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

The incidence, pathogenesis and management of the most common biliary complications are summarized, with an emphasis on non-anastomotic biliary strictures (NAS) and potential strategies to prevent NAS after liver transplantation. NAS have variable presentations in time and localization, suggesting various underlying pathogeneses. Early-onset NAS (presentation within 1 year) have shown to be largely related to ischemia-induced bile duct injury, whereas late-onset NAS (≥1 year after orthotopic liver transplantation (OLT)) have more immune-mediated causes. Cytotoxic hydrophobic bile salts and impaired biliary HCO$_3^-$ secretion may also play a role in the occurrence of NAS. Recently, insufficient biliary epithelial regeneration capacity after transplantation has also been suggested to play a major role in the pathogenesis of NAS. A potential strategy to prevent NAS has been proposed to be preservation by machine perfusion instead of classical static cold storage. Although machine perfusion has been shown to be a better preservation method for the liver parenchyma, efficacy in preventing ischemic injury of the biliary epithelium is largely unknown. The potential advantages of machine perfusion are very promising as it may provide better protection of the vulnerable bile ducts against ischemiareperfusion injury. Clinical trials will be needed to demonstrate the impact of machine perfusion in reducing the incidence of biliary complications, especially NAS, after OLT.
INTRODUCTION

Despite an increase in the number of donor livers obtained from donation after cardiac death (DCD) in some European countries and the USA in the recent years, donation after brain death (DBD) remains to be the major source of organs for orthotopic liver transplantation (OLT) in most western countries. In general, patient and graft survival rates after DBD liver transplantation are higher compared with DCD liver transplantation, mainly because of an increased rate of biliary complications after DCD liver transplantation (1,2). However, biliary complications are also the most frequent and troublesome complication after DBD liver transplantations. Biliary complications occur in 10-40% of patients and have an important impact on graft survival, hospital readmissions, the need for re-interventions and the overall costs of care (3-5). Among the variety of biliary complications occurring after OLT, bile leakage and bile duct strictures (anastomotic and nonanastomotic) are the most common. Relative minor (and infrequent) biliary complications, such as sphincter of Oddi dysfunction, external compression by peribiliary cysts, neuma or lymphomas, have also been reported (6,7). An overview of the various types of biliary complications, their incidence and management is provided in Table 1.

In this review, the most common biliary complications will be discussed with an emphasis on the pathogenesis of nonanastomotic biliary strictures (NAS) and on the potential preventive role of oxygenated machine perfusion as a potential strategy to reduce the incidence of NAS after OLT. In this review, we have used the term “NAS” for biliary strictures that can be found in the intrahepatic or extrahepatic bile ducts of the donor liver in the presence of a patent hepatic artery. Ischemic-type biliary lesions or strictures and ischemic cholangiopathy are also used in the literature for this type of injury.

BILE LEAKAGE

Bile leakage has been reported in 2-21% of the patients after OLT. It can be categorized as early and late events, and may be anastomotic or non-anastomotic in site, that is, occurring at the T-tube tract exit site or at the cut surface of the graft in case of living donor or partial liver graft transplantation (5-7). Bile leakage can later result in biloma because of extravasation of bile into the hepatic parenchyma or the abdominal cavity. Depending on the size of the leakage and the patient being asymptomatic or symptomatic, bile leaks can be managed conservatively, nonsurgically or surgically(8,9).

BILE DUCT STRICTURES (ANASTOMOTIC AND NONANASTOMOTIC)

Bile duct strictures can be categorized into anastomotic strictures and nonanastomotic strictures (NAS). A combined presentation of both anastomotic strictures and NAS is not uncommon. Solitary strictures at the site of biliary anastomosis have been reported in 9-12% of patients after liver transplantation (10-12) , with the majority occurring within the first 12 months.
### Table 1: Biliary Complications after Liver Transplantation

