Biliary Strictures after Liver Transplantation

Risk Factors, Diagnosis, Treatment and Outcome

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Whether life is worth living all depends on the liver of it

William James, 1842 – 1910

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Chapter 1

General introduction

Currently orthotopic liver transplantation (OLT) is considered the only definite treatment option for end-stage liver failure. During the first OLTs performed worldwide from the 60s to the 90s, several difficulties prolonging immediate survival after liver transplantation were encountered, of which technical and mechanical issues (i.e., thrombosis, ischemic injury and operative haemorrhage) as well as rejection and infection were the most important.[1] In the latter decades, numerous advances in pre-, intra-operative and post-transplantation procedures have been made to improve clinical outcomes and, nowadays, excellent 1- and 3-year patient survival rates up to 90% and 80% have been achieved.[2-4] Therefore, the focus has shifted towards improving longer term post-transplantation care and managing long-term complications and consequences of OLT.

The success of liver transplantation has led to a relative shortage of suitable donor organs, since the number of patients waitlisted for liver transplantation increases annually, while the number of eligible donors has not changed substantially. This has resulted in an estimated waitlist mortality of 12% to 20%.[5;6] This emphasizes the need for new strategies to potentially enhance the donor pool. To effectuate this, protocols to accept grafts from extended criteria donors have been implemented in the Netherlands.[3] Extended criteria include, among others, the acceptance of older donors (>65 years), grafts with moderate steatosis and split grafts.[7;8] Those pretransplant factors are related to general transplantation outcomes, and in case of extended criteria donor (ECD) livers, careful selection and evaluation of those factors is thus needed to select those grafts eligible for transplantation, with optimal post-transplant outcomes and minimised complication rates. A recently developed and useful tool to strongly predict the failure-free survival is the Eurotransplant Donor Risk Index (ET-DRI).[9;10] Donor and graft characteristics that are included in the ET-DRI are donor age, cause of death (trauma, anoxia, CVA, other), graft type (standard, partial/split, donation after circulatory death -DCD-), cold ischemia time, gamma-glutamyltransferase levels, donor location and whether or not the graft was allocated as a rescue organ. The use of the ET-DRI is now strongly advised to be taken into account in the allocation process.

When the number of donation after brain death (DBD) liver transplantations decreased, the three Dutch liver transplant centers were one of the earlier adaptors of transplantation of livers donated after circulatory death (DCD). As mentioned, DCD is one of the ECD factors. In DCD, an obligatory no touch period

to ensure irreversible death is included in the donation procedure.[11;12] This leads to an additional donor warm ischemia time (DWIT)[13], in which the continued energy consumption of the graft at body temperature leads to adenosine triphosphate depletion and a shift from an aerobic to an anaerobic metabolism, which may result in a more injured graft.[14] In DCD, DWIT precedes a period of cold ischemia time (CIT) and recipient warm ischemia time (RWIT). The additional DWIT in DCD procedures is in contrast to donation after brain death (DBD), in which only CIT and RWIT are present. CIT starts with a cold flush of the donor liver and ends after the removal of the donor liver from ice. The cold preservation during this period is extremely important as aerobic and anaerobic metabolic processes are decreased, which can thus minimalize ischemia-related injury during the donor and recipient operations and transportation of the graft. On the other hand, the anoxic CIT may also incur damage by itself. CIT is then followed by a recipient warm ischemia time, which is defined as the time from end of CIT until reperfusion of the donor liver in the recipient. Numerous studies have demonstrated an inferior graft outcome of DCD grafts as compared to DBD grafts in the past, mainly due to higher rates of primary non-function and biliary and vascular complications as a result of the additional donor warm ischemia time.[2; 15-19]

In view of the above, oxygenated machine perfusion (MP) has become a promising intervention, to improve preservation of especially ECD -including DCD- livers.[20;21] Whether cold -(D)HOPE- or warm -NMP- machine perfusion or combinations thereof will be optimal needs to be established.[22] Recently, also promising results with normothermic regional perfusion (NRP) in DCD liver donors were obtained. Machine preservation may also render nontransplantable livers transplantable, and in that case a normothermic phase for viability testing appears important. In rat models mitochondrial and nuclear injury, reflected by biochemical and histological parameters, decreased after a short period of end-ischemic cold oxygenated machine perfusion after cold storage.[23;24] Several animal as well as human studies suggested that this improvement is the result of restoring metabolic energy and adenosine triphosphate (ATP) depletion that have been known to develop during anoxic cold storage and subsequent rewarming and reperfusion.[25;26] In humans, increased graft viability after normothermic machine perfusion of discarded human donor livers has been demonstrated. Meanwhile, the first results of human studies of transplanted grafts after MP have been described, with excellent results.[21;27]

Recent studies have indicated that careful selection and emerging surgical and preservation techniques, such as oxygenated machine preservation, may lead to improved results in terms of graft and possibly also patient survival. This is important since more than 40% of OLTs in the Netherlands now are from DCD donors.

Biliary complications

The reverse side of the use of more grafts from donation after circulatory death is that some complications associated with DCD may become more prevalent. Among the most frequent complications that occur are biliary strictures. Post-transplant biliary strictures can, based on their location, be classified into two types, i.e., 1) anastomotic biliary strictures and 2) non-anastomotic biliary strictures.[28] Anastomotic biliary strictures (AS) are, by definition, located at the surgical notch of the bile duct anastomosis, which is either a duct-to-duct biliary anastomosis or a choledochojejunostomy. They have been reported to develop in 9% to 38% of patients and their development is generally accepted to be associated with a preceding bile duct leakage (or biloma), higher donor age, and especially local ischemia leading to fibrotic tissue – which seems related to surgical technique –.[29-35]

Non-anastomotic biliary strictures (NAS) are more heterogeneous strictures located intra- and extrahepatically, at least 1 cm above the anastomosis. Although they can occur anywhere in the biliary tree, experience learns that they are preferentially located in the extrahepatic common hepatic duct and at the bifurcation of the left- and right hepatic duct. However, severe intrahepatic stricturing also occurs. The incidence varies widely, probably as the result of lack of a clear definition. Incidences up to 30% in certain subpopulations have been reported, which underlines the importance of this post-transplant complication.[36;37]

As to date, clarification of the exact pathophysiological mechanism of the development of NAS remains one of the greatest challenges. Biliary strictures were first described after hepatic artery thrombosis (HAT), which led to the hypothesis that intra- or extrahepatic biliary strictures were the result of ischemia-related injury.[38;39] Further research revealed that these so-called ischemic type biliary lesions (ITBL) usually occur in the absence of HAT, and nowadays one of the factors defining NAS is the exclusion of HAT as a cause. More recently the term ‘post-transplant cholangiopathy’ was proposed for NAS or ITBL. Factors that have been argued to contribute to the pathogenesis of NAS can be subdivided into a) pretransplant

status of the graft and donor characteristics, b) ischemia-reperfusion injury, c) immunological factors, d) procedure-related complications and injury and e) cytotoxic effects.

a) Pretransplant status of the graft and donor characteristics include factors that represent the quality of the donor liver and are associated with post-transplant outcome, e.g., the amount of steatosis and donor age.[40;41] As mentioned above, the ET-DRI -with DCD included- is a strong predictor for graft survival after OLT.[9]

b) Ischemia-reperfusion injury (IRI) and procedure-related injury are two closely related factors that are associated with the development of biliary strictures.[42-44] For example, in case of anastomotic biliary strictures, -despite general agreement on this issue- a higher incidence has been described by some when a duct-to-duct anastomosis instead of a choledochojejunostomy has been performed.[33] The exact pathophysiological mechanism of NAS development in relation to IRI and procedure-related factors has been subject to debate. As previously mentioned, the finding that biliary strictures more frequently developed in patients with post-procedural hepatic artery thrombosis led to the hypothesis that ischemia-related injury (IRI) was a key factor in the development of NAS. One fact that supports this theory is the finding that NAS more frequently occurred after donation after circulatory death, probably as a result of the additional donor warm ischemia time.[45] In order to reduce ischemic injury, ischemia times are therefore kept as short as possible. Some believed that, because cholangiocytes are solely dependent on the hepatic artery, microthrombi that develop during the cold and warm ischemia time may lead to a reduced blood flow in the peribiliary plexus, and subsequently to ischemic injury.[44;46-48] In the current setting of procurement of donor livers, the preservation fluids University of Wisconsin (UW) solution and Histidine-tryptophan-ketoflutarate (HTK) solution are used to assure adequate flow and to flush out possible microthrombi in the circulation of the liver. Two studies suggested that adding a thrombolytic agent to the preservation fluid may dissolve those microthrombi and thus reduce the incidence of NAS.[48;49] However this has not been confirmed in properly performed randomised controlled trials. Furthermore, other studies could not confirm the presence of microthrombi in donor livers.[50;51]

Another mechanism which may be relevant is the concept that not the loss of biliary epithelium, but mainly the post-ischemic impaired regeneration capacity

from stem cells within the biliary glands results in cholestasis and the formation of fibrosis. This interesting finding, should however be confirmed in larger studies.[50]

c) Immunological factors that were recognized as related to NAS are ABO-incompatibility, CC chemokine receptor loss of function and, in some studies, cytomegalovirus viraemia was an additional risk.[52-54] A deletion of the chemokine CCR5-Δ32 gene leads to a loss of function of the CC chemokine receptor 5 and subsequently to impaired chemotaxis of T-cells. Op den Dries et al. showed that patients with this genetic mutation have a four-fold higher risk of developing NAS, especially those patients transplanted for primary sclerosing cholangitis (PSC), i.e., a probable auto-immune disease with biliary strictures.[54;55] In general, PSC is often also considered a risk factor for NAS. However, whether this is the result of NAS/ITBL or that the post-transplant biliary strictures are in fact a recurrence of PSC (which is endoscopically and radiologically difficult to distinguish from NAS) is often not easy to determine.

It is well known that -in contrast to hepatocytes- cholangiocytes have ABH antigens on their surface. After inferior results -especially due to severe biliary damage- became apparent ABO-incompatible (ABOi) liver transplantations were stopped in Eurotransplant. Only recently for some urgent indications ABOi OLT was restarted in some countries in selected cases within special protocols.

Much more is known regarding immunological factors related to hepatic IRI. Hepatic ischemia leads to a sterile immune response due to release of damage-associated molecular patterns (DAMPs) from apoptotic and necrotic cells. These activate innate immune cells, especially the sentinel Kupffer cells and dendritic cells.(Figure 1, adapted from Zhai et al.[60]) Chemokines and cytokines are then released. And especially after reperfusion T cells, monocytes, neutrophils and platelet are attracted and adhere to the activated sinusoidal endothelium. Neutrophils are mainly responsible for severe tissue damage after reperfusion by ROS and RNS production, and also by e.g. MMP9 production. During anoxia accumulation of electron transport chain substrate succinate occurs, with ATP depletion due to mitochondrial damage. This is related to reverse mitochondrial electron transport and HMGB1 release in the early reperfusion phase with ROS production and tissue damage following. This knowledge has to be translated further into clinical interventions for prevention and treatment of hepatic IRI.[56-60]

Figure 1. The distinct stages of liver ischemia–reperfusion injury

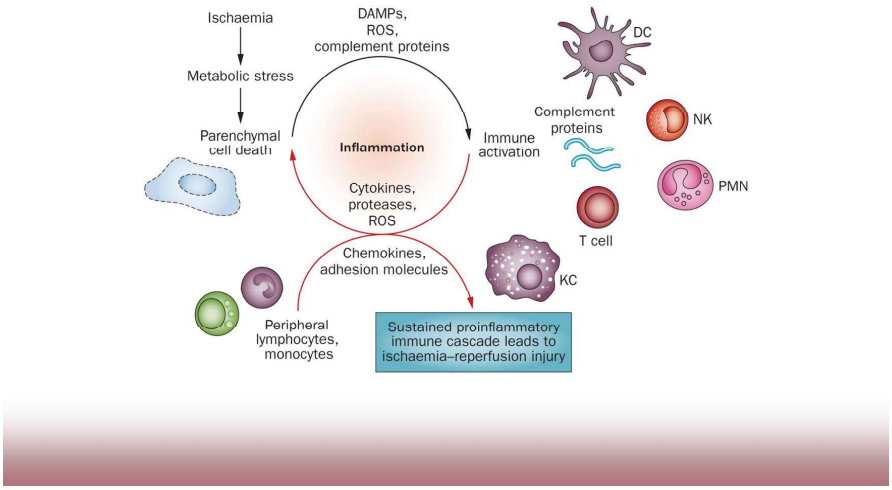

Figure 1. Ischemic injury, a localized process of hepatic metabolic disturbances, results from glycogen consumption, lack of oxygen supply and ATP depletion. The cell-death-released DAMPs, activation of complement (a group of proteins that are involved in tissue injury and/or repair) induced by tissue injury and mitochondrial ROS production triggered by oxygenation all contribute to liver immune activation after reperfusion, which involves multiple liver nonparenchymal cell types, including Kupffer cells, dendritic cells, T cells, NK cells and neutrophils (PMNs). The ischemia–reperfusion-activated proinflammatory immune cascade sustains itself by recruiting peripheral immune cells from the circulation, and is responsible for the ultimate liver reperfusion injury. Abbreviations: DAMPs, danger-associated molecular patterns; DC, dendritic cells; KC, Kupffer cells; NK, natural killer cell; PMN, polymorphonuclear cells; ROS, reactive oxygen species.
Figure adapted from Zhai, Y. et al. (2012) Ischaemia–reperfusion injury in liver transplantation—from bench to bedside Nat. Rev. Gastroenterol. Hepatol. doi:10.1038/nrgastro.2012.225

d) Procedure-related factors, such as inadequate flush-out, have been mentioned above. Adequate flush-out and reperfusion can be assured using arterial back table pressure perfusion.[47] Due to the high viscosity of the preservation fluids an inadequate perfusion of the biliary-tree small arteries was assumed. However, increasing the pressure on the perfusate bag may lead to an uncontrolled arterial high-pressure perfusion of the graft leading to injury by shear stress to the sinusoidal endothelium. The controlled flush in the donor and during the arterial back table procedure aims to prevent this. Initially there appeared to be some evidence that the use of HTK solution may slightly decrease the risk of NAS development

as compared to UW solution.[61;62] However, it finally came out that results with HTK were inferior, and its use in Eurotransplant has largely been abandoned.[63]

e) Ultimately, cytotoxic processes, e.g., altered bile salt composition, have been associated with NAS. DCD animal models demonstrated that the bile salt composition after a donor warm ischemia time of ≥ 30 minutes differed from the composition after shorter ischemia times, with a higher bile salt-to-phospholipids ratio. Impaired secretion of certain bile salts may be toxic to the biliary epithelium through this altered composition. Graft steatosis sensitizes cholestatic rats to liver injury by dysregulating bile acid synthesis and transport, leading to a shift towards a more cytotoxic hepatic bile acid composition and enhanced trafficking of bile salts from the liver to the systemic circulation.[64] Also biliary bicarbonate secretion can be impaired by IRI, resulting in a decreased protection of biliary epithelium by the 'bicarbonate umbrella.'[65] Most likely, the altered bile salt-to-phospholipids ratio is the result of an imbalance between hepatobiliary transporter proteins which secrete bile salts and multidrug-resistance protein 3 which secretes less phospholipids.[65-67] Indeed, studies with machine perfusion (MP) in discarded livers have resulted in a significantly better bile production after a period of machine perfusion.[68] Whether bile composition and bicarbonate secretion have a more normal profile after MP remains to be elucidated.

As yet, many (mainly immunological) risk factors for NAS, are still unknown and subject to research projects worldwide. NAS development is therefore considered a multifactorial process.

Diagnosis of NAS

Another challenging issue is the diagnosis of NAS. Biliary strictures are usually suspected after the clinical manifestation of cholestasis and cholestasis-related symptoms, such as jaundice, pruritus and (repeated episodes of) bacterial cholangitis. These symptoms are associated with frequent hospital readmissions.[69;70] Biochemically, elevated serum levels of bilirubin, alkaline phosphatase and gamma glutamyl transferase may indicate that a post-transplant biliary complication is present.

An early and adequate diagnosis of NAS is important to start appropriate treatment and hopefully prevent clinical symptoms and long-term complications, such as graft failure and the need for retransplantation. Whereas abdominal ultrasound (US) would be the first choice to detect prestenotic bile duct dilatation in a non-transplanted graft, several studies described a low sensitivity of US in detecting NAS, possibly because distal biliary strictures in a transplanted graft have the tendency to result in less prestenotic dilatation than strictures in a non-transplanted graft. However, bile duct dilatation and elevated GGT after OLT are quite specific for biliary problems and warrant cholangiography.[71-74]

In the recent years, magnetic resonance cholangiopancreatography (MRCP) has evolved as an upcoming technique to non-invasively detect biliary complications. However, the role of MRCP in detection of NAS and AS after OLT has not yet been firmly established and validated. Moreover, while MRCP ensures an increasingly improving visualization of the biliary tree, it has the disadvantage of not visualizing emptying of bile ducts as in direct cholangiography and the inability to treat the strictures within the same procedure.[75-77] Direct cholangiography is therefore considered the most sensitive and specific imaging technique to detect biliary strictures. After detecting the presence and exact location of the biliary strictures a balloon dilatation or stenting procedure with endoscopic retrograde cholangiopancreatography (ERCP) or percutaneous transhepatic cholangiography with drainage (PTCD) can be performed. In general, high success rates of cholangiographic treatment can be achieved, although non-anastomotic biliary strictures tend to be more difficult to treat than anastomotic biliary strictures.[69;78-82] Several studies have reported that, besides the type and location of biliary strictures, other factors are also important to determine cholangiographic treatment outcome. For example, whereas a more aggressive treatment approach with multiple stents seems to be effective, a pretransplant Model for End-stage Liver Disease score of ≥ 10, a stent retention time of ≥ 12 months, a higher recipient age, a prolonged operation time, late treatment and the simultaneous presence of other bile duct complications is associated with worse outcome.[30;78;83-86] In case of endoscopic treatment failure of AS or NAS in the donor choledochal or hepatic duct, surgical revision of the anastomosis is also a definite treatment option. The only effective surgical option for intrahepatic NAS is retransplantation, which is required in up to 18% of the patients with NAS.[70;87]

In conclusion, even though tremendous improvements have been made with the treatment of patients with irreversible liver injury and liver failure, orthotopic liver transplantation and the associated biliary complications remain a challenge for clinicians in daily practice. Further research to reduce the incidence of biliary strictures and optimize diagnosis and treatment is necessary.

Outline of the studies described in this thesis

The general aim of this thesis is to elucidate several aspects of (non-)anastomotic biliary strictures after orthotopic liver transplantation. The studies that have been performed are described in the three parts of this thesis:

Part A: Risk factors
Part B: Diagnosis
Part C: Treatment and outcome

Part A: Risk factors

As previously mentioned, the pathogenesis of the development of non-anastomotic biliary strictures is multifactorial and still only partially unravelled. Current research projects primarily have their focus on preventing ischemia-reperfusion and immunological injury. Ischemia-reperfusion injury both affects the biliary epithelium and the liver parenchyma. The relationship between these two was unknown. Accepted markers for the degree of IRI after OLT are (peak) serum levels of alanine aminotransferase (ALT) and aspartate-aminotransferase (AST) in the first days after OLT, of which ALT is more liver specific than AST. Increased levels of ALT can be found in the first postoperative week, with a peak ALT within 1 to 3 days after OLT. The relationship between peak ALT and the development of NAS after OLT was investigated and described in **chapter 2**.

With the advent of modern immunosuppression the rate of patients with acute cellular rejection after OLT has decreased to below 20%, and chronic rejection to about 1%, which is much lower than in the 90s. Until recently, it was assumed that (hyper)acute rejection after OLT did not occur because liver grafts were able to absorb preformed donor specific antibodies (DSA). However, more recent studies demonstrated that both preformed and de novo DSA are associated with graft loss after OLT.[88;89] The presence of DSA against the human leukocyte antigen

(HLA) after organ transplantation now has been associated with acute -antibody mediated- rejection and shorter graft survival.[90;91] Moreover, de novo DSA with a high mean fluorescence intensity (MFI) seems to be related to chronic rejection after OLT.[92] The biliary epithelium is known to express both HLA class I and class II molecules after OLT and earlier studies have demonstrated that preformed DSA are associated with bile duct complications and preservation injury in general.[93-96] Therefore, the relationship between preformed and de novo DSA formation, NAS development and graft failure after OLT was investigated in this thesis. The results of this clinical study are described in **chapter 3**.

Matrix metalloproteinases (MMPs) encompass a group of different proteolytic enzymes involved in tissue remodelling and repair. They can be subdivided into collagenases, stromelysins, gelatinases and membrane-type MMPs. MMP-2 and MMP-9 (both gelatinases) are capable of degrading different collagen types, of which mainly type IV collagen may be important because this type of collagen is present in basement membranes. Damage to the basement membrane of the bile ducts may produce irreversible injury.[97-99] MMP-9 is released massively from neutrophils during hepatic reperfusion after ischemia in human liver transplantation.[100;101] The activity of MMP-2 and MMP-9 is regulated by several processes, including the activity of tissue-inhibitors of matrix metalloproteinases (TIMPs) and, because collagen type IV, proMMP-2 and proMMP-9 are important substrates for MMP-7, also MMP-7 activity. Several genetic polymorphisms in the promotor region of MMP-2, MMP-7, MMP-9 and their associated TIMPs are known to alter the mRNA transcription of these MMPs, which may lead to a reduced degradation process of collagen type IV. Since MMPs and TIMPs play a role in ischemia-reperfusion injury[100-103], we hypothesized that gene polymorphisms in the MMP-2, MMP-7, MMP-9, TIMP-1 and TIMP-2 promotors may have an effect on NAS development. In **chapter 4** this possible relationship was further investigated.

Part B: Diagnosis

Early diagnosis of biliary strictures is essential to start appropriate treatment early, and where possible prevent severe complications. While ultrasonography is insensitive after OLT, dilated bile ducts on ultrasonography and/or elevated GGT warrant further investigation.[74] The current golden standard to diagnose NAS, i.e., endoscopic retrograde cholangiopancreatography (ERCP) and percutaneous transhepatic cholangiography (PTC), are invasive and can be associated with serious complica-

tions, such as post-procedural cholangitis, pancreatitis, perforation and bleeding. To prevent unnecessary invasive procedures and their associated complications, non-invasive screening tools are needed. Magnetic resonance cholangiopancreatography (MRCP) is such a safe, non-invasive diagnostic tool to visualise the biliary tree. However, even though some experience with the use of MRCP to detect biliary strictures has been gained, no universal criteria have been established to describe the presence and severity of non-anastomotic biliary strictures post-OLT. A validated classification would be useful to justify the performance of invasive cholangiography, but could also be used within the setting of clinical trials, e.g. with machine perfusion, where non-invasive diagnostic methods are preferred. In **chapter 5** a new cholangiographic classification, the 'Leiden Biliary Stricture Classification' (LBSC), -derived from a validated PSC ERCP scoring system- to score biliary strictures after OLT by MRCP was developed and validated after OLT.

Another non-invasive tool that is frequently used in liver disease, and now also more and more after OLT, is transient elastography (TE). TE is used to assess liver stiffness as a surrogate marker of liver fibrosis in patients with chronic liver disease. The technique was originally developed to prevent costly and invasive liver biopsies, which were formerly required to detect and classify liver fibrosis, e.g. according to the METAVIR classification. In addition, TE is also increasingly used to stratify patients with e.g. HCV virus infections for treatment with antiviral agents, based on the stage of fibrosis.[104-107] Recently, it appeared that TE can also accurately determine fibrosis after OLT, especially for viral hepatitis.[108;109] We therefore hypothesized that TE values might be increased in NAS patients and investigated TE in patients with and without NAS after OLT. The results are described in **chapter 6**.

Part C: Treatment and outcome

Timely resolution of biliary strictures, assuring adequate drainage, is important in all cases. The results from endoscopic and radiologic intervention that have been described after AS are variable, while for NAS very few data on outcome of such interventions exist. **Chapter 7** investigates the long-term outcome of endoscopic and percutaneous cholangiographic treatment of both anastomotic and non-anastomotic biliary strictures after DBD and DCD OLTs. Furthermore, we discuss factors and/or predictors that are associated with treatment failure or worse endoscopic outcome.

Because of the increased graft and patient survival after OLT, the emphasis is now shifting towards other clinical outcomes, e.g., patients well-being and quality of life (QOL). Clinical symptoms of biliary strictures themselves or due to invasive treatments, and coping with postoperative complications may affect perceived QOL in patients after OLT. In **chapter 8** the results of a cross-sectional study evaluating the QOL in patients with and without biliary strictures, as assessed by validated health-related quality of life questionnaires, is described.

In **chapter 9** the main results of the different studies described in this thesis are summarized and discussed, focusing on future perspectives.

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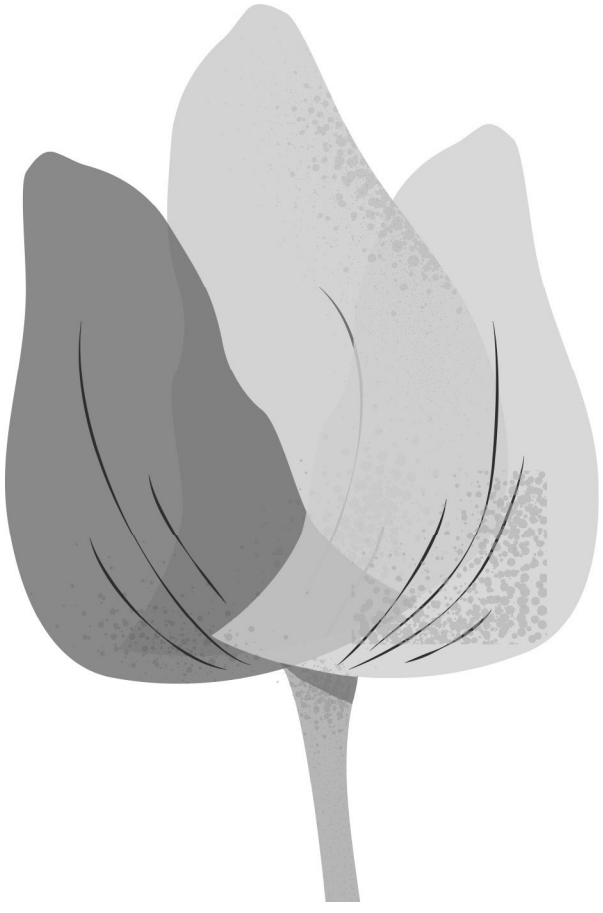
Part A

Risk factors



Chapter 2

High peak alanine aminotransferase determines extra risk for non-anastomotic biliary strictures after liver transplantation with donation after circulatory death

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Abstract

Introduction Orthotopic liver transplantation (OLT) with donation after circulatory death (DCD) often leads to a higher first week peak alanine aminotransferase (ALT) and a higher rate of biliary non-anastomotic strictures (NAS) as compared to donation after brain death (DBD).

Patients and Methods This retrospective study was to evaluate whether an association exists between peak ALT and the development of NAS in OLT with livers from DBD (*n*=399) or DCD (*n*=97) from two transplantation centers. Optimal cut-off value of peak ALT for risk of development of NAS post DCD-OLT was 1300 IU/l.

Results The four-year cumulative incidence of NAS after DCD-OLT was 49.5% in patients with a high ALT peak post-OLT, compared to 11.3% in patients with a low ALT peak. (p<0.001). No relation between peak ALT and NAS was observed after DBD-OLT. Multivariate analysis revealed peak ALT ≥1300 IU/l [adjusted hazard ratio (aHR) = 3.71, confidence interval (CI) (1.26 – 10.91)] and donor age [aHR = 1.04, CI 1.00 – 1.07] to be independently associated with development of NAS post DCD-OLT. A peak ALT of <1300 IU/l carries a risk for NAS similar to DBD-OLT.

Conclusion Thus, in DCD-OLT, but not in DBD-OLT, peak ALT discriminates patients at high or low risk for NAS.

Introduction

Orthotopic liver transplantation (OLT) has evolved into a routine treatment for advanced liver disease with excellent short and long-term survival.[1;2] An increasing number of patients eligible for liver transplantation and a decreasing number of donors after brain death (DBD) have led to the extension of criteria for acceptance of potential liver grafts in the last decade.[3;4] OLT with livers from donation after circulatory death (DCD) has become common in the Netherlands and the United Kingdom. However, OLT with a liver from a DCD donor carries a high risk for development of non-anastomotic biliary strictures (NAS).[5] NAS can occur in up to 13 – 34% after OLT with a DCD donor and is considered a major cause of morbidity and reduced graft survival.[6-8] Early recognition of an increased risk to develop NAS may be valuable for timely intervention.

Besides genetic factors, such as CCR5Δ32 and matrix-metalloproteinase 2 polymorphism, several ischemic parameters have been related to the development of NAS. For example, cold ischemia time (CIT) and warm ischemia times have been described as potential predictors of NAS in DCD and DBD donors, but clinical use of these parameters for predicting NAS has remained controversial.[9-13] Livers from DCD have an inevitable donor warm ischemia time (DWIT) between cardiac arrest and organ preservation, which may lead to a higher degree of ischemia-reperfusion injury (IRI) in the first week after OLT. It is likely that the higher incidence of NAS after OLT with DCD livers is largely the results of the additional ischemia-reperfusion injury due to the DWIT.[14-16] We therefore hypothesized that a relation may exist between injury of the liver parenchyma, reflected by first week post-OLT peak aminotransferases, and the risk for NAS development during follow-up, especially after DCD-OLT, as evaluated in a cohort from two independent centers.

Patients and Methods

A total of 519 first consecutive OLTs for chronic liver diseases were performed in two liver transplantation centers. After exclusion of 23 patients with primary non-function or a minimum follow-up of less than 7 days, 496 patients were included in the analysis. From the Leiden University Medical Center (LUMC, center A) a total of 174 OLTs could be included in the time-period of October 2001 until March 2011

next to 322 OLTs from the University Medical Center Groningen (UMCG, center B) performed in the time-period of July 2000 until June 2012. This included OLTs using livers from DBD as well as DCD donors (Figure 1). Patient follow-up was determined as time of transplantation until the first occurring event (i.e., development of NAS, retransplantation, or death) or in case of no event, until July 2012.

In 2001 a national protocol was introduced in the Netherlands regarding the acceptance of organs from donation after circulatory death.[17] In this protocol only Maastricht category 3 donors below 55 years of age, with a DWIT below 30 minutes, a maximum of 15 minutes between respiratory withdrawal and cardiac arrest a body mass index of <28, and a mean arterial pressure of <50 mm Hg for maximum 15 minutes before cardiac arrest were accepted. DCD donors were therefore selected in both centers according to this protocol.

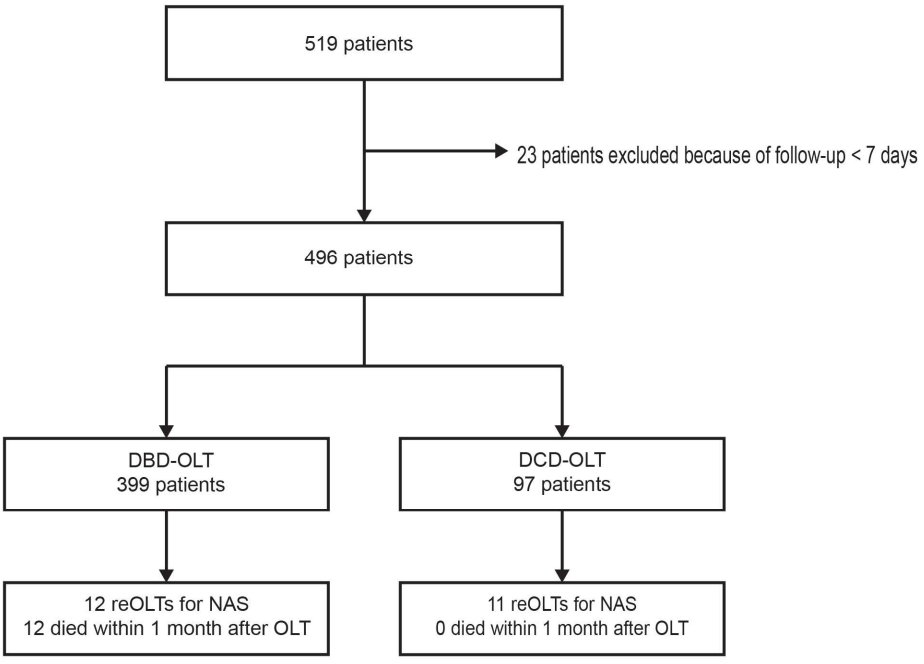

Figure 1. Flowchart of patient inclusion and exclusion.

Donor surgery

In case of DCD donors a donor warm ischemia time (DWIT) was measured, defined as the time between circulatory arrest and cold flush with preservation fluid in the donor. Cold ischemia time was defined as the time between cold flush with preservation fluid in the donor and removal of the liver from ice during the transplantation procedure. The recipient warm ischemia time was defined as the time of removal of the donor liver from ice until reperfusion of the donor liver in the recipient. In both centers, University of Wisconsin (UW) preservation fluid was used to flush out DBD liver grafts and in case of DCD liver grafts mainly histidine-tryptophan-ketoglutarate (HTK) was used.

Recipient surgery and routine follow-up

In both centers OLT with standard technique of 'piggy-back' cavo-caval anastomosis, porto-portal and hepatic artery to hepatic artery anastomosis was performed in most recipients. In some cases the hepatic artery was anastomosed to the aorta via an iliac conduit. A duct-to-duct biliary anastomosis -over an 8-12 Ch stent in center A, no stent in center B- was performed if possible. The biliary stent was removed endoscopically with endoscopic retrograde cholangiography (ERC) at 6 weeks or earlier as indicated. In general, post-transplantation care for both centers was comparable. Centers only differed in the use of prophylactic antibiotics, as was previously described.[18] In center A a combination of Gentamycin, Cefuroxime, Penicillin G and Metronidazole was used with an additional 3 weeks of selective digestive tract decontamination (Polymyxin/Neomycin, Norfloxacin and Amfotericin B). In center B patients received prophylactic Amoxicillin-Clavulanate and Ciprofloxacin. In the first year blood liver biochemistry was performed daily in the first two weeks, weekly in the following two weeks, monthly thereafter in the first year, and then every three months. In both cohorts, ultrasound (US) was performed routinely on day 0, 1 and 7, and subsequently at 3, 6, 12 months and yearly after OLT. ERC or magnetic resonance cholangiography (MRC) and other imaging studies were performed when indicated. A liver biopsy was performed per protocol at 6 months in center A, and further as indicated in both centers. Pre-OLT baseline parameters, including laboratory model for end-stage liver disease (MELD) scores were evaluated (n=449). Due to missing variables 49 MELD scores could not be computed (center A n=7, center B n=41).

IRI and NAS

The degree of both ischemic and reperfusion hepatocellular injury was evaluated by postoperative serum levels of alanine aminotransferase (ALT). Serum ALT was determined during the 7 consecutive days after OLT and measured by routine biochemical methods. The highest level of the first peak ALT was evaluated individually. Since there is no clear definition of NAS in the literature we have used the definition with the most clinical relevance. NAS was defined as follows: any *treated* stricture or irregularity of the intra- or extrahepatic bile ducts occurring at least 1 cm above the anastomosis post-OLT. Analysis of NAS development was performed in both the combined cohort as well as the individual cohorts. For timing the first endoscopic or percutaneous treatment for NAS, i.e., balloon dilatation and/or a performed stenting procedure, was used. Non-anastomotic biliary strictures that did not require intervention and anastomotic strictures were not included in the definition of NAS for the current study. Because of a different pathophysiological mechanism, biliary strictures as a result from hepatic artery thrombosis or arterial complications were not considered NAS and were excluded.

Statistical analyses

Statistical analysis was performed using IBM SPSS 20.0. A Mann-Whitney U test was used to calculate differences in medians, and Fisher's exact test was done for categorized variables. The optimal cut-off-value for peak ALT is defined as the point with the most significant split for association with NAS or no NAS as determined by log-rank test. Using the calculated cut-off value, a peak ALT below this value was considered as mild IRI, whereas a peak ALT above this value was considered as severe IRI. Cumulative incidence curves were established using one minus survival incidence rates according to the Kaplan-Meier method and risk factor analysis was performed using univariate and multivariate stepwise forward Cox regression analysis. In case of a p-value of <0.20 in the univariate analysis the parameter was taken into account in the multivariate analysis. A p-value of <0.05 was considered statistically significant.

Retrospective studies are approved by the institutional review board by legislation and the study was performed according to the guidelines of the Helsinki and the Istanbul declaration.

Results

A total number of 496 OLTs with a minimum follow-up of 7 days were performed in both centers, with 399 DBD donor livers and 97 DCD donor livers. Median follow-up from OLT until development of NAS was 4.4 months (range 0.3 – 58). Donor and recipient variables are presented in Table 1.

Table 1. Baseline characteristics. Data presented as median (range) for continuous variables and percentage (number) for categorized variables.

Characteristic	DBD (*n*=399)	DCD (*n*=97)	*p*
Donor age (median, range)	50 (14 - 86)	44 (14 - 65)	**<0.01**
Donor gender % (n)			**<0.05**
Male	49.4 (197)	60.8 (59)	
Female	50.6 (202)	39.2 (38)	
Recipient age (median, range)	53 (17 - 70)	54 (19 - 69)	0.62
Recipient gender % (n)			0.16
Male	60.9 (243)	69.1 (67)	
Female	39.1 (156)	30.9 (30)	
MELD (median, range)	16 (6 - 40)	14 (6 - 40)	0.42
Diagnosis preOLT % (n)			0.87
ALD	16.8 (67)	15.5 (15)	
HCC	10.5 (42)	11.3 (11)	
PSC	16.8 (67)	17.5 (17)	
PBC	4.8 (19)	7.2 (7)	
HBV	5.5 (22)	2.1 (2)	
HCV	9.5 (38)	11.3 (11)	
AIH	6.3 (25)	4.1 (4)	
Metabolic	7.3 (29)	7.2 (7)	
Other	22.6 (90)	23.7 (23)	
NAS % (n)	13.3 (53)	30.9 (30)	**<0.01**
CIT (median, 25 – 75% range)	503 (420 - 619)	460 (407 - 517)	**<0.01**
RWIT (median, 25 – 75% range)	40 (34 - 49)	40 (34 - 47)	0.44
DWIT (median, 25 – 75% range)	-	17 (14 - 20)	
Peak AST (median, range)	1006 (64 - 14750)	2557 (200 - 19590)	**<0.01**
Peak ALT (median, range)	719 (69 - 8242)	1525 (106 - 11105)	**<0.01**

DBD = Donation after Brain Death, DCD = Donation after Circulatory Death, MELD = Model for End-Stage Liver Disease, OLT = Orthotopic Liver Transplantation, ALD = Alcoholic Liver Disease, HCC = Hepatocellular Carcinoma, PSC = Primary Sclerosing Cholangitis, PBC = Primary Biliary Cirrhosis, HBV = Hepatitis B Virus, HCV = Hepatitis C Virus, AIH = Auto-Immune Hepatitis, NAS = Non-anastomotic Biliary Strictures, CIT = Cold Ischemia Time (min), RWIT = Recipient Warm Ischemia Time (min), DWIT = Donor Warm Ischemia time (min), AST = Aspartate aminotransferase (IU/l), ALT = Alanine aminotransferase (IU/l)

Donor and surgical variables

Median donor age for DCD donors was significantly lower compared to DBD donors in the combined cohort (44 vs. 50 years respectively, $p<0.001$). Cold ischemia time (CIT) was shorter for DCD donors than for DBD donors (DCD 460 min vs. DBD 503 min; $p=0.003$), but recipient warm ischemia time (RWIT) did not differ for both types of donors ($p=0.44$). In case of DCD-OLT, median donor warm ischemia time from cardiac arrest to flush (DWIT) was 17 minutes. As DWIT is considered a risk factor for the development of NAS, DWIT was evaluated separately for patients who developed NAS and patients without NAS, however this was not significantly different (NAS 17 minutes vs. no NAS 16 minutes, $p=0.62$). In order to evaluate injury of the liver parenchyma peak ALT was evaluated. The median time point of the first peak ALT was found to be at 1 day after OLT. Median peak ALT was significantly higher after OLT using DCD donors than after DBD-OLT (DCD 1525 IU/l vs. DBD 719 IU/l; $p<0.001$). Similar results were observed for median peak AST (DCD 2557 IU/l vs. DBD 1006 IU/l; $p<0.001$). For each cohort individually, similar results were obtained (data not shown). Because of the strong correlation between peak AST and peak ALT (Pearson's coefficient=0.86, $p<0.001$) and a more explicit cut-off value and higher AUC in the ROC-curve for peak ALT further analysis was performed for peak ALT only.

Other serum markers, such as bilirubin, alkaline phosphatase and gamma-glutamyltransferase, were not included in the analyses because no optimal cut-off value could be determined.

To determine the potential influence of blood transfusion and kidney function on peak ALT level correlation tests were performed. However, no correlation was found between peak ALT and creatinine level ($p=0.715$) or estimated glomerular filtration rate ($p=0.400$) and between peak ALT and the volume of erythrocyte, fresh frozen plasma and cellsaver transfusion ($p=0.284$, $p=0.173$ and $p=0.241$ respectively).

IRI and NAS

In the combined cohorts NAS developed in 31% after DCD-OLT and in 13% after DBD-OLT ($p<0.001$). The incidence of NAS was not statistically different between center A and center B ($p=0.37$). Optimal cut-off value of serum ALT for NAS was calculated using log-rank statistics and a receiver operating characteristic (ROC)

curve for DCD-OLT (*n*=97) and was established at ≥1300 IU/L (Figure 2A and 2B). Using the calculated cut-off value, a low peak ALT of <1300 IU/L was considered mild IRI whereas a high peak ALT of ≥1300 IU/L was considered severe IRI. Using this cut-off point sensitivity of peak ALT level to predict NAS was 87%, specificity 54%, the positive predictive value 46% and the negative predictive value 90%. A cut-off value of ≥1300 IU/l corresponded with the highest Youden Index of 0.41 and a positive Likelihood Ratio of 1.87. After DCD-OLT, severe IRI preceded NAS development in 46% cases compared to 10% in the mild IRI group (p<0.001). (Table 2). Four-year cumulative incidence of NAS development was 49.5% in case of severe IRI compared to 11.3% when mild IRI occurred (log-rank p=0.001; Figure 3). In 11% of DCD-OLT cases retransplantation for NAS was needed. When the cumulative incidence was calculated for each cohort individually, similar results were obtained. In both cohorts severe IRI was significantly associated with NAS development after DCD-OLT. (Data not shown) No optimal cut-off value of peak ALT could be obtained for DBD-OLT. (Figure 4) Therefore, the threshold for severe IRI of ≥1300 IU/l was also applied to the DBD-cohort. However, no association could be found between peak ALT ≥1300 IU/L and NAS after DBD-OLT, neither in the individual cohorts nor in the combined group (p=0.74). Based on the cut-off value determined for DCD-OLT four-year cumulative incidence of NAS after DBD-OLT in the combined group was 14.3% when peak ALT was ≥1300 IU/L compared to 13.7 % when peak ALT was <1300 IU/L (log-rank p=0.94).

