Summary, general discussion and future perspectives
Chapter 1 provides a short introduction of the facts and figures in liver transplantation. Furthermore, the aims of this thesis are discussed. These aims were to evaluate the molecular and biochemical mechanisms of bile duct injury after liver transplantation.

In Chapter 2 the literature regarding the causes and consequences of non-anastomotic strictures (NAS) is reviewed. The aim of this chapter was to describe the current knowledge about the pathophysiological mechanisms, the clinical presentation, and the treatment of NAS. NAS is a radiological diagnosis, characterized by intrahepatic strictures and dilatations on a cholangiogram. NAS were first described after liver transplantation in association with hepatic artery thrombosis (HAT). In case of early HAT after liver transplantation the biliary tree becomes ischemic and eventually necrotic, resulting in a typical cholangiographic picture of biliary strictures, dilatations and intraductal cast formation. However, these cholangiographic abnormalities of strictures and dilatations can also be seen in patients who do not have a hepatic artery thrombosis, so the term ischemic-type biliary lesions emerged. In this thesis the term NAS was used to describe intrahepatic biliary strictures and dilatations in the confirmed absence of HAT. The incidence of NAS varies around 15% in different series. Several risk factors for NAS have been identified, strongly suggesting a multifactorial origin. Main categories include ischemia related injury, immunological induced injury and cytotoxic injury by bile salts. However, in many cases no specific risk factor can be identified. The clinical presentation of patients with NAS is often not specific. Symptoms may include fever, abdominal complaints and increased cholestatic liver function tests. The diagnosis is made by imaging studies of the bile ducts. Treatment starts with relieving symptoms of cholestasis and dilatation of the stenosed bile ducts by endoscopic retrograde cholangiopancreaticography (ERCP) or percutaneous transhepatic cholangiodrainage (PTCD), if possible followed by stenting. Eventually up to 50% of the patients with NAS will require a re-transplantation or may die. In selected cases, a re-transplantation can be avoided or delayed by surgical intervention.

In the clinical study described in Chapter 3 we aimed to identify clinical risk factors for the development of NAS after liver transplantation. A total of 487 adult liver transplants with a median follow-up of 7.9 years were studied. All imaging studies of the biliary tree were reviewed.
Localization of NAS at first presentation was categorized into 4 anatomical zones of the biliary tree. Severity of NAS was semiquantified as mild, moderate, or severe. NAS developed in 81 livers (16.6%). In 85% of the cases, anatomical localization of NAS was around or below the bifurcation of the common bile duct. The severity of biliary strictures was classified as mild in 43 (55%) and as moderate to severe in 35 (45%) of the cases. The cumulative incidence of moderate to severe NAS in the entire population of liver transplant recipients was 7.3%. A large variation was observed in the time interval between liver transplantation and first presentation of NAS (median 4.1 months; range 0.3-155 months). NAS presenting early (<1 year) after liver transplantation were associated with preservation related risk factors. Cold and warm ischemia times were significantly longer in patients with early NAS compared with NAS presenting late (>1 year) after transplantation, and early NAS were more frequently located in the central bile ducts. NAS presenting late after transplantation were more frequently found in the periphery of the liver and were more frequently associated with immunological factors, such as primary sclerosing cholangitis as the indication for liver transplantation. By separating cases of NAS on the basis of the time of presentation after transplantation, we were able to identify differences in risk factors, indicating different pathogenic mechanisms depending on the time of initial presentation.