<table>
<thead>
<tr>
<th>Complication</th>
<th>Incidence</th>
<th>Management</th>
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<tbody>
<tr>
<td>Bile duct stricturesa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anastomotic</td>
<td>9-12%</td>
<td>Endoscopic/Percutaneous dilatation or stenting</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Roux-en-Y hepatico-jejunostomy</td>
</tr>
<tr>
<td>Non-anastomotic</td>
<td>1-30%</td>
<td>Endoscopic dilatation/stenting</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PTC drainage/ dilatation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Surgical resection of extrahepatic bile duct +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Roux-en-Y hepatico-jejunostomy</td>
</tr>
<tr>
<td>Bile Leakage</td>
<td>2-21%</td>
<td>Conservative therapy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PTC drainage</td>
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<tr>
<td></td>
<td></td>
<td>Endoscopic stenting/sphinctrotomy</td>
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<tr>
<td></td>
<td></td>
<td>Nasobiliary drainage</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hepatico-jejunostomy</td>
</tr>
<tr>
<td>Bacterial cholangitis</td>
<td>10%</td>
<td>Antibiotic therapy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treat strictures if present</td>
</tr>
<tr>
<td>Common bile duct filling defects</td>
<td>5-70%</td>
<td>Endoscopic removal</td>
</tr>
<tr>
<td>Stones/Casts/ Sludge</td>
<td>0-7%</td>
<td>Endoscopic sphinctrotomy/stenting</td>
</tr>
<tr>
<td>Sphincter of Oddi dysfunction</td>
<td></td>
<td></td>
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<tr>
<td>External compression of biliary tract</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cystic duct mucocele</td>
<td>Rare</td>
<td>Surgical excision of cystic duct remnant</td>
</tr>
<tr>
<td>Periductal lymphoma</td>
<td>Rare</td>
<td>Treat underlying lymphoma + endoscopic stenting</td>
</tr>
<tr>
<td>Periductal neuroma</td>
<td>Rare</td>
<td>Amputation neuroma</td>
</tr>
<tr>
<td>Kinking of redundant bile duct</td>
<td>Rare</td>
<td>Surgical resection and re-anastomosing</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Endoscopic stenting</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Roux-en-Y hepatico-jejunostomy</td>
</tr>
</tbody>
</table>

Abbreviations used: PTC: percutaneous transhepatic cholangiography

after transplantation (5,10-12). Major predictive risk factors for development of anastomotic strictures include donor age, prior anastomotic bile leak, duct-to-duct anastomosis and sex mismatch with a female donor male recipient (5,10). Local ischemia caused by the surgical techniques is believed to be the main underlying mechanism, leading to fibrotic scarring of anastomosis. To minimize the risk of local ischemia at the end of donor choledochal duct, the bile duct should remain surrounded by sufficient amount of tissue (3). Uncomplicated anastomotic strictures can generally be treated successfully with endoscopic or percutaneous dilatation and stenting (13,14). Resection of the stenotic anastomosis and construction of a Roux-en-Y hepaticojejunostomy is indicated if nonsurgical treatment fails (10,15). Recurrent anastomotic strictures occurring in approximately 20% of patients can still be treated effectively with dilatation and stenting (16).

In contrast to the anastomotic strictures, NAS may occur at any location of the biliary tree (extra-hepatic as well as intrahepatic) but is usually limited to large bile ducts in a multi-
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focal pattern. The reported incidence of NAS in patients receiving a DBD liver graft varies between 1 and 20% (11,17,18). Being frequently therapy resistant, NAS remain to be the most challenging biliary complication. Because of poor prognosis of NAS, nonsurgical interventional treatments such as endoscopic dilatation and stenting, percutaneous transhepatic cholangiography drainage and dilatation are usually considered. In case of extrahepatic NAS, surgical resection of the diseased part and construction with a hepaticojejunostomy can be successful(4,6). In up to 50% of the patients with NAS, retransplantation may be the only treatment option(4). Therefore, NAS are the most troublesome complication after OLT and are associated with high rates of morbidity, mortality and overall costs of care (19). In the current shortage of available donor livers for OLT, a better understanding of pathogenesis and better strategies to prevent NAS after transplantation are needed.