Besides higher incidences of NAS, a peak ALT of ≥ 1300 IU/l was also associated with worse patient and graft survival. Overall, mortality or retransplantation rate was 36% of the patients with severe IRI, as compared to 26% of the patients with mild IRI (p=0.03). Twenty-three patients were previously excluded due to primary non-function of the graft, early mortality and a follow-up of less than 7 days. This included 16 DBD-OLTs (3.9% of all DBD-OLT) and 7 DCD-OLTs (6.7%). Ten out of 16 DBD-OLTs and 6 out of 7 DCD-OLTs had a peak ALT of ≥1300 IU/l, indicating that severe IRI may also be associated with early graft loss. Mortality within 1 month was 1.5% for the patients with mild IRI and 4.2% for patients with severe IRI (all after DBD-OLT), which did not differ between the groups (p=0.12).

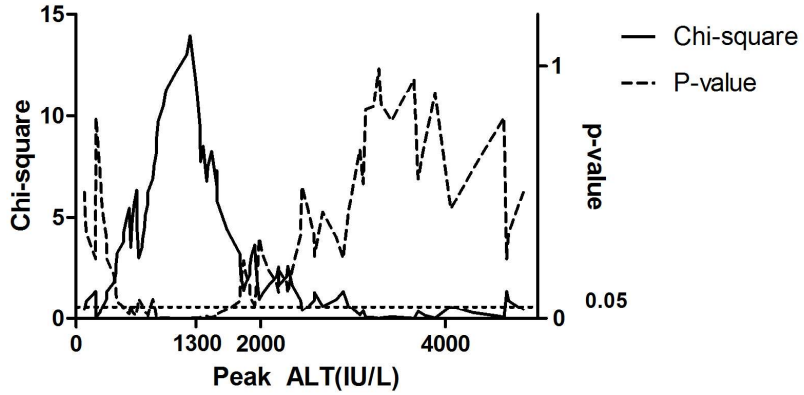

Figure 2A. Optimal ALT cut-off point for NAS after DCD-OLT.
Calculation of the optimal ALT cut-off (≥1300 IU/L) for prediction of NAS in DCD-OLT using the combined cohorts.

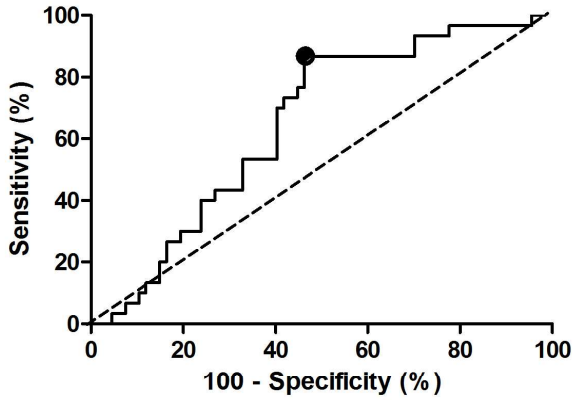

Figure 2B. ROC curve of peak ALT for NAS after DCD-OLT.

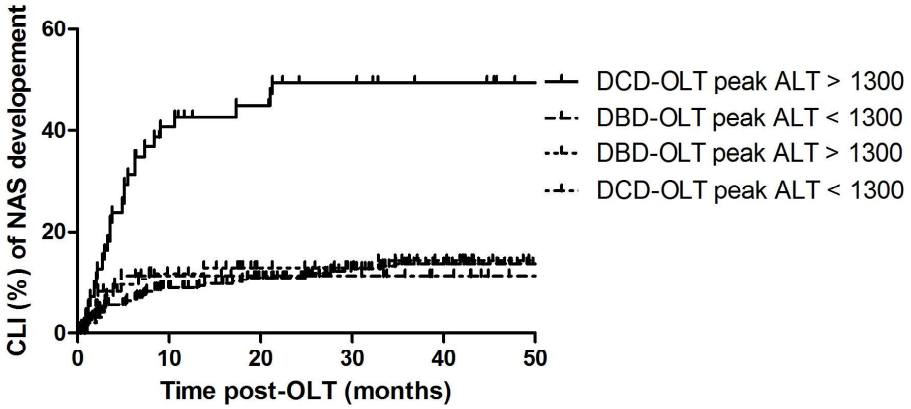

Figure 3. Development of NAS post OLT.
Cumulative incidence (CLI) of non-anastomotic biliary strictures (NAS) development after orthotopic liver transplantation (OLT) with livers from donation after cardiac death (DCD) reached 49.5% at 48 months when peak alanine aminotransferase (ALT) ≥1300 IU/L (n=57) compared to 11.3% when peak ALT <1300 IU/L (n=40) (p<0.001). CLI rates of NAS after DBD-OLT did not differ between recipients with mild or severe ischemia-reperfusion injury (IRI) or DCD-OLT with peak ALT < 1300 IU/l. CLI rates were calculated using one minus survival incidence rates with the Kaplan-Meier test and compared using log-rank test.

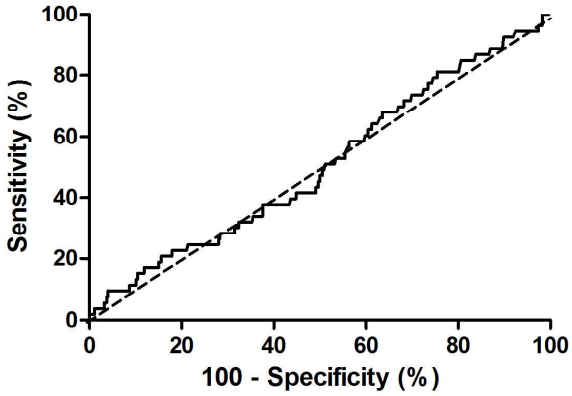

Figure 4. ROC curve of peak ALT for NAS after DBD-OLT.

Table 2. Development of NAS respective of degree of ischemia-reperfusion injury (IRI). Numbers are presented as % (n).

Combined cohorts (center A and center B)				
Graft type	DBD		DCD	
	No NAS	NAS	No NAS	NAS
Mild IRI	86.2 (250)	13.8 (40)	90.0 (36)	10.0 (4)
Severe IRI	88.1 (96)	11.9 (13)	54.4 (31)	45.6 (26)
	p=0.74		**p<0.001**	

DBD = Donation after Brain Death, DCD = Donation after Circulatory Death,
NAS = Non-anastomotic Biliary Strictures, IRI = Ischemia-Reperfusion Injury.
Mild IRI was defined as peak ALT ≤ 1300 IU/l and severe IRI as peak ALT > 1300 IU/l.

Primary Sclerosing Cholangitis and NAS

Because radiodiagnostic features of NAS resemble the diagnostic criteria for primary sclerosing cholangitis (PSC) distinguishing NAS and recurrent PSC can be difficult. Especially late occurrence of NAS might in fact be recurrent PSC. Therefore, the analysis was also performed after the exclusion of all patients with PSC (n=84). After the exclusion of patients with PSC the four-year cumulative incidence of NAS after DCD-OLT was 52.4% in case of severe IRI compared to 13.5% when peak ALT was < 1300 IU/l (log-rank p=0.002). For DBD-OLT four-year cumulative incidence was 9.0% when peak ALT was <1300 IU/l compared to 11.4% IU/l when peak ALT was ≥1300 IU/l (log-rank p=0.49).

Univariate and Multivariate analysis

Cox regression analysis for risk of NAS development was performed for OLT with DCD and DBD donors separately. For DCD-OLT, donor age, MELD score, CIT and peak ALT ≥1300 were significantly associated with NAS in the univariate analysis at the p<0.20 value and were thus included in the multivariate analysis. PSC as indication for OLT, as well as the center at which OLT was performed were not associated with development of NAS in the univariate analysis for DCD-OLT. Multivariate analysis showed peak ALT ≥1300 and donor age to be independently associated factor for the development of NAS after DCD-OLT, adjusted for MELD score and CIT (adjusted hazard ratio (aHR) for peak ALT ≥ 1300 = 3.71, confidence interval (CI) = 1.26 – 10.91, p=0.017) (Table 3). Multivariate analysis revealed PSC as indication for OLT to be the only independently associated parameter for the development of NAS after DBD-OLT (aHR = 2.37, CI 1.32 – 4.26, p=0.004). (Table 4)

Table 3. Univariate and multivariate analysis of risk factors for development of NAS after DCD-OLT.

Variables DCD-OLT		%	Univariate analysis		Multivariate analysis	
			HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Donor age	Continuous		1.04 (1.00 – 1.07)	**0.01**	1.04 (1.00 – 1.07)	**0.04**
Donor gender	Male Female (reference)	60.8 39.2	1.36 (0.65 – 2.85) 1	0.42		
Donor ICU stay (days)	Continuous		1.00 (0.85 – 1.18)	0.97		
Recipient age at OLT	Continuous		1.01 (0.98 – 1.04)	0.65		
Recipient gender	Male Female (reference)	69.1 30.9	1.21 (0.57 – 2.58) 1	0.62		
MELD score	Continuous		1.03 (0.97 – 1.07)	0.17	1.03 (0.99 – 1.07)	0.22
Peak ALT	Severe Mild (reference)	58.841.2	4.90 (1.71 – 14.05) 1	**0.003**	3.71 (1.26 – 10.91)	**0.02**
DWIT	Continuous		1.01 (0.98 – 1.04)	0.49		
CIT	Continuous		1.00 (1.00 – 1.01)	0.19	1.00 (1.00 – 1.01)	0.32
RWIT	Continuous		0.98 (0.94 – 1.02)	0.28		
PSC as indication	Other (reference) PSC	82.5 17.5	1 0.47 (0.14 – 1.55)	0.21		
Study Center	Center B Center A (reference)	60.8 39.2	0.66 (0.32 – 1.36) 1	0.26		
Preservation solution	UW HTK (reference)	33.0 67.0	1.06 (0.50 – 2.27) 1	0.88		
MAP ≤ 55 mmHg (during reperfusion)	No Yes (reference)	48.3 51.7	1.28 (0.54 – 3.02) 1	0.57		

HR = Hazard Ratio, CI = Confidence Intervals, OLT = Orthotopic Liver Transplantation, ICU = Intensive Care Unit, MELD = Model for End-stage Liver Disease, ALT = Alanine aminotransferase, DWIT = Donor Warm Ischemia Time, RWIT = Recipient Warm Ischemia Time, CIT = Cold Ischemia Time, PSC = Primary Sclerosing Cholangitis, UW = University of Wisconsin, HTK = Histidine-Tryptophan-Ketoglurarate, MAP = Mean Arterial Pressure

Table 4. Univariate and multivariate analysis of risk factors for development of NAS after DBD-OLT.

Variables DBD-OLT		%	Univariate analysis		Multivariate analysis	
			HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Donor age	Continuous		1.01 (0.99 – 1.03)	0.38		
Donor gender	Male	49.4	0.95 (0.55 – 1.62)	0.85		
	Female (reference)	50.6	1			
Recipient age at OLT	Continuous		0.99 (0.97 – 1.01)	0.15	0.99 (0.97 – 1.01)	0.25
Recipient gender	Male	60.9	1.89 (1.02 – 3.47)	**0.04**	1.82 (0.98 – 3.39)	0.06
	Female (reference)	39.1	1			
MELD score	Continuous		1.00 (0.97 – 1.04)	0.89		
Peak ALT	Mild (reference)	72.7	1			
	Severe	27.3	1.03 (0.55 – 1.92)	0.94		
CIT	Continuous		1.00 (1.00 – 1.00)	0.77		
RWIT	Continuous		1.01 (0.99 – 1.04)	0.34		
PSC as indication	Other (reference)	83.2	1			
	PSC	16.8	2.71 (1.53 – 4.78)	**0.001**	2.37 (1.32 – 4.26)	**0.004**
Study Center	Center B	65.9	1.29 (0.71 – 2.34)	0.41		
	Center A(reference)	34.1	1			

HR = Hazard Ratio, CI = Confidence Intervals, OLT = Orthotopic Liver Transplantation, MELD = Model for End-stage Liver Disease, ALT = Alanine aminotransferase, DWIT = Donor Warm Ischemia Time, RWIT = Recipient Warm Ischemia Time, CIT = Cold Ischemia Time, PSC = Primary Sclerosing Cholangitis

Discussion

The present study describes a strong and independent association between a serum peak ALT of ≥1300 IU/l and the development of NAS after liver transplantation with donation after circulatory death (DCD-OLT). A higher donor age was also independently associated with NAS after DCD-OLT.

In the last decade, transplantation of livers from DCD donors has become common in the Netherlands and United Kingdom in order to decrease wait-list mortality. However, these grafts are known to be associated with higher complications rates, such as primary non-function, ischemia-reperfusion injury and nonhepatic complications such as the development of end-stage renal disease during follow-up after OLT.[19;20] It is therefore important to balance the risk for post-transplant complications after DCD-OLT and the survival benefit for an individual patient. In 2012, Jay and al. reported improvements in effectiveness for DCD-OLT when MELD scores were >15 as compared to lower MELD scores. However, for patients with a MELD score of 15 to 20 a high cost-effectiveness ratio, due to high direct medical costs, was reported.[21] The price of donation after circulatory death was further discussed by van der Hilst et al.[22] That study described a significantly higher cost per life year for DCD grafts, mainly due to a higher complication rate and a longer ICU stay.

Similar patient survival was reported for DCD-OLT and DBD-OLT after a national protocol for accepting DCD organs was implemented in the Netherlands in 2001, with currently 30-40% of OLTs with a DCD donor.[17]

Biliary complications are among the most frequent complications after DCD-OLT leading to considerable morbidity and mortality. A recent meta-analysis of O'Neill et al. including 1619 patients with DCD-OLT showed an Odds Ratio for biliary complications of 2.4 as compared to DBD-OLT.[23] NAS is considered the most challenging biliary complication because the strictures are often located beyond the scope of endoscopic treatment and resistant to therapy. It has been hypothesized that ischemia-reperfusion injury (IRI) may play an important role in the development of NAS. IRI is the combined result of ischemia, reperfusion, status of the graft (e.g., steatosis or not) and the immune reactions of the recipient during and after reperfusion. DCD grafts are known to be more prone to IRI due to an additional donor warm ischemia time (DWIT) and to have more biliary complica-

tions after OLT than DBD grafts.[24] In order to reduce ischemic injury and hopefully compensate for DWIT-induced injury, ischemia times, especially cold ischemia time, are kept shorter for DCD-OLT compared to DBD-OLT. However, ischemia times are not indicative for injury induced during reperfusion and there is evidence that most IRI-induced hepatic injury develops after restoration of blood flow, at least partially due to an excess of reactive oxygen species.[25;26] Serum peaks of transaminases within the first 7 days after OLT reflect, more than ischemia times, liver parenchyma injury since it includes both periods of ischemia and reperfusion. OLT with DCD donors have higher peak AST and ALT levels post-operatively than DBD-OLT. The current data shows that severe IRI, defined as peak serum ALT ≥1300 IU/L post OLT, is strongly associated with the increased incidence of NAS after DCD-OLT, with a four-year cumulative incidence rate of NAS in this group of 49.5%. In the multivariate analysis peak ALT ≥1300 IU/L was independently associated with the development of NAS after DCD-OLT. Furthermore, our study showed a higher donor age to be an independent risk factor for the development of NAS after DCD-OLT. This is an important finding as advancing age is generally accepted to be associated with more hepatic steatosis, whereas Baccarani et al. described hepatic steatosis to be a risk factor for posttransplant biliary complications.[27] However, donor age as a risk factor remains subject to debate in previous studies. Whereas some studies showed an advancing age to be associated with higher incidences of NAS, others have reported similar results for DCD-OLT when younger and older donors were compared.[7;8;28;29]

In literature, DWIT has also been associated with the development of NAS. This may be the result of continued energy consumption of the graft at body temperature during DWIT. Due to an inadequate tissue perfusion this energy consumption may lead to adenosine triphosphate depletion and a shift from aerobic to an anaerobic metabolism, which may result in a more injured graft. In the present study, likely due to a low variability of DWIT between the patients, DWIT was not an independent risk factor. DWIT was defined as the time between circulatory arrest and cold flush with preservation fluid in the donor. This is slightly different than some other studies where DWIT is defined as the time between cessation of cardiopulmonary support and cold flush.[30] However, the absolute time period of DWIT is not the only risk factor of DWIT for the development of NAS. Because of different characteristics of the graft, such as steatosis and immune responses of the donor and recipient, some grafts may be more prone to injury from DWIT than

others and the actual effect of DWIT on the graft may be different even though the time period is not different. Furthermore, Op den Dries et al.[31] recently evaluated peribiliary plexus injury. DBD livers showed a significantly higher percentage of grafts without any peribiliary vascular plexus injury than DCD livers (18% vs. 0%). Injury in the deep peribiliary glands was also more severe and more prevalent in patients that developed NAS, compared to patients without NAS.

We therefore hypothesize that the higher peak ALT and increased incidence of NAS in a subgroup of DCD-OLT patients are different presentations of the same ischemia-reperfusion injury due to the donor warm ischemia time (DWIT) combined with warm ischemia between respiratory withdrawal and cardiac arrest in the donor.

After DBD-OLT, peak ALT was not associated with the development of NAS, whereas PSC was a risk factor in these patients. After DCD-OLT with peak ALT below 1300 IU/l the risk of NAS was similar to DBD-OLT. Therefore, a peak ALT below 1300 IU/l could be used as a negative predicting factor in the development of NAS. Mangus et al.[32] have recently demonstrated that the pre-transplant donor peak ALT is not related to post-transplant ALT levels. Moreover, a higher donor peak ALT was not indicative for early graft function and 1-year survival. Close monitoring of recipient peak ALT may therefore be an important first marker to predict clinical patient and graft outcome after transplantation and allow a more intensive follow-up.

Reducing IRI in DCD-OLT to the extent that peak ALT is below 1300 IU/l will probably diminish the incidence of NAS to the incidence after DBD-OLT (14%), but may not completely eliminate NAS, as other pathophysiological mechanisms might be involved. This is consistent with the idea that NAS is most likely the result of a complex mechanism involving ischemic, immunologic and toxic processes which all affect the biliary tree or its vascular supply.[33-35] Current preservation solutions and techniques may be insufficient.[36] Several attempts are being made to improve preservation and reduce IRI of liver grafts using machine liver perfusion and/or abdominal regional perfusion, but also fibrinolytic agents are used to dissolve possible microthrombi in the donor liver.[37-39]

The current study has certain limitations. The sample size is relatively small. It is clear that these novel findings need to be replicated in larger cohorts. Furthermore, we used only ALT and not AST as marker for IRI occurring in the liver, since AST is derived from mitochondria in liver cells but is also produced in heart, skeletal muscles and brain cells. After surgery, AST can also be elevated due to damage of the abdominal muscles during surgery, and this makes it less specific as a parameter of IRI after OLT. However, most cases of NAS developed within the first 6 months after OLT. Whether a more intensive follow-up and earlier intervention in patients with high peak ALT after DCD-OLT might prevent retransplantation remains to be established.

In conclusion, our data show that serum peak ALT ≥1300 IU/l and a higher donor age are strongly and independently associated with the development of clinically relevant NAS after DCD-OLT, with peak ALT below 1300 IU/l predicting a risk similar to DBD-OLT. In DCD-OLT it can thus be used in classifying patients as high-risk or low-risk (similar to DBD-OLT) for developing NAS. The current data indicate that the higher risk of NAS after DCD as compared to DBD is likely the result of cases of DCD-OLT with more severe IRI due to warm ischemia in the donor. This enables the use of peak ALT below 1300 IU/l as target for future interventions aimed at prevention of NAS and a peak ALT of ≥1300 IU/l as a justification for a more intensive follow-up in DCD-OLT.

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Chapter 3

No association of donor specific anti-HLA antibodies with non-anastomotic biliary strictures but both are independent risk factors for graft loss after liver transplantation

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Abstract

Introduction Donor-specific alloantibodies (DSA) have been associated with rejection and shorter graft survival after orthotopic liver transplantation (OLT). We examined the role of DSA in non-anastomotic biliary strictures (NAS) after OLT.

Patients and Methods Patients receiving first OLT who developed NAS (*n*=68) and a control group without NAS (*n*=83), with pre-OLT and 12 months post-OLT serum samples were included. DSA were specified using the Luminex single antigen test. Risk factors for NAS and graft survival were analysed.

Results The presence of preformed DSA was not significantly different between patients with NAS and controls (p=0.89). After 12 months, 26.5% of NAS patients and 16.9% of controls had generated de novo DSA (p=0.15). Neither de novo class I DSA nor de novo class II DSA were associated with NAS. De novo DSA generally developed after the diagnosis of NAS. Time-dependent regression analysis identified both NAS (aHR 8.05, CI 3.28 – 19.77, p<0.01) and de novo class II DSA (aHR 2.84, CI 1.38 – 5.82, p<0.01) as independent risk factors for graft loss.

Conclusion Preformed or de novo DSA were not associated with the development of NAS. However, NAS as well as de novo class II DSA were independent risk factors for graft loss after OLT.

Introduction

The presence of donor-specific alloantibodies (DSA) against human leukocyte antigen (HLA) has been associated with (hyper)acute rejection and shorter graft survival after organ transplantation in general.[1;2] Yet, after orthotopic liver transplantation (OLT), preformed DSA can be absorbed by the graft and hyperacute rejection as a result of DSA is rare.[3-5] While ABO-compatibility is required, the presence of DSA or a positive HLA crossmatch is not considered a contraindication to OLT.[6] Despite the absence of hyperacute rejection, recent studies have shown that DSA may be a more relevant predictor for patient and graft outcome after OLT than previously assumed. In a large study cohort preformed class II DSA were associated with both early graft loss and rejection.[7] In addition, Kaneku et al. recently found that de novo DSA, formed after OLT, were detected in 8% of the patients and were associated with a significantly impaired patient and graft survival.[8] Furthermore, it has been suggested that de novo DSA with a high mean fluorescence intensity (MFI) are associated with chronic rejection after OLT.[9]

Non-anastomotic biliary strictures (NAS) can be defined as intra- and extrahepatic lesions of the biliary tree more than 1 cm above the anastomosis, characterized by bile duct strictures and dilatations.[10;11] Reported incidences of treated NAS vary between 9% and 31%. NAS is considered a major cause of morbidity and reduced graft survival after OLT.[12] Whereas the complete pathogenesis of NAS is still unclear, several risk factors such as ischemia-reperfusion injury[13], grafts donated after cardiac death and damage from bile salt toxicity have been identified.[14;15] Besides these factors, a contribution of immune-mediated cholangiocyte injury has been proposed.[16] This is supported by reports on an increased incidence of NAS in cases of ABO-incompatibility, underlying disease with assumed autoimmune aetiology -such as primary sclerosing cholangitis (PSC) and autoimmune hepatitis- in patients with a genetic chemokine receptor 5 loss of function, and after cytomegalovirus viremia (CMV).[17-20] The biliary epithelium has been shown to express HLA class I and class II molecules after OLT[21] and the presence of antibodies against these antigens may therefore possibly be a factor contributing to NAS development. Indeed, earlier studies demonstrated that the presence of preformed DSA leading to a positive cytotoxic crossmatch was associated with bile duct complications in general and/or with preservation injury, which is an

important risk factor for NAS development.[10;22-24]. Therefore, the aim of the present study was to evaluate the relationship between preformed and de novo DSA with development of NAS and with graft survival after OLT.

Patients and Methods

Patients receiving ABO-compatible OLT in two Dutch transplantation centers between 2000 and 2014 who developed NAS (*n*=68) and a control group of OLT patients without NAS matched for recipient age, recipient gender, aetiology, and acute rejection, and transplanted in the same time period (*n*=83), of whom pre-OLT and 12 months post-OLT serum samples were available, were selected for the present study. Duration of follow-up was at least one year for all patients. Presentation was with bacterial cholangitis, jaundice and/or itching in around one third of cases, all with cholestatic liver enzymes. In two thirds of patients no symptoms were present while elevated serum alkaline phosphatase and gamma glutamyl transferase, and bilirubin in some, prompted further investigation. In all NAS patients, the diagnosis was confirmed with direct cholangiography, i.e., endoscopic retrograde cholangiography or percutaneous transhepatic cholangiography. In all cases diagnosed with NAS biliary strictures more than 1 cm above the biliary anastomosis were present as determined by at least two endoscopists and conformed in a multidisciplinary radiology meeting. Moreover, a strict definition of NAS was used, which required that the biliary strictures should have been treated at least once by dilatation and/or stenting by ERCP or PTC, ensuring that the strictures were considered clinically significant. In this cohort only a minority underwent MRI-MRCP first. In all of these cases NAS was intrahepatic and most often diffuse. Some were only perihilar, a minority in addition had extrahepatic non-anastomotic biliary strictures. Only patients without vascular (e.g. hepatic artery thrombosis) or other biliary complications were included. Hepatic artery thrombosis or stenosis and other complications were excluded by Doppler-ultrasound or CT angiography in all cases.

Graft survival was determined as time of transplantation until graft loss (retransplantation or death) or in case of no event, until the end of the study (June 2014). Time to NAS was determined as time from transplantation until detection of NAS

or in case of no event until graft loss or the end of the study (June 2014). Demographic and clinical data of liver transplant recipients, like age, gender, aetiology of liver disease and post-transplant complications, were derived from the electronic patient charts. Immunosuppression in both centers was similar, consisting of induction with basiliximab and Methylprednisolone, followed by Tacrolimus, or -in some cases- Ciclosporin micro emulsion, Prednisolone for 6 months and in some cases addition of Mycophenolate Mofetil, Azathioprine or -after 3 months- Everolimus or Sirolimus.

The study was approved by the Medical Ethics Committee (Protocol B14.014) and in accordance with the Declaration of Helsinki. All patients gave informed consent to donate pre-transplantation and post-transplantation blood samples for research purposes, without given preference to any explicit clinical variables. Only patients with a minimum age of 18 years who gave informed consent and donated a blood sample were included in the study.

HLA typing and determination of anti-HLA class I and class II alloantibodies
Patient and donor DNA samples were genotyped using either the sequence-specific oligonucleotide probe (PCR/SSOP) technique for HLA-DR and-DQ, or the reverse SSO method on a suspension array platform using microspheres as a solid support to immobilize oligonucleotide probes (for HLA-A and-B: Lifecodes from Immucor Transplant Diagnostics Inc. Stamford, CT, USA). Serum samples from recipients were screened for the presence of anti-HLA alloantibodies using the Lifecodes Lifescreen Deluxe (LMX) kit, according to the manufacturer's manual (Immucor Transplant Diagnostics Inc. Stamford, CT, USA). Samples that were positive for either HLA class I (HLA-A or HLA-B) or HLA class II (HLA-DQ or HLA-DR) antibodies were further analysed with a Luminex Single Antigen assay, using LABscreen HLA class I and class II antigen beads (One Lambda, Canoga Park, GA, USA). Briefly, 4 µL of LABscreen beads and 20 µL of serum were mixed in a test well, protected from light. Serum samples were incubated for 30 min. at room temperature on a rotating platform (150 rpm), followed by repeated washings with 260 µL wash buffer (1X). Afterwards, each sample was incubated for 30 min. with a goat anti-human PE conjugated antibody (1:100 wash buffer) at room temperature, protected from light, and subsequently washed 5 times with wash buffer. Samples were measured using a Luminex 100 reader (Luminex 100,

Luminex Corporation, 's-Hertogenbosch, the Netherlands). LABscreen negative control serum (LS-NC, One Lambda) was used as a negative control. Antibodies detected with a mean fluorescence intensity (MFI) of >5000 were considered positive, as DSA levels above this cut-off value are associated with clinically relevant outcomes after OLT.[7;8]

Statistical analysis

Statistical analysis was performed using SPSS version 20.0 for windows (SPSS Inc. Chicago, IL, USA). Continuous data were analysed with the Student t-test or Mann-Whitney U test. Chi-square test was performed for categorized data. A risk factor analysis for NAS and for graft loss was performed using univariate and (for univariate factors with $p<0.10$) multivariate Cox regression analysis for baseline factors with forward selection and backward exclusion. Both NAS and de novo class II DSA were considered risk factors for graft loss and as these predictors occur in the course of time, instead of being baseline variables, these factors were taken into account as time-dependent covariates in a time-dependent Cox regression analysis. A p-value of <0.05 was considered statistically significant.

Results

Patients

In total 68 patients with non-anastomotic biliary strictures (NAS) and 83 controls without NAS after first OLT were included in the present study. Median age of all recipients at time of transplantation was 55 (IQR 46 – 61) years. In 31 patients (20.5%) OLT was performed with a graft from donation after circulatory death (DCD). Median time from OLT to diagnosis of NAS was 5.5 months (IQR 1.6 – 12.8). Baseline characteristics of patients with and without NAS are expressed in Table 1. No significant differences were found between patients with and without NAS, including the incidence of acute cellular rejection, with the exception of DCD-OLTs: as expected, NAS developed more frequently in recipients who received a DCD graft as compared to a liver after DBD donation ($p<0.01$).

Table 1. Baseline characteristics. Data are presented as median (interquartile range) for continuous variables. Categorized data are presented as number (percentage).

	NAS (*n*=68)	Controls (*n*=83)	*p*-value
Donor age	47 (37 – 55)	47 (36 – 58)	0.980
Donor gender			0.364
Male	37 (54.4)	39 (47.0)	
Female	31 (45.6)	44 (53.0)	
Recipient age	55 (45 – 62)	54.0 (47 – 60)	0.928
Recipient gender			0.429
Male	46 (67.6)	51 (61.4)	
Female	22 (32.4)	32 (38.6)	
Etiology			0.854
Viral	7 (10.3)	11 (13.3)	
ALD	13 (19.1)	13 (15.7)	
PSC	13 (19.1)	17 (20.5)	
HCC	14 (20.6)	21 (25.3)	
Other*	21 (30.9)	21 (25.3)	
CMV mismatch (D+/R-)	28 (47.5)	44 (53.7)	0.467
Episode of acute rejection	14 (20.6)	12 (14.5)	0.321
Donation after cardiac death	24 (35.3)	7 (8.4)	**0.000**
Donor BMI	24 (23 – 27)	24 (22 – 26)	0.573
Recipient BMI	25 (23 – 28)	25 (23 – 28)	0.731
DWIT (min) in DCD	20 (16 – 29)	17 (13 – 20)	0.645
CIT (min)	438 (382 – 558)	452 (369 – 546)	0.444
RWIT (min)	33 (26 – 42)	31 (24 – 34)	0.148

NAS = Non-anastomotic Biliary Strictures; ALD = Alcoholic Liver Disease; PSC = Primary Sclerosing Cholangitis; HCC = Hepatocellular Carcinoma; CMV = Cytomegalovirus; BMI = Body Mass Index; DWIT = Donor Warm Ischemia Time; CIT = Cold Ischemia Time; RWIT = Recipient Warm Ischemia Time.
*Other indications include: auto-immune hepatitis, non-alcoholic steatohepatitis and polycystic liver disease.

Donor-specific antibodies and NAS

Preformed DSA were detected in 10.3% (*n*=7) of the NAS patients and in 9.6% (*n*=8) of the controls (Table 2). The presence of preformed class I DSA or preformed class II DSA was not significantly different between patients with NAS and controls in chi-square test. Female patients were more likely to have generated DSA before transplantation (female 20.4% vs. male 4.1%, $p<0.01$). DSA did not develop more frequently in patients transplanted with a DCD graft (DBD 22.5% vs. DCD 16.1%, $p=0.44$). One year after OLT, 32 patients (21%) generated de novo DSA. In 29 out of 32 patients (90.6%) who generated de novo DSA, the newly developed antibodies were directed against the HLA class II antigens of the donor, in most of the cases against HLA-DQ (69% DQ only, 14% DR only and

17% both DQ and DR). Overall, the development of DSA post-transplantation was not related to NAS development, as 26.5% of NAS patients and 16.9% of the controls had de novo DSA 1 year after OLT (p=0.15). Neither de novo class I DSA, nor de novo class II DSA were significantly more often present in patients with NAS, as compared to controls (Table 2). The incidence of alloantibodies directed at antigens of the individual loci, i.e., HLA-A, HLA-B, HLA-DQ and HLA-DR, was also not different between the two groups (data not shown). We further analysed the cumulative MFI of DSA between patients with NAS and controls, however, no significant difference was found (13601 vs. 10925, p=0.27).

Table 2. Prevalence of preformed and de novo DSA (MFI>5000) in patients with NAS and controls. Data are presented as n (%).

	NAS (n=68)	Controls (n=83)	p-value
Preformed			
Any DSA	7 (10.3)	8 (9.6)	0.893
Class I	4 (5.9)	7 (8.4)	0.548
Class II	2 (2.9)	1 (1.2)	0.447
Class I and II	1 (1.5)	0	0.268
De novo			
Any DSA	18 (26.5)	14 (16.9)	0.151
Class I	3 (4.4)	0	0.053
Class II	15 (22.1)	14 (16.9)	0.420

Uni- and multivariate analysis of pre-OLT risk factors for developing NAS are shown in Table 3. As expected a DCD donor was a risk factor for NAS both in univariate and multivariate analysis, while a donor with a positive CMV IgG status was a risk factor in univariate analysis with only a trend in multivariate analysis. Preformed DSAs overall and class I and II apart were not risk factors for NAS, and likewise the other examined factors were not a risk for developing NAS (Table 3). Uni- and multivariate analysis for baseline risk factors were also examined after exclusion of DCD OLT. In DBD OLT a trend existed for RWIT as a risk factor for NAS both in uni- and multivariate analysis and no significant risk factors for NAS were found here (Table 4). In the whole cohort and in DBD OLT there was also no relationship between DSAs against various HLA antigens and NAS with mean fluorescence intensity (MFI) of >1000 (instead of >5000) as cut-off for presence of DSA (Tables 3 and 4).

Table 3. Univariate and multivariate analysis of risk factors for NAS (all patients).

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Donor age	1.00 (0.98 – 1.02)	0.835		
Donor gender male	0.93 (0.56 – 1.59)	0.826		
CMV IgG donor positive	0.54 (0.32 – 0.92)	**0.023**	1.61 (0.93 – 2.78)	0.088
CMV mismatch (D pos/R neg)	0.73 (0.35 – 1.53)	0.409		
CMV IgG recipient positive	0.77 (0.42 – 1.43)	0.403		
Recipient age	1.01 (0.99 – 1.04)	0.404		
Recipient gender male	1.21 (0.73 – 1.01)	0.472		
Gender mismatch	0.89 (0.53 – 1.51)	0.669		
BMI donor	1.01 (0.93 – 1.09)	0.854		
OLT indication grouped*	(0.66 – 1.24)	0.540		
OLT indication PSC	1.36 (0.61 – 2.13)	0.690		
Roux-en-Y anastomosis	0.65 (0.34 – 1.27)	0.208		
MELD-score recipient	1.01 (0.95 – 1.08)	0.791		
Donor type DCD	3.02 (1.82 – 4.76)	**0.000**	2.67 (1.47 – 4.87)	**0.001**
Preformed DSA Type I (>5000)	1.01 (0.41– 2.51)	0.988		
Preformed DSA Type II (>5000)	0.62 (0.15 – 2.56)	0.152		
Preformed DSA Type I (>1000)	1.22 (0.52 – 2.82)	0.650		
Preformed DSA Type II (>1000)	1.13 (0.36 – 3.62)	0.832		
CIT	1.00 (0.99 – 1.01)	0.507		
RWIT	1.01 (0.99 – 1.03)	0.221		

HR = Hazard Ratio; CI = Confidence Intervals; OLT = Orthotopic Liver Transplantation; CMV = Cytomegalovirus, DBD = Donation after Brain Death; DCD = Donation after Circulatory Death; BMI = Body Mass Index; NAS = Non-anastomotic Biliary Strictures; DSA = donor specific antibodies against HLA (MFI>5000); ad *) viral, alcoholic, PSC, or other underlying liver disease.

Table 4. Univariate and multivariate analysis of risk factors for NAS (DCD excluded).

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Donor age	1.00 (0.98 – 1.02)	0.936		
Donor gender male	0.76 (0.41 – 1.42)	0.392		
CMV IgG donor positive	1.45 (0.78 – 2.68)	0.238		
CMV IgG recipient positive	1.30 (0.62 – 2.74)	0.491		
Recipient age	0.99 (0.96 – 1.02)	0.505		
Recipient gender male	1.06 (0.58 – 1.94)	0.858		
Gender mismatch	0.86 (0.46 – 1.61)	0.642		
BMI donor	1.01 (0.92 – 1.12)	0.802		
OLT indication grouped*	(0.28 – 2.08)	0.897		
OLT indication PSC	1.02 (0.50 – 2.08)	0.964		
Roux-en-Y anastomosis MELD-score recipient	1.77 (0.54 – 5.82)	0.345		
	1.02 (0.91 – 1.13)	0.784		
Preformed DSA Type I (>5000)	0.96 (0.34– 2.70)	0.933		
Preformed DSA Type II (>5000)	0.52 (0.12 – 2.15)	0.366		
Preformed DSA Type I (>1000)	0.90 (0.35 – 2.30)	0.829		
Preformed DSA Type II (>1000)	1.31 (0.32 – 5.46)	0.708		
CIT	1.00 (0.99 – 1.00)	0.244		
RWIT	1.02 (0.99 – 1.03)	0.079	1.02 (0.99 – 1.03)	0.079

HR = Hazard Ratio; CI = Confidence Intervals; OLT = Orthotopic Liver Transplantation; CMV = Cytomegalovirus, DBD = Donation after Brain Death; DCD = Donation after Circulatory Death; BMI = Body Mass Index; NAS = Non-anastomotic Biliary Strictures; DSA = donor specific antibodies against HLA (MFI>5000); ad *) viral, alcoholic, PSC, or other underlying liver disease.

Longitudinal analysis in a subgroup of NAS patients (*n*=20) showed that pre-existing DSA against class I HLA usually disappeared within the first 6 months after transplantation. De novo class I DSA tended to develop early after OLT, but were usually already cleared from the circulation at 12 months. Class II DSA were more likely to be persistent (data not shown). Importantly, in NAS patients, de novo DSA generally developed after the diagnosis of NAS (Figure 1, patients 1,2,5,7,8,9,10,11,12,13,14,16,19).

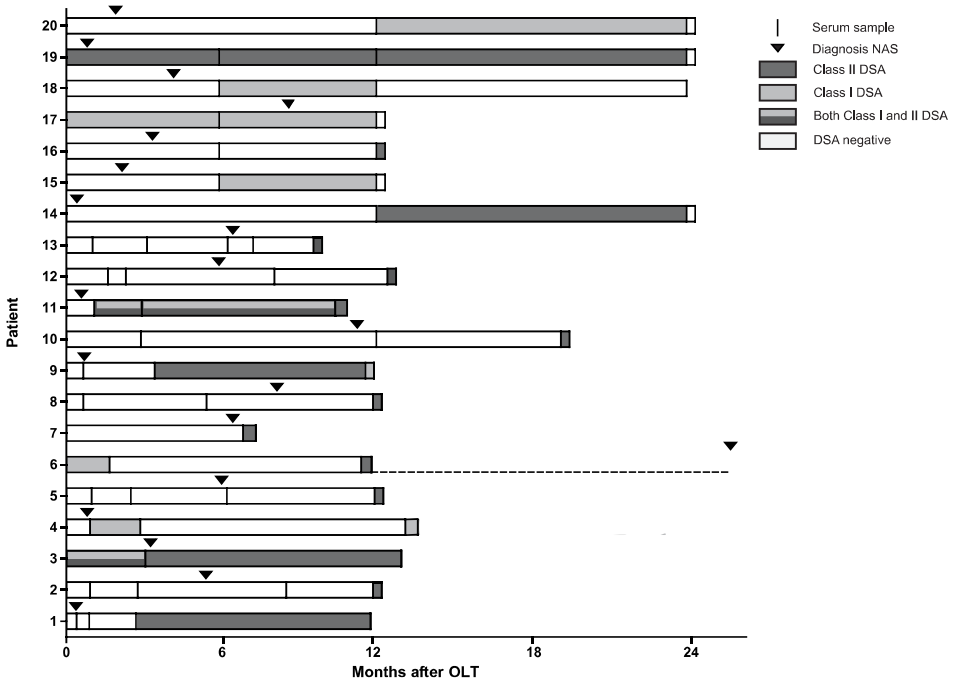

Figure 1. Donor-specific alloantibodies (DSA) status during follow-up in patients with non-anastomotic biliary strictures (NAS). Each patient is represented by a bar. The presence of class I and class II DSA is represented by different shades of gray. Repeated measurements were only available in a subgroup of NAS patients (*n*=20)

DSA and graft survival

In total, 19 (12.6%) patients died and 15 (9.9%) patients required a retransplantation during follow-up. Univariate and multivariate analysis of possible baseline risk factors for graft survival revealed male sex and donor BMI as independent risk factors for graft loss. Time-dependent Cox regression analysis identified NAS (aHR = 8.05, 95% CI 3.28 – 19.77, $p<0.01$) and de novo class II DSA (aHR = 2.84, 95% CI 1.38 – 5.82, $p<0.01$) as independent risk factors for graft loss (Table 5). De novo DSA after OLT had a similar impact on graft survival in patients with NAS and in controls ($p=0.10$).

Table 5. Univariate and multivariate analysis of pre-OLT risk factors for graft loss.

Variables		Univariate analysis		Multivariate analysis	
		HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Recipient age at OLT	Continuous	1.02 (0.99 – 1.05)	0.254		
Recipient gender	Male	2.47 (1.01 – 6.06)	**0.049**	2.69 (1.07 – 6.79)	**0.036**
Recipient BMI	Continuous	1.04 (0.96 – 1.13)	0.320		
Donation	DCD	1.35 (0.55 – 3.35)	0.515		
Donor age	Continuous	1.02 (0.99 – 1.04)	0.259		
Donor gender	Male	1.35 (0.65 – 2.78)	0.420		
Donor BMI	Continuous	1.11 (0.99 – 1.25)	**0.063**	1.09 (0.96 – 1.24)	0.175
CMV mismatch	Yes (D+/R-)	0.65 (0.29 – 1.45)	0.288		
Rejection	Yes	0.80 (0.30 – 2.10)	0.650		
Pre-OLT DSA	Class I	1.69 (0.22 – 12.92)	0.612		
Pre-OLT DSA	Class II	0.73 (0.10 – 5.51)	0.760		
NAS	Time-dependent			8.05 (3.28 -19.77)	**< 0.010**
De novo DSA class II	Time-dependent			2.84 (1.38 – 5.82)	**< 0.010**

HR = Hazard Ratio; CI = Confidence Intervals; OLT = Orthotopic Liver Transplantation; CMV = Cytomegalovirus, DBD = Donation after Brain Death; DCD = Donation after Circulatory Death; BMI = Body Mass Index; NAS = Non-anastomotic Biliary Strictures

In the NAS group 15 retransplantations were done for NAS with recurrent bacterial cholangitis (*n*=13), infection unrelated to NAS (*n*=1) and chronic rejection (*n*=1). In the NAS group 13 patient died; causes of death were recurrent cholangitis (*n*=1), chronic rejection (*n*=1), cholangitis from recurrent PSC (*n*=1), pulmonary embolism (*n*=1), multi-organ failure (*n*=1), malignancy (*n*=2), infection unrelated to NAS (*n*=2), unknown (*n*=4). In the control group without NAS no patient underwent retransplantation and 6 died; causes of death were infection (*n*=2), malignancy (*n*=2), and unknown (*n*=2).

