Chapter 4

Machine Perfusion in Liver Transplantation as a Tool to Prevent Non-Anastomotic Biliary Strictures: Rationale, Current Evidence and Future Directions

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ABSTRACT
The high incidence of non-anastomotic biliary strictures (NAS) after transplantation of livers from extended criteria donors is currently a major barrier to widespread use of these organs. This review provides an update on the most recent advances in the understanding of the etiology of NAS. These new insights give reason to believe that machine perfusion can reduce the incidence of NAS after transplantation by providing more protective effects on the biliary tree during preservation of the donor liver. An overview is presented regarding the different endpoints that have been used for assessment of biliary injury and function before and after transplantation, emphasizing on methods used during machine perfusion. The wide spectrum of different approaches to machine perfusion is discussed, including the many different combinations of techniques, temperatures and perfusates at varying time points. In addition, the current understanding of the effect of machine perfusion in relation to biliary injury is reviewed. Finally, we explore directions for future research such as the application of (pharmacological) strategies during machine perfusion to further improve preservation. We stress the great potential of machine perfusion to possibly expand the donor pool by reducing the incidence of NAS in extended criteria organs.
INTRODUCTION

Orthotopic liver transplantation is currently the only available life-saving treatment for patients with end-stage liver disease. Unfortunately, the number of organs needed for transplantation greatly surpasses the supply, prompting strict selection criteria for transplant candidates and long waiting lists for those patients that reach candidate status. As a result of this shortage about 15% of patients die while on the waiting list. Additionally, according to studies of death certificates, about 60,000 patients die of liver disease annually in the United States; many of whom could (theoretically) have been treated with a liver transplant. When only waitlist mortality is considered, the magnitude of the problem of end-stage liver disease is grossly underestimated. In fact, only about 1.5% of liver disease related mortality concerns waitlisted patients.

The grave shortage of livers available for transplantation causes the transplant community to continuously push the boundaries to increase the availability of organs. As a part of this effort, criteria for donor liver selection have been progressively expanded, allowing the usage of more suboptimal or compromised grafts. Livers from donors that fall outside of standard criteria, also known as ‘extended criteria donors (ECDs), are increasingly considered for transplantation during the past decades. For example, in the United Kingdom 42% of transplanted livers now come from donation after circulatory death (DCD) donors, as opposed to donation after brain death (DBD). The most important other criteria that are being widened include donor age, blood type ABO incompatibility, steatosis, and infectious diseases in the donor. The increased acceptance of livers from ECDs in the past two decades has contributed significantly to the expansion of the donor organ pool.

Today, the survival rate of patients that receive a DCD liver graft is approximately similar to DBD recipients. However, the transplantation of DCD liver grafts is associated with around 10% lower 1-year graft survival rate, compared to DBD livers and a markedly higher incidence of biliary complications. The incidence of non-anastomotic biliary strictures (NAS) varies between 4 and 15% after transplantation of DBD livers, but can be as high as 30%-50% after transplantation of DCD grafts. The occurrence of NAS in a donor liver critically impacts patients’ long-term survival, rate of re-transplantation, quality of life and the cost of care. Synonyms for NAS that are frequently used in the literature are ischemic–type biliary lesions and ischemic cholangiopathy. In general, all three names refer to the same clinical entity characterized by a combination of narrowing and dilatations (or even intraparenchymal leakage) of the larger intra- and extrahepatic donor bile ducts, either with or without intraluminal sludge and cast formation, and in the presence of a patent hepatic artery (Figure 1A and B). Severity and distribution of NAS along the biliary tree may vary considerably among patients and clinical symptoms range from no symptoms to recurrent jaundice and/or life-threatening cholangitis with subsequent need for re-transplantation.