VARIABLE PRESENTATIONS OF NONANASTOMOTIC BILIARY STRICTURES

Presentations of NAS vary extensively in anatomical localization and severity as well as in time of occurrence. Only 50% of the cases of NAS present within the first year after OLT, constituting early-onset versus late-onset (>1 year after OLT) NAS (20). Early-onset NAS have shown to occur mostly around the hepatic bifurcation and common bile duct. In contrast to early-onset NAS, late-onset NAS are more frequently identified in the periphery of the liver, affecting small bile ducts in a more diffuse pattern(20). It seems that early-onset and late-onset NAS not only develop at different anatomical locations, but also are associated with different risk factors. Retrospective studies have suggested that early- onset NAS are prominently associated with ischemia-related injury, whereas late-onset NAS have been more frequently associated immunological factors(20,21).

VARIABLE PATHOGENESIS OF NONANASTOMOTIC BILIARY STRICTURES

NAS are thought to have a multifactorial cause, which can be grouped based on three relevant types of biliary injury: ischemia-related injury, immune- mediated injury and cytotoxic injury caused by hydrophobic bile salts (3,22).

Ischemia-related injury
Prolonged ischemia time (>13 h) during static cold storage (SCS) of the graft has been identified as a main risk factor for the development of biliary strictures (17,23). However, to reduce the detrimental impact of cold ischemia, the period of SCS has nowadays become shorter with an average of 810 h (20). Oxygenation of biliary epithelial cells (cholangiocytes) is fully dependent on the hepatic artery, in contrast to the hepatocytes, which are oxygenated via both the hepatic artery and the portal vein. Hepatic artery thrombosis and stenosis is, therefore, very detrimental for cholangiocytes and the bile duct wall stroma, and may result in the development of massive bile duct necrosis after transplantation (24). Moreover, injury of the peribiliary microvasculature has also been shown to have an important role in the occurrence of NAS after
transplantation (25). Thus, adequate flush out of the graft and the peribiliary vasculature with preservation fluid is crucial for effective preservation of microvasculature and subsequently the bile duct. Some studies have suggested that liver preservation with high-viscosity preservation fluids may lead to higher rates of biliary strictures, because of insufficient flush out and preservation of the peribiliary microvasculature (26). However, it seems that per- fusion with low-viscosity preservation fluids such as histidinetryptophanketoglutarate solution alone is not sufficient for effective perfusion of the peribiliary microvasculature (27). Additional arterial back-table pressure perfusion may be, therefore, essential for ample perfusion of the peribiliary microvasculature and decreased occurrence of NAS after OLT as consequence (27,28). Another well-known risk factor for the development of NAS is warm ischemia during organ procurement, especially during the period between cardiac arrest and cold perfusion in case of a DCD procedure. The incidence of NAS after transplantation of livers from DCD donors is as high as 30%, which is much higher than the incidence of NAS after DBD liver transplantation (1-20%) (18,29,30).

The method of graft reperfusion during implantation has also been shown to have an impact on the occurrence of NAS. Retrograde reperfusion via the inferior vena cava while constructing the portal vein anastomosis has been associated with an increased risk of developing NAS, probably because of the relative warm ischemia of the bile ducts during implantation (31). Some investigators have advocated arterial reperfusion instead of portal vein reperfusion or simultaneous reperfusion via the portal vein and hepatic artery instead of sequential reperfusion (32); however, there is no strong evidence supporting any of these strategies and initial reperfusion via the portal vein is mostly preferred because of technical simplicity (33).