The population of patients suffering from NAS as described in Chapter 3 is further studied in Chapter 4. The aim of this particular study was to describe the treatment, and identify risk factors for radiological progression of bile duct abnormalities, recurrent cholangitis, biliary cirrhosis and retransplantation in patients with NAS. Progression of disease was noted in 68% of cases in whom follow-up radiology was available. Radiological progression was more common in patients with early NAS (≤ 1 year) and with one or more episodes of bacterial cholangitis, and less prevalent in patients with extrahepatic biliary abnormalities. Recurrent bacterial cholangitis (3 or more episodes) was more frequently seen in patients with a Roux-en-Y anastomosis. Severe fibrosis or cirrhosis developed in 23 cases, especially in cases with peripheral biliary abnormalities. Graft survival, but not patient survival, was influenced by the presence of NAS. Thirteen patients (16%) were retransplanted for NAS. The conclusion of the study is that especially patients with a hepatico-jejunostomy, those with an early diagnosis of NAS, and those with NAS presenting at the level of the peripheral branches of the biliary tree, are at risk for progressive disease with severe outcome.
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In Chapter 5 we took a closer look at the role of bile composition in the development of bile duct injury after liver transplantation, in a porcine model of non heart-beating liver transplantation. After non-heart-beating (NHB) liver transplantation, the occurrence of NAS is a serious and often encountered complication. Bile salt toxicity has been identified as an important factor in the pathogenesis of bile duct injury and cholangiopathies in general. The role of bile salt toxicity in the development of biliary strictures after NHB liver transplantation was, however, unclear. In a porcine model of NHB liver transplantation, we studied the effect of different periods of warm ischemia in the donor on bile composition and subsequent bile duct injury after transplantation. After induction of cardiac arrest in the donor, liver procurement was delayed for 0 min (group A), 15 min (group B), and 30 min or more (group C). Subsequently livers were transplanted after 4 hr of cold preservation. In the recipients, bile flow was measured, and bile samples were collected daily to determine the phospholipids-to-bile salt ratio. Severity of bile duct injury was semi quantified by using a histological grading scale. Survival after transplantation was directly related to the duration of warm ischemia in the donor. The phospholipids-to-bile salt ratio in bile produced early after transplantation was significantly higher in group C, compared with group A and B. Histopathologic examination showed the highest degree of bile duct injury in group C. Based on these results, it was concluded that prolonged warm ischemia in NHB donors is associated with the formation of toxic bile after transplantation, characterized by a low biliary phospholipids-to-bile salt ratio. These data suggest that bile salt toxicity contributes to the pathogenesis of bile duct injury after NHB liver transplantation.

The previous chapter, as well as other studies from our group, have indicated that bile formation early after liver transplantation may be disturbed, resulting in more cytotoxic bile with a relatively low phospholipids-to-bile salt ratio. It was unknown whether bile toxicity is also involved in the pathogenesis of NAS, a disease of the larger bile ducts. If bile composition is involved in the pathogenesis of NAS, one would expect that the bile composition in the first week after liver transplantation is different in those patients who will develop NAS than in patients who will not develop NAS. We tested this hypothesis in a prospective clinical study, described in Chapter 6. In this study, bile production and composition within one week after liver transplantation were correlated with the subsequent development of NAS in a large cohort of adult liver transplant recipients. In 111 adult liver transplants bile samples were
collected daily after transplantation for determination of bile composition. Expression of bile transporters was studied perioperatively. NAS were detected in 14 patients (13%) within one year after transplantation. Patient and donor characteristics and postoperative serum liver enzymes were similar between patients who developed NAS and those who did not. Secretions of bile salts, phospholipids and cholesterol were significantly lower in patients who developed NAS. In parallel, biliary phospholipids-to-bile salt ratio was lower in patients developing NAS, suggestive for increased bile cytotoxicity. There were no differences in bile salt pool composition or in hepatobiliary transporter mRNA expression.

Although patients who develop NAS were initially clinically indiscernible from patients who did not develop NAS, the biliary bile salts and phospholipids secretion, as well as biliary phospholipids-to-bile salt ratio in the first week after transplantation, was significantly lower in the former group. This supports the concept that bile cytotoxicity is involved in the pathogenesis of NAS.

In the previous chapter we have shown that altered bile composition, with a lower phospholipids-to-bile salt ratio is associated with NAS after liver transplantation. Hepatobiliary transporter proteins are responsible for the biliary secretion of phospholipids and bile salts. Aim of Chapter 7 was to assess whether variations in the genes in the donor encoding for these transporters are associated with the occurrence of NAS in the recipient. Without transplantation, genetic variations itself may not result in bile duct injury. However, early after transplantation, when the graft is still recovering from I/R injury, these variations might be a critical second factor in the sequence of events leading to bile duct injury. A similar phenomenon can be found in other diseases, such as intrahepatic cholestasis of pregnancy (ICP), where patients with a genetic variation in hepatobiliary transporters display an abnormal phenotype only during pregnancy.

