CHAPTER 11

Summary, Discussion and Future Perspectives

SUMMARY

Donor organ viability is closely linked to transplant success. Oxygenated machine perfusion is gaining increasing attention as an alternative method of organ preservation, as it can be used to improve and assess organ viability of ECD organs prior to transplantation. This may ultimately lead to transplantation of more and better quality donor organs. In this thesis, I aimed to study the effects of oxygenated machine perfusion on both donor livers and limbs in more detail. We have investigated the effects of machine perfusion on endothelial activation and function, and studied the effects of new perfusion solutions on graft function both *ex situ* and *in vivo*. In this chapter, the main findings of the studies described in this thesis are summarized and discussed. Furthermore, this chapter is concluded by a section on future perspectives. A general introduction and outline of this thesis is given in **chapter 1**.

PART A: OXYGENATED MACHINE PERFUSION AND TRANSPLANTATION OF HUMAN LIVERS

In **chapter 2** we discussed the potential impact of machine perfusion as an alternative method of organ preservation in DCD liver transplantation. In an effort to overcome the pressing organ shortage, DCD donors are being increasingly utilized as a new source of donor organs. The success of DCD liver transplantation depends, however, heavily on preservation methods to maintain viability of the organ prior to transplantation. In this chapter we briefly addressed the definition and terminologies used in DCD donation, discussed basic principles of organ preservation and outlined the renewed interest in machine perfusion. We aimed to present a comprehensive overview of the different machine perfusion modalities, including differences in timing, temperature, vascular route of perfusion (single or double), and flow characteristics (pulsatile or non-pulsatile). We concluded that machine perfusion holds many advantages over static cold preservation. However, all forms of machine perfusion serve their own purpose and all have their advantages and disadvantages. Therefore there is not one 'holy grail' type of machine perfusion. We concluded that ultimately a combination of different methods of machine perfusion might be used, specialized for the needs per organ. Interestingly, recent studies are also investigating the use of machine perfusion to effectuate ischemia free organ transplantation (IFOT) (1, 2). In the next decades the interesting technique of IFOT could become the preferred method of organ preservation, especially for ECD organs.

In **chapter 3** we investigated the effect of end-ischemic oxygenated HMP on endothelial cell function of ECD livers. This study included donor livers that were declined for transplantation and transported to our center using SCS. After SCS, all livers underwent 6 hours of viability testing (NMP), either directly upon arrival (controls) or preceded by a 2 hours of HMP. In both groups, liver biopsies were taken upon arrival and after NMP, and perfusion samples were taken during NMP. At the end of NMP, relative mRNA expression of the flow-inducible transcription factor Krüppel-like-factor 2 (KLF2), and its downstream genes endothelial nitric oxide synthase (eNOS) and thrombomodulin (TM) were significantly higher in HMP-preserved livers compared to controls. The increased phosphorylation of eNOS via KLF2 might explain, at least partially, the increased NO production observed in HMP livers in this study, and subsequent higher flows in the HMP group at all time points. Endothelial injury was assessed by the release of TM into the perfusate. At the end of NMP, TM levels were significantly higher in controls compared to HMP livers. Furthermore, we developed an histological injury score to assess microvascular injury of endothelial cells lining the vasculature and the vessel walls of hepatic arteries, portal veins and central veins in hematoxylin and eosin stained liver biopsies. After six hours of NMP, injury of the arterial branches was significantly lower HMP-preserved livers compared to controls. This chapter therefore indicates that a short period of oxygenated HMP increases endothelial cell viability after SCS and subsequent NMP of ECD livers.

In **chapter 4** we studied the potassium and sodium shifts during end-ischemic oxygenated machine perfusion and subsequent reperfusion *ex situ* or *in vivo*. This study was prompted by the unexpected observation that, during our first clinical series of end-ischemic oxygenated HMP (in this study referred to as DHOPE), reperfusion of HMP-preserved liver grafts led to severe hypokalemia in three out of ten transplant recipients. We found that during HMP, livers released potassium and took up sodium. The extent of potassium and sodium changes during HMP were in line with the clinical observation that recipient potassium levels decreased upon reperfusion of a HMP-preserved liver, while levels increased after reperfusion of a SCS-preserved liver. Furthermore, comparable shifts in potassium and sodium levels were observed during *ex situ* warm reperfusion of HMP-preserved livers. This study showed that reperfusion of hypothermic machine perfused livers can lead to decreased blood potassium or even hypokalemia in the recipient. With increasing clinical use of HMP as an alternative (or complementary) method of organ preservation, anesthesiologists and surgical teams should be prepared for potassium shifts during HMP transplantation that are opposite to those seen during transplantation of SCS-preserved livers.

