Studies on bile duct Injury and the protective role of oxygenated machine perfusion in liver transplantation

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CHAPTER 11

Summary and General Discussion

Bile duct complications, and most importantly non-anastomotic biliary complications (NAS), remain to be a major limiting factor after liver transplantation. Non-anastomotic biliary complications usually occur due to multifocal strictures at intrahepatic or extrahepatic bile ducts and patients present with features of biliary obstruction. In this thesis we aimed to obtain a better understanding of the origin of bile duct injuries and the potential underlying etiologies that lead to NAS after liver transplantation. Moreover we investigated the role of oxygenated machine perfusion in improving the quality of organ before transplantation in order to potentially reduce the development of post-transplantation complications including NAS. In this chapter, our main findings of the studies described in this thesis are summarized and the future perspectives for further investigations are discussed.

Part A: Bile Duct Injury in Liver Transplantation

In **chapter 1** a general introduction of this thesis is provided, including the aims of each chapter. Various types of biliary complications that can occur after transplantation are discussed in **chapter 2**, providing the pathogenesis, clinical presentation and clinical management of these complications. In summary, bile leakage and bile duct strictures including anastomotic strictures and NAS are the most common biliary complications that occur after transplantation. While bile leakage and anastomotic strictures can usually be managed successfully with non-surgical or surgical approaches, NAS are the most therapy-resistant complications with long-term sequelae. These complications can be categorized into early-onset and late-onset NAS, which likely have a different underlying pathogenesis. Early-onset NAS are more ischemia-related while late-onset NAS are frequently associated with immune-mediated injuries including a loss of function mutation in the chemokine receptor CCR5 (CCR5Δ32 polymorphism). Ischemic bile duct injuries during static cold storage (SCS) of the grafts may contribute to a high incidence of NAS after transplantation, especially in suboptimal (extended criteria donor) livers. However, Its still not fully known what the origin of bile duct injuries during liver transplantation is. From all the livers that are transplanted, whether DBD or DCD, at the most up to 30% develop NAS after transplantation while the majority of these livers have existing severe biliary injury at the time of transplantation (2). This necessitates more studies to provide a deeper insight on the origin of bile duct injuries and the role of repair mechanisms during transplantation. In addition, it is still not known whether the degree of injury at the distal end of extrahepatic bile duct represents the rest of the biliary tree. In the next two chapters these research questions are investigated.

In **chapter 3**, a new perspective on the origin of bile duct injuries after transplantation is discussed. For decades, it was believed that the degree of bile duct injury, featured as loss of biliary epithelial lining, contributed to the development of NAS after transplantation. The majority of biliary epithelial loss was suggested to occur after transplantation due to ischemia-reperfusion injury, immunological injury, or the cytotoxicity caused by hydrophobic bile salts. However three histopathological studies of bile duct biopsies taken during transplantation of

DBD donor liver at the time of transplantation showed severe loss of biliary epithelial lining (in up to 90% of all grafts) (2,4,5). On the other hand, NAS occurs only in about 10% of DBD and up to 30% of the DCD recipients. These findings suggest that insufficient regeneration of the biliary epithelium after transplantation might have an important role in development of NAS after transplantation. However, all the histopathological studies of bile ducts have been performed on biopsies taken from the distal end of the extrahepatic bile duct. It is not known whether the histological injury found at the distal end of the extrahepatic bile duct represents the degree of injury in the rest of the biliary tree including the intrahepatic bile duct.
In **chapter 4**, we investigated the degree of histological injury of bile ducts at different levels of the biliary tree in human donor livers that were declined for transplantation. Biopsies were collected from the distal end of extrahepatic bile duct and from the intrahepatic bile ducts at two levels (sectoral and segmental bile ducts). Bile duct injury was evaluated based on a systematic histological scoring system focusing on the loss of biliary epithelial lining, mural stroma necrosis, and injury of peribiliary vascular plexus and glands. This study demonstrated that the degree of histological injury detected at the distal end of extrahepatic bile duct of a donor liver represents the degree of injury in the proximal parts of the biliary tree, including intrahepatic bile ducts. These findings demonstrate that biopsies taken from the distal end of extrahepatic bile ducts are a valuable tool for studies focusing on bile duct injury of donor livers during transplantation.
In **chapter 5**, the effect of a functional deficiency of the chemokine receptor CCR5 on T cell-mediated immune responses during obstructive bile duct injury is explored using the model of bile duct ligation (BDL) in CCR5-null mice. CCR5 loss of function led to a significantly decreased recruitment of T_{Regs} to the liver with an unaltered Th-17 immune response after BDL. This resulted in an imbalance of the Th-17/T_{Regs} ratio towards an enhanced Th-17-mediated immune response. High levels of IL-17 secreted by Th-17 type T cells have been shown to contribute to liver damage after ischemia reperfusion. These studies suggested that an imbalance in the Th-17/T_{Regs} ratio due to lower presence of T_{Regs} might be responsible for enhanced bile duct injury and a higher incidence of NAS in recipients carrying the CCR5Δ32 polymorphism. Indirectly, this study confirms the role of an immune response in the pathogenesis of NAS.