NAS is a feared and severe complication in DCD liver transplantation, resulting in a considerable number of donor livers that are currently being rejected for transplantation, while these livers could potentially be used if a protective method against the development of NAS existed. Machine perfusion in liver transplantation as a tool to prevent non-anastomotic biliary strictures
Figure 1. Radiological and histological examples of bile duct injury in a donor liver. (A) Cholangiogram revealing mild non-anastomotic strictures in a donor liver. (B) Cholangiogram showing severe non-anastomotic strictures in a donor liver. (C) Histology of a donor bile duct (hematoxylin & eosin staining) with approximately 50% detachment and loss of the luminal epithelium (lumen indited by #), but well preserved bile duct wall stroma (asterix) and peribiliary glands (dotted lines). (D) Histology of a donor bile duct (hematoxylin & eosin staining) with a well preserved arteriole (arrow) as part of the PVP. (E and F) Histology of a donor bile duct (hematoxylin & eosin staining) with necrosis of bile duct wall stroma (asterix), arteriolonecrosis, and loss of epithelial cells in the peribiliary glands (dotted lines). The arrow indicates a peribiliary gland with a complete loss of epithelial cells.
perfusion is a promising technique that has gained renewed interest in the past decade as a tool to optimize and assess livers for transplantation. Protection of the biliary tree as a specific target of machine perfusion has, however, been underexposed in the literature. In this review we provide an update on the most recent advances in the understanding of the etiology of NAS and present current evidence supporting the hypothesis that machine perfusion reduces bile duct injury during transplantation, providing a tool to reduce the incidence of NAS. Moreover, we provide an overview of endpoints used for evaluation of the biliary tree in the setting of machine perfusion.

**Risk factors and pathogenesis of NAS**

Traditionally, the degree of biliary injury that occurs during and after transplantation was considered to be the main determining factor for NAS. Warm and cold ischemia, reperfusion injury, bile salt toxicity, and immune-mediated injury have been identified as the most important contributors to this multifactorial process of biliary injury. However, three independent histological studies focusing on the common bile duct of donor livers revealed that in 86%-88% of cases the bile duct epithelium is already significantly injured at the end of cold storage. This observation indicates that the bile ducts of almost every donor liver suffer substantial injury during graft preservation, yet only a minority of them develop NAS after transplantation, suggesting that the (lack of) regenerative capacity of the donor bile duct may be more important than the initial degree of injury.

This new concept of the pathogenesis of NAS has incited interest into the mechanisms involved in biliary regeneration. In a study of 128 liver transplants, op den Dries et al have demonstrated a strong association between injury of the peribiliary glands (PBG) and the peribiliary vascular plexus (PVP) at the time of transplantation and the later development of NAS in liver transplant recipients. The PBG are glandular structures that are abundantly present in the wall of the extrahepatic and major intrahepatic bile ducts, which are the most common locations of stricture formation. Moreover, the PBG have been shown to be a niche for progenitor cells that can proliferate upon biliary injury. The fact that i) extensive epithelial regeneration is necessary after transplantation in the majority of liver grafts; ii) proliferation is observed in the PBG upon biliary injury and iii) loss of PBG integrity at the time of transplant is associated to the development of NAS post-transplant strongly suggests that the PBG are a key component required for successful recovery from the extensive and multifactorial biliary injury that occurs during transplantation. Possibly, the absence of a healthy epithelial barrier due to the failure of cholangiocyte regeneration increases (prolonged) bile salt cytotoxicity and inflammation eventually leading to NAS.

The PVP is essential for the viability of the bile duct as it provides blood with nutrients and oxygen to the bile ducts. Blood flow through the PVP is mainly supplied by the hepatic artery and thrombosis of the hepatic artery early after transplantation generally leads to necrosis of the larger donor bile ducts. Even in the presence of a patent artery, dysfunction and preservation injury of the PVP is believed to play a role in the etiology of NAS. These recent findings suggest a critical role of the PVP and the PBG in biliary regeneration and the pathogenesis of NAS. Histology of bile
ducts with various degrees of injury of the luminal biliary epithelium, the PBG, and the PVP are presented in Figure 1C-F. The regenerative capacity of the bile duct may be negatively influenced by known risk factors for NAS, such as donor age and donor warm ischemia (as occurs in DCD donors), as well as toxic bile salts that may not only affect the luminal epithelium, but also the PBG and PVP. Especially when the luminal biliary epithelium is absent, bile salts can easily enter the bile duct wall.

Figure 2. Proposed pathogenesis of non-anastomotic biliary strictures (NAS) after liver transplantation. During warm (in DCD donors) and cold ischemia (in both DCD and DBD livers) bile duct injury occurs almost universally, characterized by injury and loss of the luminal biliary epithelium. This type of injury alone does not necessarily lead to NAS. Critical components of the bile duct wall are the peribiliary glands (PBG) and the peribiliary vascular plexus (PVP). Severe injury to the PBGs and PVP has been associated with the development of NAS suggesting that adequate preservation of these structures is essential for a timely regeneration of the biliary epithelium. After transplantation, biliary bile salts may cause additional damage to the remnant luminal epithelium and diffuse into the bile duct wall, causing injury to the PBG and PVP. Secondary influx of immune cells leads to inflammation with subsequent fibrosis and scarring of the bile ducts resulting in NAS. Machine perfusion of donor livers may reduce the incidence of NAS after transplantation by reducing the amount of injury to the various components of the bile duct wall.
stroma and cause injury to these essential structures. Achieving protection or improved recovery of these critical components of the bile duct wall by machine perfusion could therefore contribute to the prevention of NAS. Here, preventing injury altogether would be the ultimate target, because when regeneration is not needed it also cannot fail. If this cannot be accomplished, protection of the PVP and PBG to facilitate successful regeneration through tissue oxygenation and preservation of progenitor cells in the PBG are obvious subsidiary goals. Not surprisingly, optimal preservation of the PVP and PBG has become an important endpoint in machine perfusion studies.\textsuperscript{38-40} The proposed pathogenesis of NAS and the various targets for machine perfusion are summarized in Figure 2.

