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. 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. 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 blood 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. 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 hepatobiliary viability criteria during NMP. The primary endpoint, graft survival at 3 months, was 100%. Furthermore, postoperative peak ALT was lower in our recipients than in the studies that compared SCS to NMP only, which may have been attributable to the DHOPE-induced amelioration of ischemia-reperfusion injury. 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, 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. 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.\textsuperscript{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.\textsuperscript{5,11}

In \textbf{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 hepatoc- and 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 hepatoc- and cholangiocellular injury and good
hepatoc- and cholangiocellular function. Levels of HDmiR-122 in perfusate correlated
more strongly with hepatoc- and 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.

\textbf{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.\textsuperscript{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\textsuperscript{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.\textsuperscript{14} Previous
research has shown that PEGs may play an important role in the preservation of
liver and intestine, which closely resembles bile ducts.\textsuperscript{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. 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. Subsequently, it is likely that optimized livers will be preserved using subzero non-freezing techniques, allowing for the long-term storage of transplant organs.

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