Of 458 procedures in adults, cryopreserved splenocytes were available form the donors and used for genotyping. The following genes were studied: bile salt export pump (ABCB11), transporter of phospholipids (ABCB4) and transporter of glutathione and bilirubin (ABCC2). Four to five tagging single nucleotide polymorphisms (SNPs) with an equal physical distribution per gene were selected using HapMap data. Haplotypes were constructed using an Expectation-Maximization algorithm to estimate haplotype frequencies. NAS was detected in 77 patients (16%) after transplantation. Patients who received a donor liver with ABCB4 haplotype AGGTA developed NAS almost twice as often (28%) as donor livers with other
haplotypes (15%) (p=0.007). Analysis in a multivariate Cox regression model showed AGGTA haplotype of ABCB4 from the donor to be an independent risk factor for NAS (p=0.004, OR=2.23, 95% CI= 1.29 – 3.85). ABCB11 and ABCC2 haplotypes or single SNPs, were not associated with NAS. These data indicate that a common haplotype in the transporter of phospholipids (ABCB4) in donor livers is independently associated with a two-fold increased risk for NAS after liver transplantation. Transport of phospholipids into the bile in livers which are carriers of this risk haplotype might be altered in the time period early after transplantation.

Upregulation of heme oxygenase-1 (HO-1) has been considered an adaptive and protective mechanism against ischemia/reperfusion (I/R) injury. In Chapter 8 we studied the role of endogenous HO-1 expression in human liver transplants in relation to early postoperative hepatobiliary injury and dysfunction. Before transplantation, median HO-1 mRNA levels were 3.4-fold higher (range: 0.7–9.3) in donors than in normal controls. Based on the median value, livers were divided into two groups: low and high HO-1 expression. There were no differences in donor characteristics, donor serum transaminases or cold ischemia time between the two groups. Postoperatively, however, serum transaminases were significantly lower and the bile salt secretion was higher in the group with an initial low HO-1 expression, compared to the high expression group. Immunofluorescence staining identified Kupffer cells as the main localization of HO-1.

To study possible effects of HO-1 induction upon reperfusion, we categorized groups based on the ability to increase HO-1 expression during reperfusion of the liver graft. In this analysis, serum AST levels immediately after liver transplantation were significantly lower in the group with an increase in HO-1 expression compared to livers without upregulation of HO-1 upon reperfusion. These findings suggest that the ability to induce HO-1 expression at the time of graft reperfusion may confer hepatobiliary protection. Further research will be necessary to determine which is more important: a low expression of HO-1 before liver transplantation, or the ability to induce HO-1 at the time of graft reperfusion.

In the previous chapter, the endogenous regulation of HO-1 during human liver transplantation was studied. None of the clinical variables analyzed in this study could explain the variation in initial expression of HO-1 in the donor livers. We therefore hypothesized that genetic variations
may be responsible for the differences in HO-1 expression and subsequent outcome after liver transplantation. The inducibility of HO-1 is modulated by a (GT)$_n$ polymorphism and a single nucleotide polymorphism (SNP) A(-413)T in the promoter. Both a short (GT)$_n$ allele and the A-allele have been associated with increased HO-1 promoter activity. In Chapter 9, a study is described in which HO-1 genotype in the donor was tested and correlated with outcome in 308 adult patients. For (GT)$_n$ genotype, livers were divided into two classes: short alleles (<25 repeats; class-S) and long alleles (≥ 25 repeats; class-L). For the A(-413)T SNP, livers were grouped as A-carriers (AT or AA) versus TT-genotype livers. In a subset of each group, hepatic mRNA expression was correlated with genotypes. Graft survival at 1 year was significantly better for A-allele genotype compared to TT-genotype (84% versus 63%, p=0.004). Graft loss due to primary dysfunction occurred more frequently in TT-genotype compared to A-receivers (p=0.03). No differences were found for the occurrence of NAS in both groups. Recipients of a liver with TT-genotype had significantly higher serum transaminases after transplantation. Hepatic HO-1 mRNA levels were significantly lower in TT genotype livers compared to the A-allele livers (p=0.03). No differences were found for any outcome variable between class S and LL-variant of the (GT)$_n$ polymorphism. Haplotype analysis indicated the dominance of the A(-413)T SNP over the (GT)$_n$ polymorphism.

