Protection of Bile Ducts in Liver Transplantation:  
*Looking Beyond Ischemia*

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ABSTRACT

Biliary complications, especially nonanastomotic strictures (NAS), are a major cause of morbidity after orthotopic liver transplantation. Of all donor and recipient characteristics known to increase the risk of developing NAS, the role of prolonged ischemia times is most extensively described in the literature. However, there is increasing evidence that several other, non-ischemia-related factors play a critical role in the pathogenesis of NAS as well. The clinical presentation of NAS may vary considerably among liver transplant recipients, including large variations in time of occurrence, and in location and severity of the strictures. Additional underlying causes such as bile salt toxicity and immune-mediated injury are believed to explain the wide spectrum of biliary strictures after orthotopic liver transplantation. Current and emerging insight in the pathogenesis of NAS and potential targets to reduce biliary injury and preserve bile ducts are discussed in this overview.
BILIARY COMPLICATIONS AFTER LIVER TRANSPLANTATION

Orthotopic liver transplantation (OLT) has proven to be a successful treatment for patients with end-stage chronic or acute liver failure. Despite excellent 1-year patient and graft survival rates (85%-90% and 75%-85%, respectively), liver transplantation brings along the risk of complications such as infection, rejection of the graft, primary non-function or initial poor function of the graft, vascular complications and biliary complications. Of all liver transplant recipients, 10% to 40% develop biliary complications that are associated with mortality rates of 8% to 15% (1-3). There is a variety of biliary complications that can occur after OLT, the most common being bile leakage and bile duct strictures.

Bile leakage can occur at various sites and intervals after transplantation. The majority of postoperative leaks occur at the site of anastomosis or at the T-tube insertion site. Another common site for leakage is the resection surface of the graft in case of living donor or split donor transplantation. Depending on the size of the leak, bile leakage can be managed successfully conservatively, non-surgically or surgically (4-7).

Bile duct strictures are grouped into anastomotic biliary strictures and nonanastomotic biliary strictures (NAS). Anastomotic strictures are isolated strictures at the site of the bile duct anastomosis. They result mainly from surgical technique and local ischemia, leading to fibrotic scarring of the anastomosis (8). Most anastomotic biliary strictures are treated with dilatation and stenting. Sometimes surgical revision or conversion to a Roux-en-Y hepatico-jejunostomy is required, all associated with excellent long term results (9-11).

Compared with anastomotic biliary strictures, NAS present much more heterogeneously and are regarded the most troublesome biliary complication after OLT. NAS are strictures at any location in the biliary system of the transplanted liver (both intrahepatic and extrahepatic). NAS were first described after OLT as a cholangiographic image of biliary strictures and dilatations caused by ischemia after hepatic artery thrombosis (HAT). However, such cholangiographic abnormalities of strictures and dilatations can also be seen in patients who do not have biliary ischemia caused by HAT, and the name first given to this last subgroup of strictures was “ischemic-type biliary lesions” (Figure 2.1). Alternative names used in the literature are “ischemic cholangiopathy” or the more general term “nonanastomotic biliary strictures”, which is used in this overview.
The reported incidence of NAS after OLT varies between 1% and 20% (2, 3, 12, 13), which can partly be explained by variations in the definition of NAS used. Although most types of biliary complications can usually be treated successfully (surgically or by endoscopic techniques), or run a self-limiting course, NAS remain the most challenging type of biliary complication as they are frequently therapy resistant and often associated with long-term consequences. Therapeutic options are endoscopic dilatation or stenting, percutaneous transhepatic cholangiography drainage or dilatation, or surgical resection followed by construction of a hepatico-jejunostomy. Because of limited therapeutic options and therapy resistance, up to 50% of patients with NAS die or require retransplantation (2). High mortality rates and the impact on the already undersized pool of donor organs make NAS a complication in desperate need for better preventive strategies and treatment options. Therefore, it is important to understand the pathogenesis of NAS and discover ways in which development of NAS after OLT can be prevented.

Knowledge about the pathogenesis of NAS is slowly emerging from clinical and experimental studies performed during the last decade. Several risk factors have been identified, strongly suggesting a multifactorial origin (12). Although a true causative relationship may not have been
proven for many putative risk factors, most current evidence is circumstantial and based on observational studies. In general, proposed mechanisms underlying the pathogenesis of NAS can be divided into three categories: (a) ischemia-related injury, (b) immune-mediated injury, and (c) cytotoxic injury induced by hydrophobic bile salts. These mechanisms are summarized in Table 2.1 and are discussed in more detail below.