Discussion

The present study demonstrated that both NAS and DSA against HLA class II generated post-transplantation have a significant impact on graft survival. Yet, neither preformed, nor de novo DSA directed against class I or class II HLA antigens are associated with an increased risk for NAS development. Preformed DSA often disappear after OLT, while de novo DSA were more often generated after than before the diagnosis of NAS.

Antibody-mediated rejection (AMR) as a result of DSA is a serious event in kidney, lung and heart transplant recipients, associated with an increased risk of graft loss.[1;2;26] Conversely, liver allografts were presumed to be highly resistant to DSA, mainly due to the liver's ability to absorb circulating anti-HLA antibodies. Indeed, in the current study preformed DSAs usually disappeared within a year after OLT -with class II persisting longer than class I-. This property was even presumed to be one of the factors providing a protective effect against acute renal allograft rejection in simultaneous liver-kidney transplantations and may be the result of release of soluble MHC complexes from the liver.[27] However, a recent study demonstrated that renal allograft protection by the liver is incomplete in case of preformed class II DSA and that, subsequently, despite some protection both grafts remain at risk for rejection.[28] In addition, preformed and de novo DSA are increasingly recognized as a cause of rejection and allograft loss after OLT.[7;8] This is probably the result of the ability of DSA to bind to endothelial cells in the portal triads leading to complement activation. Consistent with previous literature[8;29], DSA formed after OLT were, in the majority of cases, directed against class II antigens, mainly against HLA-DQ. Serial measurements demonstrated that anti-

bodies against class II antigens tended to appear later after transplantation and to remain longer in the circulation compared to antibodies against class I antigens. Class II DSA have previously been associated with graft loss.[30]. The present study shows that after OLT this is only the case for de novo class II DSA. The prevalence of preformed and de novo DSA in this study cohort was 9.9% and 21.2 % respectively. This is consistent with previous studies in which preformed DSA were detected in 5 – 22% of the liver transplantation candidates.[7;31;32]. For de novo DSA, the reported prevalence varies between 8 and 24%.[8;29;33]

Bile duct complications after OLT are frequent, and a major cause of morbidity and mortality. The role of DSA in the occurrence of bile duct complications after transplantation was unclear. Under physiological conditions, biliary epithelial cells highly express HLA class I antigens, whereas there is only a weak expression of class II antigens.[34] However, as a result of ischemia during OLT a sterile immune response is initiated, with elevated levels of pro-inflammatory cytokines (e.g., interferon-γ and interleukins) and infiltration of inflammatory cells into the biliary epithelium, which can result in an upregulation of HLA class II antigens.[21;35;36]. Ischemia-reperfusion injury (IRI) of the biliary epithelium is also considered as one of the most important risk factors for NAS development. The effect of IRI is not limited to cholangiocytes only. In a previous study, we showed that severe IRI to hepatocytes, as reflected by a high peak alanine aminotransferase, was also an important risk factor for NAS development.[10] Interestingly, Gugenheim et al. demonstrated an upregulation of hepatic class I antigen expression after a period of normothermic ischemia in a rat model.[37]

It could therefore be expected that a relation exists between IRI, the presence of antibodies against donor HLA antigens expressed on hepatocytes and biliary epithelium and bile duct complications, such as the development of non-anastomotic biliary strictures. In the present study we could not find an association between preformed or de novo generated DSA and NAS. According to the present data de novo DSA were also not related to NAS. De novo DSA -most of class II- generally developed after the diagnosis of NAS, supporting the conclusion that de novo DSA may worsen NAS, but probably are not involved in the initiation of NAS. This is in accordance with a study performed by Iacob et al.[38] In that study, post-transplant DSA in relation to both anastomotic and non-anastomotic biliary strictures was evaluated. The presence of post-transplant HLA class II DSA was

related to the development of anastomotic strictures, but no association was found between the presence of alloantibodies and the occurrence of NAS. Yet, because of the cross-sectional design of that study, it remained unclear whether these DSA were persistent or formed after transplantation, and several studies -including the current one- have demonstrated that predominantly *de novo* DSA are relevant in predicting transplantation outcome.[8;9] Furthermore, the levels of DSA were not described by Iacob et al. In the present study, only DSA with a MFI of >5000 were considered positive, as mainly high MFI DSA are related to an inferior clinical outcome after OLT.[7;9] Sensitivity analysis was done in the current study for MFI>1000 with similar outcomes. Other studies regarding this topic were not specific to NAS. For example, Takeya et al. found an association between DSA and heterogeneous group of bile ducts complications, consisting of patients with biliary obstructions, biliary strictures and focal necrosis of the bile ducts.[23] DSA have also been suggested to be related to ductopenia[39], which is most likely the result of chronic and irreversible rejection.[22;40;41]

A limitation of the present study is that from the majority of patients post-transplant DSA could only be measured one year after transplantation. Therefore, the influence of de novo DSA that are transiently present, but are cleared from the circulation in the first year after transplantation, may have been missed in our analysis. This might have resulted in underestimation of the contribution of transient de novo class I DSA, which in a subgroup of patients that we analysed longitudinally developed early after OLT but were cleared from the circulation within the first year after transplantation. Therefore, a prospective study with repeated DSA measurements during follow-up is required to provide information on a possible association between transiently appearing de novo DSA and NAS development. Another problem is that radiodiagnostic features of NAS resemble the diagnostic criteria for PSC. Therefore, it may be difficult to distinguish NAS and recurrence of PSC after OLT. Since graft failure occurred in only four PSC patients (two without DSA and two with de novo class II DSA), and since PSC was not a risk factor for NAS in this study, we consider it unlikely that this has influenced our results.

According to the original Banff guidelines, the diagnosis of antibody-mediated rejection after kidney transplantation requires three criteria: morphologic evidence of tissue injury, serologic evidence of DSA formation or other anti-donor endothelial antigens, and evidence of antibody interaction with vascular endothelium, e.g.,

C4d deposition in the peritubular capillaries.[42] Recently, the criteria were revised and acute and chronic antibody-mediated rejection may now be diagnosed in the absence of C4d deposition. However, in this case additional evidence of current or recent antibody interaction with the vascular endothelium must be present.[43]

In conclusion, the present study is the first study that assessed the relationship of DSA with non-anastomotic biliary stricture development and graft survival after orthotopic liver transplantation. Neither preformed DSA nor de novo DSA generated within the first year after liver transplantation were associated with the development of NAS. However, time-dependent analysis revealed that both NAS and de novo class II DSA developing after liver transplantation were independently associated with graft loss.

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Chapter 4

Matrix metalloproteinase/ inhibitor genotypes and the development of non-anastomotic biliary strictures after orthotopic liver transplantation

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Submitted

Abstract

Introduction Non-anastomotic biliary strictures (NAS) are a common complication after orthotopic liver transplantation (OLT). Matrix metalloproteinases (MMPs) encompass a family of more than 25 different proteolytic enzymes involved in several physiological and pathological processes, including matrix degradation. Genetic polymorphisms of MMPs and tissue inhibitors of MMPs (TIMPs) were assessed in relation to NAS.

Patients and Methods A total of 314 OLT donor/recipients combinations were included in this single center study. Several MMP-2, MMP-7, MMP-9, TIMP-1 and TIMP-2 gene (promotor) single nucleotide polymorphisms (SNPs) were evaluated in donors and recipients in relation to NAS development, using PCR-RFLP or PCR-ARMS analysis.

Results The cumulative incidence of NAS within the first 4 years after OLT was 19.0% (n=51). In this population, donor and recipient genotype in the evaluated MMP and TIMP genes were not associated with a higher incidence of NAS ($0.27 < p < 0.97$). In addition, NAS patients did not have significantly more mismatches between donor and recipient in any of the evaluated SNPs ($0.10 < p < 0.90$). Cox's regression analysis identified donation after circulatory death [aHR = 3.77, CI 2.16 – 6.57, $p<0.01$] as the only independent risk factor for NAS development.

Conclusion No relation was found between the presence of MMP-2, MMP-7, MMP-9, TIMP-1 and TIMP-2 gene polymorphisms in liver transplant donors and/or recipients, and the development of non-anastomotic biliary strictures.

Introduction

Biliary complications, such as bile duct leakage and biliary strictures, are among the most frequent complications after orthotopic liver transplantation (OLT). Biliary strictures can, based on their anatomical location, be further subdivided into anastomotic strictures (AS) and non-anastomotic strictures (NAS).[1] Even though clinical presentation and treatment of both types of biliary strictures is similar, the pathophysiological mechanism of their development is rather different. Anastomotic strictures are associated with a higher donor age, local ischemia and surgical technique, whereas NAS is the result of a more complex mechanism, probably including pretransplant graft status, ischemia-reperfusion injury, bile salt toxicity and immunological factors.[2;3] In addition, genetic factors involved in the immune system have also been associated with a higher incidence of NAS. For example, it has been demonstrated that a deletion of the chemokine CCR5-Δ32 gene leads to a loss of function of the CC chemokine receptor 5 and subsequently to impaired chemotaxis of T-cells. Op den Dries et al. showed that patients with this genetic mutation have a four-fold higher risk of developing NAS, especially those patients transplanted for primary sclerosing cholangitis, i.e., a probable auto-immune disease with biliary strictures.[4]

Matrix metalloproteinases (MMPs) encompass a family of more than 25 different proteolytic enzymes involved in several physiological processes, including matrix degradation for tissue remodelling and repair. According to their structure and substrate preference, MMPs are further subdivided into collagenases, stromelysins, gelatinases and membrane-type MMPs. The activity of MMPs is, among other processes, regulated by tissue-inhibitors of matrix metalloproteinases (TIMPs).[5;6]

The gelatinases –MMP-2 and MMP-9– are capable of degrading collagen type I, IV, V, VII and X, of which mainly type IV collagen is present in the basement membranes. Damage to basement membranes may make bile duct damage irreversible even in the presence of progenitor cells. Polymorphisms in the promotor region of MMP-2 lead to a decreased mRNA transcription, and may therefore reduce the degradation process of collagen.[7] Indeed, probably as a result of this mechanism, we showed previously that a MMP-2 (-1306 C/T) gene promotor polymorphism in either donor and/or recipient is associated with a significantly higher incidence of NAS after OLT.[8] It is therefore interesting to evaluate genetic poly-

morphisms in other relevant MMP and TIMP genes. The development of NAS is often preceded by severe ischemia-reperfusion injury.[9] Several human and animal studies showed that MMP-9 and TIMP-1 play an important role in ischemia-reperfusion injury.[10-13] As TIMPs reversely inhibit active MMPs, we hypothesized that gene polymorphisms in the TIMP-1 and TIMP-2 promotors may also have an effect on NAS development.

In addition, collagen type IV, proMMP-2 and proMMP-9 are important substrates for MMP-7.[14] Therefore, the risk of NAS development as a result of genetic polymorphisms in the MMP-2, MMP-7 and MMP-9 genes, as well as the TIMP-1 and TIMP-2 genes, were evaluated in the present study.

Material and Methods

In total, 314 patients with a minimum follow-up of 7 days, and of whom both donor and recipient DNA was available, were included in the study. The present cohort consisted of the previously described 147 patients that were transplanted in the Leiden University Medical Center between 1992 and 2006[8], complemented with 167 patients, transplanted between 2006 and 2013. The cohort included 249 grafts from donation after brain death (DBD) and 65 grafts of donation after circulatory death (DCD).

Patient follow-up was determined as time of transplantation until the first occurring event (i.e., development of NAS, retransplantation, or death) or in case of no event, until a maximum of 4 years after OLT. Non-anastomotic biliary strictures were defined as follows: any stricture or irregularity requiring treatment, located in the intra- or extrahepatic bile ducts occurring at least 1 cm above the anastomotic site, as previously described.[9] Biliary strictures located at the anastomosis until 1 cm above were considered anastomotic strictures, and not included in the analysis. Treatment was defined as endoscopic or percutaneous treatment, i.e., balloon dilatation and/or performed stenting procedures, or –also in case of NAS without endoscopic or radiological treatment options– retransplantation.

The study was performed in accordance with the guidelines of the Medical Ethics Committee of the LUMC and the Declaration of Helsinki. All patients on the wait-

ing list for liver transplantation were requested to donate a blood sample to the liver disease biobank for research purpose only, without given preference to any explicit clinical variables. Only patients with a minimum age of 18 years during the study who gave informed consent and donated a blood sample were included in the study.

Genomic DNA of recipients was extracted by routine methods from peripheral blood. Donor DNA, isolated from blood or spleen tissue samples, was provided by the Eurotransplant Reference Laboratory or freshly isolated. In case no blood or tissue sample was available, liver biopsy tissue of the allograft in the recipient was used to evaluate donor DNA. DNA extraction from liver biopsies was performed with the Qiagen DNA Mini Kit.

Genotype frequencies were determined by restriction fragment length polymorphism (RFLP) polymerase chain reaction (PCR), or in case of MMP-2 by tetra primer amplification refractory mutation system (ARMS) PCR.[8] For RFLP genotyping, the region containing the SNP of interest was amplified with outer primers. Thereafter, a RFLP analysis with a specific restriction enzyme was performed to produce base pair fragments of different lengths. The genotypes are identified from differences in migration pattern on agarose gels. Specification of outer primers and restriction enzymes used for the analysis of SNPs in MMP and TIMP genes are described in Table 1. The MMP-2 SNP was determined by ARMC-PCR, the principles of which are previously described by Ye et al.[15]

Statistical analysis

Statistical analysis was performed using SPSS version 20.0 for windows (SPSS Inc. Chicago, IL, USA). Mann-Whitney U tests and Chi-square tests were performed for continuous data and categorized data respectively. A risk factor analysis was performed using Cox regression analysis. To compare SNP frequencies between patients with and without NAS, the p-value was corrected by Bonferroni correction for multiple testing. Therefore, a p-value of <0.01 was considered a significant difference. Post-hoc power analysis was performed with G*Power.[16]

Table 1. Specification of primers and restriction enzymes used for analysis of SNPs in MMPs and TIMPs with size of PCR products on agarose gel and corresponding genotype.

Gene/SNP	Forward and reverse primers	Restriction enzyme	Products (bp)/genotype
MMP-2 – 1306C/T	ACCAGACAAGCCTGAACTTGTCTGA TGTGACAACCGTCTCTGAGGAATG ATATTCCCCACCCAGCACGCT GCTGAGACCTGAAGAGCTAAAGAGTTG		542+379; 542+379+211; 542+211 CC; CT; TT
MMP-9 – 1562C/T	ATGGCTCATGCCCGTAATC TCACCTTCTTCAAAGCCCTATT	SpH I	352;352+208+144; 208+144 CC; CT; TT
TIMP-1 – 372C/T	GCACATCACTACCTGCAGTC GAAACAAGCCCACGATTTAG	BssS I	175; 20+155+175; 20+155 TT or T; CT; CC or C
TIMP-2 – 303C/T	CCTCCTCGGCAGTGTGTG TAGGAACAGCCCCACTTCTG	TspR I	16+112; 16+24+97; 16+24+97+112 CC; CT; TT
MMP-7 – 181A/G	TGGTACCATAATGTCCTGAATG TCGTTATTGGCAGGAAGCACACAATGAATT	EcoRI	150; 120+30+150; 120+30 AA; AG; GG
MMP-7 – 153C/T	ACGAATACATTGTGTGCTTCCTGCCAATCA TTTATATAGCTTCTCAGCCTCG	NlaIII	158; 158+127+32; 127+32 CC, CT, TT

SNP=Single Nucleotide Polymorphism; MMP=Matrix Metalloproteinase; TIMP=Tissue Inhibitor Metalloproteinase; PCR=Polymerase Chain Reaction.
NOTE. Analysis of MMP-2 – 1306C/T was performed by Amplification Refractory Mutation System (ARMS) PCR with four oligonucleotide primers, but no restriction enzyme.

Results

A total of 314 OLT donor/recipients combinations were included in the study. The cumulative incidence of NAS within the first 4 years was 19.0% (*n*=51). Donor, recipient and procedure variables in relation to NAS are presented in Table 2. No significant differences were found between patients with and without NAS, with the exception of donation after circulatory death, which was more frequent in patients who developed NAS. Median interval from OLT until NAS development was 3.7 months (interquartile range 2.4 – 7.0 months). Follow-up time was not different in patients with NAS, as compared to controls (46 months vs. 48 months, *p*=0.631).

All observed genotype frequencies of the evaluated SNPs in donors and recipients were in Hardy-Weinberg equilibrium ($p>0.05$). The donor and recipient genotype frequencies of MMP-2, MMP-9, MMP-7, TIMP-1 and TIMP-2 SNPs in relation to NAS are presented in Table 3. The presence of genetic polymorphisms was not associated with a higher incidence of NAS ($0.27 < p < 0.97$). Therefore, these genetic polymorphisms were not included in the further risk factor analysis. With the allele frequencies found in the present study, post-hoc analysis revealed a power of 79.8%, which should be sufficient to detect differences of interest.

Cox regression analysis for risk of NAS development (Table 4) revealed donation after circulatory death [aHR = 3.77, CI 2.16 – 6.57, $p<0.01$] as the only risk factor associated with NAS development. Because DCD-OLT was a strong predictor for NAS, DBD-OLTs and DCD-OLTs were evaluated separately. Donor and recipient genotype frequencies of the evaluated SNPs in DBD-OLTs ($0.13 < p < 0.96$) and DCD-OLTs ($0.20 < p < 0.93$) were not related to NAS.

Furthermore, the influence of a mismatch in MMP or TIMP genotype between donor and recipient was analysed. NAS patients did not have significantly more mismatches between donor and recipient in any of the evaluated SNPs ($0.10 < p < 0.90$). In general, when both donor and recipients had a wildtype genotype of the evaluated SNPs the incidence of NAS was not different as compared to patients in which donor and/or recipient had a genotype polymorphism ($0.23 < p < 0.91$).

Table 2. Comparison of donor, recipient and procedure variables between patients with and without NAS after OLT. Values are expressed as percentages (numbers), unless otherwise specified.

	Total (*n*=314)	NAS (*n*=51)	No NAS (*n*=263)	*p*-value
Donor age (years, median, interquartile range)	47 (35 – 56)	47 (40 – 55)	47 (33 – 56)	0.679
Gender				0.280
Male	51.9 (163)	58.8 (30)	50.6 (133)	
Female	48.1 (151)	41.2 (21)	49.4 (130)	
Recipient age (years, median, interquartile range)	51 (44 – 59)	50 (41 – 57)	52 (44 – 59)	0.240
Gender				0.845
Male	67.8 (213)	66.7 (34)	68.1 (179)	
Female	32.2 (101)	33.3 (17)	31.9 (84)	
Primary liver disease				0.275
ALD	26.4 (83)	33.3 (17)	25.1 (66)	
Viral	26.1 (82)	17.6 (9)	27.8 (73)	
PSC	13.4 (42)	17.6 (9)	12.5 (33)	
PBC	5.1 (16)	7.8 (4)	4.6 (12)	
Other disease	29.0 (91)	23.5 (12)	30.0 (79)	
MELD score (median, interquartile range)	19 (13 – 24)	21 (10 – 24)	18 (13 – 24)	0.218
OLT procedure				**0.000**
DBD	79.3 (249)	54.9 (28)	84.0 (221)	
DCD	20.7 (65)	45.1 (23)	16.0 (42)	
DWIT (minutes, median, interquartile range)	17 (13 – 20)	18 (12 – 19)	16 (13 – 20)	0.521
CIT (minutes, median, interquartile range)	563 (470 – 693)	559 (445 – 650)	563 (472 – 698)	0.875
RWIT (minutes, median, interquartile range)	35 (31 – 42)	35 (31 – 43)	35 (31 – 41)	0.727

NAS = Non-anastomotic biliary strictures, OLT = Orthotopic Liver Transplantation, ALD = Alcoholic Liver Disease, HCV = Hepatitis C Virus, HBV = Hepatitis B Virus, PSC = Primary Sclerosing Cholangitis, MELD = Model for End-Stage Liver Disease, DBD = Donation after Brain Death, DCD = Donation after Circulatory Death. DWIT = Donor Warm Ischemia Time, CIT = Cold Ischemia Time, RWIT = Recipient Warm Ischemia Time

Table 3. Genotype distributions at SNP loci in donors and recipients (%).

		Recipient			Donor		
Protein/SNP	Genotype	NAS	No NAS	*p*-value	NAS	No NAS	*p*-value
MMP-2 -1306 C/T	CC	64.7	61.8	0.267	56.9	50.8	0.414
	CT	35.3	33.2		35.3	44.3	
	TT	0	5.0		7.8	5.0	
MMP-9 -1562 C/T	CC	78.0	79.0	0.559	76.0	77.0	0.888
	CT	22.0	19.1		24.0	22.6	
	TT	0	1.9		0	0.4	
TIMP-1* +372 C/T (male)	C	48.5	45.2	0.732	51.7	49.1	0.803
	T	51.5	54.8		48.3	50.9	
TIMP-1* +372 C/T (female)	CC	11.8	23.7	0.539	19.0	26.5	0.586
	CT	47.1	43.4		52.4	53.8	
	TT	41.2	32.9		28.6	19.7	
TIMP-2 +303 C/T	CC	72.5	73.9	0.974	68.6	73.5	0.500
	CT	25.5	24.4		31.4	25.2	
	TT	2.0	1.7		0	1.3	
MMP-7 -181 A/G	AA	31.4	32.8	0.953	31.4	26.9	0.561
	AG	49.0	46.6		39.2	47.5	
	GG	19.6	20.6		29.4	25.6	
MMP-7 -153 C/T	CC	86.0	91.5	0.386	86.0	85.5	0.806
	CT	14.0	8.1		14.0	13.7	
	TT	0	0.4		0	0.9	

*TIMP-1 372C/T is localised on the X chromosome. Therefore allele frequencies of males and females are expressed separately.

Because radiodiagnostic features of NAS resemble the diagnostic criteria for primary sclerosing cholangitis (PSC), it may be difficult to distinguish NAS and recurrence of PSC after OLT. However, pathophysiological mechanisms of NAS and recurrent PSC may be different, and the presence of MMP and/or TIMP gene polymorphisms may have a different effect on the development of NAS. Therefore, the analysis was also performed after exclusion of patients with PSC (n=42). The exclusion of these patients did not lead to an altered SNP distribution of patients with or without NAS (data not shown).

Unfortunately, we could not replicate the association previously found between MMP-2 genetic polymorphisms and NAS development. (Supplementary Figure 1). However, when we compared the baseline characteristics of the first and second cohort, we found that several important characteristics differed between both cohorts, whereas the frequency distribution of the MMP/TIMP SNPs were found to be comparable (data not shown), except for a minor difference in the MMP-2

genotype distribution of the donors. (Supplementary Table 1) For example, donors and recipients were significantly older in the second cohort (p<0.01) and recipients had a higher MELD score at time of transplantation (p<0.01). OLTs with a graft from donation after circulatory death, were significantly more often performed in cohort 2 (p<0.01). A trend towards a higher incidence of PSC patients in the first cohort was found (17.0 vs 10.2%, p=0.08). When only those patients with NAS and a MMP-2 gene polymorphism were selected, cold ischemia time was longer (median, 634 vs. 532 minutes, p<0.05) in the first cohort than in the second cohort.

Table 4. Results of the Cox regression analysis of risk factors for development of NAS.

Variables		HR (95% CI)	*p*-value
Donor age	Continuous	1.01 (0.99 – 1.03)	0.382
Donor gender	Male Female (reference)	1.48 (0.85 – 2.58) 1	0.169
Recipient age at OLT	Continuous	0.99 (0.97 – 1.01)	0.302
Recipient gender	Male Female (reference)	0.97 (0.54 – 1.74)	0.920
MELD score	Continuous	1.01 (0.99 – 1.03	0.409
DWIT	Continuous	1.03 (0.91 – 1.18)	0.610
CIT	Continuous	1.00 (0.99 – 1.00)	0.775
RWIT	Continuous	1.01 (0.98 – 1.03)	0.648
PSC as indication	Other PSC (reference)	1.41 (0.687 – 2.90) 1	0.349
DCD	No (reference) Yes	1 3.77 (2.16 – 6.57)	**0.000**

HR = Hazard Ratio; CI = Confidence Intervals; NAS = Non-anastomotic Biliary Stricture; OLT = Orthotopic Liver Transplantation; MELD = Model for End-stage Liver Disease; DWIT = Donor Warm Ischemia Time; RWIT = Recipient Warm Ischemia Time; CIT = Cold Ischemia Time; PSC = Primary Sclerosing Cholangitis; DCD = Donation after Circulatory Death

Discussion

In the present study, functional SNPs in the promotor or exon region of several matrix metalloproteinases and their specific inhibitors were assessed to evaluate a possible association of these polymorphisms with the development of NAS. The results suggest that no relation exists between the presence of MMP-2, MMP-7, MMP-9, TIMP-1 and TIMP-2 gene polymorphisms in liver transplant donors and

recipients and the development of non-anastomotic biliary strictures. In a previous study, we found that a MMP-2 (-1306 C/T) gene promotor polymorphism in either donor and/or recipient is associated with a significantly higher incidence of NAS after OLT.[8] These results could not be reproduced in the present study.

There are several possible explanations for our negative findings. First of all, the actual effect of MMPs and TIMPs gene polymorphisms on MMP and TIMP function is not entirely clear. As a result of their matrix degrading ability, previous studies showed MMP and TIMP levels to be associated with various pathological processes, such as tumour progression and the severity and outcome of haemorrhagic diseases.[17-19] It would therefore be expected that processes that alter activity or mRNA transcription of MMPs and TIMPs, such as gene promotor polymorphisms, may be related to further progression of pathological processes. However, some SNPs that have been described in the literature may be less functional than previously assumed. For example, the frequently described MMP-2 1306 C/T polymorphism results in less mRNA transcription, but subsequently, the serum levels of MMP-2 are not changed. The same phenomenon was seen for the MMP-9 1562 C/T polymorphism.[7;8] Lorente et al. described patients with the T allele of the TIMP-1 372 C/T polymorphism to have a higher serum level of TIMP-1. In the same study a significant correlation was found on TIMP-1 level and MMP-9 level.[20] It would therefore be expected that this specific polymorphism in the TIMP gene would also result in alterations of serum MMP-9 level. Yet, a direct relation between TIMP-1 372 genotype and MMP-9 level has not been described so far. Therefore, probably as a result of a compensatory mechanism, a lower mRNA transcription rate does not always lead to altered serum levels, which can prevent possible negative effects on disease progression related to genetic variations.

Secondly, MMPs in serum predominantly exist as free (inactive) pro-MMP or pro-MMP complexed with TIMPs. For NAS development, the local effects of active MMPs in the extracellular matrix of liver tissue are particularly important. It is known that high MMP serum levels are not always associated with high local tissue concentrations of MMP. For example, a non-significant correlation coefficient *r* of 0.07 between serum MMP-2 level and MMP-2 concentration in normal colorectal tissue has been found.[21] Murawaki et al.[22] demonstrated significantly higher MMP-2 serum and hepatic tissue levels in patients with liver cirrhosis as compared to non-cirrhotic patients. In fibrosis and cirrhosis, the hepatic stellate cells release

active MMP-2 into the extracellular matrix, leading to high levels of MMP-2 in the local tissue. However, it remains unclear whether there is any correlation in tissue and serum level in patients with a relatively healthy graft after transplantation, and, more important, whether the effects of MMP and TIMP gene polymorphisms on serum levels are similar to alterations in local tissue.

Thirdly, in addition to the effect on MMP and TIMP levels, the direct effect of genetic polymorphisms on disease development and progression is also controversial. Whereas Langers et al., for example, described single nucleotide polymorphism MMP-2 1306 C/T to be associated with worse survival in colorectal cancer patients, Li et al demonstrated in a meta-analysis that a CT or TT genotype is protective against digestive cancers in 9067 patients, with a more protective effect in the Asian than in the European population.[23;24] This suggests that, besides MMPs and TIMPs, other factors are also involved and that MMP genotypes may have different effects in different ethnical groups.

For liver disease specifically a similar phenomenon was found. Several human and animal studies have demonstrated an important role of MMP levels in hepatic ischemia and reperfusion injury.[10;13] Hamada et al.[11] described that MMP-9 deficiency or inhibition was associated with less leukocyte recruitment and less hepatic ischemia reperfusion injury in a mouse model, as compared to wild-type mice. Specific MMP-9 inhibition was therefore hypothesized to potentially be a target in order to reduce ischemia-reperfusion injury during OLT. The absence of an influence of genetic polymorphisms for MMPs and TIMPs on NAS does not exclude a role of MMP and TIMP levels or local activity in NAS development. With regard to the role of MMP gene polymorphisms in liver disease controversies are also present. Zhai et al. found polymorphisms in the MMP-2 promotor not to be associated with hepatocellular carcinoma (HCC) risk.[25] Conversely, in a study performed by Wu et al. C tot T transition appeared to have a beneficial effect in patients transplanted for hepatocellular carcinoma, as their HCC recurrence rate was lower and the recurrence free survival longer.[26] In the present study no association was found in the genotype distribution of MMP-7 between recipients/donors and the development of NAS. Very little is known about the role of MMP-7 gene polymorphisms in the development or progression of liver diseases. Qiu et al. evaluated MMP-7 gene polymorphisms in relation to HCC in a case-control study including 434 patients with HCC and 480 control subjects. MMP-7 153 C/T

was found not to be polymorphic in that study, and no association could be found between MMP-7 181A/G gene polymorphism and HCC.[27] In another study, a G to A transition at nucleotide 457 of MMP-7 was found to be strongly associated with the development of liver cirrhosis.[28] However, this has not been confirmed in other studies.

It is possible that the susceptibility for a specific disease related to the presence of a genetic polymorphism is altered by interactions with other risk factors. For example, the pathophysiological mechanism of NAS development is complex and very likely multifactorial. Immunological factors, pretransplant graft status, ischemia-reperfusion injury and bile salt toxicity are probably involved.[2;3;29] Possibly, other donor-, recipient-, transplant- or graft-related factors are more relevant in NAS development than MMP genotype. These risk or protective factors could mask or modify the susceptibility for NAS development related to MMP genotypes.

The assumption that other risk or protective factors may be involved may partially explain why we could not reproduce the association described in the previous study. In that study we showed that the presence of a MMP-2 (-1306 C/T) gene promotor polymorphism in either donor and/or recipient was associated with a significantly higher incidence of NAS after OLT.[8] When we compared the baseline characteristics of the first and second cohort, we found several important differences between both cohorts, which supports the presumption that (the interactions with) other factors are important and may mask or skew the relation found between MMP gene polymorphism and NAS development. Subgroup analysis in patients without certain risk factors –e.g. excluding PSC and DCD-OLTs– did not affect the observations.

It is also possible that, in the previous study, we found a premature association which was a bias due to confounding factors or by chance. Large study groups are required in the assessment of genetic associations in complex diseases, in which the evaluated factor may only have a modest contribution to the pathogenesis, such as MMPs and TIMPs in NAS. The evaluation of multiple SNPs may also increase the risk of statistical type I errors. Furthermore, in case-control studies, findings of spurious associations between genetic variations and disease outcome are common. This is mainly due to the fact that genetic variations between cases and controls are solely ascribed to a certain disease, whereas in fact there is a

known disparity in allele frequencies in and between populations.[30,31] Because the sample size of the first and second cohort was comparable, we may have found a spurious association in the first cohort, or by chance, while we may not have found an association in this cohort.

In conclusion, several human and animal studies showed that MMP-9 and TIMP-1 play an important role in ischemia-reperfusion injury,[10-13] and that such injury plays a role in development of NAS. However, the present study suggests that no relation exists between the presence of MMP-2, MMP-7, MMP-9, TIMP-1 and TIMP-2 gene polymorphisms in liver transplant donors and recipients and the development of non-anastomotic biliary strictures. This may partially be explained by the interaction with other risk factors that are likely to be more relevant or that alter the susceptibility to develop NAS or obscure the genetic impact of MMPs.

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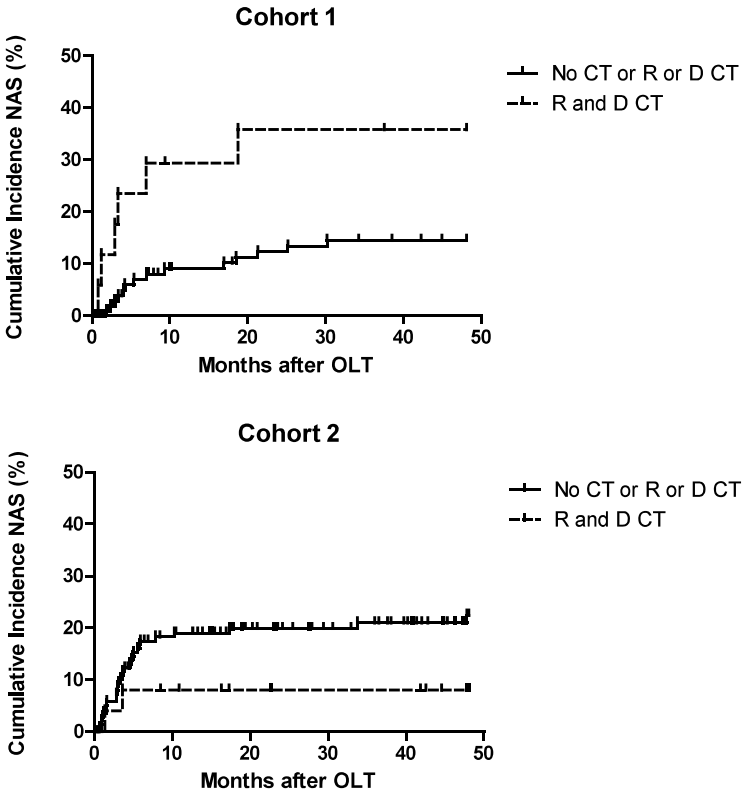
Supplemental data

Supplementary table 1. Comparison of donor, recipient and procedure variables between cohort 1 and cohort 2. Values are expressed as percentages (numbers), unless otherwise specified.

	Cohort 1 (*n*=147)	Cohort 2 (*n*=167)	*p*-value
Donor age (years, median, interquartile range)	43 (29 – 53)	49 (40 – 58)	**0.006**
Gender			0.403
Male	54.4 (80)	49.7 (83)	
Female	45.6 (67)	50.3 (84)	
Recipient age (years, median, interquartile range)	49 (42 – 56)	54 (46 – 62)	**0.005**
Gender			0.253
Male	64.6 (95)	70.7 (118)	
Female	35.4 (52)	29.3 (49)	
Primary liver disease			**0.022**
ALD	18.4 (27)	33.5 (56)	
Viral	27.2 (40)	25.1 (42)	
PSC	17.0 (25)	10.2 (17)	
PBC	6.8 (10)	3.6 (6)	
Other disease	30.6 (45)	27.5 (46)	
MELD score (median, interquartile range)	15 (11 – 22)	22 (18 – 24)	**0.000**
OLT procedure			**0.000**
DBD	89.8 (132)	70.1 (117)	
DCD	10.2 (15)	29.9 (50)	
CIT (median, interquartile range)	624 (509 – 811)	537 (459 – 635)	**0.003**
RWIT (median, interquartile range)	36 (32 – 42)	35 (31 – 41)	0.193
MMP-2- 1306 C/T donors			0.061
CC	46.3 (68)	56.6 (94)	
CT	49.7 (73)	36.7 (61)	
TT	4.1 (6)	6.6 (11)	
MMP-2 -1306 C/T genotype distribution			0.675
No CT	36.7 (54)	40.4 (67)	
Donor or recipient CT	49.0 (72)	44.0 (73)	
Donor and recipient CT	14.3 (21)	15.7 (26)	

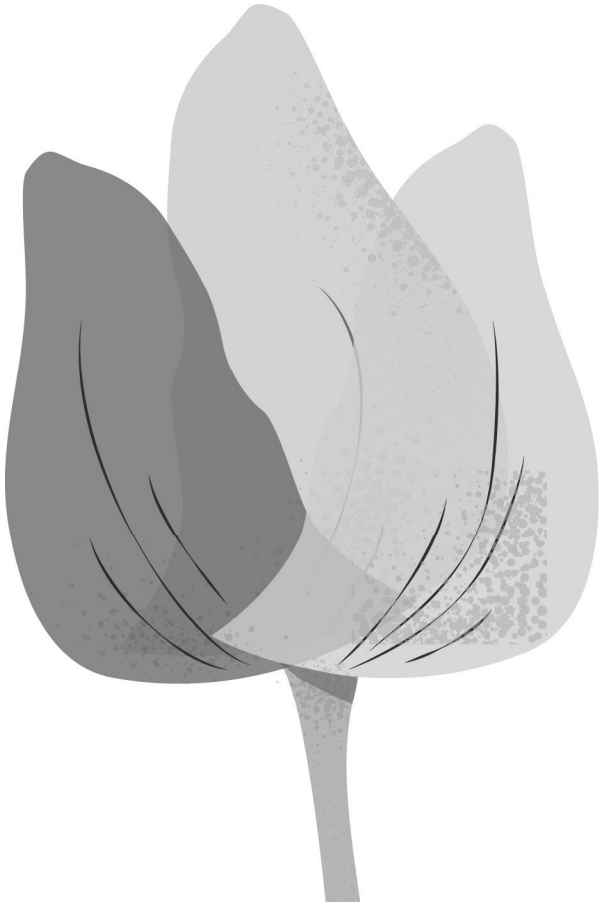
NAS = Non-anastomotic biliary strictures, OLT = Orthotopic Liver Transplantation, ALD = Alcoholic Liver Disease, HCV = Hepatitis C Virus, HBV = Hepatitis B Virus, PSC = Primary Sclerosing Cholangitis, MELD = Model for End-Stage Liver Disease, DBD = Donation after Brain Death, DCD = Donation after Circulatory Death. DWIT = Donor Warm Ischemia Time, CIT = Cold Ischemia Time, RWIT = Recipient Warm Ischemia Time



Suppl. Figure 1. Cumulative incidence of NAS within 48 months after OLT related to the presence of MMP-2 CT gene polymorphism in recipient (R) and/or donor (D) in cohort 1 and cohort 2. (cohort 1: Log Rank p=0.01; cohort 2: Log Rank p=0.14)

Part B

Diagnosis



Chapter 5

Value of magnetic resonance cholangiopancreatography in assessment of non-anastomotic biliary strictures after liver transplantation

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Abstract

Introduction Non-anastomotic biliary strictures (NAS) remain a frequent complication after orthotopic liver transplantation (OLT). The aim of this study was to evaluate whether Magnetic Resonance Cholangiopancreatography (MRCP) could be used to detect NAS and to grade the severity of biliary strictures.

Patients and Methods In total, 58 patients after OLT from two Dutch transplantation centers in whom endoscopic (ERCP) or percutaneous cholangiography (PTC) and MRCP were performed within less than 6 months apart were included in the study. Of these patients, 41 had NAS and 17 were without NAS based on ERCP or PTC and follow-up. Four radiologists – two from each center – used an adapted validated classification –termed 'Leiden Biliary Stricture Classification' (LBSC)– to evaluate the MRCP examinations independently. In this classification, NAS severity is assessed in four hepatobiliary regions. Interobserver agreement of the severity score for each region was calculated with the kappa statistics.

Results Optimal cut-off value of the LBSC to detect the presence of NAS with MRCP was calculated at ≥ 3 points for all readers. Applying this cut-off sensitivity for each reader was > 90%, with a specificity of 50-82%, positive predictive value of 86-91%, and negative predictive value of 80-100%. MRCP performance was better in evaluation of the intrahepatic than of the extrahepatic bile ducts. The additional value of MRCP for grading severity of NAS was limited.

Conclusion MRCP with the LBSC is a reliable tool to detect or exclude NAS after OLT. Currently, MRCP cannot be used to reliably grade the severity of these strictures.

Introduction

Biliary tract complications, such as anastomotic strictures (AS) or non-anastomotic biliary strictures (NAS), remain a frequent complication after orthotopic liver transplantation (OLT).[1] NAS is considered the most challenging complication as the biliary strictures can be located both in the intra- and extrahepatic bile ducts. Frequently, these strictures can be treated endoscopically or radiologically. Inadequate treatment, however, may lead to cholestasis, cholangitis and eventually graft failure with the need for retransplantation.[2] Early diagnosis and appropriate treatment of NAS can prevent this in most cases and is therefore important during follow-up.

Direct cholangiography by endoscopic retrograde cholangiopancreatography (ERCP) or percutaneous transhepatic cholangiography (PTC) is invasive and can be associated with major complications, such as post-procedural cholangitis, pancreatitis, perforation and/or bleeding in 1-4% of cases.[3] Therefore, magnetic resonance cholangiopancreatography (MRCP) is increasingly used as a non-invasive tool to monitor the presence of both AS and NAS after OLT.[4-6] MRCP is a safe, non-invasive technique to visualise the entire pancreatic and biliary tree without the use of exogenous contrast.[7] However, with regard to NAS specifically, no universal radiologic criteria have been established to describe the presence and severity of the biliary strictures. Rajaram et al.[8] previously described a validated ERCP/PTC cholangiographic scoring model for primary sclerosing cholangitis (PSC), a cholestatic disease with biliary strictures resembling NAS. As yet, it is unknown whether MRCP using this model could also be applied post-OLT in order to detect NAS. The aim of this retrospective study was to evaluate whether MRCP, using a modification of this validated scoring model, can be used as a diagnostic tool to detect or exclude NAS, and whether it can predict the severity of the biliary strictures.

Material and Methods

Between August 2005 and July 2013, a total of 68 liver transplant recipients were referred for MRCP in two Dutch liver transplantation centers. MRCP was not part of the standard protocol after OLT, but was performed on indication. After the

exclusion of 3 scans with non-diagnostic quality and 7 incomplete scans, MRCPs of 58 patients could be included in the analysis (center A: *n*=32; center B: *n*=26). Overall, 41 patients had NAS as diagnosed and confirmed with direct cholangiography, with a maximum interval between direct cholangiography and MRCP of 6 months (center A: *n*=21; center B: *n*=20). The 17 patients without NAS (center A: *n*=11; center B *n*=6) were included as 'no NAS' group. The presence or absence of NAS was also confirmed by follow-up.

Recipient surgery

In both centers, OLT with standard technique of 'piggy-back' side-to-side cavo-caval anastomosis, and end-to-end porto-portal and hepatic artery to hepatic artery anastomosis was performed. A duct-to-duct biliary anastomosis – in center A over an 8-12 Ch stent – was performed if possible. The biliary stent was removed with ERCP in center A at 6 weeks after OLT. Only in a minority of included recipients, i.e., 18.8% of patients (*n*=6) in center A and 19.2% of patients (*n*=5) in center B, a choledochojejunostomy was performed. This was performed because patients were transplanted for PSC (*n*=8) or Caroli disease (*n*=2). In 1 patient the indication was not available.