Part B: Oxygenated Machine Perfusion: A Potential Strategy to Improve Organ Quality Prior to Transplantation

Machine perfusion is a technique of organ preservation that is being used as an alternative to SCS or in combination with SCS (7-9). During machine perfusion the organ is perfused with an oxygenated or non-oxygenated perfusion fluid at hypothermic temperature (at 0-10℃), subnormothermic temperature (at $\pm$21℃) or normothermic temperature (at 37℃) (10-12). Most of the investigations on machine perfusion of liver grafts have focused on preservation of the liver parenchyma (10,11) but there is not enough data on the effect of machine perfusion on the preservation of bile ducts. *Op den Dries et al.* have shown that hypothermic oxygenated machine perfusion of porcine DCD liver grafts is a favorable alternative to SCS by provid-

ing a better preservation of peribiliary vascular plexus, but it does not prevent the bile ducts from losing their biliary epithelial lining (13). An alternative strategy is to preserve the grafts at normothermic temperature (at 37℃) during which the delivery of oxygen and nutrients to the liver provides full metabolic support of the grafts. In **chapter 6** the effect of normothermic oxygenated machine perfusion (NMP) as an alternative to SCS on bile duct preservation was investigated in both DCD and non-DCD (living donor) rat livers. The liver grafts were preserved for 3 hrs by either NMP or SCS followed by 2 hrs of *ex-situ* reperfusion. NMP of liver grafts provided a better preservation of the function and morphology of the biliary epithelium compared to SCS. The prominent beneficial effect of NMP on bile duct preservation was particularly observed in DCD livers. By reducing biliary injury, NMP could have an important impact on the utilization of DCD livers and may improve outcome after transplantation. These findings provide a strong stimulus for a clinical trial of NMP in human DCD liver transplantation.

Technical feasibility of NMP of human donor liver grafts has been shown (14). In **chapter 7** a detailed step-by-step protocol for *ex-situ* normothermic machine perfusion of human donor livers is provided. In this protocol a perfusion device was used that enables pressure controlled dual perfusion of the liver providing a pulsatile arterial flow and a continuous flow through the portal vein. The perfusion fluid is oxygenated by membrane oxygenators and the livers can be perfused at various temperatures (10-37℃). The ability to perfuse donor livers at different temperatures and the opportunity to add extra agents to the perfusion fluid during organ perfusion, offers the potential to assess and improve the organ quality before transplantation. Therefore, this method can considerably increase the number of available organs for transplantation.

One of the advantages of *ex-situ* NMP of livers is the possibility of viability evaluation of the grafts. Nowadays, many suboptimal liver grafts of which the majorities are DCD livers are declined for transplantation based on the current clinical criteria. Given the opportunity of viability assessment of livers by machine perfusion, it is necessary to identify markers during machine perfusion that can predict liver function after transplantation. In the transplantation center, we should ideally be able to evaluate the quality of the liver within a short period of time (1-3 hrs) to allow sufficient time for patient selection from the waiting list and preparation of the recipient for liver transplantation. In **chapter 8**, criteria for viability assessment of human donor livers based on bile production, as a marker of liver function, were investigated. Twelve human donor livers that were declined for transplantation due to various reasons were perfused for 6 hrs using *ex-situ* NMP. The amount and rate of bile production by the livers were evaluated and correlated with hepatobiliary function and injury during 6 hrs of NMP. Bile production by livers during a short duration of 2.5 hrs of NMP was identified as a discriminating predictor of liver viability after 6 hrs of NMP. This study suggests that normothermic perfusion of ECD livers allows assessment of graft viability prior to transplantation, which opens new avenues for donor organ selection, therapeutic interventions and preconditioning. This may not only improve organ quality and function, but will also lead to a considerable expansion of the number of organs available for transplantation.

NMP, despite the many advantages that it offers, is associated with more complexity when compared to other machine perfusion modalities such as hypothermic machine perfusion.