\textbf{Assessment of the impact of machine perfusion on the biliary tree}

Accurately assessing the effect of machine perfusion on the biliary tree poses a significant challenge. Biliary function and injury are frequently used as surrogate endpoints for the occurrence of NAS. However, in general the overall degree of biliary injury does not correlate very well with the development of NAS.\textsuperscript{26} Unfortunately, NAS do not easily occur after liver transplantation in rats and therefore this definitive endpoint is only obtainable in transplantation studies of large animals or humans, which are more complex and expensive. Nevertheless, a worthy set of endpoints, based on the current understanding of the etiology of NAS, is available for evaluating the biliary tree in the setting of machine perfusion (Table 1).

As discussed previously, multiple studies have indicated an important role of the PVP and PBG in the etiology of NAS.\textsuperscript{24,27,37} Therefore, the effects of machine perfusion on these structures are of particular relevance. They can be investigated using standard light microscopy and a standardized semi-quantitative scoring system.\textsuperscript{24,25,27} This approach is significantly more objective compared to free-format pathological interpretation and has emerged as an important endpoint.\textsuperscript{38,39} More sophisticated methods such as immunohistochemistry may also be used. For example, Ki67 and CK19 are markers for proliferation in the PBG and the bile duct epithelium respectively.\textsuperscript{32,38,41} The expression of von Willebrand Factor on the vascular endothelium indicates endothelial cell activation that can lead to platelet aggregation in the PVP.\textsuperscript{38}

Bile salt toxicity is known to be another important pillar in the mechanism of NAS.\textsuperscript{18} The epithelial lining forms a specialized barrier to protect the bile duct from the detergent effects of bile. Hepatocytes excrete phospholipids that form mixed micelles, neutralizing the detergent effect of bile salts. When this barrier is impaired by ischemia/reperfusion induced injury or dysfunction, toxic bile salts can cause additional harm to the biliary epithelium, or when this is already absent, to the unprotected sub-mucosal stroma and the deeper located structures such as PBG and PVP.\textsuperscript{33,42} Consequently, the ratio of bile salts to phospholipids in bile during perfusion is an important marker of the endogenous protective mechanisms against bile salt toxicity. The same is true for the biliary concentration of bicarbonate (\(\text{HCO}_3^-\)), which prevents the protonation of biliary glycine-conjugated bile salts and uncontrolled cell entry of the corresponding bile acids through cell membranes.
by raising the pH, a phenomenon known as the “bicarbonate umbrella.” Bicarbonate is actively secreted into the bile by the biliary epithelial cells (or cholangiocytes) and, therefore, biliary bicarbonate concentration can also be used as a biochemical marker of cholangiocyte function. Moreover, disruption or imbalance of bile salt transportation, which is an adenosine 5’-triphosphate (ATP) dependent process, can contribute to injury by the intracellular accumulation of hydrophobic bile salts.

The production of bile involves a cascade of steps that requires good function of all its components, including both hepatocytes and cholangiocytes. Therefore, the rate of bile production during machine perfusion, in combination with the characteristics of its composition, is an excellent

Table 1. Overview of endpoints suitable for studying biliary preservation by machine perfusion