In both cohorts, ultrasound and serum liver biochemistry was performed routinely at least on day 0, 1 and 7, and subsequently at 3, 6, 12 months and yearly after OLT. ERCP/PTC procedures or MRCP and other imaging studies were performed as indicated. Follow-up protocols were similar for donation after brain (DBD) and circulatory (DCD) death OLTs.

MRCP description

MRCP was performed using a Philips 1.5T scanner (center A) and a GE 1.5T scanner (center B). Single shot fast spin echo (SSFSE) sequences with thick (2D) and thin slab multislice (3D) techniques in coronal planes were performed using a phased array body coil. Additional axial MR images were obtained using a SSFSE sequence. For 2D-MRCP, thick slabs (40 mm) through the porta hepatis in coronal and coronal oblique planes were planned rotating around a point anterior to the portal vein. 3D MRCP was performed with 1.8 mm thick slices, field-of-view 260 mm, matrix size 260 x 260, resulting in a resolution of 1.8 x 1 x 1 mm.

Non-anastomotic biliary strictures

In all cases, the diagnosis of NAS was confirmed by invasive cholangiography, i.e., ERCP or PTC –which is considered the golden standard– and was confirmed during follow-up. The following definition of NAS was used: NAS was considered as any endoscopically or percutaneously *treated* stricture or irregularity of the intra- or extrahepatic bile ducts occurring at least 1 cm above the anastomosis post-OLT, as previously described.[9] Therefore, only those biliary strictures that were severe enough to cause clinical symptoms or biochemical abnormalities and to require treatment were considered NAS. To enhance the comparability between MRCP and invasive cholangiography, the time interval between both was limited to 6 months in this study. In case of no NAS or the absence of treatment, the bile ducts were considered normal. Therefore, 17 patients could be included in the no NAS group.

Evaluation of biliary strictures on MRCP

In each center, MRCPs were evaluated by two independent, experienced (> 5 years of relevant experience) radiologists with their field of expert in abdominal imaging and MRCP reading. Images were selected and provided by the research coordinator. The participating radiologist retrospectively evaluated the MRCPs for research purpose only, after completion of the study, in one setting. Therefore, the radiologists were blinded to indications for MRCP, clinical findings, laboratory results, biopsy findings or other imaging results or outcome. The presence and localization of biliary strictures was noted and categorized into 4 different hepatobiliary regions (Figure 1): at the anastomosis until 1 cm above the anastomosis (AS, region A), the donor common bile duct and the common hepatic duct until 2 cm above the bifurcation (region B), the hepatic bile ducts (region C) and peripheral bile ducts (region D). Regions C and D were further subdivided as left- and right-sided. A detailed description of this 'Leiden Biliary Stricture Classification', or LBSC, here used for evaluating MRCPs at each hepatobiliary region, is presented in Table 1. The LBSC is a modification of the 'Amsterdam Classification', which is validated for scoring biliary strictures on ERCP/PTC in PSC.[8;10] After modification, the classification system was more appropriate for MRCP interpretation.

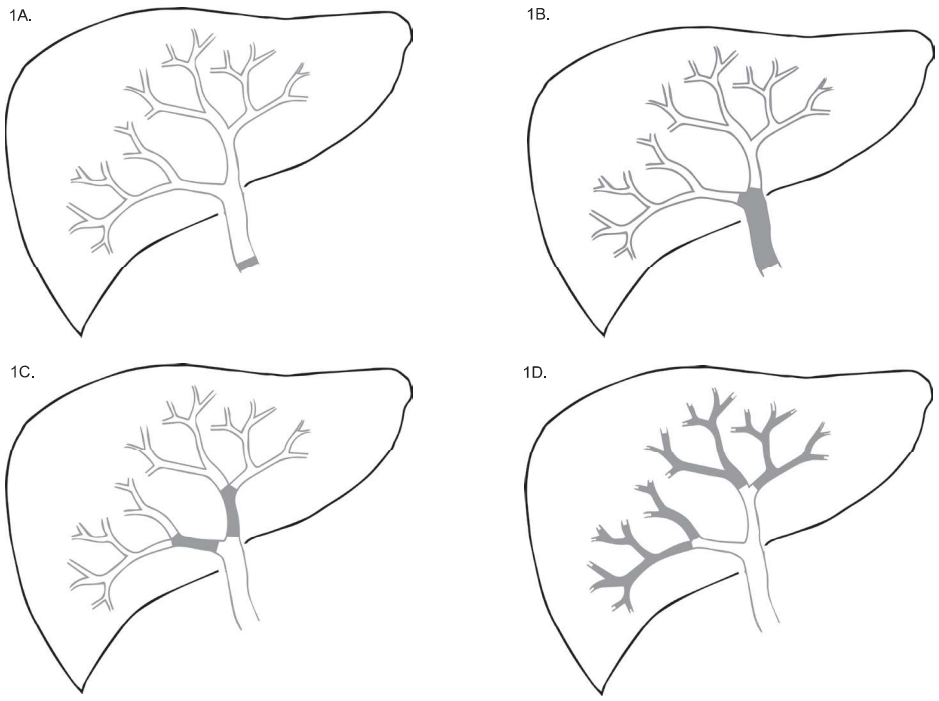

Figure 1. The Leiden Biliary Stricture Classification. The presence and localization of biliary strictures is determined and categorized into: the anastomosis until 1 cm above the anastomosis (Region A, Figure 1A), the donor common bile duct and the common hepatic duct until 2 cm above the bifurcation (Region B, Figure 1B), the left and right hepatic bile ducts (Region C, Figure 1C) and the left- and right sided peripheral bile ducts (Region D, Figure 1D).

Statistical analysis

Statistical analysis was performed using SPSS version 20.0 for Windows (SPSS Inc. Chicago, IL, USA). For normally distributed variables the Student t-test was used. Mann-Whitney U test, or when appropriate, Kruskall-Wallis test, was performed for non-normally distributed variables. Categorized data were analysed with the Chi-square test and presented in percentages (numbers). Receiver operating characteristic (ROC) curves were constructed to determine the optimal sensitivity and specificity. The level of intra-observer agreement between ERCP/PTC and MRCP and the interobserver agreement between radiologists was calculated with the statistic kappa (κ) and defined as follows: κ value of 0, no agreement; κ value of 0.01-0.40, poor agreement; κ value of 0.41-0.60, fair agreement; κ value of 0.61-0.80, good agreement; κ value of 0.81-1.00, excellent agreement.

Table 1. Leiden Biliary Stricture Classification (LBSC) for classifying cholangiographic abnormalities in patients after liver transplantation.

Hepatobiliary region	Score	Cholangiographic abnormalities
A Anastomosis + 1 cm above	0 1 2 N/A	No visible abnormalities Caliber changes ≤ 50% Caliber changes 50-75% Choledochojejunostomy
B Donor common bile duct and common hepatic ducts	0 1 2 3	No visible abnormalities Slight irregularity of duct contour without stenosis Segmental stenosis Stenosis of almost the entire length of the duct or multiple strictures
C_{Left} Left hepatic duct until 2 cm proximal from common duct	0 1 2 3	No visible abnormalities Slight irregularity of duct contour without stenosis Segmental stenosis Stenosis of almost the entire length of the duct or multiple strictures
C_{Right} Right hepatic duct until 2 cm proximal from common duct	0 1 2 3	No visible abnormalities Slight irregularity of duct contour without stenosis Segmental stenosis Stenosis of almost the entire length of the duct or multiple strictures
D_{Left} Peripheral	0 1 2 3	No visible abnormalities One or multiple strictures with normal caliber of bile ducts or minimal dilatation Multiple strictures with dilatation, sludge and/or decreased aborisation Severe pruning with only central branches seen
D_{Right} Peripheral	0 1 2 3	No visible abnormalities One or multiple strictures with normal caliber of bile ducts or minimal dilatation Multiple strictures with dilatation, sludge and/or decreased aborisation Severe pruning with only central branches seen

N/A = not applicable.

Retrospective studies are approved by the institutional review board by legislation. The study was performed in accordance with the guidelines of the Helsinki and Istanbul declaration.

Results

Within the study period, a total of 210 OLTs were performed in center A and 379 OLTs in center B. DBD-OLTs accounted for 72.9% of the OLTs in center A and 81.3% of the OLTs in center B. In general, anastomotic strictures were diagnosed in 19.5% of patients and non-anastomotic biliary strictures in 18.6% of patients. The incidence of NAS after DCD-OLT was significantly higher than after DBD-OLT (36.8% vs. 11.8%, $p<0.01$).

In the present study, 41 patients with non-anastomotic biliary strictures and 17 patients without NAS were included. Of the 58 included patients, 42 (72.4%) were male and 16 female (17.8%). Mean age of the patients was 56.0 years (range 26.6 – 76.3). In 22 patients (37.9%) OLT was performed with a DCD graft. Median follow-up from OLT until the initial MRCP was 7.5 months (range 0 – 78 months). MRCP was performed for several indications. In the majority of NAS patients (61%), patients were already diagnosed and treated before the initial MRCP. In this group, patients were referred for MRCP because sudden changes in clinical presentation (e.g. elevations in serum biochemistry or clinical symptoms) had led to the suspicion of progression of the disease or previous unsuccessful treatment (n=25). In case the presence or absence of biliary strictures had not yet been confirmed, patients were referred for MRCP because clinical presentation led to a suspicion of bile duct abnormalities (n=26). This was correct in 16 cases, whereas no abnormalities were found in 10 cases. In the remaining cases, patients were referred for other indications, e.g. pathology of the pancreas or monitoring recurrence of hepatocellular carcinoma (n=7).

None of the patients in the no NAS group developed NAS during follow-up until the end of the study (median 73 months, range 16 – 127 months). Because NAS usually presents within the first year after OLT, it is not likely that these patients would develop NAS later on during follow-up.

Patient characteristics of the two individual centres are presented in Table 2 and were not statistically different between the centers, with the exception of the simultaneous presence of AS.

In center A, but not in center B, a duct-to-duct biliary anastomosis is preferably performed over an 8-12 Ch stent, which is removed after 6 weeks with a routine ERCP. In some patients, elevated serum biochemistry or clinical symptoms may result in the performance of an ERCP earlier than 6 weeks. In patients with biliary strictures, a routine ERCP was performed in 15 patients. Irregular bile ducts or NAS were already described at routine ERCP in 7 of these patients. In the remaining cases, 4 patients had developed strictures at the anastomotic site, but not yet at the non-anastomotic regions, 3 patients had developed bile duct leakages, and only one ERCP was considered normal. In 6 cases, routine ERCP was not performed because the biliary stent had already migrated to the small intestines and there was no other indication to perform ERCP (*n*=2) or no stent had been placed because a choledochojejunostomy was performed *(n*=4).

MRCP and biliary strictures

The radiologist classified the quality of MRCPs as 'very good'' in 68% of cases, whereas the remaining MRCPs were classified as 'moderate' quality. After blinded evaluation using the LBSC, the correct classification of presence and location of biliary strictures was determined by comparing the MRCP results to direct cholangiography. Primarily, the results were obtained in a cohort of center A and afterwards validated in a cohort from center B. Overall, the readers correctly assigned ≥1 point(s) at hepatobiliary region A in 83% (50 – 89%) of patients with an anastomotic stricture, based on direct cholangiography. The results of region A were not included in further analyses since the evaluation of AS was not the purpose of this study. For categories B,C (left and right) and D (left and right) zero to three points each, so a maximum of 15 points in total, could be obtained. The distribution of the reported total LBSC scores was not statistically different between the readers (p=0.52), indicating a comparable severity of NAS between both centres. Optimal cut-off point for MRCP using the LBSC to predict NAS was calculated using a ROC curve. The area under the ROC curve was excellent (>0.80 for each reader; figures not shown). For each reader, a cut-off of ≥ 3 points served as the best predictor for treatment requirement and this was therefore the most clinically relevant cut-off. The cut-off point was first determined in the cohort

of center A and afterwards validated in the cohort of center B. Applying this cut-off value, sensitivity, specificity, positive predictive value and negative predictive value were determined. (Table 3).

Table 2. Patients characteristics. Data are presented as % (n), unless otherwise specified.

	Center A (*n*=32)	Center B (*n*=26)	*p*-value
Age (years, mean, SD)	57.6 ± 11.8	54.1 ± 11.6	0.27
Age at MRCP (years, mean, SD)	52.5 ± 11.6	50.5 ± 11.6	0.51
Gender			0.20
Male	65.6 (21)	80.8 (21)	
Female	34.4 (11)	19.2 (5)	
Etiology			0.55
ALD	34.4 (11)	23.1 (6)	
Viral	21.9 (7)	15.4 (4)	
PSC	21.9 (7)	34.6 (9)	
AIH	3.1 (1)	0 (0)	
Other	18.8 (6)	26.9 (7)	
Choledochojejunostomy	18.8 (6)	19.2 (5)	0.96
Anastomotic strictures	37.5 (12)	88.5 (23)	<0.01
Indication MRCP			0.10
Suspicion of progression or recurrence (MRCP after diagnosis)	46.9 (15)	38.5 (10)	
Suspicion of bile duct abnormalities (MRCP before diagnosis)	34.4 (11)	57.7 (15)	
Other indication	18.8 (6)	3.8 (1)	
Diagnosis before initial MRCP	71.4 (15)	50.0 (10)	0.16
Interval between OLT and NAS diagnosis (months, mean, SD)	5.0 ± 6.1	7.8 ± 9.6	0.26
Interval between MRCP and ERCP/PTC (months, mean, SD)	0.4 ± 3.0	0.1 ± 1.8	0.69
Interval between OLT and MRCP (months, median, range)	7.9 (0 – 72)	7.3 (2 – 78)	0.79
Time between MRCP and diagnosis (months, median, range)	-1.6 (-12.2 – 5.9)	0.2 (-78.0 – 2.5)	0.87

SD = Standard Deviation, MRCP = Magnetic Resonance Cholangiopancreatography, ALD = Alcoholic Liver Disease, Viral = Hepatitis B Virus and/or Hepatitis C Virus, PSC = Primary Sclerosing Cholangitis, AIH = Autoimmune Hepatitis, ERCP = Endoscopic Retrograde Cholangiopancreatography, PTC = Percutaneous Transhepatic Cholangiography, OLT = Orthotopic Liver Transplantation

Table 3. Sensitivity, specificity, positive predictive value, negative predictive value for each reader. Data are expressed as percentage.

	Center A		Center B	
	Reader 1	Reader 2	Reader 3	Reader 4
Overall (*cut-off ≥ 3*)				
Sensitivity	100	91	100	95
Specificity	82	72	50	67
Positive Predictive Value	91	86	87	91
Negative Predictive Value	100	80	100	80
Intrahepatic (*cut-off ≥ 3)*				
Sensitivity	80	75	100	85
Specificity	100	83	50	83
Positive Predictive Value	100	88	87	94
Negative Predictive Value	75	67	100	63
Extrahepatic (*cut-off ≥ 1*)				
Sensitivity	95	100	90	80
Specificity	67	50	50	67
Positive Predictive value	83	77	86	89
Negative Predictive value	89	100	60	50

In order to determine the overall ability of MRCP using the LBSC to detect NAS, both cohorts were combined using the mean score of both radiologists. When the cut-off point of LBSC ≥ 3 was applied to the radiologists' mean scores sensitivity was 98%, specificity 65%, positive predictive value 87% and negative predictive value 92%. (Figure 2) The readers reported the presence of casts, biliary stones, purulence and sludge in the bile ducts in 8 patients (20%) with NAS, which was confirmed in 7 out of these cases (88%) on subsequent direct cholangiography. The intra-observer agreement between MRCP and ERCP/PTC, as calculated by the statistic kappa, for each reader was 0.15 – 0.22 (region B), 0.22 – 0.53 (region CL), 0.29 – 0.86 (region CR). The hepatobiliary region D was located beyond the reach of ERCP and therefore presence or absence of casts in this region was difficult to interpret.

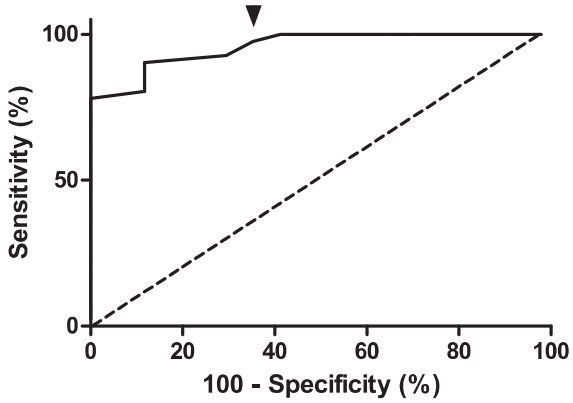

Figure 2. ROC curve of mean MRCP scores for NAS. The arrowhead indicates the position of the cut-off value of 3 points. (*n*=58).

Intrahepatic (region C + D) and extrahepatic (region B) bile ducts were evaluated separately. Similar to the calculation of the overall optimal score, the cut-off value for the intra- and extrahepatic bile ducts was determined using a ROC curve. For all readers, the optimal score was set at 3 out of the 12 points that could maximally be obtained for the intrahepatic hepatobiliary region, and 1 out of 3 points for the extrahepatic hepatobiliary region. Table 3 describes the sensitivity, specificity, positive predictive value and negative predictive value of intra- and extrahepatic NAS detection for each reader.

Interobserver agreement on severity

To evaluate whether MRCP using the LBSC can be used to describe not only presence or absence, but also the severity of NAS, interobserver agreement kappa (κ) of the severity scores was calculated for each specific hepatobiliary region. As MRCP appeared not to be distinctive in the stages 'no abnormalities' and 'slight irregularities' (0 or 1 point), these two stages were combined for all hepatobiliary regions. Agreement on severity of NAS was poor for all specific regions, i.e., κ in center A 0.31 for hepatobiliary region B, 0.41 and 0.37 for hepatobiliary region C (left and right respectively) and 0.31 and 0.32 for hepatobiliary region D (left and right respectively). For Center B, the kappa for each hepatobiliary region was as follows: 0.22 for hepatobiliary region B, 0.68 and 0.42 for hepatobiliary region C (left and right respectively) and 0.19 and 0.37 for hepatobiliary region D (left and right respectively).

In center A, a second or third MRCP was available in 14 patients. The assessment of these MRCPs confirmed that the severity of strictures is difficult to interpret, and the progression or effect of treatment could not reliably be evaluated.

Discussion

The present study showed that, with the use of the 'Leiden Biliary Stricture Classification' (LBSC), four independent readers could obtain a sensitivity, positive predictive value and negative predictive value of MRCP for NAS detection or exclusion of > 80% in two independent study cohorts. In the LBSC, we combined the modified 'Amsterdam Classification', a validated cholangiographic prognostic model for PSC, with a classification into four different hepatobiliary regions –and left and right–, as shown in Figure 1.[8;10]

Non-anastomotic biliary strictures remain a challenging complication after OLT. The presence of NAS may result in cholestasis and cholestasis-related symptoms (e.g., jaundice, pruritus, cholangitis) and, in case of inadequate treatment, graft failure with the need for retransplantation.[11] Whereas currently direct cholangiography is the imaging technique of choice when bile duct abnormalities are suspected, the use of MRCP has become a promising diagnostic tool for this purpose. Several authors have reported similar results in diagnostic accuracy between MRCP and ERCP for the detection of biliary complications after OLT.[5;12;13] However, the majority of these studies focussed on the ability of MRCP to detect biliary strictures in general, thereby including the presence of anastomotic strictures in the definition. With regard to NAS specifically, data have only been obtained without a clear classification and in small sample sizes, in which the diagnostic accuracy of MRCP is reported to be lower. Collettini et al.[14] described a sensitivity for the detection of NAS of 89-100%, which was accompanied by a moderate specificity of 50%. In a study performed by Kinner et al., a sensitivity of 67-100% and a specificity of 50-88% (depending on the type of biliary anastomosis) for MRCP to detect NAS in patients with a clinical suspicion of biliary strictures was reported. In addition, determination of the exact localisation of biliary strictures appeared to be difficult, as Zoepf et al.[4] compared the results to ERCP and reported that MRCP localized NAS correctly in only 22% of cases. The present study confirmed difficulties in determining the exact location of biliary strictures. This may possibly partly be

explained by difficulties in distinguishing strictures located at the anastomosis or in the extrahepatic bile ducts. However, the excellent sensitivity and positive and negative predictive values suggest that MRCP using the LBSC can, overall, reliably detect or exclude NAS after OLT on MRCP imaging.

Several studies, including machine preservation techniques, are currently ongoing or being planned with the aim of preventing NAS after OLT.[15] In clinical trials, the use of invasive procedures with a high risk of procedure-related complications and morbidity, such as ERCP or PTC procedures, is less justified and MRCP with the proposed classification may therefore be an alternative.

In accordance with previously described literature, the corresponding specificity in the present study was moderate and varied between 50 and 82%. When the extrahepatic and intrahepatic bile ducts were evaluated separately, specificity improved for the evaluation of intrahepatic bile ducts. These differences in interpretation of intra- and extrahepatic bile ducts are probably the result of better visualisation of the intrahepatic ducts as compared to the extrahepatic ducts on MRCP. Moreover, in our experience, sludge and biliary cast formation, which can be present mainly in the common hepatic duct and common bile duct, are difficult to distinguish from NAS on MRCP. This issue is supported by a study from Hoeffel et al., who reported similar observations.[16] This may limit the accuracy of MRCP to exclude or detect the exact location and length of biliary strictures, especially extrahepatic strictures. In addition, because 37.5% of cases in center A and 88.5% cases in center B showed anastomotic strictures on the ERCP, the congestion as a result of these strictures further impaired the interpretation of the extrahepatic bile ducts.

Direct cholangiography has the advantage that the use of contrast can both visualise the bile ducts and determine drainage function. Because bile drainage function cannot be measured with T2-weighted MRCP technique and since distal biliary strictures in a transplanted graft have the tendency to result in less prestenotic dilatation than strictures in a non-transplanted liver graft[17], the absence of prestenotic dilatation post-OLT may possibly influence the detection rate for strictures with MRCP. This suggests that imaging details of NAS acquired with MRCP can support the decision to perform a more invasive cholangiographic procedure and justify the associated risk of treatment-related complications, but that MRCP cannot fully replace direct cholangiography for diagnostic purposes. Conversely, the absence

of NAS on MRCP (LBSC score <3) may be a reason to abstain from or defer invasive cholangiography. In patients with a total LBSC score of ≥ 3 points, direct cholangiography may be indicated, or a more intensive follow-up regimen and early intervention in case of symptoms may be appropriate. However, whether this strategy leads to better outcomes and graft survival remains to be established.

In addition to distinguishing the presence of NAS requiring treatment (≥ 3 points on the LBSC) from minor irregularities (<3 points) that may not be of clinical relevance, it may be of interest to assess the progression over time of these bile duct irregularities to a more severe, clinically relevant stricture. Therefore, we determined whether the severity of strictures can be determined using this classification. Unfortunately, the interobserver agreement for grading biliary strictures severity was poor in this study. This is in accordance with a study performed by Moff et al.[18] In that study, two experienced radiologists independently evaluated MRCPs of 36 PSC patients in order to describe the severity of strictures in PSC using the Amsterdam Classification model. The statistic kappa for interobserver agreement for grading the severity of extrahepatic strictures was 0.23, and 0.07 for intrahepatic bile ducts. This implies that other imaging techniques and serum biochemical markers, i.e., bilirubin, alkaline phosphatase and gamma-glutamyltransferase, remain important in follow-up and the decision whether invasive treatment is indicated.

In the present study, we have not used a T1-weighted contrast-enhanced MR cholangiography using hepatobiliary contrast agents, such as Gd-EOB-DTPA. This is a recently emerged technique that is useful for delineating the anatomy of biliary-enteric anastomoses and detecting biliary complications, e.g., biliary strictures, intraductal stones and bile duct leakages, and it may provide additional functional information in grading biliary obstructions.[19] A future study, using these agents, could show optimising results and be of additional value, although comparative studies between T2 weighted MRCP and T1-weighted contrast-enhanced MR cholangiography which actually have proven this in a large patient group are scarce.[20-22] In addition, Duarte et al.[23] described the use of pineapple juice with gadopentetate dimeglumine as a promising contrast-enhancing agent in the evaluation of the biliary tree. An increase in concentration of manganese, i.e., a paramagnetic substance present in pineapple juice, increases signal intensity on T1 weighted images. This is especially beneficial as it may improve the visualis-

ation of the biliary tree, mainly by suppression of the digestive tube signal. The effect persisted in the entire biliary tree (both intra- and extrahepatic). This may improve the specificity of the LBSC.

A possible limitation is the retrospective study design, because most of the patients included in the analysis had been treated for NAS prior to the initial MRCP. Yet, the diagnostic accuracy in the present study is probably not influenced by this, as the readers were blinded to clinical data and were not informed whether the biliary strictures had already been diagnosed with invasive cholangiography. The majority of MRCPs were performed for the suspicion of biliary complications. However, it would be interesting to evaluate the use of MRCP with our classification model in patients without a clinical indication on fixed time points after OLT in a prospective study. The LBSC is universal, and can not only be applied to MRCPs, but also to ERCPs and PTCs.

In conclusion, MRCP using the LBSC is a reliable tool to detect or exclude NAS after OLT. It may also be used to plan the optimal treatment prior to endoscopic or percutaneous cholangiographic treatment or in the setting of clinical trials –e.g. with machine preservation– where non-invasive procedures are desired. The value of MRCP for follow-up for the progression of NAS is limited, since grading severity with MRCP is difficult and reproducibility for this purpose is low.

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Chapter 6

Non-anastomotic biliary strictures with but not without cholestasis are associated with increased liver stiffness after liver transplantation

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Submitted

Abstract

Introduction Transient Elastography (TE) has been used as surrogate marker of liver fibrosis in hepatitis C but not with non-anastomotic biliary strictures (NAS) after orthotopic liver transplantation (OLT). The aim was to investigate the relationship of TE after OLT with NAS.

Patients and Methods 97 unselected patients (NAS *n*=22; controls *n*=75) with minimum follow-up of one year and TE performed 0.5- 5 years after OLT were included. In a subgroup of 36 patients liver biopsies were available. Possible correlations between baseline factors, NAS and other post-OLT complications, including cholestasis, with TE values were tested, with $p<0.05$ as level of significance.

Results Liver stiffness was significantly increased in patients with NAS (median kPa 8.2, IQR 6.1 – 11.8), as compared to controls (median kPa: 6.1, IQR 4.6 – 7.7, $p=0.025$). In NAS patients with cholestasis (*n*=12) median TE was 10.7 kPa, which was significantly ($p<0.05$) higher than in controls (*n*=75) without NAS and no cholestasis, who had median TE values of 6.3 kPa (*n*=17), while in patients with NAS but without cholestasis after treatment (*n*=12) the median TE of 7.5 kPa did not differ from the controls. No relation of TE with other patient characteristics and aminotransferases AST or ALT, peak-ALT in first week after OLT, or (in a subset with liver biopsy, with none or minimal fibrosis and no rejection at time of TE) with histological parameters was found.

Conclusion After OLT without severe fibrosis liver stiffness is increased in NAS with persistent cholestasis, but not without cholestasis after treatment.

Introduction

Among the most frequent complications after orthotopic liver transplantation (OLT) are non-anastomotic biliary strictures (NAS). These often lead to cholestasis, cholangitis and, in some cases, graft failure.[1;2] In grafts from donation after circulatory death (DCD) –now over onethirds of liver donors in the Netherlands– we and others noticed that the incidence of NAS can be up to 30%, leading to morbidity and more retransplantations, but still with excellent patient survival.[3-6] Endoscopic retrograde cholangiopancreatography (ERCP) or percutaneous transhepatic cholangiography (PTC) are used to diagnose the presence of and treat NAS.[7] Magnetic resonance cholangiopancreaticography (MRCP) is also increasingly used for diagnosis after OLT. Transient Elastography (TE) can assess liver stiffness and has been used as a surrogate marker of liver fibrosis in patients with chronic liver disease, for example in chronic hepatitis C virus (HCV).[8] Recently, it appeared that TE can also accurately determine fibrosis after OLT, especially in the case of recurrent HCV.[9-12] Normal TE values after OLT have yet to be established. Furthermore, it is unknown if there is a relationship between TE values and presence or absence of NAS after OLT.

In a cohort of patients after OLT with and without NAS we assessed TE values and investigated the relationship of NAS with other factors, including cholestasis.

Patients and Methods

All consecutive adult patients who underwent deceased donor orthotopic liver transplantations at the Leiden University Medical Center between January 2009 and June 2015, who had a follow-up of one year or more after OLT, and of whom a TE performed at least 6 months after OLT was available, were included in the study. No other selection criteria were used. As of 2015 TE was part of the protocol 6-month and yearly investigations.

Demographic and clinical data, including age, gender, etiology of liver disease and posttransplant complications, including NAS, were derived from the electronic patient charts and database. Other clinical data consisted of recurrence of HCV infections, biopsy proven and treated rejection, peak alanine aminotransferase

(ALT) after OLT as marker for ischemia-reperfusion injury, and serum biochemical results, including serum bilirubin, aspartate aminotransferase (AST), ALT, alkaline phosphatase (ALP) and gammaglutamyl transferase (GGT), at the time of elastography. If a liver biopsy had been performed less than 1.5 year apart from the fibroscan results were compared to fibroscan results. Cholestasis was defined as serum alkaline phosphatase, gamma glutamyl transferase or bilirubin of ≥ 2 times the upper reference limit at time of TE.

The diagnosis of NAS was always based on direct cholangiography (ERCP or PTC), demonstrating one or more biliary strictures at least one centimeter above the bile duct anastomosis. In our diagnosis of NAS, only cases requiring treatment by balloon dilatation and/or placement of a stent were included, indicating that the biliary strictures were clinically relevant.

Transient elastography (TE) was performed on the right side using the Fibroscan 502 Touch (Echosens, Paris) by five well-trained operators. Details of the examination procedures have previously been described and were followed.[13;14] The M probe was used in patients with a normal posture, whereas the XL probe was used in patients with a body mass index of ≥ 30 if examination with the M-probe was unsuccessful. A successful examination included at least 10 consecutive successful measurements. Success rate was defined as the ratio of the number of successful measurements to the total number of measurements. The results were considered reliable when at least 10 measurements were successful, the success rate was >60% and the interquartile range (IQR)/ median was below 30%. The study protocol was approved by the Medical Ethics Committee (IRB approval and Protocol number C15.031) of the LUMC and was conducted in accordance with the revised Declaration of Helsinki.

Statistical analysis

Statistical analysis was performed using SPSS version 20.0 for windows (SPSS Inc. Chicago, IL, USA). Student t-test, ANOVA or Mann-Whitney U test was performed for continuous data. Categorized data was analysed using the Chi-square test. With multivariate linear regression analysis possible correlations between baseline parameters and post-OLT complications with liver stiffness were tested. A p-value of <0.05 was considered statistically significant.

Results

Within the study period, a total of 199 OLTs were performed in the Leiden University Medical Center. Out of these, 97 patients (22 with NAS, and 75 without NAS) fulfilled the inclusion criteria. The excluded patients did not have follow-up of one year or more after OLT, or no TE performed at least 6 months after OLT was available. No other in- or exclusion criteria were used. No difference in rate of NAS existed between included and excluded patients. Donation after cardiac death accounted for 47.4% of these OLTs. The overall incidence of NAS in this cohort was 19.2%. As expected, the incidence of NAS was significantly higher after DCD-OLT than after DBD-OLT (28.8% vs. 13.9%, p=0.013). No cases of acute hepatitis (as defined by AST or ALT levels ≥ 2x the upper reference limit at the time of TE) were included in the study. Only three of the included patients had elevated transaminases of ≥ 1.5x the upper reference limit at the time of TE. This was the result of an episode of acute rejection (n=1), and non-alcoholic fatty liver disease with steatosis but no inflammation on liver biopsy (n=2). TE was performed with the M probe in 74.2% (n=72) of the patients, and the XL-probe in 25 patients. The success rate was 85% and the mean IQR/median 16%.

Patient and transplant characteristics of patients with and without NAS are presented in Table 1. Median time from OLT to TE was not significantly different between patients with or without NAS. Median time from OLT to NAS diagnosis was 3.0 months (IQR 1.0 – 5.3). Median time from NAS diagnosis to TE was 1.9 years (IQR 0.8 – 3.7). The success rate of TE in NAS patients was 87% as compared to 85% in the controls (p=0.970). The M probe was used in 77.3% of NAS patients and in 73.3% of the controls, which was not significantly different (p=0.710). In patients with NAS, 95.5% (n=21) of the TEs were performed after the diagnosis of NAS, the other 4.5% within 3 month before the diagnosis.

Liver stiffness was significantly increased in patients with NAS (median kPa 8.2, IQR 6.1 – 11.8), as compared to controls (median kPa: 6.1, IQR 4.6 – 7.7, p=0.025) (Figure 1). However, using a ROC curve the accuracy of liver stiffness as positive or negative predictor for NAS was limited (Figure 2A), while it was slightly better as a predictor for cholestasis (these were all patients in whom cholestasis persisted after treatment of NAS) (Figure 2B).

Table 1. Comparison of patient and OLT characteristics between patients with and without NAS after OLT. Values are expressed as percentages (numbers), unless otherwise specified.

	NAS (*n*=22)	No NAS (*n*=73)	p-value
Recipient age (years, median, IQR)	55 (44 – 63)	57 (49 – 64)	0.501
Gender			0.614
Male	63.6 (14)	69.3 (52)	
Female	36.4 (8)	30.7 (23)	
Primary liver disease			0.665
ALD	40.9 (9)	30.7 (23)	
HBV	0	9.3 (7)	
HCV	13.6 (3)	18.7 (14)	
PSC	13.6 (3)	13.3 (10)	
AIH	9.1 (2)	5.3 (4)	
Other disease	22.7 (5)	22.7 (17)	
OLT procedure			0.083
DBD	36.4 (8)	57.3 (43)	
DCD	63.6 (14)	42.7 (32)	
Simultaneous presence of anastomotic biliary stricture	54.5 (12)	12.0 (9)	**0.000**
Previous episode of rejection	27.3 (6)	6.7 (5)	**0.007**
Time from OLT to TE (years, median, IQR)	2.5 (1.0 – 4.0)	1.9 (1.1 – 3.0)	0.430

OLT = Orthotopic Liver Transplantation, NAS = Non-anastomotic Biliary Stricture, IQR = Interquartile Range, ALD = Alcoholic Liver Disease, HBV = Hepatitis B Virus, HCV = Hepatitis C Virus, PSC = Primary Sclerosing Cholangitis, AIH = Auto-immune Hepatitis, DBD = Donation after Brain Death, DCD = Donation after Cardiac Death, TE = Transient Elastography

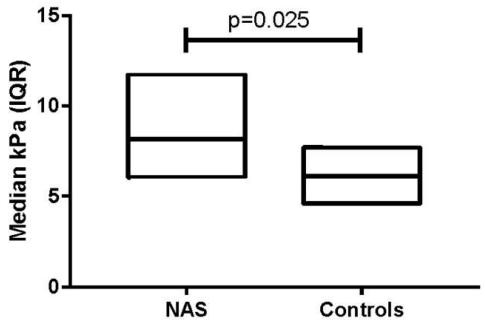

Figure 1. Median TE values of patients with versus without NAS (with interquartile range IQR) after orthotopic liver transplantation (OLT). Liver stiffness was significantly increased in patients with NAS (median kPa 8.2, IQR 6.1 – 11.8), as compared to controls (median kPa: 6.1, IQR 4.6 – 7.7, *p*=0.025).

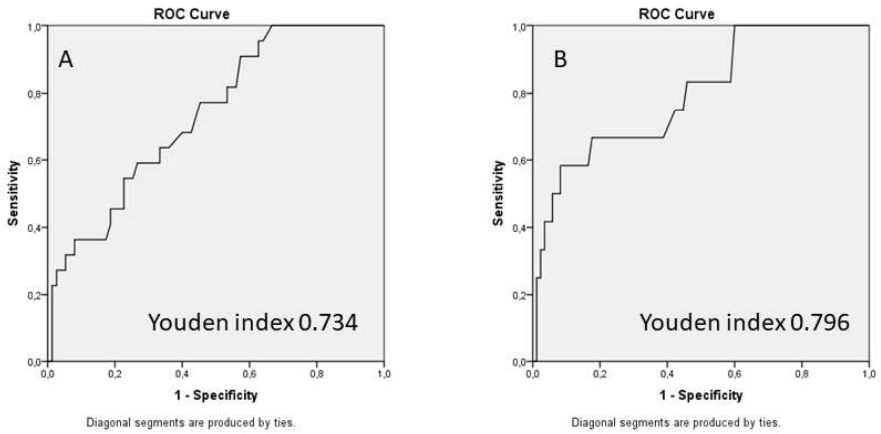

Figure 2. Receiver operating characteristics (ROC) curve of median TE values demonstrates moderate accuracy of TE for indicating A) non-anastomotic biliary strictures (NAS), and B) cholestasis.

To assess normal liver stiffness over time after OLT, only control patients after OLT without NAS were selected and stratified into four groups depending on the time from OLT until TE. The groups consisted of patients in which elastography was performed between 6 months - 1 year (*n*=14), 1 - 2 years (*n*=26), 2 - 3 years (*n*=11) and ≥ 3 years after OLT (*n*=24). Interestingly, median kPa values were relatively stable over time, i.e., 6 months – 1 year: 6.9; 1-2 years: 5.7; 2-3 years: 6.1; >3 years: 5.8 kPa (p=0.645). When analysed per year after OLT, for each year apart the difference in TE between patients with and without NAS was not statistically significant in each time interval after OLT ($0.282 < p < 0.683$). This probably is due to the small numbers in each yeargroup.

TE was not related to any of the baseline parameters. Using the Mann Whitney U test the median TE was not different with different types of donation (DBD: 6.9 kPa vs. DCD: 6.3 kPa), with or without AS (7.3 vs. 6.6 kPa), with or without recurrence of HCV (6.9 kPa vs. 6.7 kPa), and whether first-week peak-ALT was below or above 1300 IU/l (6.7 kPa vs.7.0 kPa). However, patients who had a previous episode of biopsy proven and treated rejection had a significantly higher TE than those who did not have such a previous rejection episode (8.6 kPa vs. 6.5 kPa, p<0.05) (Figure 3). Univariate linear regression analysis did show a relation of TE with peak-ALT in the first week after OLT as marker of ischemia-reperfusion injury (R=0.209, p=0.04), with previous rejection (R=0.313, p=0.002), and at time of TE

measurement with AST (R=0.203, p=0.046), with ALP (R=0.261, p=0.010), with cholestasis as defined (R=0.420, p<0.001), but not with ALT (R=0.020, p=0.846), with GGT (R=0.137, p=0.182) or total bilirubin (R=0.033, p=0.749). TE had no statistical relationship to any histological parameter in the subset with biopsies. In multivariate linear regression analysis only cholestasis at time of elastography was related to the measured TE values (R=0.420, p<0.001).

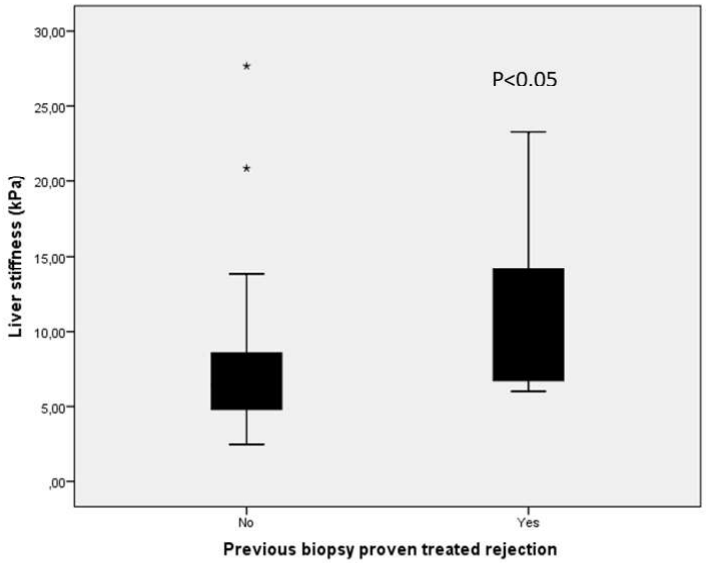

Figure 3. In patients with previous biopsy-proven treated acute cellular rejection transient elastography values were higher (median 8.60 kPa, IQR 8.70) than in patient without previous rejection (median 6.45 kPa, IQR 3.83)(p<0.05).

Cholestatic serum markers (alkaline phosphatase, gamma glutamyl transferase or bilirubin) of ≥ 2 times the upper reference limit at time of TE were present in 54.5% of the NAS patients. In NAS patients with persistent cholestasis after treatment (n=12) median TE was 10.7 kPa, which was significantly (Mann-Whitney p<0.05) higher than in controls (n=75) with no NAS and no cholestasis who had median TE values of 6.3 kPa, while in patients with NAS but without cholestasis after treatment (n=10) the median TE of 7.5 kPa did not differ from the controls (Figure 4). Median first-week peak-ALT in patients who later had persistent cholestasis after treatment for NAS was 1890 IU/l, which was significantly (p<0.05) higher than the median peak-ALT of 681 IU/l in patients who did not develop NAS or cholestasis, while median peak-ALT of 1266 IU/l in patients developing NAS in whom cholesta-

sis resolved with treatment did not differ significantly from both groups (Figure 5). Serum alkaline phosphatase values at the time of elastography were higher in patients who had experienced previous acute cellular rejection than in those who had not had previous acute cellular rejection (p<0.05) (Figure 6). When only those patients without previous rejection were selected, NAS was still associated with increased liver stiffness (NAS: 8.7 kPa; controls: 6.7 kPa, p=0.044). After exclusion of patients with previous rejection still NAS with persistent cholestasis had significantly higher TE values (9.6 kPa) than patients without NAS or cholestasis (TE 6.2 kPa) (Mann Whitney U test p<0.05), while the median TE of 7.0 kPa in NAS without cholestasis (after treatment) was not different from the latter group (Supplementary Figure 1).