The main conclusion of this study was that the HO-1 promoter polymorphism A(-413)T is associated with outcome after liver transplantation. The TT variant is linked with worse graft survival, more primary dysfunction, increased I/R injury and reduced HO-1 mRNA levels. Furthermore we provided evidence for a greater functional relevance of the A(-413)T SNP over the (GT)$_n$ polymorphism.
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Part I: Non-anastomotic biliary complications after liver transplantation

The specific aims of this section were to describe the various forms of NAS and the accompanying clinical risk factors as well as to study the risk factors for progression of NAS. We found a difference in risk factors for NAS presenting early (≤1 year) and NAS presenting late (>1 year) after transplantation. NAS occurring early after transplantation were correlated with prolonged ischemia times. NAS occurring late after transplantation were more strongly associated with immunological risk factors. These data suggest that there are different subtypes of NAS that have different etiologies. This aspect should be considered in future studies.

The following groups of patients were found to have an increased risk for disease progression: patients with a hepaticojejunostomy, those with an early diagnosis of NAS, and those with NAS presenting at the level of the peripheral branches of the biliary tree. In clinical practice it is important to identify these patients for a close follow up and early intervention.

Our newly proposed classification system for NAS is a promising tool to better classify patients with NAS. However, to become useable and successful in the currently expanding international field of liver transplantation, our system should be validated. This would enable us to confirm our findings on the relevance of the localization of NAS and subsequent consequences for prognosis and management. Aligning many different international centres with different protocols, facilities and expertise for a prospective study into this classification system might be complex and time consuming. Therefore and second best, this validation could be achieved by retrospectively studying other cohorts of liver transplant patients by reviewing the images of the biliary tree and correlating the classification with risk factors and level of progression.

It is likely that recurrent PSC may have been accountable for the occurrence of late NAS in a number of patients. On the basis of radiological evaluation, however, recurrent PSC cannot be distinguished from a late presentation of NAS. Although some of our patients fit well within the definition of recurrent PSC, more than half of our patients who presented with NAS late after transplantation were not transplanted for PSC. In an attempt to reduce the occurrence of early NAS, it remains important to focus on a further reduction of ischemic times, in particular
the cold ischemia time. However, many centres have already put a lot of effort in this, and it is questionable whether a substantial further reduction of cold ischemic time is feasible. New perspectives in preservation of the liver graft might realize these assiduously sought-after improvements of graft quality. Maintaining organ viability via (normothermic) machine perfusion during preservation might be effective in reducing postoperative bile duct injury. The central concept behind (normothermic) perfusion is to maintain normal function of the liver during the whole period of preservation and enable immediate graft function and protect the vulnerable biliary epithelial cells from I/R injury. Currently great efforts are being taken to better understand the concepts of machine perfusion, as well as to find the ideal preservation fluid and to create possibilities to implement machine perfusion in daily practice (1).

Part II: Bile physiology after liver transplantation
The specific aims of this second section were to evaluate the contribution of bile composition to the development of bile duct injury. We found supporting evidence that toxic bile, characterized by a low phospholipids-to-bile salt ratio, contributes to the development of bile duct injury, not only at a microscopic level but also at a macroscopic level, like NAS.

The questions whether bile duct injury and toxic bile composition are not just two consequences of the same underlying factor, has been studied previously by our group. Using mice heterozygous for disruption of the Mdr2 gene (equivalent of human MDR3), Hoekstra et al. confirmed that there is indeed a cause-effect relationship between toxic bile formation and bile duct injury after liver transplantation and ruled out the possibility that toxic bile composition and bile duct injury both result from the same underlying factor (2). In this study it was demonstrated that endogenous bile salts act synergistically with I/R in the origin of bile duct injury in vivo.

The question of which role toxic bile plays in bile duct injury is of great interest. What is exactly happening on a cellular level? What is happening on epithelial level? What is the sequence of events before the epithelial cells are damaged so severely that we can detect it by radiological examination? Fickert et al proposed a very appealing mechanism similar to the pathogenesis of primary sclerosing cholangitis in humans. They stated that due to a lack of phospholipids the nonmicellar-bound, free bile acids might damage the tight junctions and basement membranes of the epithelial lining, leading to leakage of potentially toxic bile acids
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into the periductal area. As a result the inflammatory response is induced, ultimately resulting in fibrosis and narrowing of the biliary ducts (3).