In **chapter 5** we aimed to develop a new preservation solution for *ex situ* NMP without the need of human blood products. We sequentially replaced red blood cells (RBCs) with HBOC-201, an acellular hemoglobin-based oxygen carrier, and fresh frozen plasma with the colloid solution gelofusine. Livers perfused with RBCs and fresh frozen plasma were used as controls. We found that livers that were perfused with HBOC-201 had significantly higher hepatic ATP content, cumulative bile production and vascular flows (both portal and arterial), compared to controls. Furthermore, perfusate levels of ALT were lower in HBOC-201 perfused livers compared to controls. During NMP, livers that were perfused with a HBOC-201 based solution, performed at least similar (and for some biomarkers of liver function even superior) to perfusion based on RBCs. We subsequently showed that NMP of human livers can effectively be performed with HBOC-201 and gelofusine. Currently, the first clinical series of extended criteria donor liver perfusion prior to transplantation is being executed with a HBOC-201-based perfusion solution (3).

Chapter 6 describes a sub study of the previous chapter. In chapter 6, we studied the effect of HBOC-201 on endothelial cell function of donor livers during *ex situ* NMP. The reason to further study the effect of HBOC-201 on endothelial cell function, were the serious hemodynamic complications that have been reported in patients that received HBOCs to treat hemorrhagic shock in the setting of a clinical trial. It was thought that the acellular Hb molecules caused vasoconstriction because of 'nitric oxide scavenging'. The main finding of this study was that cumulative NO production did not decrease during ex situ NMP with a HBOC-201 based perfusion solution. At the end of 6 hours of NMP, cumulative NO levels were comparable with between HBOC-201 and RBC perfused livers. The limited or absent NO scavenging in this study might be due to the polymerization of HBOC-201. HBOC-201 polymerization has been performed as a strategy to prevent extravasation of HBOCs molecules, which limited NO scavenging. Furthermore, we found that the relative expression of hypoxia-inducible factor HIF-2a was significantly higher in HBOC-201 livers, compared to RBC-perfused livers. This might suggest that HBOC-201-perfused livers are more resistant to oxidative damage compared to RBC-perfused livers.

In **chapter 7** we aimed to study the *in vitro* effects of recombinant soluble human thrombomodulin (ART-123) on coagulation and fibrinolysis in plasma samples of patients with end-stage liver disease undergoing liver transplantation. ART-123 is used clinically as an anticoagulant and anti-inflammatory agent in the treatment of disseminated intravascular coagulation. We were, however, interested in ART-123 because of the promising results that were reported in preclinical studies that ART-123 efficiently reduced I/R injury of donor livers. Plasma samples were collected of 10

patients undergoing liver transplantation, during and in the days after transplantation. To set reference values, samples of 10 healthy controls were also included in this study. Different concentrations of ART-123 were added to plasma samples and peak thrombin generation and clot lysis times were determined. Compared to controls, we found that plasma of patients was profoundly resistant to the anticoagulant action of ART-123. This might be explained by low levels of protein C, protein S, and elevated levels of factor VIII in patients undergoing liver transplantation. Furthermore, ART-123-dependent prolongation of clot lysis times did not differ from healthy controls. This study suggested that ART-123 is unlikely to provoke bleeding in patients undergoing liver transplantation, but future clinical studies are needed to confirm the safe use of ART-123 during liver transplantation.

PART B: OXYGENATED MACHINE PERFUSION AND TRANSPLANTATION OF LIMBS

Chapter 8 provided a comprehensive overview of novel strategies that may enable extended preservation of vascularized composite allotransplantation (VCA) grafts. In summary, the current method of static cold preservation is considered inadequate. Major advancements in the field of VCA regarding matching, desensitization, and potential tolerance induction all require grafts to be kept viable up to hours or days. The potential of machine perfusion as a new method of preservation is discussed in this chapter. Also, advantages and disadvantages of more extreme preservation techniques, such as cryopreservation approaches, are discussed. The composite nature of VCA grafts remains a complicating factor, as all tissue types have their own degree of susceptibility to (cold) ischemia and cryoprotectant agents. We believe that in the current scope of extended preservation protocols, high subzero approaches of VCA grafts seem a promising alternative that can greatly impact the clinical application of VCA as a reconstructive option for patients worldwide.