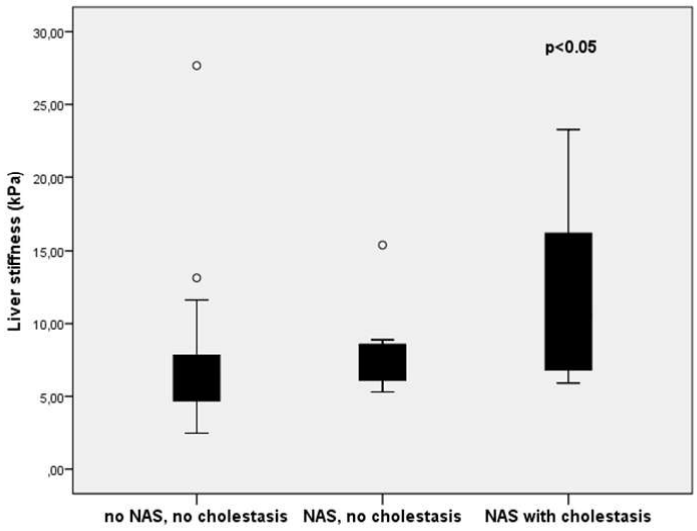

Figure 4. Median liver stiffness was higher in patients with NAS and cholestasis present at time of elastography (n=12, median TE 10.65 kPa, IQR 10.65) than in controls without NAS or cholestasis (n=75, median TE 6.30 kPa, IQR 3.20) (p<0.05). TE in patients with NAS but without cholestasis after treatment (n=10, median TE 7.55 kPa, IQR 2.72) did not differ from both other groups.

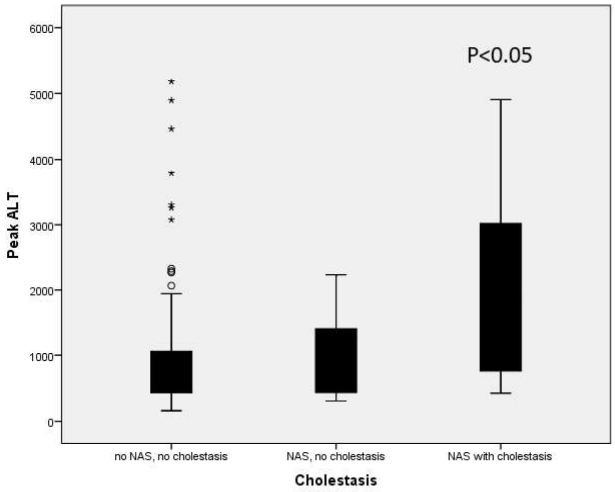

Figure 5. Median first-week peak-ALT in patients who later had persistent cholestasis after treatment for NAS was 1890 IU/l (IQR 2324 IU/l), which was significantly higher than the median peak-ALT in patients who did not develop NAS or cholestasis of 681 IU/l (IQR 712 IU/l) (p<0.05). Median peak-ALT of 1266 IU/l (IQR 1128 IU/l) in patients developing NAS in whom cholestasis resolved with treatment did not differ significantly from both groups.

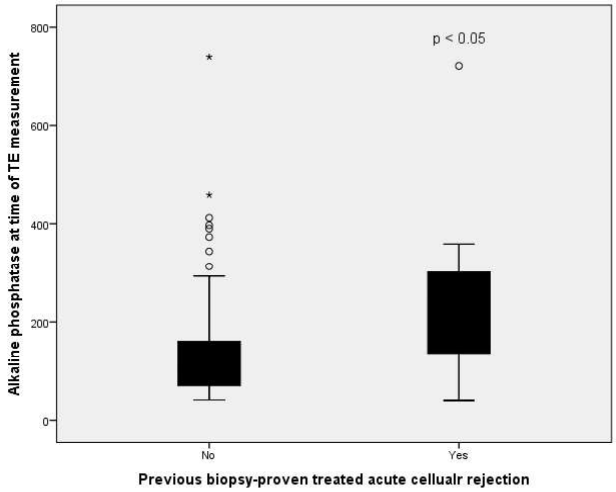

Figure 6. Serum alkaline phosphatase values at the time of elastography were higher in patients who had experienced previous acute cellular rejection (median 254 IU/l (IQR 193 IU/l) than in those who did not have previous acute cellular rejection (median 105 IU/l (IQR 93 IU/l)(p<0.05).

In 36 cases a liver biopsy less than 1.5 year apart from elastography was available. In the liver biopsy fibrosis was absent (F0) in 30/36 cases, while 5/36 had portal fibrosis (F1) with TE values of 6.0, 7.8, 6.4, 6.9 and 4.7, and 1/36 had portoportal and portocentral septation (F3) on liver biopsy and a TE value of 8.6. Considering the RAI rejection score 9/36 had RAI>3 with TE values of 6.9, 9.1, 4.5, 4.7, 6.1, 7.4, 10.9, 4.7 and 16.6 kPa, measured much later. Only 5/36 biopsies had abnormal bile ducts including one with frank bile duct necrosis. Hepatic arteries did not show abnormalities. Out of these 36 cases with biopsies less than 1.5 years apart 13 (33%) had a TE value above 9.0. Out of these 13 cases rejection RAI score according to Banff criteria was 0-3 in eleven, 4 in one, and 6-7 in one patient (not at time of elastography). Cholestasis was visible in the liver biopsy in 2/13 cases, slight steatosis in 4/13 cases, pericentral ischemia-reperfusion injury in 2/13 cases. In this limited number of cases with biopsies there was no correlation of any of the histological characteristics, i.e. fibrosis, rejection score, steatosis, pericentral damage, or abnormal bile ducts with the TE values. Results were similar if limited to the 24 biopsies with interval to elastography less than one year.

In patients not developing NAS, a measurable HCV-RNA titer was present in 9 out of 14 patients with pre-OLT HCV-RNA presence (64.3%). Median TE value of patients without NAS and with HCV recurrence was 6.3 (IQR 4.5 – 9.5) as compared to 6.0 (IQR 4.6 – 7.6) of the other controls. In two patients, HCV-RNA was detectable at time of TE. TE values of these patients were 9.8 and 7.5 kPa. In the NAS group only one out of three patients with HCV before OLT had recurrence of HCV after OLT. Median TE value in this patient was 8.5 kPa. When patients with HCV-RNA present after OLT were excluded, NAS with persistent cholestasis was still associated with increased liver stiffness with median TE of 10.7 kPa (IQR 8.3 – 15.9) as compared to controls: 6.3 kPa (IQR 5.9 – 7.6) (Mann Whitney $p<0.05$), while median TE of 7.3 kPa (IQR 5.6 – 10.3) for those with NAS but no cholestasis after treatment did not significantly differ from controls.

Discussion

In the present study –in a cohort of patients without severe fibrosis or current rejection or hepatitis– after orthotopic liver transplantation, increased liver stiffness as measured by transient elastography (TE) was associated with the presence

of non-anastomotic biliary strictures (NAS) with persistent cholestasis after treatment. Patients with NAS in whom cholestasis had resolved with dilatation and/or stenting had TE values similar to those without NAS or cholestasis. No significant relation between TE and baseline parameters, including DCD, was present. Liver stiffness in this cohort was not related to current aminotransferases in multivariate analysis. However, there was a relationship of current TE values with peak-ALT in the first week after OLT as a marker of ischemia-reperfusion injury. The explanation is that patients who developed NAS with persisting cholestasis had higher first-week peak ALT as a marker of more severe ischemia-reperfusion injury.

The relationship of NAS with cholestasis to TE values persisted when patients with HCV after OLT were excluded. Patients without NAS had low TE values after OLT, which were not different at different times after OLT. The presence of NAS may lead to cholestasis. In the native liver cholestasis can also be associated with increased TE values.[15] If cholestatic serum markers do not normalize after treatment of biliary strictures, it is also more likely that fibrosis due to persisting cholestasis could develop. In the subgroup with liver biopsies less than 1.5 years apart from elastography no relation of TE values with histological parameters was detected. All but one patient had none or only minimal liver fibrosis with grade F0-1 and only one had F3 on liver biopsy, and that patient had a TE value of 8.6 kPa. Therefore severe fibrosis as the reason for elevated TE in those with NAS and cholestasis was unlikely. It has been demonstrated in small series, especially with HCV recurrence, but also in a series with other graft disease, that after OLT TE values accurately reflects the degree of liver fibrosis.[10;16] However, in contrast to many NAS patients, these HCV patients usually were not cholestatic. Data from protocol biopsy studies after OLT demonstrate that in general no significant fibrosis is present in most adult patients in the first years after OLT in the absence of recurrent HCV. After exclusion of HCV patients results did not change in the current study, and HCV had been eradicated with direct antiviral drugs at the time of elastography. Therefore HCV did not influence the results.

The relationship of first-week peak ALT and later TE values is not surprising: Ischemia-reperfusion injury is considered one of the most important risk factors for NAS, and as recently demonstrated in DCD OLT first-week peak-ALT above 1300 IU/l is strongly related to development of NAS.[17-19] Whether this is mainly because of direct injury to the biliary epithelium or due to ischemia-related impaired

regeneration capacity from stem cells within the biliary glands or due to injury to the peribiliary plexus is yet unknown. As a result of the additional donor warm ischemia time in DCD grafts, these livers are subject to more ischemia and therefore are more prone to NAS development than DBD grafts. Schlegel et al.[20] previously demonstrated that, among other immunological effects, DCD grafts showed increased Kupffer cell and endothelial cell activation. Moreover, cytokeratin 19, alfa smooth muscle actin II and sirius red staining confirmed increased proliferation of cholangiocytes, increased activation of myofibroblasts and the presence of fibrosis in these grafts. This may indicate that ischemia-reperfusion injury can on the one hand induce direct injury to the biliary epithelium which may lead to NAS and on the other hand it may lead to liver fibrosis. Recently it was shown that not the direct injury to the biliary epithelium, but mainly the injury to peribiliary structures is associated with NAS.[21] In a study consisting of 128 transplanted livers, the vast majority showed loss of biliary epithelium, but this was not related to NAS development. However, peribiliary vascular injury, necrosis of the periluminal stroma and deep peribiliary plexus injury during OLT was significantly associated with NAS development during follow-up. It is possible that the subsequent inflammation and impaired regeneration capacity as a result of injury to peribiliary structures may lead to increased cholestasis in these NAS patients. In the liver biopsies of the current patients with NAS only in a minority necrosis of the smaller bile ducts was seen and no severe fibrosis (except in one patient) was seen. Apparently it was only the cholestasis that was related to TE in these patients.

Recently, we demonstrated that the use of MRCP with a validated scoring system for NAS can reliably detect or exclude NAS after OLT.[22] The current data indicate that increased liver stiffness strengthens the clinical suspicion for the presence of NAS with cholestasis. Whether after OLT elastography –in addition to the combined cholestatic liver enzymes with bilirubin and MRCP– really helps in the decision making whether or not to perform further diagnostic procedures may be the subject of future studies.

It has been reported that during acute rejection, liver stiffness is associated with the severity of acute cellular rejection.[23] In the present study, previous episodes of rejection were related to increased liver stiffness. Since rejection usually occurs in an early phase after OLT, and since chronic rejection has become rare, no long-term complications of acute cellular rejection such as cholestasis and fibrosis due

to ductopenia, as seen in the past, were expected in the current cohort.[24] In this cohort past acute cellular rejection was more prevalent in patients developing NAS, especially those patients with persisting cholestasis, and that condition was related with elevated liver stiffness. The higher prevalence of previous rejection in the patients with NAS may have been a coincidence: Since NAS has not been related to acute cellular rejection in the literature it is unlikely that a direct relation of previous acute cellular rejection with later NAS would exist. When only those patients without previous rejection were selected, NAS with cholestasis was still associated with increased liver stiffness.

Weaknesses of this study are a limited number of patients, and a limited number of patients with liver biopsies, while time after OLT varied. This limits the possibility to strongly validate elastography with histological characteristics. However, it does give an indication of the stability of TE values over time. In this cohort, except for one case, no severe fibrosis was present. It is therefore unknown if the relationship of TE with NAS and cholestasis holds in patients with more severe fibrosis. The advantage is that fibrosis and inflammation were not confounding factors in this study. A strength is also that baseline characteristics, biochemical and cholangiography data were complete. Another strength of this study is the strict definition of NAS, including the requirement of treatment, so that only patients with clinically relevant strictures were included and patients with and without persisting cholestasis after dilatation and stenting.

In conclusion, the presence of non-anastomotic biliary strictures after liver transplantation with persistent cholestasis after treatment is associated with increased liver stiffness. If cholestasis has resolved with treatment for NAS liver stiffness was not different from patients without NAS and no cholestasis. After OLT NAS with cholestasis appears an important cause of increased TE values and should prompt further investigation, since in patients without NAS or severe fibrosis and relatively low aminotransferases TE values seem quite stable over time. Larger studies with simultaneous liver histology and repeated measurements over time are required to further validate these results and establish the role of elastography after liver transplantation.

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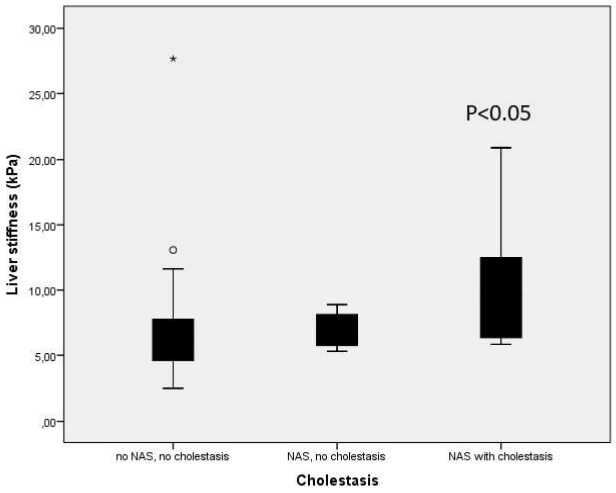
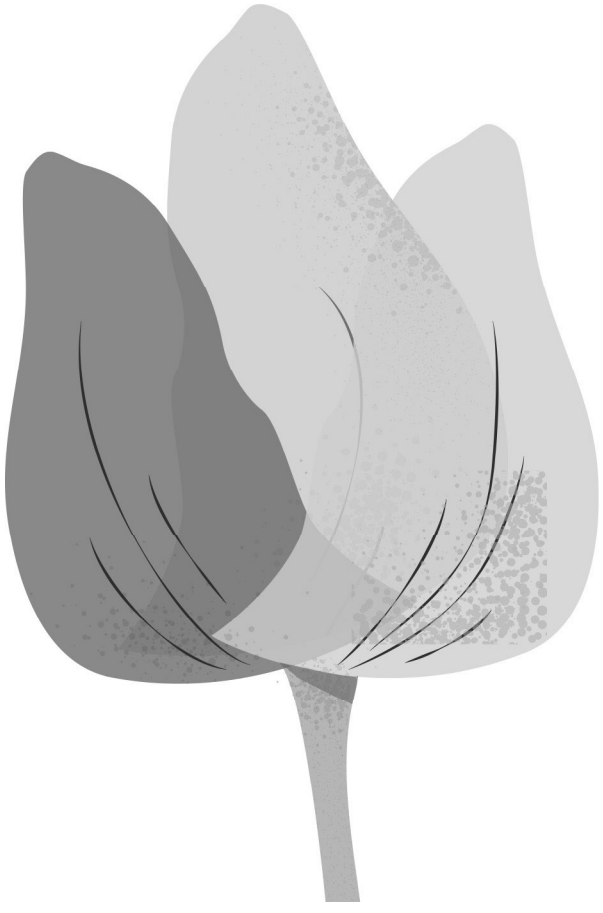
Supplemental data



Supplementary Figure 1. After exclusion of patients with previous rejection still median liver stiffness is higher in patients with NAS and cholestasis present at time of elastography (*n*=8, median TE 9.55 kPa, IQR 7.00) than in controls without NAS or cholestasis (*n*=70, median TE 6.20 kPa, IQR 3.23) ($p<0.05$). TE in patients with NAS but without cholestasis after treatment (*n*=8, median TE 6.95 kPa, IQR 2.68) did not differ from those without NAS or NAS with cholestasis.

Part C

Treatment and outcome



Chapter 7

Long-term outcome of cholangiographic treatment of biliary strictures after orthotopic liver transplantation

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Submitted

Abstract

Introduction Anastomotic (AS) and non-anastomotic (NAS) biliary strictures after orthotopic liver transplantation (OLT) can cause morbidity and graft failure. Strictures can be treated with endoscopic (ERCP) or percutaneous transhepatic cholangiography (PTC) or surgically. In this single center study, management and long-term outcome of these biliary strictures over the last ten years is described.

Patients and Methods A total of 285 OLTs were retrospectively analysed, 31% were OLT with donation after circulatory death. The presence of AS and/or NAS was confirmed with direct cholangiography and exclusion of hepatic artery thrombosis. Cholangiographic treatment of biliary strictures was analysed and considered successful if patients did not require further treatment at least 1 year after the last procedure, if adequate drainage function was achieved and if clinical symptoms were absent. Factors determining success and risk factors for AS and NAS were assessed.

Results In total, 92/285 patients (32.3%) developed post-transplant biliary strictures, of which 13.3% AS only, 9.1% NAS only and 9.8% simultaneous NAS and AS. For AS 183 ERCPs and for NAS 154 ERCPs with intervention were performed. Successful cholangiographic treatment was achieved in 60.5% of patients with only AS, 34.6% of patients with only NAS and 32.1% of the patients with combined AS and NAS. Location of the biliary strictures, timing of treatment and the coexistence of other biliary complications affected therapeutic success rate. Patients with NAS required significantly more often reOLT as compared to patients without NAS ($p<0.01$).

Conclusion Treatment of biliary strictures remains a challenge. However, early treatment of biliary strictures with balloon dilatation and/or stent placement can obviate or defer re-interventions or retransplantations in the majority of cases.

Introduction

Biliary strictures frequently occur after orthotopic liver transplantation (OLT). Based on their anatomical location, they can be subdivided into anastomotic biliary strictures (AS) and non-anastomotic biliary strictures (NAS). Anastomotic strictures occur in 9-38% of patients with a duct-to-duct biliary anastomosis and their development is generally accepted to be associated with local ischemia, surgical techniques, preceding bile duct leakage or higher donor age.[1-7] Reported incidences of NAS after OLT vary between 11% and 31%, among others depending on whether the liver was donated after brain death (DBD) or circulatory death (DCD). NAS is thought to be the result of a complex mechanism, including pretransplant status of the graft, immunological factors, ischemia, and bile salt toxicity.[8-11]

Both types of biliary strictures may result in cholestasis and cholestasis-related symptoms, e.g., pruritus, jaundice and cholangitis.[12] Especially repeated episodes of cholangitis can result in frequent hospital admissions and graft failure. Previous studies have demonstrated that retransplantation is required in up to 16% of these patients, depending on the location and therapy-resistance of the strictures.[13;14] To prevent this, the timely resolution of biliary strictures, assuring adequate drainage, is important in all cases.

Treatment options for biliary strictures include dilatation or stenting with endoscopic retrograde cholangiopancreatography (ERCP) or percutaneous transhepatic cholangiography with drainage (PTC). In addition, in case of endoscopic failure, surgical revision of the anastomosis or retransplantation can be required. In the literature, successful endoscopic treatment of AS was achieved in 68 – 94% of the patients.[15;16] Little is known of the success rates of endoscopic or percutaneous cholangiography for non-anastomotic biliary strictures, as only small cohorts of NAS patients have been evaluated.[12;17;18] In the present study, we describe parameters of long-term outcome of endoscopic and percutaneous cholangiographic treatment of biliary strictures after DBD and DCD OLTs from a single center over ten years. Furthermore, risk factors for the development of biliary strictures were assessed.

Patients and Methods

From January 2005 until December 2014, a total of 292 liver transplantations were performed in the Leiden University Medical Center, of which 285 (97.6%) orthotopic and 7 auxiliary liver transplantations. Auxiliary liver transplants were excluded.

Donor and recipient surgery

As a result of the implementation of a national protocol in 2001 regarding the acceptance of organs, both grafts from donation after brain (DBD) and circulatory (DCD) death are accepted for OLT in the Netherlands. In case of DCD donors, a donor warm ischemia time was present, defined as the time between circulatory arrest and cold flush with preservation fluid in the donor. Cold ischemia time was defined as the time between cold flush in the donor and removal of the liver from ice during the transplantation procedure. The recipient warm ischemia time was defined as the time between removal of the graft from ice until reperfusion in the recipient. University of Wisconsin preservation fluid was used to flush out DBD liver grafts and in case of DCD liver grafts, mainly histidine-tryptophan-ketoglutarate was used. The Eurotransplant Donor Risk Index (ET-DRI) was calculated according to the formula as described by Braat et al.[19]

In general, OLT with standard technique of 'piggy-back' cavo-caval anastomosis, porto-portal and hepatic artery to hepatic artery anastomosis was performed. Preferably, a duct-to-duct biliary anastomosis over an 8 – 12 Ch stent was performed during OLT.

Patient follow-up

Routine immunosuppression was used, mainly Basiliximab and Methylprednisolone induction, followed by Tacrolimus or in some Ciclosporin microemulsion, Prednisolone and in some cases Mofetil Mycophenolate, Sirolimus or Everolimus was added in order to reduce the dose of calcineurin inhibitors if required by renal insufficiency.

In the first year, blood liver biochemistry was performed daily in the first 2 weeks, weekly in the following two weeks and then every 1-3 months. Ultrasound was performed routinely on day 0, 1 and 7, and subsequently at 3, 6 and 12 months,

and yearly after OLT. Six weeks after transplantation, the biliary tree was assessed and the plastic stent that was inserted during OLT was removed endoscopically with ERCP.

Graft survival was defined as time of transplantation until the first occurring event (i.e., retransplantation or death) or, in case of no event, until the end of the study (July 2015). The development of biliary strictures was defined as any stricture or irregularity, requiring treatment, located at the post-OLT anastomotic site (AS) –including the first centimetre above– or in the intra- or extrahepatic bile ducts occurring at least 1 cm above the anastomosis (NAS) after the exclusion of hepatic artery thrombosis (HAT). NAS was categorized into the donor common bile duct and the common hepatic duct until 2 cm above the bifurcation (extrahepatic) and the hepatic ducts and peripheral bile ducts (intrahepatic), as described elsewhere.[20] Time of diagnosis was defined as the date of the first endoscopic or percutaneous treatment, i.e., balloon dilatation and/or a performed stenting procedure. Cholangiographic treatment of biliary strictures was considered successful if patients did not require further treatment at least 1 year after the last procedure, if drainage function was described during the last procedure and if clinical symptoms were absent. In case a stricture recurred after successful treatment as defined, this was termed a recurrent stricture.

Dilatation during ERCP was performed using Hurricane (Boston Scientific, Marlborough, MA, USA) balloons (6, 8, or 10 mm diameter) at 8-11atm during 1-2 minutes, depending on location and type of stricture. In very tight strictures over-the-wire Soehendra dilatation catheters (4-7Fr, 5-8,5Fr) (Cook Medical, Winston-Salem, NC, USA) were used to facilitate subsequent balloon dilatation. For extraction of pre-stenotic debris or casts regular Cook and Olympus extraction balloons were used. Different types of endoscopic stents were used to treat biliary strictures. For anastomotic biliary strictures, patients were treated with 7-10 Fr plastic stents (Cook Medical, Winston-Salem, NC, USA or Olympus Medical, Tokyo, Japan), Niti-S Kaffes intraductal metallic covered stent (Taewoong Medical Co LTd, Gyeonggi-do, South Korea) or fully-covered Hanarostent (MI-tech, Seoul, South Korea). Non-anastomotic biliary strictures were treated with balloon dilatation and to the discretion of the endoscopist also with either 7-10 Fr plastic stents (Olympus Medical) or 12-20cm, 8,5Fr Johlin Wedge stents (Cook Medical, Winston-Salem, NC, USA).

Statistical analysis

Statistical analysis was performed using SPSS version 20.0 for Windows (SPSS Inc. Chicago, IL, USA). Data are expressed as median (interquartile range) or as percentage. Mann-Whitney U test was used to compare continuous data. Categorized data were analysed with the Chi-square test. Survival curves were analysed according to the Kaplan-Meier method and compared using the Log-Rank (Mantel-Cox) test. Risk factor analysis was performed using univariate and multivariate Cox regression analysis. In case of a p-value of <0.20 in the univariate analysis, the risk factor was taken into account in the multivariate analysis. A p-value of less than 0.05 was considered a statistical difference.

The study was performed according to the Helsinki guidelines. Retrospective studies are approved by the institutional review board by legislation.

Results

Between January 2005 and December 2014, 285 orthotopic liver transplantations were performed in the Leiden University Medical Center. Of these, 251 (88.1%), were first grafts, whereas 31 and 3 were second or third grafts respectively. The majority of recipients were male, i.e., 204 patients (71.6%). The extension of acceptance criteria for donor livers in the last decade has led to an increase of OLTs with grafts from donation after circulatory death (DCD). In this cohort, DCD-OLTs accounted for 89 (31.2%) of the performed OLTs. Other recipient characteristics, including the Eurotransplant Donor Risk Index (ET-DRI), are presented in table 1. ET-DRI was significantly higher after DCD-OLT, than DBD-OLT (2.0 vs. 1.7, $p<0.01$) In total, 92 recipients (32.3%) developed biliary strictures, of which 38 (13.3%) AS only, 26 (9.1%) NAS only and 28 (9.8%) simultaneous NAS and AS.

Anastomotic biliary strictures

In general, anastomotic biliary strictures developed early after transplantation, with a median follow-up from OLT until the first treatment of 2.5 months (interquartile range 1.5 – 6.3 months). No significant difference was found in type of donation regarding development of AS (DBD 21.9% vs. DCD 25.8%, $p=0.469$).

Table 1. Baseline characteristics. Data are presented as median (interquartile range) for continuous variables. Categorized data are presented as percentage (number).

Patient characteristics	(*n*=285)
Age at OLT	55 (47 – 62)
Recipient gender	
Male	71.6 (204)
Female	28.4 (81)
Etiology	
ALD	31.9 (91)
Viral	24.6 (70)
PSC	10.9 (31)
Cryptogenic	5.6 (16)
AIH	4.9 (14)
Metabolic	3.9 (11)
PBC	2.5 (7)
ALF	2.1 (6)
Other*	13.7 (39)
With HCC**	29.8 (85)
ET-DRI	1.8 (1.5 – 2.0)
Donation after cardiac death	31.2 (89)
DWIT (min)	17 (12 – 20)
CIT (min)	550 (470 – 635)
RWIT (min)	35 (30 – 41)
Duct-to-duct anastomosis	96.5 (275)

NAS = Non-anastomotic Biliary Strictures; ALD = Alcoholic Liver Disease; PSC = Primary Sclerosing Cholangitis; AIH = Auto-immune Hepatitis; ALF = Acute Liver Failure; PBC = Primary Biliary Cirrhosis; DWIT = Donor Warm Ischemia Time; CIT = Cold Ischemia Time; RWIT = Recipient Warm Ischemia Time; HCC = Hepatocellular Carcinoma;
*Other indications include: non-alcoholic steatohepatitis and polycystic liver disease.
**Categorized into primary etiology

In 40 out of 66 patients (61%) who developed AS during follow-up, abnormalities were already present during the 6-week routine ERCP. These abnormalities included significant strictures or irregularities at the anastomotic site (*n*=34) or anastomotic leakages (*n*=6). ERCP at 6 weeks did not yet reveal abnormalities in 12/66 (18%) patients who developed AS during follow-up. In the remaining 14/66 cases (21%), ERCP was not performed because no biliary stent had been placed during OLT or the stent had already migrated to the small intestines and there was no other indication to perform the procedure.

Within the study period, a total of 183 ERCPs with intervention were performed in patients with anastomotic biliary strictures. In the majority of these procedures (122/183, 66.7%) a combined balloon dilatation and placement of a stent was performed. In the remaining cases, either only balloon dilation (26/183, 14.2%)

or only stents placement (35/183, 19.1%) was performed. Successful endoscopic treatment of AS, defined as described above, was achieved in 30 patients (45.5%) with ERCP only. Successful treatment of AS with PTC only was achieved in an additional 2 (3%) patients. None of the 10/66 (15%) patients who were treated with both endoscopic and percutaneous procedures fulfilled successful treatment criteria as defined after treatment of the anastomotic stricture, probably as a result of a complex stricture. Median follow-up time from last treatment of AS until the end of follow-up was 4.2 years (interquartile range 1.7 – 6.4). Even though patients with successful treatment had a considerable follow-up, the possibility of recurrence of the anastomotic strictures could not entirely be ruled out. In the present cohort, 3 patients developed a recurrent anastomotic stricture (1, 2.5 and 3 years after last treatment respectively). Those patients were all under current treatment and therefore not yet considered as successfully treated at end of follow-up. In 78.1% of the patients with successful cholangiographic treatment, the laboratory biochemical liver function tests normalized within months after the transplantation.

Several factors were associated with a better outcome after AS treatment by ERCP and/or PTC. Cholangiographic success rate was higher in patients with AS only (60.5%), as compared to patients with simultaneous AS and NAS (32.1%, $p<0.03$). In patients with anastomotic leakages the success rate was significantly lower as compared to patients without preceding bile leaks (27.8% ($n=5$) vs. 56.2% ($n=27$), $p<0.04$). Resolution of AS was also higher in patients who received a first treatment within 3 months after OLT, as compared to late treatment (60.5% ($n=23$) vs. 32.1% ($n=9$), $p<0.03$).

In general, patients with AS underwent a median of 3 endoscopic or percutaneous procedures with dilatation and/or stent placement (interquartile range 1 – 4 procedures) during follow-up. Patients with endoscopically successful treatment required a median of 2.5 (interquartile range 2 – 4) procedures during the entire treatment period. No significant difference in endoscopic success rate was found between patients who were solely treated with multiple plastic stents and patients who were at least once treated with a fully covered metal Kaffes- or Hanarostent (49.1% ($n=27$) vs. 45.5% ($n=5$), $p=0.826$).

In addition to the 32 patients who achieved successful endoscopic or percutaneous treatment of AS, 11/66 (16.7%) patients underwent successful surgical revision

or retransplantation and 5 patients died within 1 year after the last endoscopic procedure. In the majority of deaths (4/5), the cause of death was not stricture-related, i.e., intracerebral bleeding (*n*=1), multi-organ failure after correction of an abdominal incisional hernia (*n*=1), endocarditis (*n*=1) or renal failure (*n*=1) and cholangiosepsis (*n*=1). Eighteen patients are considered as under current treatment, i.e., the follow-up after the last treatment was less than 1 year.

Simultaneous presence of sludge (53%), biliary stones (18.2%), bilomas requiring drainage (6.0%) and leakage (27.3%) was frequent in all patients with AS.

Non-anastomotic biliary strictures

In the present cohort, 54 patients developed NAS, of which 28 with simultaneous AS. As expected, the incidence of NAS was significantly higher after OLT using DCD donors than after DBD-OLT (33.3% (*n*=30) vs. 11.9% (*n*=24), $p<0.01$). Median follow-up from OLT until the diagnosis and first treatment of NAS was 3.1 months (interquartile range 1.6 – 5.2 months), which did not differ between DBD-OLTs and DCD-OLTs. NAS was located only in the extrahepatic bile ducts (*n*=9), only in the intrahepatic bile ducts (*n*=7) or both intra- and extrahepatic (*n*=38).

Routine ERCP at 6 weeks after OLT showed signs of NAS in only 18 patients (33.3%) with irregular bile ducts or non-anastomotic strictures. In the remaining 36 cases developing NAS, 9 patients at 6 weeks ERCP had developed only strictures at the anastomotic site, but not yet at the non-anastomotic regions, while 8 patients had developed bile duct leakages, 9 ERCPs were considered normal and in 10 patients routine 6-week ERCP was not performed. These findings confirmed that AS usually presents earlier than NAS.

Within the study period a total of 154 ERCPs with intervention for NAS were performed in 54 patients. Treatment consisted of balloon dilatation (31.1%, *n*=48) or stent placement (13.6%, *n*=21) or combined dilatation and stent placement (55.2%, *n*=85). NAS is considered more troublesome than AS, as non-anastomotic biliary strictures are often resistant to therapy. This was confirmed in the present study by a lower cholangiographic success rate for NAS as compared to AS: successful treatment of NAS with ERCP procedures was achieved in 17/54 patients (31.5%), in 5 cases combined with percutaneous treatment) and 1 patient who was treated with percutaneous cholangiography only. This included 9 out of

26 patients (34.6%) with NAS only and 9 out of 28 patients (32.1%) with both AS and NAS. Interestingly, patients who presented with NAS within 3 months after OLT had a trend towards a lower success percentage than patients who presented with late NAS after 3 months (22.2% vs. 44.4%, p=0.08). This is probably due to a more severe character of the biliary strictures. Successful treatment was achieved in 44.4% of the patients with only extrahepatic biliary strictures, in 14.3% of the patients with only intrahepatic biliary strictures and in 34.2% of the patients with both intra- and extrahepatic biliary strictures. Normalisation of liver biochemistry after successful cholangiographic treatment occurred in only 55.5% of these patients. Median follow-up from last treatment for NAS until the last follow-up date was 2.2 years (interquartile range 1.5 – 3.1). Recurrence of non-anastomotic strictures occurred in 2 patients (after 2 and 2.5 years respectively). Both of the patients with recurrence were under current treatment and thus not considered as successfully treated.

During follow-up, patients with NAS underwent a median of 3 endoscopic or percutaneous intervention procedures (interquartile range 2 – 7 procedures). To achieve endoscopic success, a median of 5.5 (interquartile range 2 – 8) procedures was required. Different stent types were used in the treatment of NAS. No significant difference in endoscopic success rate was found between patients who were treated with plastic stents only (33.3%) and patients who were at least once treated with a Johlin Wedge stent (37.5%, p=0.819) – used for more proximal strictures.

Out of the patients not yet responding to endoscopic or percutaneous treatment (n=36), 12 patients underwent retransplantation because of biliary strictures. Seven patients died before they could achieve successful management of their strictures, of which two of cholangiosepsis and 5 of non-stricture related causes, and 17 patients are still receiving treatment.

Simultaneous presence of sludge (70.4%), biliary stones (20.4%), bilomas requiring drainage (13.0%) and leakages 19 (35.2%) was frequent among patients with NAS.

Besides cholangiographic evaluations, several laboratory and radiological parameters were evaluated during follow-up in patients with NAS. Bile duct dilatation on the performed ultrasound was present in 18.6%, 26.2% and 28.1% of NAS

(with or without AS) cases at 3, 6 and 12 months after OLT respectively. Before treatment, serum levels above the reference values of alkaline phosphatase and gamma glutamyl transferase were present in up to 78% and 94% of patients respectively, indicating that laboratory parameters can be important indicators for bile duct abnormalities.

Radiodiagnostic features of NAS resemble the diagnostic criteria for primary sclerosing cholangitis. It may be difficult to distinguish NAS from recurrent PSC in a transplanted graft, and some of the NAS cases may in fact be recurrent PSC. Therefore, the cholangiographic success rate for patients transplanted for PSC were compared to patients transplanted for other etiologies. Cholangiographic success in patients with PSC was not significantly lower than in patients transplanted for other etiologies (22.2% vs. 35.6%, p=0.439).

In the routine complication registry and charts, complications were recorded in 6.2% (n=21) of the performed procedures, which included perforation (n=5), pancreatitis (n=3), cholangitis (n=2), post-sphincterotomy bleeding (n=9), and cardiac arrhythmia (n=2).

Donor and recipient-associated risk factors

Recipient-associated risk factors for AS and NAS were evaluated separately. For anastomotic strictures, no significant risk factors could be found in the univariate analysis, with the exception of a trend towards a higher incidence of AS in patients with a higher ET-DRI in multivariate analysis. (Table 2) Primary sclerosing cholangitis, ET-DRI and DCD-OLTs were associated with NAS development in univariate analysis at the p<0.20 level. In multivariate analysis, DCD-OLT and ET-DRI were independent risk factors for NAS development (p<0.01) (Table 3).

Table 2. Univariate and multivariate analysis of risk factors for development of AS after OLT.

Variables OLT		Univariate analysis		Multivariate analysis	
		HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Recipient age at OLT	Continuous	0.99 (0.98 – 1.02)	0.870		
Recipient gender	Male	1.71 (0.93 – 3.14)	**0.084**	1.53 (0.83 – 2.82)	0.174
	Female (reference)	1			
ET-DRI	Continuous	1.91 (0.97 – 3.73)	**0.061**	1.83 (0.94 – 3.56)	0.077
MELD score	Continuous	1.02 (0.98 – 1.05)	0.438		
DWIT	Continuous	1.00 (0.91 – 1.10)	0.977		
CIT	Continuous	1.00 (0.99 – 1.00)	0.579		
RWIT	Continuous	0.99 (0.96 – 1.01)	0.234		
Type of anastomosis	Duct-to-duct (reference)	1			
	Choledochojejunostomy	0.75 (0.18 – 3.06)	0.687		
PSC as indication	Other (reference)	1			
	PSC	0.59 (0.24 – 1.47)	0.254		
Donation	DBD (reference)	1			
	DCD	1.33 (0.80 – 2.22)	0.267		

HR = Hazard Ratio, CI = Confidence Intervals, OLT = Orthotopic Liver Transplantation, ET-DRI = Eurotransplant Donor Risk Index, MELD = Model for End-stage Liver Disease, ALT = Alanine aminotransferase, DWIT = Donor Warm Ischemia Time, RWIT = Recipient Warm Ischemia Time, CIT = Cold Ischemia Time, PSC = Primary Sclerosing Cholangitis,

Table 3. Univariate and multivariate analysis of risk factors for development of NAS after OLT.

Variables OLT		Univariate analysis		Multivariate analysis	
		HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Recipient age at OLT	Continuous	0.99 (0.97 – 1.02)	0.769		
Recipient gender	Male	1.24 (0.67 – 2.32)	0.494		
	Female (reference)	1			
ET-DRI	Continuous	4.39 (2.19 – 8.78)	**0.000**	2.30 (1.00 – 5.26)	**0.050**
MELD score	Continuous	1.02 (0.98 – 1.07)	0.252		
DWIT	Continuous	0.97 (0.90 – 1.06)	0.512		
CIT	Continuous	1.00 (0.99 – 1.00)	0.668		
RWIT	Continuous	1.01 (0.98 – 1.03)	0.667		
PSC as indication	Other (reference)	1			
	PSC	1.74 (0.85 – 3.57)	**0.128**	1.55 (0.76 – 3.19)	0.232
Donation	DBD (reference)	1			
	DCD	3.36 (1.95 – 5.76)	**0.001**	2.36 (1.23 – 4.53)	**0.010**

HR = Hazard Ratio, CI = Confidence Intervals, OLT = Orthotopic Liver Transplantation, ET-DRI = Eurotransplant Donor Risk Index, MELD = Model for End-stage Liver Disease, DWIT = Donor Warm Ischemia Time, CIT = Cold Ischemia Time, RWIT = Recipient Warm Ischemia Time, PSC = Primary Sclerosing Cholangitis, DBD =

Patient and graft survival

None of the included patients were lost to follow-up. Overall 1-, 5- and 10-years graft survival was 88%, 73% and 66% respectively. No significant difference (p=0.44 and p=0.27) was found between patient and graft survival of DBD vs. DCD. (Figure 1) Early retransplantation (< 2 weeks) for graft failure was also not significantly different between DCD-OLT and DBD-OLT (DCD 20.2% vs. DBD 15.3%, p=0.304). Patients with non-anastomotic biliary strictures required significantly more retransplantations as compared to patients without NAS (22.2% vs 7.6%, p<0.01), whereas this was not the case for patients with AS (13.6 vs. 9.3%, p=0.31). Retransplantation for non-anastomotic biliary strictures was also more frequent in the DCD group (DCD 15.7% vs. DBD 7.7%, p=0.04). Patient survival was not related to the presence of NAS (p=0.232) or AS (p=0.175) as compared to patients without biliary strictures.

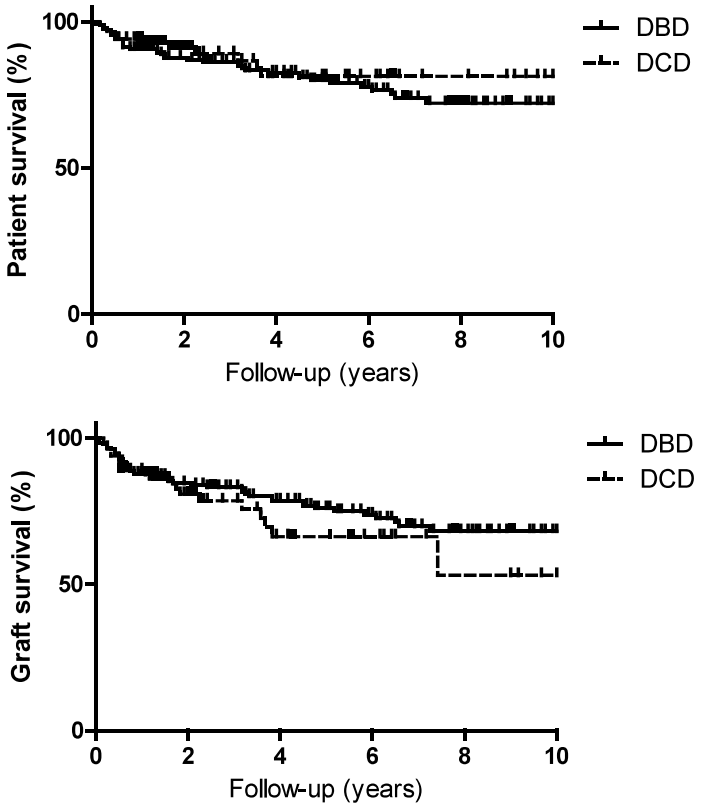

Figure 1. Patient and graft survival with regard to donation after brain and cardiac death.

Discussion

The present study describes the development and cholangiographic management of biliary strictures after orthotopic liver transplantation with long-term outcome and its determinants in a single center with a relatively high rate of DCD-OLTs. Post-transplant biliary strictures remain a frequent complication with an incidence of 23.2% for anastomotic strictures and 18.9% for non-anastomotic biliary strictures in this cohort. This is in accordance with previous studies, where incidences between 9–38% for AS and incidences between 11–31% for NAS were reported.[2;7;9;11;21;22] The present study further revealed that AS usually presented earlier than NAS and was usually easier to treat with less procedures. Successful treatment was, among other factors, dependent on co-existence of other biliary complications, timing of treatment and location of the biliary strictures.

Anastomotic strictures

The development of AS has been associated with donor- and procedure-related risk factors, such as donor age, postoperative bile duct leakages and a performed duct-to-duct anastomosis.[1;2;5] To assess donor-related risk factors, the ET-DRI was evaluated in relation to AS development in the current study. A trend was found in the multivariate analysis towards a higher incidence of AS in patients with a higher ET-DRI. Recipient-associated variables are not frequently mentioned as risk factor for the development of AS. Indeed, in the present study, we could not find a significant association between any of the tested recipient variables and the development of AS.

After diagnosis of AS, endoscopic or percutaneous cholangiographic balloon dilatation and/or the placement of a stent is usually considered the first step in treatment of biliary strictures. Several studies demonstrated an endoscopic success rate between 68% and 94% in patients with anastomotic biliary strictures.[15;16;18;23] In the present study, successful cholangiographic management could be achieved in up to 60.5% for patients with AS, which is slightly lower than the reported rates of the studies mentioned above. However, we used a strict definition for strictures and of success, which should last for more than a year. Follow-up time after the last treatment procedure was less than 1 year in most studies, and therefore not representative for long-term outcomes. In addition, in the present study it became clear that the success rates were significantly influenced by several other factors,

including the presence of other bile duct complications, such as the simultaneous presence of both anastomotic and non-anastomotic biliary strictures and bile duct leakages, but this may have been the case in other studies as well. Differences in endoscopic success rate between patients with an hepaticojejunostomy and duct-to-duct anastomosis could not be assessed as only 2 patients with an hepaticojejunostomy developed AS in the present study.