Therapeutic strategies to modify intrahepatic cholestasis and to prevent bile duct injury after OLT may include the administration of the hydrophilic bile salt ursodeoxycholic acid. Daily oral administration of ursodeoxycholic acid is a well-known therapy to reduce bile salt toxicity by replacement of the hydrophobic bile salts in the bile salt pool (4,5). Although the exact mechanisms underlying its cytoprotective effect are not fully understood, it may reduce bile salt–induced injury by replacing the toxic hydrophobic biliary bile salts. In addition, it has been shown to stimulate canalicular transport and biliary excretion, enhancing bile flow and reducing the exposure time of biliary epithelium to toxic bile salts (4). The potentially beneficial effects of ursodeoxycholic acid make this drug an interesting strategy to prevent NAS. Current experimental and clinical research provides strong support for a prospective clinical trial focusing on the abilities of ursodeoxycholic acid to prevent NAS early after liver transplantation.

Another interesting therapeutic target could be the MDR3 gene, given the key role of biliary phospholipids in protecting bile duct epithelium from potentially toxic, aggressive biliary content (5). Administration of fibrates, statins, or peroxisome proliferators in mice, have been shown to stimulate biliary phospholipid secretion by the induction of MDR3 making bile less toxic (7-9). Further research in this direction seems justified.

Part III: HO-1 and hepatobiliary injury after liver transplantation

The specific aim of the third section was to study the role of HO-1 in relation to postoperative hepatobiliary injury and graft function. We showed that upregulation of HO-1 during liver transplantation correlates with better hepatobiliary function after transplantation. Furthermore, we demonstrated that patients possessing a polymorphism that is associated with reduced HO-1 expression on mRNA level have a worse hepatobiliary function after transplantation and an increased risk of graft loss on the long run. The role of HO-1 as a cytoprotective protein was confirmed by these studies.

It was noted that HO-1 is already upregulated in many livers from brain death donors. The variations in the observed upregulation of HO-1 mRNA levels could not be explained by a larger number of marginal donors in the group with high HO-1 expression. Moreover, factors associated with major hemodynamic alterations in the donor and several surgical variables
were similarly distributed amongst the donor groups with initial low expression of HO-1, compared to the donor group with initial high expression of HO-1. To find an explanation for these differences we studied two functional polymorphisms in the promoter region of the gene: a (GT)$_n$ polymorphism and the single nucleotide polymorphism A(-413)T SNP. The finding that the A(-413)T SNP exerts its effect not only in the immediate moments after the transplant procedure, but has also consequences in the longer term (figure 4 in chapter 9), is interesting. This could indicate that not only attenuation of I/R injury by a favourable HO-1 phenotype is beneficial but that HO-1 mediated processes may also play a role in later phases after the transplantation.

Clinical application of interventions in the HO-1 system should be considered. However, we should bear in mind that the beneficial effects of HO-1 may have a narrow therapeutic window as shown in chapter 8. Highly overexpressed HO-1 displays pro-oxidant properties secondary to iron accumulation, and may therefore be harmful instead of cytoprotective. It would be very interesting to focus this research on the specific effect of HO-1 on biliary epithelial cells which are especially vulnerable for I/R injury. We know from the study described in chapter 8 that human HO-1 in the liver is mainly located in the Kupffer cells, and not abundantly present in biliary epithelial cells. Strategies to enter HO-1 in these cells might be of great interest to study whether HO-1 over expression could protect the bile ducts from injury resulting from I/R injury or bile toxicity.

In summary, new insights are provided into the molecular and biochemical mechanisms of bile duct injury after liver transplantation. We have proposed a classification system of NAS based on the localization and severity of the biliary abnormalities. This classification system appeared valuable in identifying different etiologies of NAS and also allowed the identification of patients with NAS who are more at risk for complications or disease progression. Toxic bile composition, characterized by a low phospholipids-to-bile salt ratio was discovered as a contributing mechanism in the development of bile duct injury and NAS after liver transplantation. Further interventional studies aimed at prevention of NAS based on the principle of this altered bile composition are warranted. Finally, we have demonstrated a cytoprotective role of HO-1 in liver transplantation, opening new avenues for the development of novel preventive strategies or therapies.
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References


