Rationale and aims of the thesis
Orthotopic liver transplantation (OLT) is an established treatment option for patients with end-stage liver disease. Survival following OLT has improved substantially over the years. Increasing waiting list mortality due to organ shortage, has led to the use of compromised or “extended criteria donors” (1). Donation after cardiac death (DCD) is potentially an important source of additional donor organs (2, 3). However, several studies have shown a higher incidence of bile duct complications after DCD liver transplantation compared to donation after brain death (DBD) liver transplantation (4, 5).

Bile duct complications are an important cause of morbidity and graft loss after OLT, occurring between 10-30% of the patients (6, 7). Biliary complications comprise both leakage and strictures. Isolated strictures at the bile duct anastomosis must be distinguished from non-anastomotic biliary strictures (NAS). The latter are considered as one of the most troublesome biliary complications and can be found in up to 20% of the patients after OLT (8).

NAS may present at multiple locations in the biliary tree and are frequently resistant to therapy (9). NAS are identified by radiological imaging studies and defined as any stricture, dilatation or irregularity of the intra- or extrahepatic bile ducts of the liver graft, either with or without biliary sludge formation. NAS are diagnosed after exclusion of hepatic artery thrombosis (HAT) by either Doppler ultrasound or conventional angiography. HAT after OLT has been shown to result in ischemic injury of the bile duct, due to the fact that the bile ducts completely depend on the arterial circulation for their blood supply. Because of the resemblance of intrahepatic biliary strictures occurring after HAT, NAS that appear in the absence of occlusion of the hepatic artery are also called ischemic-type biliary lesions (ITBL). Although prolonged ischemia time has been identified as an independent risk factor for this type of injury, in many patients with NAS an apparent risk factor cannot be identified.

The aim of this thesis is to identify clinical risk factors for NAS, by analyzing donor and patient characteristics, as well as surgical variables in relation to postoperative outcome after OLT. Furthermore, we have performed clinical studies and experimental studies in mice to unravel the role of bile salt toxicity in the pathogenesis of hepatobiliary injury after OLT.
The thesis is divided in two parts. The first part focuses on the clinical risk factors for the development of NAS and in the second part we investigate the role of toxic bile salts and bile secretory function in the development of hepatobiliary injury after OLT.

**Part I. Non-anastomotic biliary strictures after liver transplantation**

The specific aims of this section are to describe the various risk factors for NAS. Chapter 2 provides an overview of the literature on the causes and consequences of NAS. Here we discuss three types of biliary injury, which have been identified as putative mechanisms in the development of NAS: preservation-related injury, immunological injury, and injury due to bile salt toxicity. In addition to these three putative mechanisms of biliary injury, NAS has also been associated with Roux-Y choledochojejunostomy. Roux-Y choledochojejunostomy is frequently used as an alternative to a duct-to-duct reconstruction of biliary drainage in OLT and although it is a rather secure type of biliary drainage it may facilitate ascending bacterial migration and cholangitis, which could lead to the development and progression of NAS. Previous studies have suggested that the use of a Roux-Y choledochojejunostomy is associated with a higher risk of developing NAS. This technique, however, is preferentially used in patients transplanted for primary sclerosing cholangitis (PSC) and PSC itself has also been associated with a higher incidence of NAS. The aim of chapter 3 was to determine whether Roux-Y choledochojejunostomy is an independent risk factor for NAS in a cohort of 486 consecutive adult liver transplantations.

Transplantation of livers from DCD donors has been associated with lower graft survival rates and a higher incidence of bile duct complications (4, 5, 10). In chapter 4 the results of a multicenter study in The Netherlands on differences in outcome after OLT with DBD and DCD liver grafts are described. Clinical risk factors for graft loss and NAS were identified in a cohort of 526 adult liver transplantations performed in the three Dutch transplant centers.