In the current data the success rate of treatment of AS started within three months was almost twice the success rate if intervention started more than three months after OLT. It is possible that the early strictures are easier to treat, or there may have been some overtreatment in the early phase. The latter may be due to the routine ERCPs at six weeks in this cohort, performed during surgical stent removal. On the other hand this may have been the key to the successful early treatment. Since this was not a randomized controlled trial of ERCP or not at six weeks the possible benefits of this early ERCPs are unclear.

In general, it was reported that an increased short- and long-term success rate can be achieved with a more aggressive approach, in which multiple stents are used, as Costamagna et al. proposed.[24;25] Biliary strictures treated with combined balloon dilatation and stenting procedures and 10 Fr diameter stent placement were reported to be more likely to respond, whereas a pretransplant Model for End-Stage Liver Disease score of ≥ 10 and a stent retention time of ≥ 12 months were significantly associated with a worse endoscopic outcome.[15] Other predictors for an inferior outcome in the literature were recipient age, high blood transfusion requirements during OLT, a prolonged operation time, late treatment and the simultaneous presence of other bile duct complications.[2;26;27]

Non-anastomotic biliary strictures

Non-anastomotic biliary strictures are considered even more troublesome than AS. This is mainly due to their diffuse character and higher resistance to therapy. Indeed, in the current data there was a lower treatment success rate in patients with NAS than with AS, with a trend to a lower success rate when NAS appeared within the first 3 months after OLT. Success rate was higher for extrahepatic NAS and for combined extra- and intrahepatic NAS as compared to only intrahepatic NAS. There was more reOLT in patients with than in patients without NAS. Success rate was similar in patients with only NAS or combined NAS and AS. Since

only onethirds of patients developing NAS has features of NAS at the 6-weeks ERCP and early intervention was not favourable in NAS, such a routine ERCP seems to have no place for diagnosis of NAS. Instead, ERCP on indication only is now the standard, especially now that diagnosis of biliary strictures after OLT is reliable with MRCP combined with a validated scoring system.[20]

As compared to previous studies, Graziadei et al.[18] found complete resolution of clinical symptoms and cholestatic parameters with ERCP in 21% in a cohort of 19 patients. Resolution of clinical symptoms, but persistent biochemical cholestasis, was found in an additional 42%. Tabibian et al.[17] described a success rate of 73% (11/15) for NAS patients. However, this was achieved with a relative short follow-up (median 13 months), whereas we found recurrence of NAS one year after the last treatment in 11.1% of cases. Within one year after the last cholangiographic treatment, even higher recurrence rates of NAS have been reported.[28] The presence of multiple non-anastomotic biliary strictures, recurrent strictures or early presentation has been associated with unsuccessful outcomes in previous studies.[28;29] In accordance with the treatment of anastomotic biliary strictures, the combination of balloon dilatation and stent placement is usually considered the best regimen for NAS. Yet, Zoepf et al.[30] demonstrated that a combination of balloon dilatation and stent placement was successful in 31%, whereas a balloon dilatation alone was successful in 91% of NAS cases. However, due to its retrospective study design a selection bias could not be excluded: less severe strictures may have been more frequently treated with balloon dilatation alone, whereas in more severe strictures, a combined approach with stent placement may have been the treatment of choice. Possibly, favourable endoscopic outcomes can also be associated with stent type. However, we failed to demonstrate differences in outcome with regard to different stent types, possibly as a result of a low sample size. In previous literature, stricture resolution and a shorter treatment duration with a covered stent have been demonstrated, but spontaneous migration of the stent and difficulties to remove fully covered stents due to overgrowth have also been reported in small case series.[31-34] In case of endoscopic failure, percutaneous transhepatic cholangiography is usually considered as a feasible alternative.[12;35]

A possible limitation of the present study is that treatment procedures were retrospectively analysed. However, we used a strict definition for the presence of biliary strictures and severity and location of the strictures was clearly noted in the patient

chart. Furthermore, we used a strict definition of treatment success, which should last for more than a year.

Even though complete resolution of biliary strictures cannot be achieved in all patients, the current data support that it is important to treat AS and NAS and a reasonable success rate of cholangiographic treatment can be achieved in two thirds of AS and one third of NAS patients. Timely endoscopic or percutaneous cholangiographic treatment may reduce episodes of cholangitis and progression to graft failure, and can lead to relatively low rates of retransplantation as in the current study.

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Chapter 8

Quality of life, anxiety and depression after orthotopic liver transplantation in relation to biliary strictures and compared to healthy controls

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Submitted

Abstract

Introduction Quality of life (QOL) after liver transplantation (LT) has been compared to QOL before LT and usually not to healthy controls, and impact of biliary strictures after LT on QOL has not been tested.

Patients and Methods We compared QOL after LT with versus without biliary strictures and with healthy controls in a cross-sectional study with validated QOL questionnaires > 3 months after LT.

Results 142 patients provided 71 matched controls. Scores were adjusted for age, gender, marital status, (un)employment, ethnicity and known or self-reported comorbidity. After LT QOL was impaired on all subscales of SF-36, MFI-20 and EQ-5D questionnaires compared to controls (p<0.001). Patients with (n=55) versus without (n=87) biliary strictures showed similar QOL scores on the SF-36 and MFI-20. Only in the first four years after LT, patients with biliary strictures (n=21) showed a trend towards a worse QOL on SF-36 and MFI-20. Patients with biliary strictures reported more symptoms of depression and anxiety on the EQ-5D (p<0.04) and LDSI (Odds ratio 2.07 [1.01 – 4.26], p<0.05).

Conclusion In conclusion, after liver transplantation quality of life was below normal. Patients with biliary strictures reported significantly more anxiety and depression and had a trend towards lower QOL in the first 4 years.

Introduction

With excellent one- and three-year patient survival rates after liver transplantation (LT) of 90% and 80%, respectively, enhancing the quality of life (QOL) has become an important target of patient-care.[1] Prior studies demonstrated an improvement in QOL after as compared to before transplantation.[2-4] However, some studies found that health-related quality of life (HRQOL) scores of transplanted patients remain lower in comparison to the general population, even in a subgroup analysis performed among patients with a follow-up > 20 years after LT.[5;6] These results suggest that, even with long-term follow-up, health issues remain present. Several attempts have been made to identify factors that may independently contribute to a decreased QOL in patients after LT. These factors can be subdivided into personal factors (age and gender), socioeconomic factors (like unemployment and marital status), and disease-related factors (like recurrence of the original disease and duration of follow-up).[6-8]

Biliary strictures, divided into anastomotic (AS) and non-anastomotic biliary strictures (NAS), are frequent complications after LT and may have an effect on patients' perception of general well-being and QOL. AS occur in 6-23% and reported incidences of NAS vary between 9% and 31%.[1;9-11] These patients often need invasive procedures, such as repetitive endoscopic or percutaneous biliary dilatation and stenting procedures, and sometimes surgical intervention or retransplantation.[12;13] Also, major complications, such as bacterial cholangitis, postprocedural pancreatitis and bleedings can occur in 1-4% of endoscopic cholangiography procedures.[14] We hypothesized that clinical symptoms of biliary strictures, invasive treatment procedures and their associated risk of complications, may affect perceived quality of life in patients after LT. Therefore, differences in HRQOL were assessed between patients and healthy controls, and between patients with or without (a history of) biliary strictures for which intervention was required, as evaluated by validated HRQOL questionnaires.

Patients and Methods

Study population

A cross-sectional case-control study in the Leiden University Medical Center (LUMC), a tertiary referral center for liver disease, with generic and liver-disease specific quality of life questionnaires, was performed in March 2014. A total of 228 patients, i.e., all LT survivors since 1992, with a minimum follow-up of ≥ 3 months after LT were sent a general background questionnaire and four validated HRQOL questionnaires, i.e., Short-Form 36 (SF-36), Multidimensional Fatigue Index-20 (MFI-20), Euroqol EQ-5D and adjusted Liver Disease Symptom Index 2.0 (LDSI). These parameters have been cross-culturally validated and have been used previously in liver transplant studies. Quality of Life was defined as in the reference populations for these questionnaires. Patients were requested to complete the set of questionnaires and return them in a provided envelope. Non-responders were encouraged to complete and return the questionnaires by a reminder letter three months after the initial request. In case of missing or incomplete data, patients received a request per mail to complete the missing question(s). All questionnaires returned before August 2014 were included in the study. Clinical data, e.g., age, gender, etiology of liver disease and posttransplant complications, including the presence of anastomotic and/or non-anastomotic biliary strictures was derived from the electronic patient charts. Posttransplant complications further included (episodes of) rejection, infection and/or sepsis, malignancies, such as post-transplant lymphoproliferative disorders, hepatic artery thrombosis and bile duct leakages. Biliary strictures were defined as follows: any stricture or irregularity requiring treatment, located at the anastomotic site (anastomotic stricture or AS) or in the intra- or extrahepatic bile ducts occurring at least 1 cm above the anastomotic site (non-anastomotic strictures or NAS), as previously described.[15] Treatment was defined as endoscopic or percutaneous treatment, i.e., balloon dilatation and/or performed stenting procedures, or in case of biliary strictures without endoscopic treatment options, surgical revision of the anastomosis and retransplantation.

In order to compare patients and controls with a more or less similar background and socioeconomic status, patients were requested to provide a control person of similar age and, if possible, gender. Controls received an identical set of questionnaires with exception of the LDSI, as this liver-disease specific questionnaire is not appropriate for healthy subjects.

The study protocol was approved by the Medical Ethics Committee of the LUMC and is in accordance with the Declaration of Helsinki. Only patients and controls with a minimum age of 18 years who gave written informed consent to participate were included in the study. No compensation was given for participation in the study.

Questionnaires

HRQOL was assessed using different disease-specific or generic questionnaires. Patients and controls were sent a separate questionnaire to evaluate personal factors and socioeconomic status. Participants were also requested to report current comorbidity that restricted them in their usual activities.

Short Form-36 (SF-36)

The SF-36 is a validated instrument for the measurement of QOL in different medical conditions, and is widely used to asses HRQOL in patients after LT. The questionnaire consists of 36 items measuring the perception on patients own functional status and general well-being during the last month.[16-19] The questionnaire includes both questions and statements related to different subscales, i.e., 1) physical functioning, 2) social functioning, 3) limitations in usual role activities because of physical health problems, 4) limitations in usual role activities because of emotional problems, 5) general mental health (psychological distress and well-being), 6) vitality (energy and fatigue) 7) pain and 8) general health perception. Per subscale the missing data was evaluated. When half or less of the questions were missing, the missing question was substituted by the personal mean score of the specific subscale. If more than half of the questions were missing the subscale was excluded from the analysis. The proportion of substituted data was < 2% for all questions. A higher score indicates a better quality of life.

Multidimensional Fatigue Index 20 (MFI-20)

The MFI-20 is a self-report questionnaire including 20 statements to assess fatigue.[20] The statements are scored on a five-point scale varying from “yes, that is true” to “no, that is not true”. The MFI-20 consists of five different domains, measuring 1) General Fatigue, 2) Physical Fatigue, 3) Reduced Activity, 4) Reduced Motivation and 5) Mental Fatigue. A higher score indicates more fatigue.

EQ-5D

The EQ-5D is a short questionnaire containing 5 questions, each measuring a different dimension of QOL (mobility, self-care, usual activities, pain/discomfort, anxiety/depression).[21] Limitations per dimension can be noted in three categories: no problems, moderate problems and extreme problems. The EQ-5D also contains a visual analogue scale (VAS) measuring self-perception on a scale from 0 to 100.

Liver Disease Symptom Index 2.0

The Liver Disease Symptom Index is a disease-specific questionnaire for patients with chronic liver disease, which can be used as a complementary questionnaire to generic QOL questionnaires.[22] The LDSI contains 18 statements. Nine statements assess specific symptoms related to liver disease that were present in the previous week. The nine other statements assess the hindrance experienced as a result of the these symptoms. The items can be scored on a five-point scale from 'not at all' to 'to a high extent'.

Statistical analysis

Statistical analysis was performed using SPSS version 20.0 for windows (SPSS Inc. Chicago, IL, USA). For normally distributed variables the Student t-test was used to compare patients and controls and patients with or without biliary strictures. Mann-Whitney U test was performed for non-normally distributed variables. Continuous variables are presented as mean ± standard deviation (SD). Categorized data was analysed with the Chi-square and presented in percentages.

Based on existing literature age, gender, marital status, unemployment, ethnicity and known or self-reported comorbidity were considered factors with a potential influence on HRQOL scores. Using linear regression possible correlations between each subscale and the factor was tested. In case of a p-value of <0.20 the factor was taken into account in the multiple linear regression analysis. Reported subscale scores of the SF-36, MFI-20 and EQ-5D VAS were adjusted for independent factors related to HRQOL using the standardized β. A p-value of less than 0.05 was considered statistically significant.

Results

A total of 142 patients (62.3%) completed and returned the questionnaires. No significant differences in gender, disease aetiology, median follow-up after LT, the presence or absence of biliary strictures and complications during follow-up between responders and non-responders was found. Median age of responders was higher than of non-responders (61 years vs. 57 years, p=0.03).

Patients and controls

The 142 patients who returned the questionnaires provided 75 controls. The questionnaires of 4 control subjects were excluded from the analysis due to too much missing information in the responses. Therefore, 71 controls were included in the analysis. Overall, patients were slightly older than controls (median age 61 vs. 57 years, p=0.02) and a significant higher percentage of patients was male (p=0.02). However, when only those patients who provided a control subject were taken into account, median age did not differ significantly (59 vs. 57 years, p=0.62). A similar result was found for gender frequency, i.e., 57.7% of patients was male vs. 51.4% of controls (p=0.45). Patients characteristics are presented in Table 1. Because (mean) scale scores of all questionnaires did not differ between patients with or without a provided control subject, all returned questionnaires were included (Supplementary Fig 1). Factors related to HRQOL were assessed by linear regression in relation to SF-36, MFI-20 and EQ-5D VAS in patients after OLT with and without biliary strictures and controls (data not shown) and adjusted scores were compared. Adjusted QOL scores on all SF-36 and MFI-20 subscales were significantly impaired in patients compared to their controls (p<0.001). Specific mean scores are provided in Table 2. Furthermore, patients showed an increased frequency of reporting any problems in mobility, self-care, usual activities and the presence of pain/discomfort and anxiety/depression on the EQ-5D questionnaire compared to their controls (p<0.001). Mean adjusted VAS score of patients was 40.4 ± 22.4 compared to 63.5 ± 23.9 for controls (p<0.001).

Table 1. Characteristics of patients and controls (%, unless otherwise specified).

	Patients (*n* = 142)	Own controls (*n* = 71)	*p*-value	Strictures (*n* = 55)	No strictures (*n* = 87)	*p*-value
Age (years, median, range)	61.3 (20 – 74)	57.0 (18 – 72)	0.02	58.9 (31 – 72)	61.6 (20 – 74)	0.50
Sex			0.02			0.16
Male	67.6	51.4		74.5	63.2	
Female	32.4	48.6		25.5	36.8	
Marital status			0.34			0.45
Married/living together	69.7	78.6		65.5	72.4	
Single/widow(er)/divorced	30.3	21.4		34.5	27.6	
Employed	23.9	50.7	<0.001	20.0	26.4	0.38
Self-reported comorbidity	69.0	39.4	<0.001	67.3	70.1	0.72
Etiology						0.29
ALD	25.4			38.2	17.2	
HCV	12.7			12.7	12.6	
HBV	7.0			7.3	6.9	
PSC	9.2			7.3	10.3	
AIH	3.5			5.5	2.3	
Other	42.2			29.0	50.7	
Current complications	11.3			14.5	9.2	0.33
Complications during follow-up						
Episode(s) of rejection	24.6			34.5	18.4	0.03
Infections	45.8			49.1	43.7	0.53
Other	19.0			27.3	13.8	<0.05
Age at OLT (years, median, range)	54.2 (19 – 69)			54.3 (24 – 69)	54.0 (19 – 68)	0.66
Follow-up (months, median, range)	59.5 (3 – 258)			62 (5 – 190)	56 (3 – 258)	0.63
Number of reOLTs						0.007
None	88			76.4	95.4	
1	10.6			20	4.6	
2	0.7			1.8	0	
3	0.7			1.8	0	

ALD = Alcoholic Liver Disease, HCV = Hepatitis C Virus, HBV = Hepatitis B Virus, PSC = Primary Sclerosing Cholangitis,AIH = Autoimmune Hepatitis, OLT = Orthotopic Liver Transplantation.

Table 2. Quality of Life parameters in OLT patients compared to controls. Scores of the SF-36 and MFI-20 are expressed as adjusted mean scale scores ± standard deviation.

Questionnaire	Patients (*n*=142)	Own controls (*n*=71)	*p*-value
SF-36			
Physical functioning	37.6 ± 25.7	68.7 ± 26.1	<0.001
Social functioning	53.8 ± 25.9	77.6 ± 23.9	<0.001
Role limitations due to physical problems	33.6 ± 31.5	72.3 ± 27.4	<0.001
Role limitations due to emotional problems	59.5 ± 34.8	85.5 ± 21.4	<0.001
General mental health	52.2 ± 23.4	67.7 ± 21.6	<0.001
Vitality	35.1 ± 22.1	57.8 ± 24.5	<0.001
Pain	48.9 ± 28.6	72.2 ± 29.0	<0.001
General health perception	26.8 ± 22.4	53.3 ± 28.4	<0.001
MFI-20			
General fatigue	17.6 ± 9.1	10.2 ± 6.4	<0.001
Physical fatigue	17.7 ± 9.3	9.7 ± 6.6	<0.001
Reduced activity	17.3 ± 8.7	10.8 ± 6.9	<0.001
Reduced motivation	13.4 ± 7.5	8.7 ± 5.0	<0.001
Mental fatigue	14.3 ± 4.6	11.1 ± 3.7	<0.001

SF-36: Short Form 36, MFI-20: Multidimensional Fatigue Index-20

Quality of life in patients with biliary strictures

Out of 142 patients, 55 patients had (a history of) biliary strictures that required at least one endoscopic or percutaneous treatment procedure. This included 25 patients with AS only, and 30 patients with NAS, of whom 22 patients had both AS and NAS. Patients with or without biliary strictures did not differ in age, gender, socioeconomic status and disease etiology (Table 1). Furthermore, 23.6% of patients (*n*=13) with biliary strictures required at least one retransplantation, compared to 4.6% of patients (*n*=4) without biliary strictures (*p*<0.001). Indications for retransplantation in this group were: the presence of therapy-resistant biliary strictures (*n*=5), repeated cholangitis (*n*=2), hepatic artery thrombosis (HAT) (*n*=3), progressive graft failure (*n*=2) and bile duct leakage (*n*=1).

Mean scale scores on the SF-36 and MFI-20 between patients with or without biliary strictures were highly comparable. Adjusted mean scale scores are provided in Table 3. Similar results were obtained when AS and NAS were evaluated separately. Because biliary strictures usually occur within the first years after LT a

subgroup analysis of patients with a follow-up of ≤ 4 years was performed. In this group, patients with biliary strictures (*n*=21) showed a trend towards a worse QOL on the SF-36 ($0.16 < p < 0.51$), as compared to patients without biliary strictures (*n*=39). (Figure 1). The same trend was found on the MFI-20 ($0.09 < p < 0.79$). (Figure 2).

Table 3. Quality of Life parameters in patients with or without biliary strictures. Scores of the SF-36 and MFI-20 are expressed as adjusted mean scale scores ± standard deviation.

Questionnaire	Biliary Strictures (*n*=55)	No Biliary Strictures (*n*=87)	*p*-value
SF-36			
Physical functioning	33.8 ± 25.4	40.0 ± 25.7	0.16
Social functioning	51.5 ± 27.5	55.4 ± 25.0	0.39
Role limitations due to physical problems	30.1 ± 32.4	35.8 ± 30.9	0.30
Role limitations due to emotional problems	55.9 ± 38.2	61.8 ± 32.4	0.78
General mental health	51.1 ± 24.3	52.8 ± 23.0	0.66
Vitality	34.2 ± 21.2	35.7 ± 22.8	0.69
Pain	47.5 ± 29.9	49.8 ± 27.9	0.65
General health perception	25.0 ± 20.9	27.9 ± 23.4	0.46
MFI-20			
General fatigue	18.8 ± 8.5	16.8 ± 9.5	0.21
Physical fatigue	18.5 ± 8.5	17.1 ± 9.8	0.38
Reduced activity	18.6 ± 8.2	16.5 ± 9.0	0.16
Reduced motivation	14.5 ± 6.9	12.7 ± 7.8	0.15
Mental fatigue	14.3 ± 4.3	14.3 ± 4.9	0.96

SF-36: Short Form 36, MFI-20: Multidimensional Fatigue Index-20

Overall, reported frequencies on the different EQ-5D categories were similar for mobility, selfcare, usual activities and pain/discomfort. However, the percentage of patients within higher categories of anxiety and depression were statistically different ($p<0.04$). (Figure 3) Noted frequencies in the categories 'no problems', 'moderate problems' and 'extreme problems' in the EQ-5D for patients with biliary strictures were 61.1%, 39.9% and 0%, compared to 73.6%, 21.8% and 4.6% for patients without biliary strictures. When the subgroups of exclusively AS only (*n*=25) or NAS only (*n*=8) and both NAS and AS (*n*=22) were compared to patients without any biliary strictures a similar trend was seen, although not significant due to the small subgroups, ($p=0.10$, $p=0.82$, $p=0.13$ respectively).

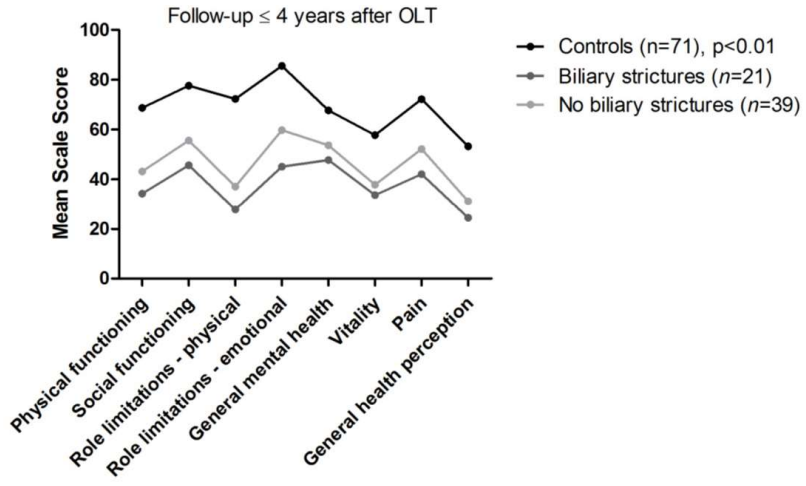

Figure 1. Short-Form 36. Follow-up ≤ 4 years after OLT.
Mean Scale Scores on the Short-Form 36 patients with or without biliary strictures and a follow-up ≤ 4 years after orthotopic liver transplantation. On all subscales, patients with biliary strictures tend to report a worse QOL, as compared to patients without biliary strictures. Physical functioning p=0.23; Social functioning p=0.16; Role limitations- physical p=0.29; Role limitations – emotional p=0.14; General mental health p=0.38; Vitality p=0.51; Pain p=0.20; General Health Perception p=0.29.

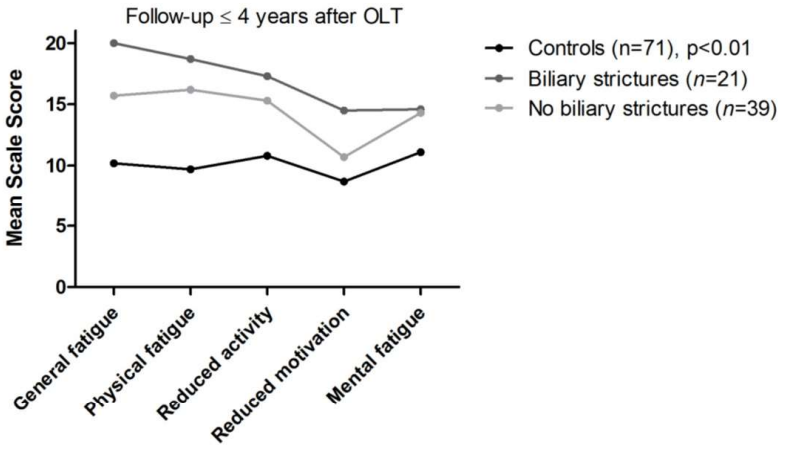

Figure 2. Multidimensional Fatigue Index-20. Follow-up ≤ 4 years after OLT.
Mean Scale Scores on the Multidimensional Fatigue Index-20 patients with or without biliary strictures and a follow-up ≤ 4 years after Orthotopic Liver Transplantation. On all subscales, patients with biliary strictures tend to report a worse QOL, as compared to patients without biliary strictures. General fatigue p=0.09; Physical fatigue p=0.35; Reduced activity p=0.42; Reduced motivation p=0.14; Mental fatigue p=0.79.

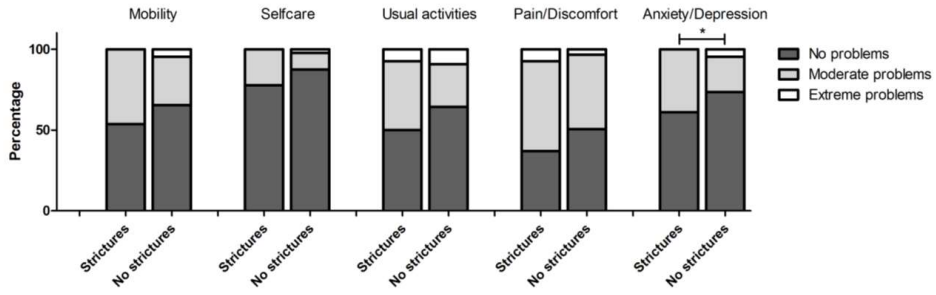

Figure 3. EQ-5D in patients with and without biliary strictures.
Reported frequencies on the EQ-5D in the categories 'no problems', 'moderate problems' and 'extreme problems' for patients with (*n*=55) and without (*n*=87) biliary strictures. *p<0.04

Liver Disease Symptom Index 2.0

The LDSI was completed and returned by 133 transplanted patients. In the original design, the presence of symptoms related to liver disease could be scored on a five-point scale, with a higher score indicating more severe symptoms. However, due to a low variability in reported severity, the five-point scale was simplified to a two-point scale, indicating whether the symptom was present or not. Overall, the presence of fatigue (64.7%), depression (39.1%), fear of complications of the LT (34.6%) and worries about the impact of the LT on the family situation (30.8%) were most frequently noted. Reported frequencies of the presence of pruritus, fever, fear of complications and complication-related treatment, fatigue, worries about the impact of the LT on the family situation and jaundice were not statistically different between patients with (*n*=50) or without (*n*=83) biliary strictures ($0.23 < p < 0.82$). However, decreased appetite was significantly more often reported in relation to biliary strictures (34.7% vs. 15.7%, $p<0.02$). Odds ratio for decreased appetite was 2.86 [CI 1.24 – 6.59]. Furthermore, depression was reported in 50% of the patients with biliary strictures, whereas patients without biliary strictures reported depression in 32.5% of cases (Odds ratio 2.07, CI [1.01 – 4.26], $p<0.05$). When AS and NAS were evaluated separately, in each subgroup similar frequencies of depression were found as in the combined cohort, namely 45% and 50% respectively.

In general, the presence of disease- and treatment-related worries may contribute to the high frequency of depression in patients after LT. This was expressed by patients with depression reporting higher frequencies of fear of complications

(65.4% vs. 14.8%, $p<0.001$), fear of complication-related treatment (33.3% vs. 3.7%, $p<0.001$) and worries about the impact of the LT on the family situation (59.6% vs. 12.3%, $p<0.001$), as compared to patients without depression. The character of depression appeared highly comparable between patients with or without biliary strictures as the frequencies of reported disease- and treatment-related worries that contributed to the depression did not differ between the groups ($0.61 < p < 0.84$).

The hindrance on usual activities of patients who reported symptoms on the LDSI was compared in relation to biliary strictures. Even though the incidence of worries about the impact of LT on the family situation was not different in patients with or without biliary strictures, patients with biliary strictures reported significantly more hindrance of this symptom (Odds ratio 13.9, CI [1.6 – 121.4], $p=0.005$). Hindrance of symptoms other than worries about the family situation did not differ between the groups.

Discussion

The results of the present study reveal an impaired health-related quality of life in patients after LT compared to patient-provided controls. Patients with or without biliary strictures reported similar scores on SF-36 and MFI-20, but more often symptoms of anxiety and depression, especially in the first years, on both the EQ-5D and LDSI questionnaires.

In the last decades, patient and graft survival after LT have increased significantly, mainly as a result of surgical and medical advancement. Focus on outcomes is therefore shifting towards patients' well-being and QOL. Prior studies have demonstrated an improvement in various dimensions of HRQOL when posttransplantation patients and equivalent waitlisted patients were compared.[3;23] Domains associated with physical health were most frequently affected in decompensated cirrhotic patients preLT, and these were also the domains with the greatest improvements after transplantation.[3;24;25] Other studies evaluated changes after LT compared to the patients' own preoperative status: improvement was found especifically in the subscales of personal functioning, social functioning and general health.[2;4].

However, even though an important improvement of HRQOL is achieved both shortly after LT and after a long-term (>10 years] follow-up, transplanted patients still scored significantly lower as compared to the general population. This is particularly the case on the subscales physical functioning, social functioning and general health.[5;6;26] The present study confirmed these differences between patients and their controls, even though our patients have been evaluated with a considerable follow-up (median 5.0 years after LT), whereas most HRQOL-affecting complications can be expected within the first years after transplantation. Some socioeconomic factors, such as unemployment and comorbidity, are generally related to worse HRQOL scores and may be more frequent in a group of transplanted patients. [7;19;26] Therefore in the present study, all continuous scores were adjusted for several predictors that are associated with HRQOL, i.e., age, gender, unemployment, marital status and comorbidity. Adjusting HRQOL scores for personal and socioeconomic factors associated with worse HRQOL is important to strengthen the result of a study using HRQOL instruments, but may have a greater effect on patients than on controls. For example, comorbidity was strongly associated with mean HRQOL scores on all domains of the SF-36 and MFI-20 in the multiple linear regression analysis. As 69% of patients and only 39% of controls reported at least one comorbidity that negatively affected HRQOL, mean scale scores of patients may be more affected by adjustment for this factor than their controls. In addition, unemployment (patients 76.1%, controls 49.3%) was associated with worse HRQOL scores on all subscales of MFI-20 and the subscales of (role limitations due to) physical functioning, vitality and general mental health on the SF-36 (p<0.03). The adjustment for multiple factors may also explain the relatively low mean scale scores on the SF-36 and high scale scores on the MFI-20 for patients in this study.

The present study is the first to focus on HRQOL of patients with biliary strictures after LT. Anastomotic and non-anastomotic biliary strictures are among the most frequent biliary complications after LT. The development of AS is generally accepted to be caused by a higher donor age, local ischemia and/or surgical techniques, whereas NAS is thought to be the result of a complex mechanism, including pretransplant status of the graft, immunological factors, ischemia-reperfusion injury -including donation after circulatory death- and bile salt toxicity.[9;10;27] Even though pathophysiological mechanisms of both types of biliary strictures are somewhat different, clinical presentation is very similar. A substantial number of

patients with biliary strictures report clinical symptoms, such as pruritus, cholangitis and jaundice, which may negatively affect HRQOL scores. Furthermore, the presence of biliary strictures is associated with repeated cholangitis and hospital admissions.[28;29] A recent study demonstrated that the presence of pruritus in patients with primary sclerosing cholangitis (PSC, a disease with biliary strictures) was associated with worse HRQOL scores on the majority of SF-36 subscales.[30] As the intensity of pruritus increased, mean scores of the SF-36 decreased. In the present study mean scale scores of the SF-36 did not differ between patients with or without biliary strictures requiring treatment. This is in accordance with the non-transplant results described by Gotthardt et al. In that study the presence of a dominant biliary stricture was not associated with impaired QOL scores on the SF-36 in PSC patients.[30] The presence of biliary strictures is also associated with frequent invasive treatment procedures. Management of anastomotic strictures consists of repeated endoscopic or percutaneous dilatation and/or stenting, and in some cases surgical revision of the anastomosis including Roux-and Y hepaticojejunostomy or even re-LT. Non-anastomotic strictures are considered the most challenging biliary strictures because of their diffuse character and resistance to therapy. Some strictures can successfully be treated by endoscopic or percutaneous dilatation and stenting, however retransplantation is required in 3-18% of cases.[1;13;28;31] Endoscopic treatment can have adverse effects like postprocedural pancreatitis, cholangitis or bleeding.[14] The similar results on the SF-36 and MFI-20 with or without biliary strictures may be due to the fact that with treatment cholestasis had improved in most patients. Indeed, in patients with a follow-up ≤ 4 years after LT, when more patients were still under treatment, all mean scale scores of the SF-36 were lower in patients with biliary strictures, though not significantly. This interesting observation should be further elaborated upon by increasing the sample size in this subgroup in future studies.

Patients with biliary strictures reported a higher incidence of anxiety and depression on both the EQ-5D and LDSI. Anxiety and depression as important contributors to low HRQOL scores in LT recipients have previously been described.[32-34] It was recently found that in our country anxiety and depression, as well as posttraumatic stress, are frequent both short-term and years after LT, while anxiety and depression are also common on the waitlist for LT.[35-37] The current study demonstrated that in patients with posttransplant biliary strictures the incidence of anxiety and depression appears to be higher in the first years after LT. Miller et al. also

reported the importance of evaluating patients before and after transplantation for anxiety and depressive symptoms, as pre-transplant anxiety scores predicted posttransplant anxiety and there was a trend towards predicting posttransplant depression.[33] Therefore adequate patient education and counselling is mandatory, taking patients' worries and illness perception into account. Regular screening for symptoms of depression may detect psychological issues and lead to adequate treatment in an early stage to improve HRQOL.

This study has some limitations: The approach taken to select the health control group might have introduced some selection bias. On the other hand with this approach controls with the same age from the same background were selected. The number of the two groups was unbalanced. However, no differences were detected between patients with or without an own control. The samples are not large. Further validation in other centers is needed for generalizability of findings beyond the setting where the study was conducted. In many patients treatment for biliary strictures had finished at the time of these measurements with normalization of liver enzymes in half of these patients. We tried to adjust for this by separately analyzing the first four years after transplantation when most of these treatments are performed. On the SF-36, mean scale score of general mental health did not differ between patients with or without biliary strictures. However, mental health on the SF-36 is measured on a six-point scale, focusing on how frequent symptoms were present during the last 4 weeks. Therefore, the SF-36 is more a reflection of the impairment of mental health, whereas the EQ-5D and LDSI measure solely the presence of symptoms of depression and anxiety. Unfortunately, the Hospital Anxiety and Depression Scale questionnaire was not used in this study, as this may confirm the results found on the EQ-5D and LDSI, and this certainly should be done in future studies.

In conclusion, our data show that HRQOL scores remain lower in patients after LT as compared to their matched, healthy controls, even after long-term follow-up. This may partially be due to lower employment rates and more comorbidity in patients after LT. Patients with biliary strictures tend to have a lower HRQOL in the first years after LT. Even though this trend improves after long-term follow-up, patients with biliary strictures reported more symptoms of anxiety and depression. Disease and treatment-related worries may contribute to the high frequency of depressive symptoms in patients after LT, and should therefore be addressed during consultations.

Acknowledgements

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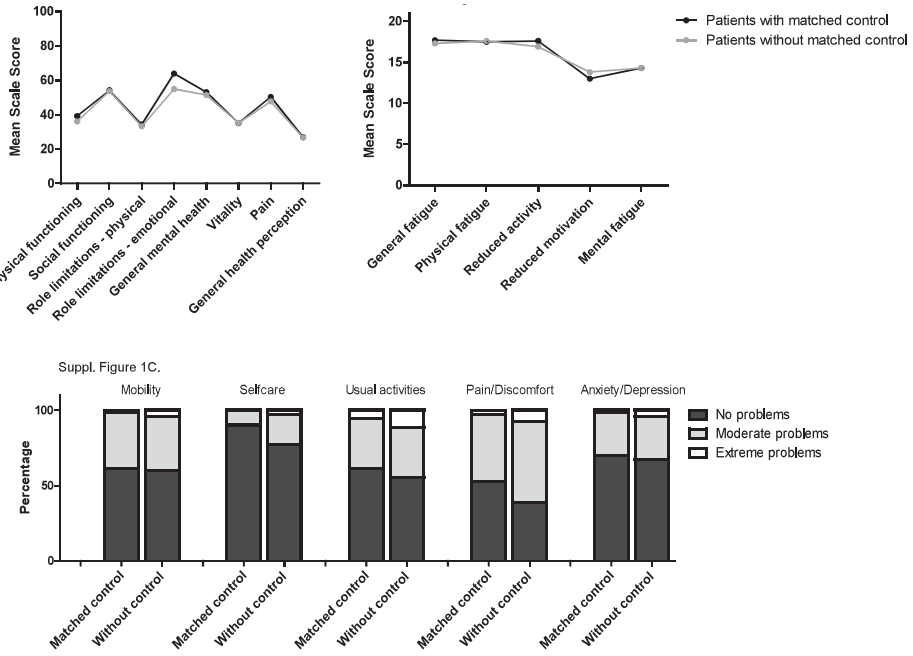
Supplemental data



Supplemental Figure 1. Mean scale scores on the Short-Form 36 (1A) and Multidimensional Fatigue Index-20 (1B) of patients with (n=71) versus without (n=71) a provided matched control subject ($0.08 < p < 0.97$). Reported frequencies on the EQ-5D (1C) in the categories 'no problems', 'moderate problems' and 'extreme problems' for patients with and without a matched control subject. Frequencies did not differ between the groups ($0.08 < p < 0.60$).

Chapter 9

Summarizing discussion

The occurrence of non-anastomotic biliary strictures (NAS) is a troublesome complication after orthotopic liver transplantation (OLT).[1] Strictures located in the biliary tree may lead to cholestasis, which can result in clinical symptoms, e.g., pruritis, fever from bacterial cholangitis and jaundice. In case of inadequate treatment -e.g. due to difficult to reach strictures- persistent cholestasis may lead to graft failure and retransplantation. Re-OLT for NAS is required in up to 18% of cases according to the literature.[2-4] Even though the complete pathophysiological pathway that leads to NAS development is unclear, several risk factors are known. Among those risk factors are ischemia-reperfusion injury, immunological risk factors and pretransplant status of the graft.

Ischemia-reperfusion injury and NAS

It has long been known that ischemia-reperfusion injury (IRI) is related to NAS development. The first reports to describe this association noticed an increased incidence of biliary strictures after the presence of a post-transplant hepatic artery thrombosis.[5;6] Furthermore, it has been described that grafts that are exposed to more severe ischemic injury, e.g., after a procedure with long ischemia times, NAS occurred more frequently.[7-10] Ischemia times are therefore kept as short as possible. However, logistics often dictate the ischemia times. Also, because of shortage of suitable donor organs, grafts donated after circulatory death (DCD) are increasingly used. These grafts are, in contrast to grafts from donation after brain death (DBD), exposed to more severe ischemic injury due to an additional donor warm ischemia time (DWIT) in which metabolic processes shift from aerobic to anaerobic. Numerous reports have confirmed the association between NAS and DCD.[11-14]

The degree of IRI is clearly visible in liver biopsies, but is also reflected by the peak alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in the first post-operative week, of which peak ALT is considered the most sensitive. In **chapter 2** we analyzed the relationship between peak ALT within the first 7 days after OLT and the development of NAS in a cohort of 399 DBD livers and 97 DCD livers from two transplantation centers, i.e., the Leiden University Medical Center and University Medical Center Groningen. A log rank test was performed to determine the optimal cutoff-value for peak ALT. Using the calculated cutoff-value, mild IRI was defined as a peak ALT of <1300 IU/L, whereas a high peak ALT of ≥ 1300 IU/L was considered severe IRI. Median peak ALT was significantly higher

after OLT using DCD donors than after DBD-OLT. After DCD-OLT, severe IRI was strongly and independently associated with NAS development, as after a peak ALT of ≥ 1300 IU/L NAS developed in 46% of cases, compared to 10% of cases with mild IRI. Four-year cumulative incidence of NAS development was 49.5% in case of severe IRI, compared to 11.3% when mild IRI occurred. This association was also found when the cohorts of the two transplantation centers were analyzed individually. After DBD-OLT, the incidence of NAS was 13% and no association could be found between peak ALT and NAS development after DBD-OLT. Interestingly, the incidence of NAS after DCD-OLT with mild IRI was similar to the incidence after DBD-OLT. These findings imply that reducing IRI after DCD-OLT to the extent that peak ALT is below 1300 IU/L will significantly reduce the incidence of NAS to the incidence of DBD-OLT. Furthermore, peak ALT can be used to classify patients as high-risk or low-risk for developing NAS, which may be useful to include patients in a more intensive follow-up program. However, the presence of NAS after DBD-OLT and DCD-OLT with only mild IRI, also shows that other factors than IRI are also responsible for NAS development.

Donor specific antibodies and NAS development

Immunological factors are also recognized to be important in NAS development, especially those factors related to hepatic IRI. As a result of ischemia, damage-associated molecular patterns (DAMPs) are released which leads to the activation of Kuppfer cells, dendritic cells, T cells and neutrophils, and subsequently to the release of chemokines, cytokines, matrix metalloproteinases (MMPs) and reactive oxygen species (ROS).[15-17] This sterile immune response ultimately results in a severe tissue damage and an upregulation of human leukocyte antigen (HLA) class molecules on the biliary epithelium.[18-20] In general, the presence of donor-specific alloantibodies (DSA) against HLA molecules is associated with (hyper)acute rejection and shorter graft survival after organ transplantation.[21-24] However, it is unknown whether DSA against the upregulated HLA molecules are also related to increased injury of the biliary epithelium after IRI. In **chapter 3** we evaluated the role of preformed and de novo DSA in the pathogenesis of NAS in patients after ABO-compatible OLT. Therefore, we determined the presence of HLA class I and class II DSA in pre-OLT and 12 months post-OLT serum samples of patients with and without NAS. A time-dependent analysis was performed to assess a possible relation between DSA formation and NAS development. A longitudinal analysis was performed to determine the timeline of DSA formation. Overall, preformed

DSA (mainly class I DSA) were detected in 10% of the patients. Preformed DSA frequently disappeared after OLT, probably due to absorption in the liver. One year after OLT, 21% of patients generated de novo DSA. In the vast majority, the newly developed antibodies were directed against the HLA class II (HLA-DQ and HLA-DR) antigens of the donor. Neither preformed DSA nor DSA generated de novo within the first year after OLT were associated with an increased risk for NAS development. In fact, longitudinal analysis revealed that, in NAS patients, de novo DSA generally developed after the diagnosis of NAS. Similar results were found when class I and class II DSA were analysed individually. However, time-dependent analysis revealed that both NAS as well as de novo class II DSA are independent risk factors for graft loss after OLT. No association was found between de novo class I DSA and graft loss. Graft loss after DSA formation is probably the result of DSA that bind to endothelial cells in the portal triads leading to complement activation. DSA against class II HLA are probably more capable of initiating a clinically relevant immune response than class I DSA. This can possibly be explained because class II antigen presenting cells are abundant in the liver.