The reason is the high metabolic support required for the liver during NMP and higher risks of liver damage in case of technical failure. Bringing the technique of machine perfusion to clinical usage in the multidisciplinary setting of transplantation requires a more logistic simplicity and safety. Hypothermic oxygenated machine perfusion at the end of SCS is an alternative approach that allows safe transportation of the donor liver graft to the transplantation center and at the same time helps the graft resuscitate from SCS by restoring hepatic ATP (9,17). In **chapter 9**, the efficacy of end-ischemic hypothermic oxygenated machine perfusion (HMP) of human donor livers is discussed. Six DCD human livers that were declined for transplantation were exposed to 2 hrs of HMP followed by hepatobiliary viability assessment during 6 hrs of *ex-situ* NMP and were compared to 12 donor livers that underwent 6 hrs of *ex-situ* NMP without prior HMP. Hepatic ATP content increased more than 15-fold higher during HMP. During NMP the livers that received HMP displayed significantly better hepatobiliary function when compared to livers without prior HMP. Hepatic ATP content and cumulative bile production during *ex-situ* NMP was significantly higher in livers with prior HMP. This study suggested that a short period of 2 hrs hypothermic (12 oC) arterial and portal oxygenated perfusion of donor livers after traditional cold preservation restores cellular energy levels, resulting in less injury and better hepatobiliary function upon subsequent NMP. This study is the first to indicate that end-ischemic HMP improves viability of human ECD livers. In **chapter 10** data from the clinical utilization of dual hypothermic oxygenated machine perfusion (DHOPE) to resuscitate DCD liver grafts prior to transplantation is provided. Ten consecutive patients aged $\geq$18 years old undergoing DCD liver transplantation at University Medical Center Groningen (UMCG) were included in this study. Upon arrival in the transplantation center, the DCD livers underwent 2 hrs of DHOPE prior to implantation. The recipients were followed up for a median of 8 months post-transplantation and the outcomes were compared to matched historical recipients who had received a DCD liver after conventional SCS. All the DHOPE preserved livers showed excellent early function and the median postoperative peak of ALT was significantly lower when compared to historical controls. Only one case of NAS (10%) was observed in recipients of DCD livers that underwent DHOPE. This was significantly lower compared to 7 cases of NAS (35%) observed among the controls. This first clinical study of end-ischemic DHOPE in DCD liver transplantation demonstrated that this technique is safe, can restore cellular energy levels, and reduces reperfusion injury. Our data suggest that DHOPE may reduce the incidence of NAS after DCD liver transplantation. Our research group is currently leading a multicenter randomized clinical trial to validate the above findings.

CONCLUSIONS AND FUTURE PERSPECTIVES

The aim in this thesis was to provide a better understanding of the etiologies underlying the bile duct injuries that occur during liver transplantation, which may lead to development of NAS after liver transplantation. Moreover, the beneficial role of oxygenated machine perfusion in bile duct preservation and in improving the quality of organs prior to transplantation has been explored.

However, as the investigations go on new challenges and questions arise necessitating the

continuation of research in this field. As a conclusion of this thesis it is worthwhile discussing some of the issues that remained unanswered and propose potential new strategies and opportunities to resolve these issues.
Among the multifactorial etiologies of NAS, the role of CCR5 functional deficiency in immune-mediated bile duct injury was explored. Obstructive cholestasis is the clinical presentation of NAS. Lower hepatic influx of T_{Regs} and enhanced Th-17 immune response after obstructive biliary injury, which is due to CCR5 functional deficiency, provide a potential mechanistic explanation for the association of NAS with CCR5Δ32 polymorphism. However, a limitation of these findings is that theyre based on a model of bile duct ligation, as there is no established model of NAS. Therefore, it is difficult to link these findings to development of NAS. It is evident that NAS formation is a progressive process and the immunological disturbance caused by CCR5 functional deficiency might be responsible for NAS formation from the early phases until the time that it becomes clinically evident. Therefore it is of interest to verify this mechanism in a liver transplantation model.
Since a new era has opened in the perspective of ischemia-mediated bile duct injury during transplantation (3), it is evident that the current organ preservation method of SCS is not sufficient to protect the bile ducts and livers with suboptimal quality. High rates of NAS and early graft dysfunction after transplantation of ECD livers, especially DCD livers, have limited the utilization of this type of donor grafts. Machine perfusion is a technique of organ preservation with a great potential to improve the quality of organs and prevent preservation injury prior to transplantation. With the ongoing technical improvements in the field of machine perfusion in recent years and its promising results, this technique offers opportunities for a groundbreaking change in the field of organ transplantation. In this thesis we demonstrated some of the benefits of oxygenated machine perfusion in improving organ quality and preventing bile duct injury. We also reported a first clinical utilization of this technique in our transplantation center. However, there are still several aspects in using machine perfusion of organ before transplantation that were not discussed in this thesis.
Future investigations should focus on the following questions:

1) When is the best time to perfuse the liver?

During the procedure of organ donation, liver ischemic damage starts right after the aortic cross clamp in DBD and circulatory arrest in DCD donors and the injury increases during SCS when the organ is transported to the transplantation center. Therefore the strategies to use oxygenated machine perfusion to reduce ischemic injury of the liver includes three phases:

A Machine perfusion of the organ in the donor center: Normothermic regional perfusion (NRP) is a technique currently under investigation by Fondevila *et al.* and Oniscu *et al.* which involves isolation of the sub-diaphragmatic aorta from the systemic circulation and perfusion of abdominal organs with a continuous flow at 37℃ prior to SCS. The concept of this strategy is based on reducing vasoconstrictive effects and therefore improving the subsequent cold flush as well as restoring the livers cellular energy and preparing it for the period of SCS (8,12). However, the liver might still be ATP depleted at the time of transplantation after SCS.