Table 2.1. Mechanisms of biliary epithelial injury after liver transplantation

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<thead>
<tr>
<th>Ischemia-related injury caused by</th>
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<td>• Warm and cold ischemia associated with organ preservation</td>
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<td>• Warm ischemia in donation after cardiac death donors</td>
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<td>• Inadequate and preservation of the peribiliary capillary plexus</td>
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<th>Immune-mediated injury due to</th>
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<td>• ABO-incompatible transplantation</td>
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<td>• CMV-infection*</td>
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<td>• Female organs in male recipients</td>
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<td>• Chemokine receptor CCR5-Δ32 polymorphism</td>
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<th>Cytotoxic effect of hydrophobic bile salts caused by</th>
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<td>• Inadequate flush out of bile from the bile ducts at organ retrieval</td>
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<td>• High biliary bile salt/phospholipid ratio after transplantation</td>
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<td>• Impaired cholehepatic shunt with intracellular accumulation of bile salts in cholangiocytes</td>
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<td>• Impairment of the protective HCO\textsubscript{3}\textsuperscript{-} umbrella at the canalicular membrane of cholangiocytes</td>
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* CMV, cytomegalovirus.

NOT ALL PRESENTATIONS OF NAS ARE EQUAL

NAS is a heterogeneous group of bile duct strictures and not all presentations of NAS are equal. A large variation is observed in the time interval between liver transplantation and the first presentation of NAS, ranging from 0.3 to 155 months (14, 15). Location and severity also differ between patients. NAS may be confined to the hepatic bifurcation (large bile ducts), but may also present as a more diffuse type, affecting the small peripheral bile ducts of the liver (Figure. 1). One large clinical study reported differences in anatomical localization and underlying causes of NAS presenting early (< 1 year) versus late (> 1 year) after OLT (15). In the group with early presentation of NAS, the majority of lesions were found around the bifurcation and the extrahepatic bile duct. On the contrary, biliary abnormalities in the group
with late presentation of NAS were more frequently located in the periphery of the liver. Moreover, NAS presenting with within 1 year after OLT were associated with ischemia-related variables, such as a longer cold and warm ischemia time, compared with NAS occurring more than 1 year after OLT. The latter group was more frequently associated with variables that may explain an immunological cause. It has, therefore, been suggested that ischemia-mediated mechanisms are frequently responsible for the development of biliary strictures early (<1 year) after transplantation, whereas immune-mediated mechanisms play an important role in the pathogenesis of strictures occurring beyond the first year (15).

**ISCHEMIA AS A CAUSE OF NAS**

**Ischemia-reperfusion injury.**

Bile ducts entirely depend on arterial blood supply for oxygenation. Hepatocytes, on the other hand, are supplied by both the hepatic artery and the portal vein. For this reason, HAT is specifically detrimental for bile duct epithelial cells (cholangiocytes) and may result in massive bile duct necrosis (16). Additionally, cholangiocytes are highly susceptible to reoxygenation after anoxia (reperfusion injury) (17). Reoxygenation of anoxic liver cells improves survival of hepatocytes but causes increased cell death of cholangiocytes. The increased susceptibility to reoxygenation injury by cholangiocytes is associated with increased production of toxic reactive oxygen species by cholangiocytes during reoxygenation with concomitant low basal levels of the antioxidant glutathione in these epithelial cells (17).

Ischemic preconditioning (IPC) has been proposed as an attractive surgical strategy to decrease liver ischemia-reperfusion injury. Thus far, there is significant controversy regarding the use of IPC in the liver transplant setting (18). However, the effect of IPC on survival of cholangiocytes during liver transplantation has not been studied, as outcome parameters used to determine liver injury in previous studies were mainly hepatocyte-specific.

**Cold ischemia**

The duration of cold ischemic storage of a graft is strongly associated with development of biliary strictures. Prolonged cold ischemia time (>13 h) greatly increases the occurrence of NAS after transplantation (13). However, with the shorter periods of cold ischemia in OLT these days (on average 8-10 h), the association between cold ischemia time and the development of NAS has become less evident (15).
Warm ischemia

One of the best-known risk factors for NAS is donation after cardiac death (DCD). Warm ischemic time in the donor in addition to subsequent cold ischemia-reperfusion injury is believed to result in increased damage to biliary epithelial cells. Grafts from DCD donors are used to increase the number of organs available for liver transplantation, and approximately 20% of the livers available for transplantation in the United States and many European countries is currently derived from a DCD donor (20, 21). The use of DCD grafts and restrictive criteria can result in patient and graft survival rates similar to those of donation after brain death liver transplantation; however, this is associated with a higher risk of biliary strictures. The incidence of NAS after DCD is 10% to 30% compared with a 1% to 10% incidence of NAS after donation after brain death (20, 22-26).