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Variable</th>
<th>Level of evidence*</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-anastomotic biliary strictures (NAS)</td>
<td>ERCP or MRCP (gold standards)</td>
<td>IIa</td>
<td>21,115</td>
</tr>
<tr>
<td></td>
<td>PTCD</td>
<td>IIa</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td>Plastination casting (animal model)</td>
<td>III</td>
<td>117</td>
</tr>
<tr>
<td>Established risk factors for NAS</td>
<td>H&amp;E staining/systematic light microscopic evaluation of common bile duct biopsies at time of transplantation</td>
<td>III</td>
<td>24,27</td>
</tr>
<tr>
<td></td>
<td>PVP injury</td>
<td>III</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Deep PBG injury</td>
<td>III</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Mural stroma necrosis</td>
<td>III</td>
<td>24,25,27</td>
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<tr>
<td></td>
<td>Cholangiocyte loss</td>
<td>III</td>
<td>25</td>
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<tr>
<td></td>
<td>Bile salt / phospholipid ratio in bile</td>
<td>III</td>
<td>33,42</td>
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<tr>
<td></td>
<td>Cholesterol in bile</td>
<td>III</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>Cholangiocyte specific microRNAs</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>In bile</td>
<td>III</td>
<td>42,52</td>
</tr>
<tr>
<td></td>
<td>In preservation fluid</td>
<td>III</td>
<td>118</td>
</tr>
<tr>
<td>Indicators of biliary injury with unknown correlation to NAS</td>
<td>Immunohistochemistry of bile duct biopsies</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Markers of apoptosis</td>
<td>V</td>
<td>109</td>
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<tr>
<td></td>
<td>Ki-67 protein</td>
<td>V</td>
<td>32,38</td>
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<tr>
<td></td>
<td>Von Willebrand Factor</td>
<td>V</td>
<td>38</td>
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<tr>
<td></td>
<td>Loss of cytokeratin 19 (CK19) positive cells</td>
<td>V</td>
<td>41</td>
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<tr>
<td></td>
<td>Cholangiocyte function markers in bile</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Bicarbonate</td>
<td>V</td>
<td>38,50,74</td>
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<tr>
<td></td>
<td>pH</td>
<td>V</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td>V</td>
<td>119</td>
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<tr>
<td></td>
<td>Cholangiocyte injury markers in bile</td>
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<td></td>
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<tr>
<td></td>
<td>Gamma-glutamyltransferase</td>
<td>III</td>
<td>38,50,77,98,120</td>
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<tr>
<td></td>
<td>Lactate dehydrogenase</td>
<td>III</td>
<td>38,50,98,120</td>
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* Level of evidence assessed according to Oxford 2011 levels of evidence.

Abbreviations: ERCP, Endoscopic Retrograde Cholangiopancreatography; MRCP, Magnetic Resonance Cholangiopancreatography; PTCD, percutaneous cholangiography drainage; H&E, hematoxillin and eosin; PVP, peribiliary vascular plexus; PBG, peribiliary gland.
Machine perfusion in liver transplantation as a tool to prevent non-anastomotic biliary strictures

Glucose is initially present in the canalicular bile at a concentration equal to plasma and is subsequently reabsorbed by the cholangiocytes of the bile ducts. A low glucose concentration (< 1.0 mmol/L) in the common bile duct bile, therefore, indicates adequately functioning biliary epithelial cells, and can be used as a target for assessing bile duct integrity during machine perfusion.

Markers of cellular injury, such as lactate dehydrogenase and gamma-glutamyltransferase, can be measured in bile and perfusate. Although they may reflect the degree of biliary injury, it is unknown whether high biliary levels of these injury markers are associated with the later development of NAS. One method that holds promise is the detection of cholangiocyte specific micro RNAs in bile that have been associated with the development of NAS.

Obviously, the incidence of NAS during follow up after transplantation of machine-perfused grafts is the most relevant endpoint in determining the effect of machine perfusion on biliary complications. Randomized clinical trials are needed to elucidate the effect of machine perfusion on biliary complications in humans.

Machine perfusion: a spectrum of techniques
The term ‘Machine perfusion’ is very broad; it covers a spectrum of techniques with mechanical perfusion as their common denominator. Fundamentally, the configuration of machine perfusion depends on three parameters: the timing and duration of its application, and the perfusion temperature. In order to prevent endothelial shear stress, pressure controlled perfusion is considered to be a safer method compared to flow controlled perfusion.

Perfusion through the portal vein only is feasible at low temperatures. But from the perspective of biliary preservation, arterial perfusion and the delivery of oxygen to the bile ducts seems paramount because the biliary system depends mainly on the arterial circulation. Additionally, oxygenated perfusion of the arterial system has been described to attenuate arteriolonecrosis of the PVP and therefore may contribute to preservation of the vasculature to the bile ducts. On the other hand, it should be noted that in contrast to traditional belief, blood is also supplied to the bile ducts by the portal vein and insufficient portal perfusion should also be considered a risk factor for the development of NAS. An argument against cannulation and perfusion of the hepatic artery is the potential risk of causing intima injury or vascular dissection. This risk can be minimized by using a cylindrical aortic segment for cannulation, which can be cut off and discarded at the end of perfusion before implantation.