B Machine perfusion of the graft from the donor center and during transportation to the transplantation center: This strategy which is currently being pursued by Friend *et al.* is based on replacing the entire period of cold storage by NMP (7). Although this technique might be the ultimate approach to organ preservation, it carries a risk of failure and requires a complex multidisciplinary organization. An accidental technical failure leading to disconnection of the liver from the perfusion device during the transportation might result in disastrous warm ischemic damage.

C Machine perfusion of the graft at the transplantation center prior to implantation: This strategy is based on the transportation of the graft using conventional SCS and improving the quality of an organ by a short period of oxygenated hypothermic machine perfusion prior to implantation at the transplantation center. This approach, which is investigated by Dutkowski *et al.* (17) and our group at the UMCG, has the advantage of safer transportation of the graft using the conventional SCS. Moreover, the liver is resuscitated immediately before being implanted in the recipient.

Although all the above strategies have shown promising results in phase 1 clinical studies, the effectiveness of each approach and whether they can reduce the occurrence of NAS after transplantation should be investigated in randomized clinical trials.

2) What is the optimal temperature for machine perfusion of the liver?

Donor liver grafts can be perfused hypothermically (at 0-10℃), subnormothermically (at 20-30℃) or normothermically (at 37℃) (9,15,17,20-23). Hypothermic machine perfusion (HMP) is a relatively simple and safe approach. HMP in a porcine DCD liver model showed a better preservation of peribiliary vascular plexus when compared to SCS (13). The first clinical studies using HMP of donor livers (**chapter 10**) have shown improvements in the early graft function and showed reduced delayed graft function (17,20). However, the effectiveness of HMP in reducing the occurrence of NAS after transplantation is still unknown. Subnormothermic machine perfusion (SNMP) offers a higher metabolic support of the liver and has shown advantages in hepatic ATP restoration after SCS (23). The impact of SNMP on bile duct preservation is yet to be studied. In **chapter 6** of this thesis we have shown that normothermic machine perfusion (NMP) prevents bile duct preservation injury in a rat model of DCD. An alternative strategy is gradual increase of the temperature during machine perfusion. Minor *et al.* have shown that controlled oxygenated rewarming of the liver by machine perfusion up to 20℃ may result in a better hepatobiliary function when compared to SCS or only SNMP (24). Choosing the right temperature for machine perfusion also depends on the reason for which the liver is perfused. In **chapter 8** of this thesis we have demonstrated that *ex-situ* NMP can be used for viability assessment of the liver prior to transplantation based on the bile production by the liver. Predicting the viability of the liver before transplantation requires mimicking the metabolic status of the liver after transplantation. This can be achieved only during NMP when the liver has almost full metabolic activity. More research is needed in this area to find out the most effective modality of machine perfusion.

3) What is the best perfusion fluid?

The content of the perfusion fluid serves to provide enough nutrients to the organ during machine perfusion. The exact composition of the perfusion fluid thus depends on the metabolic needs of the liver, which differ based on the temperature of the perfusion. During HMP a perfusion fluid as simple as machine perfusion solution-Belzer UW (**chapter 9** and **10**) seems to be sufficient. Higher temperatures of perfusion require a better metabolic support of the liver. Bruinsma *et al.* have used Williams medium E for SNMP of donor livers (23), which contains essential amino acids and electrolytes. In our studies on NMP we tried to mimic physiology by using a perfusion fluid based on packed red blood cells (RBC), fresh frozen plasma (FFP) and albumin (15,16,22).

The temperature of the perfusion fluid also defines the need for oxygen carriers in the perfusion fluid. Oxygen solubility is temperature-dependent. Therefore, during HMP and SNMP there seems to be enough oxygen delivery to the graft by dissolved free oxygen (23,25). NMP on the other hand requires an oxygen carrier such as RBCs for sufficient oxygen delivery to the graft. Since the accessibility to blood products is relying on donation, the use of artificial oxygen carriers and plasma replacements may be a potential alternative. Although Fontes *et al.* have chosen to use a hemoglobin-based oxygen carrier during SNMP of liver grafts (26), it is still unknown whether it is essential to use oxygen carriers at temperatures lower than 37°C.

4) What is the optimal duration of machine perfusion?

