RBC, which are mostly used for NMP, cannot be used at low temperatures due to increasing lipid membrane stiffness and the risk of hemolysis. In contrast to RBC, HBOC can be used at low temperatures. In addition, HBOC-201, used in this study, has a lower oxygen affinity than human Hb in erythrocytes and thus gives off the oxygen more easily. In the cold, the affinity of HBOC-201 for oxygen increases, similar to that of human Hb in erythrocytes, but is still less.

HBOC-201 has previously been used in experimental and pre-clinical studies on *ex-situ* liver machine perfusion, but not in clinical practice.[14,16,17] In a study with discarded human livers, NMP with an HBOC-201-based perfusion fluid resulted in similar outcome compared to NMP with RBC, indicating HBOC-201 as a suitable alternative for RBC.[14] Our group reported higher ATP concentration, and cumulative bile production in discarded human livers undergoing NMP with an HBOC-201-based solution, compared to perfusion with an RBC-based perfusion fluid.[16] Altogether these preclinical studies and the currently presented first clinical application indicate that HBOC-201 can be used as a substitute for RBC in fluids for machine perfusion of donor organs.

A limitation of this series is a lack of a control group. However, livers of suboptimal quality with a perceived high risk of primary non-function or early allograft dysfunction were included in the current study, making it unethical, in our opinion, to transplant these livers without resuscitation and functional assessment. Furthermore, this study cannot discriminate between the beneficial effects of DHOPE, COR and NMP separately. Finally, extrahepatic bile duct biopsies of the two livers that were secondary declined for transplantation, based on their failure to produce bile with a pH>7.45 during NMP, were taken.

Yet, biopsies of higher level bile ducts were not taken. Although we have previously shown that the degree of histological injury of the extrahepatic bile duct of a donor liver after cold storage is representative for the degree of injury of the proximal biliary tree, including larger intrahepatic ducts[29], we do not formally know whether this is also true after NMP.

A potential limitation of HBOC is its susceptibility to a conversion into methemoglobin, especially in the venous phase with low oxygen saturation. In contrast to erythrocytes, HBOC do not contain NADH-dependent enzyme methemoglobin reductase, which is responsible for converting methemoglobin back to hemoglobin. We have noted a gradual increase in methemoglobin during NMP, but not during DHOPE when the perfusion fluid was oxygenated with an FiO_2 of 100% (**Figure 3S**). In a separate experiment, we have noted that the percentage of methemoglobin can be corrected or slowed down by the addition of extra HBOC-201, glutathione or vitamin C to the perfusion fluid (supporting material **Figure 3S**). However, we do not prefer the use of vitamin C due to its effects on the pH and osmolality of the perfusion fluid.



Supporting figure 3. Data on methemoglobin formation reduction strategies. Three machine perfusion procedures are shown in this figure. The line with the shaded dots depicts machine perfusion with HBOC-201 without a liver connected to the perfusion circuit. The addition of >500mg Vitamin C (ascorbic acid) resulted in a decrease of methemoglobin. The other two lines represent two livers that were included in the clinical trial. One of these lives was transplanted and the other one not. Temperature and oxygenation were according to the protocol, as described in the methods. In the transplanted liver, the addition of one unit of HBOC-201 decreased the percentage of methemoglobin. For the non-transplanted liver both >200mg Vitamin C and >600 mg glutathione (GSH) resulted in a decrease of methemoglobin. Based on these observations we prefer to add an extra unit of HBOC-201 or GSH to the perfusion fluid, if methemoglobin increases above 20% and NMP needs to be continued for more than an hour. *Abbreviations: Vit C, vitamin C (ascorbic acid); GSH, glutathione; HBOC-201, hemoglobin-based oxygen carrier-201; Tx, transplantation.*

In conclusion, this first clinical experience demonstrates the feasibility of combined hypo- and normothermic machine perfusion after traditional static cold storage of suboptimal liver grafts. The combination of oxygenated hypothermic and normothermic perfusion protects livers against ischemia-reperfusion injury and enables hepatobiliary viability assessment prior to transplantation. The use of a novel HBOC-201-based perfusion fluid eliminated the need to change perfusion fluid during the various temperature phases and appeared to be a safe alternative for RBC as oxygen carrier in *ex situ* donor organ machine perfusion. This new protocol of *ex situ* machine perfusion provides a tool to safely expand the pool of organs for liver transplantation.

ACKNOWLEDGEMENTS

We are grateful to Zafiris Zafirelis (HBO_2 Therapeutics) for providing HBOC-201 free of charge and for his advice on the use of this product. Moreover, we want to thank our organ perfusionists (Rinse Ubbink, Maureen Werner, Gert-Jan Pelgrim and Leonie Venema) for their help during the machine perfusion procedures.

REFERENCES

1. Karangwa SA, Dutkowski P, Fontes P, et al. Machine perfusion of donor livers for transplantation: A proposal for standardized nomenclature and reporting guidelines. *Am J Transplant*. 2016;16(10):2932-2942.
2. van Rijn R, Karimian N, Matton APM, et al. Dual hypothermic oxygenated machine perfusion in liver transplants donated after circulatory death. *Br J Surg*. 2017.
3. Dutkowski P, Polak WG, Muiesan P, et al. First comparison of hypothermic oxygenated PErfusion versus static cold storage of human donation after cardiac death liver transplants: An international-matched case analysis. *Ann Surg*. 2015;262(5):1.
4. Perera T, Mergental H, Stephenson B, et al. First human liver transplantation using a marginal allograft resuscitated by normothermic machine perfusion. *Liver Transpl*. 2016;22(1):120-124.
5. Watson CJ, Kosmoliaptsis V, Randle LV, et al. Preimplant normothermic liver perfusion of a suboptimal liver donated after circulatory death. *Am J Transplant*. 2016;16(1):353-357.
6. Schlegel A, Rougemont O, Graf R, Clavien PA, Dutkowski P. Protective mechanisms of end-ischemic cold machine perfusion in DCD liver grafts. *J Hepatol*. 2013;58(2):278-286.
7. Mergental H, Perera M, Laing RW, et al. Transplantation of declined liver allografts following normothermic ex-situ evaluation. *Am J Transplant*. 2016;16(11):3235-3245.
8. Sutton ME, op den Dries S, Karimian N, et al. Criteria for viability assessment of discarded human donor livers during ex vivo normothermic machine perfusion. *PLoS One*. 2014;9(11):e110642.
9. Boteon YL, Laing RW, Schlegel A, et al. Combined hypothermic and normothermic machine perfusion improves functional recovery of extended criteria donor livers. *Liver Transpl*. 2018. doi: 10.1002/lt.25315 [doi].
10. Westerkamp AC, Karimian N, Matton AP, et al. Oxygenated hypothermic machine perfusion after static cold storage improves hepatobiliary function of extended criteria donor livers. *Transplantation*. 2016;100(4):825-835.
11. op den Dries S, Karimian N, Sutton ME, et al. Ex vivo normothermic machine perfusion and viability testing of discarded human donor livers. *Am J Transplant*. 2013;13(5):1327-1335.
12. Watson CJE, Kosmoliaptsis V, Pley C, et al. Observations on the ex situ perfusion of livers for transplantation. *Am J Transplant*. 2018; doi: 10.1111/ajt.14687.
13. Selzner M, Goldaracena N, Echeverri J, et al. Normothermic ex vivo liver perfusion using steen solution as perfusate for human liver transplantation: First north american results. *Liver Transpl*. 2016;22(11):1501-1508.

14. Laing RW, Bhogal RH, Wallace L, et al. The use of an acellular oxygen carrier in a human liver model of normothermic machine perfusion. *Transplantation*. 2017;101(11):2746-2756.

15. Buttari B, Profumo E, Rigano R. Crosstalk between red blood cells and the immune system and its impact on atherosclerosis. *Biomed Res Int*. 2015;2015:616834.

16. Matton APM, Burlage LC, van Rijn R, et al. Normothermic machine perfusion of donor livers without the need for human blood products. *Liver Transpl*. 2018 ;24(4):528-538.

17. Fontes P, Lopez R, van der Plaats A, et al. Liver preservation with machine perfusion and a newly developed cell-free oxygen carrier solution under subnormothermic conditions. *Am J Transplant*. 2015;15(2):381-394.

18. Koetting M, Luer B, Efferz P, et al. Optimal time for hypothermic reconditioning of liver grafts by venous systemic oxygen persufflation in a large animal model. *Transplantation*. 2011;91(1):42-47.

19. Hardison WG, Wood CA. Importance of bicarbonate in bile salt independent fraction of bile flow. *Am J Physiol*. 1978;235(2)

20. Imber CJ, St Peter SD, Lopez de Cenarruzabeitia I, et al. Advantages of normothermic perfusion over cold storage in liver preservation. *Transplantation*. 2002;73(5):701-709.

21. Watson CJ, Kosmoliaptsis V, Randle LV, et al. Normothermic perfusion in the assessment and preservation of declined livers prior to transplantation: Hyperoxia and vasoplegia - important lessons from the first 12 cases. *Transplantation*. 2017;101(5):1084-1098.

22. Weeder PD, van Rijn R, Porte RJ. Machine perfusion in liver transplantation as a tool to prevent non-anastomotic biliary strictures: Rationale, current evidence and future directions. *J Hepatol*. 2015;63(1):265-275.

23. Olthoff KM, Kulik L, Samstein B, et al. Validation of a current definition of early allograft dysfunction in liver transplant recipients and analysis of risk factors. *Liver Transpl*. 2010;16(8):943-949.

24. Hohenester S, Wenniger LM, Paulusma CC, et al. A biliary HCO3- umbrella constitutes a protective mechanism against bile acid-induced injury in human cholangiocytes. *Hepatology*. 2012;55(1):173-183.

25. Nasralla D, Coussios CC, Mergental H, et al. A randomized trial of normothermic preservation in liver transplantation. *Nature*. 2018;557(7703):50-56.

26. van Rijn R, van Leeuwen OB, Matton APM, et al. Hypothermic oxygenated machine perfusion reduces bile duct reperfusion injury after transplantation of donation after circulatory death livers. *Liver Transpl*. 2018;24(5):655-664.

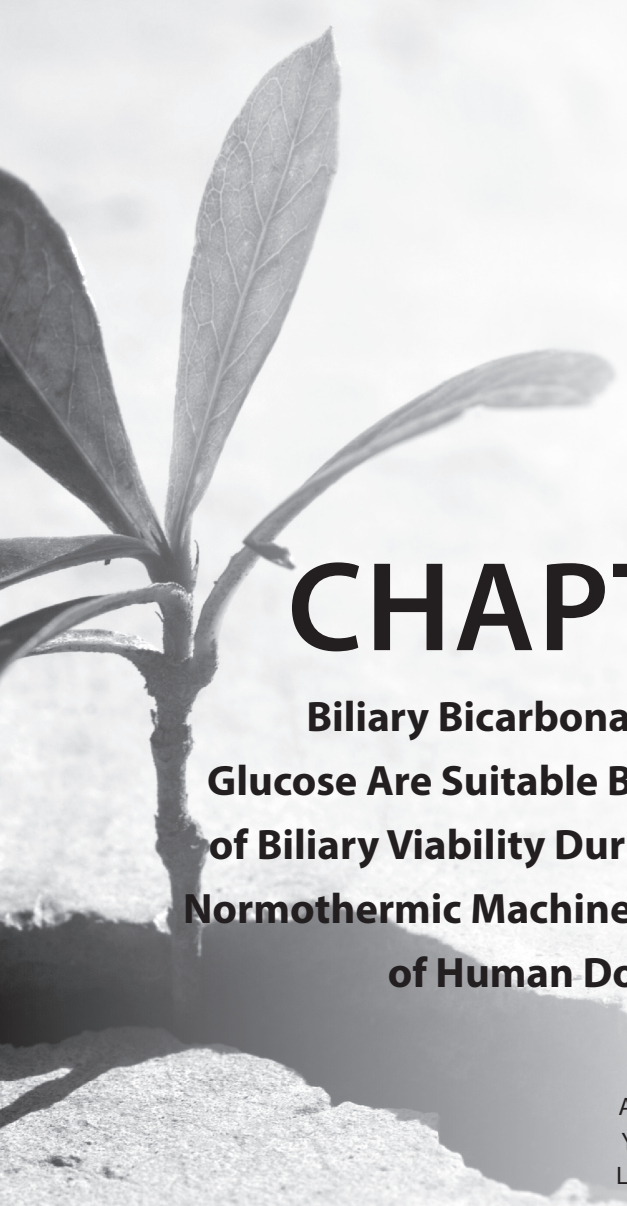
27. Minor T, Efferz P, Fox M, Wohlschlaeger J, Luer B. Controlled oxygenated rewarming of cold stored liver grafts by thermally graduated machine perfusion prior to reperfusion. *Am J Transplant*. 2013;13(6):1450-1460.

28. Hoyer DP, Mathe Z, Gallinat A, et al. Controlled oxygenated rewarming of cold stored livers prior to transplantation: First clinical application of a new concept. *Transplantation*. 2016;100(1):147-152.

29. Karimian N, Weeder PD, Bomfati F, et al. Preservation injury of the distal extrahepatic bile duct of donor livers is representative for injury of the intrahepatic bile ducts. J Hepatol. 2015;63(1):284-7

30. Schaubel DE, Sima CS, Goodrich NP, et al. The Survival Benefit of Deceased Donor Liver Transplantation as a Function of Candidate Disease Severity and Donor Quality. *Am J Transplant.* 2008; 8: 419-425.

31. Braat AE, Blok JJ, Putter H, et al. The Eurotransplant donor risk index in liver transplantation: ET-DRI. *Am J Transplant*. 2012;12(10):2789-2796.



6

CHAPTER

Biliary Bicarbonate, pH and Glucose Are Suitable Biomarkers of Biliary Viability During Ex Situ Normothermic Machine Perfusion of Human Donor Livers

Alix P.M. Matton
Yvonne de Vries
Laura C. Burlage
Rianne van Rijn
Masato Fujiyoshi
Vincent E. de Meijer
Marieke T. de Boer
Ruben H.J. de Kleine
Henkjan J. Verkade
Annette S. H. Gouw
Ton Lisman
Robert J. Porte

Transplantation. 2019; 103:1405-1413.

ABSTRACT

Background: *Ex situ* normothermic machine perfusion (NMP) can be used to assess viability of suboptimal donor livers prior to implantation. Our aim was to assess the diagnostic accuracy of bile biochemistry for the assessment of bile duct injury (BDI).

Methods: In a preclinical study, 23 human donor livers underwent 6 hours of end-ischemic NMP to determine biomarkers of BDI. Livers were divided into groups with low or high BDI, based on a clinically relevant histological grading system. During NMP, bile was analyzed biochemically and potential biomarkers were correlated with the degree of BDI. Receiver operating characteristics curves were generated to determine optimal cut-off values. For clinical validation, identified biomarkers were subsequently included as viability criteria in a clinical trial (n=6) to identify transplantable liver grafts with low BDI.

Results: Biliary bicarbonate and pH were significantly higher and biliary glucose was significantly lower in livers with low BDI, compared to high BDI. The following cut-off values were associated with low BDI: biliary bicarbonate >18 mmol/L (*P*=0.002), biliary pH >7.48 (*P*=0.019), biliary glucose <16 mmol/L (*P*=0.013), and bile/perfusate glucose ratio <0.67 *(P*=0.013). In the clinical trial, 4 out of 6 livers met these criteria and were transplanted, and none developed clinical evidence of post-transplant cholangiopathy.

Conclusions: Biliary bicarbonate, pH, and glucose during *ex situ* NMP of liver grafts are accurate biomarkers of BDI and can be easily determined point-of-care, making them suitable for the pre-transplant assessment of bile duct viability. This may improve graft selection and decrease the risk of post-transplant cholangiopathy.

INTRODUCTION

The gap between the demand and availability of donor livers for transplantation has stimulated the use of extended-criteria donor livers, including steatotic, elderly, and donation after circulatory death (DCD) liver grafts.[1] These types of donor livers, however, have a higher risk of developing postoperative complications. Especially post-transplant cholangiopathy, or non-anastomotic biliary strictures (NAS), occur in 13 – 35% of DCD grafts, compared to 1 – 24% of livers donated after brain death.[2-7] Although the pathogenesis of NAS is not fully understood, the degree of histological bile duct injury (BDI) at the time of transplantation has been identified as a strong predictor of the development of NAS after transplantation.[8-10]

In an attempt to expand the donor liver pool, researchers are increasingly using *ex situ* machine perfusion of liver grafts prior to transplantation. When performed at 37°C, normothermic machine perfusion (NMP) renders the organ metabolically active, which allows for viability testing and the selection of potentially transplantable organs.[11-14] Currently, criteria for the selection of suitable donor livers during NMP include bile production, lactate clearance, vascular flows, macroscopic appearance of the liver, transaminase concentration and pH buffering capacity.[11-14] These parameters, however, mainly reflect hepatocellular function and injury, and do not provide information about cholangiocellular function or injury. Identification and determination of the diagnostic accuracy of biomarkers of BDI is clinically relevant as they may provide a missing tool in the selection of liver grafts with an anticipated low risk of NAS. Low biliary pH, bicarbonate and high biliary glucose during NMP have recently been associated with the development of NAS in three cases, and with histological bile duct stroma necrosis in five research livers.[14] The authors reported that a biliary pH ≤7.4 during NMP was able to discriminate between these livers, but have provided no other cut-off values and have not reported on the implementation of these biliary parameters clinically.

Cholangiocytes lining the bile duct lumen and peribiliary glands actively contribute to the composition of bile by secretion of bicarbonate via cystic fibrosis transmembrane regulator (CFTR) and chloride-bicarbonate anion exchanger 2 (AE2).[15,16] As a result of this, bile in the extrahepatic bile duct has a pH ranging between 7.5 and 8.1 and bicarbonate concentration of 12 to 55 mmol/L.[17,18] Moreover, cholangiocytes actively reabsorb glucose from bile via sodium-dependent glucose transporter, SGLT1, expressed on their apical plasma membrane domain, and another glucose transporter, GLUT1, on their basolateral domain.[19,20] This results in very low biliary glucose concentrations under physiological conditions.[21,22] These transporters are ATP-dependent, and studies have shown that ischemia leads to diminished function of SGLT1 in cholangiocytes, with subsequent decreased glucose reabsorption.[23]

To determine the added value of specific biomarkers of biliary viability during NMP, we first examined whether histological BDI correlates with markers of

hepatocellular injury. As this correlation was poor, we proceeded to identify biomarkers of BDI that can be easily assessed point-of-care. Our aim was to determine the diagnostic accuracy of biliary pH, bicarbonate, glucose and lactate dehydrogenase (LDH) concentration[24-27], to discriminate between livers with a high or low degree of BDI.

MATERIALS AND METHODS

Donor Livers

Twenty-three human donor livers that were declined for transplantation nationwide and offered for research were used to determine suitable biomarkers of BDI during NMP in a preclinical study. All livers underwent six hours of NMP for viability testing after static cold storage, as described previously.[28] In brief, livers underwent NMP at 37°C using a pressure-controlled perfusion device (Liver Assist, Groningen, the Netherlands), providing continuous portal flow and pulsatile arterial flow. For the preclinical study, informed consent was obtained from relatives of the donors and the study protocol was approved by the Medical Ethical Committee of the University Medical Center Groningen (METc, #2012.068) and by the Dutch Transplantation Foundation (NTS). For validation purposes, the identified cut-off values were prospectively applied to six human livers in an ongoing clinical trial as described below.

Histological Analysis

Two biopsies of the common or proper hepatic bile duct were collected: one during the back-table procedure prior to NMP and one at the end of NMP (proximally of the biliary catheter). All biopsies were fixed in formalin and paraffin-embedded. Slides were stained with hematoxylin and eosin (H&E) and assessed by light microscopy using an established, clinically relevant, histological BDI grading system[10] (**Table 1**). The presence of stroma necrosis, injury of the extramural peribiliary glands, and injury of the perivascular plexus in pre-transplant bile duct biopsies have been found to predict the development of NAS after transplantation.[10] For this reason, we selected these three histological parameters for the present study. The range of the total BDI score was 0 – 7 (**Table 1**). Biopsies were independently scored in duplicate by two investigators (APMM and YdV) without knowledge of clinical or NMP data under supervision of an expert liver pathologist (ASHG), who resolved any discrepancies between the researchers' scores.

Table 1. Histological Bile Duct Injury Scoring System*.

Grade	Bile Duct Wall Stroma Necrosis	Extramural Peribiliary Glands	Peribiliary Vascular Plexus
0	No stroma necrosis	No loss or injury of cells	No vascular lesions
1	≤25% stroma necrotic	≤50% loss or injury of cells	≤50% of vessels necrotic
2	>25% and ≤50% stroma necrotic	>50% loss or injury of cells	>50% of vessels necrotic or no longer visible
3	>50% stroma necrotic		-

** Significant histological predictors of NAS were selected from op den Dries et al.*[10]

Bile Sample Analyses

During NMP, bile samples were collected every 30 min under mineral oil (to prevent the exchange of CO_2 between the sample and ambient air, which would influence the pH and bicarbonate concentration) for immediate point-of-care determination of pH, bicarbonate and glucose concentration using an ABL90 FLEX analyzer (Radiometer, Brønhøj, Denmark). Additional bile samples were stored at -80°C and later analyzed for LDH using routine laboratory technique for standard patient care.

Hepatocellular Injury and Function

Alanine aminotransferase (ALT) concentration, lactate clearance and bile production are commonly used parameters to assess hepatocellular injury and function during NMP.[11-14] ALT and lactate concentrations in perfusate were analyzed as described previously.[28] A peak ALT concentration <6,000 U/L was considered to reflect low hepatocellular injury. A lactate concentration of ≤2.5 mmol/L and cumulative bile production of ≥5 mL/kg liver within 2.5 h of NMP were considered to reflect good hepatocellular function.

Validation Cohort

Identified biomarkers of BDI were subsequently included as selection criteria, in addition to hepatocellular injury and function criteria, in a clinical trial of end-ischemic NMP for viability assessment of high-risk donor livers that were declined for transplantation nationwide based on perceived suboptimal quality (www.trialregister.nl; NTR5972). The study protocol was approved by the Medical Ethical Committee (METc #2017.281) and all patients gave written informed consent.

Statistics

Continuous variables were presented as median with interquartile range (IQR) and were compared between groups using the Mann-Whitney U test. Correlations were calculated using the Spearman's rank correlation test. Receiver operating characteristics (ROC) curves were generated for biliary pH, bicarbonate, glucose, and LDH concentration, as well as for the bile/perfusate glucose concentration ratio and the delta between perfusate and biliary glucose concentration (perfusate minus biliary glucose) to illustrate diagnostic ability of the binary BDI-score. The first time point with an area under the ROC curve (AUC-ROC) >0.80 and a P <0.05 for each potential biomarker was selected to determine the cut-off value with the highest sensitivity and specificity to discriminate livers with low BDI from those with high BDI. Positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (LR+) and negative likelihood ratio (LR-) with corresponding 95% confidence interval (CI) were calculated using cross tabulation. Level of significance was set at P-value <0.05. All statistical analyses were performed using IBM SPSS version 23.0 (Chicago, IL, USA).

RESULTS

Donor Liver Characteristics

In the pre-clinical study, 18 livers were from DCD donors and 5 livers were donated after brain death (DBD). The overall median (IQR) cold ischemia time was 8.1 (7.0 – 9.3) h and warm ischemia time (from withdrawal of life support until *in situ* cold flush) in DCD livers was 38 (33 – 42) min.

Bile Duct Histology

The degree of BDI per histological item in livers with low or high BDI is summarized in **Table 2**. Representative examples of H&E staining of bile ducts with low or high BDI are presented in **Figure 1**. The median BDI score before NMP was 4.0 (3.0 – 7.0) and 6.0 (6.0 – 7.0) after NMP, with 8 livers showing no change in the BDI score over time. The mean BDI score of the two biopsies ranged between 3 – 7, with 4.75 as the median of the range. This value was chosen to divide livers into a group with a low and a group with a high BDI score.

Table 2. Comparison of Bile Duct Injury Score per Histological Item.

	Livers with a Low BDI Score (n=9)	Livers with a High BDI Score (n=14)
Stroma Necrosis (Grade 0 – 3)	2.0 (1.5 - 2.0)	3.0 (2.6 – 3.0)
Extramural Peribiliary Glands (Grade 0 – 2)	1.0 (1.0 – 1.0)	2.0 (1.5 – 2.0)
Peribiliary Vascular Plexus (Grade 0 – 2)	1.0 (1.0 – 1.5)	2.0 (2.0 – 2.0)

Variables are presented as median and (interquartile range). *Abbreviations: BDI, bile duct injury.*

Hepatocellular Function and Injury Correlate Poorly with BDI

Overall, there was a weak correlation between peak ALT concentration in perfusate during NMP, a marker of hepatocellular injury, and the degree of BDI (Spearman r 0.424, *P*=0.044) (**Figure 2A**). In livers with extremely high ALT levels (>6,000 U/L), the BDI score was also high in 7 out of 8 cases (88%). However, when ALT levels were <6,000 U/L and livers could potentially be considered for transplantation based on an acceptable degree of hepatocellular injury[14], the correlation between ALT and BDI was very poor (Spearman r 0.192, *P*=0.493) with 7 out of 15 (47%) livers still having a high BDI score.

Similarly, 4 out of 9 (44%) livers with good lactate clearance also had high BDI (**Figure 2B**). There was no correlation between BDI and lactate concentration (Spearman r 0.349, *P*=0.103). Likewise, 10 out of 18 (56%) livers with high bile production also had high BDI. There was no correlation between BDI and cumulative bile production (Spearman r -0.291, *P*=0.178) (**Figure 2C**). Furthermore, there was no correlation between (cumulative) bile production and biliary pH, bicarbonate, LDH and the glucose and bile/perfusate glucose ratio (**SDC, Figure S1**).These findings indicate that markers of hepatocellular injury and function poorly predicted the degree of BDI, especially in cases that could potentially be considered for transplantation based on hepatocellular criteria. This supports the need for specific biomarkers of BDI during NMP.

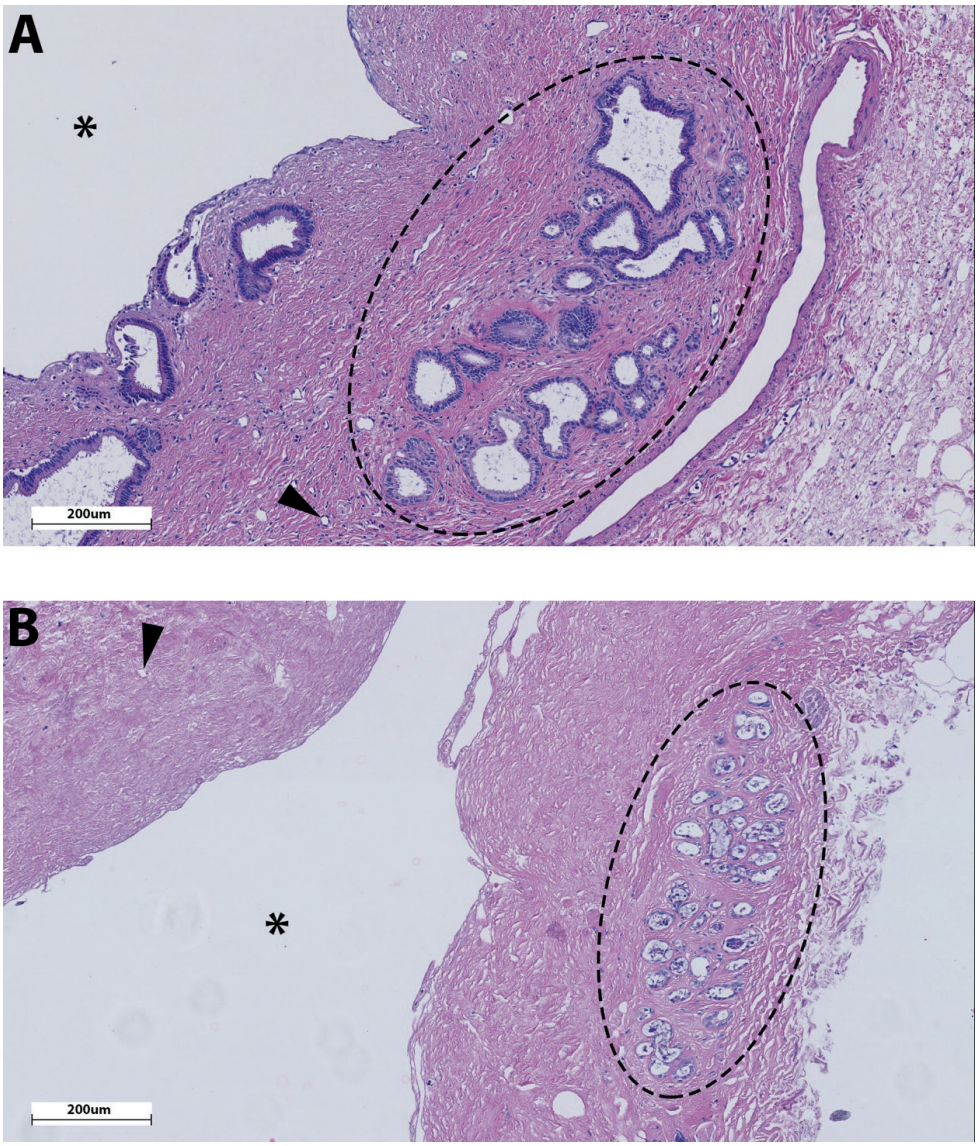


Figure 1. **Representative histological H&E staining of an extrahepatic bile duct biopsy with a low histological bile duct injury (BDI) score (A) and a high BDI score (B).** (A) Intact extramural peribiliary glands (encircled), intact vasculature (e.g. arrowhead pointing to vital arteriole in stroma) and no signs of stroma necrosis. Note that also the periluminal peribiliary glands are largely intact. (B) Severe injury to the extramural peribiliary glands with loss of cells (encircled), necrotic (arrowhead) or absent vessels and diffuse stroma necrosis. Note the denuded epithelial lining of the bile duct lumen (asterisk), as is the case in >90% of all donor bile ducts prior to transplantation.[8-10]

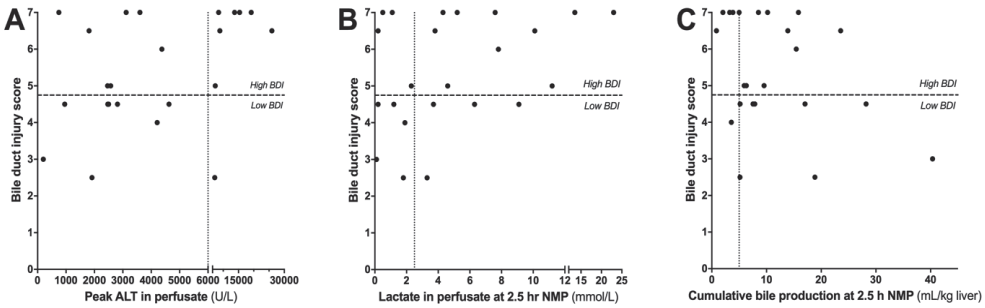


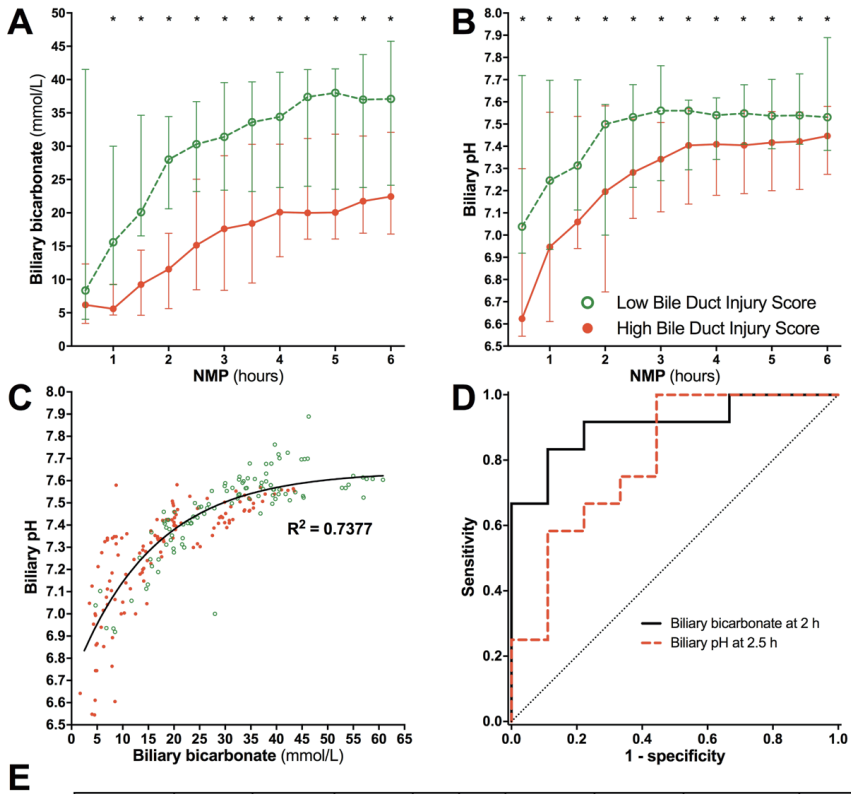
Figure 2. Poor correlation between hepatocellular injury and function and bile duct injury (BDI). (A) Peak ALT concentration in perfusate during normothermic machine perfusion (NMP) correlated poorly with BDI. This was especially true for livers with ALT <6,000 U/L, which are potentially transplantable based on this hepatocellular criterion, of which nearly half had simultaneous high BDI. Livers with very high ALT (>6,000 U/L) frequently had very high BDI scores. (B) Nearly half of the livers with good lactate clearance, also had a high BDI score. (C) There was no correlation between bile production and BDI, with over half of livers with high bile production also having high BDI. *Abbreviations: ALT, alanine-aminotransferase; BDI, bile duct injury.*

Biliary pH and Bicarbonate Correlate Significantly with BDI

Biliary pH and bicarbonate concentration during NMP were significantly higher in livers with low BDI, compared to livers with high BDI (**Figure 3A-B**). As expected, biliary pH and bicarbonate were strongly correlated (**Figure 3C**). The line of best fit (R^2=0.7377) demonstrated that in the lower range of biliary bicarbonate small increases lead to a relatively large increase in pH, while at higher bicarbonate concentrations (>30 mmol/L) pH remained relatively stable.

Since bile production can be low or absent during the first 2 h of NMP, we only used bile samples collected after 2 h to determine optimal cut-off values to discriminate livers with low BDI from those with high BDI (**SDC, Table S1**). Already after 2 h, a biliary bicarbonate concentration of 18 mmol/L discriminated between low and high BDI with an AUC-ROC of 0.91 (*P*=0.002) and a sensitivity, specificity, PPV, NPV >80% (**Figure 3D-E**).

The earliest time point at which biliary pH discriminated livers with low BDI from those with high BDI was 2.5 h (**SDC, Table S1**). At this time point, the optimal cut-off value for pH was 7.48, with an AUC-ROC of 0.81 (*P*=0.019) (**Figure 3D-E**).



E

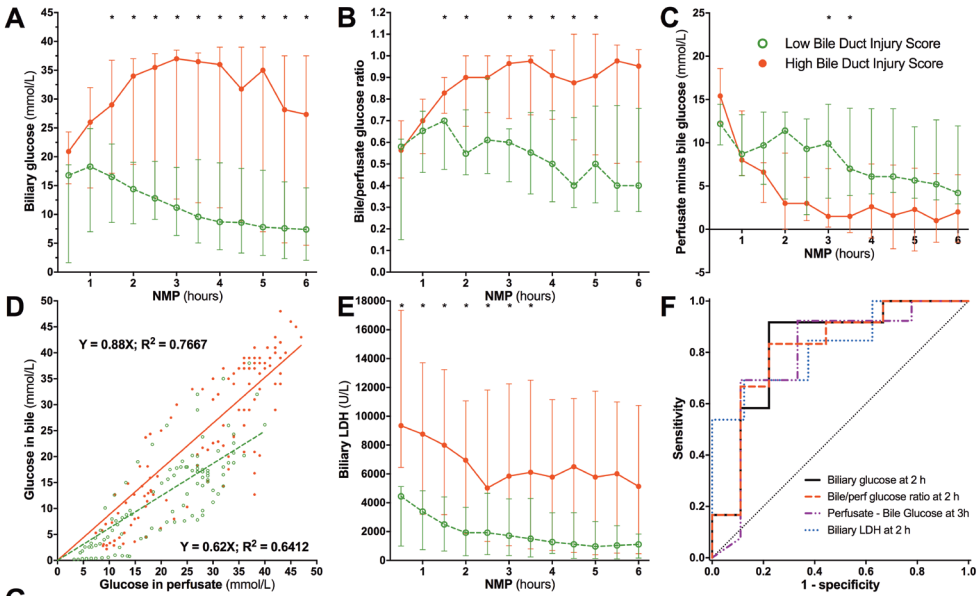
	Time of NMP	Value	AUC-ROC	*P* - value	Sens	Spec	PPV	NPV	LR+ (95% CI)	LR- (95% CI)
Biliary Bicarbonate	2.0 h	18 mmol/L	0.91	0.002	83%	89%	91% (61 – 98%)	80% (53 – 94%)	7.50 (1.16 – 48.43)	0.19 (0.05 – 0.68)
Biliary pH	2.5 h	7.48	0.81	0.019	75%	67%	75% (53 – 89%)	67% (40 – 86%)	2.25 (0.84 – 6.00)	0.38 (0.13 – 1.11)

Figure 3. Biliary bicarbonate and pH produced during normothermic machine perfusion (NMP) correlated significantly with bile duct injury (BDI). Biliary bicarbonate concentration (A) and biliary pH (B) were significantly higher in livers with a low BDI score, compared to livers with a high BDI score at each time point during NMP. (C) Biliary pH and bicarbonate concentration were strongly correlated with each other. The line of best fit shows that in the lower range of biliary bicarbonate, small increases in biliary bicarbonate led to relatively large increases in biliary pH. At biliary bicarbonate >30 mmol/L, biliary pH remained relatively stable despite further increases in bicarbonate concentration. (D) ROC curves and (E) statistical analyses of biliary bicarbonate and pH used to discriminate high and low bile duct injury at the earliest time points. * p <0.05. More detailed results, including calculations for all time points of bile collection during 6 h of normothermic machine perfusion, are provided in **SDC, Table S1**. *Abbreviations: AUC-ROC, area under the curve of the receiver operating characteristics curve; CI, confidence interval; LDH, lactate dehydrogenase; LR+, positive likelihood ratio; LR-, negative likelihood ratio; NPV, negative predictive value; PPV, positive predictive value; Sens, sensitivity; Spec, specificity.*

Biliary Glucose and Bile/Perfusate Glucose Ratio Correlate Significantly with BDI

Glucose concentrations were lower in bile of livers with low BDI, compared to livers with high BDI score (**Figure 4A**). While biliary glucose gradually decreased in livers with low BDI, biliary glucose increased in livers with high BDI, generally remaining >20 mmol/L. In livers with high BDI, the median bile/glucose concentration ratio increased to 1 and remained stable throughout perfusion. In contrast, in livers with low BDI the median ratio was always ≤0.7 and gradually declined over time (**Figure 4B-C**). Another way to study the relation between biliary and perfusate glucose is by calculating the difference between perfusate and biliary glucose concentration (delta).[14] In livers with high BDI, the delta was lower compared to livers with low BDI, reaching significance at only 3 and 3.5 h NMP (**Figure 4C**). After several hours of NMP, negative delta values were reached.