**Immune-mediated injury**

Many studies have suggested a role for immunological factors in the development of NAS. For example, transplantation of ABO incompatible donor livers has shown to be associated with higher incidence of NAS (34). The reason for this immune- mediated biliary injury is thought to be the existence of blood-type antigens on the biliary epithelium of the donor bile ducts, therefore biliary epithelium cells could be targeted by ABO antibodies (34,35). Moreover, pre-existing autoimmune diseases such as primary sclerosing cholangitis (PSC) are associated with a higher incidence of NAS (36). Additional evidence for immune-mediated injury of the bile ducts as a mechanism leading to NAS is based on the strong association between a loss-of- function mutation in the chemokine receptor CCR5 (CCR5Δ32) in recipients and a high incidence of NAS (37,38). CCR5 has an important role in chemotaxis of regulatory T cells and biliary epithelial cells express its ligands in the liver. CCR5Δ32 polymorphism has been associated with functional immune system changes, including impaired attraction of regulatory T cells. The association between the occurrence of NAS and CCR5Δ32 polymorphism provides evidence on the role of immune system in development of NAS (37,38). The risk of developing NAS is even higher in patients transplanted for PSC who also have a CCR5Δ32 mutation (3).
Cytotoxic hydrophobic bile salts
In contrast to hydrophilic bile salts, which are cyto-protective, hydrophobic bile salts may cause injury to hepatocytes and cholangiocytes because of their detergent properties. The role of hydrophobic bile salts in the occurrence of NAS after transplantation can be explained through the following mechanisms: insufficient flush out of bile from the bile ducts during organ procurement; altered bile composition after transplantation, leading to a relatively high biliary bile salt to phospholipid ratio; intracellular accumulation of hydrophobic bile salts because of a disturbed cholehepatic shunt after transplantation and impaired biliary HCO$_3^-$ secretion after transplantation. Studies have shown that early after transplantation, the composition of bile changes toward higher ratio between hydrophobic bile salts and phospholipids(39). This may be explained by differed expression and function of the transporters bile salt export pump (BSEP) and multidrug resistance protein 3 (MDR3), which results in higher bile salt and phospholipid ratio in the bile. Disturbed protective micelle formation by a lower concentration of phospholipids allows injury of the cholangiocytes by hydrophobic bile salts, resulting in increased incidence of NAS (40).

In addition to the elevated bile salt to phospholipid ration, reuptake of bile acids by cholangiocytes as part of the cholehepatic shunt may be disturbed after transplantation, causing accumulation of toxic bile salts inside cholangiocytes. This phenomenon has been explained by the disbalance in function and expression of transporters responsible for reuptake of bile salts by cholangiocytes from the bile (apical sodium-dependent bile acid transporter or ASBT) and the basolateral transporters responsible for secretion of bile salts into the peribiliary vascular plexus (organic solute and steroid transporters OSTa and OSTb). This could lead to cellular accumulation of the hydrophobic bile salts and subsequent intracellular injury of cholangiocytes, in particular, in the large bile ducts (3).

Experimental studies have shown that biliary HCO$_3^-$ secretion prevents bile-salt-induced cholangiocyte injury by alkalizing the bile close to the apical membrane of the cholangiocytes, leading to deprotonation of hydrophobic bile salts. This protects the cells against penetration of toxic bile salts in the cholangiocytes (41). The main transporters involved in biliary bicarbonate secretion are the cholangiocyte Cl$^-$/HCO$_3^-$ exchanger AE2 and transporter cystic fibrosis transmembrane conductance regulator (CFTR), which are ATP dependent. Not only ATP levels but also the expression of these transporters are disturbed because of ischemia during transplantation, which will lead to a diminished function of biliary “HCO$_3^-$ umbrella” (42).

LACK OF REGENERATION: A NEW CONCEPT OF PATHOGENESIS OF NONANASTOMOTIC BILIARY STRICTURES

Recent retrospective studies on histological examinations of biopsies taken from the distal end of extrahepatic bile duct at the time of transplantation have shown signs of severe injury characterized by biliary epithelial loss, mural stroma necrosis, and injury of the peribiliary vasculature (25,43). Despite the universal biliary epithelial injury of large bile ducts of donor livers (about 90%), only a minority (up to 15% in DBD liver grafts) of recipients develop NAS
after transplantation. This has brought up a new concept of pathogenesis of NAS based on insufficient regeneration and repair capacity of the biliary epithelium after transplantation in certain livers (44). More studies will be needed to investigate better the true relevance of this new finding and examine whether stimulation of the regenerative capacity of the biliary epithelium may result in a reduction of NAS.