Machine perfusion can be applied at different time points during the process of procurement, transportation, and transplantation, as summarized in Figure 3. The earliest possible application of machine perfusion in the chain of events is in situ normothermic regional perfusion (NRP) in the donor, immediately after circulatory arrest. It offers the advantage of restoring cellular levels of ATP that have become depleted during the period of warm ischemia by restoration of normothermic circulation in the donor prior to standard cold storage. The technique has been successfully used
Investigators from the Hospital Clinic in Barcelona have reported 42 liver transplant procedures after NRP of DCD donors with a biliary complication rate of 17%. Another group, from the University of Michigan, has described 13 liver transplant procedures using this method with an incidence of biliary complications of 14%. While these outcomes are very hopeful, both studies lacked a control group and were not randomized. Also, in one report 38% of donor livers were discarded because of technical complications, illustrating the complexity of NRP. Randomized controlled trials are necessary to assess the clinical benefits of NRP.

Ex situ machine perfusion of the liver can be performed at the donor site before cold storage (pre-storage perfusion), at the receiving hospital after cold storage prior to implantation (end-ischemic perfusion), or throughout the preservation period (preservation perfusion) (Figure 3). The advantage of pre-storage perfusion is that it has the ability to restore the energy charge of the liver prior to subjecting it to cold ischemia, potentially reducing the synergistic accumulation of injury caused by subsequent warm and cold ischemia. However, accomplishing on-site machine perfusion in donor hospitals is troubled by several logistical challenges.

These challenges are even more pronounced in preservation perfusion, which requires a machine perfusion device that is portable and self-sufficient for the duration of the preservation period. Theoretically, continuous preservation perfusion is the most optimal choice because it eliminates the need for cold storage. Normothermic preservation perfusion has been successfully used in a clinical trial including liver transplantation of ten patients. Although continuous normothermic preservation perfusion may prove to be the most effective preservation method in the future, it is questionable whether complete elimination of cold preservation is necessary and cost-effective in the current era.

End-ischemic perfusion offers the advantage that it can be performed in the environment of the transplant center with all the logistic support needed for successful perfusion. Although logistically appealing, irreversible injury may have occurred already to the liver and the biliary tree, which might have been preventable or treatable upon earlier intervention. Finally, through both

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Figure 3. Schematic presentation of the various dimensions of machine perfusion. Abbreviations: DCD: donation after circulatory death, NRP: normothermic regional perfusion.
preservation perfusion and end-ischemic perfusion, the organ can be optimized immediately prior to implantation, which is not the case for pre-storage perfusion. In general, machine perfusion requires specific surgical training and the direct costs are higher compared to simple cold storage. However, the technique could be cost-effective and even lead to lower overall costs due to shorter postoperative length of stay and reduction of the incidence of NAS, but studies of cost-effectiveness are needed to clarify this. Promisingly, renal machine perfusion has been proven to be cost-effective.\textsuperscript{67,68}

**Temperature**

The cellular rate of respiration is greatly dependent on the temperature, following an exponential curve.\textsuperscript{69} Simple static cold storage at 0-4 °C is based on this principle and reduces the metabolic rate of the liver to about 5% of the physiological level. Because of this relationship, the temperature used during machine perfusion dictates the calibration of most other perfusion parameters that should be optimized to meet the dynamics and metabolic needs of the organ at a given temperature.

**Hypothermic machine perfusion**

Under hypothermic conditions (0-10°C), the use of an acellular perfusion fluid at low pressures is most common. The use of red blood cells or an oxygen carrier is not necessary, because adequate uptake of oxygen into the fluid can be achieved through diffusion at low temperatures.\textsuperscript{70} Hypothermic machine perfusion (HMP) is safe because in case of pump failure, the organ falls back on the ‘default’ conditions of static cold preservation. The low metabolic activity that is associated with hypothermic perfusion conditions also forms a limitation, because it does not allow a functional assessment of the liver. For example, the liver does not produce bile during HMP, which is an important indicator of hepatobiliary function and allows biochemical assessment of biliary markers of biliary epithelial cell function and injury.\textsuperscript{50,71}