The earliest time point at which biliary glucose and the bile/perfusate glucose ratio could discriminate livers with low BDI from those with high BDI was 2 h (**SDC, Table S1**). At this time point, the optimal cut-off values were 16 mmol/L and 0.67, respectively (both AUC 0.82; *P*=0.013) (**Figure 4F-G**). The delta between perfusate and biliary glucose did not result in an AUC > 0.80 at any time point, though at 3 h NMP an AUC of 0.79 resulted in a cut-off value of 2.3 mmol/L (*P*=0.030, **Figure 4F-G**, **SDC, Table S1**).



G

	Time of NMP	Value	AUC-ROC	*P* - value	Sens	Spec	PPV	NPV	LR+ (95% CI)	LR- (95% CI)
Biliary Glucose	2.0 h	16 mmol/L	0.82	0.013	92%	78%	85% (62 – 95%)	88% (51 – 98%)	4.12 (1.20 – 14.17)	0.11 (0.02 – 0.72)
Bile/Perfusate Glucose Ratio	2.0 h	0.67	0.82	0.013	83%	78%	83% (59 – 95%)	78% (49 – 93%)	3.75 (1.08 – 13.07)	0.21 (0.06 – 0.80)
Delta Perfusate Bile Glucose	3.0 h	2.3 mmol/L	0.79	0.023	69%	89%	79% (59 – 90%)	86% (46 – 98%)	2.75 (1.07 – 7.04)	0.13 (0.02 – 0.86)
Biliary LDH	2.0 h	3689 U/L	0.83	0.014	69%	88%	90% (58 – 98%)	64% (43 – 81%)	5.54 (0.85 – 35.90)	0.35 (0.15 – 0.83)

Figure 4. Biliary glucose, bile/perfusate glucose ratio and biliary LDH correlated significantly with bile duct injury (BDI) during normothermic machine perfusion (NMP). (A) Biliary glucose concentration, inversely reflecting biliary epithelial cell function, was significantly lower in livers with low BDI, compared to livers with high BDI at nearly each time point during NMP. (B) In livers with high BDI, the bile/perfusate glucose concentration ratio was higher compared to livers with low BDI. (C) The delta between perfusate and biliary glucose concentration was lower in livers with high BDI, even reaching negative values at the end of NMP. (D) Livers with high BDI have relatively higher biliary glucose in relation to perfusate glucose concentrations, compared to livers with a low BDI. This resulted in a steeper slope in high BDI livers (slope 0.88), compared to low BDI livers (slope 0.62). (E) Biliary LDH concentration, a marker of biliary epithelial cell injury, was significantly higher at each time point in livers with a high BDI score compared to livers with a low BDI score. (F) ROC curves and (G) statistical analyses of biliary glucose, bile/perfusate glucose ratio, perfusate - biliary glucose delta and biliary LDH used to discriminate high and low bile duct injury at the earliest time points**.** * p <0.05. More detailed results, including calculations for all time points of bile collection during 6 h of normothermic machine perfusion, are provided in **SDC, Table S1**. *Abbreviations: AUC-ROC, area under the curve of the receiver operating characteristics curve; CI, confidence interval; LDH, lactate dehydrogenase; LR+, positive likelihood ratio; LR-, negative likelihood ratio; NPV, negative predictive value; PPV, positive predictive value; Sens, sensitivity; Spec, specificity.*

Biliary LDH Correlates Significantly with BDI

Biliary LDH concentration, a marker of biliary epithelial cell injury, was >2-fold higher in livers with high BDI, compared to livers with low BDI (**Figure 4E**). In both groups biliary LDH concentration declined gradually during NMP. The earliest time point with a significant AUC-ROC for biliary LDH was 2 h NMP (**SDC, Table S1**), with an optimal cut-off value of 3,689 U/L and an AUC of 0.83 (*P*=0.014) (**Figure 4F-G**).

Clinical Validation of Biliary Biomarkers during NMP

Based on the preclinical research data, we included biliary pH as one of the criteria for hepatobiliary viability assessment in a clinical trial of end-ischemic NMP of high-risk livers that were initially declined nationwide for transplantation. So far, 6 DCD livers (median Eurotransplant-Donor Risk Index[30] (ET-DRI): 2.9 (IQR 2.7 – 2.9)) have been included in this trial of which 4 (median ET-DRI: 2.9, (IQR 2.8 – 3.0); median United Kingdom-DCD risk score[31] 6.0 (IQR 5.8 – 7.5)) met all selection criteria for transplantation during NMP, including a biliary pH >7.48 within 2.5 h of NMP. In addition, biliary bicarbonate and glucose bile/perfusate ratio were within the ranges identified in the preclinical study (**Figure 5**). The recipients of these four liver grafts had an uneventful recovery and none of them developed clinical evidence of post-transplant cholangiopathy at a median follow-up of 8.3 months (IQR 7.6 – 10.1). Of the two livers that were declined for transplantation, the first fulfilled all hepatocellular criteria and was declined only on the basis of bile biochemistry (at 2.5 h NMP: cumulative bile production 45 mL (≥10 mL within 2.5 h NMP and ≥4 mL in preceding hour); perfusate lactate 0.3 mmol/L (<1.7 mmol/L); perfusate pH 7.38 (7.35 – 7.45)).The second liver was also declined based on hepatocellular criteria (cumulative bile production 57 mL; perfusate lactate 3.5 mmol/L; perfusate pH 7.25). At 2.5 h NMP, the perfusate ALT concentration was approximately 4,000 U/L and 6,000 U/L respectively. The retrospectively determined median BDI score for the transplanted livers was 3.2 (IQR 2.8 – 3.5) and 4.3 (3.0, 5.5) for the non-transplanted livers.

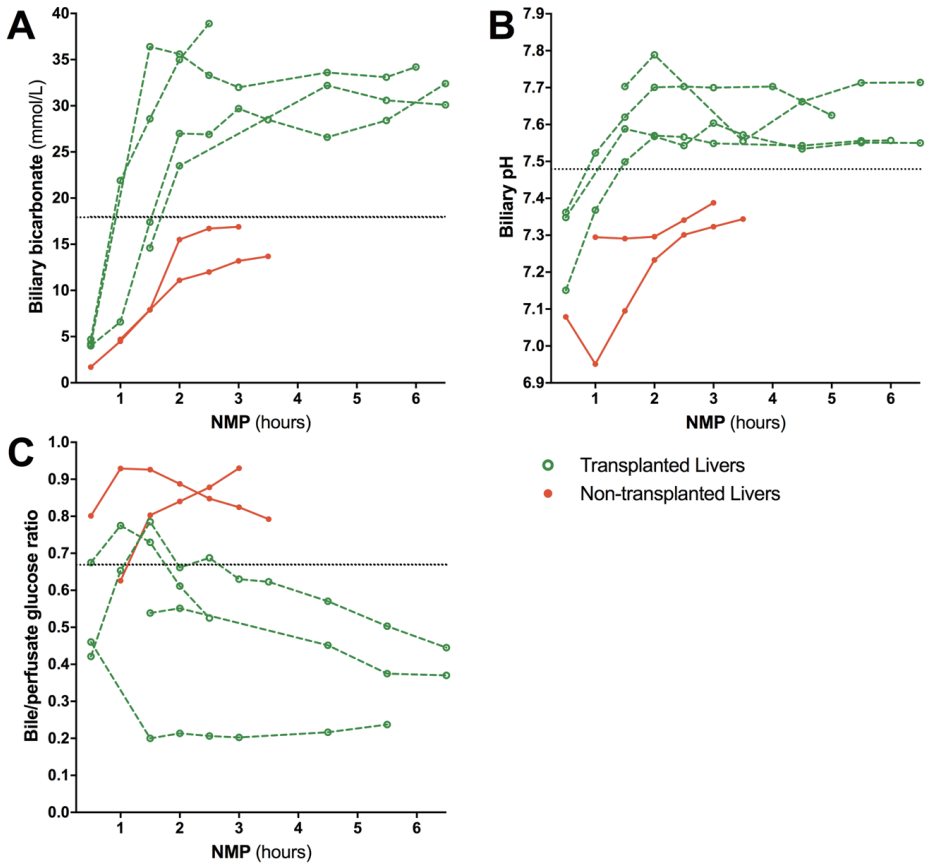


Figure 5. Clinical validation of biliary biomarkers during *ex-situ* normothermic machine perfusion (NMP) of transplanted and non-transplanted livers. Six livers that were declined for transplantation nationwide underwent end-ischemic NMP to assess their viability for transplantation in a clinical trial. According to protocol, viability assessment occurred within 2.5 h NMP. Four livers fulfilled the hepatobiliary criteria and were transplanted. There was no clinical evidence of post-transplant cholangiopathy at a median of 8.3 months (IQR 7.6 – 10.1) follow-up. Dotted lines indicate biliary biomarker cut-off values established in the preclinical study.

Table S1. Optimal Biliary Biomarker Cut-off Values per Time Point of NMP Based on AUC-ROC Analyses.

Biomarker	Number of Cases*	Cut-off Value	Time of NMP	AUC-ROC	*P* - value	Sens	Spec	PPV	NPV	LR+ (95% CI)	LR- (95% CI)
Biliary Bicarbonate Concentration	***21***	***18***	***2.0 h***	***0.91***	***0.002***	***83%***	***89%***	***91% (61 – 98%)***	***80% (53 – 94%)***	***7.50 (1.16 –***	***0.19 (0.05 –***
	21	27	2.5 h	0.86	0.006	83%	78%	83% (59 –95%)	78% (49 –93%)	3.75 (1.08 –	0.21 (0.06 – 0.80)
	21	26	3.0 h	0.83	0.012	75%	78%	82% (56 – 94%)	70% (45 –87%)	3.38 (0.95 –	0.32 (0.11 – 0.91)
	21	32	3.5 h	0.81	0.019	83%	67%	77% (56 – 90%)	75% (44 – 92%)	2.50 (0.96 – 6.52)	0.25 (0.07 – 0.96)
	20	32	4.0 h	0.80	0.025	82%	67%	75% (53 – 89%)	75% (44 – 92%)	2.45 (0.94 – 6.44)	0.27 (0.07 – 1.04)
	19	31	4.5 h	0.79	0.034	80%	67%	73% (50 – 88%)	75% (44 – 92%)	2.40 (0.91 – 6.36)	0.30 (0.08 – 1.13)
	19	32	5.0 h	0.78	0.041	80%	67%	73% (50 – 88%)	75% (44 – 92%)	2.40 (0.91 – 6.36)	0.30 (0.08 – 1.13)
	19	32	5.5 h	0.77	0.050	80%	67%	73% (50 – 88%)	75% (44 – 92%)	2.40 (0.91 – 6.36)	0.30 (0.08 – 1.13)
	19	32	6.0 h	0.78	0.041	80%	67%	73% (50 – 88%)	75% (44 – 92%)	2.40 (0.91 – 6.36)	0.30 (0.08 – 1.13)
Biliary pH	21	7.32	2.0 h	0.75	0.051	75%	78%	82% (56 – 94%)	70% (45 – 87%)	3.38 (0.95 –	0.32 (0.11 – 0.91)
	21	***7.48***	***2.5 h***	***0.81***	***0.019***	***75%***	***67%***	***75% (53 – 89%)***	***67% (40 – 86%)***	***2.25 (0.84 – 6.00)***	***0.38 (0.13 –***
	21	7.45	3.0 h	0.81	0.017	75%	78%	82% (56 – 94%)	70% (45 – 87%)	3.38 (0.95 –	0.32 (0.11 – 0.91)
	21	7.46	3.5 h	0.79	0.028	75%	78%	82% (56 – 94%)	70% (45 – 87%)	3.38 (0.95 –	0.32 (0.11 – 0.91)
	20	7.51	4.0 h	0.80	0.025	91%	67%	77% (57 – 90%)	86% (47 – 98%)	2.73 (1.06 – 7.00)	0.14 (0.02 – 0.93)
	19	7.53	4.5 h	0.85	0.010	90%	67%	75% (54 – 89%)	86% (47 – 98%)	2.70 (1.05 – 6.96)	0.15 (0.02 – 1.02)
	19	7.50	5.0 h	0.79	0.034	90%	67%	75% (54 – 89%)	86% (47 – 98%)	2.70 (1.05 – 6.96)	0.15 (0.02 – 1.02)
	19	7.51	5.5 h	0.81	0.022	80%	67%	73% (50 – 88%)	75% (44 – 92%)	2.40 (0.91 – 6.36)	0.30 (0.08 – 1.13)
	19	7.51	6.0 h	0.77	0.045	80%	67%	73% (50 – 88%)	75% (44 – 92%)	2.40 (0.91 – 6.36)	0.30 (0.08 – 1.13)
Biliary Glucose Concentration	***21***	***16***	***2.0 h***	***0.82***	***0.013***	***92%***	***78%***	***85% (62 – 95%)***	***88% (51 – 98%)***	***4.12 (1.20 –***	***0.11 (0.02 –***
	21	27	2.5 h	0.77	0.039	67%	89%	89% (55 – 98%)	67% (47 – 82%)	6.00 (0.91 –	0.38 (0.16 – 0.86)
	21	24	3.0 h	0.81	0.015	69%	88%	90% (58 – 98%)	64% (43 – 80%)	5.54 (0.85 –	0.35 (0.15 – 0.83)
	22	23	3.5 h	0.81	0.015	69%	89%	90% (58 – 98%)	67% (46 – 82%)	6.23 (0.95 –	0.35 (0.15 – 0.81)
	22	22	4.0 h	0.81	0.016	69%	89%	90% (58 – 98 %)	67% (46 – 82%)	6.23 (0.95 –	0.35 (0.15 – 0.81)
	21	23	4.5 h	0.79	0.028	67%	100%	100%	69% (50 – 83%)	n.a.[†]	0.33 (0.15 – 0.74)
	20	21	5.0 h	0.82	0.015	73%	100%	100%	75% (53 – 89%)	n.a.	0.27 (0.10 – 0.72)
	19	20	5.5 h	0.80	0.027	70%	100%	100%	75% (54 – 89%)	n.a.	0.30 (0.12 – 0.77)
	19	19	6.0 h	0.80	0.027	70%	100%	100%	75% (54 – 89%)	n.a.	0.30 (0.12 – 0.77)

Biomarker	Number of Cases	Cut-off Value	Time of NMP	AUC-ROC	*P* - value	Sens	Spec	PPV	NPV	LR+ (95% CI)	LR- (95% CI)
Bile/Perfusate Glucose Ratio	***21***	***0.67***	***2.0 h***	***0.82***	***0.013***	***83%***	***78%***	***83% (59 – 95%)***	***78% (49 – 93%)***	***3.75 (1.08 –***	***0.21 (0.06 –***
	21	0.70	2.5 h	0.71	0.110	83%	78%	83% (59 – 95%)	78% (49 – 93%)	3.75 (1.08 –	0.21 (0.06 – 0.80)
	22	0.66	3.0 h	0.78	0.030	85%	78%	85% (61 – 95%)	78% (48 – 93%)	3.81 (1.10 –	0.20 (0.05 – 0.74)
	22	0.70	3.5 h	0.77	0.035	85%	78%	85% (61 – 95%)	78% (48 – 93%)	3.81 (1.10 –	0.20 (0.05 – 0.74)
	22	0.73	4.0 h	0.76	0.042	77%	78%	83% (59 – 95%)	70% (45 – 87%)	3.46 (0.98 –	0.30 (0.10 – 0.85)
	21	0.72	4.5 h	0.77	0.039	75%	67%	75% (53 – 89%)	67% (40 – 86%)	2.25 (0.84 – 6.00)	0.38 (0.13 – 1.11)
	20	0.74	5.0 h	0.75	0.063	82%	78%	82% (56 – 94%)	78% (49 – 93%)	3.68 (1.05 –	0.23 (0.06 – 0.86)
	19	0.78	5.5 h	0.75	0.066	70%	89%	88% (51 – 98%)	73% (50 – 88%)	6.30 (0.95 –	0.34 (0.13 – 0.89)
	19	0.72	6.0 h	0.73	0.086	80%	78%	80% (53 – 93%)	78% (49 – 93%)	3.60 (1.02 –	0.26 (0.07 – 0.93)
Biliary LDH Concentration	***21***	***3689 U/L***	***2.0 h***	***0.83***	***0.014***	***69%***	***88%***	***90% (58 – 98%)***	***64% (43 – 81%)***	***5.54 (0.85 –***	***0.35 (0.15 –***
	22	4710 U/L	2.5 h	0.78	0.03	69%	78%	82% (55 – 94%)	64% (42 – 81 %)	3.12 (0.87 –	0.40 (0.16 – 0.96)
	22	2393 U/L	3.0 h	0.79	0.025	69%	67%	75% (53 – 89%)	60% (37 – 79%)	2.08 (0.77 – 5.60)	0.46 (0.18 – 1.18)
	22	3696 U/L	3.5 h	0.79	0.021	69%	89%	90% (58 – 98%)	67% (46 – 82%)	6.23 (0.95 –	0.35 (0.15 – 0.81)
	21	2749 U/L	4.0 h	0.72	0.088	67%	78%	80% (53 – 94%)	64% (42 – 81%)	3.00 (0.83 –	0.43 (0.18 – 1.03)
	21	2818 U/L	4.5 h	0.74	0.065	67%	78%	80% (53 – 94%)	64% (42 – 81%)	3.00 (0.83 –	0.43 (0.18 – 1.03)
	20	2210 U/L	5.0 h	0.73	0.087	64%	78%	78% (49 – 93%)	64% (43 – 80%)	2.86 (0.78 -	0.47 (0.20 - 1.10)
	20	2614 U/L	5.5 h	0.75	0.063	64%	78%	78% (49 – 93%)	64% (43 – 80%)	2.86 (0.78 -	0.47 (0.20 -1.10)
	20	2445 U/L	6.0 h	0.69	0.149	64%	89%	88% (51 – 98%)	67% (47 – 82%)	5.73 (0.86 -	0.41 (90.18 -

Bold and italic values are the chosen cut-off points based on the first bile sample with an AUCROC >0.80 and a P-value <0.05. * Values do not add up to the total of 23 due to livers not producing bile at every time point, or occasional errors in reporting values by the ABL90 FLEX analyzer. † When specificity is 100%, calculating the LR+ is mathematically impossible. *Abbreviations: AUC-ROC, area under the curve of the receiver operating characteristics curve; CI, confidence interval; LDH, lactate dehydrogenase; LR+ positive likelihood ratio; LR-, negative likelihood ratio; NMP, normothermic machine perfusion; n.a., not applicable; NPV, negative predictive value; PPV, positive predictive value; Sens, sensitivity; Spec, specificity.*

DISCUSSION

In the present study, we have determined the diagnostic accuracy of the previously described biomarkers of biliary injury and viability, which can be easily assessed point-of-care during *ex situ* NMP of human donor livers. Biliary bicarbonate concentration >18 mmol/L, biliary pH >7.48, biliary glucose concentration <16 mmol/L, bile/perfusate glucose concentration ratio <0.67, and biliary LDH concentration <3,689 U/L within 2.5 h of NMP were strongly associated with low histological BDI. These findings have important clinical implications as the proposed biomarkers allow transplant teams to stratify livers grafts during *ex situ* NMP based on the risk of BDI, and potentially also that of post-transplant cholangiopathy. Biliary bicarbonate had the highest predictive value out of all of the studied biomarkers. This is likely because biliary bicarbonate is less influenced by other, non-biliary factors.

Ex situ NMP is increasingly being applied and explored as a tool to assess viability of liver grafts that were initially declined for transplantation based on a perceived high risk of early graft failure.[11-14] In most centers, selection criteria are currently based on hepatocellular injury and function. The risk of post-transplant graft failure, however, is not only determined by the degree of hepatocellular injury, but also by the presence of biliary injury. Especially DCD livers have an increased risk of developing biliary complications.[32] In a recent clinical study of 12 initially declined liver grafts that were identified as transplantable during end-ischemic NMP, 25% developed post-transplant cholangiopathy despite adequate hepatocellular function during pre-transplant *ex situ* NMP.[13] These clinical data are in line with our observation that some livers with a low degree of hepatocellular injury can still have a high degree of BDI, which can be missed when no specific biliary viability criteria are used. This difference in hepatocellular and cholangiocellular injury can be explained by the greater susceptibility of cholangiocytes to ischemia-reperfusion injury and a slower post-ischemic recovery of intracellular ATP, compared to hepatocytes.[23,33]

The parameters we have selected as potential biomarkers of biliary viability were all based on the known physiological function of healthy bile duct epithelium. Two important physiological functions of biliary epithelium are active secretion of bicarbonate and reabsorption of glucose, leading to an alkalotic pH and very low biliary glucose concentrations at the level of the extrahepatic bile duct.[17,18] We have previously suggested that these parameters could potentially serve as biomarkers of bile duct viability during machine perfusion.[26,34,35] In the current study, we demonstrated that biliary pH, bicarbonate and glucose concentration indeed strongly correlate with histological BDI of liver grafts during NMP, confirming observations made earlier this year.[14] In three independent clinical studies, the histological degree of BDI before liver transplantation has been identified as a significant predictor of the development of NAS after transplantation.[8-10]

Our findings suggest that biliary epithelial cells of livers with high BDI are unable to secrete sufficient amounts of bicarbonate to raise the biliary pH. Increasing the biliary pH helps biliary epithelial cells to protect themselves against the toxic effects of hydrophobic bile salts, which is also known as the "biliary bicarbonate umbrella".[36] Low biliary pH and bicarbonate, therefore, not only reflect biliary injury/dysfunction, but may also contribute to additional biliary injury due to an absent bicarbonate umbrella.[37] Biliary pH and bicarbonate were not linearly correlated, which is explained by the fact pH varies on a logarithmic scale, whereas bicarbonate does not. The pH values we observed in liver grafts with low BDI are within the normal range of biliary pH, which varies between 7.5 and 8.1 in the common bile duct.[17] The same is true for biliary bicarbonate concentration, which normally ranges widely between 12 and 55 mmol/L.[18] Interestingly, Watson et al. recently reported three patients that developed cholangiopathy after the transplantation of livers that were unable to produce bile with a pH >7.4 during NMP.[14] This preliminary clinical observation is in line with the identified biomarkers of BDI in the current study in preclinical livers as well as the clinical validation cohort.

While biliary glucose also correlated significantly with the degree of biliary injury, the interpretation of glucose values is slightly more complex. Post-ischemic reperfusion of a liver graft almost universally results in a pronounced increase of glucose levels in the perfusion fluid due to glycogenolysis.[38] This contributes to a higher glucose concentration in the primary bile produced by hepatocytes, which affects the reabsorption of glucose from bile when it passes from the canaliculi to the common bile duct. Even when the biliary epithelium is intact, glucose reabsorption via SGLT1 and GLUT1 becomes insufficient when biliary glucose concentrations are too high, a phenomenon analogous to the renal threshold for glucose in kidney tubuli.[22,39] In other words, high glucose concentration in the perfusion fluid (which is frequently seen during NMP) may affect the biliary reabsorption of glucose. Biliary glucose levels should therefore be viewed in relation to glucose levels in the perfusion fluid. To illustrate this, we have correlated glucose concentrations in bile with those in the perfusion fluid. The ratio between glucose in bile and perfusion fluid was almost 1 in livers with high BDI, while it was ≤0.7 in livers with low BDI. Others have reported the delta between perfusate and biliary glucose levels,[14] though in our hands this did not result in a usable criterion. Our findings regarding biliary glucose are in line with an *in vitro* study with rat cholangiocytes, in which ATP-depleted cholangiocytes showed prolonged dysfunction of biliary glucose transporter SGLT1 and diminished glucose reabsorption.[23] Similarly, in an experimental study in rabbits, higher glucose concentrations were found in bile of livers that had suffered from warm and cold ischemia, compared to livers that had not been ischemic.[40]

The observations made for biliary bicarbonate, pH and glucose were supported by the data on biliary LDH concentrations. Based on animal experiments, biliary LDH has previously been proposed as a marker of biliary epithelial injury.[24] In the current study, biliary LDH was indeed significantly higher in livers with high

BDI, compared to livers with low BDI. In all livers, however, biliary LDH concentration declined gradually during NMP, which can be explained by an early washout of LDH from dead cholangiocytes when bile flow recurs. This is in line with previous research, where biliary LDH correlated with the length of cold ischemia in a rat liver model and previous studies that reported lower LDH concentrations in machine perfused livers compared to cold stored livers.[25,27,41]

This study has some limitations. We took biopsies from the extrahepatic bile duct to assess the degree of histological injury, while the intrahepatic bile ducts can also be involved in post-transplant cholangiopathy. However, in a previous study, we have demonstrated that histological injury (in particular injury to the peribiliary vascular plexus, extramural peribiliary glands and stroma necrosis) assessed at the level of the extrahepatic bile duct also reflects the degree of injury in the intrahepatic bile ducts, as deep as the segmental bile ducts.[42] Furthermore, there may have been some degree of sampling error in bile duct biopsies. To minimize this, the average BDI of two biopsies was taken. Furthermore, our preclinical data included 5 DBD livers, whereas the clinical livers were all DCD. However, when we excluded the DBD livers in the preclinical study, similar results were obtained. Lastly, some livers produce very little or no recorded bile during NMP. This raises the question as to whether or not such livers can be transplanted safely. We would advocate an approach tailored to the specific donor liver and recipient. For example, when assessing a liver with a high risk of developing NAS, production of good quality bile during NMP would be essential to allow cholangiocyte viability assessment prior to transplantation. On the other hand, for livers with a low risk of NAS, cholangiocyte viability assessment may not always be necessary, as has been reported in the literature.[14]

Implementation of the identified biomarkers in larger clinical trials of NMP with longer follow-up, in which both DCD and DBD livers are included, is necessary to confirm their utility in decreasing the incidence of post-transplant cholangiopathy. Machine perfusion has the potential to provide opportunities to ameliorate BDI, for instance through stem cell therapy. These cholangiocellular criteria could be applied to identify livers that require additional pharmacological interventions, and be used to assess the efficacy of such treatments. In conclusion, biliary bicarbonate, pH, glucose, and LDH during *ex situ* NMP are accurate biomarkers of clinically relevant, histological biliary injury of livers grafts. These biomarkers can be easily determined point-of-care, making them suitable for the assessment of bile duct viability during NMP and the potential identification of donor livers with a low risk of developing post-transplant cholangiopathy.

ACKNOWLEDGEMENTS

The authors would like to thank all donors and donor relatives, as well as the Dutch Transplantation Society and its staff, for enabling the use of donor livers for research.

REFERENCES

1. Jochmans I, van Rosmalen M, Pirenne J, Samuel U. Adult liver allocation in Eurotransplant. *Transplantation.* 2017;101:1542-1550.
2. de Vries Y, von Meijenfeldt FA, Porte RJ. Post-transplant cholangiopathy: Classification, pathogenesis, and preventive strategies. *Biochim Biophys Acta.* 2017.
3. Meurisse N, Vanden Bussche S, Jochmans I, et al. Outcomes of liver transplantations using donations after circulatory death: a single-center experience. *Transplant Proc.* 2012;44:2868-2873.
4. Jay CL, Lyuksemburg V, Ladner DP, et al. Ischemic cholangiopathy after controlled donation after cardiac death liver transplantation: a meta-analysis. *Ann Surg.* 2011;253:259-264.
5. Dubbeld J, Hoekstra H, Farid W, et al. Similar liver transplantation survival with selected cardiac death donors and brain death donors. *Br J Surg.* 2010;97:744-753.
6. Pine JK, Aldouri A, Young AL, et al. Liver transplantation following donation after cardiac death: an analysis using matched pairs. *Liver Transpl.* 2009;15:1072-1082.
7. Chan EY, Olson LC, Kisthard JA, et al. Ischemic cholangiopathy following liver transplantation from donation after cardiac death donors. *Liver Transpl.* 2008;14:604-610.
8. Hansen T, Hollemann D, Pitton MB, et al. Histological examination and evaluation of donor bile ducts received during orthotopic liver transplantation--a morphological clue to ischemic-type biliary lesion? *Virchows Arch.* 2012;461:41-48.
9. Brunner SM, Junger H, Ruemmele P, et al. Bile duct damage after cold storage of deceased donor livers predicts biliary complications after liver transplantation. *J Hepatol.* 2013;58:1133-1139.
10. op den Dries S, Westerkamp AC, Karimian N, et al. Injury to peribiliary glands and vascular plexus before liver transplantation predicts formation of non-anastomotic biliary strictures. *J Hepatol.* 2014;60:1172-1179.
11. Sutton ME, op den Dries S, Karimian N, et al. Criteria for viability assessment of discarded human donor livers during ex vivo normothermic machine perfusion. *PLoS One.* 2014;9:e110642.
12. Mergental H, Perera MT, Laing RW, et al. Transplantation of Declined Liver Allografts Following Normothermic Ex-Situ Evaluation. *Am J Transplant.* 2016;16:3235-3245.
13. Watson CJE, Kosmoliaptsis V, Randle LV, et al. Normothermic Perfusion in the Assessment and Preservation of Declined Livers Before Transplantation: Hyperoxia and Vasoplegia-Important Lessons From the First 12 Cases. *Transplantation.* 2017;101:1084-1098.

14. Watson CJE, Kosmoliaptsis V, Pley C, et al. Observations on the ex situ perfusion of livers for transplantation. *Am J Transplant.* 2018.

15. Cohn JA, Strong TV, Picciotto MR, Nairn AC, Collins FS, Fitz JG. Localization of the cystic fibrosis transmembrane conductance regulator in human bile duct epithelial cells. *Gastroenterology.* 1993;105:1857-1864.

16. Martinez-Anso E, Castillo JE, Diez J, Medina JF, Prieto J. Immunohistochemical detection of chloride/bicarbonate anion exchangers in human liver. *Hepatology.* 1994;19:1400-1406.

17. Sutor DJ, Wilkie LI. Diurnal variations in the pH of pathological gallbladder bile. *Gut.* 1976;17:971-974.

18. Boyer JL. Bile formation and secretion. *Compr Physiol.* 2013;3:1035-1078.

19. Lazaridis KN, Pham L, Vroman B, de Groen PC, LaRusso NF. Kinetic and molecular identification of sodium-dependent glucose transporter in normal rat cholangiocytes. *Am J Physiol.* 1997;272:G1168-74.

20. Masyuk AI, Masyuk TV, Tietz PS, Splinter PL, LaRusso NF. Intrahepatic bile ducts transport water in response to absorbed glucose. *Am J Physiol Cell Physiol.* 2002;283:C785-91.

21. Schein CJ, Zumoff B, Kream J, Cassouto J, Hellman L. A blood-bile glucose barrier in man. *Gastroenterology.* 1968;54:1094-1097.

22. Guzelian P, Boyer JL. Glucose reabsorption from bile. Evidence for a biliohepatic circulation. *J Clin Invest.* 1974;53:526-535.

23. Doctor RB, Dahl RH, Salter KD, Fouassier L, Chen J, Fitz JG. ATP depletion in rat cholangiocytes leads to marked internalization of membrane proteins. *Hepatology.* 2000;31:1045-1054.

24. Vajdova K, Smrekova R, Kukan M, Lutterova M, Wsolova L. Bile analysis as a tool for assessing integrity of biliary epithelial cells after cold ischemia--reperfusion of rat livers. *Cryobiology.* 2000;41:145-152.

25. Vairetti M, Ferrigno A, Rizzo V, et al. Correlation between the liver temperature employed during machine perfusion and reperfusion damage: role of Ca2+. *Liver Transpl.* 2008;14:494-503.

26. op den Dries S, Karimian N, Sutton ME, et al. Ex vivo normothermic machine perfusion and viability testing of discarded human donor livers. *Am J Transplant.* 2013;13:1327-1335.

27. Liu Q, Nassar A, Farias K, et al. Sanguineous normothermic machine perfusion improves hemodynamics and biliary epithelial regeneration in donation after cardiac death porcine livers. *Liver Transpl.* 2014;20:987-999.

28. Matton APM, Burlage LC, van Rijn R, et al. Normothermic machine perfusion of donor livers without the need for human blood products. *Liver Transpl.* 2018;24:528-538.

29. Laing RW, Mergental H, Yap C, et al. Viability testing and transplantation of marginal livers (VITTAL) using normothermic machine perfusion: study protocol for an open-label, non-randomised, prospective, single-arm trial. *BMJ Open.* 2017;7:e017733-2017-017733.

30. Braat AE, Blok JJ, Putter H, et al. The Eurotransplant donor risk index in liver transplantation: ET-DRI. *Am J Transplant.* 2012;12:2789-2796.

31. Schlegel A, Kalisvaart M, Scalera I, et al. The UK DCD Risk Score: A new proposal to define futility in donation-after-circulatory-death liver transplantation. *J Hepatol.* 2018;68:456-464.

32. Blok JJ, Detry O, Putter H, et al. Longterm results of liver transplantation from donation after circulatory death. *Liver Transpl.* 2016;22:1107-1114.

33. Noack K, Bronk SF, Kato A, Gores GJ. The greater vulnerability of bile duct cells to reoxygenation injury than to anoxia. Implications for the pathogenesis of biliary strictures after liver transplantation. *Transplantation.* 1993;56:495-500.

34. Bruinsma BG, Yeh H, Ozer S, et al. Subnormothermic machine perfusion for ex vivo preservation and recovery of the human liver for transplantation. *Am J Transplant.* 2014;14:1400-1409.

35. Weeder PD, van Rijn R, Porte RJ. Machine perfusion in liver transplantation as a tool to prevent non-anastomotic biliary strictures: Rationale, current evidence and future directions. *J Hepatol.* 2015;63:265-275.

36. Beuers U, Hohenester S, de Buy Wenniger LJ, Kremer AE, Jansen PL, Elferink RP. The biliary HCO(3)(-) umbrella: a unifying hypothesis on pathogenetic and therapeutic aspects of fibrosing cholangiopathies. *Hepatology.* 2010;52:1489-1496.

37. Op den Dries S, Sutton ME, Lisman T, Porte RJ. Protection of bile ducts in liver transplantation: looking beyond ischemia. *Transplantation.* 2011;92:373-379.

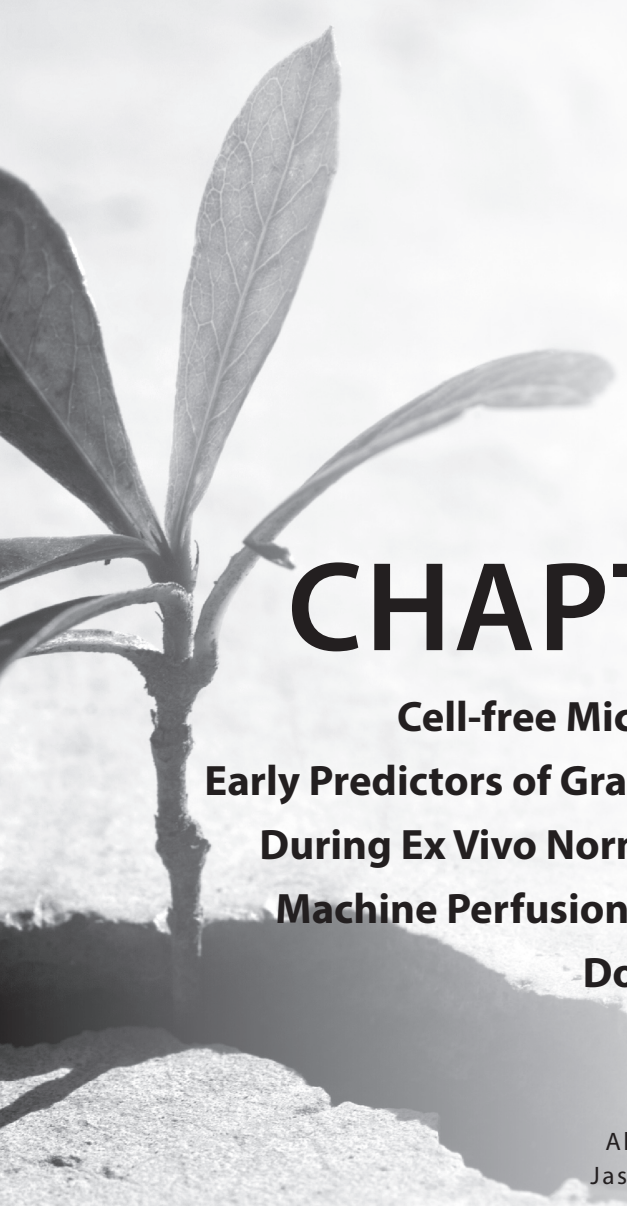
38. Villalobos-Molina R, Saavedra-Molina A, Devlin TM. Effect of hypoxia and reoxygenation on metabolic pathways in rat hepatocytes. *Arch Med Res.* 1998;29:219-223.