The decision how long an organ should be perfused to have the best results depends on several aspects. Schlegel *et al.* have shown that HMP for a duration of 2 hrs increased hepatic ATP but there is no extra benefit if livers are perfused for a longer time period (9). Westerkamp *et al.* have shown in a rat model of DCD that only a short period of end-ischemic oxygenated machine perfusion regardless of the temperature provides better preservation of biliary epithelial function and morphology (27). Machine perfusion at higher temperature using a perfusion fluid which is richer in nutritional contents allows a longer perfusion time (7,15,22,28,29). During machine perfusion the graft is perfused in a closed circuit with a certain concentration of nutrients in the perfusion fluid. Longer duration of machine perfusion might require renewal of the perfusion fluid as well as a dialysis system to remove waste products such as urea and damage associated molecular patterns (DAMPs) that are washed out of the liver but accumulate in the perfusion fluid.

5) Can we assess bile duct viability during *ex-situ* machine perfusion?

In **chapter 8** of this thesis we discussed viability assessment of the liver parenchyma during *ex-situ* NMP (16). However, we are still unable to evaluate bile duct viability during *ex-situ* machine perfusion. In our studies we have used the concentration of bicarbonate in the bile as a biomarker of biliary epithelial cell function and the concentration of gamma-glutamyl transferase (gamma-GT) and alkaline phosphatase (ALP) in the bile as biomarkers of biliary epithelial injury. However the latter enzymes may also reflect hepatocellular injury. A recent study reported that cholangiocyte-specific microRNAs may be more specific biomarkers to predict biliary strictures after transplantation (30). A potential alternative tool might be development

of non-invasive imaging techniques combined with near infrared fluorescence and visible light cholangioscopy (31) or thedevelopment of ultrasound devices that allow real-time assessment of bile ducts during *ex-situ* machine perfusion.

6) Can we prolong the duration of liver preservation beyond the current limitations?

Currently, the duration of liver preservation by SCS (0-4℃) is limited to up to 12-14 hrs, although beyond 10 hrs transplant success decreases significantly (32). The apparent limitation is the amount of metabolic activity that takes place at low temperatures and the lack of nutrients and oxygen required for that level of metabolism in the current SCS preservation solutions such as the UW solution (33). An approach to extend the duration of liver preservation is to reduce the metabolic activity as much as possible by cooling down the liver to subzero temperatures, hence improving preservation quality, but avoiding ice formation using a preservation solution supplemented with cell stabilizers (34). Berendsen *et al.* have developed a novel approach to supercool rat donor livers to subzero temperatures using a combination of oxygenated machine perfusion with a controlled cooling system. They have extended the duration of viable rat liver preservation to up to 72 hrs with 100% transplantation survival (35). Scaling up such a groundbreaking technique to human donor livers potentially increases the number of viable organs for transplantation and enables global organ sharing due to viable extended preservation time.

In summary, the studies described in this thesis have provided a better understanding of the underlying pathophysiology of bile duct injuries during and after transplantation; Approaches to improve the quality of organ prior to transplantation, prevention of bile duct injuries during organ preservation and a better organ selection for transplantation have been discussed. Moreover, the safety and feasibility of dual hypothermic oxygenated machine perfusion (DHOPE) of DCD liver grafts prior to transplantation has been reported. I hope that the technique of machine perfusion will be used extensively in clinical practice to increase the number of suitable organs for transplantation and expand global access to such a life-saving therapy.

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Nederlandse Samenvatting

Galwegcomplicaties, in het bijzonder non-anastomotische galwegstricturen (NAS), zijn een veel voorkomend en moeilijk behandelbaar probleem na levertransplantatie. Kenmerkend voor deze complicatie is het ontstaan van vernauwingen van de galwegen in of net buiten de lever, waarbij patinten zich vaak presenteren met symptomen van galwegobstructie. De doelstelling van dit proefschrift is om de oorzaken van galwegschade en de onderliggende etiologie van NAS beter te begrijpen. Daarnaast hebben we de rol van geoxygeneerde machineperfusie onderzocht in het verbeteren van orgaankwaliteit voor transplantatie en het potentieel voorkomen van complicaties na transplantatie, inclusief NAS. In dit hoofdstuk worden de belangrijkste resultaten van dit proefschrift samengevat en besproken.

Deel A: Galwegschade tijdens levertransplantatie

Hoofdstuk 1 is een algemene inleiding tot dit proefschrift, inclusief de doelstellingen van ieder hoofdstuk. De pathogenese, het klinische beeld en de behandelmogelijkheden van verschillende types galwegcomplicaties die na levertransplantatie kunnen optreden, worden besproken in **hoofdstuk 2**. Samengevat zijn galwegstricturen en gallekkage de meest voorkomende complicaties van alle galwegcomplicaties na levertransplantatie. Hoewel gallekkage en een strictuur van de anastomose gewoonlijk succesvol behandeld kunnen worden, is NAS vaak therapieresistent en meestal is re-transplantatie de enige effectieve behandeling. Niet iedere manifestatie van NAS is identiek; er wordt onderscheid gemaakt tussen NAS vroeg na transplantatie en NAS laat na transplantatie. Gedacht wordt dat deze variaties in manifestatie (in ieder geval deels) te verklaren zijn door verschillen in onderliggende oorzaken. Hoewel NAS vroeg na levertransplantatie grotendeels geassocieerd wordt met een ischemie-gerelateerde pathogenese, wordt NAS laat na levertransplantatie vaak toegeschreven aan een immuungemedieerde oorzaak, zoals een verlies-van-functie mutatie in de chemokine receptor CCR5 (CCR5-Δ32), welke leidt tot veranderingen in het immuunsysteem. Ischemie-gerelateerde galwegschade tijdens de veelgebruikte methode van orgaanpreservatie door middel van het bewaren op ijs “static cold storage” (SCS), draagt mogelijk bij aan de incidentie van NAS na transplantie, in het bijzonder in het geval van een suboptimale (extended criteria donor) lever. Echter, de oorsprong van galwegschade tijdens levertransplantatie wordt nog niet volledig be-