A large number of studies using HMP in animal models have been performed; however, few studies have evaluated the effects of HMP on the biliary system.\textsuperscript{53} Using a non-arterialized model of DCD liver transplantation in rats, Schlegel et al\textsuperscript{41} compared one hour of end-ischemic hypothermic oxygenated machine perfusion to cold storage alone. After four weeks of follow up, animals from the HMP group presented with superior histological appearance of the intrahepatic bile ducts and significantly lower serum gamma-glutamyltransferase, alkaline phosphatase and bilirubin.\textsuperscript{41} In another experimental study, our group has compared 4 hours of hypothermic oxygenated perfusion to cold storage of DCD pig livers, followed by 2 hours of normothermic blood reperfusion to simulate transplantation. Although, no differences in biliary epithelial function or injury were observed, oxygenated HMP did lead to superior preservation of the PVP, which may have a positive impact on blood supply to the bile ducts and their regenerative capacity after transplantation.\textsuperscript{39}

Guarerra and coworkers, who were the first to describe successful clinical transplantation of HMP preserved liver grafts in 2010\textsuperscript{54}, recently reported the outcome of the first 31 cases performed
in their center.\textsuperscript{70} Apart from generally improved outcome parameters (shorter length of stay, lower postoperative serum injury markers etc.), the number of biliary strictures in the HMP preserved group was significantly lower compared to matched controls (10\% after HMP vs. 33\% after SCS). It should be noted that the perfusion system used by Guarerra and coworkers does not provide active oxygenation of the perfusion fluid and the duration of perfusion was not standardized. Dutkowski et al\textsuperscript{55} have applied oxygenated HMP, or Hypothermic Oxygenated PErfusion (HOPE), to eight locally procured DCD livers before transplantation and compared the outcomes with matched DBD controls. The rate of biliary complications was identical between the two groups; however, the numbers were too low to draw any robust conclusion on the incidence of biliary strictures. Despite the fact that livers were perfused through the portal vein only and the study lacks a DCD control group, it suggests a possible protective effect of HOPE against bile duct injury. A clinical trial with hypothermic oxygenated dual perfusion of DCD livers (through both the portal vein and hepatic artery) was recently initiated in our center (Netherlands Trial Registry, NTR4493; www.trialregister.nl). Final assessment of the efficacy of HMP (either oxygenated or non-oxygenated) in reducing biliary injury and subsequent NAS should come from large multi-center randomized clinical trials. Several initiatives to organize such trials have been taken (i.e. ClinicalTrials.gov, ID: NCT01317342).

\textit{Subnormothermic machine perfusion}

Subnormothermic machine perfusion (SNMP, 20–30 °C) is a middle-of-the-road approach between HMP and normothermic machine perfusion (37 °C, NMP).\textsuperscript{73} An advantage of SNMP is its relative simplicity compared to NMP. By inducing a metabolic rate of approximately 25\% of physiological levels, it allows for functional assessment of the liver.\textsuperscript{74} To prevent depletion of nutrients, SMP requires the use of a nutrient enriched perfusate. Surprisingly, an oxygen carrier is not always used; SMP has been performed both with and without red blood cells or an artificial oxygen carrier.\textsuperscript{73,75,76} The question whether the use of an oxygen carrier at subnormothermic temperatures is essential, therefore, remains a matter of debate and this issue requires further research.

SNMP has been tested in rats with positive effects on bile production and decreased release of biliary enzymes.\textsuperscript{77,78} Using a hepatocyte cell culture medium as a perfusate, Bruinsma et al\textsuperscript{74} performed SNMP of seven human livers that were declined for transplantation. During 3 h of end-ischemic SNMP, increasing bile flow and biliary bicarbonate excretion were seen. Moreover, the ratio of bile salts to phospholipids in bile decreased over time, indicating improvement of hepatocellular secretory function and a decrease in bile toxicity. Unfortunately, this study had no control group or reperfusion phase; therefore, it provides little substantiation for SNMP beyond proof of feasibility.\textsuperscript{74}

Knaak et al\textsuperscript{79} have performed liver transplantation after SNMP of DCD pig livers, comparing it to static cold storage. The three-hour perfusion phase in this experiment, using Steen's solution with washed red blood cells at 33 °C, was preceded by four hours, and followed by three hours of cold ischemia time. The investigators first studied bile output of the liver during eight hours of in situ reperfusion. Subsequently, ten animals (five vs. five) were transplanted and monitored for
seven days. Lactate dehydrogenase in the bile was found to be significantly lower immediately after transplantation in the SNMP group. Moreover, cold stored grafts presented with substantial bile duct necrosis after seven days, but those that preserved with SNMP did not. This was also reflected in the serum levels of alkaline phosphatase during the survival period, which were significantly higher in the cold storage group. This study indicates that SNMP has protective effects on the bile ducts in a DCD model of pig liver transplantation.