39. Lawrence RD. Renal Threshold for Glucose: Normal and in Diabetics. *Br Med J.* 1940;1:766-768.

40. Habib MM, Hafez TS, Parkes HG, Seifalian AM, Fuller BJ, Davidson BR. A comparison of bile composition from heart-beating and non-heart-beating rabbit organ donors during normothermic extracorporeal liver perfusion: experimental evaluation using proton magnetic resonance spectroscopy. *Transplant Proc.* 2004;36:2914-2916.

41. Westerkamp AC, Karimian N, Matton AP, et al. Oxygenated Hypothermic Machine Perfusion After Static Cold Storage Improves Hepatobiliary Function of Extended Criteria Donor Livers. *Transplantation.* 2016;100:825-835.

42. Karimian N, Weeder PD, Bomfati F, Gouw AS, Porte RJ. Preservation injury of the distal extrahepatic bile duct of donor livers is representative for injury of the intrahepatic bile ducts. *J Hepatol.* 2015;63:284-287.



7

CHAPTER

Cell-free MicroRNAs as Early Predictors of Graft Viability During Ex Vivo Normothermic Machine Perfusion of Human Donor Livers

Alix P.M. Matton*
Jasmijn W. Selten*
Henk P. Roest
Jeroen de Jonge
Jan N.M. IJzermans
Vincent E. de Meijer
Robert J. Porte&
Luc J.W. van der Laan&

*Equally contributing authors
& Shared senior authorship

Submitted for publication.

ABSTRACT

Background: Cell-free microRNAs (miRs) have emerged as early and sensitive biomarkers for tissue injury and function. This study aimed to investigate whether the release of hepatocyte-derived microRNAs (HDmiRs) and cholangiocyte-derived miRs (CDmiRs) correlate with hepato-cholangiocellular injury and function during oxygenated normothermic machine perfusion (NMP).
Methods: Donor livers (n=12) declined for transplantation were subjected to 6 h NMP after a static cold storage period. Perfusate and bile samples were analyzed using qRT-PCR for HDmiR-122 and CDmiR-222. Spearman correlations were performed between miR levels and currently available indicators and classic markers.
Results: Both HDmiR-122 and CDmiR-222 levels in perfusate at 30 min of NMP strongly correlated with hepatocyte injury (peak perfusate AST) and cholangiocyte injury (peak biliary LDH). In bile, only CDmiR-222 correlated with these injury markers. Regarding hepato-cholangiocellular function, both miRs in perfusate correlated with total bilirubin, while HDmiR-122 (in perfusate) and CDmiR-222 (in bile) correlated with bicarbonate secretion. The relative ratio (HDmiR-122/CDmiR-222) and AST (perfusate, 30 min) significantly correlated with cumulative bile volume.
Conclusion: Early levels of HDmiR-122 and CDmiR-222, in perfusate and/or bile, are predictive of excretory functions and hepato-cholangiocellular injury after 6 h NMP. These miRs may represent new biomarkers for graft viability and function during machine perfusion.

INTRODUCTION

To address the global shortage of donor livers, the use of "extended criteria" donor livers, which include steatotic, elderly, and donation after circulatory death (DCD) livers, has substantially increased.[1,2] Organs from these donors typically are more susceptible to ischemic damage, resulting in an increased incidence of primary non-function, early allograft dysfunction (EAD) and biliary complications such as non-anastomotic strictures (NAS).[3-5]

Normothermic machine perfusion (NMP) is a technique whereby donor livers are perfused *ex situ*. At 37°C, NMP renders the organ metabolically active, offering a window for assessing the viability of the graft. Several research groups have proposed putative markers of organ viability in this context, but their utility in predicting outcome thus far is limited.[6-8] The lack of reliable, objective measurements to determine organ quality prior to transplantation limits clinicians in predicting graft performance following transplantation and possibly improving graft function during machine perfusion.

In the field of biomarker discovery, small non-coding RNAs, in particular microRNAs (miRs), have emerged as sensitive, specific and stable markers for cell function, cell stress and cell injury.[9] Several studies have recently investigated the release of miRs in relation to cellular injury resulting from liver transplantation.[10,11] It was shown that not only levels of released miRs differ between damaged and normal functioning hepatocytes and cholangiocytes but also that, during impaired excretory function and injury of hepatocytes, the liver shows polarized release of extracellular hepatocyte-derived miRs (HDmiRs) and cholangiocyte-derived miRs (CDmiRs) into both bile and serum, suggesting active rather than passive underlying release mechanisms.[12]

The aim of the current study was to assess whether miRs in the perfusate and bile of normothermically perfused liver grafts correlate with, and are predictive of, hepatocellular and cholangiocellular injury and liver function as measured by currently available indicators and classic markers.

MATERIALS AND METHODS

Donor Livers

Between August 2012 and August 2014, twelve consecutive human donor livers that were declined for transplantation were included in this study after informed consent had been obtained. Following a period of static cold storage (SCS), livers underwent 6 h of NMP for viability assessment. This cohort has been previously described in the study by Westerkamp et al. 2016 ("SCS only" group).[13]

The study protocol was approved by the medical ethical committee of the University Medical Center Groningen (UMCG) (METC 2012.068) and the Dutch Transplantation Foundation (NTS).

Machine Perfusion

Upon arrival at the UMCG, NMP was performed using the Liver Assist (Organ Assist, Groningen, the Netherlands) with a perfusion solution based on packed red blood cells and fresh frozen plasma as described previously.[13] Bile and perfusion fluid samples were obtained at baseline prior to connecting the liver and at 30 min intervals, and essentially deprived of cells by centrifugation for 5 min at 2700 rpm. Supernatants were subsequently stored at -80°C until further use.

Assessment of Hepatobiliary Function and Injury

Lactate, glucose, bicarbonate and total bilirubin were measured using an ABL800 FLEX analyzer (Radiometer, Brønhøj, Denmark). Aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were measured using routine biochemical methods. Cumulative bile production, measured gravimetrically, was expressed as mL bile/kg liver.

RNA Isolation

Fifty µl of essentially cell-free bile or cell-free perfusion fluid was supplemented with 150 µl PBS and total RNA was extracted using the Qiagen miRNeasy kit (Qiagen, Venlo, the Netherlands), essentially as described previously.[12] Samples were spiked with 200 amol of artificial *Caenorhabditis elegans* miR-39 (Cel-miR-39, Sigma Aldrich, Zwijndrecht, the Netherlands) during the lysis procedure to monitor loss during workup. RNA was eluted using 50 µl of nuclease-free water and stored at -80°C until further use.

Reverse transcription and real-time polymerase chain reaction (qRT-PCR)

Complement DNA (cDNA) synthesis was performed using the Taqman microRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) as suggested by the manufacturer with minor modifications as described previously.[14] To eliminate qRT-PCR inhibition by heparin, the RT reactions were co-incubated with 6 IU heparinase (New England Biolabs, Ipswich, MA, USA).[15] One hundred amol of synthetic Cel-miR-54 was added to monitor the presence of PCR inhibiting components that co-eluted with total RNA, and/or incomplete heparin degradation. cDNA samples were diluted to 100 µl with nuclease-free water and stored at -20°C until further use. PCR reactions were performed in duplicate essentially as described by the manufacturer with the following modifications: 6 µl Taqman 2x Universal PCR Master Mix was mixed with 0.5 µl microRNA-specific PCR primer (Applied Biosystems), 0.5 µl nuclease-free water, and 5.0 µl of the diluted cDNA. The mature sequences of the miRs analyzed, both endogenous and synthetic, are summarized in **Table 1**.

Table 1. Mature microRNA sequences and assays.

microRNA	Mature sequence	Assay ID
hsa-miR-122	UGGAGUGUGACAAUGGUGUUUG	002245
hsa-miR-222	AGCUACAUCUGGCUACUGGGU	002276
cel-miR-39	UCACCGGGUGUAAAUCAGCUUG	000200
cel-miR-54	UACCCGUAAUCUUCAUAAUCCGAG	001361

Statistical Analysis

Levels of endogenous miRs in perfusate and bile, represented as relative values (2^{-Cq}), were normalized using the relative values of the spiked-in synthetic Cel-miR-39 as described by Vandesompele et al. with the highest relative value set to 1.[16] Continuous variables were presented as median with interquartile rage (IQR) and compared between groups using the Mann-Whitney U-test. In order to determine the predictive value of both miRs, relative miR levels in perfusate at 30 min and in bile at 2 h were correlated with hepato-cholangiocellular parameters using the Spearman correlations test. The level of significance was set at $p<0.05$. All statistical analyses were performed using SPSS software version 22.0 for Windows (SPSS, Inc., Chicago, IL) and GraphPad Prism 5.0 (GraphPad Software, La Jolla California USA).

RESULTS

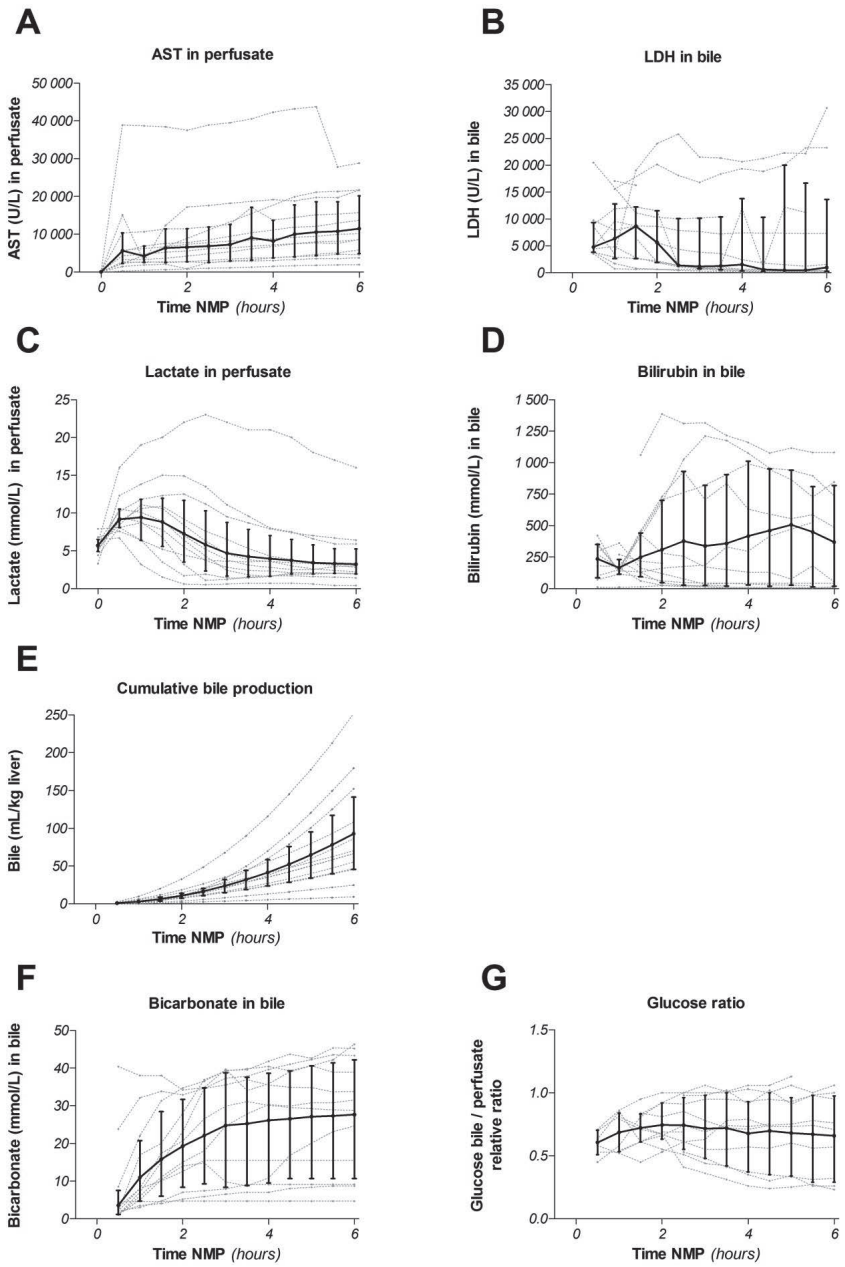
Hepato-cholangiocellular injury and function parameters during NMP

An overview of donor characteristics has previously been published by Westerkamp et al. ("SCS-only" group) and is provided in **Table 2**.[13] **Supplementary Figure 1** shows the levels of hepatocellular injury and function parameters during 6 h of NMP. AST in perfusate and LDH in bile reflect hepatocyte and cholangiocyte injury, respectively. Lactate in perfusate, bilirubin in bile, and (cumulative) bile production reflect hepatocyte function during NMP. Bicarbonate in bile and the bile/perfusate glucose ratio are indicators of cholangiocyte secretory function and cholangiocyte resorptive function, respectively.[17]

Table 2. Donor characteristics.

Donor characteristics	**n = 12**
Age (years)	61 (52-64)
Gender	
Male	8 (67%)
Female	4 (33%)
Body mass index (kg/m2)	27.3 (24.5-36.0)
Type of donor liver	
DCD	9 (75%)
DBD	3 (25%)
Cause of death	
Cardiovascular	2 (16%)
Post-anoxia	5 (42%)
Trauma	5 (42%)
Reasons livers were declined for transplantation	
DCD + age > 60 years	5 (42%)
DCD + high BMI	5 (42%)
DCD + high transaminases	2 (16%)
Type of preservation solution	
UW solution	9 (75%)
HTK solution	3 (25%)
Time between withdrawal of life support treatment and circulatory arrest (min)[a]	20 (4-46)
Time between circulatory arrest and cold flush in situ (min)[a]	18 (12-22)
Cold ischemia time (hr: min)[b]	9:04 (7:01-11:14)
Donor risk index	2.79 (2.24-3.21)
Weight of liver (kg)	2.11 (1.81-2.30)

Data represent median with interquartile ranges for continuous variables or numbers (percentages) for categorical variables. [a] The total of both periods can be defined as the total donor warm ischemia time during DCD donation. [b] Period between cold flush out of the liver in the donor and start of machine perfusion. *Abbreviations: DCD, donation after circulatory death; DBD, donation after brain death; SCS, static cold storage; UW, University of Wisconsin; HTK, histidine-tryptophanketoglutarate; BMI, body mass index.*



Supplementary Figure 1. Dynamics of hepato-cholangiocellular injury and function parameters during NMP. Levels of AST in perfusate (hepatocyte injury, A), LDH in bile (cholangiocyte injury, B), lactate in perfusate, total bilirubin in bile and cumulative bile production (hepatocyte function, C-E), bicarbonate in bile (cholangiocyte excretory function, F) and bile/perfusate glucose ratio (cholangiocyte resorptive function, G) during 6 h NMP.

Dotted lines; Data of each individual sample. Solid lines; median with interquartile range (IQR) of all samples.

Hepatocyte- and cholangiocyte-derived miRs in perfusate and bile during NMP

Figure 1A-B shows the relative values of HDmiR-122 in perfusate and in bile during NMP, and **Figure 1C-D** shows the relative values of CDmiR-222 in the same fluids. Overall, relative HDmiR-122 values were higher than relative CDmiR-222 values. **Figure 1E-F** illustrates the relative ratios between HDmiR-122 and CDmiR-222 in the respective fluids. Both HDmiR-122 and CDmiR-222 levels in perfusate initially increased during the first 30 min of perfusion and the median values showed a slight, but not significant, decrease when relative values were determined in perfusate samples taken after 6 h NMP (**Figure 1A-C**). The HDmiR-122/CDmiR-222 ratio in perfusate decreased over time in most livers after an initial peak at 30 min of perfusion (**Figure 1E**). Relative levels of HDmiR-122 in bile showed a different trend with a median continuous increase during perfusion (**Figure 1B**). The mean relative value of CDmiR-222 in bile however, although also not significantly, decreased during the 6 h of perfusion (**Figure 1D**). The HDmiR-122/CDmiR-222 ratio in bile therefore increased in most livers, indicating that there was relatively more HDmiR-122 than CDmiR-222 in bile towards the end of perfusion after 5.5 h of perfusion ($p<0.001$) (**Figure 1F**).

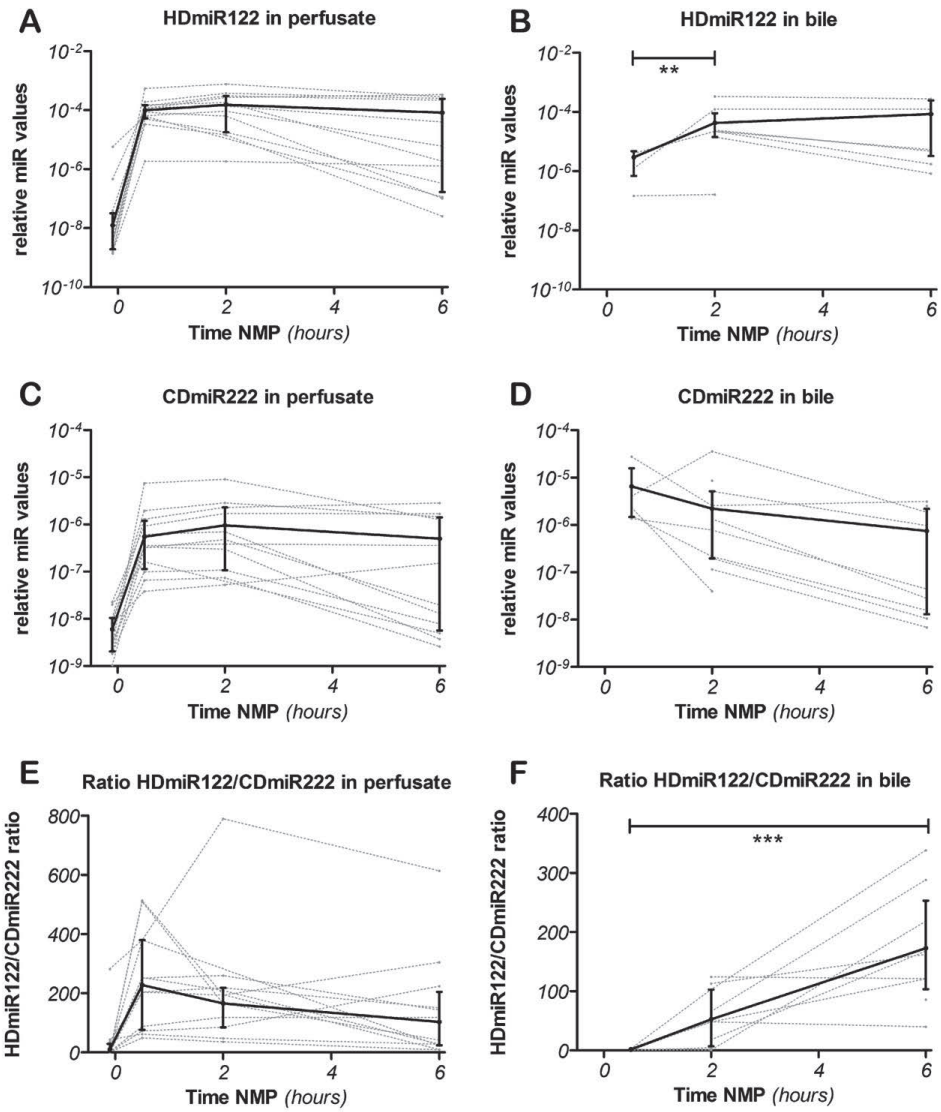


Figure 1. Dynamics of HDmiR-122 and CDmiR-222 levels in perfusate and bile during NMP. Relative values of HDmiR-122 (A, B), CDmiR-222 (C, D), and the HDmiR-122/CDmiR-222 ratio (E, F) in perfusate (A, C, E), and bile (B, D, F). In perfusate, median values of both miRs peaked after 2 h of NMP and slowly declined thereafter (A, C). In bile, median relative values of HDmiR-112 significantly increase between 0.5 and 2 h (B) . The miR ratio in bile significantly increase over time during NMP. MiR levels are represented as relative values (2^{-Cq}). Solid lines and bars: Median ± IQR, dotted lines: relative miR value profiles for each individual sample. **p<0.01, ***p<0.001

Early miR release into perfusate is predictive of late hepato-cholangiocellular parameters

Peak HDmiR-122 levels precede peak serum AST levels in predicting the onset of hepatic injury from acute rejection.[14] To investigate this observation during NMP, the relative values of HDmiR-122 and CDmiR-222 in perfusate early during machine perfusion (30 min) were correlated to peak AST levels within 6 h of NMP. A positive correlation was observed for both HDmiR-122 and CDmiR-222 with peak AST levels during the 6 h perfusion period (HDmiR-122 r=0.90, p<0.001; CDmiR-222 r=0.78, p<0.01) (**Figure 2A**). A similar observation was made for peak LDH in bile, which also showed a positive and significant correlation with HDmiR-122 (r=0.76, p<0.01) and CDmiR-222 (r=0.63, p=0.04) (**Figure 2B**). No significant correlation was observed between early miR levels and lactate at 6 h of NMP and cumulative bile production (HDmiR-122 r=0.45, p=0.15 and r=-0.39, p=0.21, respectively; CDmiR-222 r=0.30, p=0.34 and r=-0.50, p=0.10, respectively) (data not shown). There was, however, a significant negative correlation between early miR levels in perfusate and bilirubin levels in bile after 6 h of NMP (HDmiR-122 r=-0.72, p=0.02; CDmiR-222 r=-0.78, p=0.01) (**Figure 2C**). Bicarbonate concentration in bile at 6 h, an indicator of cholangiocyte function, correlated significantly and negatively with early HDmiR-122 levels (r=-0.65, p=0.02) in perfusate and nearly reached significance with early CDmiR-222 (r=-0.54, p=0.07) (**Figure 2D**). The bile/perfusate glucose ratio did not show a correlation with early miR levels in perfusate (HDmiR-122 r=0.23, p=0.55; CDmiR-222 r=0.20, p=0.61) (data not shown).

CDmiR-222 release into bile is predictive of later hepato-cholangiocellular parameters, except for hepatocellular- and cholangiocyte resorptive functions

The relative values of CDmiR-222 in bile at 2 h NMP correlated very strongly with peak AST in perfusate and peak LDH in bile (r=0.88, p<0.001 and r=0.81, p=0.003, respectively) (**Figure 3A-B**). HDmiR-122 in bile correlated less strongly, and nearly significantly, with peak AST but not with peak LDH in bile (r=0.61, p=0.05 and r=0.46, p=0.16, respectively). None of the hepatocellular function parameters, lactate in perfusate, total bilirubin in bile, or total bile production, correlated with either HDmiR-122 (r=0.21, p=0.54; r=-0.56, p=0.09 and r=0.03, p=0.96 ,respectively) or CDmiR-222 in bile (r=0.47, p=0.14; r=-0.50, p=0.14 and r=-0.15, p=0.20, respectively) (data not shown). Bicarbonate in bile, however, did show a strong and negative correlation with CDmiR-222 in bile (r=-0.89, p<0.001) (**Figure 3C**), while the bile/perfusate glucose ratio, which is higher in livers with poor cholangiocyte resorptive function, correlated strongly and positively with both HDmiR-122 (r=-0.70, p=0.04) and CDmiR-222 in bile (r=0.72, p=0.03) (**Figure 3D**).

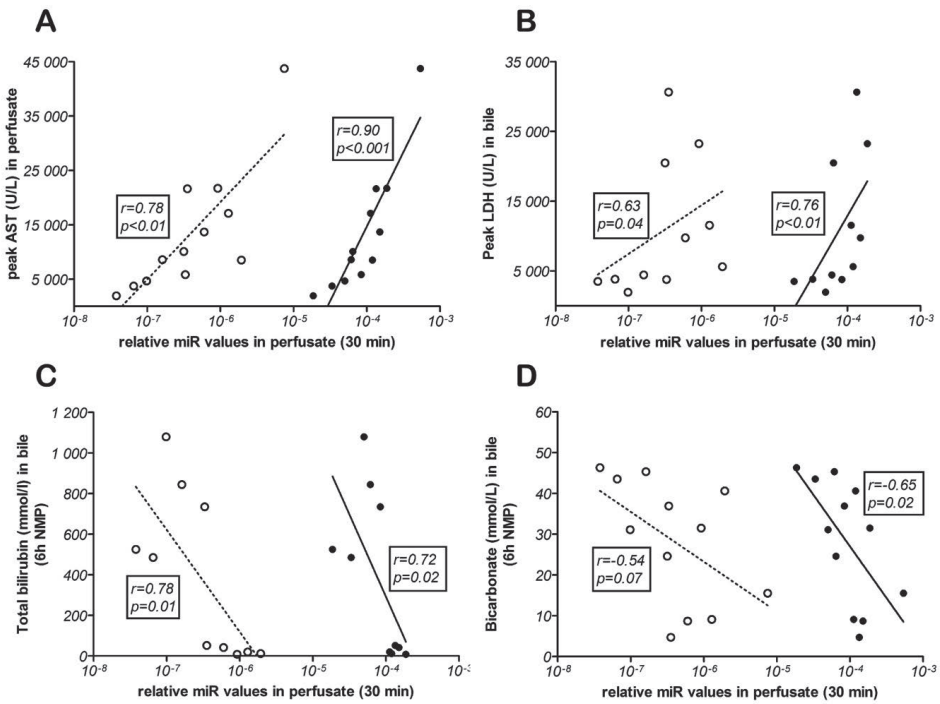


Figure 2. Early relative HDmiR-122 and CDmiR-222 levels in perfusate are predictive for hepato-cholangiocellular injury at the end of NMP. Relative levels for HDmiR-122 (filled fots) and CDmiR-222 (empty dots) in perfusate at 30 min correlated with hepato-cholangiocellular injury markers, peak AST in perfusate (A) and peak LDH in bile (B) at 6 h of NMP. Total bilirubin in bile at 6 h NMP correlated with both HDmiR-122 and CDmiR-222 at 30 min (C). HDmiR-122, but not CDmiR-222, at 30 min correlated with bicarbonate levels in bile at 6 h NMP (D). Spearman's correlation coefficient (r) and p-values are indicated.

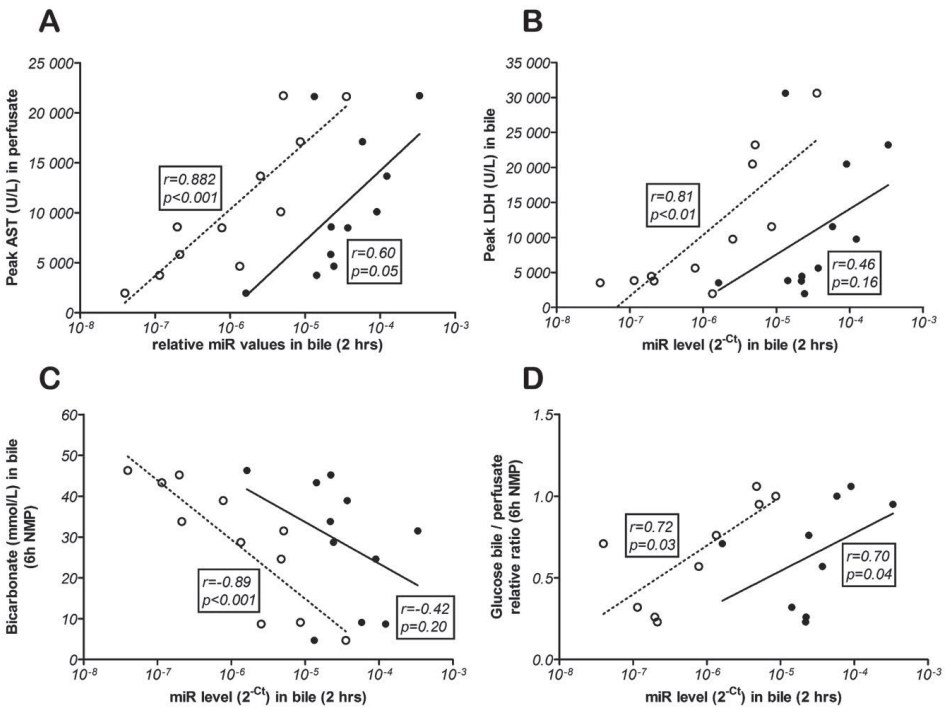


Figure 3. CDmiR-222 levels in bile at 2 h of NMP are predictive of hepato-cholangiocellular injury and function at the end of NMP. Relative CDmiR-222 (filled dots) values, but not relative HDmiR-122 values (empty dots), at 2 h of NMP correlated with peak AST in perfusate (A), and with LDH in bile (B) at 6 h of NMP. In the case of bicarbonate in bile at 6 h NMP, also only CDmiR-222, and not HDmiR-122, at 2 h showed a strong, but negative, correlation (C), while both miRs were predictive of the bile/perfusate glucose ratio at 6 h (D). Spearman's correlation coefficient (r) and p-values are indicated.

The relative HDmiR-122/CDmiR-222 ratio in perfusate correlate with cumulative bile production

Cumulative bile production during NMP has been considered a good parameter of liver function in non-transplanted research livers as other clinical post-transplantation outcomes are not available.[13] Therefore, we correlated the previously mentioned clinical markers in perfusate and bile, as well as the HDmiR-122/CDmiR-222 ratio, to the cumulative bile production after 6 h of perfusion (**Figure 4**). Early AST levels were negatively correlated to cumulative bile production (r=-0.76, p=0.01) as is shown in **Figure 4A**. This was however, not the case for LDH (r=-0.58, p=0.10), lactate (r=-0.25, p=0.46), total bilirubin in bile (r=0.32, p=0.41), bicarbonate in bile (r=-0.21, p=0.51) or HDmiR-122 or CDmiR-222 levels in perfusate (r=-0.39, p=0.21; r=-0.5, p=0.10, respectively) and bile (r=-0.08, p=0.99; r=-0.42, p=0.20, respectively) (data not shown). Interestingly, the cumulative bile production at 30 min was not predictive for the cumulative

bile production 5.5 h later (r=0.28, p=0.38) (not shown). Cumulative bile production did correlate significantly with the ratio of the relative HDmiR-122/CDmiR-222 values in perfusate after 30 min (r=0.67, p=0.01) (**Figure 4B**).

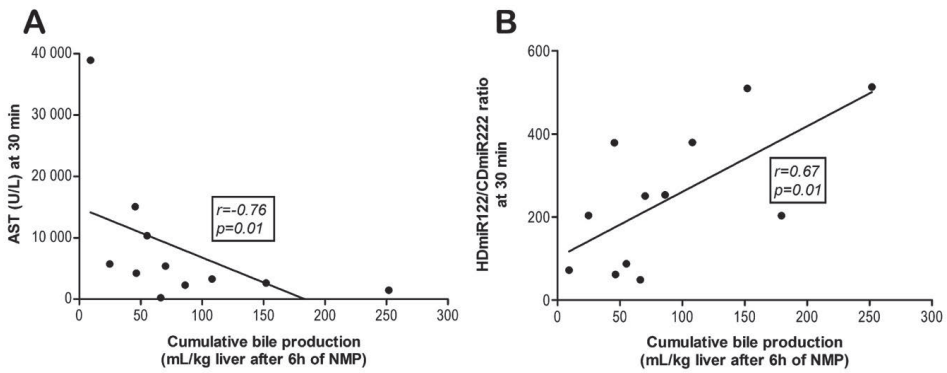


Figure 4. AST and the miR ratio are predictive of good liver function, as measured by cumulative bile production during NMP. Cumulative bile production after 6 h plotted against clinical parameter AST (A) and the relative HDmiR-122/CDmiR-222 ratio (B) measured after 30 min of NMP. Only these two parameters showed a good correlation with the cumulative bile production after 6 h. Spearman's correlation coefficient (r) and p-values are indicated.

DISCUSSION

The aim of this study was to assess whether hepatocyte- and cholangiocyte-derived miR levels in perfusate and bile were predictive of function and injury after 6 h of *ex situ* NMP of human liver grafts. This is the first study to report on miRs at different time points during NMP of human donor livers.

Overall, the current study showed that poor quality liver grafts with high hepato-cholangiocellular injury (defined as high AST and LDH peaks in perfusate and bile, respectively) and low hepato-cholangiocellular function (reflected by high lactate levels in perfusate, low levels of bilirubin in bile, low total bile production and a low bile/perfusate glucose ratio) had higher levels of HDmiR-122 and CDmiR-222 in both perfusate and bile. As expected, early relative values of HDmiR-122 in perfusate showed a strong correlation with hepatocyte injury[10,12,14], but also with cholangiocyte injury after 6 h of perfusion. A similar observation, although less pronounced, was made for CDmiR-222 in perfusate. In bile. On the other hand, HDmiR-122 did not correlate with either hepatocyte or cholangiocyte injury. CDmiR-222, however, showed an even stronger correlation with both these injury markers than was observed for this miR in perfusate. In terms of hepatocyte function, both HDmiR-122 and CDmiR-222 in perfusate correlated significantly and negatively with bilirubin in bile, but lactate and cumulative bile production did not. The relative ratio of HDmiR-122/CDmiR-222 values after 30 min was an early predictor of cumulative bile production after 6 h of perfusion and thereby a predictor of the function of non-transplanted (research) livers.

Machine perfusion is a technique that is rapidly making its way into the clinic and can be applied for various purposes. NMP not only offers the possibility to perform viability testing of high-risk livers in order to select transplantable organs[8,17-21], but also results in a lower rate of organ discard and lower levels of graft injury after NMP compared to SCS.[22] In the early detection of hepatic injury during machine perfusion, miRs in perfusate offer several advantages. miRs can withstand harsh environmental conditions, and because the perfusate circulates through the entire liver during machine perfusion, miR levels in perfusate provide a better and non-invasive representation of the entire liver compared to miR levels measured in total RNA samples from biopsies.[10] Furthermore, miRs have been shown to be earlier and more sensitive markers of liver damage than more conventional markers such as AST or ALT, and machine perfusion potentially allows for an even more representative and dynamic evaluation of the liver graft.[14,23,24] In addition, machine perfusion can be used for the resuscitation of grafts but fast and predictive biomarkers, such as miRs, are necessary to assess the effects of the treatment provided.[25-28] Especially HDmiR-122, which is involved in several processes in the liver such as lipid metabolism, has the potential to serve as a biomarker to monitor the effect of interventions, or as a therapeutic target itself.[10-12,29-32]

Studies have shown that HDmiR-122 levels in graft preservation fluid are higher in DCD grafts and in livers that went on to develop EAD and NAS.[10, 11] Higher

HDmiR-122 levels in perfusate were also measured in a porcine DCD NMP model with increasing warm ischemia times.[10] Moreover, patients with high serum AST levels after transplantation, as well as patients with acute liver failure and chronic hepatitis C infection, showed higher serum HDmiR-122 levels.[12,29] Our finding that HDmiR-122 levels in perfusate strongly and positively correlated with hepatocellular injury during NMP is entirely in line with the currently available literature.

In addition to being present in perfusate, HDmiR-122 is also present in bile. HDmiR-122 was present in bile of patients after liver transplantation and levels decrease upon episodes of liver injury.[12] These findings are confirmed in this study, with higher levels of HDmiR-122 in bile from livers with higher AST levels.

In our study, HDmiR-122 levels in both perfusate and bile were highest at 2 h of NMP and declined thereafter, a phenomenon that has not been described before. As the perfusion solution was contained in a closed circuit, this suggests that HDmiR-122 is internalized or otherwise metabolized during NMP.[33,34] This phenomenon, although less pronounced, was also observed for CDmiR-222. This might be due to an active reuptake process and decreased passive release of miRs by better functioning and less injured grafts. However, to confirm this hypothesis further research is warranted.[33,34]

Although not as extensively studied as HDmiR-122, CDmiR-222 has been shown to be abundantly expressed in biliary epithelium.[12,35] Elevated levels have been reported in serum of patients with primary sclerosing cholangitis, in tissue of hepatocellular carcinoma and in primary colorectal cancer lesions with liver metastases.[36-38] CDmiR-222 was also found to be lower in the preservation solution of grafts that developed NAS after transplantation.[11] In the present study, CDmiR-222 levels in both perfusate and bile correlated strongly and positively with hepato-cholangiocellular injury, and, in bile, with cholangiocyte function. These results are in concordance with a previous study showing that higher CDmiR-222 levels in bile were associated with high hepatocyte injury and poor hepatocyte excretory function. The finding that both miRs were excreted into bile and strongly correlated with cellular excretory function, has been, as already mentioned, described previously.[12]

Subsequently, we observed that the HDmiR-122/CDmiR-222 ratio correlated with cumulative bile production during NMP, with significantly higher ratios in livers with better bile production. The relative ratio of hepatocyte-derived and cholangiocyte-derived miRs represents a relative degree of hepatocellular injury using an internal stabilization factor. Elevated HDmiR-122/CDmiR-222 ratios in the flushing solution of liver grafts, obtained prior to implantation, have previously been associated with NAS, EAD, graft loss and DCD livers in humans[10,11], and has, in a porcine DCD model, been directly correlated with increasing WIT.[10] Future research is necessary to determine whether miR levels in bile and perfusate during NMP (but also at other temperatures) can be used to predict clinical outcomes, such as NAS or EAD.