Preservation

Insufficient flush out of the microvasculature of the liver (peribiliary capillary plexus) is believed to play a role in the development of NAS. A sufficient flush out is needed for adequate exposure of cholangiocytes and endothelium to preservation fluids. The preservation fluid prevents injury of these cells and subsequent formation of microthrombi in the small arteries as a result of cell necrosis and local coagulation. With that, it prevents prolonged hypoxia of the bile ducts. The use of high-viscosity preservation solution is believed to result in an insufficient flush out of the peribiliary capillary plexus, leading to a higher incidence of NAS, compared with low-viscosity preservation solutions (27). Lower viscosity could provide a faster initial flush during organ retrieval, resulting in faster cooling and improved washout of blood cells. Equally, arterial back-table pressure perfusion used to achieve sufficient perfusion of the peribiliary plexus significantly decreased the occurrence of NAS after transplantation, compared with standard perfusion methods (28). In contrast with the potentially favorable effect of low-viscosity solutions such as histidine-tryptophan-ketoglutarate solution on the biliary system, this type of solution has recently been associated with reduced graft survival in deceased donor livers, especially those from DCD donors (29). The cardiac standstill in DCD donors is not only believed to result in warm ischemic injury but also in the formation of thrombi in the microvasculature of the donor organ. Animal studies have shown that the use of a fibrinolytic agent in a preflush before preservation of a DCD liver markedly improves flush out of such microthrombi and improves microperfusion with University of Wisconsin solution (30).

Potential targets

An obvious target to reduce ischemia-induced bile duct injury in OLT is keeping ischemic times as short as possible. Moreover, low-viscosity organ preservation solution and arterial
back-table pressure perfusion with or without thrombolysis seem to be beneficial. Adequately powered randomized controlled trials (RCTs) with long follow-up periods are required to evaluate the long-term effect of histidine-tryptophan-ketoglutarate and University of Wisconsin solutions. The safety and side-effects of thrombolysis in DCD donation should be studied more extensively and future studies should also focus more on bile duct related outcome parameters (31). In the future, normothermic or hypothermic machine perfusion could potentially greatly decrease ischemic injury to the liver graft. Some positive results have been shown in this field, however, more research and experience are required (32).

In addition, more research on the role of IPC in the protection of cholangiocytes against ischemia is warranted.

**IMMUNOLOGICAL FACTORS AS A CAUSE OF NAS**

NAS has been associated with various immunological processes. Although the exact role of the immune system in the pathogenesis of NAS remains unclear, there are several studies suggesting a role for the immune system in the pathogenesis of NAS.

For example, ABO-incompatible liver transplantation has been associated with the development of NAS. This may be explained by a persisting ABH antigen expression in the intrahepatic biliary system of the hepatic allograft, resulting in a biliary system more susceptible to immunologic injury, or by vascular occlusion and subsequent ischemic injury caused by endothelial injury, or both. Increased bile duct damage after transplantation across the ABO barrier will eventually result in a higher rate of NAS (33-35). Moreover, preexisting diseases with a presumed autoimmune component, such as primary sclerosing cholangitis and autoimmune hepatitis, have been associated with a higher incidence of NAS (36-38).

In one retrospective study, a female to male donor/recipient match was significantly associated with late occurrence of NAS (15). It was suggested that the higher incidence of NAS in male recipients of a female graft is related to immunological processes.

Biliary complications develop significantly more often in the presence of preceding or concomitant cytomegalovirus (CMV) viremia, especially in conjunction with primary CMV infection (38-39). CMV inclusions have been detected in a histopathologic specimen of bile duct strictures from a liver transplant patient, in whom biliary strictures developed during CMV
infection. However, the exact mechanisms explaining the relationship between CMV infection and NAS are still to be elucidated. Thus far, it is unclear whether CMV injures the biliary epithelium in a direct manner by infecting biliary epithelial cells, or in an indirect manner by immune attack evoked against infected biliary epithelial cells. Alternatively, it is potentially possible that CMV infection causes ischemic injury of the biliary epithelium by injury of endothelial cells of the peribiliary capillary plexus and subsequent formation of microthrombi and inadequate oxygenation of the biliary epithelium.