grepen. Ondanks dat de overgrote meerderheid van donorlevers wordt getransplanteerd met significant beschadigde galwegen, “slechts” $\leq$30% van deze levers ontwikkelt NAS na transplantatie (1). Om dit te kunnen begrijpen zijn studies nodig om beter inzicht te geven in de oorzaak van galwegschade en de rol van reparatiemechanismen tijdens en na transplantatie. Daarnaast is het onduidelijk of de schade die wordt gezien in de extrahepatische perifere galwegen representatief is voor schade in de galwegen dichterbij en in de lever. In de twee hier opvolgende hoofdstukken worden deze onderzoeksvragen bestudeerd.

In **hoofdstuk 3** wordt een nieuwe visie op de pathogenese van NAS besproken. Jarenlang werd er verondersteld dat slechts enkele galwegepitheelcellen beschadigd raken of verloren gaan tijdens de koude preservatietijd, en dat de meeste galwegschade ontstaat na levertransplantatie als gevolg van reperfusieschade, immunologische oorzaken en hydrofobe galzouttoxiciteit. Echter, drie histopathologische studies lieten uitgebreide galwegepitheelschade gezien in de meerderheid (tot 90%) van de extrahepatische galwegbiopten die verzameld waren aan het einde van de koude preservatietijd (1-3). Daarentegen ontstaat NAS slechts in (tot) 10% van de DBD en (tot) 30% van de DCD donorlevers. Blijkbaar is verlies van galwegepitheel zeer frequent aanwezig in humane donorlevers, maar slechts een minderheid ontwikkelt galwegstricturen na levertransplantie. Deze bevinding suggereert dat een ontoereikende regeneratieve capaciteit van het galwegepitheel een belangrijke rol speelt in de ontwikkeling van NAS na transplantatie. Een belangrijke vraag die beantwoordt moet worden, is of de schade die werd gezien in de distale extrahepatische galwegbiopten in de hierboven genoemde studies, representatief is voor de galwegen in de rest van de galwegboom, dichterbij en in de lever (waar de meerderheid van NAS ontstaat). In **hoofdstuk 4** hebben we dit onderzocht door galwegschade op verschillende niveaus in de galwegboom te bestuderen in humane donorlevers, die waren afgekeurd voor transplantatie. Biopten werden verzameld van het distale einde van de extrahepatische galweg en de intrahepatische galwegen op twee niveaus. De biopten werden lichtmicroscopisch bestudeerd aan de hand van een systematisch scoringssysteem. De hoeveelheid histologische schade in de distale extrahepatische galweg bleek representatief te zijn voor de hoeveelheid galwegschade in de meer proximale galwegboom, inclusief de intrahepatische galwegen. Hieruit kon geconcludeerd worden dat biopten van de distale extrahepatische galweg een waardevol instrument is voor studies die zich focussen op galwegschade van donorlevers tijdens levertransplantatie.

In **hoofdstuk 5** focust zich op de rol van het immuunsysteem in de ontwikkeling van NAS na levertransplantatie. Een verlies-van-functie mutatie in de chemokine receptor CCR5 (CCR5Δ32), welke gedacht wordt te leiden tot veranderingen in het immuunsysteem, werd onderzocht in een muizenmodel. In het bijzonder, het effect van CCR5 deficintie op de T-cell-gemedieerde immuunrespons werd onderzocht in een model van galwegligatie (afbinden van de galweg) in CCR5 deficinte (CCR5-null) muizen. In het galwegligatie model, leidde CCR5 deficintie tot een significant verminderde rekrutering van T_{Regs} naar de lever met een ongewijzigde Th-17 imuunrespons. Dit resulteerde in een verstoord evenwicht van het Th-17/ T_{Regs} ratio met een versterkt Th-17-gemedieerd immuunrespons tot gevolg. Uit voorgaand onderzoek naar ischemie-reperfusie schade van de lever is bekend dat hoge IL-17 waarden bijdragen aan toegenomen leverschade na ischemie-reperfusie. Deze studie suggereert dat een verstoord evenwicht van het Th-17/ T_{Regs} ratio, door een verminderde aanwezigheid van

T_{Regs}, verantwoordelijk zou kunnen zijn voor toegenomen galwegschade en een hogere incidentie van NAS in ontvangers die CCR5 $\triangle$32 dragers zijn. Indirect bevestigt deze studie de rol van het immuunsysteem in de ontwikkeling van NAS na levertransplantatie.