Several limitations should be considered in this study. Livers were not transplanted after perfusion, hence post-transplantation data are not obtainable. Instead, the clinical parameters for hepato-cholangiocellular injury and function are used as mere viability markers to correlate with relative miR values.[13,21,22] Another limitation is the relatively small number of liver grafts included in this study due to a minimal availability of research livers. To avoid further stratification and reduced numbers, both DCD and DBD livers were included. Despite these small numbers and graft type heterogeneity, there were still significant differences observed in miR detection in perfusate and bile, which supports the utility of relative miR values to serve as potential, dynamic, biomarkers. Lastly, the measurement of miRs is currently a time-consuming process, although techniques are under development to make clinical applications possible.[39] Nonetheless, the predictive properties of these miRs might aid in the detection of transplantable livers or the effects of the efforts to improve the graft during machine perfusion. Therefore, the dynamics of miRs during perfusion in a closed circuit shown in this study can give new insights in the kinetics of miRs as biomarkers in general.

In conclusion, this study shows that HDmiR-122 and CDmiR-222 levels in bile and perfusate, already at early stages of NMP, are predictive of late classic markers of injury and function. Furthermore, the relative ratio of hepatocyte-derived and cholangiocyte-derived miRs is predictive of the cumulative bile production during perfusion. It will be of utmost importance to determine whether miR levels maintain a steady-state profile or whether they will change during pre-implant interventions and what the level profiles will be for (normothermic) machine perfused liver grafts once they are used for transplantation.

REFERENCES

1. Jochmans I, van Rosmalen M, Pirenne J, Samuel U. Adult Liver Allocation in Eurotransplant. Transplantation. 2017: 101(7): 1542.
2. Barshes NR, Horwitz IB, Franzini L, Vierling JM, Goss JA. Waitlist mortality decreases with increased use of extended criteria donor liver grafts at adult liver transplant centers. Am J Transplant. 2007: 7(5): 1265.
3. Blok JJ, Detry O, Putter H, Rogiers X, Porte RJ, van Hoek B, et al. Longterm results of liver transplantation from donation after circulatory death. Liver Transpl. 2016: 22(8): 1107.
4. de Vries Y, von Meijenfeldt FA, Porte RJ. Post-transplant cholangiopathy: Classification, pathogenesis, and preventive strategies. Biochim Biophys Acta Mol Basis Dis. 2018: 1864(4 Pt B): 1507.
5. Meurisse N, Vanden Bussche S, Jochmans I, Francois J, Desschans B, Laleman W, et al. Outcomes of liver transplantations using donations after circulatory death: a single-center experience. Transplant Proc. 2012: 44(9): 2868.
6. Verhoeven CJ, Farid WRR, De Jonge J, Metselaar HJ, Kazemier G, Van Der Laan LJW. Biomarkers to assess graft quality during conventional and machine preservation in liver transplantation. J Hepatol. 2014: 61(3): 672.
7. Sutton ME, den Dries SO, Karimian N, de Boer MT, Wiersema-Buist J, Gouw ASH, et al. Criteria for Viability Assessment of Discarded Human Donor Livers during Ex-Vivo Normothermic Machine Perfusion. Liver Transplantation. 2014: 20: S240.
8. Watson CJE, Kosmoliaptsis V, Pley C, Randle L, Fear C, Crick K, et al. Observations on the ex situ perfusion of livers for transplantation. Am J Transplant. 2018: 18(8): 2005.
9. Farid WR, Verhoeven CJ, de Jonge J, Metselaar HJ, Kazemier G, van der Laan LJ. The ins and outs of microRNAs as biomarkers in liver disease and transplantation. Transpl Int. 2014: 27(12): 1222.
10. Selten JW, Verhoeven CJ, Heedfeld V, Roest HP, de Jonge J, Pirenne J, et al. The Release of MicroRNA-122 During Liver Preservation is Associated with Early Allograft Dysfunction and Graft Survival After Transplantation. Liver Transpl. 2017: 23(7): 946.
11. Verhoeven CJ, Farid WR, de Ruiter PE, Hansen BE, Roest HP, de Jonge J, et al. MicroRNA profiles in graft preservation solution are predictive of ischemic-type biliary lesions after liver transplantation. J Hepatol. 2013: 59(6): 1231.
12. Verhoeven CJ FW, Roest HP, Ramakrishnaiah V, de Ruiter PE, de Jonge J, Kwekkeboom J, Metselaar HJ, Tilanus HW, Kazemier G, Ijzermans JNM and van der Laan LJW. Polarized release of hepatic microRNAs into bile and serumin response to cellular injury and impaired liver function. Liver int. 2015.

13. Westerkamp AC, Karimian N, Matton AP, Mahboub P, van Rijn R, Wiersema-Buist J, et al. Oxygenated Hypothermic Machine Perfusion After Static Cold Storage Improves Hepatobiliary Function of Extended Criteria Donor Livers. Transplantation. 2016: 100(4): 825.

14. Farid WR, Pan Q, van der Meer AJ, de Ruiter PE, Ramakrishnaiah V, de Jonge J, et al. Hepatocyte-derived microRNAs as serum biomarkers of hepatic injury and rejection after liver transplantation. Liver Transpl. 2012: 18(3): 290.

15. Roest HP, Verhoeven CJ, de Haan JE, de Jonge J, Ijzermans JNM, van der Laan LJW. Improving Accuracy of Urinary miRNA Quantification in Heparinized Patients Using Heparinase I Digestion. J Mol Diagn. 2016.

16. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002: 3(7): RESEARCH0034.

17. Matton APM, de Vries Y, Burlage LC, van Rijn R, Fujiyoshi M, de Meijer VE, et al. Biliary Bicarbonate, pH and Glucose Are Suitable Biomarkers of Biliary Viability During Ex Situ Normothermic Machine Perfusion of Human Donor Livers. Transplantation. 2018.

18. Mergental H, Perera MT, Laing RW, Muiesan P, Isaac JR, Smith A, et al. Transplantation of Declined Liver Allografts Following Normothermic Ex-Situ Evaluation. Am J Transplant. 2016.

19. Ravikumar R, Jassem W, Mergental H, Heaton N, Mirza D, Perera MT, et al. Liver Transplantation After Ex Vivo Normothermic Machine Preservation: A Phase 1 (First-in-Man) Clinical Trial. Am J Transplant. 2016: 16(6): 1779.

20. Watson CJ, Kosmoliaptsis V, Randle LV, Russell NK, Griffiths WJ, Davies S, et al. Preimplant Normothermic Liver Perfusion of a Suboptimal Liver Donated After Circulatory Death. Am J Transplant. 2016: 16(1): 353.

21. Mergental H, Stephenson BTF, Laing RW, Kirkham AJ, Neil DAH, Wallace LL, et al. Development of Clinical Criteria for Functional Assessment to Predict Primary Nonfunction of High-Risk Livers Using Normothermic Machine Perfusion. Liver Transpl. 2018: 24(10): 1453.

22. Nasralla D, Coussios CC, Mergental H, Akhtar MZ, Butler AJ, Ceresa CDL, et al. A randomized trial of normothermic preservation in liver transplantation. Nature. 2018: 557(7703): 50.

23. Shaked A, Chang BL, Barnes MR, Sayre P, Li YR, Asare S, et al. An ectopically expressed serum miRNA signature is prognostic, diagnostic, and biologically related to liver allograft rejection. Hepatology. 2017: 65(1): 269.

24. van der Meer AJ, Farid WR, Sonneveld MJ, de Ruiter PE, Boonstra A, van Vuuren AJ, et al. Sensitive detection of hepatocellular injury in chronic hepatitis C patients with circulating hepatocyte-derived microRNA-122. J Viral Hepat. 2013: 20(3): 158.

25. Guarrera JV, Henry SD, Samstein B, Odeh-Ramadan R, Kinkhabwala M, Goldstein MJ, et al. Hypothermic machine preservation in human liver transplantation: The first clinical series. Am J Transplant. 2010: 10(2): 372.

26. Dutkowski P, Schlegel A, de Oliveira M, Mullhaupt B, Neff F, Clavien PA. HOPE for human liver grafts obtained from donors after cardiac death. J Hepatol. 2014: 60(4): 765.

27. van Rijn R, Karimian N, Matton APM, Burlage LC, Westerkamp AC, van den Berg AP, et al. Dual hypothermic oxygenated machine perfusion in liver transplants donated after circulatory death. Br J Surg. 2017: 104(7): 907.

28. Boteon YL, Boteon A, Attard J, Mergental H, Mirza DF, Bhogal RH, et al. Ex situ machine perfusion as a tool to recondition steatotic donor livers: Troublesome features of fatty livers and the role of defatting therapies. A systematic review. Am J Transplant. 2018: 18(10): 2384.

29. Dubin PH, Yuan H, Devine RK, Hynan LS, Jain MK, Lee WM, et al. Micro-RNA-122 levels in acute liver failure and chronic hepatitis C. J Med Virol. 2014: 86(9): 1507.

30. Jopling CL. Liver-specific microRNA-122: Biogenesis and function. RNA Biology 2012: 9(2):

31. Ruoquan Y, Wanpin N, Qiangsheng X, Guodong T, Feizhou H. Correlation between plasma miR-122 expression and liver injury induced by hepatectomy. J Int Med Res. 2014: 42(1): 77.

32. Wen J, Friedman JR. miR-122 regulates hepatic lipid metabolism and tumor suppression. J Clin Invest. 2012: 122(8): 2773.

33. Winter J, Jung S, Keller S, Gregory RI, Diederichs S. Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat Cell Biol. 2009: 11(3): 228.

34. Zhou M, Hara H, Dai Y, Mou L, Cooper DK, Wu C, et al. Circulating Organ-Specific MicroRNAs Serve as Biomarkers in Organ-Specific Diseases: Implications for Organ Allo- and Xeno-Transplantation. Int J Mol Sci. 2016: 17(8).

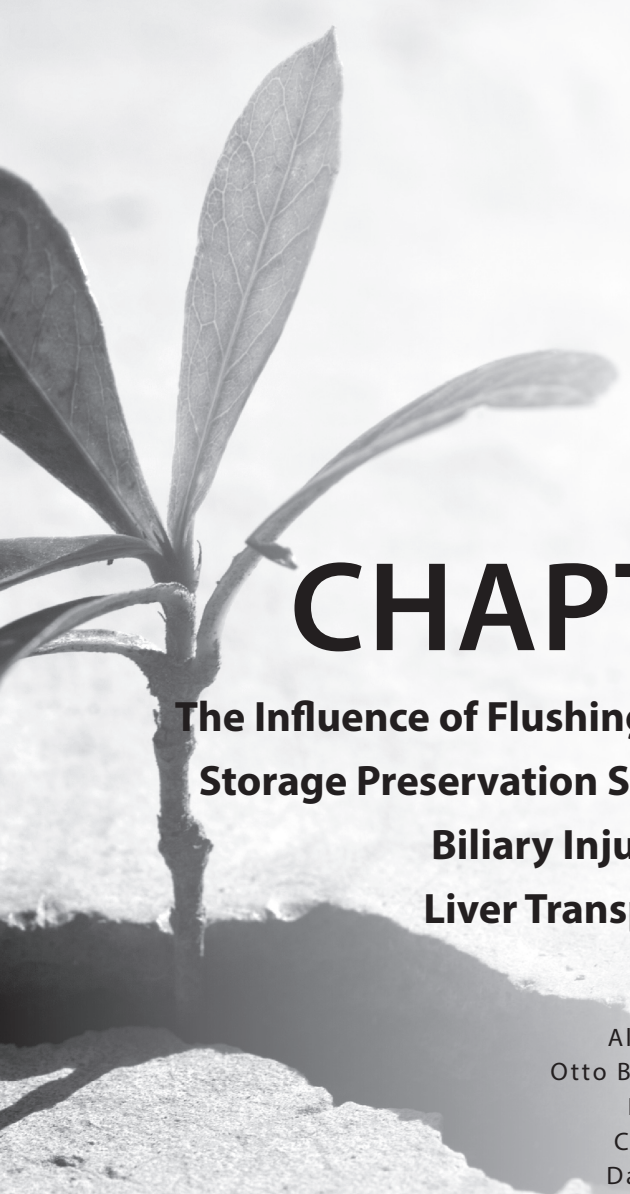
35. Chen L, Yan HX, Yang W, Hu L, Yu LX, Liu Q, et al. The role of microRNA expression pattern in human intrahepatic cholangiocarcinoma. J Hepatol. 2009: 50(2): 358.

36. Bernuzzi F, Marabita F, Lleo A, Carbone M, Mirolo M, Marzioni M, et al. Serum microRNAs as novel biomarkers for primary sclerosing cholangitis and cholangiocarcinoma. Clin Exp Immunol. 2016: 185(1): 61.

37. Karakatsanis A, Papaconstantinou I, Gazouli M, Lyberopoulou A, Polymeneas G, Voros D. Expression of microRNAs, miR-21, miR-31, miR-122, miR-145, miR-146a, miR-200c, miR-221, miR-222, and miR-223 in patients with hepatocellular carcinoma or intrahepatic cholangiocarcinoma and its prognostic significance. Mol Carcinog. 2013: 52(4): 297.

38. Iida M, Hazama S, Tsunedomi R, Tanaka H, Takenouchi H, Kanekiyo S, et al. Overexpression of miR221 and miR222 in the cancer stroma is associated with malignant potential in colorectal cancer. Oncol Rep. 2018: 40(3): 1621.

39. Cheng Y, Dong L, Zhang J, Zhao Y, Li Z. Recent advances in microRNA detection. Analyst. 2018: 143(8): 1758.



8

CHAPTER

The Influence of Flushing and Cold Storage Preservation Solution on Biliary Injury Prior to Liver Transplantation

Alix P.M. Matton*
Otto B. van Leeuwen*
Reinier de Vries
Corey M. Eymard
Danielle Detelich
Vincent E. de Meijer
Annette S. H. Gouw
James F. Markmann
Ton Lisman
Korkut Uygun
Heidi Yeh
Robert J. Porte

*Equally contributing authors

In preparation for submission.

ABSTRACT

Background: The development of non-anastomotic strictures (NAS) of the biliary tree remains one of the most feared complications after liver transplantation. Static cold storage (SCS) is the golden standard for donor liver preservation, though worldwide no consensus has been reached regarding the optimal flushing and preservation solution for protecting against bile duct injury (BDI).Our aim was to investigate biliary tree injury after flushing and preserving using University of Wisconsin (UW) or histidine-tryptophan-ketoglutarate (HTK) solution, the two most commonly used preservation solutions. Polyethylene glycols (PEGs) are promising compounds in organ preservation and their ability in preserving the biliary tree were also investigated.

Methods: Sixteen human donor livers that were declined for transplantation were transported to our center using SCS in UW (standard practice). Upon arrival, the common bile duct was dissected, cut into 6 equal segments, flushed and preserved in 5 different ice-cold preservation solutions for a median of 8 hours. Study groups were UW solution, UW + PEG15-20 solution, HTK solution, HTK + PEG15-20 solution, and HTK + PEG35 solution. Histological BDI was assessed using a clinically relevant scoring system.

Results: Bile preservation in HTK solution led to significantly higher total BDI compared to baseline, whereas the other study groups had no significant increases in BDI compared to baseline. The addition of PEG15-20 and PEG35 to HTK resulted in slightly less, but non-significant, stroma necrosis, peribiliary gland injury and peribiliary vascular plexus injury.

Conclusion: The present study suggests that HTK solution is worse than UW solution in protecting the biliary tree. The addition of PEGs to preservation solutions may be a readily implementable and affordable method to protect the biliary tree and warrants further investigation.

INTRODUCTION

The formation of non-anastomotic strictures (NAS), or post-transplant cholangiopathy, of the biliary tree remains one of the most feared complications following liver transplantation.[1] Especially following transplantation of donation after circulatory death (DCD) livers, NAS are seen in 13 – 35% of grafts, compared to 1 – 24% of grafts in livers donated after brain death (DBD).[1-5] Histological damage of the bile duct at the time of transplantation has been directly linked to the formation of NAS after transplantation.[6]

Flushing of the bile ducts during procurement is a crucial step in preserving the biliary tree, mainly to remove bile, which contains cytotoxic and detergent hydrophobic bile salts. Flushing protocols, however, vary widely across the world, ranging from flushing with saline to flushing with the preservation solution itself, which includes University of Wisconsin (UW) solution, histidine-tryptophan-ketoglutarate (HTK) solution, Celsior solution and Institut Georges Lopez-1 (IGL-1) solution.

Compared to UW solution, HTK solution has been reported to lead to improved protection of the biliary tree in rat studies[7,8], and has retrospectively and prospectively been reported to lead to lower incidences of biliary complications and/or NAS in clinical studies.[9-13] Other studies found no differences in the rate of NAS formation after transplantation between both preservation solutions.[14-15]

A compound that could potentially aid in the protection of bile ducts are polyethylene glycols (PEGs). PEGs are FDA approved water-soluble non-toxic non-immunogenic compounds that are used in a variety of applications, including cosmetics, foods and drugs.[16] These polymers, which consist of variable repeats of ethylene glycols resulting in a range of molecular weights with various shapes, are generally considered to have low toxicity via all routes of administration.[16]

Recently, the use of PEGs in organ preservation has been discovered as a new therapeutic tool to protect pancreas, small bowel, kidney and liver grafts from ischemia-reperfusion injury. PEG35 has been used as an additive to preservation solution in rat models, which resulted in improved protection of liver grafts and protection against ischemia-reperfusion injury.[17-20] PEGs have also been used for the protection of intestinal tissue, the architecture and vulnerable epithelial lining of which closely resembles that of human bile duct. Just like biliary epithelium, intestinal epithelium is exposed to similar toxins such as bile salts, and it is morphologically composed of similar tissue with stem cell crypts in the tissue wall around the lumen. PEG15-20 was shown to provide cytoprotective effects against bile salt injury in *in vitro* studies on intestinal epithelium.[21,22]

Overall, different studies have provided conflicting results regarding the optimal preservation solution for protecting the bile ducts. Therefore our aim was to compare the effect of five different preservation solutions, based on UW or HTK solution with and without PEG15-20 or PEG35, on biliary injury during static cold storage (SCS).

MATERIALS & METHODS

Study Groups

The five preservation solution study groups consisted of 1. UW solution (Belzer UW Cold Storage Solution, Bridge to Life Ltd.; "UW only group"), 2.UW + PEG15-20 5% (Polyethylene glycol Bisphenol A Epichlorohydrin Copolymer, Sigma-Aldrich #P2263; "UW + PEG15-20 group"), 3. HTK solution (Custodiol HTK Solution, Essential Pharmaceuticals, LLC; "HTK only group"), 4. HTK solution + PEG15-20 5%; "HTK + PEG15-20 group") and 5. HTK solution + PEG35 5% (Polyethylene glycol 35,000, Sigma-Aldrich #81310; "HTK + PEG35 group").

Donor livers

Sixteen human donor livers that were declined for transplantation for various reasons were obtained from the New England Donor Services with consent for research from the relatives. This study was exempted by the institutional review board of Massachusetts General Hospital.

Bile duct processing

All livers were procured according to standard practice, which included flushing of the extrahepatic bile ducts with saline solution through the gall bladder. All livers were transported to Shriners Hospital for Children by static cold storage in UW solution. Upon arrival, the present study commenced. The extrahepatic common bile duct was dissected and cut transversally into 6 equal bile duct segments of approximately 4mm in length, taking care to minimize manipulation of the bile duct. To wash away the UW solution and possible bile remains, each bile duct segment was gently flushed in ice cold preservation solution of its allocated study group, and subsequently placed in five conical tubes containing 20mL fresh ice cold preservation solution of each group. One segment was immediately placed in formalin (baseline). Care was taken to place the segments in different study groups, per liver, to avoid anatomical bias. After at least 8 hours of cold storage, the bile ducts were removed from the preservation solutions and stored in formalin, followed by 70% ethanol within 48 hours for subsequent paraffin embedding and hematoxylin & eosin (H&E) staining.

Histological bile duct injury (BDI)

The degree of histological BDI was assessed on the following components: epithelial lining, stroma necrosis, intramural and extramural PBGs and the peribiliary vascular plexus (PVP) (**Table 1**). These relevant components were selected from the formerly described scoring system by op den Dries et al. 2014, with slight modifications (more subcategories for epithelial lining, stroma

necrosis and PBGs).[6] Sections were scored in a double blinded fashion (APMM and OBvL).

Table 1. Histological Bile Duct Injury Scoring System.*

Grade	Epithelial Lining	Bile Duct Wall Stroma Necrosis	Intramural Peribiliary Glands	Extramural Peribiliary Glands	Peribiliary Vascular Plexus
0	No loss	No stroma necrosis	No loss or injury of cells	No loss or injury of cells	No vascular lesions
1	≤25% loss or injury of cells	≤25% stroma necrotic	≤25% loss or injury of cells	≤25% loss or injury of cells	≤50% of vessels necrotic
2	>25% and ≤50% loss or injury of cells	>25% and ≤50% stroma necrotic	>25% and ≤50% loss or injury of cells	>25% and ≤50% loss or injury of cells	>50% of vessels necrotic or no longer visible
3	>50% and ≤75% loss or injury of cells	>50% and ≤75% stroma necrotic	>50% and ≤75% loss or injury of cells	>50% and ≤75% loss or injury of cells	-
4	>75% loss or injury of cells	>75% stroma necrotic	>75% loss or injury of cells	>75% loss or injury of cells	-

** Modified from Op den Dries et al.[6]*

Statistics

Continuous variables were presented as median with interquartile range (IQR) and were compared between groups using the Mann-Whitney U test. All statistical analyses were performed using IBM SPSS version 23.0 (Chicago, IL, USA).

RESULTS

Donor Livers

Donor livers from 16 human donors were included in this study. Donor characteristics are summarized in **Table 2**. Twelve livers were derived from DCD and 4 from DBD donors.

Table 2. Donor liver characteristics.

Age (years)		**45 (37 - 53)**
Sex	Male	10 (63%)
	Female	6 (38%)
BMI (kg/m^2)		31 (25 - 35)
Type of donor	DCD	12 (75%)
	DBD	4 (25%)
Cause of death	Anoxia	6
	CVA	5
	Trauma	5
Donor warm ischemia time (min)[*]		23 (13 - 47)
Cold ischemia time prior to inclusion (h)[†]		13.6 (8.6 - 16.1)
Reason declined for transplantation	DCD + age	5
	(Expected) steatosis	5
	DWIT > 30 min	2
	Excessive alcohol abuse	2
	Other[‡]	2

Variables are presented as median and (interquartile range). [*]Time between withdrawal of life support until aortic cold flush (DCD only). [†]Time between aortic cold flush and inclusion in study preservation solution. [‡]One pediatric DCD liver (not transplanted routinely) and one cirrhotic liver. *Abbreviations: BMI, body mass index; DBD, donation after brain death; DCD, donation after circulatory death; CVA, cerebrovascular accident; DWIT, donor warm ischemia time.*

Total bile duct injury score and total significant NAS predictors, before and after preservation

At baseline, a median total BDI score of 13.0 (IQR 11.0 – 14.5) increased to 14.5 (11.5 – 16.3) in the UW group, 14.0 (13.0 – 16.0) in the UW + PEG15-20 group, 15.5 (14.3 – 18.0) in the HTK group, 15.5 (12.3 – 17.25) in the HTK + PEG15-20 group and 13.0 (9.0 – 19.0) in the HTK + PEG35 group (**Figure 1A**). In short, HTK solution led to the greatest increase in total BDI injury from baseline, when compared to the other preservation solutions. The only statistically significant difference was between baseline and HTK ($p = 0.016$), though the difference between baseline and HTK + PEG15-20 nearly reached significance ($p = 0.061$).

Three components of BDI have previously been reported to be strongly associated with the development of NAS after transplantation (stroma necrosis, extramural PBG injury and PVP injury).[6] **Figure 1B** shows the total BDI score of these components. Again, HTK solution was significantly worse compared to baseline ($p = 0.020$), whereas the other solutions were not. The addition of PEG15-20 and PEG35 to HTK solution led to a non-significantly lower grade of BDI, whereas with UW solution the addition of PEG15-20 had no effect.

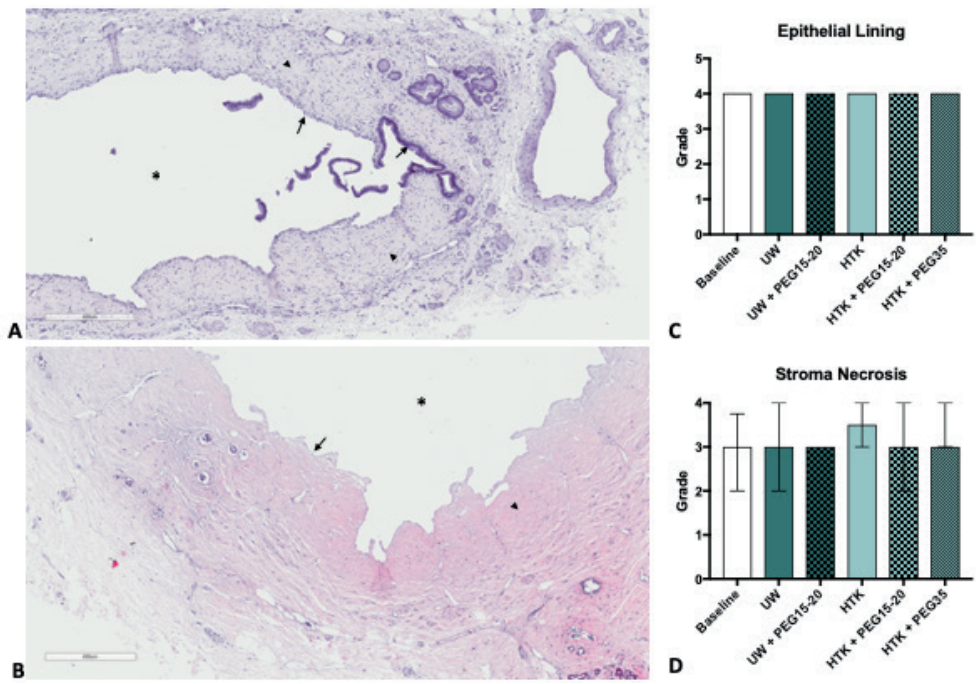


Figure 1. Bile duct injury increased most in HTK solution stored bile ducts. (A) Total bile duct injury and (B) the combined individual histological components that predict NAS after transplantation were significantly higher in bile ducts that were stored in HTK solution. The addition of PEG15-20 to both UW and HTK and PEG35 to HTK solution resulted in a moderate non-significant improvement in preservation. *Abbreviations: HTK solution, histidine-*

tryptophan-ketoglutarate; PEG, poly-ethylene glycol; NAS, non-anastomotic strictures; UW, University of Wisconsin solution.

Individual histological components

Figure 2A is an example of a bile duct segment with partial loss of epithelial lining and a low degree of stroma necrosis, while **Figure 2B** is an example with complete loss of epithelial lining and severe stroma necrosis. The epithelial lining was >75% injured or gone in 81% of bile ducts at baseline, and increased to 94% in the UW + PEG15-20 group, 100% in the HTK group, and 88% in both the HTK + PEG15-20 and HTK + PEG35 groups. There were no significant differences between the groups regarding epithelial lining, though HTK scored the highest and nearly reached significance compared to baseline ($p = 0.074$) (**Figure 2C**).

Remarkably, the same degree of stroma necrosis was present, with a median grade of 3 (between 50 – 75% stroma necrosis), at baseline and in all groups except for HTK, which had a median grade of 3.5 (**Figure 2D**). This difference, compared to baseline, also nearly reached significance ($p = 0.083$).

Figure 2. Epithelial lining and stroma necrosis were similar at baseline and in all study groups. (A) H&E section indicating severe loss of epithelial lining (arrows) and ≤25% stroma necrosis. (B) Complete loss of epithelial lining and 100% stroma necrosis. (C) Epithelial lining was already highest at baseline (grade 4). (D) Stroma necrosis was similar at baseline and in all study groups, except for the HTK preserved group which was non-significant higher. The asterisk indicates the bile duct lumen. *Abbreviations: H&E, hematoxylin & eosin; HTK solution, histidine-tryptophan-ketoglutarate; PEG, poly-ethylene glycol; UW, University of Wisconsin solution.*

Figure 3A-B shows intramural and extramural PBGs that were very well preserved (panel A) and highly injured with loss of cells and resultant cavities (panel B). Intramural and extramural PBG injury was lowest at baseline, with a median grade of 3 and 1.5 respectively (**Figure 3C-D**). Intramural PBG injury was nearly significantly higher in the UW group compared to baseline ($p = 0.061$) (**Figure 3C**). Extramural PBG injury, on the other hand, was nearly significantly higher in the HTK group compared to baseline ($p = 0.077$) (**Figure 3D**). The average of intramural and extramural PBG injury also nearly reached significance between UW and HTK solution, when compared to baseline ($p = 0.070$ and $p = 0.080$, respectively) (**Figure 3E**). The median average PBG injury was slightly lower in both UW and HTK solutions with PEG15-20 and PEG30 added, though this did not reach significance.

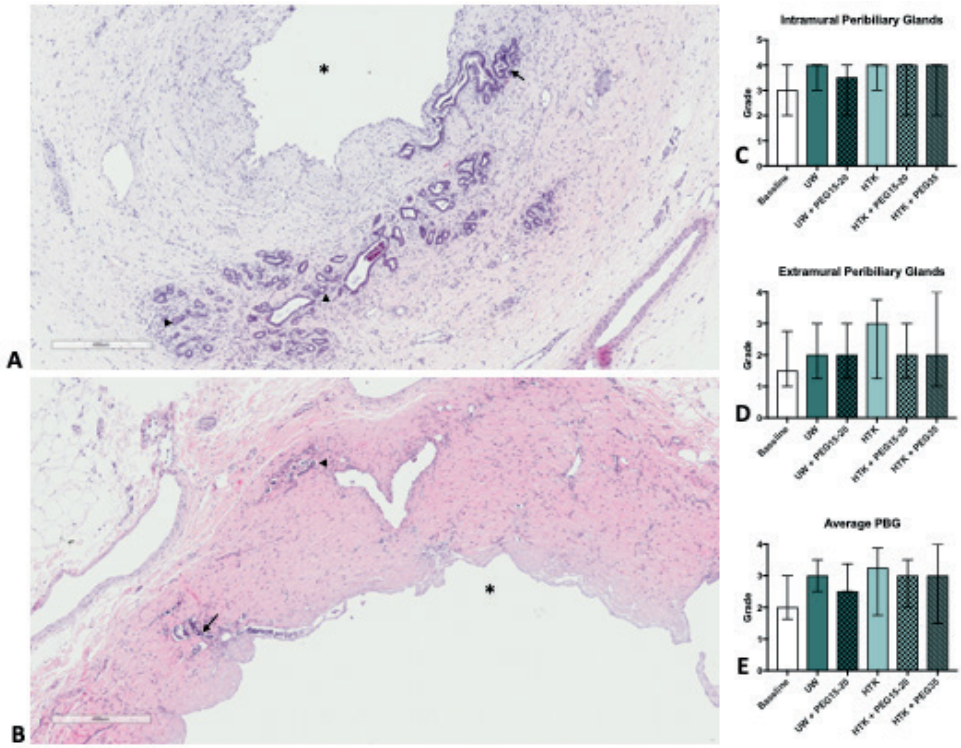


Figure 3. PBG injury was non-significantly higher in HTK solution preserved bile ducts. (A) H&E section indicating ≤25% loss or injury of intramural (arrows) and extramural (arrowheads) peribiliary gland cells. (B) Complete loss of intramural (arrows) and extramural (arrowheads) peribiliary gland cells. There were no significant differences in intramural (C) or extramural (D) PBG injury between baseline and the five study groups, though it was non-significantly higher in the HTK preserved bile ducts. (E) There were no significant differences in average intramural and extramural PBG injury between baseline and the study groups, though again the HTK group was worst. Addition of PEG15-20 to both UW and HTK solution, and PEG35 to HTK solution, lead to a non-significant slight improvement in average PBG injury. The asterisk indicates the bile duct lumen. *Abbreviations: H&E, hematoxylin & eosin; HTK solution, histidine-tryptophan-ketoglutarate; PBG, peribiliary glands; PEG, poly-ethylene glycol; UW, University of Wisconsin solution.*

Figure 4A-B show a low and high degree of PVP injury, respectively. Also here, the degree of PVP injury was significantly higher in the HTK group, with a median grade of 1.25, compared to baseline (0.75, p = 0.031) (**Figure 4C**). All other groups had the same median PVP injury score of 1.0, though it was nearly significantly higher in the UW group compared to baseline (0.079).

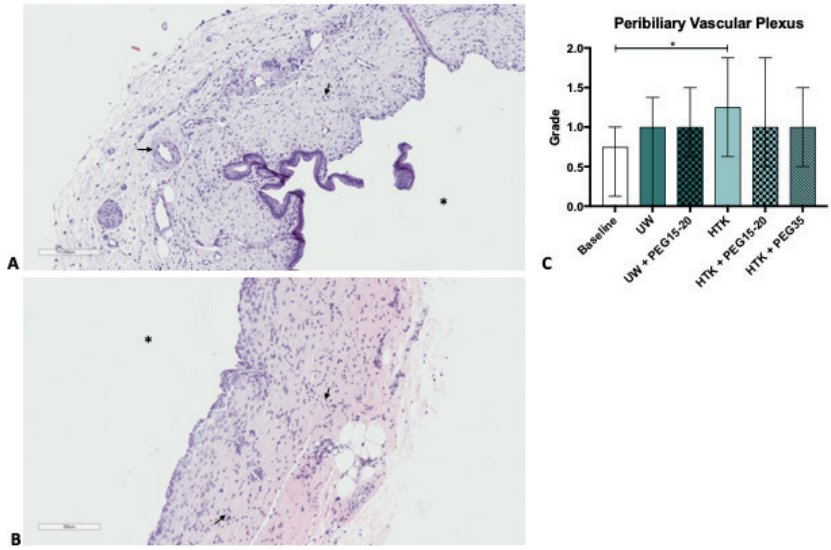


Figure 4. PVP injury was highest in HTK solution preserved bile ducts. (A) H&E section showing intact vessels of the PVP. (B) Severe PVP injury with >50% of vessels necrotic or no longer visible. (C) PVP injury was significantly higher in the HTK solution preserved bile ducts compared to baseline, while all other groups had a similar median injury score. The asterisk indicates the bile duct lumen. *Abbreviations: H&E, hematoxylin & eosin; HTK solution, histidine-tryptophan-ketoglutarate; PEG, poly-ethylene glycol; PVP, peribiliary vascular plexus; UW, University of Wisconsin solution.*

DISCUSSION

In this study, static cold preservation of human bile duct segments in HTK solution, but not UW solution, resulted in worsening of the degree of histological injury, compared to baseline. The addition of PEG15-20 to both solutions, and PEG35 to HTK solution, resulted in a slight but non-significant improvement in biliary preservation.

The degree of histological bile duct injury at the time of transplantation has been correlated with the risk of NAS development after transplantation.[6] In particular, injury to the extramural peribiliary glands, peribiliary vascular plexus and stroma necrosis are histological features that significantly and strongly predict the development of NAS after transplantation.[6] For this reason, the present study was focused on determining the optimal preservation solution to protect against biliary injury during SCS.

Earlier studies have provided conflicting results regarding the optimal preservation solution to protect the liver and biliary tree during SCS. In rat studies, the use of HTK solution was suggested to be superior in protecting the bile ducts, compared to UW solution.[7,8] One prospective and multiple retrospective human studies reported significantly lower incidences of NAS or

biliary complications using HTK preserved grafts, when compared to UW.[9-13,23] Two other studies, one retrospective and one prospective, reported no significant differences in the rate of NAS formation after transplantation between HTK and UW preserved livers.[14,15] A large database study also showed that HTK preserved livers, especially DCD, had lower graft survival compared to UW (biliary complications were not separately reported, though a major cause of graft failure after DCD transplantation is NAS).[24] In contrast with most of the literature, however, the present study showed that static cold storage of human bile ducts for 8 hours in HTK solution resulted in significant deterioration of the biliary morphology. This deterioration of bile duct histology was not seen after 8 h SCS in UW solution.

There are two proposed reasons for HTK solution to be superior in preserving the biliary tree. The viscosity of HTK solution is the same as water, with the average velocity being three times higher than UW solution under the same perfusion pressure, therefore leading to more thorough flushing of the bile ducts and removal of toxic bile salts and other substances. Secondly, temperature reduction is faster in HTK than in UW solution.[7] In the present study, however, the extrahepatic bile duct segment was severed into relatively small segments, thereby leading to thorough flushing of all segments. Furthermore, all segments were already ice cold at the start of this study. These potential benefits of HTK solution may have therefore been masked by the current study design.

In our study, the addition of PEG15-20 to both UW and HTK solution led to a slight improvement in biliary preservation, compared to UW and HTK solution alone, though this did not reach significance. When assessing the significant NAS predictors (stroma necrosis, extramural PBG injury and PVP injury), HTK + PEG35 was the most promising group with the same median biliary injury score after SCS as baseline.