Another line of evidence for an immune-mediated mechanism of bile duct injury and NAS formation was provided by a recently described association between a loss-of-function mutation in the chemokine receptor CCR5 (CCR5-Δ32) and the development of NAS after OLT. Retrospective studies have shown that liver transplant recipients carrying CCR5-Δ32 are at much greater risk of developing NAS after transplantation compared with CCR5 wild-type recipients (40, 41). This risk is even higher in recipients with CCR5-Δ32 transplanted for primary sclerosing cholangitis (PSC). Moreover, late development of NAS is significantly more present in recipients carrying CCR5-Δ32 (41). A possible explanation for this may be functional changes in the immune system resulting from the CCR5-Δ32 mutation, including impaired attraction of regulatory T cells to the site of injury (42-44).

Potential targets
Because the exact role of the immune system in the pathogenesis of NAS remains unclear, this aspect requires further research and is not yet ready for targeted therapy. At this stage, only anti-CMV prophylaxis to prevent NAS in liver recipients who are seronegative for CMV is supported by scientific data, as de novo CMV infections are clearly associated with a higher incidence of NAS.

THE ROLE OF BILE SALTS IN THE DEVELOPMENT OF NAS

Another potential factor in the pathogenesis of bile duct injury after liver transplantation is bile salt toxicity. Hydrophobic bile acids can induce damage to hepatocytes and cholangiocytes based on their detergent effects towards lipid cellular membranes or by intracellular cytotoxic effects and induction of apoptosis.

Inadequate Flush Out of Bile During Cold Ischemia
Experimental studies in pigs have indicated that hydrophobic bile salts are able to induce
damage to the biliary epithelium of the liver graft during cold ischemia when hydrophobic bile salts are added to the preservation solution. This type of injury is characterized by loss of microvilli, cell-surface erosions, and cell death (45-47). Based on these observations the importance of adequate flush out of the biliary tract, to remove bile during the backtable procedure, has been emphasized.

**Altered Composition of Bile After Liver Transplantation**

Hepatic secretion of bile salts, phospholipids, and cholesterol is an active process mediated by specific hepatobiliary transporter proteins located in the canalicular cell membrane of hepatocytes. Although the bile salt export pump (gene ABCB11) is largely responsible for the secretion of bile salts, the multidrug resistance protein 3 (gene ABCB4) is responsible for the secretion of phospholipids (48).

Experimental and clinical studies have provided increasing evidence that the expression and function of these transporters may be impaired after OLT, resulting in an abnormal bile composition that has been associated with bile duct injury and NAS after OLT.

A well-functioning graft immediately starts to secrete bile salts on graft reperfusion, but the overall biliary secretion of bile salts remains low within the first week after OLT. Restoration of biliary secretion of phospholipids, however, may recover even slower. These differences may be explained by differences in the expression and function of the transporters bile salt export pump and multidrug resistance protein 3, resulting in a higher bile salt/phospholipid (BS/PL) ratio in the bile during the first days after OLT (Figure 2.2A,B). The formation of protective mixed micelles is therefore impaired and free hydrophobic bile salts may cause injury of the cellular lipid membranes of cholangiocytes through their detergent activities. Compelling evidence that this high BS/PL ratio is correlated with the development of biliary injury and NAS has been provided in experimental animal studies (46, 49, 50) and two prospective clinical studies (51, 52).
Figure 2.2  Schematic presentation of the hepatobiliary transporter proteins responsible for the uptake and secretion of bile components by hepatocytes and cholangiocytes. (A) Normal situation. (B) Reduced expression or functional impairment of hepatocellular transporters for bile salts and phospholipids (BSEP and MDR3) may result in abnormal bile composition with a relatively high bile salt/phospholipid ratio. This has been associated with increased bile duct injury and development of nonanastomotic strictures. (C) Unbalanced alteration in ASBT, OST α, and OST β, the main transporters involved for the cholehepatic shunt, may result in accumulation of toxic bile salts inside cholangiocytes. (D) Finally, a functional impairment of the secretion of HCO₃⁻ by cholangiocytes may diminish the protective effect of an alkaline environment at the canalicular membrane of cholangiocytes, making them more susceptible to uncontrolled membrane permeation of hydrophobic bile salts (full description of this hypothesis is provided in ref. 56). BS, bile salt; PL, phospholipid; BSEP, bile salt export pump; MDR3, multidrug resistance protein 3 (phospholipid flippase); ASBT, apical sodium-dependent bile acid transporter; OST, organic solute and steroid transporter; AE2, anion exchanger 2; CFTR, cystic fibrosis transmembrane conductance regulator.