Matrix Metalloproteinases and NAS development

As previously mentioned, IRI may lead to a massive release of matrix metalloproteinases (MMPs), especially MMP-9. MMP-2 and MMP-9 (both gelatinases) are capable of degrading different collagen types, of which mainly type IV collagen may be important because this type of collagen is present in basement membranes. Damage to the basement membrane of the bile ducts may produce irreversible injury.[25-28]

The activity of MMPs is regulated by tissue inhibitors of matrix metalloproteinases. Several genetic polymorphisms in the promotor region of MMP-2, MMP-7, MMP-9 and their associated TIMPs are known to alter the mRNA transcription of these MMPs, which may lead to a reduced degradation process of collagen type IV. **Chapter 4** therefore evaluates the risk of NAS development as a results of genetic polymorphisms in the MMP-2, MMP-7 and MMP-9 genes, as well as the TIMP-1 and TIMP-2 genes. Genetic variations in both donors and recipients were determined by extracting DNA from peripheral blood and subsequently perform a restriction fragment length polymorphism (RFLP) polymerase chain reaction (PCR) or tetra primer amplification refractory mutation system (ARMS) PCR. We did not find genetic polymorphisms in either donor and/or recipient in the assessed

MMPs and TIMPs to be associated with a higher incidence of NAS. In addition, NAS patients did not have significantly more mismatches between donor and recipient in any of the evaluated single nucleotide polymorphisms (SNPs). However, the absence of a relation in this study may not completely exclude a role of MMPs and/or TIMPs in NAS development. Possible explanations for our negative findings are a lack of knowledge of the exact (local) function of MMPs, TIMPs and the evaluated SNPs, compensatory mechanisms which prevent alterations in serum MMP and TIMP levels in patients with genetic variations and interactions with other risk factors that alter the susceptibility for NAS. Additional studies to determine the exact functional mechanism should therefore be performed.

Diagnosis and follow-up of NAS

Apart from the challenges to unravel the pathophysiological mechanisms of NAS development, are the difficulties to diagnose and treat NAS in an early stage. Currently, direct cholangiography by endoscopic retrograde cholangiopancreatography (ERCP) or percutaneous transhepatic cholangiography (PTC) is the golden standard for diagnosis. However, these procedures are associated with major complications.[29;30] Therefore, we evaluated magnetic resonance cholangiopancreatography (MRCP) as a non-invasive tool to monitor the presence or absence of NAS after OLT. **Chapter 5** describes a new scoring model, the Leiden Biliary Stricture Classification (LBSC), which is a modification of a validated scoring model for ERCP/PTC to detect the severity of biliary strictures in patients with primary sclerosing cholangitis. Patients with and without NAS from two liver transplantation centers in whom MRCP and ERCP/PTC (as reference) were performed within less than 6 months were assessed. The MRCP was evaluated by two independent, blinded abdominal radiologists. The presence, localization and severity of biliary strictures was noted and categorized into 4 different hepatobiliary regions. For the non-anastomotic regions a maximum of 15 points could be obtained. The optimal cut-off point, as determined using a ROC curve, was ≥ 3 points. When this cut-off point was applied to the radiologists mean scores, a sensitivity of 98%, specificity of 65%, positive predictive value of 87% and negative predictive value of 92% was achieved. MRCP using the LBSC is thus a reliable tool to detect or exclude NAS after OLT. This is a useful finding, as MRCP can be used to noninvasively diagnose or exclude NAS and plan the optimal treatment prior to direct cholangiography, thereby preventing unnecessary procedures. MRCP performance was better in the evaluation of the intrahepatic than of the extrahepatic bile ducts. The interobserver agreement for

grading biliary strictures severity was poor, indicating that the value of MRCP with current techniques for follow-up of NAS progression is limited. However, for diagnosing or excluding NAS an MRCP with application of the LSBC proved very useful.

Transient Elastography (TE) is an upcoming non-invasive tool in the follow-up of patients after OLT. TE can reliably measure liver fibrosis, by assessing liver stiffness.[31] Even though normal TE values have not been established, its use has especially proven valuable to monitor the recurrence of hepatitis C virus (HCV). Because it is yet unknown whether NAS is associated with fibrosis of the transplanted liver, in **chapter 6** we assessed TE values in a cohort of patients with and without NAS. Interestingly, liver stiffness was significantly increased in patients with NAS with persistent cholestasis after treatment. Patients with NAS in whom cholestasis had resolved with dilatation and/or stenting had TE values similar to those without NAS or cholestasis. The relation persisted when patients with HCV after OLT were excluded from the analysis. No association between increased TE values and the presence of anastomotic biliary strictures (AS), recurrence of HCV, type of donation and previous episodes of rejection was found. However, there was a relationship of current TE values with peak-ALT in the first week after OLT as a marker of ischemia-reperfusion injury. Unfortunately, we could not find an optimal liver stiffness cut-off value for the prediction of NAS. In controls, median TE values were relatively stable over time. These data indicate that repeated TE may be helpful during follow-up of patients with and without NAS after OLT.

Treatment and outcome of biliary strictures

In **chapter 7** we focused on (longterm) outcomes of the cholangiographic treatment of biliary strictures after OLT. Biliary strictures located at the anastomotic site or in the choledochal or hepatic ducts can usually be treated with an endoscopic procedure in which a balloon dilatation and/or the placement of a stent is performed. In case of more intrahepatic strictures, PTC with drainage is usually the treatment option of choice.

We found that, in the majority of patients with AS, cholangiographic abnormalities were already present in the first weeks after OLT. Successful treatment of AS was achieved in 45.5% of the patients that were treated with ERCP only and 3% of patients that were treated with PTC only. Cholangiographic success rate was higher in patients who received early treatment, whereas the simultaneous

presence of other biliary complications, i.e., NAS or anastomotic leakages was associated with a worse outcome. Patients with endoscopic success required a median of 2.5 procedures.

NAS was found to develop slightly later than AS. Treatment of NAS in the majority of cases consisted of combined balloon dilatation and stent placement. However, even though an aggressive approach with multiple stenting was performed in most patients, cholangiographic success rate in NAS was lower than in AS, i.e., 31.5%. A higher success rate was found for extrahepatic than for intrahepatic biliary strictures. Furthermore, a trend was found towards a lower success percentage in patients with early presentation, probably due to a more severe character of the biliary strictures. Not only was success rate lower in patients with NAS as compared to AS, patients also required more cholangiographic procedures. In general, complications after cholangiographic procedures were rare. This study therefore indicates that timely cholangiographic treatment is important in all cases to prevent cholangitis and progression to graft failure.

From the literature it is known that perceived quality of life in general improves after OLT for end-stage liver disease. Even though biliary strictures may partly be asymptomatic and complications of their treatment are rare in our center, it is not unthinkable that their presence and related interventions may have an effect on patients' perception of general well-being and quality of life (QOL). Therefore, **chapter 8** focussed on differences in QOL in patients with and without biliary strictures. In addition, patients after OLT were compared to matched healthy controls to evaluate the impact of a liver transplantation. In a cross-sectional case-control study, a general background questionnaire and four validated QOL questionnaires were evaluated.

Overall, we found that QOL was significantly worse in post-OLT patients, as compared to matched healthy controls. Patients after OLT with and without biliary strictures reported similar QOL. In addition, no difference was found when AS and NAS were evaluated separately. Because biliary strictures usually occur within the first year after OLT, a subgroup analysis of patients with a follow-up of ≤ 4 years was performed. In this subgroup, patients with biliary strictures had lower QOL scores in all domains, although this was, probably due to a small sample size, not statistically significant in any of the subscales.

However, patients with biliary strictures reported significantly more anxiety and depression on both the EQ-5D and Liver Disease Symptom Index questionnaires. Surprisingly, worries about biliary strictures and invasive treatment procedures did not negatively affect QOL scores, as the frequencies of reported disease- and treatment-related worries that contributed to depression did not differ between patients with and without biliary strictures. The results indicate that reducing anxiety and depression should be an important target in patient-care. This could possibly be achieved by adequate patient education and counselling during consultations.

General conclusion and future perspectives

Orthotopic liver transplantation is considered the only definite treatment option for end-stage liver failure. In the past decades, the many improvements regarding surgical technique, immunosuppression and post-transplantation care have made OLT a treatment with very acceptable patient and graft survival.[11;12;32] Even though post-transplant complications are to be taken into account, in patients with advanced chronic liver disease the benefits of OLT often far outweigh the risk of complications.

This thesis describes several aspects and the clinical importance of one of the most frequent complications after OLT, i.e., the development of biliary strictures. Biliary complications are known to have a significant clinical and financial impact on post-OLT care.[33] We attempted to facilitate diagnosing their presence by proposing the use of non-invasive screening tools, i.e., MRCP and transient elastography. We also demonstrated that most biliary strictures can be well-treated, although NAS seems to be more difficult to treat and more therapy-resistant than AS. Much is known about the development of anastomotic biliary strictures and therefore is seems that currently the greatest challenge is unravelling the pathways that lead to NAS formation. This knowledge is essential to change donation procedures in order to prevent NAS development after future OLTs. Their multifactorial etiology makes it difficult to have a complete insight in this pathway, but with the current knowledge risk factors that seem to have the greatest contribution in this process are immunological factors resulting from ischemia and reperfusion.[15-17;34-36]

This thesis confirmed that, even though strict protocols for donation after cardiac death are applied in the Netherlands, the incidence of NAS is still significantly

higher after this type of donation, than after donation after brain death.[12] This is probably due to the additional warm ischemia time leading to more severe IRI, but other factors may also contribute to this process. We have shown that severe IRI, as reflected by a peak ALT above 1300 IU/L, is associated with NAS development after DCD-OLT, but not after DBD-OLT. The main focus in the future should therefore be on improving the quality of DCD grafts. Following the current results a peak ALAT below 1300 IU/l may serve as a surrogate endpoint in studies aiming at prevention of NAS in DCD OLT. This can possibly be achieved by keeping ischemia times as short as possible and by a careful selection of grafts, but the greatest improvement in graft quality can be expected from machine perfusion.[37;38] This technique can likely not only improve the quality of donated grafts, but may also make grafts suitable for donation that would otherwise be discarded, thereby increasing the donor pool and decreasing waitlist mortality. The optimal conditions for machine preservation need yet to be established and currently several studies are ongoing on this subject. One of the most important questions that needs to be answered is whether machine perfusion should be normothermic, hypothermic or a combination thereof. However, excellent results have been described so far in pilot studies, and the expectation is that machine preservation will prove useful for prevention of NAS in further studies.[37;38]

Another possibility for decreasing the incidence of biliary complications theoretically might be improvement of the allocation process. However, currently, no screening tool is available to predict the risk of biliary strictures post-OLT, but recently the Eurotransplant Donor Risk Index was introduced as a valuable tool to predict the outcome of liver transplantations in general.[39;40] Since immunological factors are also important in the development of NAS, it would be interesting to see whether this could be incorporated in the allocation process. Unfortunately, so far we could not find an association between immunological factors of donor and recipient (i.e., donor specific antibodies and mismatches in SNPs of matrix metalloproteinases) and NAS formation. However, it is not unthinkable that matching donor and recipient for other (yet unknown) factors may reduce the incidence of NAS.

Until dramatic decreases in the incidence of biliary strictures can be achieved, high risk patients should be monitored and screened on a regular basis. We demonstrated that magnetic resonance pancreatocholangiography may have an

important role in non-invasively detect or exclude the presence of biliary strictures. We have also shown that early treatment can dissolve biliary strictures in many cases and this may possibly prevent repeated episodes of cholangitis, hospital readmissions and progression to graft failure. Furthermore, patients with biliary strictures report increased symptoms of anxiety and depression, which can hopefully be prevented by timely and adequate treatment.

In conclusion, this thesis demonstrated that the development, diagnosis and treatment of biliary strictures remains a complex issue and should be taken into account in future studies related to OLT.

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Chapter 10

Nederlandse samenvatting

Levertransplantatie is momenteel de standaard behandeling voor eindstadium leverfalen. Het Leids Universitair Medisch Centrum (LUMC) voert in een programma levertransplantaties uit sinds 1992, na in 1966 de eerste levertransplantatie in Nederland te hebben verricht. De grote verbeteringen op het gebied van pre-operatieve, peroperatieve en postoperatieve kennis en technieken hebben ertoe geleid dat de 1- en 3-jaars overleving na levertransplantatie in ons land inmiddels ruim 90% en 80% bedraagt. Het accent verschuift nu langzaam van het overleven van de operatie en de periode direct daarna richting het verbeteren van de lange-termijn overleving, het minimaliseren en beter behandelen van complicaties en het verbeteren van de kwaliteit van leven.

De keerzijde van het succes van levertransplantatie is dat de laatste jaren steeds meer patiënten met eindstadium leverfalen in aanmerking komen voor een levertransplantatie. Het relatieve tekort aan donoren neemt hierdoor verder toe en heeft ertoe geleid dat de wachtlijst voor een donorlever, en daarmee ook de mortaliteit op de wachtlijst, aanzienlijk is toegenomen. Een potentiële oplossing hiervoor is het uitbreiden van het donoraanbod, bijvoorbeeld door de recent aangekondigde wijziging van de donorwet, maar ook door organen te accepteren van de zogenoemde 'extended criteria donors' (ECD), bijvoorbeeld van oudere donoren. Omdat deze levers vaak van iets mindere kwaliteit zijn, dienen deze organen met zorg geselecteerd te worden, waarbij gestreefd wordt naar een optimale uitkomst van de transplantatie, met een zo klein mogelijk risico op complicaties. Met de recent beschikbaar gekomen Eurotransplant Donor Risk Index (ET-DRI) kan, op basis van enkele kenmerken van de donor en de te doneren lever, ingeschat worden wat de kans is op orgaanfalen na de transplantatie. Dit kan helpen om een acceptabele afweging te maken of het orgaan geschikt is voor donatie.

Sinds 2001 worden in Nederland vanwege afname van het aantal hersendode (DBD) donoren levers van donoren met een circulatiestilstand (DCD) getransplanteerd binnen een streng protocol. Een belangrijk verschil tussen DCD en DBD donatie is dat er bij DBD donatie nog een intacte circulatie is, waardoor de lever nog voorzien wordt van zuurstofrijk bloed. Indien DCD levers echter goed geselecteerd worden, onder andere door slechts categorie III donoren te selecteren volgens de in 2001 in gebruik genomen 'Maastricht criteria', zijn deze organen goed te gebruiken voor donatie, waarbij een vergelijkbare patiëntoverleving na transplantatie bereikt kan worden als bij DBD levers.

Voor een succesvolle levertransplantatie is het van belang om de conditie van donororganen voorafgaand aan de transplantatie te optimaliseren en de ischemische schade tot een minimum te reduceren. Ischemie-reperfusie schade (IRI) ontstaat wanneer de lever (tijdelijk) niet of onvoldoende van zuurstofrijk bloed wordt voorzien. De preservatie en het transport van de donorlever dient daarom efficiënt te verlopen, zodat de preservatietijden, zoals de koude ischemie tijd (CIT), minimaal zijn. In het geval van DCD donoren, is er -als gezegd- een additionele ischemie tijd die optreedt gedurende de circulatiestilstand (donor warme ischemie tijd (DWIT)). DCD levers zijn daarom gevoeliger voor IRI dan levers van hersendode donoren.

Machineperfusie is een recent beschikbaar gekomen veelbelovende techniek om de preservatie van met name DCD organen te optimaliseren. In verschillende humane studies zijn er sterke aanwijzingen dat de kwaliteit van de donorlever met deze techniek aanzienlijk verbetert, waarbij het in enkele gevallen mogelijk bleek organen die aanvankelijk niet transplantabel werden geacht, toch in aanmerking te laten komen voor transplantatie. Door toenemend gebruik van machinepreservatie zal in de toekomst het succespercentage van levertransplantaties waarschijnlijk nog verder stijgen.

Galwegcomplicaties na levertransplantatie

Ondanks de goede patiëntenoverleving blijft een levertransplantatie een grote en risicovolle operatie, waarbij complicaties kunnen optreden. De ontwikkeling van galwegstricturen (vernauwingen) na een levertransplantatie is een relatief veelvoorkomende complicatie. Galwegstricturen worden, op basis van hun lokalisatie, ingedeeld in anastomotische stricturen (AS) die zich op de chirurgische naad bevinden en niet-anastomotische stricturen (NAS) die zich in de hoger gelegen galboom bevinden, minimaal 1 cm boven de chirurgische naad.

Anastomotische galwegstricturen ontstaan volgens de literatuur in 9 - 38% van de patiënten en zijn vaak het gevolg van een chirurgische complicatie waarbij er een gallekkage of verlittekening is ontstaan ter plaatse van de chirurgische naad in de ductus choledochus. Ook is het ontstaan van AS geassocieerd met een hogere leeftijd van de donor. De chirurgische naad is relatief makkelijk te benaderen met een endoscopische procedure, waardoor vaak een succesvolle behandeling mogelijk is.

Het ontstaan van niet-anastomotische galwegstricturen is veel complexer, omdat meerdere factoren hieraan bijdragen, zoals de kwaliteit van het donororgaan voorafgaand aan transplantatie en ischemische, immunologische en toxische factoren. Het vaak diffuse karakter van de niet-anastomotische galwegstricturen maakt dat deze ook moeilijker te behandelen zijn. Over het succes van endoscopische behandeling van NAS is weinig bekend.

Ischemische schade en niet-anastomotische galwegstricturen

Al geruime tijd is bekend dat IRI geassocieerd is met het ontstaan van NAS. Daarnaast is bekend dat, vanwege de ischemische schade die ontstaat tijdens de DWIT, de incidentie van NAS na een DCD levertransplantatie hoger is dan na een DBD levertransplantatie. Om de mate van ischemische schade vast te stellen kunnen de leverenzymen ALT en AST vervolgd worden. Postoperatief zullen deze leverenzymen stijgen als gevolg van het afsterven van hepatocyten tijdens de DWIT, CIT en reperfusiefase, waarbij er in de eerste drie dagen -meestal binnen 24 uur- na levertransplantatie een piekwaarde bereikt wordt. Deze piekwaarde geeft een goede reflectie van de mate van IRI. In **hoofdstuk 2** wordt de relatie beschreven tussen de piek ALT en de incidentie van NAS na DCD levertransplantatie. In een cohort van 399 DBD en 97 DCD levertransplantaties afkomstig uit het Leids Universitair Medisch Centrum en het Universitair Medisch Centrum Groningen werd de optimale cut-off waarde van de piek ALT als voorspeller voor het ontstaan van NAS na een DCD levertransplantatie bepaald. Deze waarde werd vastgesteld op 1300 IU/L, waarbij een waarde onder 1300 IU/L werd beschouwd als milde IRI, en een waarde boven 1300 IU/L als ernstige IRI. In het cohort van DCD levertransplantaties bleek ernstige IRI een sterke en onafhankelijke voorspeller voor het ontstaan van NAS, waarbij NAS zich ontwikkelde in 46% van de patiënten met een piek ALT ≥1300 IU/L en slechts in 10% van de patiënten met een piek ALT ≤1300 IU/L. De 4-jaars cumulatieve incidentie van NAS ontwikkeling was 49.5% in het geval van ernstige IRI en 11.3% van de patiënten met milde IRI. De gevonden associatie bleek ook aanwezig wanneer de twee transplantatiecentra's afzonderlijk van elkaar werden geanalyseerd. Na DBD levertransplantaties was de incidentie van NAS 13%, waarbij er geen relatie gevonden werd met de hoogte van de piek ALT. Deze resultaten suggereren dat het verlagen van IRI na DCD levertransplantaties tot een piek ALT van ≤1300 IU/L de incidentie van NAS significant kan verlagen, waarbij mogelijk vergelijkbare resultaten als na DBD levertransplantatie bereikt kunnen worden. Daarnaast kan de piek ALT gebruikt

worden om een risico-inschatting te maken voor de ontwikkeling van NAS. Dit zou wellicht een intensiever follow-up programma rechtvaardigen in de hoog-risico patiëntengroep. Ook kan de piek ALT waarde gebruikt worden als surrogaatparameter in studies die het doel hebben de incidentie van NAS te verminderen.

Donor-specifieke anilichamen en NAS ontwikkeling

Het ontstaan van NAS bij patiënten met slechts geringe IRI suggereert ook dat andere factoren, zoals immunologische schade, een rol spelen in het ontstaan van NAS. Als gevolg van ischemische schade zullen zuurstofradicalen en ontstekingscellen, zoals de Kuppfercellen en neutrofielen, geactiveerd worden. De steriele immuunrespons die zo ontstaat zal er ook toe leiden dat er een upregulatie plaatsvindt van zowel klasse I als klasse II van het humaan leukocyten antigenen (HLA). De combinatie van HLA antigenen is voor iedereen vrijwel uniek, waardoor het afweersysteem van de ontvanger antistoffen (donor-specifieke antilichamen (DSA)) kan vormen tegen de lichaamsvreemde HLA antigenen die aanwezig zijn op de donorlever. De vorming van DSA na transplantatie is geassocieerd met acute en chronische afstotingsreacties en falen van het transplantaat. Ook in het galwegepitheel vindt een upregulatie plaats van HLA als gevolg van de ischemische schade tijdens transplantatie. In **hoofdstuk 3** werd daarom de rol van DSA in het ontstaan van NAS onderzocht. Hierover bestonden vrijwel geen gegevens. De aanwezigheid van DSA voorafgaand aan de transplantatie en 12 maanden na de transplantatie werd bepaald in serum van patiënten met en zonder NAS. In 10% van de patiënten waren DSA (met name klasse I) aanwezig voorafgaand aan de transplantatie. Echter, na transplantatie bleken deze DSA in het merendeel van de patiënten uit de circulatie te verdwijnen door absorptie van de lever. In de 12 maanden na transplantatie had 21% van de patiënten de novo DSA gevormd, dus na transplantatie, welke met name gericht waren tegen klasse II HLA. Het ontstaan van NAS bleek geen relatie te hebben met de aanwezigheid van DSA voorafgaand aan transplantatie of met de vorming van de novo DSA. Zowel het ontstaan van NAS als de vorming van de novo klasse II DSA waren onafhankelijke voorspellers voor het ontwikkelen van transplantaatfalen. Een mogelijke verklaring hiervoor die in de literatuur wordt genoemd, is dat DSA waarschijnlijk in staat zijn om te binden aan endotheelcellen in de lever, wat zou kunnen leiden tot activatie van het complementsysteem met op de lange termijn weefselschade en transplantaatfalen.

Matrix Metalloproteinases en het ontstaan van NAS

De activatie van Kuppfercellen en neutrofielen als gevolg van IRI leidt tot activering van de stellaatcellen en daarmee de productie en activatie van matrix metalloproteinases (MMPs), met name MMP-2 en MMP-9. MMPs zijn een groep eiwitklievende enzymen die een belangrijke rol spelen in het afbreken en hermodelleren van bindweefsel. MMP-2 en MMP-9 zijn in staat om collageen type IV af te breken, wat een belangrijk bestanddeel is van de basaalmembranen in de galwegen. De activiteit van MMPs wordt onder andere gereguleerd door tissue inhibitors of matrix metalloproteinases (TIMPs). Verschillende genetische varianten (single nucleotide polymorfismen, (SNPs)) in de promotor regio van MMPs en TIMPs zijn van invloed op de mRNA transcriptie, waardoor mogelijk de activiteit van MMPs, en dus de afbraak van collageen type IV, verminderd wordt. In **hoofdstuk 4** werd de relatie tussen het ontstaan van NAS en verschillende SNPs in de promotorregio van MMP-2, MMP-7, MMP-9 en de TIMP-1 en TIMP-2 genen onderzocht. Er werd geen relatie gevonden tussen genetische variaties in zowel donoren als ontvangers van een levertransplantatie en het ontstaan van NAS. NAS patiënten bleken ook niet vaker een mismatch te hebben tussen donor en ontvanger dan controles in de onderzochte SNPs. Wellicht zou het ontbreken van een associatie in de huidige studie echter verklaard kunnen worden door compensatoire mechanismen die voorkomen dat een genetische variatie leidt tot verminderde MMP activiteit of door interacties met andere risicofactoren die het risico op NAS ontwikkeling veranderen.

Diagnose en follow-up van NAS

Het vroegtijdig stellen van de diagnose NAS is een uitdaging, maar van essentieel belang om een tijdig goede behandeling te kunnen starten en daarmee ernstige complicaties te voorkomen. Een klinische verdenking op galwegstricturen ontstaat wanneer de patiënt symptomen van stase van galzouten (cholestase) ervaart, zoals jeuk, buikpijn, geelzucht of (herhaalde episodes van) galwegontstekingen. Deze symptomen leiden tot frequente ziekenhuisopnames. Als uiting van cholestase kunnen de leverenzymen bilirubine, alkalisch fosfatase (AF) en gamma glutamyl transferase (GGT) in het serum verhoogd zijn. Echografisch kunnen uitgezette galwegen zichtbaar zijn, al is dit vaak niet zo en kan de exacte lokalisatie van de galwegstricturen vaak niet worden vastgesteld met echografie. De definitieve diagnose wordt dan gesteld met endoscopische retrograde cholangio- en pancreaticografie (ERCP) of percutane cholangiografie met drainage

(PTCD). Hierbij worden de galwegen bereikt via een endoscoop of door direct aanprikken via de lever. Hoewel deze procedures als voordeel hebben dat er direct een behandeling kan worden gestart, zijn ze invasief en kunnen er soms ernstige complicaties optreden. Om onnodige invasieve procedures en complicaties te voorkomen is het daarom wenselijk eerst een goede screening uit te kunnen voeren met non-invasieve methoden, waarvoor de magnetische resonantie cholangio- en pancreaticografie (MRCP) zich goed lijkt te lenen. Ondanks het feit dat MRCP een goede methode is om de galwegen te visualiseren en er enige ervaring is met het detecteren van galwegcomplicaties, waren er tot op heden geen universele criteria voor het vaststellen van de diagnose NAS na OLT met behulp van MRCP. In **hoofdstuk 5** wordt daarom een nieuw scoringssysteem, de Leiden Biliary Stricture Classification (LBSC), voorgesteld om NAS betrouwbaar te kunnen aantonen of uitsluiten met MRCP. Patiënten na OLT met en zonder NAS, afkomstig uit het LUMC of het Erasmus Medisch Centrum (EMC) werden geïncludeerd als er zowel een ERCP/PTCD (gouden standaard) als een MRCP verricht waren binnen een tijdsperiode van 6 maanden. De MRCP werd beoordeeld door twee onafhankelijke, geblindeerde radiologen. De aanwezigheid, lokalisatie en ernst van de galwegstricturen werd beoordeeld en gescoord in 4 verschillende hepatobiliaire regio's. In het geval van NAS konden maximaal 15 punten verkregen worden. De optimale cut-off waarde werd vastgesteld op ≥ 3 punten. Door de resultaten van de MRCP en ERCP/PTCD met elkaar te vergelijken en deze cut-off waarde toe te passen werd een sensitiviteit van 98%, een specificiteit van 65%, een positief voorspellende waarde van 87% en een negatief voorspellende waarde van 92% bereikt. Indien gecombineerd met de LBSC kan een MRCP dus in betrouwbare mate de diagnose NAS vaststellen of uitsluiten. Deze non-invasieve methode kan daarom na OLT van vaste waarde zijn om de optimale behandeling te plannen en onnodige, risicovolle procedures te vermijden. Ook is MRCP op grond van bovenstaande zeer geschikt in studies die tot doel hebben de incidentie van NAS te verlagen. MRCP bleek betrouwbaarder de intrahepatische dan de extrahepatische galwegen te kunnen visualiseren. Het beoordelen van de ernst van de galwegstricturen bleek met de huidige MRCP techniek nog niet betrouwbaar en reproduceerbaar, waardoor MRCP nu nog niet geschikt is voor beoordelen van progressie van stricturen in de tijd, maar dit zal ongetwijfeld snel verbeteren met de snel voortschrijdende techniek.

Transiente Elastografie (TE) is een veelbelovende nieuwe techniek voor de follow-up van patiënten na een levertransplantatie. TE wordt gebruikt om via een elasticiteitsmeting de hoeveelheid littekenweefsel (fibrose) in patiënten met een chronische leverziekte te meten, bijvoorbeeld bij patiënten met een hepatitis C virus infectie. Referentiewaarden voor het bepalen van de mate van fibrose na een levertransplantatie zijn nog niet vastgesteld. In **hoofdstuk 6** werd met transiente elastografie aangetoond dat levers van patiënten met NAS en persisterende cholestase significant meer bindweefsel (leverfibrose) bevatten dan levers van patiënten zonder NAS of patiënten met NAS zonder cholestase (door bijvoorbeeld adequate behandeling). Er werd geen associatie gevonden tussen verhoogde TE waarden en AS, terugkerende hepatitis C infecties, IRI of episodes van afstoting. Dit suggereert dat de verhoogde waarden die gevonden zijn in patiënten met NAS het gevolg zijn van cholestase en in mindere mate van meer leverfibrose. In een controlegroep zonder NAS bleek bovendien dat de TE waarden stabiel blijven over de tijd, waardoor TE dus ook geschikt is voor de lange-termijn follow-up na levertransplantatie.

Behandeling en uitkomsten van galwegstricturen

In **hoofdstuk 7** werden de lange termijn uitkomsten van endoscopische of radiologische behandelingen van zowel anastomotische als niet-anastomotische galwegstricturen onderzocht. In het geval van AS bleek een succesvolle behandeling met ERCP en/of PTCD mogelijk in 60.5% van de patiënten. AS ontstaat relatief snel na de levertransplantatie, waarbij in het merendeel van de patiënten al binnen enkele weken afwijkingen in de galwegen zichtbaar waren. Een vroege behandeling (≤ 3 maanden) leidt tot een hoger succespercentage van endoscopische behandelingen dan late therapie. De gelijktijdige aanwezigheid van meerdere galwegcomplicaties was geassocieerd met een slechtere uitkomst van endoscopische behandeling. Zo werd bij patiënten met zowel AS als NAS slechts een succesvolle behandeling bereikt in 32.1%. Bij patiënten waarbij AS voorafgegaan werd door een lekkage op de chirurgische naad was het succespercentage 27.8%. Voor een succesvolle uitkomst waren mediaan 2.5 procedures vereist.

NAS ontwikkelt zich in het algemeen in een later stadium dan AS en is, mede gezien het vaak diffuse karakter en de ernst en moeilijke bereikbaarheid moeilijker behandelbaar. In vergelijking met AS werd niet alleen een lager succespercentage behaald (31.5%) met ERCP/PTCD behandeling, maar bleken hier ook meer

procedures (mediaan 5.5) voor nodig te zijn. Extrahepatische (dus buiten de lever gelegen) galwegstricturen zijn endoscopisch makkelijker benaderbaar dan intrahepatische (binnen de lever gelegen) galwegstricturen, wat de kans op een succesvolle behandeling vergroot. Deze studie onderschrijft het belang voor het vroegtijdig behandelen van galwegstricturen om ernstige complicaties en falen van de donorlever te voorkomen.

Het is bekend dat de kwaliteit van leven verbetert na levertransplantatie bij eindstadium leverfalen. Het is bovendien invoelbaar dat de impact van een levertransplantatie en de aanwezigheid van klinische symptomen van galwegstricturen van invloed zijn op de door patiënten ervaren kwaliteit van leven. Daarnaast spelen herhaalde ziekenhuisopnames, de angst voor progressie van de stricturen en de angst voor behandelprocedures en geassocieerde complicaties een belangrijke rol in het welbevinden van patiënten met galwegstricturen, ook indien deze stricturen asymptomatisch zijn. **Hoofdstuk 8** beschrijft in de eerste plaats de verschillen in kwaliteit van leven van patiënten na een levertransplantatie in vergelijking met gematchte gezonde controles. Hiervoor werden patiënten en controles gevraagd een algemene vragenlijst en 4 gevalideerde kwaliteit van leven vragenlijsten in te vullen. De door patiënten ervaren kwaliteit van leven was significant lager dan die van gezonde controles. Bovendien bleken patiënten met en zonder galwegstricturen een vergelijkbare kwaliteit van leven te rapporteren in alle domeinen van de SF-36 en de Multidimensional Fatigue Inventory vragenlijst. Omdat galwegstricturen met name voorkomen in de eerste jaren na levertransplantatie werd een subgroep analyse verricht van patiënten met een follow-up duur van ≤ 4 jaar. In deze subgroep analyse bleek de kwaliteit van leven lager in patiënten met galwegstricturen. Dit resultaat was echter vanwege de kleine groepsgrootte niet significant.

Patiënten met galwegstricturen rapporteerden echter op zowel de EQ-5D vragenlijst als de Liver Disease Symptom Index vragenlijst significant meer angst en depressie. Zorgen over de galwegstricturen en invasieve behandelprocedures hadden geen negatief effect op de kwaliteit van leven. Er was namelijk geen verschil in de gerapporteerde frequentie van ziekte- en behandelgerelateerde zorgen bij patiënten met en zonder galwegstricturen. Het lijkt echter van belang om gevoelens van angst en depressie altijd bespreekbaar te maken bij controlebezoeken op de polikliniek of tijdens ziekenhuisopnames. Adequate patiëntenvoorlichting met

extra aandacht voor angst en depressie zou zinvol kunnen zijn om de kwaliteit van leven te verbeteren, met name bij patiënten met galwegstricturen.

Conclusie en toekomstperspectief

Levertransplantatie is momenteel de enige behandeling voor patiënten met eindstadium leverfalen. De verbeteringen op het gebied van chirurgische technieken, immuunsuppressie en goede follow-up na transplantatie hebben geleid tot een zeer acceptabele patiëntenoverleving. Voor patiënten met een ernstige leverziekte wegen de voordelen van een levertransplantatie in het algemeen op tegen de kans op complicaties van de procedure.

Dit proefschrift beschrijft verschillende aspecten en de klinische relevantie van een frequent voorkomende complicatie na levertransplantatie, namelijk het ontstaan van postoperatieve galwegstricturen. Het proefschrift beschrijft ontwikkeling en validatie van non-invasieve methodes om patiënten te screenen op galwegstricturen en het diagnosticeren ervan te vergemakkelijken. Daarnaast werd aangetoond dat galwegstricturen vaak goed te behandelen zijn, alhoewel de behandeling van NAS vaak minder succesvol verloopt dan de behandeling van AS. Momenteel is het een grote uitdaging om het ontstaan van NAS te voorkomen, aangezien dit een multifactorieel proces is.

In dit proefschrift wordt bevestigd dat, hoewel er strikte protocollen zijn voor DCD donatie procedures, het voorkomen van galwegstricturen na OLT met deze vorm van donatie nog steeds hoger is dan na OLT met een lever na DBD donatie. Dit is zeer waarschijnlijk het gevolg van de additionele warme ischemie tijd die kan leiden tot ernstige IRI. Andere factoren zullen echter ook bijdragen aan het verhoogde risico op NAS na DCD levertransplantaties. We hebben aangetoond dat ernstige IRI, weergegeven door een piek ALT van ≥ 1300 IU/L, geassocieerd is met het vaker ontstaan van NAS na een DCD levertransplantatie. Deze associatie was niet aanwezig na een DBD levertransplantatie. Het verbeteren van de kwaliteit van DCD levers is daarom een belangrijk aandachtsgebied. Een dergelijke verbetering kan wellicht bereikt worden door korte ischemietijden en strenge selectie van de donorlevers. Goede resultaten kunnen daarnaast verwacht worden van de momenteel in onderzoek zijnde machinepreservatie. Deze techniek kan mogelijk de kwaliteit van aangeboden levers verbeteren, maar kan er waarschijnlijk ook toe leiden dat eerder afgekeurde levers beschikbaar komen voor de donorpool. Dit

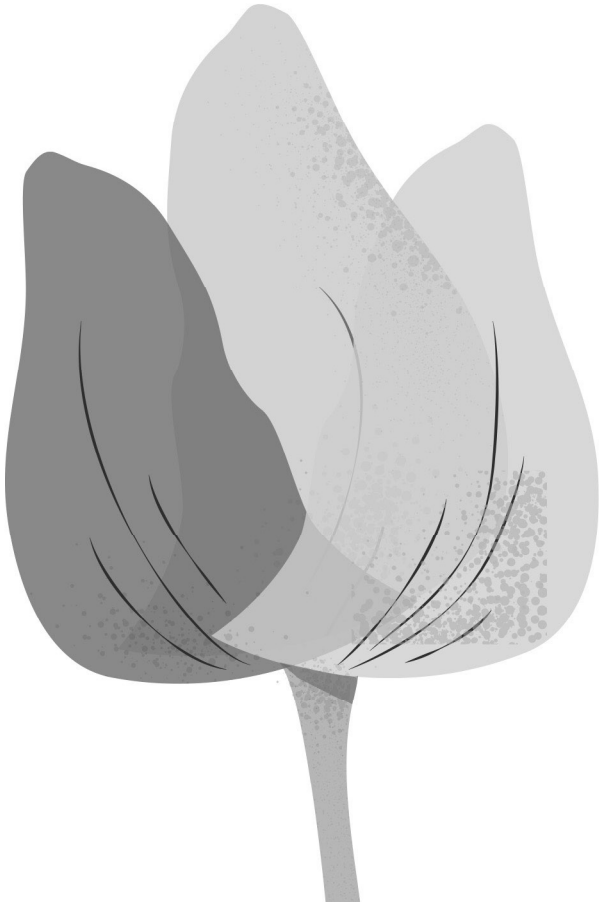
zal dan zowel gevolgen hebben voor het donoraanbod als voor de mortaliteit op de wachtlijst. Momenteel worden veel studies verricht om techniek en uitkomsten van machinepreservatie vast te stellen

Een andere mogelijkheid om de ontwikkeling van galwegstricturen te voorkomen is het optimaliseren van het allocatieproces. Momenteel is er geen mogelijkheid om de kans op galwegstricturen post-transplantatie te voorspellen. Het is niet bekend of het kunnen voorspellen van de kans op orgaanfalen na transplantatie, zoals met de ET-DRI bereikt kan worden, leidt tot een hoger succespercentage van levertransplantaties met minder (galweg)complicaties. Ondanks de bevinding dat er in dit proefschrift geen associatie werd gevonden tussen NAS en de immunologische factoren DSA en genetische variaties of mismatches in MMPs, is het mogelijk dat andere immunologische factoren wel een belangrijke rol spelen bij NAS. Dit geldt bijvoorbeeld voor de steriele ontstekingstreactie die het gevolg is van ischemie en reperfusie. Verder onderzoek naar dergelijke factoren zal mogelijk leiden naar nieuwe preventieve maatregelen.

Totdat er een forse daling in de incidentie van galwegstricturen is bereikt, dienen hoogrisicopatiënten goed gecontroleerd en gescreend to worden. Het gebruik van MRCP lijkt een betrouwbare manier om de aanwezigheid galwegstricturen aan te tonen of uit te sluiten. Daarnaast werd aangetoond dat een vroege behandeling van galwegstricturen de kans op een succesvolle behandeling vergroot er daarmee mogelijk (herhaalde episodes van) cholangitis, ziekenhuisheropnames en progressie naar orgaanfalen voorkomt. Omdat patiënten met galwegstricturen vaker symptomen van angst en depressie rapporteren kan een succesvolle behandeling van stricturen en nog betere begeleiding mogelijk deze gevoelens deels voorkomen.

Concluderend, toont dit proefschrift de complexiteit van het ontstaan van galwegstricturen, maar ook de uitdaging van het diagnosticeren en behandelen ervan. Het optimaliseren van de kwaliteit van aangeboden donorlevers lijkt op korte termijn de meest haalbare strategie om het ontstaan van galwegstricturen zo veel mogelijk te kunnen voorkomen.

Appendices



Chapter 11

Flushing the liver with urokinase before transplantation does not prevent non-anastomotic biliary strictures

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Abstract

Introduction The aim of the present study was to assess whether flushing the donor liver with urokinase immediately before implantation reduces the incidence of non-anastomotic biliary strictures (NAS) after liver transplantation, without causing increased blood loss, analyzed as a historical cohort study.

Patients and Methods Between January 2005 and October 2012, all liver (re-)transplantations were included. Of the 185 liver transplant recipients included, 63 donor livers between January 2010 and October 2012 received urokinase (study group), whereas the donor liver of 122 consecutive recipients, who served as a historical control group, between January 2005 and January 2010 did not receive urokinase.

Results Basic donor (Eurotransplant donor risk index) and recipient (age, body mass index, laboratory Model for End-Stage Liver Disease score) characteristics did not significantly differ in both groups. Thirty-three recipients developed NAS: 22 in the control group (18%), 11 (17.5%) in the study group (p=0.68). Analyzed separately for donation after circulatory death (p=0.42) or donation after brain death (p=0.89), there was no difference between the groups in incidence of NAS. Of all recipients developing NAS, 7 (21%) needed re-transplantation, all others were treated conservatively. Autologous blood transfusion requirements did not differ significantly between both groups (p=0.91), whereas interestingly, more heterologous blood transfusions were needed in the control group (p< 0.001). This study has its limitations by its retrospective character. A multi-institutional prospective study could clarify this issue.

Conclusion Arterial flushing of the liver with urokinase immediately before implantation did not lead to a lower incidence of NAS in this study, nor did it lead to increased blood loss.

Introduction

Biliary complications are a well-known, major cause of morbidity and graft failure in recipients after liver transplantation.[1;2] The most troublesome are the so-called non-anastomotic biliary strictures (NAS), with an incidence of 5-15% reported in most current studies,[3;4] and in up to 30% of patients receiving a liver from donation after circulatory death (DCD).[5]

With direct treatment of strictures, by using endoscopic or percutaneous cholangiographic dilatations and stenting, more than 50% of patients with NAS can be treated successfully.[6-11]

The pathophysiology of NAS development still remains unknown. Over the years, several risk factors have been indicated, suggesting that its origin may be multifactorial. In addition to immunological injury and bile salt-induced injury, it is suggested that ischemic injury to the peribiliary vascular plexus plays a critical role.[12] During the donor procedure, the peribiliary arterial plexus may not be completely flushed out. Because the blood supply to the biliary tract is solely dependent on arterial inflow, these microcirculatory disturbances in the peribiliary plexus may lead to obstruction and may subsequently result in insufficient bile duct preservation.[13;14]

Three previous studies with historical controls suggest that adding a thrombolytic agent, such as urokinase or tissue plasminogen activator (tPA) to the preservation fluid (after trimming of the donor liver, on the back table, or before completion of the portal vein anastomosis) seems to reduce the incidence of NAS. The hypothesis was that this might be the result from dissolving microthrombi in the micro-vascular system of the biliary tree.[15-17] The aim of the present study is to assess whether flushing the donor liver with urokinase directly before to transplantation reduces the incidence of NAS without causing increased blood loss.