The addition of PEGs, which are FDA approved water-soluble non-toxic non-immunogenic compounds, to both HTK and UW solution was tested in this study because of their promising protective effects and their potentially relatively simple introduction into the clinic. PEG35 has been used as an additive to preservation solution in rat models, resulting in improved protection of liver grafts and protection against ischemia-reperfusion injury.[18-20,25] In another rat study, intravenous PEG35 administration during liver procurement, followed by UW cold storage, resulted in less liver injury, protection of mitochondria, and upregulation of cytoprotective factors during normothermic machine perfusion.[26] The addition of PEG35 has also been shown to be a crucial step in the technique of supercooling, whereby both hepatocytes and whole rat livers were preserved to temperatures below freezing.[27,28] In our experience, the addition of PEG35 to HTK solution (unfortunately it was not added to UW solution, due to the limited number of bile duct segments that could be obtained per liver) led to a non-significant reduction of injury of the histological components that were significant predictors for NAS.

PEGs have also been used for the protection of intestinal tissue, which closely resembles that of human bile duct in that it is exposed to similar toxins such as bile salts, and is morphologically composed of similar tissue with stem cell crypts in the tissue wall around the lumen. PEG15-20 has been shown to prevent *P. aeruginosa*-induced barrier disruption and to provide cytoprotective effects against bile acid injury in *in vitro* human intestinal studies.[21,22] In another study using a mouse model for intestinal transplantation, the effect of preserving the graft in HTK with and without PEG15-20 showed superior preservation when using HTK + PEG15-20.[29] In our study, the addition of PEG15-20 to HTK solution led to a non-significant reduction of injury in the predictors for NAS, while its addition to UW solution did not lead to any changes, except for a slight improvement in overall PBG preservation.

Both HTK and UW solution are widely used across the world for the preservation of livers. In some countries, such as Germany, only HTK solution is used.[30] The current study provides evidence against the use of HTK solution in terms of protecting the biliary tree. Especially in the case of DCD grafts, protection of the biliary tree is crucial in reducing the risk of NAS after transplantation. Further studies are required to determine the safety and efficacy of the addition of PEGs, such as PEG15-20 or PEG35, to either preservation solutions. More detailed analyses, including histological stem cell and proliferation markers of the PBGs, are currently being performed and will be reported as a sub-study. This is particularly important as the PBGs are niches of stem cells with proliferative capacity that play a role in the development of NAS.[31] The promising literature results regarding the addition of PEGs to preservation solutions, their relatively easy implementation into the clinic and the results of the present study combined, provide a strong incentive to further investigate their use.

There are some limitations of the present study. First of all, all livers were flushed with and stored in UW solution and bile ducts were flushed with saline solution according to standard practice of the procuring centers. Despite flushing of each bile duct segment in the allocated preservation solution, the foregoing saline flush and UW solution storage may have resulted in changes in the bile duct tissue. Subsequent switching to another preservation solution may have caused more damage and may have acted in favor of the UW solution storage group. Furthermore, as mentioned, some of the benefits of HTK solution including its low viscosity and high temperature reduction capacity may have been masked due to the study design.

In conclusion, the present study suggests that HTK solution is worse in protecting the biliary tree against preservation injury, compared to UW solution, and may therefore may be less suitable for preserving grafts that carry a high risk of biliary complications. Given that increasing numbers of DCD livers are being transplanted worldwide and that biliary injury plays a crucial role in the development of NAS after transplantation, it is critical that preservation solutions are used that protect the biliary tree. The addition of PEG15-20 and PEG35 to the preservation solutions resulted in a (non-significant) reduction of

biliary injury and warrants further investigation, especially as their implementation would be relatively easy to introduce into the clinic.

REFERENCES

1. de Vries Y, von Meijenfeldt FA, Porte RJ. Post-transplant cholangiopathy: Classification, pathogenesis, and preventive strategies. Biochim Biophys Acta 2018;1864:1507-1515.
2. Meurisse N, Vanden Bussche S, Jochmans I, Francois J, Desschans B, Laleman W, et al. Outcomes of liver transplantations using donations after circulatory death: a single-center experience. Transplant Proc 2012;44:2868-2873.
3. Jay CL, Lyuksemburg V, Ladner DP, Wang E, Caicedo JC, Holl JL, et al. Ischemic cholangiopathy after controlled donation after cardiac death liver transplantation: a meta-analysis. Ann Surg 2011;253:259-264.
4. Dubbeld J, Hoekstra H, Farid W, Ringers J, Porte RJ, Metselaar HJ, et al. Similar liver transplantation survival with selected cardiac death donors and brain death donors. Br J Surg 2010;97:744-753.
5. Chan EY, Olson LC, Kisthard JA, Perkins JD, Bakthavatsalam R, Halldorson JB, et al. Ischemic cholangiopathy following liver transplantation from donation after cardiac death donors. Liver Transpl 2008;14:604-610.
6. op den Dries S, Westerkamp AC, Karimian N, Gouw AS, Bruinsma BG, Markmann JF, et al. Injury to peribiliary glands and vascular plexus before liver transplantation predicts formation of non-anastomotic biliary strictures. J Hepatol 2014;60:1172-1179.
7. Cui DX, Yin JQ, Xu WX, Chai F, Liu BL, Zhang XB. Effect of different bile duct flush solutions on biliary tract preservation injury of donated livers for transplantation. Transplant Proc 2010;42:1576-1581.
8. Spiegel HU, Schleimer K, Freise H, Diller R, Drews G, Kranz D. Organ preservation with EC, HTK, and UW, solution in orthotopic rat liver transplantation. Part II. Morphological study. J Invest Surg 1999;12:195-203.
9. Heidenhain C, Pratschke J, Puhl G, Neumann U, Pascher A, Veltzke-Schlieker W, et al. Incidence of and risk factors for ischemic-type biliary lesions following orthotopic liver transplantation. Transpl Int 2010;23:14-22.
10. Welling TH, Heidt DG, Englesbe MJ, Magee JC, Sung RS, Campbell DA, et al. Biliary complications following liver transplantation in the model for end-stage liver disease era: effect of donor, recipient, and technical factors. Liver Transpl 2008;14:73-80.
11. Canelo R, Hakim NS, Ringe B. Experience with hystidine tryptophan ketoglutarate versus University Wisconsin preservation solutions in transplantation. Int Surg 2003;88:145-151.
12. Pirenne J, Van Gelder F, Coosemans W, Aerts R, Gunson B, Koshiba T, et al. Type of donor aortic preservation solution and not cold ischemia time is a major determinant of biliary strictures after liver transplantation. Liver Transpl 2001;7:540-545.

13. Li S, Stratta RJ, Langnas AN, Wood RP, Marujo W, Shaw BW,Jr. Diffuse biliary tract injury after orthotopic liver transplantation. Am J Surg 1992;164:536-540.

14. Moench C, Otto G. Ischemic type biliary lesions in histidine-tryptophan-ketoglutarate (HTK) preserved liver grafts. Int J Artif Organs 2006;29:329-334.

15. Erhard J, Lange R, Scherer R, Kox WJ, Bretschneider HJ, Gebhard MM, et al. Comparison of histidine-tryptophan-ketoglutarate (HTK) solution versus University of Wisconsin (UW) solution for organ preservation in human liver transplantation. A prospective, randomized study. Transpl Int 1994;7:177-181.

16. Pasut G, Panisello A, Folch-Puy E, Lopez A, Castro-Benitez C, Calvo M, et al. Polyethylene glycols: An effective strategy for limiting liver ischemia reperfusion injury. World J Gastroenterol 2016;22:6501-6508.

17. Bessems M, Doorschodt BM, Dinant S, de Graaf W, van Gulik TM. Machine perfusion preservation of the pig liver using a new preservation solution, polysol. Transplant Proc 2006;38:1238-1242.

18. Hata K, Tolba RH, Wei L, Doorschodt BM, Buttner R, Yamamoto Y, et al. Impact of polysol, a newly developed preservation solution, on cold storage of steatotic rat livers. Liver Transpl 2007;13:114-121.

19. Yagi S, Doorschodt BM, Afify M, Klinge U, Kobayashi E, Uemoto S, et al. Improved preservation and microcirculation with POLYSOL after partial liver transplantation in rats. J Surg Res 2011;167:e375-83.

20. Mosbah IB, Zaouali MA, Martel C, Bjaoui M, Abdennebi HB, Hotter G, et al. IGL-1 solution reduces endoplasmic reticulum stress and apoptosis in rat liver transplantation. Cell Death Dis 2012;3:e279.

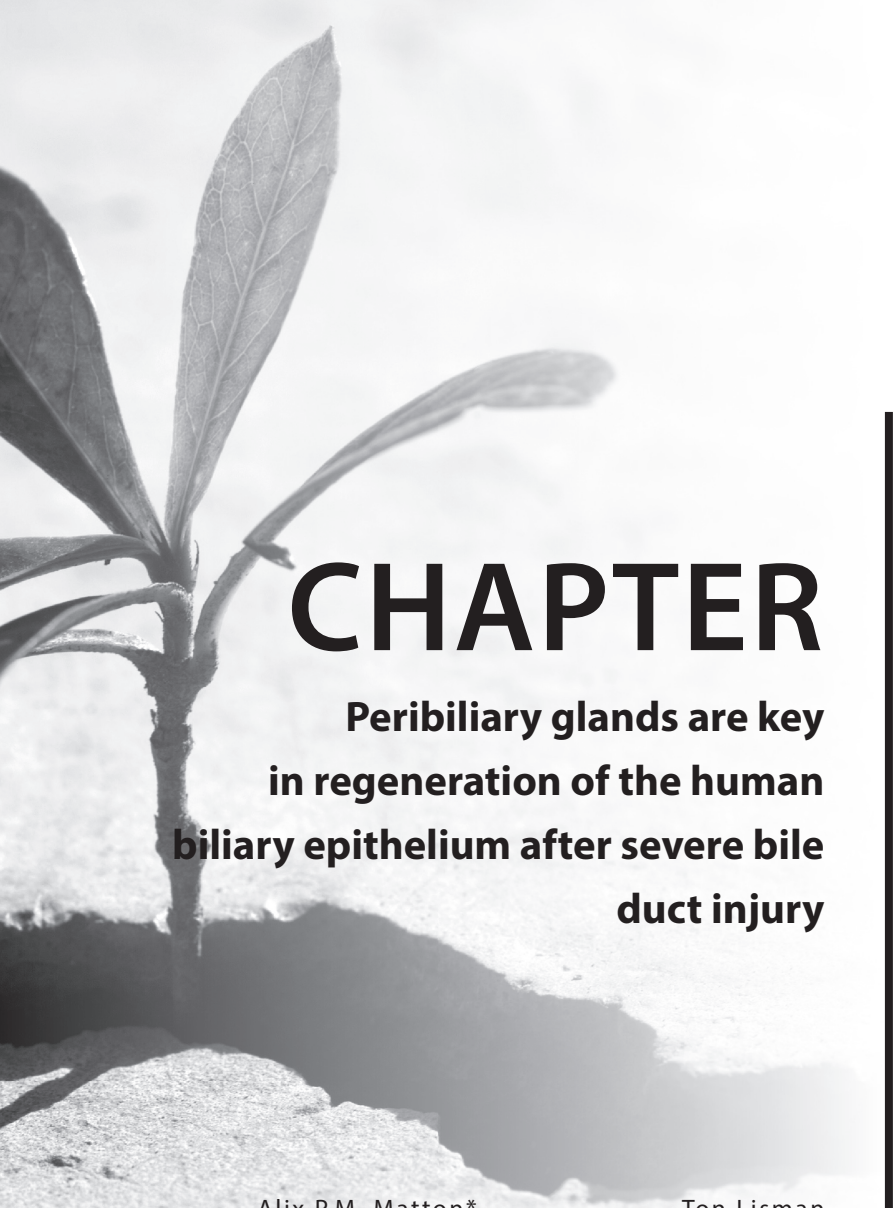
21. Edelstein A, Fink D, Musch M, Valuckaite V, Zaborina O, Grubjesic S, et al. Protective effects of nonionic triblock copolymers on bile acid-mediated epithelial barrier disruption. Shock 2011;36:451-457.

22. Wu L, Zaborina O, Zaborin A, Chang EB, Musch M, Holbrook C, et al. High-molecular-weight polyethylene glycol prevents lethal sepsis due to intestinal Pseudomonas aeruginosa. Gastroenterology 2004;126:488-498.

23. Mangus RS, Fridell JA, Vianna RM, Milgrom MA, Chestovich P, Chihara RK, et al. Comparison of histidine-tryptophan-ketoglutarate solution and University of Wisconsin solution in extended criteria liver donors. Liver Transpl 2008;14:365-373.

24. Stewart ZA, Cameron AM, Singer AL, Montgomery RA, Segev DL. Histidine-Tryptophan-Ketoglutarate (HTK) is associated with reduced graft survival in deceased donor livers, especially those donated after cardiac death. Am J Transplant 2009;9:286-293.

25. Zaouali MA, Padrissa-Altes S, Ben Mosbah I, Alfany-Fernandez I, Massip-Salcedo M, Casillas-Ramirez A, et al. Improved rat steatotic and nonsteatotic liver preservation by the addition of epidermal growth factor and insulin-like growth factor-I to University of Wisconsin solution. Liver Transpl 2010;16:1098-1111.
26. Bejaoui M, Pantazi E, Folch-Puy E, Panisello A, Calvo M, Pasut G, et al. Protective Effect of Intravenous High Molecular Weight Polyethylene Glycol on Fatty Liver Preservation. Biomed Res Int 2015;2015:794287.
27. Puts CF, Berendsen TA, Bruinsma BG, Ozer S, Luitje M, Usta OB, et al. Polyethylene glycol protects primary hepatocytes during supercooling preservation. Cryobiology 2015;71:125-129.
28. Berendsen TA, Bruinsma BG, Puts CF, Saeidi N, Usta OB, Uygun BE, et al. Supercooling enables long-term transplantation survival following 4 days of liver preservation. Nat Med 2014;20:790-793.
29. Valuckaite V, Seal J, Zaborina O, Tretiakova M, Testa G, Alverdy JC. High molecular weight polyethylene glycol (PEG 15-20) maintains mucosal microbial barrier function during intestinal graft preservation. J Surg Res 2013;183:869-875.
30. de Boer JD, Strelniece A, van Rosmalen M, de Vries E, Ysebaert D, Guba M, et al. The Effect of Histidine-tryptophan-ketoglutarate Solution and University of Wisconsin Solution: An Analysis of the Eurotransplant Registry. Transplantation 2018;102:1870-1877.
31. de Jong IEM, Matton APM, van Praagh JB, van Haaften WT, Wiersema-Buist J, van Wijk LA, et al. Peribiliary glands are key in regeneration of the human biliary epithelium after severe bile duct injury. Hepatology 2018.



9

CHAPTER

Peribiliary glands are key in regeneration of the human biliary epithelium after severe bile duct injury

Alix P.M. Matton*
Iris E.M. de Jong*
Jasper B. van Praagh
Wouter T. van Haaften
Janneke Wiersema-Buist
Louise A. van Wijk
Dorenda Oosterhuis
Raditya Iswandana
Su Suriguga
Diletta Overi
Ton Lisman
Guido Carpino
Annette S.H. Gouw
Peter Olinga
Eugenio Gaudio
Robert J. Porte

*Equally contributing authors

Hepatology. 2019; 69:1719-1734

ABSTRACT

Peribiliary glands (PBG) are a source of stem/progenitor cells organized in a cellular network encircling large bile ducts. Severe cholangiopathy with loss of luminal biliary epithelium has been proposed to activate PBG, resulting in cell proliferation and differentiation to restore biliary epithelial integrity. However, formal evidence for this concept in human livers is lacking. We, therefore, developed a novel *ex vivo* model using precision-cut slices of extrahepatic human bile ducts obtained from discarded donor livers, providing an intact anatomical organization of cell structures, to study spatiotemporal differentiation and migration of PBG cells after severe biliary injury. Post-ischemic bile duct slices were incubated in oxygenated culture medium for up to a week. At baseline, severe tissue injury was evident with loss of luminal epithelial lining and mural stroma necrosis. In contrast, PBG remained relatively well preserved and different reactions of PBG were noted, including PBG dilatation, cell proliferation and maturation. Proliferation of PBG cells increased after 24 h of oxygenated incubation, reaching a peak after 72 h. Proliferation of PBG cells was paralleled by a reduction in PBG apoptosis and differentiation from a primitive and pluripotent (Nanog+/Sox9+) to a mature (CFTR+/secretin receptor+) and activated phenotype (increased expression of HIF-1α, Glut-1, and VEGF-A). Migration of proliferating PBG cells in our *ex vivo* model was unorganized, but resulted in generation of epithelial monolayers at stromal surfaces.

Conclusion: Human PBG contain biliary progenitor cells and are able to respond to bile duct epithelial loss with proliferation, differentiation, and maturation to restore epithelial integrity. The *ex vivo* spatiotemporal behaviour of human PBG cells provides evidence for a pivotal role of PBG in biliary regeneration after severe injury.

INTRODUCTION

The peribiliary glands (PBG) of large intra- and extrahepatic bile ducts appear as a three-dimensional network of interconnected acini and ducts draining into the biliary lumen. PBG have been shown to contain cell types with pluripotent properties, typical of stem/progenitor cells.[1] Recent advances have unravelled the phenotypical heterogeneity of PBG along the extrahepatic and large intrahepatic bile ducts and PBG have been proposed as a key element in the pathophysiology of liver, pancreas, and bile duct.[2,3] In this concept, PBG are activated upon injury and driven by molecular pathways to restore the compromised integrity of the luminal biliary epithelium in various cholangiopathies.

Up to now, the activation of PBG has been observed in patients with hepatolithiasis, primary sclerosing cholangitis, and post-transplant cholangiopathies.[3-6] Our understanding of PBG and their behaviour in pathological conditions is based on findings in cross-sectional studies, animal studies, and cell cultures. These models have not provided definite proof of the functional and morphological recovery of the biliary epithelial lining by a coordinated response of PBG cells after severe injury of the large bile ducts in humans. Further studies to investigate the molecular interactions of PBG in the context of pathological conditions are hampered by a lack of experimental models in which the proliferation, migration, and maturation of human PBG cells can be studied.

The aim of the current study was to develop a novel *ex vivo* human model to recapitulate the cellular organization of the PBG niche within the human bile duct, thus allowing the spatiotemporal study of PBG proliferation, maturation and migration in regeneration processes after injuries. Specifically, we used this model to explore the role of PBG cells in the post-ischemic recovery of bile ducts, as may occur after liver transplantation.

MATERIALS AND METHODS

Donor livers

Bile duct tissue was obtained from 16 adult human donor livers that were declined for transplantation within the Eurotransplant area. All livers were procured as part of a multi-organ donation procedure using a standard technique of *in situ* cooling and flush-out with ice cold University of Wisconsin preservation fluid. During procurement, the cystic duct was ligated and bile ducts gently flushed retrograde with preservation fluid. Livers were transported to our center using static cold storage.

The use of donor livers for research was approved by the medical ethical committee of the University Medical Center Groningen and the 'Nederlandse Transplantatie Stichting', the competent authority for organ donation in the Netherlands. In all cases informed consent was obtained from donor families to use the livers for research purposes.

Preparation of bile duct segments

Upon arrival of a donor liver at our center, the large intra- and extrahepatic bile ducts were dissected and removed while maintaining constant cooling of the tissue. Surrounding (adipose) tissue was carefully removed while avoiding manipulation of the bile duct wall. Bile duct dissection occurred from the distal end of the common bile duct until proximal of the biliary bifurcation to the right and left hepatic ducts. Main parts of the right and left hepatic ducts were included (**Figure 1**). Subsequently, the dissected bile ducts were cut in segments of approximately 15 mm to be fixed in 3% low melting agarose (w/v) solution (Sigma-Aldrich, Steinheim, Germany) in 0.9% NaCl at 37 °C in cylindrical cores. The cores were positioned in an embedding unit filled with crushed ice. Due to the cooling, the agarose solidified and a solid-like structure was created, suited for slicing. Simultaneously, Krebs-Henseleit buffer (KHB) solution was prepared and kept at 0-4 °C on melting ice (7). The KHB solution was oxygenated with 95% O_2 / 5% CO_2 for 30 min and adjusted to a pH of 7.4.

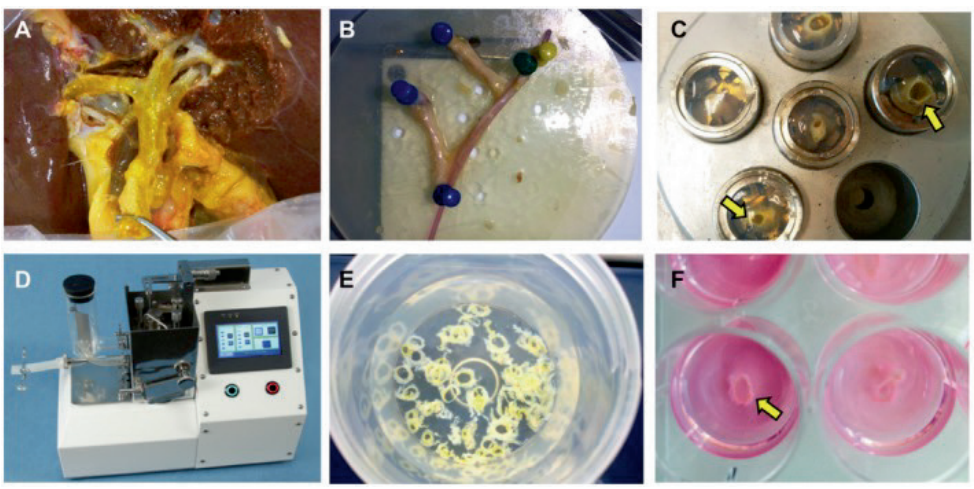


Figure 1. Photographic details of the precision-cut bile duct slicing procedure in chronological order. Panel A: A discarded liver positioned in the cold embedding unit for dissection of the large bile ducts. **Panel B:** An isolated bile duct segment including the hepatic bifurcation after dissection. A small probe is passed through the main bile duct lumen. **Panel C:** Bile duct segments embedded in cores filled with low-melting agarose. Small segments of the bile duct are situated vertically in the agarose and indicated by arrows. **Panel D:** Krumdieck tissue slicer. **Panel E:** Bile duct slices collected in cold oxygenated Krebs-Henseleit buffer solution. **Panel F:** 12-wells culture plate filled with Williams medium E, supplemented with glucose and antibiotics. Each well contains one precision-cut bile duct slice (arrow).

Precision-cut bile duct slicing procedure

A Krumdieck tissue slicer (Alabama Research and Development, Munford, AL, USA) filled with ice-cold KHB solution was used for precision-cut bile duct slicing (8)Blade and arm speed were adjusted to the tissue strength. The tissue slices were collected in ice-cold oxygenated KHB solution.

Incubation of bile duct slices

Biliary slices were transferred from the cold oxygenated KHB solution to pre-warmed oxygenated 12-wells plates filled with 1.3 ml Williams' medium E glutamax-I (Gibco, Paisley, UK) containing 1.1 g/ml of D-glucose and 2 mg/ml ciprofloxacin per well (**Figure 1**). Slices at baseline were harvested directly after the slicing procedure. Plates with slices were placed in a shaking incubator (shaking at 90 rpm at 37 °C) under continuous supply of 95% O_2 / 5% CO_2.[9] Thereafter, every 24 hours the medium was refreshed and slices were harvested for immunohistochemistry and for quantitative real time polymerase chain reaction (qRT-PCR) for up to 144 h. For immunohistochemistry, harvested slices were fixed in 10% buffered formalin and embedded in low-temperature fusion paraffin (55-57°C), 3-4 µm sections were prepared and mounted on glass slides for histological and immunohistochemical assessment. For qRT-PCR, harvested slices were preserved in 400 ml of RNAlater solution (Sigma-Aldrich, Steinheim, Germany), snap frozen and stored at -80**°**C.

Histology and immunohistochemistry

Sections were prepared for hematoxylin and eosin (H&E) staining for histological assessment. For immunohistochemistry, tissue sections were deparaffinized through a graded alcohol series and rinsed in phosphate-buffered saline (PBS, pH 7.4). Stainings for epithelial cell adhesion molecule (EpCAM) and calretinin were applied using an automated immunohistologic staining system Bench Mark Ultra of Ventana (Roche Ventana Medical Systems, Tucson, Arizona) and by applying the Ultraview DAB detection kit of the same company. Regarding all other stainings: endogenous peroxidase activity was blocked by a 30 min incubation in H_2O_2. Antigen retrieval for Cytokeratin 19 (CK19) and Ki-67 was performed with Tris/HCl pH 9.0 buffer at 80°C overnight and Tris/EDTA pH 9.0 buffer in the microwave for 15 min, respectively. Antigen retrieval for the other stainings was done using citrate at 90°C for 30 min. After cooling down of the sections and PBS wash for 5 min, first antibodies were applied for one hour (**Supplementary Table 1**). Next, for CK19 and Ki-67 a 30 min-incubation was applied with peroxidase-labeled goat anti-rabbit antibody (for CK19) or rabbit anti-mouse antibody (for Ki-67) in dilution 1:100. Rabbit anti-goat for CK19 and goat anti-rabbit for Ki-67 were used as third antibodies (1:100 dilution). For the other stainings, samples were incubated for 20 min with secondary biotinylated antibody and then Streptavidin-HRP (LSAB+ System-HRP, code K0690; Dako). The staining reaction was developed by 3,3'-diaminobenzidine (DAB), and

counterstained with hematoxylin. Images of the sections were scanned and subsequently analysed using Aperio ImageScope software (version 11.0.2.725, Aperio Technologies, Vista, CA, USA). Ki-67 stainings were used to measure the proliferation index in each slide. All PBG cells were manually counted in the slices. Proliferation index was calculated as Ki-67 positive PBG cells relative to all PBG cells in the slide:

$$\text{Proliferation index} = \frac{\text{Ki67 positive PBG cells}}{\text{All PBG cells in slide}} \text{x100}$$

For immunofluorescence, non-specific protein binding was blocked by 5% normal goat serum. Specimens were incubated with primary antibodies. Cells were washed and incubated for 1h with labelled isotype-specific secondary antibodies (anti-mouse AlexaFluor-546, anti-mouse AlexaFluor-488, anti-rabbit AlexaFluor-488, anti-goat AlexaFluor-546, Invitrogen, Life Technologies Ltd, Paisley, UK) and counterstained with 4,6-diamidino-2-phenylindole (DAPI) for visualization of cell nuclei. To perform double IF with two mouse primary antibodies, we followed a three-step protocol as previously described[10]: sections were incubated with anti-VEGF-R2; then, an anti-mouse secondary fluorescent antibody (AlexaFluor-488) was applied; finally, the antibody for CD31 was pre-labelled with a fluorophore using the APEX-594 labelling kit (Invitrogen, catalogue #A10474) and was applied to the section. All antibodies were diluted (1:50) and incubated at room temperature (RT) for 1 h. For all immunoreactions, negative controls (the primary antibody was replaced with pre-immune serum) were also included. Sections were examined in a coded fashion by Leica Microsystems DM 4500 B Light and Fluorescence Microscopy (Weltzlar, Germany), equipped with a Jenoptik Prog Res C10 Plus Videocam (Jena, Germany). Immunofluorescence stainings were also analyzed by confocal microscopy (Leica TCS-SP2). Slides were further processed with an Image Analysis System (IAS - Delta Sistemi, Rome- Italy). Expression of cleaved caspase-3 and vascular endothelial growth factor A (VEGF-A) was independently evaluated by two researchers (E.G. and G.C.) in a blinded fashion using a semi-quantative scoring system.[11] Briefly, when 0–5% of the bile ducts were positive, we assigned a negative score; a ± score was assigned when 6–10% of the bile ducts were positive; a + score was assigned when 11–30% of the bile ducts were positive; a ++ score was assigned with 31–50% of the bile ducts positive; and a +++ score was assigned when more than 50% of the bile ducts were positive. Quantification of Sox9 and PCNA expression was performed calculating the percentage positive PBG cells. Similarly, Sox9+ or Sox9- PBG cells co-expressing PCNA/cleaved caspase-3 were calculated as percentage positive PCNA/cleaved caspase-3 cells with respect to all Sox9+ or all Sox9- cells. All histological analyses were supervised by an experienced hepato-pathologist (ASHG).

Quantitative real-time PCR

Biliary mRNA expression of relevant genes was determined by qRT-PCR. RNA was isolated from bile duct slices that were snap-frozen and stored at -80°C. Total RNA was extracted using RNeasy mini kit (Qiagen, Venlo, the Netherlands)

according to the manufacturer's instructions. An additional step for purification of nucleic acid extraction in the protocol was performed using phenol-chloroform-isomylalcohol (Sigma-Aldrich, Steinheim, Germany; code 77617) and chloroform-isomylalcohol (Sigma-Aldrich, Steinheim, Germany; code 25666) after homogenization of the tissue. RNA concentrations were measured on a NanoDrop ND-1000 full spectrum (220-750 nm) spectrophotometer (Thermo Scientific, Wilmington, MA, USA). Single-stranded cDNA was synthesized using Reverse Transcriptase System (Promega, Leiden, the Netherlands) according to the manufacturer's instructions in a total volume of 20 µl. cDNA was diluted to a concentration of 2 ng/µl for qRT-PCR analysis. PCR reactions were performed in duplicate in 10-µl reaction volume containing 5 µl Taqman qPCR mastermix (Eurogentic, Liege, Belgium) and 5 µl cDNA. The following predesigned TaqMan primers were used: *CK19* (Hs00761767_S1), *Nanog* (Hs02387400_g1), and *CFTR* (Hs00357011_m1), obtained from Applied Biosystems. Thermal cycling and fluorescence detection were performed on a ViiATM 7 Real-Time PCR system (Applied Biosystems). Expression levels were corrected using GAPDH as reference gene (ΔCt) and compared with baseline (ΔΔCt). Results are displayed as fold change ($2^{-\Delta\Delta Ct}$).

Statistical analyses

Continuous variables were expressed as mean ± SEM. Categorical variables were presented as number and percentage. Continuous variables were compared between time points with a paired t-test. GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA) was used for presenting data in graphs. All statistical analyses were performed using SPSS software version 23 for Windows (SPSS, Inc., Chicago, IL).

RESULTS

Preparation of precision-cut bile duct slices

Donor characteristics of the 16 discarded donor livers used in the experiments are presented in **Supplementary Table 2.** All livers but one were obtained from donation after circulatory death donors. The main reasons that livers were declined for transplantation were advanced age or steatosis in combination with donation after circulatory death. The average length of the dissected bile duct segment was 8 cm. Per bile duct segment, a minimum of 80 bile duct slices with a thickness of approximately 300 µm were obtained (**Figure 1**).

PBG and stromal injury after ischemia and reoxygenation of human bile duct slices

The mean cold ischemic period before oxygenated incubation of precision-cut bile duct slices was 649 ± 66 min. Baseline histology of bile ducts revealed severe tissue injury with complete loss of luminal biliary epithelial lining and mural

stroma necrosis. In contrast, PBG cells were relatively well preserved with less signs of cell death at baseline (**Figure 2C** and **Figure 3A**). Morphology of PBG during incubation varied from a well-preserved cellular phenotype and architecture to total destruction of cells, causing collections of empty acini with cell debris, suggesting a process of necrosis. Some PBG showed dilatation, which appeared with or without necrotic PBG collections in the same section (**Figure 2A**). Strikingly, at baseline, PBG were organized in compact clusters with small PBG cells, while at later time points PBG acini were found to be more separated (**Figure 3A**). A significantly shorter distance between PBG was calculated at baseline (18 µm ± 0,04) compared to 72 h of incubation (73 µm ± 0,15) (P = 0.001). Immunohistochemical evidence of cleaved caspase-3 expression was found for both PBG and surrounding stromal cells, indicating the activation of apoptotic cell death (**Figure 2B**). After oxygenated incubation, expression of cleaved caspase-3 in PBG cells was significantly lower at 72 and 144 h, compared to baseline (P = 0.036) (**Figure 2B**). The percentage of vital PBG cells increased slightly after baseline and remained around 50% up to 144 h of incubation. In contrast, the percentage of vital surface epithelial cells increased from 0% to 32% during 144 h of incubation (**Figure 2C**). Some of the PBG collections were surrounded by a concentric organization of stroma (**Figure 2A**) composed of α-SMA positive (desmin and caldesmon negative) myofibroblasts (**Figure 2D** and **Figure 3B**). Immunohistochemistry for VWF showed the presence of vessels within the duct wall stroma and near PBG (**Figure 2E**). Some vessels displayed signs of damage, characterized by dilatation and loss of endothelial cells. In general, the presence of stromal cells (myofibroblasts and vessels) in the bile duct slices indicated an almost intact anatomical organization of the tissue. Furthermore, the response of PBG to hypoxic conditions was studied by investigating expression of a post-ischemic restorative response (i.e. HIF-1α, Glut-1, and VEGF-A). PBG showed increased expression of the hypoxia markers HIF-1α (at a nuclear level), Glut-1, and VEGF-A over time (**Figure 4A-C**), thus suggesting the activation of HIF-dependent and pro-angiogenic pathways. When VEGFR expression was studied, PBG were positive for VEGFR-1 and R-2, suggesting both an autocrine effect and a paracrine stimulation of neighboring VEGFR-2+ (CD31+) endothelial cells (**Figure 4D**).

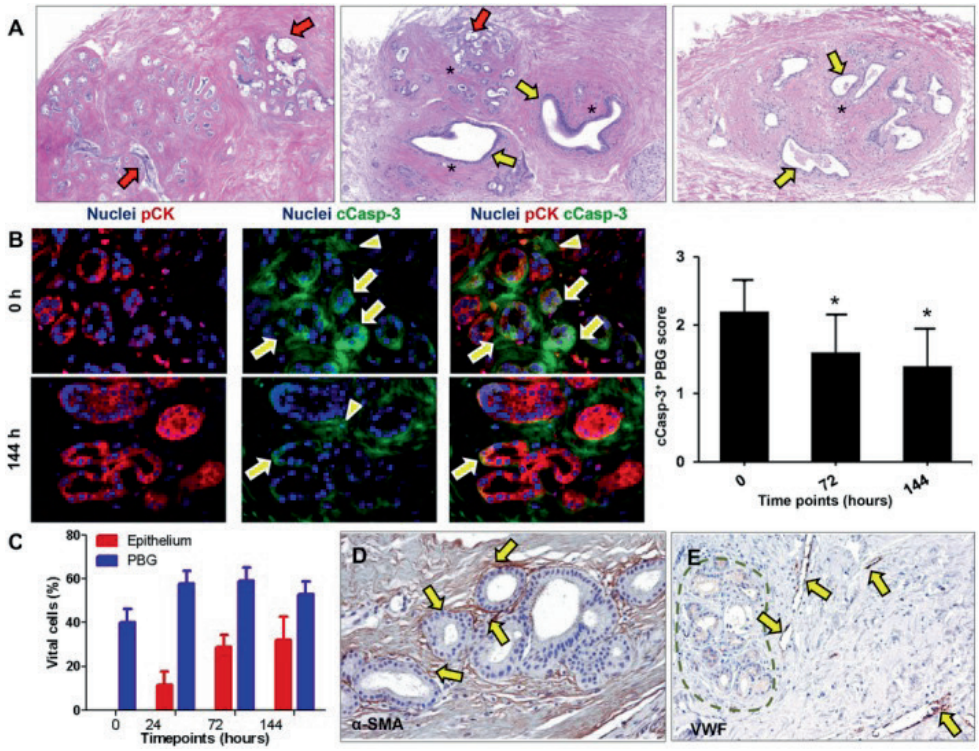


Figure 2. Bile ducts that were subjected to severe ischemia and subsequent reoxygenation showed cell death and PBG dilatation. Panel A: Hematoxylin and Eosin (H&E) staining showing necrotic PBG leaving cell debris in the empty acini (red arrows). In addition, PBG dilatation occurred in specific glands (yellow arrows). In some sections, both cell death and dilatation were present in proximate PBG collections (central image). Some PBG collections were captured in a concentric formation of stromal cells (asterisks). **Panel B:** Immunofluorescence for pan cytokeratin (pCK) and cleaved caspase-3 (cCasp-3) indicated significant less apoptosis in PBG cells after 72 h and 144 h of incubation compared with baseline (*$P < 0.05$). Cleaved caspase-3 was expressed by the PBG cells (arrows) as well as around the PBG cells in the stroma (arrowheads). Nuclei are displayed in blue. **Panel C:** Quantification of the percentage of vital cells in PBG and epithelium showing that approximately 50% of the PBG cells appeared vital during all time points, with no significant differences over time. In contrast, no luminal epithelial cells were present at baseline, yet a growth of epithelium up to 32% apparent vital cells was evident up to 144 hours of incubation. **Panel D:** Immunohistochemistry for alpha-smooth muscle actin (α-SMA) illustrates presence of myofibroblasts around PBG and provides evidence for an anatomical organization of stromal cells (arrows). **Panel E:** Immunohistochemistry for von Willebrand factor (VWF) in endothelial cells (arrows) indicating vessels throughout the stroma in proximity of PBG (dotted line). Dilated vessels with loss of endothelial cells can be observed. **Panel A-C:** Original magnification x10. **Panel D,E:** Original magnification x20.