Intracellular Accumulation of Hydrophobic Bile Salts in Cholangiocytes

At physiological concentrations, a simple detergent-like effect involving disruption of cell membranes is likely not the only explanation for bile salt-induced biliary injury after transplantation. An alternative mechanism of bile salt-mediated bile duct injury after OLT is the intracellular accumulation of hydrophobic bile salts in cholangiocytes. Intracellular accumulation of bile salts may occur when the transporters responsible for canalicular uptake and basolateral excretion of bile salts by cholangiocytes are no longer in balance (Figure. 2.2C). These cholangiocyte transporters are, under physiological circumstances, believed to be responsible for the so called cholehepatic shunt (53). Bile salt accumulation
leading to cellular damage in hepatocytes has been described in cholestatic diseases (54), and it is likely that a similar process may occur in cholangiocytes. A recent experimental study in rats has indicated that in addition to the high biliary BS/PL ratio, an ischemia-related discrepancy may occur in the recovery of the cholangiocyte bile acid reuptake transporter apical sodium-dependent bile acid transporter and the cholangiocyte basolateral export transporters (organic solute and steroid transporters (OSTs) α and β), resulting in the accumulation of toxic bile acids and subsequent injury of cholangiocytes, especially in the larger bile ducts (55).

**Impaired Biliary HCO₃⁻ Secretion as a Cause of Biliary Injury**

Biliary HCO₃⁻ secretion has been proposed as a mechanism to prevent the uncontrolled membrane permeation of hydrophobic bile salts by maintaining an alkaline pH near the apical surface of hepatocytes and cholangiocytes (56). An alkaline environment results in deprotonation of hydrophobic bile acids, making them less capable of attacking cell membranes (Figure 2D). Biliary secretion of HCO₃⁻ is largely controlled by the cholangiocyte Cl-/HCO₃⁻ exchanger AE2 and the transporter cystic fibrosis transmembrane conductance regulator, which are adenosine triphosphate-dependent. Ischemia, as inevitably occurs in transplantation, will not only result in a reduction of adenosine triphosphate levels but may also result in altered expression of AE2 and cystic fibrosis transmembrane conductance regulator, which will subsequently lead to a diminished function of the “HCO₃⁻ umbrella” protecting cholangiocytes (57). Although this mechanism has been hypothesized to contribute to the development and progression of NAS after OLT, formal evidence for this is still lacking and further research in this area is needed (56).

**Potential Targets**

The accumulating evidence that hydrophobic bile acids contribute to biliary epithelial injury after OLT provides new avenues for possible preventive strategies. Adequate retrograde flushing of the bile ducts during organ procurement alone may not be enough to avoid bile salt-mediated biliary injury. Additional therapies, such as substitution of hydrophobic bile salts by more hydrophilic bile salts, which are believed to exert cytoprotective instead of detrimental effects on cholangiocytes (i.e., ursodeoxycholic acid or nor-ursodeoxycholic acid), are an attractive and relatively simple option. However, formal evidence that substitution of (nor-) ursodeoxycholic acid in liver transplant recipients results in a reduction of the incidence of NAS is still lacking. Randomized clinical trails on the efficacy of (nor-)ursodeoxycholic acid in the prevention of NAS are needed. In addition, future research should focus on the potentially protective effects of stimulating HCO₃⁻ secretion by cholangiocytes after OLT.
CONCLUSION

NAS continues to be a serious cause of morbidity and graft loss after OLT. Not all cases of NAS are equal. Timing, localization, and severity of strictures vary between patients. Differences in the pathogenesis of NAS are believed to, at least partly, explain these variations in presentation. Although NAS occurring early after OLT are largely associated with an ischemia-related pathogenesis, NAS occurring late after OLT are believed to have a more immune-mediated origin. Endogenous bile salts may play an additional role in the pathogenesis of bile duct injury after OLT. Hydrophobic bile salts have a direct cytotoxic effect on the cholangiocytes and this may contribute to the postischemic injury of the biliary epithelium. Donor surgeons can help prevent NAS by reducing warm and cold ischemia time as much as possible, providing adequate flush out of the liver, the peribiliary plexus, and the bile ducts. Recipient (transplant) surgeons can help reducing the incidence of NAS by keeping the cold ischemia time as short as possible and by performing arterial back-table pressure perfusion of the liver graft to ensure adequate perfusion of the peribiliary capillary plexus. More studies will be needed to provide better insight in the immune-mediated mechanisms of NAS that occur late after OLT.

Recent advancements in normothermic and hypothermic machine perfusion could potentially have an important impact on incidence of NAS through a reduction of preservation injury of the bile ducts and the peribiliary capillary plexus of liver grafts.
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