Deel B: Geoxygeneerde machineperfusie: Een potentile strategie om orgaankwaliteit te verbeteren

Machineperfusie is een orgaanpreservatietechniek welke gebruikt wordt als een alternatief voor de klassieke SCS of in combinatie met SCS (4-6). Tijdens machineperfusie worden donorlevers doorspoeld (geperfuseerd) met een geoxygeneerde of niet-geoxygeneerde perfusievloeistof op lichaamstemperatuur; normotherm (37℃), kamertemperatuur; subnormotherm ($\pm$21℃) of koud; hypotherm (0-10℃) (7-9). De meeste machineperfusie studies van donorlevers hebben zich geconcentreerd op de preservatie van het leverparenchym (7, 8), maar er is onvoldoende informative over het effect van machineperfusie op de preservatie van de galwegen. Op den Dries *et al.* demonstreerden in varkenslevers dat hypotherm geoxygeneerde machineperfusie een gunstig alternatief is voor SCS, met betere preservatie van de peribiliaire vasculaire plexus, hoewel het verlies van galwegepitheel niet kon worden voorkomen (10). Een alternatief voor hypotherme machineperfusie is het perfuseren van de lever op lichaamstemperatuur, waarbij de lever wordt voorzien van complete metabole ondersteuning met zuurstof en voedingstoffen.
In **hoofdstuk 6** bestudeerden we de impact van normotherme geoxygeneerde machine perfusie (NMP) op galwegpreservatie in zowel DCD (donation after cardiac death) als non-DCD (donatie zonder voorafgaande circulatiestilstand) rattenlevers. Levers werden 3 uren gepreserveerd met SCS of NMP, gevolgd door 2 uren *ex vivo* reperfusie. Vergeleken met de klassieke SCS, resulteerde NMP in superieure preservatie van galwegepitheel morfologie en -functie. Dit gunstige effect was met name uitgesproken in de DCD donorlevers. Door het verminderen van galwegschade kan NMP een grote impact hebben op het gebruik van DCD donorlevers en zodoende de uitkomsten na transplantatie verbeteren. Deze bevindingen zijn een belangrijke stimulans om een klinische studie naar NMP in humane DCD levertransplantatie op te zetten. Het is gedemonstreerd dat NMP van humane levers technisch mogelijk is (11).
Hoofdstuk 7 beschrijft een stap-voor-stap protocol voor *ex-situ* normotherme machineperfusie van humane donorlevers. Het perfusiesysteem dat werd gebruikt voorziet de lever van een druk- en temperatuurgereguleerde pulserende doorbloeding van de arterie hepatica en een continue doorbloeding van de vena porta. De perfusievloeistof werd van zuurstof voorzien door twee kunstmatige longen en perfusie is mogelijk op verschillende temperaturen (10-37℃). De mogelijkheid om donorlevers op verschillende temperaturen te kunnen perfuseren en vloeistoffen/geneesmiddelen te kunnen toevoegen aan de perfusievloeistof, biedt de mogelijkheid tot *ex vivo* testen en mogelijk verbeteren van de leverfunctie vr transplantatie. De verbetering van orgaankwaliteit zou kunnen leiden tot een uitbreiding van het aantal voor transplantatie beschikbare donororganen.
n van de grote voordelen van NMP is de mogelijkheid tot het *ex vivo* testen van subopti-

male donorlevers die, gebaseerd op de huidige klinische criteria, niet in aanmerking komen voor transplantatie (in verband met een te groot risico op vroeg transplantaatfalen). Om machineperfusie klinisch te kunnen gebruiken voor het selecteren van transplanteerbare organen, is het noodzakelijk dat er markers worden gedentificeerd die tijdens machineperfusie adequaat de leverfunctie na transplantatie kunnen voorspellen. Wanneer NMP aan het einde van een periode van koude ischemie (SCS) wordt uitgevoerd, zou het idealiter mogelijk moeten zijn om de leverkwaliteit te onderzoeken en te voorspellen binnen 1-3 uur perfusietijd. Op deze manier zal er voldoende tijd overblijven voor het selecteren en voorbereiden van een ontvanger.