**OXYGENATED MACHINE PERFUSION: A STRATEGY TO REDUCE NONANASTOMOTIC BILIARY STRICTURES?**

NAS are associated with high rates of morbidity, mortality and subsequently higher overall costs of care after OLT. It is, therefore, necessary to develop new strategies to reduce the occurrence of NAS. The potential strategies can be defined as reduction of biliary epithelial cell injury by reduction of ischemia-induced injury; reduction of cold-induced injury; enhancement of endogenous protective mechanisms (e.g. stimulation of HCO$_3^-$ umbrella and increase the bile flow) and stimulation of regenerative strategies.

Recently, machine perfusion was shown to be a more powerful alternative as preservation method than the current method SCS (45). During machine perfusion, livers are perfused with an oxygenated (46) or non-oxygenated perfusion fluid (47) at either low temperature or normal body temperature. A relatively simple technique to revitalize grafts after the cold ischemia caused by SCS is end-ischemic hypothermic oxygenated machine perfusion (perfusion at 10°C) (48,49). A couple of studies have demonstrated that oxygenated machine perfusion can help organ recovery by improving cellular energy homeostasis. The restoration of mitochondrial function by oxygenated machine perfusion will result in increased intracellular concentration of ATP concentration and a reduction of the oxygen debt. This has been associated with less formation of reactive oxygen species after graft reperfusion and subsequent less cellular injury (48-51). Subnormothermic oxygenated machine perfusion (20°C) has also shown to have a beneficial role in recovery of the liver graft after transplantation (52). In all the above techniques, the metabolism of the liver is limited because of low temperatures. Normothermic machine perfusion, on the other hand, allows the organ to have full metabolism activity in a near physiological environment. In a recent study, technical feasibility of normothermic machine perfusion was shown in discarded human livers. In this study, a commercially available device (Liver Assist, Organ Assist, Groningen, The Netherlands) was used for machine perfusion of the liver, using continuous portal perfusion and pulsatile arterial perfusion (46). Normothermic machine perfusion provides the opportunity to assess viability and function of liver grafts before transplantation. So far, studies have only suggested that machine perfusion results in better preservation of the liver parenchyma compared with SCS. Although machine perfusion has also the potential to lower ischemic injury of the biliary epithelium, it still needs to be demonstrated whether machine perfusion indeed results in better preservation of the biliary epithelium and the peribiliary vasculature. Nevertheless, the results for protecting the hepatocyte against the cold are very promising. Moreover, during machine perfusion the status of the biliary epithelium could be investigated by measuring the biomarkers of bile duct injury in the perfusate or bile. Although detection of gamma-glutamyl transferase ($\gamma$-GT) and
alkaline phosphatase (ALP) in bile samples has been shown to correlate with the degree of biliary epithelial injury, these enzymes are not specific for cholangiocytes and may also reflect hepatocellular injury. A recent study reported that cholangiocyte-specific microRNAs can be seen as a more specific biomarker to predict post-transplant biliary strictures. MicroRNAs are stable and can be easily detected in perfusate, bile or tissue during machine perfusion prior to transplantation (53).

In addition to providing better graft preservation and a tool for selection of grafts prior to transplantation, machine perfusion also provides an opportunity to improve organ function and viability. During machine perfusion, organs can be treated with therapeutic agents such as hydrophilic bile salts (i.e. ursodeoxycholic acid and norursodeoxycholic acid) to substitute hydrophobic bile salts and increase the $\text{HCO}_3^-$ umbrella function (Figure-1) (54). The benefits of such strategies, however, have to be proven in the future studies.