**Normothermic machine perfusion**

Under normothermic conditions, the liver reaches a physiological metabolic rate and functionality. Sufficient oxygen delivery can only be achieved with the use of an oxygen carrier. This form of machine perfusion has been studied extensively in experimental models by the group of Friend and coworkers\(^{66,71,80,81}\) and enables ex situ viability testing of the liver.\(^{50}\) Some argue that it is superior to cold perfusion because it provides the closest resemblance to the normal physiological situation in vivo.\(^{82}\) However, bringing the organ up to ‘full metabolic speed’ also makes it more vulnerable. Interruption of perfusion or insufficient oxygen delivery will immediately expose the liver to warm ischemia. Remaining mindful of this risk, NMP can be safe and reliable when performed correctly.\(^{83,84,85,86}\)

NMP appears to be the category of perfusion that provides the most pronounced effects on biliary preservation. In an interesting experiment using pig DCD livers, Liu et al\(^{38}\) assessed the effect of 10 h of NMP on biliary injury and regeneration during 24 h of ex vivo reperfusion. The study showed lower release of injury markers (gamma-glutamyltransferase and lactate dehydrogenase) in bile and increased biliary bicarbonate secretion in NMP preserved livers, compared to livers that underwent conventional 10 h of static cold storage. Moreover, NMP preserved livers displayed significantly less histological injury of the PBG and PVP, produced more bile, and demonstrated lower thrombogenicity of the microvasculature. Additionally, using Ki-67 staining, these investigators observed proliferation of cholangiocytes during 24 h of reperfusion of NMP preserved grafts, which was not seen in livers preserved by static cold storage. This study provides important evidence that 10 h of NMP yields superior results with regard to biliary injury and regeneration, compared to static cold storage.\(^{38}\) It should however, be noted that this study did not include actual transplantation of machine-perfused livers and, therefore, the occurrence of NAS after transplantation was not assessed.

Boehnert et al\(^{87}\) have published an experimental study showing extraordinary biliary preservation by NMP in a DCD pig model. Although their experiment offers new perspectives on NMP, such as through the use of CT angiography, this study has been criticized for its design and difficulty to explain the outcomes.\(^{88,89}\) Our group was the first to publish on the feasibility of NMP in four discarded human livers using six hours of perfusion in an end-ischemic approach.\(^{50}\) During the six hours of NMP, bile duct histology remained well preserved and did not deteriorate. After expanding this series to a total of 12 livers, we found that bile production during NMP can be used
as a predictor of graft function, enabling viability testing. This finding is in line with previous animal studies of NMP, which have recognized bile production as a critical parameter of viability of the perfused liver.

Several (randomized) clinical trials have been initiated recently and hopefully these studies will provide evidence for the beneficial effects of machine perfusion on the incidence of biliary complications after liver transplantation. In one randomized controlled multicenter trial normothermic preservation perfusion will be compared with static cold storage (http://www.controlled-trials.com/ISRCTN39731134/). Another multicenter trial is investigating the possible protective effects of normothermic regional perfusion in DCD donors (http://www.controlled-trials.com/ISRCTN89667087/).

**Controlled rewarming**

The abrupt change in temperature that occurs upon reperfusion during transplantation of a hypothermic liver is believed to contribute to reperfusion injury. Many research groups have explored machine perfusion strategies at a constant temperature, still exposing the liver to sudden temperature changes. Minor *et al* studied the effect of controlled oxygenated rewarming (COR) of the liver from ice-cold to subnormothermic temperatures during perfusion and compared this method with HMP and SNP alone. In this experiment, pig livers were harvested and preserved by cold storage for 18 h, followed by 90 min of SNP, HMP or COR. The organs where subsequently re-perfused ex vivo normothermically with autologous blood to simulate transplantation. Energy charge restoration during COR was similar to SMP, but superior to HMP. In addition, COR was associated with significantly better hepatobiliary function (as indicated by bile production) and less injury upon reperfusion. A group from Tokyo has described similar results. Integrating controlled rewarming into perfusion protocols could potentially combine the best of cold and warm perfusion techniques and may potentially provide better protection of the bile ducts.