In **chapter 9** we developed a protocol for 6 hours of *ex situ* subnormothermic machine perfusion (SNMP). A rodent hind limb model was used as a vascularized composite allograft. We compared three different perfusion solutions all based on muscle-specific culture media, with the addition of polyethylene glycol and the acellular oxygen carrier HBOC-201. Peak oxygen extraction was superior in HBOC-201 perfused limbs, compared to the other groups. Moreover, compared to static cold preserved limbs, the cellular energy charge was significantly higher in HBOC-201 preserved limbs. Viability of the HBOC-201 perfused limbs was confirmed with a heterotopic transplantation and successful one week follow up. In summary, this chapter demonstrated that 6 hours

ex situ SNMP of rat hind limbs is feasible and results in superior tissue preservation compared with conventional cold preservation methods. Current studies are investigating extension of the preservation time of VCA grafts by implementing our SNMP protocol in a 24 hour subzero preservation protocol.

In the **addendum chapter 10**, we have provided a detailed summary of our recently developed 24 hour subzero non-freezing protocol for extended preservation of rodent limbs

The studies described in this thesis have resulted in better understanding of the effect of oxygenated machine perfusion, with novel perfusion solutions, on endothelial cell function of both livers and limbs.

In conclusion, the main findings of this thesis are:

1. End-ischemic oxygenated hypothermic machine perfusion improves endothelial cell function of extended criteria donor livers
2. End-ischemic oxygenated hypothermic machine perfusion of donor livers grafts results in potassium and sodium shifts that are opposite to those observed during transplantation of SCS-preserved livers
3. The acellular oxygen carrier HBOC-201 is good alternative for red blood cells during normothermic machine perfusion of extended criteria donor livers
4. Normothermic machine perfusion of extended criteria donor livers with a HBOC-201 based perfusion solution results in better cellular energy content, better flow and preserved endothelial cell function
5. Recombinant human soluble thrombomodulin (ART-123) is unlikely to provoke bleeding in patients undergoing liver transplantation and could potentially be used a component of *ex situ* machine perfusion solutions
6. Subnormothermic machine perfusion with a HBOC-201-based perfusion solution results in better preservation of vascularized composite allografts compared to static cold preservation

GENERAL DISCUSSION & FUTURE PERSPECTIVES

Although these studies answer questions raised in the beginning of this thesis, still many question remain and, as research continues, new challenges have arisen. In the last section of this chapter, I would like to address a few of these questions, and discuss opportunities and directions for future research.

Ad 1. Optimization of mechanical flow characteristics of machine perfusion

Over the last years, the technique of machine perfusion has truly been evolving from bench to bedside. In 2011, Guarrera *et al*. published the first clinical series on hypothermic, non-oxygenated machine perfusion of human livers (4). This year, in 2018, the first randomized controlled trial was been published, comparing post-transplant outcome of livers continuously preserved using NMP or livers preserved by SCS (5). Moreover, just recently a prospective clinical study has been initiated by our own group to study whether combined *ex situ* HMP and NMP of donor livers can be used to distinguish potentially transplantable high risk donor liver grafts from non-viable high risk donor liver grafts (www.trialregister.nl; NTR5972). With the growing clinical interest in machine perfusion, the need to study and optimize all technical aspects of this technique are more relevant than ever. Although it is nowadays acknowledged that hepatic endothelial damage represent the initial factor in hepatic I/R injury (6), only a hand full of research groups have studied the effects of different machine perfusion modalities on endothelial cell viability (7). To date, there is no consensus about the best mechanical flow characteristics: pulsatile or non-pulsatile, single or dual perfusion. Data presented in this thesis suggest that end-ischemic dual, pulsatile, oxygenated HMP improves endothelial cell function of extended criteria donor livers through upregulation of flow dependent gene KLF-2 (**chapter 3**). The protective shear stress regulatory effect of KLF-2 has been shown to be flow pattern specific (8). In cultured human endothelial cells, pulsatile flow with significant forward direction increased KLF-2 expression, while oscillatory flow with little forward direction did not (9). Recently, a preclinical study reported higher hepatic artery flows in livers perfused with pulsatile flow compared to livers perfused with a continuous flow via the hepatic artery at subnormothermic temperature (10). Moreover, this study reported better lactate clearance in livers perfused via both the portal vein and hepatic artery compared to livers only perfused via the portal vein. Several studies on renal perfusion studies report superiority of pulsatile perfusion over continuous perfusion (11, 12). In the search for the most optimal mechanical flow characteristics, it is important to realize that endothelial cell phenotype and function differ per organ type, but also per 'extended criteria donor' type. For example, the porosity and endocytic capacity of liver sinusoidal endothelial cells decreases with age (13), making livers from older donors more easily damaged by shear stress. Also, macrosteatotic livers present attenuated vasoprotective signaling pathways due to altered sinusoidal blood flow (14, 15). On the other hand, the impact of mechanical stimulation on the endothelial cells highly depends on the timing (before

or after cold ischemia), temperature, perfusion solution (viscosity) and flow rate of machine perfusion. Future studies are thus needed to align machine perfusion settings with the needs of endothelial cells per extended criteria donor organ type.