In **hoofdstuk 8** hebben we 12 afgekeurde levers gevalueerd voor transplantaatfunctie na 6 uur NMP. Zes levers vertoonden excellente leverfunctie en de andere zes levers lieten duidelijke tekenen van schade en disfunctie zien. Galproductie na 2,5 uur NMP werd in deze studie gedentificeerd als de enige 100% discriminerende voorspeller van transplantaatfunctie na 6 uur NMP. Concluderend kan gezegd worden dat normotherme machineperfusie van "extended criteria donor" (ECD) levers de mogelijkheid biedt om donorlevers te testen vr transplantatie. Dit creert nieuwe kansen voor donororgaan selectie, therapeutische interventies en preconditionering. Naast het verbeteren van donororgaankwaliteit en functie kan dit leiden tot een uitbreiding van het aantal voor transplantatie beschikbare donororganen.

NMP heeft veel voordelen ten opzichte van alternatieve machineperfusie methoden, maar het is tegelijkertijd een stuk complexer dan machineperfusie op een lagere temperatuur, zoals hypotherme machineperfusie. De metabolische ondersteuning die een lever nodig heeft om goed te functioneren op lichaamstemperatuur is relatief ingewikkeld, daarnaast is er een risico op direct significante leverschade in het geval van een technisch probleem met het systeem. Om de machineperfusie techniek in de (klinische) praktijk te kunnen brengen is logistieke eenvoudigheid en veiligheid noodzakelijk. Hypotherm geoxygeneerde machineperfusie aan het einde van de SCS is een alternatieve benadering en het staat een veilig transport van de donorlever naar het transplantatiecentrum toe, waarbij het tegelijkertijd zorgt voor ATP herstel na SCS (6, 12).

In **hoofdstuk 9** wordt de werkzaamheid van eind-ischemische geoxygeneerde hypotherme machineperfusie van humane donorlevers besproken. Zes humane DCD-donorlevers (afgekeurd voor transplantatie) ondergingen 2 uur HMP gevolgd door 6 uur *ex-situ* NMP (waarin hepatobiliaire functies werden getest), werden vergeleken met 12 DCD-donorlevers die 6 uur *ex-situ* NMP ondergingen zonder voorafgaande HMP. HMP zorgde voor een 15-voudige toename van ATP in het leverweefsel. De levers die HMP hadden ondergaan demonstreerden significant verbeterde hepatobiliair functies tijdens de daaropvolgende 6 uur NMP, vergeleken met de levers die geen HMP hadden ondergaan. ATP in het leverweefsel en de cumulatieve galproductie tijdens NMP was significant hoger in de levers die voorafgaand HMP hadden ondergaan. Deze studie liet zien dat een (relatief korte) periode van 2 uur hypotherme geoxygeneerde perfusie van donorlevers, na de traditionele koude preservatie methode, de cellulaire energieniveaus herstelt wat resulteert in minder schade en een verbeterd hepatobiliair functioneren tijdens het daaropvolgende NMP. Dit is de eerste studie die erop wijst dat eind-ischemische HMP de functionaliteit en transplanteerbaarheid van humane DCD-levers verbeterd.

De klinische toepassing van eind-ischemische hypotherme geoxygeneerde machineperfusie (DHOPE) in humane DCD-donorlevers wordt beschreven in **hoofdstuk 10**. Tien achtereenvolgende patinten ($\geq$18 jaar) die een DCD levertransplantatie ondergingen in het Universitair Medisch Centrum Groningen (UMCG) werden gencludeerd in de studie. Nadat de DCD-levers in het transplantatiecentrum aankwamen, ondergingen ze 2 uur DHOPE waarna de lever werd geplanteerd in de ontvanger. De ontvangers werden gemiddeld 8 maanden na transplantatie gevolgd en vergeleken met een vergelijkbare, historische (match) groep ontvangers die een DCD-lever hadden ontvangen zonder voorafgaande DHOPE. Alle DHOPE-levers functioneerden uitmuntend goed vroeg na transplantatie en leverschade markers zoals ALT was significant lager in vergelijking met de controles. Slechts 1 ontvanger ontwikkelde NAS (10%) in de groep DHOPE-levers. Dit was significant minder dan de 7 ontvangers in de controlegroep die NAS ontwikkelden (35%). Dit is de eerste klinische studie waarin wordt aangetoond dat eind-ischemische DHOPE in DCD-levertransplantatie veilig is, dat het de cellulaire energieniveaus herstelt en reperfusieschade verminderd. Onze data suggereert dat DHOPE de incidentie van NAS na DCD-levertransplantatie kan verminderen. Onze onderzoeksgroep leidt op dit moment een multicentre gerandomiseerde klinische studie om de bovengenoemde bevindingen te valideren.
Concluderend, in dit proefschrift werd de onderliggende pathofysiologie van galwegschade tijdens levertransplantatie bestudeerd, de rol van geoxygeneerde machine perfusie in het verbeteren van orgaankwaliteit voor transplantatie en het voorkomen van galwegschade na transplantatie. Hopelijk leiden de veelbelovende resultaten van dit proefschrift tot een toenemend gebruik van de machineperfusie techniek om de orgaanselectie voor transplantatie te verbeteren.

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