**Perfusate composition**

The composition of the perfusion fluid is guided primarily by the temperature, timing and duration of machine perfusion. Whereas at low temperatures a low-nutrient preservation solution may be used, increasing duration and temperature of perfusion necessitates more additives. Irrespective of these factors, oxygen delivery through the perfusate is a fundamental pillar of the advantages that machine perfusion has to offer to both the liver and the bile ducts. When oxygen consumption exceeds the delivery potential of dissolved oxygen in a watery fluid, red blood cells or an artificial oxygen carrier should be added. Because the hepatobiliary transporters that are responsible for bile formation are calcium dependent, it is important to titrate calcium to physiological levels for the optimization of biliary secretory function. This is especially true when blood products are added to the perfusate, which usually contain calcium-scavenging agents such as citrate. Bile salts excreted by the liver during machine perfusion do no return to the perfusate, which normally
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happens through the enterohepatic circulation in vivo. Supplementation of bile salts is therefore necessary when applying machine perfusion for extended periods of time to ensure sustained bile production.\textsuperscript{71} Another relevant aspect is the viscosity of the solution. Several studies have indicated that low-viscosity solutions may achieve superior perfusion of the peribiliary vasculature.\textsuperscript{99-102} However, the effect of low-viscosity cold flush solutions on biliary complications is controversial.\textsuperscript{103} Other constituents of machine perfusion solutions may be oncotic agents (e.g. albumin), parenteral nutrition, antibiotics, insulin, heparin, and vasoactive drugs.\textsuperscript{38,50,104} Machine perfusion also opens alleyways towards ex situ treatment of the liver to enhance the preservation even further. As these techniques are still speculative, they will be discussed as ‘future directions’.

Many combinations of perfusion temperatures, perfusates, time points and durations have been explored. For a more detailed overview of particular configurations, we suggest further reading of specific reviews.\textsuperscript{53,105-108} Which combination of these parameters provides optimal protection for the biliary tree currently remains unclear.

**Future directions**

Machine perfusion provides a platform that may facilitate the treatment of donor livers, uncovering specific targets that previously remained out of reach for the transplant surgeon. One possibility that quickly comes to mind is the addition of pharmacological agents to the perfusion fluid. Such interventions could be specifically targeted at factors that have been shown to play a role in the pathogenesis of NAS, such as bile composition, immunological factors, bicarbonate secretion, or even factors that may stimulate biliary epithelial regeneration.

With the aim of reducing the Kupffer cell-mediated insult to the liver after transplantation, one group has administered gadolinium chloride (GdCl\textsubscript{3}) to rats in an autologous rat transplant model. This intervention reduced the amount of apoptosis observed in the bile duct after warm ischemia/reperfusion injury.\textsuperscript{109} Another prospect could be the addition of hydrophilic bile salts or other compounds to stimulate a less cytotoxic bile composition.\textsuperscript{71}

Finally, it has been proposed that the infusion of mesenchymal stem cells (MSCs) into the perfusion circuit could help restore a donor organ.\textsuperscript{110} Although the exact mechanisms are unknown, MSCs are believed to have anti-inflammatory and pro-regenerative effects mostly through the excretion of paracrine factors without requiring colonization.\textsuperscript{111,112} The clinical safety of MSC infusion in liver transplant recipients has been suggested, but the efficacy is still strongly debated.\textsuperscript{113,114} Machine perfusion provides an exceptional opportunity for the ex situ application of MSCs.

**Conclusion**

Liver machine perfusion comprises a spectrum of promising techniques of liver graft preservation that are currently making the transition into clinical practice. It is anticipated that the use of machine perfusion can lead to a significant increase of the availability of transplant livers as well as a reduction in postoperative complications such as early graft dysfunction and biliary complications.
Existing data from animal models and preliminary data from (discarded) human donor livers provide promising evidence that machine perfusion has relevant protective effects on the bile ducts. In animal studies, liver machine perfusion did not only reduce the amount of injury to the bile ducts, but it also helped to restore the endogenous regenerative capacity of the biliary epithelium and bile duct wall. Whether this results in a decreased incidence of biliary complications, such as NAS, after clinical transplantation needs to be awaited. Randomized clinical trials are being initiated and will be necessary to demonstrate the efficacy of machine perfusion in clinical transplantation. Finally, machine perfusion provides a platform for the exploration of putative ex situ treatments, using interventions that are aimed at specific targets in the liver and the biliary system.

**Key points**

- Non-anastomotic biliary strictures (NAS) are a major barrier for the widespread use of extended-criteria donor livers.

- Considerable progress has been made in the understanding of NAS.

- Surrogate endpoints have been identified to study the protective effect of machine perfusion on the biliary tree.

- Machine perfusion comprises a spectrum of techniques; different forms of machine perfusion offer protective effects on the biliary tree and may prevent NAS.

- Machine perfusion holds great promise to expand the donor pool by improving the quality of extended criteria donor livers and reducing the incidence of NAS.
References


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