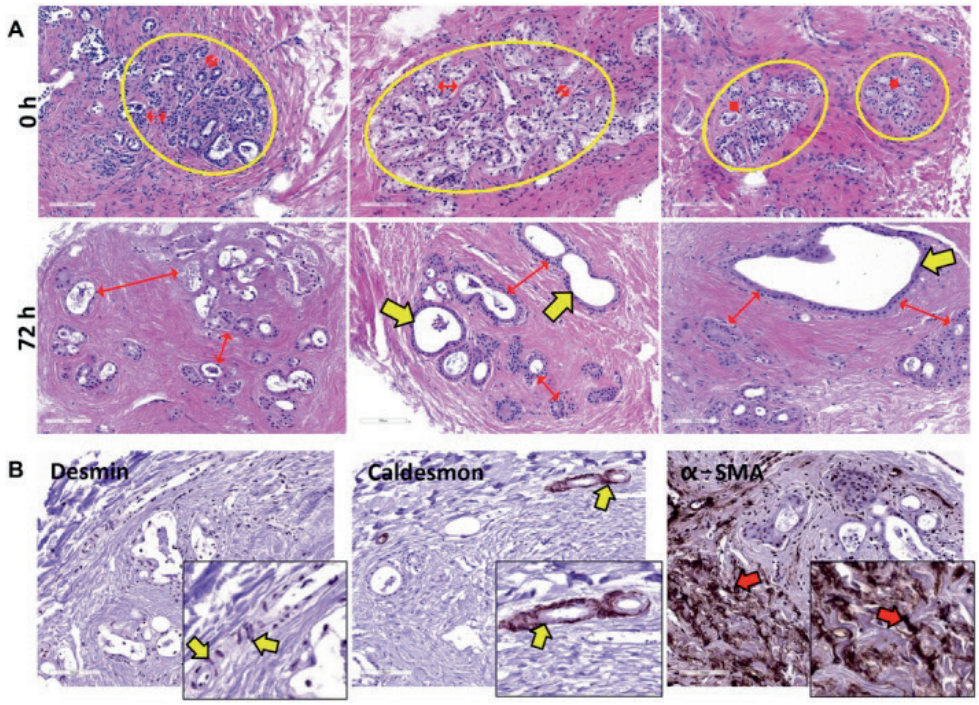


Figure 3. Stromal and PBG (re-)organization during incubation. Panel A: At baseline, small PBG acini were organized in compact clusters (yellow circles) and were located close to each other (red arrows). After 72 hours of incubation, PBG acini showed greater distance between each other (red arrows) and evident dilatation (yellow arrows). **Panel B:** Desmin and caldesmon expression was restricted to smooth muscle cells around arteries (yellow arrows), conversely, no expression of these markers was observed in alpha-smooth muscle actin (α-SMA) positive stromal cells (red arrows). These data confirm that the stromal reorganization around PBG clusters was characterized by α-SMA positive myofibroblasts (red arrows). **Panel B:** Original magnification x20.

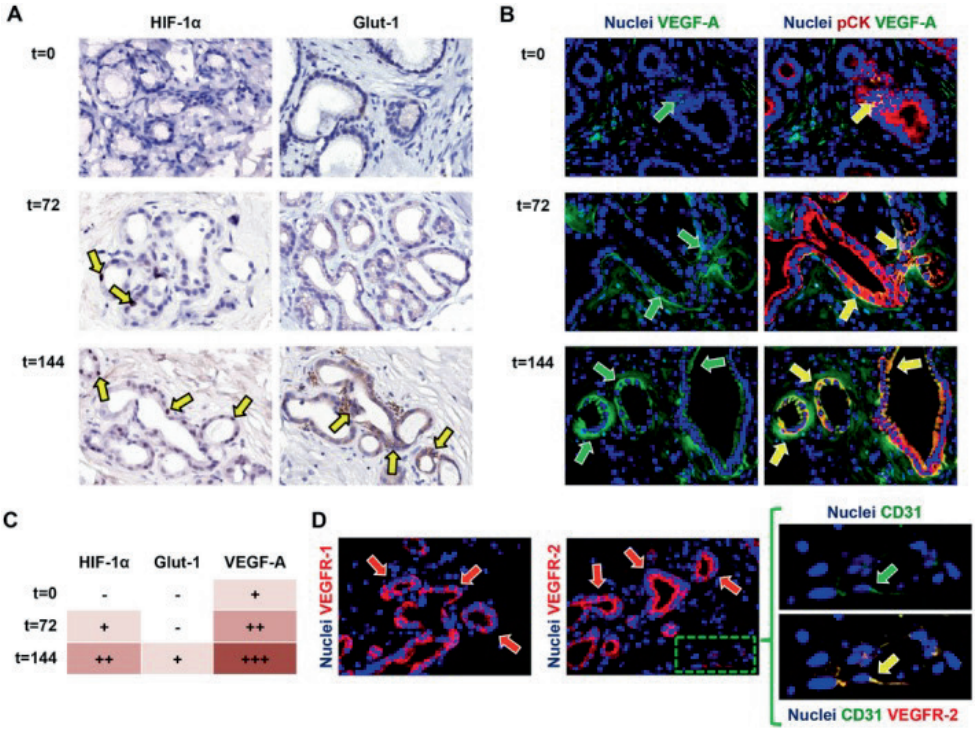
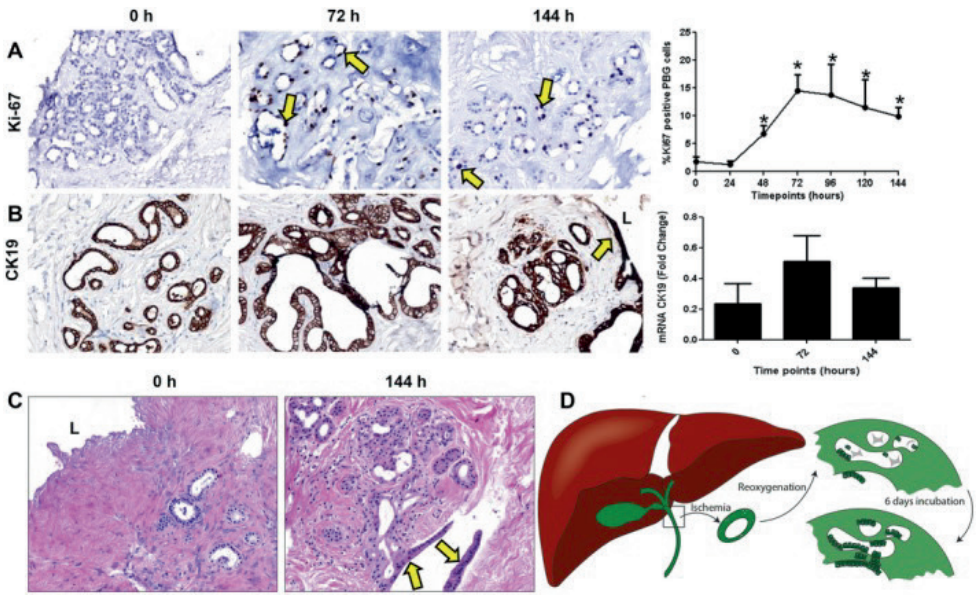


Figure 4. Activation of angiogenic factors during incubation. Panel A: Immunohistochemistry for the hypoxic response markers hypoxia-inducible factor 1 alpha (HIF-1α) and glucose transporter 1 (Glut-1) showed upregulation during incubation (yellow arrows). **Panel B:** Correspondingly, vascular endothelial growth factor A (VEGF-A) expression increased over time (green arrows) and was expressed by pan cytokeratin positive PBG cells (yellow arrows). **Panel C:** Semi-quantitative evaluation of angiogenic factor expression in PBG cells showed upregulation of HIF-1α, Glut-1, and VEGF-A over time. **Panel D:** VEGF receptors VEGFR-1 and VEGFR-2 were expressed by PBG cells (red arrows) and cluster of differentiation 31 (CD31) positive endothelial cells (green arrow) expressed VEGFR-2 at their cell membrane. Area in the box is magnified on the right and separate channels are provided. **Panel A-C:** Original magnification x40.

Proliferation of PBG cells is associated with regeneration of epithelial monolayers

Immunohistochemistry for Ki-67 revealed that whereas no proliferating PBG cells were present at baseline, during the culture period a substantial amount of proliferating cells within the PBG was observed. We quantified the extent of PBG proliferation by calculating the proliferation index (**Figure 5A**). Proliferating cells appeared within the first 24 h after reoxygenation after which the proliferation index increased and peaked at 72 h of incubation, where after the proliferation index gradually declined. From 48 h onwards the proliferation index was

significantly higher compared to baseline ($P = 0.004$). In parallel, regeneration of CK19 positive epithelium was observed after 144 h and quantification of CK19 expression showed a pattern reminiscent of the PBG proliferation index, suggesting an increase of biliary epithelial cells due to PBG cell proliferation (**Figure 5B**). In addition, epithelial monolayers in proximity of PBG collections were formed at an open space within the section after 144 h of incubation, suggesting that proliferating PBG cells migrated to create epithelial layers at any tissue surface that was encountered (**Figure 5C**). In support of this, newly formed epithelial monolayers appeared at the luminal and basolateral side of the slices at 72 h of incubation (**Figure 6A-E**). To confirm that the newly formed



monolayers at basolateral sides were epithelium and not mesothelium we stained the slices for EpCAM and calretinin, which are specific for epithelium- and mesothelium, respectively. Monolayers at all stroma surfaces were EpCAM positive and calretinin negative, confirming the regeneration of epithelium at several sides in the tissue (**Figure 6D,E**). Collectively, these findings suggest that ischemia and subsequent reoxygenation induced severe damage to biliary epithelial cells, but the residual PBG cells were able to proliferate and replenish the lost cells (**Figure 5D**).

Figure 5. PBG proliferation and regeneration after ischemia-induced epithelial cell loss. Panel A: Immunohistochemistry for Ki-67 showed mitotic activity after 24 h of incubation. PBG proliferation index increased significantly after 24 h, peaked at 72 h, and remained constant until 144 h of incubation, compared to baseline (*P < 0.05). **Panel B:** Immunohistochemistry for biliary epithelial marker cytokeratin 19 (CK19) showed that PBG and biliary epithelium expressed CK19 during all time points. The pattern of the relative number of CK19 expressing cells resembled that of proliferation. **Panel C:** Hematoxylin and Eosin (H&E) staining displaying complete loss of epithelial layer at baseline and regeneration of an epithelial monolayer after 144 h of incubation. This monolayer appeared at the surface

of an open place within the stroma (yellow arrows). **Panel D:** Schematic overview and summary of the observed biliary damage and cellular reaction after ischemia and subsequent reoxygenation. After ischemia and reoxygenation, the biliary epithelium was generally detached and PBG were necrotic. Directly after reoxygenation, PBG showed cell debris in the acini with a few residual PBG cells. After 6 days of oxygenation, proliferation, refilled PBG and regeneration of the epithelium were evident. **Panel A:** Original magnification x10. **Panel B,C:** Original magnification x20. L, lumen.

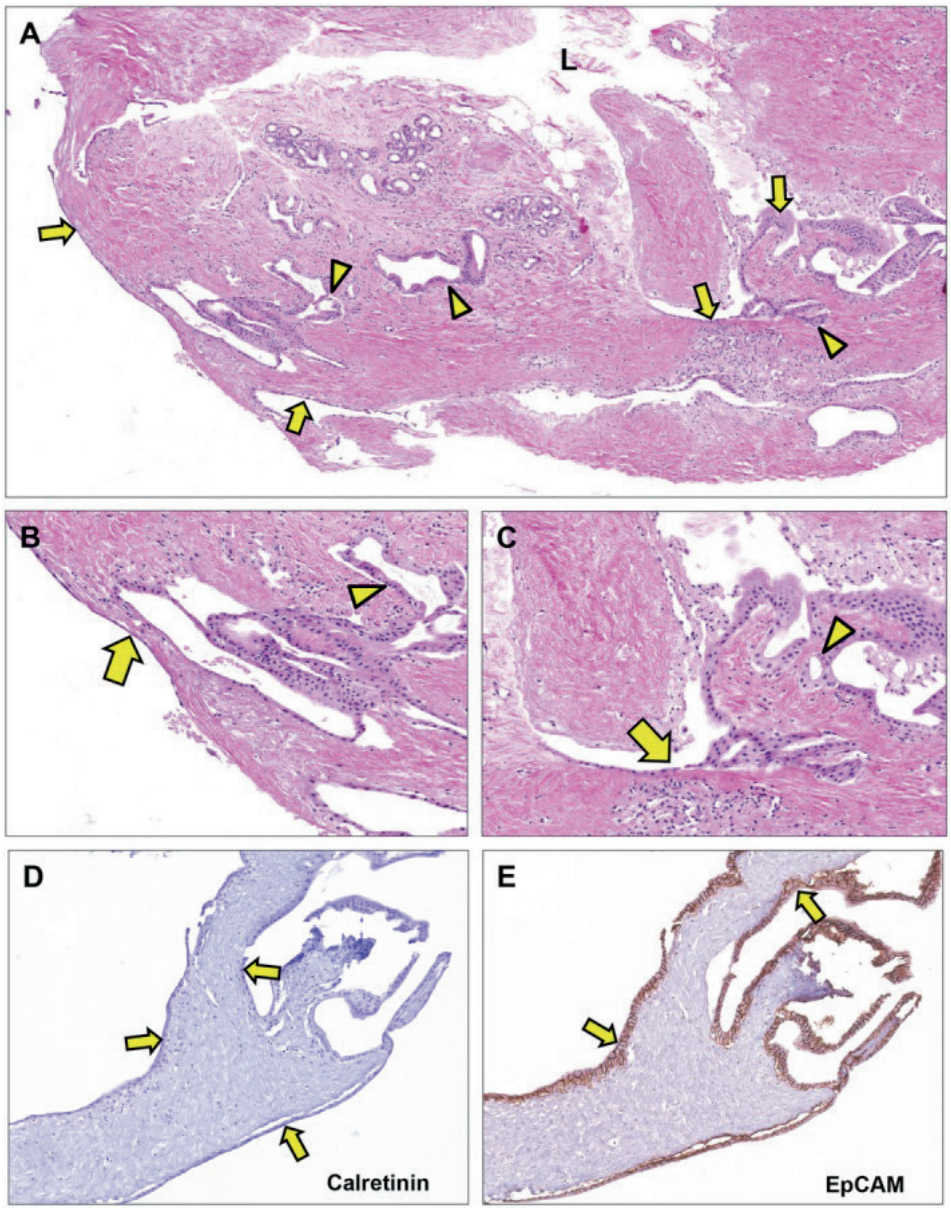


Figure 6. Epithelial regeneration after 72 hours of *ex vivo* culture. Panels A-C: Hematoxylin and eosin (H&E) staining. Epithelial lining appeared at the luminal surface as well as at the basolateral surface (arrows). These epithelial patches are in proximity of PBG (arrowheads) suggesting newly formed epithelium driven from PBG to restore the uncovered surface at several sides throughout the tissue slice. **Panel D:** Epithelial lining at basolateral and luminal side of the bile duct slice appeared negative for calretinin which is specific for mesothelium (arrows). **Panel E:** All cells lining the basolateral and luminal side of the bile duct slice expressed epithelial cell adhesion molecule (EpCAM), confirming that the (re)generated monolayers consisted of epithelial cells (arrows). **Panel A:** Original magnification x5. **Panel**

B,C: Original magnification x20. **Panel D, E:** Original magnification x15. *Abbreviations: L, lumen.*

Change of PBG phenotype indicating maturation during proliferation and regeneration

In general, 30-50% of the PBG contained Sox9-expressing cells during all time points, indicating an endoderm progenitor phenotype (**Figure 7A**). To explore maturation of proliferating and migrating PBG cells, we performed qRT-PCR to assess mRNA expression for homeobox protein Nanog, a transcription factor critically involved with self-renewal of undifferentiated stem cells, and mRNA expression of CFTR, a transporter that characterizes mature cholangiocytes. As shown in **Figure 7B**, relative Nanog gene expression decreased over time with a significant reduction at 144 h of oxygenated incubation, compared to baseline (P = 0.001). Presence of cells with nuclear Nanog positivity within PBG was also confirmed by immunohistochemistry (**Figure 7B**). In contrast to Nanog, relative CFTR gene expression increased over time and levels were significantly higher at 144 h compared to baseline (P = 0.025). Accordingly, the ratio between CFTR and Nanog gene expression increased over time, indicating an overall differentiation from primitive and pluripotent to mature phenotype (**Figure 7D**)**.** The increased gene expression of CFTR was paralleled by a progressively more extensive apical immunofluorescence staining of CFTR at 72 h and 144 h, compared to baseline (**Figure 7C**). Maturation of proliferating pluripotent PBG cells into adult cholangiocytes was confirmed by the expression of secretin receptor, which mostly appeared after 144 h (**Figure 7E**). Changes in Nanog and CFTR expression were evident at 24 h, which coincided with the initiation of PBG proliferation as demonstrated by Ki-67 expression (**Figure 5A**). Additionally, preservation of radial organized PBG has been studied in the present *in vitro* model. Deep and periluminal PBG were phenotypically characterized in terms of Sox9 and PCNA expression (**Figure 8A**). PBG located in the deeper position were characterized by a higher expression of Sox9 and PCNA compared to periluminal located PBG in continuity with surface epithelium. Surface epithelium was almost Sox9- and devoid of proliferating PCNA+ cells. In addition, Sox9+ PBG were characterized by a significantly higher proliferation index (i.e. percentage of PCNA positivity, **Figure 8B**) and lower cleaved caspase-3 expression compared to Sox9- PBG cells (**Figure 8C**), thus indicating that progenitor cells were less likely to be affected by apoptosis.

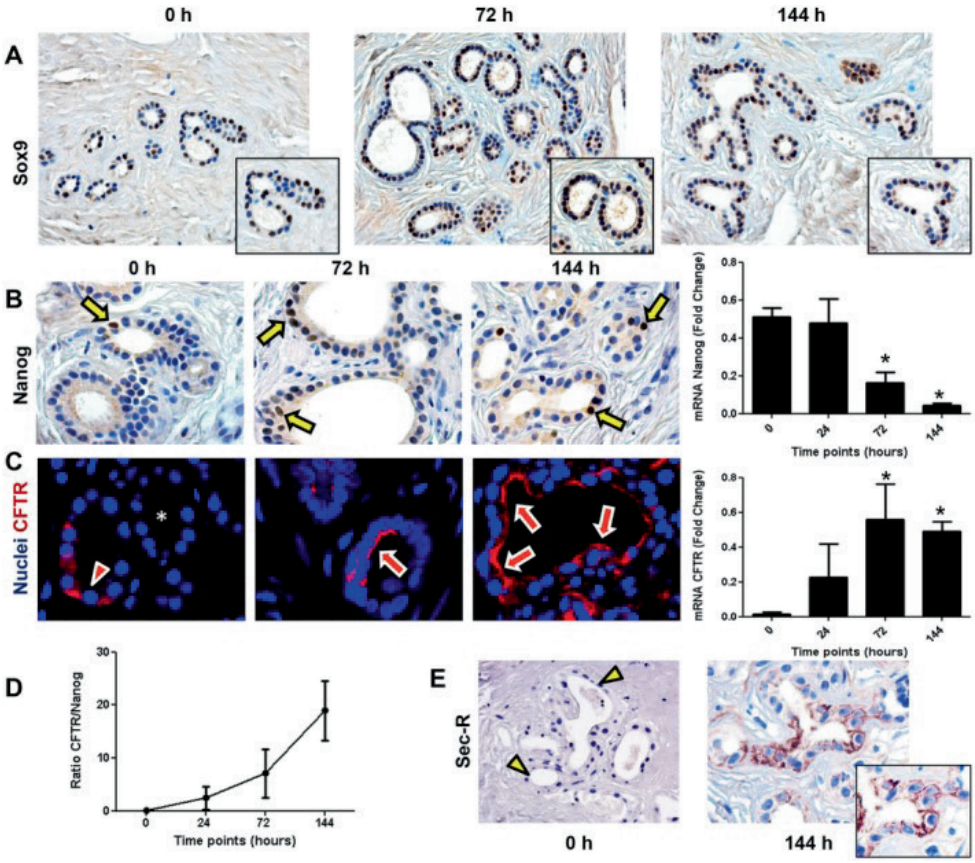


Figure 7. Cell maturation during 144 hours of oxygenated incubation of bile duct slices. Panel A: Immunohistochemistry for endoderm progenitor marker sex determining region Y-box 9 (Sox9) showed expression in PBG cells at all time points. **Panel B:** Immunohistochemistry demonstrated the presence of Nanog positive cells at a nuclear level (arrows) within PBG. Only pluripotent and primitive cells expressed Nanog; this cell population decreased over time and was significant lower after 144 h of incubation (**P* < 0.05). **Panel C:** Immunofluorescence for cystic fibrosis transmembrane conductance regulator (CFTR), expressed by mature biliary cells, showed less evident apical expression at baseline (arrowhead) and in some PBG no expression (asterisk) compared with PBG cells after 72 h and 144 h of incubation (arrows). Nuclei are displayed in blue. qRT-PCR confirmed that CFTR gene expression increased over time and was significant after 144 h of incubation compared to baseline (**P* <0.05). **Panel D:** Ratio between CFTR and Nanog increased during incubation, indicating maturation of the viable cells in the bile duct slices. **Panel E:** Immunohistochemistry for secretin receptor (Sec-R). Secretin receptor expression of PBG cells appeared after 144 h of incubation (arrow). At baseline, PBG were almost negative for secretin receptor (arrowheads). **Panel A,E:** Original magnification x20. **Panel B,C:** Original magnification x40.

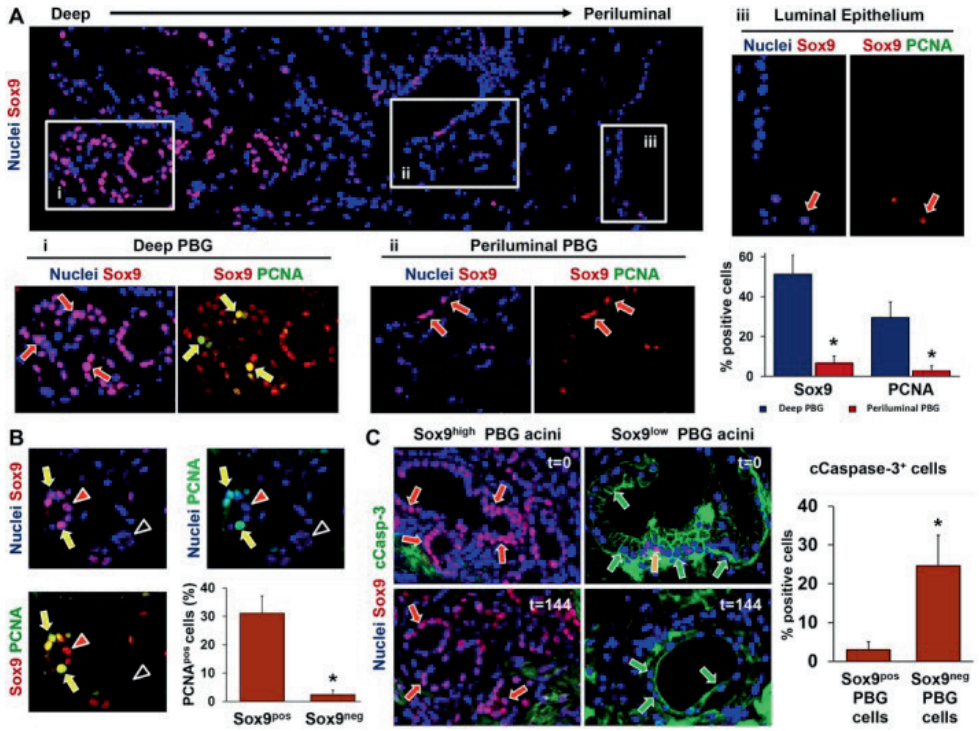


Figure 8: Immunophenotype of deep and periluminal PBG. Panel A: Immunofluorescence for sex determining region Y-box 9 (Sox9), which identifies endoderm-derived progenitor cells, showed that PBG located in deeper position (i) with respect to the luminal epithelium were characterized by higher expression of Sox9 compared to PBG acini located in a periluminal position (ii) and in continuity with luminal epithelium (iii). Sox9+ cells (red arrows) co-expressed the proliferation marker proliferating cell nuclear antigen (PCNA), merely in the deeper located PBG (i: yellow arrows). Periluminal PBG showed less Sox9 expression and almost no PCNA expression (ii: red arrows). The luminal epithelium (iii) showed rare Sox9+ cells (red arrow) and negligible levels of PCNA. The corresponding graph shows that both Sox9 and PCNA expression was significantly higher in deeper PBG, compared to periluminal PBG. **Panel B:** Specifically, PBG acini with Sox9- cells expressed no PCNA (black arrowheads) and acini with abundant Sox9 expression stained positive for PCNA (yellow arrows). Red arrowheads point towards Sox9+ cells that were PCNA negative. Almost all PCNA+ cells were Sox9+ as displayed in the graph, confirming that the proliferating cell population consisted of mainly progenitor cells. **Panel C:** PBG harboring Sox9+ cells (red arrows) were less positive for the apoptosis marker cleaved caspase-3 (cCasp-3). PBG harboring mainly Sox9- cells and a few Sox9+ cells (yellow arrow) showed marked expression of cleaved caspase-3 (green arrows). This was evident during all time points. Quantification of Sox9/cCasp-3 co-expression revealed significantly higher expression of cleaved caspase-3 in Sox9- PBG cells, compared to Sox9+ PBG cells. **Panel A:** Original magnification x10. Area in the boxes is magnified at x20. **Panel B,C:** Original magnification x20.

DISCUSSION

This study provides evidence that supports the concept of proliferation, maturation and migration of PBG-derived cells towards mature cholangiocytes after severe bile duct injury. In fact, this is the first study to provide evidence that PBG play a role in the restoration of biliary epithelial lining after severe bile duct injury in the human liver.

The main observations of this study indicate that, after severe ischemia-induced bile duct wall injury and luminal biliary epithelial cell loss, PBG cells are relatively spared and remain viable. While some PBG are affected and react with dilatation, necrosis, and apoptosis, surviving PBG respond with proliferation and regeneration of the epithelial lining, paralleled by a concentric organization of myofibroblasts and activation of HIF and VEGF signaling. During this process, PBG-derived cells change from a primitive and pluripotent phenotype located in the deeper layers of the bile duct wall to a mature biliary epithelial cell phenotype near the luminal surface.

In the present study we used a novel model to assess the spatiotemporal activation of PBG cells and biliary epithelial regeneration after severe injury, including loss of the luminal biliary epithelial lining, of large human bile ducts. Other models that have aimed to study activation of the biliary stem cell compartment and thereby attempted to recapitulate the human situation included lineage tracing studies in mice and organoid systems.[12,13] Whereas lineage tracing is restricted to animal models, organoid systems fail to capture the actual human anatomical organization of various cell types, including stromal cells, endothelium and PBG cells.[14] With the described model of precision-cut bile duct slices, we succeeded in providing an *ex vivo* situation with an intact anatomical organization of cellular structures using human tissue, avoiding the limitations of the aforementioned research models.

Until recently, PBG were mainly considered as secretory glands, producing mucus and humoral factors relevant for maintaining a healthy biliary epithelium.[15] However, over the past decade PBG cells have been characterized in more detail and stem cell properties of PBG have come to light.[1,3,16,17] Although marked proliferation of PBG in the context of large bile duct pathologies has been described for hepatolithiasis, primary sclerosing cholangitis, and after ischemia[4-6], today's view on PBG and their counterparts in the pancreas and liver suggest that several other hepato-pancreato-biliary diseases are also linked with these progenitor cell compartments.[2,18,19] In support of this, the current *ex vivo* study demonstrated the central role of PBG in bile duct recovery after severe ischemic damage.

We found a specific pattern of PBG cell proliferation with a noticeable increase in the first 24 h after reoxygenation of ischemically injured human bile ducts. These findings are in accordance with previous observations in animal models of other types of cholangiopathy. In a model of mechanically induced bile duct injury in guinea pigs, PBG have been described as crypts and glands in which all stages of mitoses can be observed 24 h after the injury, resulting in small patches

of a newly formed epithelial layers.[20] In addition, pronounced PBG cell proliferation has been described 24 h after bile duct ligation in mice.[21]

In our *ex vivo* model of human precision-cut bile duct slices, patches of newly formed epithelial cell layers appeared in the proximity of PBG collections, yet at both the basolateral and luminal side of the slices. The formation of new epithelium at the basolateral sides may, at first glance, seem contradictory to the peribiliary anatomical design in which PBG cells can replenish the luminal epithelium via small connecting tubules that run transversely through the mural stroma.[21] However, in our model of cultured, ultra-thin bile duct slices of approximately 300 µm, connecting tubules between PBG and the central lumen may be transected, resulting in a lack of mechanical guidance for migrating PBG cells. Our observations suggest that due to the lack of navigating tubules, proliferation and migration of PBG cells in our model occurred in random directions until the migrating cells encountered an open space to line its uncovered surface.

In addition to the maturation and migration of PBG cells, we also found evidence for a change from a quiescent phenotype towards a more reactive and activated phenotype. This was illustrated by the increased expression of HIF1-α, Glut-1, VEGF-A, VEGFR-1 and VEGFR-2 during 144 h of oxygenated incubation. It is well known that HIF1-α is translocated into the nucleus of the cell in response to hypoxia, leading to increased transcription of VEGF, Glut-1 and carbonic anhydrase 9 (CA9). VEGF and Glut-1 orchestrate angiogenesis and glucose metabolism, respectively.[22] Our findings suggest that hypoxia, manifest during static cold storage prior to oxygenated incubation, stimulated translocation of HIF1-α and subsequent initiated pro-angiogenic and metabolic pathways. Interestingly, PBG express VEGF-R, thus suggesting not only a paracrine effect on neighboring endothelial cells but also an autocrine/paracrine effect on PBG cells. This aspect parallels studies on mature cholangiocytes, indicating VEGF as a growth factor implicated in cholangiocyte proliferation.[23,24]

Notably, a reorganization of stromal cells was evident during incubation, with a concentric arrangement of myofibroblasts encircling PBG clusters. In addition, the distance between PBG acini increased, suggesting stromal cell expansion within the normally compact PBG clusters. At baseline, the freshly isolated donor bile ducts presented with a normal morphology of PBG clusters: small cells forming small acini in compact clusters. However, during 144 h of post-ischemic incubation, PBG started to proliferate and differentiate into mature cholangiocytes, generally recognizable by their higher cytoplasma-to-nucleus ratio.[25] In the current study, the presence of larger PBG acini could be due to differentiation towards mature (and large) cholangiocytes. Studies on hepatic stem cells have demonstrated that lineage-stage appropriate epithelial-mesenchymal partners with relevant paracrine signaling are highly effective in stimulating differentiation.[26,27] In addition, differentiation of bipotent progenitor cells towards a biliary fate in the hepatic progenitor niche, known as the Canals of Hering, requires deposition of collagens.[28,29] Thus we speculate that the observed stromal remodeling could be a consequence of proliferation

and differentiation of the progenitor stem cell compartment within PBG and activation of its stromal companion in similarity with the hepatic progenitor niche behavior.

Previous studies using *in vitro* cell cultures of isolated PBG cells have demonstrated the ability of these cells to proliferate and differentiate into mature cell types, including hepatocytes, cholangiocytes and pancreatic cells.[1] This confirms the pluripotency of PBG along the biliary tree, which is indicative of a primitive cell type. Primitive and pluripotent cells are known to be highly resistant to ischemia and other forms of damage, compared to mature cells.[30,31] This is confirmed by our findings of a relative preservation of PBG despite massive luminal epithelial cell loss and mural stroma necrosis in ischemically injured large extra- and intrahepatic bile ducts. The epithelium of the biliary tree consists of small cholangiocytes that populate the proximal side of the biliary tree and large cholangiocytes that cover the large intrahepatic bile ducts and extrahepatic bile duct.[25,32,33] Large cholangiocytes respond to damage with different intracellular pathways and are more differentiated than small cholangiocytes. Over the course of biliary damage, large cholangiocytes are the first to be affected and are sequentially replenished by proliferating small cholangiocytes. Previous experiments on the role of PBG in cholangiopathies have reported that PBG respond with low proliferation rates to mild biliary injuries whereas severe post-transplant cholangiopathy result in high rates of PBG proliferation.[17] Moreover, PBG activation corresponded with disease progression in primary sclerosing cholangitis.[4] Altogether, these findings and our current results indicate that following bile duct damage PBG may serve as a cholangiocytic regenerative reservoir and that the rate of PBG activation is influenced by the extent and progression of the defects.

The proliferation and migration of PBG cells have been associated with a maturational lineage process from the bile duct periphery to the luminal surface.[34] In the current study, we were able to confirm this by demonstrating that in ischemically injured bile ducts Sox9+/PCNA+ cells were situated in deeper located PBG. In general, our results indicated that progenitor cells with high regenerative capacities were settled in the deeper layers of the bile duct wall and were able to mature and regenerate the biliary epithelium.

The current view of the biliary tree as a heterogeneous cellular network is supported by the clinical spectrum of cholangiopathies that exclusively manifest at specific sites. Primary biliary cholangitis, for example, is restricted to the small intrahepatic bile duct and involves the hepatic progenitor compartment, whereas in primary sclerosing cholangitis the large intra- and extrahepatic bile ducts are affected, including involvement of the PBG.[35] Ischemia-reoxygenation injury, as can be found after liver transplantation, mainly affects the large cholangiocytes of the larger bile ducts. Therefore, post-transplant cholangiopathy mainly affects the larger intra- and extrahepatic bile ducts and involves damage to the PBG cell compartment, a finding that is supported by our results.[6] Unfortunately, we could not obtain sufficient numbers of bile duct slices from different parts of the large intra- and extrahepatic bile ducts to

demonstrate differences in PBG expression profiles between these different parts. Therefore, the inability to consider the longitudinal axis of biliary heterogeneity should be noted as a limitation of the current study. In addition, pathways that drive ischemia-reoxygenation-induced biliary proliferation and remodeling could have been characterized in more detail by biochemical analyses of culture medium samples. Unfortunately, we did not collect these samples in the current study.

In conclusion, we succeeded in recapitulating human biliary regeneration driven by PBG proliferation and maturation in its original anatomical cellular organization. In contrast to biliary epithelial luminal lining and stromal cells, PBG are remarkably resistant to ischemia-reoxygenation injury. After severe bile duct injury, PBG cells are able to respond with proliferation, migration and maturation to restore biliary integrity. Therefore, this study provides evidence to support the concept that PBG respond to bile duct injuries by restoration of the biliary integrity and confirms the protective role of PBG in the development of large duct cholangiopathies.

STATEMENT POLICY

None of the donor organs used in this study were obtained from executed prisoners or other institutionalized persons.

REFERENCES

1. Cardinale V, Wang Y, Carpino G, Cui CB, Gatto M, Rossi M, et al. Multipotent stem/progenitor cells in human biliary tree give rise to hepatocytes, cholangiocytes, and pancreatic islets. Hepatology 2011;54:2159-2172.
2. Lanzoni G, Cardinale V, Carpino G. The hepatic, biliary, and pancreatic network of stem/progenitor cell niches in humans: A new reference frame for disease and regeneration. Hepatology 2016;64:277-286.
3. de Jong, I E M, van Leeuwen OB, Lisman T, Gouw ASH, Porte RJ. Repopulating the biliary tree from the peribiliary glands. Biochim Biophys Acta 2018; 1864:1524-1531.
4. Carpino G, Cardinale V, Renzi A, Hov JR, Berloco PB, Rossi M, et al. Activation of biliary tree stem cells within peribiliary glands in primary sclerosing cholangitis. J Hepatol 2015; 63:1220-1228.
5. Igarashi S, Sato Y, Ren XS, Harada K, Sasaki M, Nakanuma Y. Participation of peribiliary glands in biliary tract pathophysiologies. World J Hepatol 2013;5:425-432.
6. op den Dries S, Westerkamp AC, Karimian N, Gouw AS, Bruinsma BG, Markmann JF, et al. Injury to peribiliary glands and vascular plexus before liver transplantation predicts formation of non-anastomotic biliary strictures. J Hepatol 2014;60:1172-1179.