**Part II. The role of bile salts in the pathogenesis of hepatobiliary injury after liver transplantation**

Hepatobiliary bile salt transporters play a critical role in maintaining the bile salt homeostasis and the cholehepatic circulation (11). Bile salts are absorbed from the
sinusoidal blood via basolateral membrane of hepatocytes through the Na\(^+\) -dependent taurocholate cotransporting polypeptide (NTCP) and secreted into bile via the canalicular bile salt export pump (BSEP) located in the canalicular membrane. Phospholipids are secreted into bile in human via the concerted action of the canalicular multidrug resistance 3 P-glycoprotein (MDR3) and the canalicular bile salts (12). The toxic effects of bile salts are prevented at least in part by formation of complexes (mixed micelles) with phospholipids. Bile salts are actively reabsorbed from the bile through the apical sodium-dependent bile salt transporter (ASBT) at the ductular membrane of cholangiocytes. In addition, uptake of bile salts via ASBT is linked with Cl\(^-\) and HCO\(_3\)\(^-\) secretion into bile via a ductular anion channel or cystic fibrosis transmembrane conductance regulator (CFTR) (13). Bile salts are subsequently excreted by cholangiocytes into the peribiliary vascular plexus (PBP) via the basolateral heteromeric organic solute transporter (OST), consisting of two half transporters OST-alpha/beta (Figure 1).

Bile duct injury after human OLT correlates with the formation of toxic bile early after OLT, characterized by a high bile salt/phospholipid ratio (14, 15). Whether bile salt toxicity actively contributes to hepatic injury or is an epiphenomenon could not be discriminated in these clinical studies.

In chapter 5 a study is described that was aimed to define the role of endogenous bile salt toxicity in the origin of bile duct injury and obtain new insights on the pathogenesis of cholestasis after OLT. We developed a mouse model of arterIALIZED liver transplantation, using mice heterozygous for the disruption of the Mdr2 gene (Mdr2\(^{+-}\)), a homologue of human MDR3. These mice disclose approximately half of the normal phospholipid concentration in bile, but in contrast to their homozygous (Mdr2\(^{-}\)) littermates, they do not develop bile duct injury and intrahepatic strictures under normal conditions. We hypothesized that the high biliary bile salt/phospholipid ratio in Mdr2\(^{-}\) mice contributes to bile duct injury due to cold storage and subsequent reperfusion during OLT. We therefore transplanted livers from either Mdr2\(^{-}\) mice or wild-type mice into wild-type recipients and assessed the degree of hepatobiliary injury, as well as changes in bile composition and bile secretory function in these two groups.

In chapter 6 we report on the results of a study on the involvement of bile secretory dysfunction in the pathogenesis of hepatobiliary injury after HAT. We hypothesized that biliary injury after HAT is not only a direct result of ischemia, but may also be
aggravated by changes in the bile secretory function and subsequent bile salt toxicity. In a mouse model, we have studied this hypothesis by comparing changes in hepatocyte bile transporter expression, serum markers of cholestasis (serum bile salts and bilirubin), as well as hepatic ATP-content in mice with normal arterial blood supply to the liver or with either selective disruption of the PBP or with complete dearterialization of the liver.

The purpose of the study described in chapter 7 concerns the role of cholangiocyte bile salt transporters and the cholehepatic shunt in the pathogenesis of hepatobiliary injury after OLT in mice. This study is an extension of the results described in Chapter 5. In this study, we assessed changes in gene and protein expression for Asbt and the organic solute transporters Ost-alpha/beta after transplantation of wild-type or Mdr2−/− livers to wild-type mice in relation to bile composition and biliary injury after OLT.

The aim of the study described in chapter 8 was to examine changes in the expression of the cholangiocyte transporters ASBT, OST-alpha, OST-beta, and CFTR during and after human OLT. Furthermore, to determine the role of the cholehepatic shunt in the development of bile salt induced bile duct injury after OLT, gene expressions were correlated with the biliary bile salt secretion and degree of bile duct injury. In 37 adult liver transplant recipients, biopsies were taken from the grafted liver at the end of cold storage, 3 hours after graft reperfusion and at one week after transplantation. Changes in the cholangiocyte transporter expression were assessed using real time RT-PCR and immunofluorescence staining.

Finally, in chapter 9 the results as described in the thesis are summarized and future perspectives are discussed.
Figure 1

Schematic overview of hepatobiliary transporters responsible for bile salt secretion and the cholehepatic shunt.

References