Ad 2. Optimal perfusion solution

The search for the most optimal preservation solution is as old as the field of organ transplantation itself. More than 20 years after the first randomized comparison between University of Wisconsin (UW) and Histidine-tryptophan-ketoglutarate (HTK), investigating a 'preservation solution effect' in outcome (16), the debate about the best static cold storage solution has still not 'cooled down' (17). While HTK solution has shown clinical equivalence to UW solution in liver preservation, when kept under 15 hours of cold ischemia (18), UW solution is still the cold preservation solution of first choice in most countries. In **chapter 4** of this study, we described opposite potassium shifts upon reperfusion of livers grafts that were preserved with UW and subsequently perfused with HMP. Future studies are needed to investigate the effect of preservation solutions and additional machine perfusion on outcome.

Meanwhile the development of perfusion solutions is in full progress. Machine perfusion solutions are mainly based on a colloid with electrolytes with or without an oxygen carrier, depending on the temperature and corresponding degree of cellular metabolism. Given the expectation that NMP will only be used more frequently for preservation of ECD livers prior to transplantation, the need for off the shelf available perfusion solutions is growing. As studied in **chapter 5 and 6**, we demonstrated that the use of acellular oxygen carrier HBOC-201 is a good alternative for red blood cells. Also, the combination of PEG and HBOC-201 has been shown to have beneficial effects on edema and peak oxygen extraction during SNMP of limbs (**chapter 9**). Future studies are needed to investigate whether PEG could replace or compliment the use of gelofusine in liver machine perfusion, and whether this would result in better endothelial cell preservation.

Ad 3. Improving donor organ function: potential of pharmaceutical agents

A promising advantage of *ex situ* NMP is the opportunity to not only assess but to improve organ function prior to transplantation by adding pharmaceutical agents or even stem cells. When such strategies are successful, we can eventually transplant more and better quality donor organs. As the clinical need to utilize steatotic livers growing, so is the interest in NMP as a tool to reverse steatosis prior to transplantation (19). Several groups have reported the preclinical use of 'defatting cocktails' that successfully

lowered intracellular lipid content in hepatocytes during NMP (20, 21). In the future, the technique of NMP might play a key role in defatting approaches, but further research is needed to translate these promising but scarce preliminary results to clinical application. Furthermore, while the use of NMP in VCA has been scarce so far (22–24), I believe that it holds great promise for this field as well. By optimizing extended NMP for VCA grafts we would be able to explore entirely novel concepts of tolerance induction, such as immune engineering of VCA grafts *ex situ,* thereby lowering the need of immunosuppression after transplantation.

Ad 4. Prolonging preservation time beyond the current limits

As previously pointed out in the introduction (**chapter 1**) of this thesis, the increasing demand for organ transplantation is one of the biggest challenges in organ transplantation to date. Development of an organ and tissue supply chain that meets these twenty-first century healthcare demands is needed, which requires i) enough suitable organs and tissues and ii) means to store and transport them for a variety of applications (25). Extensively discussed in **chapter 8** is the use of subzero non-freezing protocols to prolong the 'storing time' of organs. Berendsen et al. were the first to successfully utilize a subzero non-freezing protocol (26) for extended preservation of donor rat livers up to several days (27). They reported 100% graft survival (30 days follow up) with 72 hours of storage and 58% survival when preservation time was extended to 96 hours. The opportunity to 'buy time' in organ transplantation is invaluable. By extending the preservation time for hours to days or maybe even weeks in the future, we can amplify matching options, broaden the option to revive ECD organs and may gain time to implement tolerance induction protocols thereby decreasing the need for immunosuppression – benefiting millions of patients worldwide (25, 28–31). I believe that in the coming years, we may enter a new era of organ and tissue preservation, and that we may ultimately use a combination of multiple existing preservation approaches and new technologies. Continued coordinated efforts between various disciplines and institutions are needed to further explore extended preservation techniques and even the possibility of organ banking.

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