![Non-anastomotic Biliary Strictures](image)

**Pathogenesis**

**Ischemia-related:**
- Cold ischemia
- Preservation
- Warm ischemia

**Immune-mediated:**
- ABO incompatibility
- Preexisting autoimmune disease
- CCR5Δ32 polymorphism

**Bile salt Toxicity:**
- High BS/PL ratio
- Impaired BS reuptake
- Impaired $\text{HCO}_3^-$ secretion

**Potential Strategy**

- Adequate back table flush out
- Oxygenated machine perfusion

- Administer hydrophilic bile salts (UDCA, nor-UDCA)
- Induce MDR3 (statins, fibrates)
- Induce "$\text{HCO}_3^-$ umbrella"

**Hypothermic**

**Subnormothermic**

**Normothermic**

- Oxygenation
- Increase ATP content
- Viability Testing

**Figure 1. Schematic overview of the mechanisms of biliary injury during and after liver transplantation**

Machine perfusion is an attractive alternative method of organ preservation that could contribute to better preservation of the biliary tree and may subsequently result in a reduction of preservation-induced biliary complications after transplantation. ATP, adenosine triphosphate; BS, bile salts; BS/PS ratio, bile salt to phospholipid ratio; MDR3, multidrug resistance protein 3; (nor-)UDCA, (nor-)ursodeoxycholic acid; TUDCA, tauroursodeoxycholic acid.
Biliary complications remain to be a burden in liver transplantation. Biliary leakage and stricture formation are the most frequent types of biliary complications. Whereas bile leakage and anastomotic strictures can usually be managed successfully with nonsurgical or surgical approaches, NAS are frequently therapy resistant and leave long-term sequels. NAS have variable presentations in time and localization, suggesting different underlying mechanisms and pathogeneses. Early-onset NAS have shown to be more ischemia related in contrast to late-onset NAS. Late-onset NAS are more frequently immune-mediated. Cytotoxic hydrophobic bile salts play a critical role in biliary epithelial injury and occurrence of NAS. An adequate flush out of the liver, peribiliary microvasculature and the bile ducts during organ procurement could reduce biliary epithelial injury to some extent. However, bile duct injury during subsequent cold ischemic storage of a liver graft may still be substantial and may contribute to a high incidence of biliary complications after transplantation, especially in case of an extended criteria (preinjured) liver graft. Two recent histopathology studies of bile duct biopsies taken from human DBD donor livers at the time of transplantation have shown almost universal loss and injury of the biliary epithelial lining of the extrahepatic bile duct (up to 90% of all grafts). The degree of injury was strongly associated with the occurrence of NAS after transplantation. These studies have suggested that lack of complete and timely regeneration of the biliary epithelium after transplantation rather than the amount of initial bile duct wall injury may be an important underlying cause of NAS. Machine perfusion has received increasing research attention during the last years as an attractive alternative method of graft preservation prior to transplantation. Although the most optimal method of machine perfusion still needs to be determined (temperature, duration, timing, etc.), most studies have shown improved preservation of the liver parenchyma with this technique. Much less is still known about the potentially beneficial effects of machine perfusion on the bile ducts of a donor liver. Nevertheless, the capacities of machine perfusion are very promising (i.e. viability testing by biomarkers or administering therapeutic agents) and may provide a new tool to protect the vulnerable bile ducts against preservation-induced injury.
KEY POINTS

• Biliary complications remain to be the major problem after orthotopic liver transplantation.

• Nonanastomotic biliary strictures are the most challenging complication after OLT because of variability in the pathogenesis and resistance to therapy.

• The main mechanisms described for pathogenesis of NAS include ischemiareperfusion injury, immune-mediated injury, cytotoxic hydrophobic bile salts and impaired biliary bicarbonate secretion.

• Insufficient regeneration capacity of biliary epithelium has recently been proposed as a new perspective to view the pathogenesis of NAS.

• Machine perfusion has been proposed as a potential preservation strategy to reduce ischemiareperfusion injury in donor livers; however, clinical trials are needed to demonstrate whether machine perfusion can reduce the incidence of NAS after transplantation.
REFERENCES


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