7. Luangmonkong T, Suriguga S, Bigaeva E, Boersema M, Oosterhuis D, de Jong KP, et al. Evaluating the antifibrotic potency of galunisertib in a human ex vivo model of liver fibrosis. Br J Pharmacol 2017;174:3107-3117.
8. Olinga P, Schuppan D. Precision-cut liver slices: a tool to model the liver ex vivo. J Hepatol 2013;58:1252-1253.
9. Stribos EGD, Hillebrands J, Olinga P, Mutsaers HAM. Renal fibrosis in precision-cut kidney slices. Eur J Pharmacol 2016;790:57-61.
10. Carpino G, Renzi A, Cardinale V, Franchitto A, Onori P, Overi D, et al. Progenitor cell niches in the human pancreatic duct system and associated pancreatic duct glands: an anatomical and immunophenotyping study. J Anat 2016;228:474-486.
11. Glaser SS, Gaudio E, Rao A, Pierce LM, Onori P, Franchitto A, et al. Morphological and functional heterogeneity of the mouse intrahepatic biliary epithelium. Lab Invest 2009;89:456-469.
12. Nakagawa H, Suzuki N, Hirata Y, Hikiba Y, Hayakawa Y, Kinoshita H, et al. Biliary epithelial injury-induced regenerative response by IL-33 promotes cholangiocarcinogenesis from peribiliary glands. Proc Natl Acad Sci U S A 2017;114:E3815.
13. Ogawa M, Ogawa S, Bear CE, Ahmadi S, Chin S, Li B, et al. Directed differentiation of cholangiocytes from human pluripotent stem cells. Nat Biotechnol 2015;33:853-861.
14. Fatehullah A, Tan SH, Barker N. Organoids as an in vitro model of human development and disease. Nat Cell Biol 2016;18:246-254.
15. Spitz L, Petropoulos A. The development of the glands of the common bile duct. J Pathol 1979;128:213-220.
16. Irie T, Asahina K, Shimizu-Saito K, Teramoto K, Arii S, Teraoka H. Hepatic progenitor cells in the mouse extrahepatic bile duct after a bile duct ligation. Stem Cells Dev 2007;16:979-987.
17. Sutton ME, op den Dries S, Koster MH, Lisman T, Gouw AS, Porte RJ. Regeneration of human extrahepatic biliary epithelium: the peribiliary glands as progenitor cell compartment. Liver Int 2012;32:554-559.
18. Katabathina VS, Flaherty EM, Dasyam AK, Menias CO, Riddle ND, Lath N, et al. "Biliary Diseases with Pancreatic Counterparts": Cross-sectional Imaging Findings. Radiographics 2016;36:374-392.
19. Nakanuma Y, Harada K, Sasaki M, Sato Y. Proposal of a new disease concept "biliary diseases with pancreatic counterparts". Anatomical and pathological bases. Histol Histopathol 2014;29:1-10.
20. Hou CT. Repair of the extrahepatic bile-ducts after mechanical and chemical injury. J Pathol 1961;82:83-94.

21. DiPaola F, Shivakumar P, Pfister J, Walters S, Sabla G, Bezerra JA. Identification of intramural epithelial networks linked to peribiliary glands that express progenitor cell markers and proliferate after injury in mice. Hepatology 2013;58:1486-1496.

22. Weidemann A, Johnson RS. Biology of HIF-1alpha. Cell Death Differ 2008;15:621-627.

23. Gaudio E, Barbaro B, Alvaro D, Glaser S, Francis H, Franchitto A, et al. Administration of r-VEGF-A prevents hepatic artery ligation-induced bile duct damage in bile duct ligated rats. Am J Physiol Gastrointest Liver Physiol 2006;291:307.

24. Gaudio E, Barbaro B, Alvaro D, Glaser S, Francis H, Ueno Y, et al. Vascular endothelial growth factor stimulates rat cholangiocyte proliferation via an autocrine mechanism. Gastroenterology 2006;130:1270-1282.

25. Han Y, Glaser S, Meng F, Francis H, Marzioni M, McDaniel K, et al. Recent advances in the morphological and functional heterogeneity of the biliary epithelium. Exp Biol Med (Maywood) 2013;238:549-565.

26. Wang Y, Cui C, Yamauchi M, Miguez P, Roach M, Malavarca R, et al. Lineage restriction of human hepatic stem cells to mature fates is made efficient by tissue-specific biomatrix scaffolds. Hepatology 2011;53:293-305.

27. Turner RA, Wauthier E, Lozoya O, McClelland R, Bowsher JE, Barbier C, et al. Successful transplantation of human hepatic stem cells with restricted localization to liver using hyaluronan grafts. Hepatology 2013;57:775-784.

28. Williams MJ, Clouston AD, Forbes SJ. Links between hepatic fibrosis, ductular reaction, and progenitor cell expansion. Gastroenterology 2014;146:349-356.

29. Fabris L, Spirli C, Cadamuro M, Fiorotto R, Strazzabosco M. Emerging concepts in biliary repair and fibrosis. Am J Physiol Gastrointest Liver Physiol 2017;313:G116.

30. Vitale I, Manic G, De Maria R, Kroemer G, Galluzzi L. DNA Damage in Stem Cells. Mol Cell 2017;66:306-319.

31. Rocha CRR, Lerner LK, Okamoto OK, Marchetto MC, Menck CFM. The role of DNA repair in the pluripotency and differentiation of human stem cells. Mutat Res 2013;752:25-35.

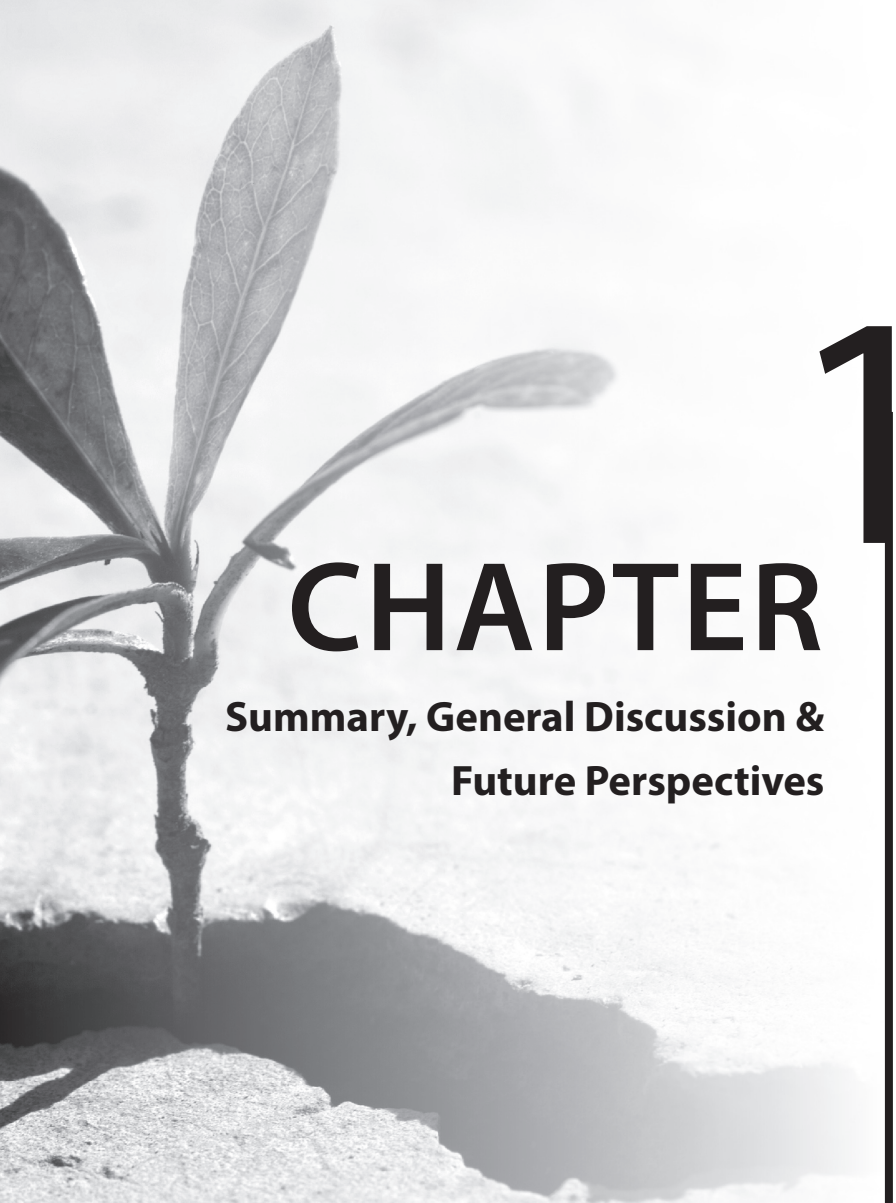
32. Marzioni M, Glaser SS, Francis H, Phinizy JL, LeSage G, Alpini G. Functional heterogeneity of cholangiocytes. Semin Liver Dis 2002;22:227-240.

33. Glaser S, Francis H, Demorrow S, Lesage G, Fava G, Marzioni M, et al. Heterogeneity of the intrahepatic biliary epithelium. World J Gastroenterol 2006;12:3523-3536.

34. Carpino G, Cardinale V, Onori P, Franchitto A, Berloco PB, Rossi M, et al. Biliary tree stem/progenitor cells in glands of extrahepatic and intraheptic

bile ducts: an anatomical in situ study yielding evidence of maturational lineages. J Anat 2012;220:186-199.

35. Carpino G, Cardinale V, Folseraas T, Overi D, Floreani A, Franchitto A, et al. Hepatic Stem/Progenitor Cell Activation Differs between Primary Sclerosing and Primary Biliary Cholangitis. Am J Pathol 2018;188:627-639.



CHAPTER 10

Summary, General Discussion & Future Perspectives

The studies in the present thesis focused on expanding the donor liver pool through the use of machine perfusion of extended criteria donor (ECD) livers, with a particular focus on the biliary tree.

In **chapter 1**, the thesis and outline of the various chapters were introduced. In **chapter 2**, ways to expand the donor liver pool were addressed.[1] One of the most impactful ways to increase the number of available donor livers for transplantation is by transplanting ECD livers. Compared to optimal standard criteria donor livers, ECD grafts are inherently of lesser quality and have endured more injury, and transplantation of these organs carries a higher risk of complications. The gold standard for preservation of donor livers, using static cold storage in which the organs are cooled and transported on ice, causes too much additional injury to these already high-risk ECD organs. Therefore, management of these grafts requires a different approach. Machine perfusion is a novel technique that provides better protection against ischemic injury and allows for the resuscitation and careful selection of donor livers. Depending on the indication for machine perfusion, it can be implemented during different phases of transplantation (procurement, transportation and upon arrival at the transplanting center), at a range of temperatures and using various preservation solutions. As machine perfusion continues to gain clinical application in the coming years, it is likely that a tailored approach will be implemented depending on various indications.

Chapter 3 describes the technique of normothermic machine perfusion (NMP) in a video article.[2] At 37°C, oxygenated NMP renders the organ metabolically active and allows for viability assessment. In order to create a physiological environment, NMP was performed using a perfusion solution based on packed red bloods cells (RBC), fresh frozen plasma (FFP) and a mix of nutrients, trace elements and antibiotics. An online video explains how NMP is performed using the Liver Assist (Organ Assist) perfusion device, which provides pressure-controlled continuous flow through the portal vein and pulsatile flow through the hepatic artery. In contrast to other perfusion devices, such as Metra (OrganOx), this device was not designed to be used during transportation and does not offer automated monitoring and adjustments of biochemistry in perfusate. The Liver Assist can therefore only be used at the procurement or implantation center. However, it is much cheaper, offers equal perfusion quality and most importantly, allows for machine perfusion at a range of temperatures.

As human blood products are scarce and their use is logistically complex, we sought to develop an NMP perfusion solution that circumvents the use of human blood products. In **chapter 4**, RBCs were first replaced by an acellular bovine hemoglobin (HBOC-201) and this modified perfusion solution was tested in six discarded donor livers. Subsequently FFPs were replaced by gelofusine and a mix of other additives and the resulting solution was tested using another six donor livers.[3] Both groups were compared to the historical cohort of twelve donor livers in which NMP was performed using a perfusion solution based on RBCs and FFPs. The livers in the HBOC-201 perfused groups displayed significantly better function, including higher cumulative bile production, portal

and arterial flows, and hepatic adenosine triphosphate (ATP) content. This was likely due to the HBOC-201 molecule's lower affinity for oxygen compared to human hemoglobin and smaller size, causing it to release oxygen more readily and allowing it to penetrate more deeply into the tissue. Disadvantages of using HBOC-201, however, include the potential formation of methemoglobin and the inability to remove HBOC-201 from samples, potentially leading to interference of spectrophotometric analyses of the perfusion fluid. Lastly, HBOC-201 and gelofusine are bovine-derived products, and future research should aim to find animal-free alternatives.

Another important property of HBOC-201 is that it, in contrast to RBCs, can be used as an oxygen carrier at lower temperatures. This allows for a perfusion protocol involving a period of dual hypothermic oxygenated machine perfusion (DHOPE), followed by controlled oxygenated rewarming (COR) and finally NMP. **Chapter 5** showed that sequential DHOPE-COR-NMP using an HBOC-based perfusion fluid offers a novel method of liver machine perfusion for combined resuscitation and viability testing of suboptimal livers prior to transplantation. In this case series, seven livers that were initially declined for transplantation were perfused using DHOPE-COR-NMP, of which five were successfully transplanted after meeting all hepato-biliary viability criteria during NMP. The primary endpoint, graft survival at 3 months, was 100%. Furthermore, post-operative peak ALT was lower in our recipients than in the studies that compared SCS to NMP only,[4,5] which may have been attributable to the DHOPE-induced amelioration of ischemia-reperfusion injury.[6-8] In addition, none of the recipients showed clinical signs of non-anastomotic biliary strictures (NAS), although as this was not the primary outcome we did not perform routine imaging and subclinical cases may have been missed. In conclusion, this study showed that sequential perfusion with DHOPE-COR-NMP using HBOC-201 is feasible and safe. Despite the promising results, larger cohorts with longer follow-up are required in order to draw any conclusions regarding the protocol's efficacy in safely increasing the number of donor livers for transplantation and reducing complications.

In **chapter 6**, we showed that biliary bicarbonate, pH, LDH, glucose and the bile/perfusate glucose ratio are accurate predictors of histological biliary injury during NMP. Histological bile duct injury was assessed using a clinically relevant scoring system and livers were divided into a group with high and low injury. Biliary biochemistry markers were subsequently analyzed for their predictive value using AUC-ROC curves. Establishing biliary viability biomarkers is very important, as up until then NMP viability assessment was mainly based on hepatocellular criteria, despite the fact that biliary injury prior to transplantation has been directly linked with the development of NAS. Especially biliary bicarbonate, rather than biliary pH as had been suggested by other groups[5], was able to predict biliary viability during NMP. Low biliary pH and bicarbonate do not only reflect biliary injury/dysfunction, but may also contribute to additional biliary injury due to an absent bicarbonate umbrella, which helps protect against the cytotoxic bile salts.[9] Since the publication of this paper, we have also found

that the delta between bile and perfusate pH and bicarbonate is even more discriminatory than their absolute values in bile. Biliary glucose is dependent on perfusate or blood glucose, and most post-ischemic livers have increased glucose levels due to glycogenolysis.[10] For this reason, it is important to determine the ratio between bile and perfusate glucose. One issue with the use of biliary biochemistry in assessing biliary viability is that some livers produce very little or no recorded bile during NMP. Donor livers that did not produce bile during NMP have, however, also been successfully transplanted and we therefore advise that especially livers with an increased risk for post-transplant cholangiopathy be assessed for biliary viability.[5,11]

In **chapter 7**, we showed that early release of hepatocyte- and cholangiocyte-derived micro-RNAs (HDmiR-122 and CDmiR-222, respectively) in perfusate and bile can predict late hepato-cholangiocellular injury and function of donor livers during NMP. These analyses were performed in a group of twelve donor livers that were declined for transplantation. Overall, levels of both miRs in perfusate and bile were lower in livers with low hepato-cholangiocellular injury and good hepato-cholangiocellular function. Levels of HDmiR-122 in perfusate correlated more strongly with hepato-cholangiocellular parameters compared to levels in bile, while levels of CDmiR-222 in bile correlated more strongly with parameters compared to levels in perfusate, confirming their cell-specific origin. The HDmiR-122/CDmiR-222 ratio was significantly higher in perfusate of low-quality livers compared to the ratio in high-quality livers. These miRs are differentially released into perfusate and bile and may in the future be used as biomarkers for assessment of graft viability during machine perfusion.

Chapter 8 compared the ability of various preservation solutions in protecting against histological bile duct injury, which has been shown to be predictive of the development of NAS after transplantation.[12] In this study, extrahepatic bile duct segments from discarded donor livers were cold stored in five different preservation solutions after a period of SCS in University of Wisconsin (UW) solution and subsequently assessed for histological bile duct injury on HE sections. Histidine-tryptophan-ketoglutarate (HTK) solution led to higher bile duct injury compared to UW solution. This carries important clinical implications as HTK solution is widely used and donor livers with a high-risk of post-transplant cholangiopathy are increasingly being transplanted. However, the benefits of HTK solution that have been described in the literature, including its low viscosity and fast temperature reduction[13], may have been masked in the current study design as the bile duct segments were small and a solution switch from UW to HTK solution may have caused additional injury, too. Furthermore, the addition of poly-ethylene glycols (PEGs) to HTK and UW solution resulted in a slight but non-significant, reduction in bile duct injury. PEGs are FDA-approved non-toxic water-soluble compounds that are widely used, including clinically, with high flexibility, hydrophilicity and protein-rejecting properties.[14] Previous research has shown that PEGs may play an important role in the preservation of liver and intestine, which closely resembles bile ducts.[15-20] Therefore the

addition of PEGs to preservation solutions may be a readily implementable and affordable method to protect the biliary tree that warrants further investigation.

In the final study reported in **chapter 9**, we established a novel method called precision-cut bile duct slices (PCBDS) to study human bile ducts, circumventing the use of laboratory animals. Using this *ex vivo* model, human extrahepatic bile ducts derived from discarded donor livers were cut into small slices and incubated for up to six days. This technique maintained intact anatomical organization of cell structures and allowed for the study of spatiotemporal differentiation and migration of peribiliary gland (PBG) cells. PBG are niches containing progenitor and stem cells embedded in the bile duct wall, which play a crucial role in the development of NAS after transplantation.[21,22] We showed that after severe bile duct injury, PBG cells were able to respond with proliferation, migration and maturation to restore biliary epithelium. This was the first study to provide evidence that PBG respond to biliary injury by restoration of biliary epithelium and confirms the protective role of PBG in the development of large duct cholangiopathies. A limitation of this study was that intra- and extrahepatic bile ducts could not be studied separately, as there is a limit to the number of PCBDS that can be obtained per liver.

FUTURE PERSPECTIVES

The studies described in the present thesis have contributed to the body of knowledge regarding the value of machine perfusion in liver transplantation. Machine perfusion in liver transplantation, and especially its role in protecting the bile ducts, holds great potential in expanding and improving the field of liver transplantation. Clearly, many questions remain unanswered and new challenges remain to be addressed, as will subsequently be discussed.

In the current thesis, we have shown that NMP can be used to expand the donor liver pool by identifying viable donor livers amongst livers initially declined for transplantation. NMP, which renders the organ metabolically active, could potentially also be used to make livers transplantable through the administration of drugs *ex situ*, if necessary also using higher dosages than would be possible *in vivo*. One area which has the potential to increase the donor pool substantially would be through the defatting of livers prior to transplantation, as currently around 40% of livers are declined for transplantation due to (expected) steatosis in the UK and Spain.[23,24] Furthermore, the regenerative capacity of the biliary tree could potentially be restored during machine perfusion by implanting or infusing PBG stem cells into injured bile ducts. Stem cells could even be obtained from a cholecystectomy of the recipient, expanded in culture and implanted into the donor liver during machine perfusion, thereby possibly also reducing the immunogenic influence in the development of NAS. MiRs should be further investigated for their use as biomarkers for hepato-cholangiocellular injury and function during machine perfusion. As miR assays are becoming more rapid to execute, these cell-specific miRs hold the potential to track processes in real time during machine perfusion. These miRs could potentially also provide useful real-time information about organ quality during organ procurement, storage, perfusion, transplantation and post-transplantation. Lastly, a perfusion fluid based on HBOC-201 is very promising as it can be used at various temperatures and allows for superior oxygenation, as the hemoglobin molecule releases its oxygen more easily and penetrates more deeply into the tissue. However, a bovine-derived solution could theoretically still lead to the transmission of infectious and immunogenic agents. Furthermore, for ethical reasons animal-free alternatives should be developed. For these reasons, synthetic oxygen carriers should be investigated as alternative oxygen carriers for machine perfusion.

As the application of machine perfusion of donor livers is growing and different variants of it are increasingly being applied around the world, it might be very worthwhile to compile all the information regarding donor characteristics, machine perfusion and transplantation information together in order to create an extensive body of knowledge regarding machine perfusion. Such a database could be used to identify the most optimal combinations between liver type and type of machine perfusion. The optimal machine perfusion temperature, solution, pressure, phase (i.e. pre-ischemic, during transportation or end-ischemic), duration and technique (e.g. dual versus single) could then be compared using large cohorts, as a frequently encountered issue with machine

perfusion studies is the relatively small sample sizes that are used due to the scarcity of human donor livers and high costs. Trials should subsequently be carried out to test the optimal application of machine perfusion for various indications, as well as to evaluate its cost-effectiveness.

Machine perfusion is likely going to, one day, replace static cold storage altogether. Until that time, however, it is of utmost importance that donor livers are preserved in an optimal preservation solution. Attention should be focused, in particular, on eliminating additional biliary injury instead of only on liver injury, as has been the main focus in literature. The addition of PEGs to preservation solutions is promising, readily implementable and relatively cheap and future studies should be focused on their use in liver preservation. The histological morphology of the bile ducts could be further investigated using electron microscopy. This was attempted for the study in chapter 8, but the bile ducts were too severely injured to be able to draw any conclusions and future studies should focus on preserving the bile ducts immediately after procurement of the liver. An experimental study that could be performed is to preserve human bile duct segments in various preservation solutions, as was done in chapter 8, and subsequently applying the PCBDS method to compare the proliferative capacity of the PBG cells *in vitro* after preservation in various solutions. Furthermore, the PCBDS model could also be used to stimulate PBG proliferation with the addition of various components. Bile acids, such as taurocholic acid, and Wnt signaling factors, which play a role in intestinal stem cell proliferation, could be used to stimulate PBG proliferation.[25,26] These factors could then be added to the biliary tree during machine perfusion to reduce or prevent the development of NAS after transplantation.

Machine perfusion will possibly lead to the establishment of organ banks where livers are stored for extended periods of time. This would allow for the elective transplantation of livers in the most optimal setting, with the recipient in optimal condition and international matching without time restrictions. Machine perfusion has the potential to allow for the resuscitation (defatting, chemotherapy etc.) and perhaps even complete de- and recellulization of organs, as is currently being investigated at Massachusetts General Hospital in Boston by Dr. K. Uygun and Dr. B. Uygun.[27] Subsequently, it is likely that optimized livers will be preserved using subzero non-freezing techniques, allowing for the long-term storage of transplant organs.[28]

In summary, the studies reported in the present thesis have contributed to the application of machine perfusion of human donor livers and include one of the first clinical applications of NMP that led to literal expansion of the donor liver pool. Moreover, this thesis has contributed to the establishment and identification of suitable biomarkers during machine perfusion, with a particular focus on protecting the biliary tree and thereby hopefully reducing the risk of NAS. Machine perfusion is an exciting and booming field that is in the starting blocks of revolutionizing organ transplantation, holding great potential to substantially increase both the quantity and quality of lives of transplant patients.

REFERENCES

1. Matton AP, Porte RJ. Opportunities for scientific expansion of the deceased donor pool. *Liver Transpl.* 2014;20 Suppl 2:S5.
2. Karimian N, Matton AP, Westerkamp AC, et al. Ex Situ Normothermic Machine Perfusion of Donor Livers. *J Vis Exp.* 2015;(99):e52688. doi:e52688.
3. Matton APM, Burlage LC, van Rijn R, et al. Normothermic machine perfusion of donor livers without the need for human blood products. *Liver Transpl.* 2018;24:528-538.
4. Mergental H, Perera MT, Laing RW, et al. Transplantation of Declined Liver Allografts Following Normothermic Ex-Situ Evaluation. *Am J Transplant.* 2016;16:3235-3245.
5. Watson CJE, Kosmoliaptsis V, Pley C, et al. Observations on the ex situ perfusion of livers for transplantation. *Am J Transplant.* 2018.
6. Schlegel A, de Rougemont O, Graf R, Clavien PA, Dutkowski P. Protective mechanisms of end-ischemic cold machine perfusion in DCD liver grafts. *J Hepatol.* 2013;58:278-286.
7. Dutkowski P, Polak WG, Muiesan P, et al. First Comparison of Hypothermic Oxygenated PErfusion Versus Static Cold Storage of Human Donation After Cardiac Death Liver Transplants: An International-matched Case Analysis. *Ann Surg.* 2015;262:764-70; discussion 770-1.
8. van Rijn R, Karimian N, Matton APM, et al. Dual hypothermic oxygenated machine perfusion in liver transplants donated after circulatory death. *Br J Surg.* 2017;104:907-917.
9. Beuers U, Hohenester S, de Buy Wenniger LJ, Kremer AE, Jansen PL, Elferink RP. The biliary HCO(3)(-) umbrella: a unifying hypothesis on pathogenetic and therapeutic aspects of fibrosing cholangiopathies. *Hepatology.* 2010;52:1489-1496.
10. Villalobos-Molina R, Saavedra-Molina A, Devlin TM. Effect of hypoxia and reoxygenation on metabolic pathways in rat hepatocytes. *Arch Med Res.* 1998;29:219-223.
11. Nasralla D, Coussios CC, Mergental H, et al. A randomized trial of normothermic preservation in liver transplantation. *Nature.* 2018;557:50-56.
12. Op den Dries, Sanna Westerkamp, Andrie Karimian, Negin Gouw, Annette S H Bruinsma, Bote Markmann, James Lisman, Ton Yeh, Heidi Uygun, Korkut Martins,Paulo Porte, Robert. Injury to peribiliary glands and vascular plexus before liver transplantation predicts formation of non-anastomotic biliary strictures. *J Hepatol.* 2014;60:1172-1179.
13. Cui DX, Yin JQ, Xu WX, Chai F, Liu BL, Zhang XB. Effect of different bile duct flush solutions on biliary tract preservation injury of donated livers for transplantation. *Transplant Proc.* 2010;42:1576-1581.

14. Pasut G, Panisello A, Folch-Puy E, et al. Polyethylene glycols: An effective strategy for limiting liver ischemia reperfusion injury. *World J Gastroenterol.* 2016;22:6501-6508.

15. Bessems M, Doorschodt BM, Hooijschuur O, van Vliet AK, van Gulik TM. Optimization of a new preservation solution for machine perfusion of the liver: which is the preferred colloid? *Transplant Proc.* 2005;37:329-331.

16. Hata K, Tolba RH, Wei L, et al. Impact of polysol, a newly developed preservation solution, on cold storage of steatotic rat livers. *Liver Transpl.* 2007;13:114-121.

17. Yagi S, Doorschodt BM, Afify M, et al. Improved preservation and microcirculation with POLYSOL after partial liver transplantation in rats. *J Surg Res.* 2011;167:e375-83.

18. Mosbah IB, Zaouali MA, Martel C, et al. IGL-1 solution reduces endoplasmic reticulum stress and apoptosis in rat liver transplantation. *Cell Death Dis.* 2012;3:e279.

19. Edelstein A, Fink D, Musch M, et al. Protective effects of nonionic triblock copolymers on bile acid-mediated epithelial barrier disruption. *Shock.* 2011;36:451-457.

20. Wu L, Zaborina O, Zaborin A, et al. High-molecular-weight polyethylene glycol prevents lethal sepsis due to intestinal Pseudomonas aeruginosa. *Gastroenterology.* 2004;126:488-498.

21. Karimian, Negin Westerkamp,Andrie Porte, Robert. Biliary complications after orthotopic liver transplantation. *Current opinion in organ transplantation.* 2014;19:209-216.

22. op den Dries S, Westerkamp AC, Karimian N, et al. Injury to peribiliary glands and vascular plexus before liver transplantation predicts formation of non-anastomotic biliary strictures. *J Hepatol.* 2014;60:1172-1179.

23. Loinaz C, Gonzalez EM. Marginal donors in liver transplantation. *Hepatogastroenterology.* 2000;47:256-263.

24. NHS Blood and Transplant. INTERIM REPORT ON LIVER TRANSPLANTATION. 2017.

25. Alpini G, Ueno Y, Glaser SS, et al. Bile acid feeding increased proliferative activity and apical bile acid transporter expression in both small and large rat cholangiocytes. *Hepatology.* 2001;34:868-876.

26. Seishima R, Barker N. A contemporary snapshot of intestinal stem cells and their regulation. *Differentiation.* 2019.

27. Uygun BE, Izamis ML, Jaramillo M, et al. Discarded Livers Find a New Life: Engineered Liver Grafts Using Hepatocytes Recovered From Marginal Livers. *Artif Organs.* 2017;41:579-585.

28. Berendsen TA, Bruinsma BG, Puts CF, et al. Supercooling enables long-term transplantation survival following 4 days of liver preservation. *Nat Med.* 2014;20:790-793.

CHAPTER 11

Dutch Summary, List of Publications, List of Contributing Authors, Acknowledgements, Biography

DUTCH SUMMARY / NEDERLANDSE SAMENVATTING

De studies in dit proefschrift zijn gericht op het uitbreiden van het aantal bruikbare donorlevers door middel van machinale perfusie van zogenaamde “extended criteria donor” (ECD) levers, met een bijzondere focus op de galwegen.

In **hoofdstuk 1** werden het proefschrift en de verschillende hoofdstukken geïntroduceerd. In **hoofdstuk 2** werden verschillende manieren besproken om de donorleverpool uit te breiden.[1] Één van de meest effectieve manieren om het aantal beschikbare donorlevers voor transplantatie te vergroten, is door ECD levers te transplanteren. In vergelijking met donorlevers die aan de optimale standaardcriteria voldoen zijn ECD levers inherent van mindere kwaliteit en hebben ze meer schade opgelopen, waardoor transplantatie van deze organen een hoger risico op complicaties met zich meebrengt. De gouden standaard voor het preserveren van donorlevers, waarbij de organen worden gekoeld en op ijs worden getransporteerd (“static cold storage”, SCS), veroorzaakt teveel extra schade aan deze reeds risicovolle ECD organen. Daarom vereist de omgang met deze transplantaten een andere aanpak. Machineperfusie is een nieuwe techniek die zowel bescherming biedt tegen ischemische schade, alsook de mogelijkheid geeft om organen te verbeteren en zorgvuldig te selecteren. Afhankelijk van de indicatie voor machineperfusie kan het worden geïmplementeerd tijdens verschillende fasen van transplantatie (tijdens uitname, transport en bij aankomst in het transplantatiecentrum), bij verschillende temperaturen en met behulp van verschillende preservatievloeistoffen. Machineperfusie zal de komende jaren blijven groeien, waardoor het waarschijnlijk is dat een op maat gemaakte aanpak zal worden geïmplementeerd, afhankelijk van verscheidene indicaties.

Hoofdstuk 3 beschrijft de techniek van normotherme machineperfusie (NMP).[2] Op 37 °C zorgt geoxygeneerde NMP ervoor dat het orgaan metabolisch actief wordt, hetgeen de mogelijkheid biedt om de kwaliteit van het orgaan te beoordelen. Om een fysiologische omgeving te creëren werd NMP toegepast met behulp van een perfusievloeistof op basis van rode bloedcellen (RBC), fresh frozen plasma (FFP) en een mix van voedingsstoffen, spoorelementen en antibiotica. Een online video legt uit hoe NMP wordt uitgevoerd met behulp van het Liver Assist (Organ Assist)-perfusieapparaat, hetgeen een druk-gestuurde continue stroom door de vena porta en een pulserende stroom door de arteria hepatica geeft. In tegenstelling tot andere perfusieapparaten, zoals Metra (OrganOx), is dit apparaat niet ontworpen om te worden gebruikt tijdens transport en biedt het geen geautomatiseerde monitoring en aanpassing van biochemie in perfusaat. De Liver Assist kan daarom alleen worden gebruikt in het uitname- of implantatiecentrum. Het is echter veel goedkoper, biedt dezelfde perfusiekwaliteit en, belangrijker nog, biedt machinale perfusie bij verschillende temperaturen.

Omdat menselijke bloedproducten schaars zijn en het gebruik ervan logistiek complex is, wilden we een NMP-perfusievloeistof ontwikkelen die het gebruik

van menselijke bloedproducten omzeilt. In **hoofdstuk 4** werden RBC's eerst vervangen door een acellulair bovine hemoglobine (HBOC-201) en deze gemodificeerde perfusievloeistof werd getest in zes voor transplantatie afgekeurde donorlevers. Vervolgens werden FFP's vervangen door gelofusine en een mix van andere additieven, en de resulterende vloeistof werd getest in nog eens zes donorlevers.[3] Beide groepen werden vergeleken met een historische cohort van twaalf donorlevers waarin NMP werd uitgevoerd met een perfusievloeistof op basis van RBC's en FFP's. De levers in de HBOC-201 geperfundeerde groepen vertoonden een significant betere functie, waaronder een hogere cumulatieve galproductie, portale en arteriële flow en hepatisch adenosinetrifosfaat (ATP)-gehalte. Dit was waarschijnlijk te wijten aan de lagere affiniteit en kleinere afmeting van het HBOC-201-molecuul voor zuurstof in vergelijking met humaan hemoglobine, waardoor het gemakkelijker zuurstof loslaat en waardoor het dieper in het weefsel kan doordringen. Nadelen van het gebruik van HBOC-201 zijn echter de mogelijke vorming van methemoglobine en het onvermogen om HBOC-201 uit monsters te verwijderen, wat mogelijk kan leiden tot interferentie van spectrofotometrische analyses van de perfusievloeistof. Ten slotte zijn HBOC-201 en gelofusine producten die uit runderen worden afgeleid. Toekomstig onderzoek moet gericht zijn op het vinden van diervrije alternatieven.

Een andere belangrijke eigenschap van HBOC-201 is dat het, in tegenstelling tot RBC's, bij lagere temperaturen als zuurstofdrager kan worden gebruikt. Dit maakt een perfusieprotocol mogelijk met een periode van duale hypotherme geoxygeneerde machineperfusie (dual hypothemic machine perfusion, DHOPE), gevolgd door gecontroleerde geoxygeneerde heropwarming (controlled oxygenated rewarming, COR) en uiteindelijk NMP. **Hoofdstuk 5** toonde aan dat sequentiële DHOPE-COR-NMP met behulp van een HBOC-gebaseerde perfusievloeistof een nieuwe methode biedt voor gecombineerde reanimatie en kwaliteitsbeoordeling van suboptimale levers voorafgaand aan transplantatie. In deze casusreeks werden zeven levers die aanvankelijk werden geweigerd voor transplantatie geperfundeerd met behulp van DHOPE-COR-NMP, waarvan er vijf met succes werden getransplanteerd nadat ze tijdens NMP aan alle hepato-cholangiocellulaire kwaliteitscriteria voldeden. Het primaire eindpunt, transplantaatoverleving na 3 maanden, was 100%. Ook was de post-operatieve piek-ALT lager in onze ontvangers dan in studies waarin SCS alleen werd vergeleken met NMP[4,5], hetgeen mogelijk te wijten is aan de door DHOPE geïnduceerde vermindering van ischemie-reperfusieschade.[6-8] Bovendien vertoonden geen van de ontvangers klinische tekenen van niet-anastomotische stricturen (NAS), hoewel dit niet de primaire uitkomst was en we geen routinematige beeldvorming uitgevoerd hebben waardoor subklinische gevallen mogelijk zijn gemist. Concluderend heeft dit onderzoek aangetoond dat sequentiële perfusie met DHOPE-COR-NMP met behulp van HBOC-201 haalbaar en veilig is. Ondanks de veelbelovende resultaten zijn grotere cohorten met een langere follow-up nodig om conclusies te kunnen trekken omtrent de effectiviteit van het protocol bij het veilig verhogen van het aantal donorlevers voor transplantatie en het verminderen van complicaties.

In **hoofdstuk 6** hebben we aangetoond dat biliaire bicarbonaat, pH, LDH, glucose en de gal/perfusaat glucoseratio nauwkeurige voorspellers zijn van histologische galwegschade tijdens NMP. Histologische galwegschade werd beoordeeld met behulp van een klinisch relevant scoresysteem en levers werden verdeeld in een groep met hoge en lage schade. Biliaire biochemiemarkers werden vervolgens geanalyseerd op hun voorspellende waarde met behulp van AUC-ROC-curves. Het vaststellen van biomarkers voor het beoordelen van galwegschade is belangrijk, omdat NMP-beoordeling van levers tot dan toe voornamelijk was gebaseerd op hepatocellulaire criteria ondanks het feit dat galwegschade voorafgaand aan transplantatie rechtstreeks is gecorreleerd aan de ontwikkeling van NAS. Vooral biliaire bicarbonaat, in plaats van biliaire pH zoals door andere groepen werd gesuggereerd[5], was geschikt om galwegschade te voorspellen tijdens NMP. Lage biliaire pH en bicarbonaat weerspiegelen niet alleen galwegschade/disfunctie, maar kunnen ook bijdragen aan extra galwegschade door een afwezige bicarbonaatparaplu, die helpt beschermen tegen cytotoxische galzouten.[9] Sinds de publicatie van dit artikel hebben we ook gevonden dat de delta tussen gal en perfusaat pH en bicarbonaat zelfs nog meer discriminerend is dan hun absolute waarden in gal. Biliaire glucose is afhankelijk van perfusaat of bloedglucose en de meeste post-ischemische levers hebben verhoogde glucosewaarden als gevolg van glycogenolyse.[10] Daarom is het belangrijk om de ratio tussen gal en perfusaatglucose te bepalen. Een probleem met het gebruik van gal-biochemie bij het beoordelen van de kwaliteit van de galwegen is dat sommige levers tijdens NMP zeer weinig of geen gemeten gal produceren. Donorlevers die tijdens NMP geen gal produceerden, zijn echter ook met succes getransplanteerd en daarom adviseren wij dat met name levers met een verhoogd risico op post-transplantatie cholangiopathie worden beoordeeld op galwegschade.[5,11]

In **hoofdstuk 7** hebben we aangetoond dat vroege afgifte van hepatocyten en cholangiocyten afgeleide micro-RNA's (respectievelijk HDmiR-122 en CDmiR-222) in perfusaat en gal late hepato-cholangiocellulaire schade en functie van donorlevers tijdens NMP kan voorspellen. Deze analyses werden uitgevoerd in een groep van twaalf donorlevers die werden afgekeurd voor transplantatie. Over het algemeen waren de niveaus van beide miR's in perfusaat en gal lager in levers met lage hepato-cholangiocellulaire schade en goede hepato-cholangiocellulaire functie. Niveaus van HDmiR-122 in perfusáat correleerden sterker met hepato-cholangiocellulaire parameters vergeleken met niveaus in gal, terwijl niveaus van CDmiR-222 in gál sterker correleerden met parameters vergeleken met niveaus in perfusaat, wat hun celspecifieke oorsprong bevestigde. De HDmiR-122 / CDmiR-222 ratio was aanzienlijk hoger in perfusaat van levers van lage kwaliteit in vergelijking met de verhouding in levers van hoge kwaliteit. Deze miR's worden differentieel vrijgegeven in perfusaat en gal en kunnen in de toekomst mogelijk worden gebruikt als biomarkers voor de beoordeling van de kwaliteit van het transplantaat tijdens machineperfusie.

Hoofdstuk 8 vergeleek het vermogen van verschillende preservatievloeistoffen om te beschermen tegen histologische galwegbeschadiging, waarvan is aangetoond dat het voorspellend is voor de ontwikkeling van NAS na transplantatie.[12] In deze studie werden extrahepatische galwegsegmenten van afgekeurde donorlevers koud bewaard in vijf verschillende preservatievloeistoffen na een periode van SCS in University of Wisconsin oplossing (UW), en vervolgens beoordeeld op histologische galwegschade op HE coupes. Histidine-tryptofaan-ketoglutaraat (HTK) oplossing leidde tot hogere galwegschade in vergelijking met UW oplossing. Dit heeft belangrijke klinische implicaties, aangezien de HTK oplossing op grote schaal wordt gebruikt en donorlevers met een hoog risico op post-transplantatie cholangiopathie in toenemende mate worden getransplanteerd. De voordelen van HTK-oplossingen die in de literatuur zijn beschreven, inclusief de lage viscositeit en snelle temperatuurreductie[13], kunnen echter in het huidige onderzoeksontwerp zijn gemaskeerd doordat de galwegsegmenten klein waren en een oplossingswisseling van UW naar HTK oplossing ook extra schade kan hebben veroorzaakt. Bovendien resulteerde de toevoeging van polyethyleenglycolen (PEG's) aan HTK en UW oplossing in een lichte maar niet-significante vermindering van galwegschade. PEG's zijn door de FDA goedgekeurde niet-toxische wateroplosbare verbindingen die op grote schaal worden gebruikt, inclusief klinisch, met hoge flexibiliteit, hydrofiliciteit en eiwitafstotende eigenschappen.[14] Uit eerder onderzoek is gebleken dat PEG's een belangrijke rol kunnen spelen bij het behoud van lever en darm, die sterk lijkt op galwegen.[15-20] De toevoeging van PEG's aan preservatievloeistoffen zou een gemakkelijk uitvoerbare en betaalbare methode kunnen zijn om de galwegen te beschermen, wat verder onderzoek rechtvaardigt.

In de laatste studie, gerapporteerd in **hoofdstuk 9**, hebben we een nieuwe methode ontwikkeld met de naam precision-cut bile duct slices (PCBDS) om humane galwegen te bestuderen, waarbij het gebruik van proefdieren werd omzeild. Met behulp van dit *ex vivo* model werden humane extrahepatische galwegen van weggegooide donorlevers in kleine plakjes gesneden en gedurende maximaal zes dagen geïncubeerd. Deze techniek handhaafde de intacte anatomische organisatie van celstructuren en maakte de studie van spatiotemporale differentiatie en migratie van peribiliary gland (PBG) cellen mogelijk. PBG zijn niches met progenitor en stamcellen ingebed in de galwegwand, die een cruciale rol spelen in de ontwikkeling van NAS na transplantatie.[21,22] We toonden aan dat na ernstige galwegschade, PBG cellen reageerden met proliferatie, migratie en differentiering om galwegepitheel te herstellen. Dit was de eerste studie die bewijs leverde dat PBG reageren op galwegschade door herstel van galwegepitheel en bevestigt de beschermende rol van PBG bij de ontwikkeling van grote galweg cholangiopathieën. Een beperking van deze studie was dat intra- en extrahepatische galwegen niet afzonderlijk konden worden bestudeerd, omdat er een limiet is aan het aantal PCBDS dat per lever kan worden verkregen.

REFERENCES

1. Matton AP, Porte RJ. Opportunities for scientific expansion of the deceased donor pool. Liver Transpl. 2014;20 Suppl 2:S5.
2. Karimian N, Matton AP, Westerkamp AC, et al. Ex Situ Normothermic Machine Perfusion of Donor Livers. J Vis Exp. 2015;(99):e52688. doi:e52688.
3. Matton APM, Burlage LC, van Rijn R, et al. Normothermic machine perfusion of donor livers without the need for human blood products. Liver Transpl. 2018;24:528-538.
4. Mergental H, Perera MT, Laing RW, et al. Transplantation of Declined Liver Allografts Following Normothermic Ex-Situ Evaluation. Am J Transplant. 2016;16:3235-3245.
5. Watson CJE, Kosmoliaptsis V, Pley C, et al. Observations on the ex situ perfusion of livers for transplantation. Am J Transplant. 2018.
6. Schlegel A, de Rougemont O, Graf R, Clavien PA, Dutkowski P. Protective mechanisms of end-ischemic cold machine perfusion in DCD liver grafts. J Hepatol. 2013;58:278-286.
7. Dutkowski P, Polak WG, Muiesan P, et al. First Comparison of Hypothermic Oxygenated PErfusion Versus Static Cold Storage of Human Donation After Cardiac Death Liver Transplants: An International-matched Case Analysis. Ann Surg. 2015;262:764-70; discussion 770-1.
8. van Rijn R, Karimian N, Matton APM, et al. Dual hypothermic oxygenated machine perfusion in liver transplants donated after circulatory death. Br J Surg. 2017;104:907-917.
9. Beuers U, Hohenester S, de Buy Wenniger LJ, Kremer AE, Jansen PL, Elferink RP. The biliary HCO(3)(-) umbrella: a unifying hypothesis on pathogenetic and therapeutic aspects of fibrosing cholangiopathies. Hepatology. 2010;52:1489-1496.
10. Villalobos-Molina R, Saavedra-Molina A, Devlin TM. Effect of hypoxia and reoxygenation on metabolic pathways in rat hepatocytes. Arch Med Res. 1998;29:219-223.
11. Nasralla D, Coussios CC, Mergental H, et al. A randomized trial of normothermic preservation in liver transplantation. Nature. 2018;557:50-56.
12. Op den Dries, Sanna Westerkamp, Andrie Karimian, Negin Gouw, Annette S H Bruinsma, Bote Markmann, James Lisman, Ton Yeh, Heidi Uygun, Korkut Martins,Paulo Porte, Robert. Injury to peribiliary glands and vascular plexus before liver transplantation predicts formation of non-anastomotic biliary strictures. J Hepatol. 2014;60:1172-1179.
13. Cui DX, Yin JQ, Xu WX, Chai F, Liu BL, Zhang XB. Effect of different bile duct flush solutions on biliary tract preservation injury of donated livers for transplantation. Transplant Proc. 2010;42:1576-1581.

14. Pasut G, Panisello A, Folch-Puy E, et al. Polyethylene glycols: An effective strategy for limiting liver ischemia reperfusion injury. World J Gastroenterol. 2016;22:6501-6508.

15. Bessems M, Doorschodt BM, Hooijschuur O, van Vliet AK, van Gulik TM. Optimization of a new preservation solution for machine perfusion of the liver: which is the preferred colloid? Transplant Proc. 2005;37:329-331.

16. Hata K, Tolba RH, Wei L, et al. Impact of polysol, a newly developed preservation solution, on cold storage of steatotic rat livers. Liver Transpl. 2007;13:114-121.

17. Yagi S, Doorschodt BM, Afify M, et al. Improved preservation and microcirculation with POLYSOL after partial liver transplantation in rats. J Surg Res. 2011;167:e375-83.

18. Mosbah IB, Zaouali MA, Martel C, et al. IGL-1 solution reduces endoplasmic reticulum stress and apoptosis in rat liver transplantation. Cell Death Dis. 2012;3:e279.

19. Edelstein A, Fink D, Musch M, et al. Protective effects of nonionic triblock copolymers on bile acid-mediated epithelial barrier disruption. Shock. 2011;36:451-457.

20. Wu L, Zaborina O, Zaborin A, et al. High-molecular-weight polyethylene glycol prevents lethal sepsis due to intestinal Pseudomonas aeruginosa. Gastroenterology. 2004;126:488-498.

21. Karimian, Negin Westerkamp,Andrie Porte, Robert. Biliary complications after orthotopic liver transplantation. Current opinion in organ transplantation. 2014;19:209-216.

22. op den Dries S, Westerkamp AC, Karimian N, et al. Injury to peribiliary glands and vascular plexus before liver transplantation predicts formation of non-anastomotic biliary strictures. J Hepatol. 2014;60:1172-1179.

23. Loinaz C, Gonzalez EM. Marginal donors in liver transplantation. Hepatogastroenterology. 2000;47:256-263.

24. NHS Blood and Transplant. INTERIM REPORT ON LIVER TRANSPLANTATION. 2017.

25. Alpini G, Ueno Y, Glaser SS, et al. Bile acid feeding increased proliferative activity and apical bile acid transporter expression in both small and large rat cholangiocytes. Hepatology. 2001;34:868-876.

26. Seishima R, Barker N. A contemporary snapshot of intestinal stem cells and their regulation. Differentiation. 2019.

27. Uygun BE, Izamis ML, Jaramillo M, et al. Discarded Livers Find a New Life: Engineered Liver Grafts Using Hepatocytes Recovered From Marginal Livers. Artif Organs. 2017;41:579-585.

28. Berendsen TA, Bruinsma BG, Puts CF, et al. Supercooling enables long-term transplantation survival following 4 days of liver preservation. Nat Med. 2014;20:790-793.

LIST OF PUBLICATIONS

Matton APM, Porte RJ. Opportunities for scientific expansion of the deceased donor pool. *Liver Transpl. 2014;20 Suppl 2:S5.*

Matton APM, Burlage LC, van Rijn R, de Vries Y, Karangwa SA, Nijsten MW, Gouw ASH, Wiersema-Buist J, Adelmeijer J, Westerkamp AC, Lisman T, Porte RJ. Normothermic machine perfusion of donor livers without the need for human blood products. *Liver Transpl 2018;24:528-538.*

Matton APM, de Vries Y, Porte RJ. Reply. *Liver Transpl 2018;24:1149-1150.*

Matton APM, de Vries Y, Burlage LC, van Rijn R, Fujiyoshi M, de Meijer VE, de Boer MT, de Kleine RHJ, Verkade HJ, Gouw ASH, Lisman T, Porte RJ. Biliary Bicarbonate, pH, and Glucose Are Suitable Biomarkers of Biliary Viability During Ex Situ Normothermic Machine Perfusion of Human Donor Livers. *Transplantation 2019;103:1405-1413.*

Matton APM, de Meijer VE, Lisman T, Porte RJ. The Authors' Reply to Letter to the Editor, Re: Biliary Bicarbonate, pH and Glucose Are Suitable Biomarkers of Biliary Viability During Ex Situ Normothermic Machine Perfusion of Human Donor Livers. *Transplantation 2019 Jul 15.* (Epub ahead of print)

Matton APM*, de Jong IEM*, van Praagh JB, van Haaften WT, Wiersema-Buist J, van Wijk LA, Oosterhuis D, Iswandana R, Suriguga S, Overi D, Lisman T, Carpino G, Gouw ASH, Olinga P, Gaudio E, Porte RJ. Peribiliary Glands Are Key in Regeneration of the Human Biliary Epithelium After Severe Bile Duct Injury. *Hepatology 2019;69:1719-1734.* *shared first authorship

Karimian N, **Matton APM**, Westerkamp AC, Burlage LC, Op den Dries S, Leuvenink HG, Lisman T, Uygun K, Markmann JF, Porte RJ. Ex Situ Normothermic Machine Perfusion of Donor Livers. *J Vis Exp 2015;(99):e52688.*

de Vries Y, **Matton APM**, Nijsten MWN, Werner MJM, van den Berg AP, de Boer MT, Buis CI, Fujiyoshi M, de Kleine RHJ, van Leeuwen OB, Meyer P, van den Heuvel MC, de Meijer VE, Porte RJ. Pretransplant sequential hypo- and normothermic machine perfusion of suboptimal livers donated after circulatory death using a hemoglobin-based oxygen carrier perfusion solution. *Am J Transplant 2019;19:1202-1211.*

Westerkamp AC, Karimian N, **Matton APM**, Mahboub P, van Rijn R, Wiersema-Buist J, de Boer MT, Leuvenink HG, Gouw AS, Lisman T, Porte RJ. Oxygenated Hypothermic Machine Perfusion After Static Cold Storage Improves Hepatobiliary Function of Extended Criteria Donor Livers. *Transplantation 2016;100:825-35.*

van Rijn R, Karimian N, **Matton APM**, Burlage LC, Westerkamp AC, van den Berg AP, de Kleine RHJ, de Boer MT, Lisman T, Porte RJ. Dual hypothermic oxygenated machine perfusion in liver transplants donated after circulatory death. *Br J Surg 2017;104:907-917.*

van Rijn R, van Leeuwen OB, **Matton APM**, Burlage LC, Wiersema-Buist J, van den Heuvel MC, de Kleine RHJ, de Boer MT, Gouw ASH, Porte RJ. Hypothermic oxygenated machine perfusion reduces bile duct reperfusion injury after transplantation of donation after circulatory death livers. *Liver Transpl 2018;24:655-664.*

de Vries Y, van Leeuwen OB, **Matton APM**, Fujiyoshi M, de Meijer VE, Porte RJ. Ex situ normothermic machine perfusion of donor livers using a haemoglobin-based oxygen carrier: a viable alternative to red blood cells. *Transpl Int 2018;31:1281-1282.*

Karangwa SA, Adelmeijer J, **Matton APM**, de Meijer VE, Lisman T, Porte RJ. Production of Physiologically Relevant Quantities of Hemostatic Proteins During Ex Situ Normothermic Machine Perfusion of Human Livers. *Liver Transpl 2018;24:1298-1302.*

Burlage LC, Hessels L, van Rijn R, **Matton APM**, Fujiyoshi M, van den Berg AP, Reyntjens KMEM, Meyer P, de Boer MT, de Kleine RHJ, Nijsten MW, Porte RJ. Opposite acute potassium and sodium shifts during transplantation of hypothermic machine perfused donor livers. *Am J Transplant 2019;19:1061-1071.*

Wolters JC, Ciapaite J, van Eunen K, Niezen-Koning KE, **Matton APM**, Porte RJ, Horvatovich P, Bakker BM, Bischoff R, Permentier HP. Translational Targeted Proteomics Profiling of Mitochondrial Energy Metabolic Pathways in Mouse and Human Samples. *J Proteome Res 2016;15:3204-13.*

Burlage LC, Karimian N, Westerkamp AC, Visser N, **Matton APM**, van Rijn R, Adelmeijer J, Wiersema-Buist J, Gouw ASH, Lisman T, Porte RJ. Oxygenated hypothermic machine perfusion after static cold storage improves endothelial function of extended criteria donor livers. *HPB (Oxford) 2017;19:538-546.*

Karangwa SA, Burlage LC, Adelmeijer J, Karimian N, Westerkamp AC, **Matton APM**, van Rijn R, Wiersema-Buist J, Sutton ME, Op den Dries S, Lisman T, Porte RJ. Activation of Fibrinolysis, But Not Coagulation, During End-Ischemic Ex Situ Normothermic Machine Perfusion of Human Donor Livers. *Transplantation 2017;101:e42-e48.*

de Vries Y, Berendsen TA, Fujiyoshi M, van den Berg AP, Blokzijl H, de Boer MT, van der Heide F, de Kleine RHJ, van Leeuwen OB, **Matton APM**, Werner MJM, Lisman T, de Meijer VE, Porte R. Transplantation of high-risk donor livers after resuscitation and viability assessment using a combined protocol of oxygenated hypothermic, rewarming and normothermic machine perfusion: study protocol for a prospective, single-arm study (DHOPE-COR-NMP trial). *BMJ Open 2019;9:e028596.*

LIST OF CONTRIBUTING AUTHORS

Aad P. van den Berg, MD, PhD
Department of Gastroenterology and Hepatology
University Medical Center Groningen
The Netherlands

Andrie C. Westerkamp, MD, PhD
Surgical Research Laboratory
Department of Surgery
University Medical Center Groningen
University of Groningen
The Netherlands

Annette S.H. Gouw, MD, PhD, Professor of Pathology
Department of Pathology and Medical Biology
University Medical Center Groningen
University of Groningen
The Netherlands

Carlijn I. Buis, MD, PhD
Department of Surgery
Section of Hepatobiliary Surgery and Liver Transplantation
University Medical Center Groningen
University of Groningen
The Netherlands

Corey M. Eymard, MD
Department of Surgery
Massachusetts General Hospital
Boston, Massachusetts, USA

Danielle Detelich, MD
Department of Surgery
Center for Engineering in Medicine
Harvard Medical School
Massachusetts General Hospital
Boston, Massachusetts, USA

Diletta Overi
Department of Anatomical, Histological, Forensic Medicine and Orthopedic Sciences
Sapienza University of Rome
Rome, Italy

Dorenda Oosterhuis
Department of Pharmaceutical Technology and Biopharmacy
University of Groningen
The Netherlands

Eugenio Gaudio, MD, PhD, Professor of Human Anatomy
Department of Anatomical, Histological, Forensic Medicine and Orthopedic Sciences
Sapienza University of Rome
Rome, Italy

Guido Carpino, MD, PhD, Associate Professor
Division of Health Sciences
Department of Movement, Human and Health Sciences
University of Rome “Foro Italico”
Rome, Italy

Heidi Yeh, MD
Department of Surgery
Massachusetts General Hospital
Harvard Medical School
Boston, MA, United States of America

Henk P. Roest, PhD
Department of Surgery
Erasmus MC – University Medical Center Rotterdam
The Netherlands

Henkjan J. Verkade, MD, PhD, Professor of Pediatrics
Section of Pediatric Gastroenterology and Hepatology
Department of Pediatrics
University Medical Center Groningen
University of Groningen
The Netherlands

Henri G.D. Leuvenink, PhD, Professor of Experimental Surgery
Surgical Research Laboratory
Department of Surgery
University Medical Center Groningen
University of Groningen
The Netherlands

Iris E.M. de Jong, BSc
Surgical Research Laboratory
Department of Surgery
University Medical Center Groningen
University of Groningen
The Netherlands
James F. Markmann, MD, PhD, Claude E Welch Professor of Surgery
Chief of the Division of Transplant Surgery
Department of Surgery
Massachusetts General Hospital
Harvard Medical School
Boston, MA, United States of America

Jan N.M. IJzermans, MD, PhD, Professor of HPB and Transplant Surgery
Department of Surgery
Erasmus MC – University Medical Center Rotterdam
The Netherlands

Janneke Wiersema-Buist
Surgical Research Laboratory
Department of Surgery
University Medical Center Groningen
University of Groningen
The Netherlands

Jasmijn W. Selten, MD
Department of Surgery
Erasmus MC – University Medical Center Rotterdam
The Netherlands

Jasper B. van Praagh, BSc
Department of Pharmaceutical Technology and Biopharmacy
University of Groningen
The Netherlands

Jelle Adelmeijer
Surgical Research Laboratory
Department of Surgery
University Medical Center Groningen
University of Groningen
The Netherlands

Jeroen de Jonge, MD, PhD
Department of Surgery
Erasmus MC – University Medical Center Rotterdam
The Netherlands

Korkut Uygun, PhD, Associate Professor of Surgery
Center of Engineering in Medicine
Organ Reengineering Laboratory
Massachusetts General Hospital
Harvard Medical School
Boston, MA, United States of America

Laura C. Burlage, MD, PhD
Surgical Research Laboratory
Department of Surgery
University Medical Center Groningen
University of Groningen
The Netherlands

Louise A. van Wijk
Department of Pharmaceutical Technology and Biopharmacy
University of Groningen
The Netherlands

Luc J.W. van der Laan, PhD, Professor Liver Regenerative Medicine
Department of Surgery
Erasmus MC – University Medical Center Rotterdam
The Netherlands

Maarten W. Nijsten, MD, PhD
Department of Critical Care
University Medical Center Groningen
University of Groningen
The Netherlands

Marieke T. de Boer, MD, PhD
Department of Surgery
Section of Hepatobiliary Surgery and Liver Transplantation
University Medical Center Groningen
University of Groningen
The Netherlands

Marius C. van den Heuvel, MD, PhD
Department of Pathology and Medical Biology
University Medical Center Groningen
University of Groningen The Netherlands

Masato Fujiyoshi, MD
Surgical Research Laboratory
Department of Surgery
University Medical Center Groningen
University of Groningen
The Netherlands

Maureen J.M. Werner, MD
Surgical Research Laboratory
Department of Surgery
University Medical Center Groningen
University of Groningen
The Netherlands

Negin Karimian, MD, PhD
Surgical Research Laboratory
Department of Surgery
University Medical Center Groningen
University of Groningen
The Netherlands

Peter Meyer, MD, PhD
Department of Anesthesiology
University Medical Center Groningen
University of Groningen
The Netherlands

Peter Olinga, PhD, Associate Professor
Department of Pharmaceutical Technology and Biopharmacy
University of Groningen
The Netherlands

Raditya Iswandana, PhD
Department of Pharmaceutical Technology and Biopharmacy
University of Groningen
The Netherlands
Faculty of Pharmacy
Universitas Indonesia
Indonesia

Reinier de Vries, MD
Department of Surgery
Center for Engineering in Medicine
Harvard Medical School
Massachusetts General Hospital
Boston, Massachusetts, USA

Rianne van Rijn, MD, PhD
Surgical Research Laboratory
Department of Surgery
University Medical Center Groningen
University of Groningen
The Netherlands

Robert J. Porte, MD, PhD, Professor of Surgery
Department of Surgery
Section of Hepatobiliary Surgery and Liver Transplantation
University Medical Center Groningen
University of Groningen
The Netherlands

Ruben H.J. de Kleine, MD
Department of Surgery
Section of Hepatobiliary Surgery and Liver Transplantation
University Medical Center Groningen
University of Groningen
The Netherlands

Sanna op den Dries, MD, PhD
Surgical Research Laboratory
Department of Surgery
University Medical Center Groningen
University of Groningen
The Netherlands

Shanice A. Karangwa, BSc
Surgical Research Laboratory
Department of Surgery
University Medical Center Groningen
University of Groningen
The Netherlands

Su Suriguga
Department of Pharmaceutical Technology and Biopharmacy
University of Groningen
The Netherlands

Ton Lisman, PhD, Professor of Experimental Surgery
Surgical Research Laboratory
Department of Surgery
University Medical Center Groningen
University of Groningen
The Netherlands

Otto B. van Leeuwen, BSc
Surgical Research Laboratory
Department of Surgery
University Medical Center Groningen
University of Groningen
The Netherlands

Vincent E. de Meijer, MD, PhD
Department of Surgery
Section of Hepatobiliary Surgery and Liver Transplantation
University Medical Center Groningen
University of Groningen
The Netherlands

Wouter T. van Haaften, MD, PhD
Department of Pharmaceutical Technology and Biopharmacy
University of Groningen
The Netherlands

Yvonne de Vries, MD
Surgical Research Laboratory
Department of Surgery
University Medical Center Groningen
University of Groningen
The Netherlands

ACKNOWLEDGEMENTS

Een proefschrift schrijven doe je natuurlijk niet alleen, hetgeen het ook zeker een stuk minder leuk had gemaakt. Over de jaren heen heb ik het voorrecht gehad om met een heleboel talentvolle, gedreven en leuke mensen samen te werken, te leren, te lachen, te dansen en te leven. Ik wil jullie allen van harte bedanken voor jullie hulp bij het tot stand komen van dit proefschrift.

Prof. dr. R.J. Porte, beste Robert, dank je wel voor alle steun en kansen die je me de afgelopen jaren hebt gegeven. Zonder jouw gedrevenheid en passie voor het vak was het machineperfusie onderzoek nooit zo ver gekomen als dat het nu is. Je holt van een (ellelange) operatie naar de poli en van een leverperfusie naar een congres aan de andere kant van de wereld. Je moet het maar kunnen! Ik heb ontzettend veel van je geleerd en voel me vereerd dat ik onder jouw toezicht en kunde mag promoveren.

Prof. dr. J.A. Lisman, beste Ton, het was ontzettend fijn om jou als tweede promotor te hebben. Een back-up waar ik altijd op terug kon vallen. Als Robert het met 24 uur per dag toch niet allemaal tijdig bijhield, reageerde jij ogenblikkelijk met zeer waardevolle feedback. Een overleg met jou was steeds inspirerend en gezellig, waarbij je je promovendi altijd weer uitdaagde. Dank je wel voor je supervisie!

Geachte leden van de beoordelingscommissie, **Prof. dr. Henri Leuvenink, Prof. dr. U. Beuers**, en **Prof. dr. J.M. Klaase**, hartelijk dank voor het beoordelen van dit proefschrift.

During the last period of my PhD, I spent eight months working with the groundbreaking research group at the Center of Engineering in Medicine, Massachusetts General Hospital, Harvard Medical School in Boston, MA, USA, under the supervision of **Prof. dr. Korkut Uygun** and **dr. Basak Uygun**. Dear Korkut and Basak, thank you very much for your hospitality, generosity and making me feel part of the MGH family. Korkut, thank you for being so understanding, from stem cells being impossible little things to giving me the space to deal with issues outside of research. **Dr. Heidi Yeh**, dear Heidi, thank you for always being such a positive and motivating person and for your help

obtaining endless IRB approvals. **Dr. Martin Yarmush**, **dr. Mehmet Toner**, **dr. Shannon Tessier**, it was an honor and pleasure working in your research facility.

De Junior Scientific Masterclass wil ik graag bedanken voor de mogelijkheid om een MD-PhD traject te volgen. **Dr. Joke van der Mark-van der Wouden**, **Jans van Aalst-Ubels** en **Maria de Vries**, dank dat jullie er steeds op gezellige en laagdrempelige wijze waren voor advies en het beantwoorden van allerlei vragen.

Lieve lotgenoten van het triadegebouw, **Laura Burlage**, **Shanice Karangwa**, **Yvonne de Vries**, **Iris de Jong**, **Anne van Erp**, **Wilma Potze, Fien von Meijenfeld**, **Simone Kleiss**, mede dankzij jullie bracht ik met plezier mijn dagen door in de meest stoffige hoek van het UMCG. Wat was het een gezellige tijd om met jullie daar te werken! **Rianne van Rijn**, **Otto van Leeuwen, Maureen Werner**, **Masato Fujiyoshi, Isabel Bruggenwirth**, **Rinse Ubbink**, **Pepijn Weeder**, ook jullie wil ik hartelijk bedanken voor al jullie inzet en gezelligheid. Ik heb het voorrecht gehad om met velen van jullie congressen in o.a. Sao Paulo, Oslo, Chicago en Boston te bezoeken. Ik kan met volle overtuiging zeggen dat ze stuk voor stuk onvergetelijk waren en dat heb ik aan jullie te danken. **Sanna op den Dries**, **Michael Sutton**, **Negin Karimian** en **Andrie Westerkamp**, jullie hebben een onmisbare bijdrage aan dit prachtige onderzoek geleverd en ik heb veel van jullie geleerd.

Jacco Zwaagstra en **Janneke Wiersema-Buist**, de vaste drijfkrachten achter het COL, wil ik in het bijzonder bedanken voor al hun hulp. Zonder Jacco's toezichthoudend oog en zonder Janneke's flexibiliteit en jarenlange ervaring zouden alle PhD studenten aan hun lot over gelaten zijn. Ik wil jullie tevens bedanken voor het regelmatig naar huis sturen van het lab nadat we nachtwerk hadden verricht. **Petra Ottens** en **Jelle Adelmeijer**, ook jullie zijn niet weg te denken bij het COL en ik wil jullie hartelijk bedanken voor alle uitleg en hulp die jullie me hebben geboden.

Prof. dr. Peter Olinga, beste Peter, jij hebt mij de mogelijkheid geboden om een alternatief onderzoeksmodel op te zetten waarbij geen dierproeven nodig waren. Het is een prachtige studie geworden waardoor ik nog blijer ben met de keuze die ik destijds heb gemaakt. Je was altijd laagdrempelig te benaderen en een ochtend met je sparren was niet alleen erg motiverend en leerzaam, maar ook gewoon gezellig. Hartelijk dank hiervoor! Ook **Iris de Jong**, **Tobias van**

Haaften, **Raditya Iswandana**, **Dorenda Oosterhuis**, **Su Suriguga** en **Theerut Luangmonkong** wil ik bedanken voor hun hulp bij het opzetten en uitvoeren van dit onderzoek.

Beste **Prof. dr. Luc van der Laan**, **Jasmijn Selten**, **Dr. Henk Roest** en **Renée Verhoeven** van het Erasmus MC in Rotterdam – hartelijk dank voor de samenwerking. De afstand en het oerwoud aan data waren behoorlijke uitdagingen, maar het resultaat was het waard.

Dr. Vincent de Meijer, **Dr. Marieke de Boer**, **Ruben de Kleine** en de rest van het HPB chirurgie team in het UMCG, hartelijk dank voor jullie bijdrage aan het machine perfusie onderzoek. Vincent, jou in het bijzonder wil ik bedanken voor je kritische en waardevolle input die je op vele studies in dit proefschrift hebt geleverd. **Prof. dr. Maarten Nijsten**, bedankt voor het delen van je uitgebreide kennis om samen tot een bruikbare perfusievloeistof te komen. **Prof. dr. Annette Gouw**, hartelijk dank voor je onmisbare kennis over de wereld van de lever achter de microscoop en bedankt dat je zelfs op een zaterdag mij een keer hebt geholpen met het scoren van coupes.

Alle Nederlandse transplantatie coördinatoren wil ik hartelijk bedanken voor hun onmisbare bijdrage aan het ter beschikking stelling van donorlevers voor onderzoek.

Zaf Zafirelis and **Greg Dubé**, thank you for offering HBOC-201 to our research group free of charge and for the numerous emails and calls to perform pre-clinical and clinical perfusions successfully.

Lieve **bestuurs- en organisatieleden van het MD-PhD congres**, dank voor een geweldige tijd! Ik had met niemand anders zo'n mooi congres willen of kunnen neerzetten.

My dear colleagues and friends in Boston, **Reinier, Peony, Shuk, Camilo, Sonal, Stephanie, Casie, Maria, Tom, Yibin, Sinan, Danielle** – thank for you the warm welcome and helping me find my way around the lab and for assisting me with experiments. I owe many fond memories of my time in Boston to you (many of which took place or started at The Hill)! **Nick**, **Erin**, **Smalls** and **Eva**, thanks for all the good times we had exploring breweries, hiking and throwing house parties. **Iris** and **Stefan**, wat was het leuk dat jullie tegelijkertijd in Boston waren!

My dearest partners in crime, 's werelds leukste paranimpfen – **Laura Burlage** en **Shanice Karangwa**. Lau'tje, Burly, DeLorraWitdeCurves, wat heb ik een geweldige tijd gehad met jou als partner in crime de afgelopen jaren! Onze levens liepen letterlijk parallel en we maakten bizar vaak dezelfde ups en downs mee. We waren zo goed op de hoogte van elkaars leven dat we makkelijk een dagje undercover hadden kunnen gaan in elkaars leven. Dank je wel voor al het lachen, gieren, brullen tijdens congressen, co-schappen, wintersport, feestjes, festivals, perfusies – en natuurlijk tijdens ons onvergetelijk avontuur in "Bahstannn", waar het lot het natuurlijk zo bracht dat we ook daar tegelijkertijd zaten. Lau'tje, je bent een prachtig mens en ik geniet altijd in volle teugen van je humor. Daarnaast heb je ook nog eens een briljant stel hersens en weet je duidelijk wat je wil – alles waar jij je toe zet zal je ongetwijfeld lukken!

Shanice, Shaniqua, GORG – I can't tell you enough how glad I am that you joined our research group. I feel like our friendship truly began during our time in Chicago in 2015, where we explored the city together, ate too much cake and laughed at every street corner. But we didn't need to be on the 96th floor of the John Hancock Tower to have a good time – girl, I even had fun spending an entire summer in Triade with you! I can't imagine anyone being more fabulous while air petting dogs that aren't there. But it's not only giggles and laughs, you're also beautiful person who truly listens and cares. Thank you for being an amazing friend that I can turn to no matter what and for being by my side today! The end is nearrrr for you too!

Dear **Shar**, **Rachel** and **Cat**, we've known each other for nearly two decades now and still we go on yearly holidays where we get to forget about everything and pretend we're 17 again. Until we're old and grey, knitting on the beach and pushing each other's wheelchairs around! Shar, thank you for being an amazing friend every step of the way. No matter how great the physical distance and how long we haven't seen each other, when we do see each other it's like nothing ever changed. Thanks for all the memorable travels, laughter, fun, advice, tears and endless phone calls we've had.

My dearest **Jaz**, **Ronja**, **Maya**, **Dani**, **Tay**, **Ondrej**, **Milan, Yuki**, **Joe**, **Jess**, **Cora**, **Neya**, **Ray**, **Iris**, **Lonneke**, **Forrest**, **Harriet**, **Lilly**, **Maleen** (and everybody else!) – you've turned my world upside down in the best of ways. Thank you all for everything you do! I probably would have finished this thesis a year earlier if I

hadn't met you all, but I also wouldn't be the liberated person I am today if it weren't for you. Jaz, you were (and will be!) the best buddy imaginable. You always go the extra mile to do the right thing and I've learned so much from you. I'm so proud of you, thank you for being you!

Lieve oud HW-genootjes, **Kiki** in het bijzonder, lieve **Lot** & **Lau**, lieve **Brigit**, **Anne-Claire** en alle andere fantastische Distaal (Fistaal?) huisgenoten – dank voor een onvergetelijke tijd met jullie allemaal! Ik heb zo hard genoten van jullie gezelschap. Tegen al mijn Groningense huisgenootjes, sorry voor alle huisavonden, bios-avonden en Sinterklaasfeesten waar ik halsoverkop werd weg gebeld voor de zoveelste donorlever. Dit is het resultaat!

Sander, **Mark**, **Wei** en **Ilka**, dank jullie wel voor alle leuke momenten die ik de afgelopen jaren met jullie heb mogen delen en voor alle support om mij door dit traject heen te slepen.

Lieve **mama en papa**, dank dat jullie er altijd voor mij geweest zijn en mij door dit traject hebben geholpen, zelfs toen er jarenlang een oceaan tussen ons in zat. En toen ik echt hele andere dingen wilde gaan doen. Jullie begrijpen mij als geen ander en ik ben heel trots dat jullie mijn super open-minded totaal niet standaard ouders zijn. **Dieter** big bro, thanks for always making laugh and telling me about things I didn't even know were things one could think about. I'm a lucky little sister!

En last but not least, lieve **Jonathan**, liefste Joon, wat ben ik blij dat je mee ging naar Zwitserland. Ik denk dat dat genoeg zegt!

BIOGRAPHY

Alix Matton was born on November 28th, 1991 in Leuven, Belgium. She moved to Sint-Pieters-Leeuw with her family when she was 7 years old and attended the Sint-Niklaasinstituut in Anderlecht. At the age of 10, Alix and her family moved to Basel, Switzerland, where she attended the International School of Basel. The last move with her family was to Hilversum, the Netherlands, at age 15, where she completed the International Baccalaureate at the International School of Hilversum. Alix started the International Bachelor of Medicine Groningen, Global Health Profile, at the University of Groningen when she was 17. She moved to Jupiter, Florida, US, for her bachelor thesis within plastic surgery in 2011. During her bachelor, Alix was further involved with medical research by participating in the Junior Scientific Masterclass (JSM) Honors program. This is where she was introduced to the research lead by Prof. dr. Robert J. Porte and Prof. dr. Ton Lisman at the Department of Surgery, Section Hepato-Pancreato-Biliary Surgery, University Medical Center Groningen (UMCG). In the summer between her bachelor and master, Alix visited Nepal to perform medical voluntary work at Chitwan Medical College. Thereafter, she began her master in 2012 with her research elective under the supervision of Prof. dr. R.J. Porte. Alix continued this research, with a focus on bile ducts and machine perfusion, after she was accepted to the JSM MD/PhD program in 2014. She refused to take part in animal experiments, which led to the establishment of a human *in vitro* model to study bile ducts. Alix was also a board member of the 4th European MD/PhD Conference and alternated full-time research with her junior medical internships at UMCG. After finishing a tropical medicine internship at Santa Casa hospital in Sao Paulo, Brazil, in 2015, Alix moved to Deventer to complete her senior medical internships at Deventer Ziekenhuis. She finished her master with her final internship at the Department of Emergency Medicine at the UMCG. After that Alix moved to Boston, US, to continue her PhD research at Massachusetts General Hospital/Harvard Medical School at the Center of Engineering in Medicine under the supervision of Prof. dr. Korkut Uygun. Here, she performed research with human stem cells from donor livers and set up a study to investigate the optimal preservation solution to preserve the bile ducts. After returning to the Netherlands, Alix took a break from medicine and dedicated her time and effort to animal rights and climate activism, two subjects that have always been very close to her heart. Alix will start working in psychiatry in the area of The Hague in September 2019.