Patients and methods

Between January 2005 and October 2012, all orthotopic liver transplantations at the Leiden University Medical Center (Leiden, the Netherlands) were included in this study. Exclusion criteria were domino, split, or auxiliary liver transplantations. Clinical information was obtained from a prospectively collected database. Covariates included donor demographics, recipient demographics, pretransplant information, intraoperative data, and postoperative outcomes. Calculated Model for End-Stage Liver Disease (labMELD) scores were included in the recipient analysis. The labMELD score was calculated using laboratory data (creatinine, bilirubin, international normalized ratio) and did not include exception points that were given for liver malignancies or other medical conditions.

On the basis of existing literature, a protocol change was made as of January 2010 to flush the donor liver with urokinase, directly before transplantation. This protocol change was approved by the institutional ethical committee.

Definition of NAS

NAS was defined as described by Ten Hove et al.[18] NAS was any stricture or irregularity of the intrahepatic or extrahepatic bile ducts of the liver graft that was at least 1 centimeter above the anastomosis, with or without dilation and with or without biliary sludge formation, and treated endoscopically with endoscopic retrograde cholangiopancreatography and dilation and/or stenting, percutaneously with percutaneous transhepatic cholangiography and biliary drainage or by surgical intervention. Therefore, all these NAS were clinically significant. Hepatic artery thrombosis (HAT) by either Doppler ultrasound, or conventional angiography, as well as isolated strictures/stenosis at the bile duct anastomosis and related dilations were, by definition, excluded from the analysis.

Operative techniques

The procurement of organs was performed as described by the Eurotransplant protocol. During procurement, the donor liver was flushed with preservation fluid under a pressure of 300 mm Hg (the type of perfusion used depended on the country where the procurement took place within the Eurotransplant region). During procurement in DCD liver allografts, 5000 IU of heparin was administered during initial organ perfusion. In donation after brain death (DBD) liver allografts, 300 IU/

kg of heparin was administered 5 minutes before cross-clamping. After procurement, the liver was sent to our hospital. Since January 1, 2010, as a change in the center protocol, after inspection of the donor liver and immediately before transplantation, the hepatic artery was flushed with 250,000 IU of manually pressurized urokinase on the back table. Hereafter, the hepatic artery was clamped to prevent backflow. After a minimum period of 10 minutes after flushing with urokinase, the hepatic artery was flushed with 500 mL of preservation fluid (histidine tryptophan ketoglutarate [HTK] or University of Wisconsin) in order to prevent systemic introduction of urokinase. Also, according to standardized protocol, the portal vein was flushed with 150 mL of albumin during caval anastomosis in order to prevent systemic introduction of urokinase. Further implantation of the liver allograft was done according to protocol. Before January 1, 2010, the same protocol was carried out, only without administration of urokinase. After consultation of the medical ethics committee, recipients did not have to give informed consent because the administration of urokinase was implemented as a new center protocol.

Statistical analysis

Continuous variables were presented as median (range) and standard deviation, whereas categorical variables were presented as number and percentage. Patient and graft survival curves and the cumulative incidence of NAS were calculated using the Kaplan – Meier method and compared using the log-rank test. Categorical variables were compared with the Pearson's chi-square test or Fisher's exact test, where appropriate. Characteristics of the donor, transplantation and recipient were analyzed using the 2-tailed Student *t*-test. Blood loss was analyzed using the Mann-Withney U-test. The level of significance was set at 0.05. Statistical analyses were performed using SPSS software version 22.0 for Windows (SPSS Inc., Chicago, IL, USA).

Power analysis

With an anticipated reduction of NAS from 45% to 10% on the basis of previous studies on DCD liver transplantation,[15-17] the power of this study would be 83.2% when comparing 28 DCD livers in the study group to 17 DCD livers in the control group.

With an anticipated reduction of NAS from 20% to 5% on the basis of previous studies concerning DBD liver transplantation, the power of this study would be 80.1% when comparing 94 DBD livers in the study group to 46 DBD livers in the control group.

Results

Of the 205 patients who received a liver transplantation between January 2005 and October 2012, 5 recipients were excluded based on missing information on receiving urokinase, 3 recipients were excluded based on protocol deviation (Figure 1). Of the 197 liver recipients remaining for the study, 127 donor livers did not receive urokinase (historic control group), and 70 donor livers received urokinase (study group). In the historic control group, 5 recipients were excluded (4 split liver transplantations, 1 domino donor), leaving 122 recipients in this group. In the study group, 7 recipients were excluded (6 split-liver transplantations, 1 domino donor), leaving 63 recipients.

Donor and recipient characteristics

Table 1 shows the basic donor and recipient characteristics of both groups. The mean Eurotransplant Donor Risk Index (ET-DRI)[19] in the control group was 1.8 ± 0.3 (range, 1 – 3.1), in the study group 1.8 ± 0.4 (range 1 – 2.6; p=0.56). Of 3 donors, the ET-DRI could not be calculated. Of the donors in the control group, 51% were female versus 48% in the study group (p=0.76). Donor body mass index was lower in the control group than in the study group. The mean cold ischemia time (CIT) of the transplanted livers in the control group was 572 ± 142 minutes (range 224 – 1090 minutes) and in the study group 535 ± 129 minutes (range 230 – 850 minutes; p=0.09). The mean first warm ischemia time in the control group was 16.7 ± 5 minutes (range 11-31 minutes), in the study group 17.6 ± 6 minutes (range 9 – 31 minutes; p=0.60). The mean labMELD score in the control group was 16.6 ± 8.7 (range 6-40), in the study group 16.6 ± 8.9 (range 6-40; p=0.99).

Biliary complications

In total, 33 (17.8 %) recipients developed NAS, of which 22 (18%) recipients were in the control group, and 11 (17.5%) were recipients in the study group. None of the recipients had evidence of hepatic artery thrombosis (HAT) or stenosis. The mean follow-up in the control group was 1543 ± 1049 days (range 1 - 3278 days) versus 675 days ± 495 days (range 1 – 1434 days) in the study group. The median number of days of follow-up was 1731 ± 1049 days (range 312 – 2356 days) in the study group versus 731 ± 495 days (range 119 – 1109) in the control group (Table 1).

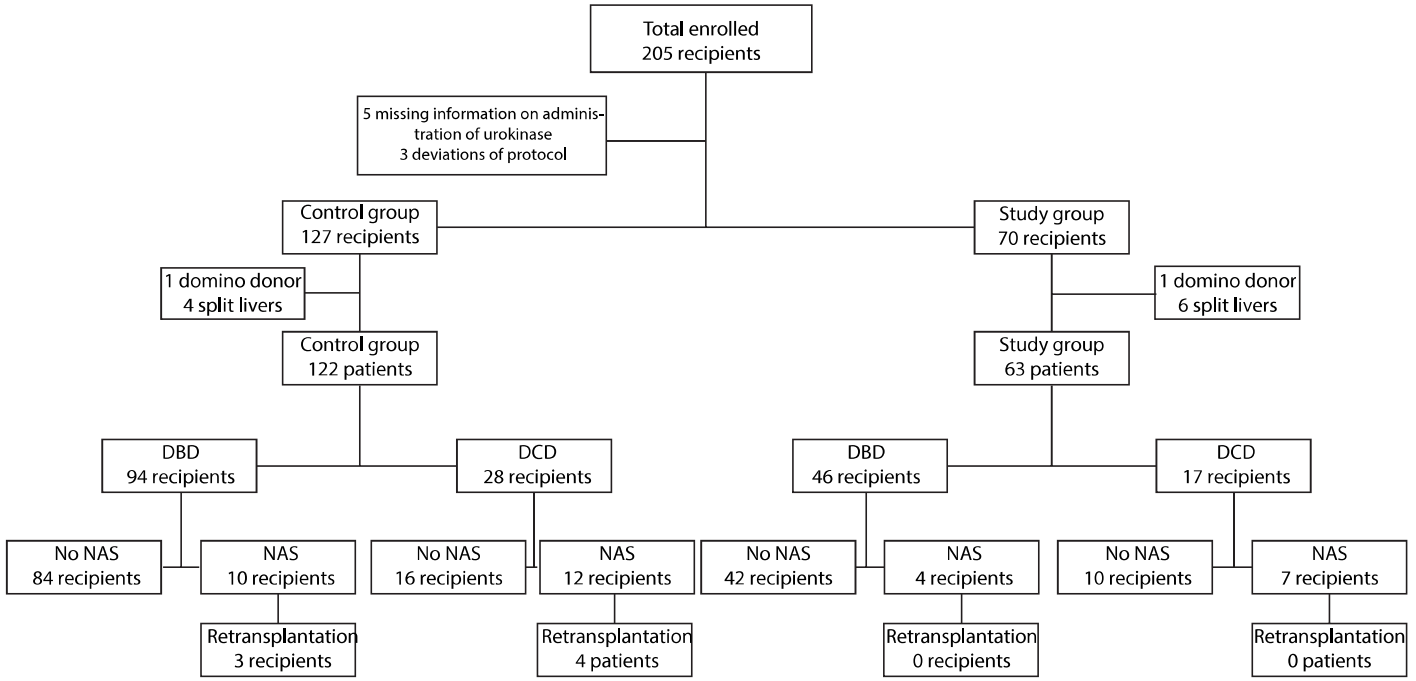

Figure 1. Study design.
NAS = non-anastomotic biliary strictures, DCD = Donation after Circulatory Death, DBD = Donation after Brain Death

Table 1. Donor, transplant and recipient characteristics.

	Urokinase group (*n*=63)	Controls (*n*=122)	*p*-value
ET-DRI	1.8 ± 0.3	1.8 ± 0.4	0.56
Donor age (years)	49.4 ± 15.0	46.9 ± 14.2	0.27
Donor BMI (kg/m^2)	25.2 ± 3.3	24.0 ± 3.3	**0.03**
First Warm Ischemia Time (min)	17.6 ± 6.0	16.7 ± 4.5	0.09
Cold Ischemia Time (min)	535.0 ± 129.0	572.0 ± 142.3	0.60
Recipient Warm Ischemia Time (min)	35.9 ± 8.5	33.8 ± 8.4	0.12
Recipient Age (years)	51.9 ± 11.6	52.3 ± 10.8	0.80
Recipient BMI (kg/m^2)	25.8 ± 4.6	26.2 ± 4.8	0.53
LabMELD	16.6 ± 8.9	16.6 ± 8.7	0.99

Data are presented as mean ± SD. SD = Standard Deviation, ET-DRI = Eurotransplant Donor Risk Index, BMI = Body Mass Index, LabMELD = Laboratory Model for End-Stage Liver Disease Score

In the control group, the mean number of days until NAS was diagnosed was 295 ± 363 days (22 – 1454 days). In the study group, the mean number of days was 189 ± 202 days (range 30 – 723 days; *p*=0.38). Graft survival, censored for death, shows equal results of both groups (*p*=0.68; Fig. 2). In the control group, the median number of days until NAS was diagnosed was 172 ± 363 days (range 71 – 346) compared to 119 ± 202 days (range 48 – 216) in the study group.

Comparison of liver transplantations from DCD-donors only also showed equal graft survival; 7 (41%) recipients in the study group developed NAS versus 12 (43%) recipients in the control group (*p*=0.42). In the control group, 10 (11%) recipients who received a liver allograft from DBD donors developed NAS versus 4 (9%) recipients in the study group. This was not different (*p*=0.89). Of all cases, 7 (21%) recipients needed retransplantation for NAS.

Postreperfusion blood loss

Table 2 shows the hematological and coagulation parameters of both groups preoperative, the activated partial thromboplastin time (aPTT) values during anhepatic phase, after reperfusion, and after surgery; the number of packed red blood cells (RBCs) transfused during the first 24 hours from incision; and volume of autologous blood transfused. Most remarkable, the mean preoperative aPTT in the control group was 34.5 ± 7.5, versus 38.5 ± 7.5 seconds in the study group ($p < 0.01$), whereas aPTT did

not differ between study group and control group in the anhepatic phase, after reperfusion and after surgery.

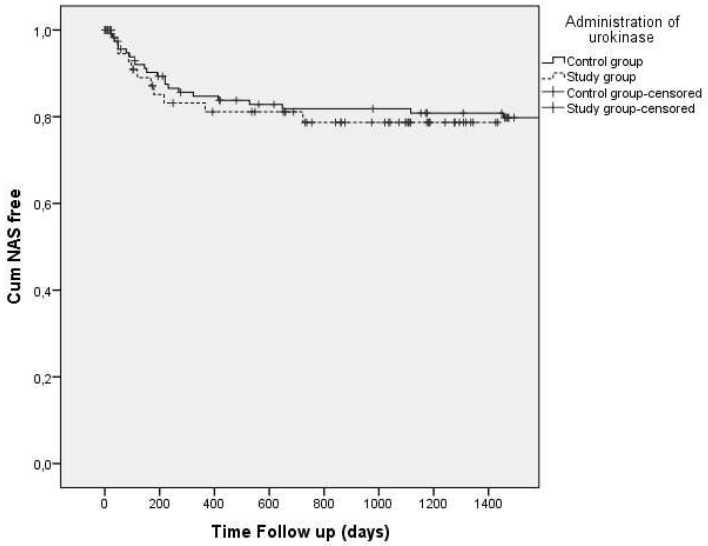

Figure 2. Graft survival, censored for death.

The mean packed RBCs transfused in the control group was 8.6 ± 7.7 versus 5.6 ± 6.2 units in the study group (p< 0.01). The mean volume of autologous blood transfused in the control group was 919 ± 1320 versus 946 ± 1166 mL in the study group (p=0.91).

Table 2. Hematological, coagulation, and transfusion parameters before, during and after surgery.

	Urokinase group (n=63)	Controls (n=122)	p-value
Platelet count before surgery (10^9/L)	128 ± 96	118 ± 90	0.47
PTT before surgery (sec)	20 ± 8	17 ± 5	**<0.01**
Fibrinogen before surgery (g/L)	3.1 ± 1.7	3.1 ± 1.7	0.97
aPTT before surgery (sec)	39 ± 8	35 ± 8	**<0.01**
aPTT anhepatic phase (sec)	45 ± 15	43 ± 11	0.27
aPTT after reperfusion (sec)	70 ± 23	72 ± 28	0.57
aPTT after surgery (sec)	51 ± 16	51 ± 18	0.92
Packet Cells (units)	5.6 ± 6.2	8.6 ± 7.7	**<0.01**
Cell Saver (mL)	946 ± 1166	919 ± 1320	0.91

Data are presented as mean ± SD. PTT = Partial Thromboplastin Time

Discussion

The present retrospective cohort study demonstrates that flushing the donor liver with urokinase immediately before liver transplantation is safe but does not prevent the development of NAS. It also did not lead to an increase of transfusion requirements or disturbed clotting.

In contrast to previous studies[15-17] describing a decrease in the rate of NAS after thrombolysis before liver transplantation, this study could not find a decrease in NAS rate. The first study by Hashimoto et al. was a retrospective study, with the injection of t-PA in the donor hepatic artery on the backtable in DCD liver transplantations, resulting in significantly less NAS development without increased blood loss.[15] Lang et al. described a prospective study with double perfusion of the donor liver with urokinase in DCD liver transplantation, resulting in significantly less NAS after year of follow-up.[16] Seal et al. described a retrospective analysis of DCD liver transplantations with an intraoperative t-PA injection, which minimized the incidence of NAS without increasing the need of intraoperative blood transfusion.[17] When looking closely at the dosage, type, and timing of the thrombolytic agent, our study has some minor differences compared to the studies previously published. In the study by Hashimoto et al[15], heparin was given to the donor before withdrawal of life support, and the liver allograft was perfused on the back table with 0.5 mg/100g graft tPA in the donor hepatic artery. Lang et al. perfused the arterial system of the donor liver twice. First, the used a dosage of 2000 mL HTK solution that contained 2 MU urokinase for perfusion through the arterial system. After trimming of the donor liver, the arterial system was perfused again with 1 MU urokinase. In the study by Seal et al.,[17] heparin was given to the donor before withdrawal of life support and, based on donor's weight and before completion of the portal vein anastomosis, 100 mg/kg tPA was perfused in the donor liver to account for variations in graft size. In our study, the donor liver was perfused through the hepatic artery after inspection on the back table, with a high fixed dose of 250,000 IU of urokinase, manually pressurized, in order to dissolve possible microthrombi.

To our knowledge, no previous studies have been published that show superiority for tPA, compared to urokinase, in low temperature circumstances.

Furthermore, the CIT in the study by Hashimoto et al.[15] and Seal et al.[17] was much shorter. They described a CIT of 422 ± 96 minutes and 5.1 ± 1.2 hours, whereas the CIT in this study was 572 ± 142 minutes in the control group and 535 ± 129 minutes in the study group. Lang et al.[16] described a CIT between 2 and 13.5 hours but did not mention a mean value.

The effect of thrombolytic agents has been reported in the experimental as well as clinical transplantation of various organs from DCD-donors,[20-23] suggesting that pretreatment with thrombolytic agents could be helpful in human liver transplantation. A possible explanation for not seeing a decrease in the rate of NAS in the current study could be the timing of the intervention. The therapeutic principle of administrating urokinase is to dissolve possible microthrombi from the peribiliary microcirculation. It may be that administrating urokinase immediately after organ procurement may be beneficial, whereas late administration of urokinase may not be able to prevent or dissolve microthrombosis. However, this hypothesis is not supported by Seal et al.,[17] who administered t-PA before completion of the portal vein anastomosis in order to limit the effects of hypothermia and dilution. The administration of urokinase in the donor liver is not part of the liver transplantation protocol in many other centers in the Eurotransplant region. For that reason, we were not able to administrate urokinase during the donation procedure.

The absence of microthrombi in the microvascular system of the biliary tree also has to be considered as a possible explanation why urokinase in our hands did not prevent NAS. In a study by Op den Dries et al.,[24] biopsies were taken from the donor bile duct in 128 liver transplant procedures. In the peribiliary plexus, thrombi were found in only 2.7% of these bile ducts from the livers that developed NAS, suggesting that thrombosis may not play a critical role in the development of NAS.[24]

In a study by Hansen et al.[25], histological evaluation of 93 donor common bile ducts, received after recirculation of the hepatic artery and before biliary end-to-end anastomosis in LT, were performed. With regard to NAS, they found that necrosis of the bile duct wall, arteriolonecrosis, vascular lesions, and intramural bleeding were statistically relevant associated factors. Thrombosis was not of statistical influence on occurrence of NAS. This also supports the theory that thrombosis does not play a critical role in the development of NAS.

Furthermore, Burlage et al.[26] believed that other factors, such as increased experience, explain the observed differences found in the study by Seal et al.[17]

The use of allogeneic and autologous blood as a measurement of blood loss has been described before by Hendriks et al.[27] Surprisingly, in this study, there was significantly more need for packed RBC transfusion in the control group, even though this group had significantly better coagulation parameters preoperatively. A possible explanation for this may be a positive effect of increasing experience within the transplantation team, as has been described previously.[27;28] During the study period, the transfusion protocol has not changed.

The extent and severity of NAS after liver transplantation determines its prognosis and management. Diffuse strictures have worse prognosis than local strictures because of a lack of therapeutic options. Even though we did not see a significant difference in the rate of NAS, the number of retransplantations due to NAS in the first year after transplantation could potentially show a difference because of different severity of NAS. Of all cases of NAS in the control group, 6 (38%) recipients required retransplantation in the first year after liver transplantation. Five (23%) recipients received a retransplantation in the first year after liver transplantation and 1 (5%) recipient was placed on the waiting list for retransplantation. In the study group, none of the recipients received a retransplantation in the first year after liver transplantation, and 1 (9%) recipient was placed on the waiting list for retransplantation. This difference was not significant (p=0.32). All other cases of NAS were treated conservatively by using balloon dilatation of the bile duct combined with the placement of an intraductal stent. The median number of interventions for NAS was 5.0 ± 2.4 (range 2.0 – 7.0) in the control group versus 4.5 ± 4.2 (range 2.0 – 8.0) in the study group.

Even though the median number of days of follow-up is shorter in the study group, compared to the control group, we do not believe this difference had an influence on the outcome because the median number of days until NAS diagnosis in both groups (172 ± 363 days [range 71 – 346 days] in the control group versus 119 ± 202 days [range 48 – 216 days] in the study group) is much shorter compared to the median number of days of follow-up in both groups (1731 ± 1049 days in the study group versus 731 ± 495 days in the control group). Because NAS is more common after DCD liver transplantation, compared to DBD liver transplan-

tation, both groups have been analyzed separately. When analyzed separately, no difference was found in the incidence of NAS after DCD liver transplantation (p=0.42). Also, no difference was found in the incidence of NAS after DBD liver transplantation (p=0.89).

This study has its limitations by its retrospective character. A multi-institutional prospective study could clarify this issue.

To conclude, arterial flushing of the donor liver with urokinase immediately before implantation did not lead to a lower incidence of NAS in this study, nor did it lead to increased blood loss.

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Appendix

Vragenlijsten

Vragenlijsten chapter 8

Achtergrond vragenlijst

De volgende vragen gaan over uw persoonlijke situatie en uw leverziekte. Zet een kruisje bij het antwoord dat het best bij uw situatie past.

1. Bent u bereid om aan dit onderzoek mee te doen?
- ❑ Ja
- ❑ Nee

Ook als u niet wilt meedoen aan ons onderzoek, verzoeken wij u, om toch de vragenlijst naar ons terug te sturen door middel van de antwoordenenvelop. U hoeft dan uiteraard de rest van de vragenlijst niet in te vullen.

2. Bent u:
- ❑ Man
- ❑ Vrouw

3. Geboortejaar:
Jaar……...

4. Wat is uw geboorteland?
- ❑ Nederland
- ❑ Nederlandse Antillen
- ❑ Suriname
- ❑ Turkije
- ❑ Marokko
- ❑ Anders, namelijk……

5. Wat is uw hoogste volledig afgemaakte opleiding?
- ❑ Ik heb geen enkele opleiding volledig afgemaakt
- ❑ Lagere school
- ❑ Lager beroepsonderwijs/Voorbereidend beroepsonderwijs (Huishoudschool, LTS, LEAO, VMBO-BB, VMBO-KB, VMBO-GL)
- ❑ Middelbaar algemeen voortgezet onderwijs (MAVO, IVO, MULO, VMBO-TL)
- ❑ Middelbare beroepsopleiding (MTS, MEAO, MHNO, INAS)
- ❑ Hoger algemeen en voorbereidend wetenschappelijk onderwijs (HAVO, VWO, HBS, MMS, GYMNASIUM, ATHENEUM)
- ❑ Hoger beroepsonderwijs
- ❑ Universiteit

6. Wat is uw burgerlijke status?

- ❑ Getrouwd / Samenwonend / Geregistreerd partnerschap
- ❑ Ongetrouwd / Weduwe of Weduwnaar / Alleenstaand

7. Welke van de volgende situaties is op u van toepassing? Indien meerdere situaties van toepassing zijn, wilt u dan aangeven welke situatie het *meest* op u van toepassing is?

- ❑ Ik heb een betaalde baan
- ❑ Ik zorg voor het huishouden (en eventueel kinderen)
- ❑ Ik ben gepensioneerd of met prepensioen
- ❑ Ik ben scholier of student
- ❑ Ik kan (gedeeltelijk) geen betaald werk doen vanwege gezondheidsredenen en ben voor …….% arbeidsgeschikt
- ❑ Ik doe geen betaald werk om een andere reden (Bijv. vanwege onvrijwillige werkloosheid of vrijwilligerswerk)

8. Geeft u een ruwe schatting van het aantal uur dat u, gemiddeld per week, besteedt aan betaald werk, vrijwilligerswerk, huishoudelijk werk en studie. Indien u geen tijd besteedt aan het desbetreffende werk of studie, vult u dan een 0 in.

Betaald werk	Vrijwilligerswerk	Huishoudelijk werk	Studie (zelfstudie en lessen)
……	…….	…….	……

9. Geeft u een ruwe schatting van het aantal uur dat u, gemiddeld per week, besteedt aan de volgende vrije tijd-activiteiten?
Indien u geen tijd besteedt aan de betreffende activiteiten-categorie, vult u dan een 0 in.

Activiteiten zonder lichamelijke inspanning. (Bijv. schaken, kaarten, puzzelen, breien, TV kijken.)	Activiteiten met lichamelijke inspanning. (Bijv. voetballen, fietsen, wandelen, tuinieren.)
……..	……..

10. Heeft u een levertransplantatie ondergaan?

- ❑ Ja, namelijk op: dag…… / maand……. / jaar……..
- ❑ Nee

11. Heeft u (andere) ziekten/aandoeningen die u belemmeren in het dagelijks functioneren? Meerdere antwoorden zijn mogelijk.
Ja, namelijk ziekten of aandoeningen van:

- ❑ hart- en vaten (bijv. hoge bloeddruk)
- ❑ het zenuwstelsel (bijv. ziekte van Parkinson)
- ❑ de luchtwegen (bijv. astma)
- ❑ de spieren
- ❑ de gewrichten (bijv. reuma)
- ❑ de urinewegen
- ❑ het maag / darmstelsel (bijv. ziekte van Crohn, colitis ulcerosa)
- ❑ suikerziekte
- ❑ het oog
- ❑ psychische aandoeningen
- ❑ overige, namelijk……..
- ❑ Nee

12. Graag willen wij van u weten of en welke medicijnen u op dit moment gebruikt.
In de onderstaande lijst worden een aantal medicijnen weergegeven. Leest u de lijst eerst rustig door.

Ik gebruik medicijnen
- ❑ Ja
- ❑ Nee

Kruist u de medicijn(en) aan die u **op dit moment** gebruikt.

Medicijnen	**Gebruik nu**
1. Interferon (Intron A, Roferon)	❑
2. PEG-interferon	❑
3. Lamivudine	❑
4. Famiciclovir	❑
5. Entecavir	❑
6. Ribavirine	❑
7. Prednison	❑
8. Tacrolimus (Prograft, Advagraf)	❑
9. Ciclosporine (Neoral)	❑
10. Mycofenolaatmofetil (Cellcept)	❑
11. Ursodeoxycholzuur (Ursochol, Ursofalk)	❑
12. Budenoside	❑
13. Furosemide (Lasix)	❑
14. Spironolacton (Aldactone)	❑
15. Propanolol (Inderal)	❑
16. Antihypertensiva (tegen hoge bloeddruk)	❑
17. Antidiabetica (tegen suikerziekte)	❑
18. Medicatie tegen luchtwegaandoeningen	❑
19. Slaapmiddelen	❑
20. Middelen tegen psychische klachten (bijv. antidepressiva, middelen tegen de angst, enz.)	❑
21. Overig, namelijk:	❑
1. …….	
2. …….	
3. …….	

Liver Disease Symptom Index 2.0

Vult u de onderstaande vragen in, als u op dit moment een leverziekte heeft of als u ooit een leverziekte heeft gehad.

Met behulp van de onderstaande vragen willen wij een indruk krijgen in welke mate u de afgelopen week bepaalde klachten had en in welke mate u hinder had van deze klachten. Zet een kruisje bij het antwoord dat het best bij uw situatie past.

Bijvoorbeeld:
Wanneer u vindt dat u de afgelopen week geen jeuk had, dan plaatst u bij vraag 1A een kruisje in het meest linkse hokje. Hoe meer jeuk u had de afgelopen week, hoe meer u het kruisje in de richting van "in hoge mate" kunt plaatsen.

Slaat u alstublieft geen vragen over en plaats telkens één kruisje bij elke vraag.

1A. In welke mate had u, de afgelopen week, jeuk?
In het geheel niet ❑ ❑ ❑ ❑ ❑ In hoge mate

1B. In welke mate werd u, de afgelopen week, door jeuk gehinderd in uw werk of in uw dagelijkse bezigheden?
In het geheel niet ❑ ❑ ❑ ❑ ❑ In hoge mate

1C. In welke mate werd u, de afgelopen week, door jeuk gehinderd in uw slaap?
In het geheel niet ❑ ❑ ❑ ❑ ❑ In hoge mate

2A. In welke mate had u, de afgelopen week, gewrichtspijnen?
In het geheel niet ❑ ❑ ❑ ❑ ❑ In hoge mate

2B. In welke mate werd u, de afgelopen week, door gewrichtspijnen gehinderd in uw werk of in uw dagelijkse bezigheden
In het geheel niet ❑ ❑ ❑ ❑ ❑ In hoge mate

3A. In welke mate had u, de afgelopen week, pijn in de rechter onderbuik?
In het geheel niet ❑ ❑ ❑ ❑ ❑ In hoge mate

3B. In welke mate werd u, de afgelopen week, door pijn in de rechter bovenbuik gehinderd in uw werk of in uw dagelijkse bezigheden?
In het geheel niet ❑ ❑ ❑ ❑ ❑ In hoge mate

4A. In welke mate was u, de afgelopen week, slaperig overdag?
In het geheel niet ❑ ❑ ❑ ❑ ❑ In hoge mate

4B. In welke mate werd u, de afgelopen week, gehinderd door slaperigheid overdag in uw werk of in uw dagelijkse bezigheden
In het geheel niet ❑ ❑ ❑ ❑ ❑ In hoge mate

5A. **In welke mate heeft u zich, de afgelopen week, zorgen gemaakt over de invloed van uw leverziekte op de thuis/gezinssituatie**
In het geheel niet ❑ ❑ ❑ ❑ ❑ In hoge mate

5B. **Hebben zorgen over de invloed van uw leverziekte op de thuis/gezinssituatie u de afgelopen week, gehinderd in uw werk of in uw dagelijkse bezigheden?**
In het geheel niet ❑ ❑ ❑ ❑ ❑ In hoge mate

6A. **In welke mate had u, de afgelopen week, een verminderde eetlust?**
In het geheel niet ❑ ❑ ❑ ❑ ❑ In hoge mate

6B. **In welke mate werd u, de afgelopen week, door verminderde eetlust gehinderd?**
In het geheel niet ❑ ❑ ❑ ❑ ❑ In hoge mate

7A. **In welke mate heeft u zich, de afgelopen week, door uw ziekte neerslachtig gevoeld?**
In het geheel niet ❑ ❑ ❑ ❑ ❑ In hoge mate

7B. **In welke mate werd u, de afgelopen week, door neerslachtigheid ten gevolge van uw ziekte, gehinderd in uw werk, uw dagelijkse bezigheden en/of uw contacten met andere mensen?**
In het geheel niet ❑ ❑ ❑ ❑ ❑ In hoge mate

8. **In welke mate was u, de afgelopen week, bang voor mogelijke complicaties van uw leverziekte?**
In het geheel niet ❑ ❑ ❑ ❑ ❑ In hoge mate

9A. **In welke mate was uw huid, de afgelopen week, ten gevolge van de uw leverziekte geel gekleurd?**
In het geheel niet ❑ ❑ ❑ ❑ ❑ In hoge mate

9B. **In welke mate hinderde een gele kleur van uw huid u, de afgelopen week, in uw werk, uw dagelijkse bezigheden en/of contacten met andere mensen?**
In het geheel niet ❑ ❑ ❑ ❑ ❑ In hoge mate

SF-36 Gezondheidstoestand

Instructie: Deze vragenlijst gaat over uw standpunt t.a.v. uw gezondheid. Met behulp van deze gegevens kan worden bijgehouden hoe u zich voelt en hoe goed u in staat bent uw gebruikelijke bezigheden uit te voeren.

Beantwoord elke vraag door het antwoord op de aangegeven wijze te markeren. Als u niet zeker weet hoe u een vraag moet beantwoorden, geef dan het best mogelijke antwoord.

1. Hoe zou u over het algemeen uw gezondheid beoordelen?

Uitstekend	1
Zeer goed	2
Goed	3
Matig	4
Slecht	5

2. Hoe beoordeelt u nu uw gezondheid over het algemeen, vergeleken met een jaar geleden?

Veel beter nu dan een jaar geleden	1
Wat beter nu dan een jaar geleden	2
Ongeveer hetzelfde nu als een jaar geleden	3
Wat slechter nu dan een jaar geleden	4
Veel slechter nu dan een jaar geleden	5

3. De volgende vragen gaan over dagelijkse bezigheden die u misschien doet op een doorsnee dag. Wordt u door uw gezondheid op dit moment beperkt bij deze bezigheden? Zo ja, welke mate? (omcirkel één cijfer op elke regel)

BEZIGHEDEN

		Ja, ernstig beperkt	Ja, een beetje beperkt	Nee, helemaal niet beperkt
a.	*Forse inspanning,* zoals hardlopen, tillen van zware voorwerpen, een veeleisende sport beoefenen	1	2	3
b.	*Matige inspanning,* zoals een tafel verplaatsen, stofzuigen, zwemmen of fietsen	1	2	3
c.	Boodschappen tillen of dragen	1	2	3
d.	Een paar trappen oplopen	1	2	3
e.	Eén trap oplopen	1	2	3
f.	Bukken, knielen of hurken	1	2	3
g.	*Meer dan een kilometer* lopen	1	2	3
h.	*Een paar honderd meter* lopen	1	2	3
i.	Ongeveer *honderd meter* lopen	1	2	3
j.	Uzelf wassen of aankleden	1	2	3

4. Heeft u in de afgelopen 4 weken één van de volgende problemen bij uw werk of andere bezigheden gehad, ten gevolge van uw lichamelijke gezondheid? (Omcirkel één cijfer op elke regel)

		Ja	Nee
a.	U besteedde *minder tijd* aan werk of andere bezigheden	1	2
b.	U heeft *minder bereikt* dan u zou willen	1	2
c.	U was beperkt in het *soort* werk of andere bezigheden	1	2
d.	U had moeite om uw werk of andere bezigheden uit te voeren (het kostte u bijv. extra inspanning)	1	2

5. Heeft u in de afgelopen 4 weken één van de volgende problemen bij uw werk of andere bezigheden gehad, ten gevolge van uw emotionele problemen (zoals depressieve of angstige gevoelens)? (omcirkel één cijfer of elke regel)

		Ja	Nee
a.	U besteedde *minder tijd* aan werk of andere bezigheden	1	2
b.	U heeft *minder bereikt* dan u zou willen	1	2
c.	U deed uw werk of andere bezigheden niet zo *zorgvuldig* als gewoonlijk	1	2

6. In hoeverre hebben uw lichamelijke gezondheid of emotionele problemen u gedurende de afgelopen 4 weken gehinderd in uw normale omgang met familie, vrienden buren of bij activiteiten in groepsverband?

Helemaal niet	1
Enigszins	2
Nogal	3
Veel	4
Heel erg veel	5

7. Hoeveel lichamelijke pijn heeft u de afgelopen 4 weken gehad?

Geen	1
Heel licht	2
Licht	3
Nogal	4
Ernstig	5
Heel ernstig	6

8. In welke mate bent u de afgelopen 4 weken door pijn gehinderd in uw normale (zowel werk buitenshuis als huishoudelijk werk)?

Helemaal niet	1
Een klein beetje	2
Nogal	3
Veel	4
Heel erg veel	5

9. Deze vragen gaan over hoe u zich voelt en hoe het met u ging in de afgelopen 4 weken. Wilt u a.u.b. bij elke vraag het antwoord geven dat het beste benadert hoe u zich voelde. Hoe vaak gedurende de afgelopen 4 weken: (omcirkel één cijfer op elke regel)

	Altijd	Meestal	Vaak	Soms	Zelden	Nooit
a. Voelde u zich levenslustig?	1	2	3	4	5	6
b. Was u erg zenuwachtig?	1	2	3	4	5	6
c. Zat u zo in de put dat niets u kon opvrolijken?	1	2	3	4	5	6
d. Voelde u zich rustig en tevreden?	1	2	3	4	5	6
e. Had u veel energie?	1	2	3	4	5	6
f. Voelde u zich somber en neerslachtig?	1	2	3	4	5	6
g. Voelde u zich uitgeput?	1	2	3	4	5	6
h. Was u een gelukkig mens?	1	2	3	4	5	6
i. Voelde u zich moe?	1	2	3	4	5	6

10. Hoe vaak hebben uw lichamelijke gezondheid of emotionele problemen u gedurende de afgelopen 4 weken gehinderd bij uw sociale activiteiten (zoals vrienden of familie bezoeken, etc.)?

Altijd	1
Meestal	2
Soms	3
Zelden	4
Nooit	5

11. Hoe JUIST of ONJUIST is elk van de volgende uitspraken voor u? (omcirkel één cijfer op elke regel)

	Volkomen juist	Grotendeels juist	Weet ik niet	Grotendeels onjuist	Volkomen onjuist
a. Ik lijk gemakkelijker ziek te worden dan andere mensen	1	2	3	4	5
b. Ik ben even gezond als andere mensen die ik ken	1	2	3	4	5
c. Ik verwacht dat mijn gezondheid achteruit zal gaan	1	2	3	4	5
d. Mijn gezondheid is uitstekend	1	2	3	4	5

A

Zet bij iedere groep in de lijst hieronder een kruisje in het hokje achter de zin die het best past bij uw eigen gezondheidstoestand vandaag.

Motiliteit

Ik heb geen problemen met lopen ❑
Ik heb enige problemen met lopen ❑
Ik ben bedlegerig ❑

Zelfzorg

Ik heb geen problemen om mijzelf te wassen of aan te kleden ❑
Ik heb enige problemen om mijzelf te wassen of aan te kleden ❑
Ik ben niet in staat mijzelf te wassen of aan te kleden ❑

Dagelijkse activiteiten *(bijv. werk, studie, huishouden, gezins- en vrijetijdsactiviteiten)*

Ik heb geen problemen met mijn dagelijkse activiteiten ❑
Ik heb enige problemen met mijn dagelijkse activiteiten ❑
Ik ben niet in staat mijn dagelijkse activiteiten uit te voeren ❑

Pijn/klachten

Ik heb geen pijn of andere klachten ❑
Ik heb matige pijn of andere klachten ❑
Ik heb zeer ernstige pijn of andere klachten ❑

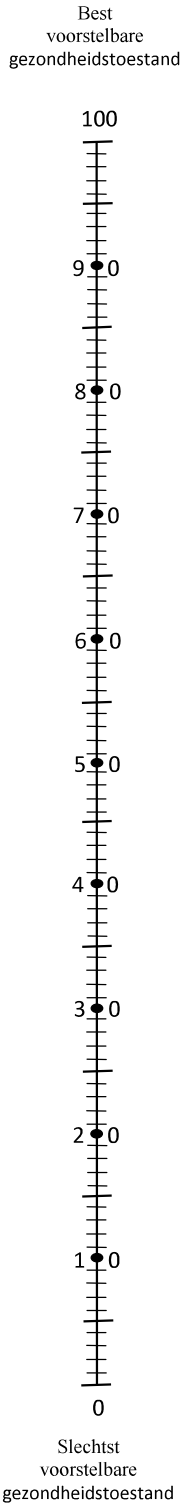
Stemming

Ik ben niet angstig of somber ❑
Ik ben matig angstig of somber ❑
Ik ben erg angstig of somber ❑

Om mensen te helpen bij het aangeven hoe goed of hoe slecht een gezondsheidstoestand is, hebben we een meetschaal (te vergeljiken met een thermometer) gemaakt. Op de meetschaal hiernaast betekent “100” de beste gezondheidstoestand die u zich kunt voorstellen en “0” de slechtste gezondheidstoestand die u zich kunt voorstellen.

We willen u vragen op deze meetschaal aan te geven hoe goed of slecht volgens u uw eigen gezondheidstoestand vandaag is. Trek een lijn van het hokje hieronder naar het punt op de meetschaal dat volgens u aangeeft hoe goed of hoe slecht volgens u uw eigen gezondheidstoestand vandaag is.

Uw gezondheidstoestand vandaag



Multidimensionele vermoeidheidsindex - 20

Met behulp van de onderstaande uitspraken, willen wij een indruk krijgen van hoe u zich de laatste dagen voelt.

Bijvoorbeeld
Wanneer u vindt dat de uitspraak voor u helemaal klopt, plaatst u dan een kruisje in het meest linkse hokje. Hoe minder u de uitspraak op uzelf van toepassing vindt, hoe meer u het kruisje naar rechts, richting 'nee, dat klopt niet', kunt plaatsen. Slaat u alstublieft geen vragen over en plaats telkens één kruisje bij elke uitspraak.

Het gaat om hoe u zich de laatste dagen voelt.

1. **Ik voel me fit.**
   Ja, dat klopt ❑❑❑❑❑ Nee, dat klopt niet

2. **Lichamelijk voel ik me tot weinig in staat.**
   Ja, dat klopt ❑❑❑❑❑ Nee, dat klopt niet

3. **Ik zit vol activiteit.**
   Ja, dat klopt ❑❑❑❑❑ Nee, dat klopt niet

4. **Ik heb zin om allerlei leuke dingen te gaan doen.**
   Ja, dat klopt ❑❑❑❑❑ Nee, dat klopt niet

5. **Ik voel me moe.**
   Ja, dat klopt ❑❑❑❑❑ Nee, dat klopt niet

6. **Ik vind dat ik veel doe op een dag.**
   Ja, dat klopt ❑❑❑❑❑ Nee, dat klopt niet

7. **Als ik ergens mee bezig ben, kan ik mijn gedachten er niet goed bijhouden.**
   Ja, dat klopt ❑❑❑❑❑ Nee, dat klopt niet

8. **Lichamelijk kan ik veel aan.**
   Ja, dat klopt ❑❑❑❑❑ Nee, dat klopt niet

9. **Ik zie er tegen op om iets te doen.**
   Ja, dat klopt ❑❑❑❑❑ Nee, dat klopt niet

10. **Ik vind dat ik weinig doe op een dag.**
    Ja, dat klopt ❑❑❑❑❑ Nee, dat klopt niet

11. **Ik kan me goed concentreren.**
    Ja, dat klopt ❑❑❑❑❑ Nee, dat klopt niet

12. **Ik voel me uitgerust.**
    Ja, dat klopt ❑❑❑❑❑ Nee, dat klopt niet

13. **Het kost me moeite ergens mijn aandacht bij te houden.**
    Ja, dat klopt ❑❑❑❑❑ Nee, dat klopt niet

14. Lichamelijk voel ik me in slechte conditie.
Ja, dat klopt ❑❑❑❑❑ Nee, dat klopt niet

15. Ik zit vol plannen.
Ja, dat klopt ❑❑❑❑❑ Nee, dat klopt niet

16. Ik ben gauw moe.
Ja, dat klopt ❑❑❑❑❑ Nee, dat klopt niet

17. Er komt weinig uit mijn handen.
Ja, dat klopt ❑❑❑❑❑ Nee, dat klopt niet

18. De zin om dingen te ondernemen ontbreekt mij.
Ja, dat klopt ❑❑❑❑❑ Nee, dat klopt niet

19. Mijn gedachten dwalen gemakkelijk af.
Ja, dat klopt ❑❑❑❑❑ Nee, dat klopt niet

20. Lichamelijk voel ik me in een uitstekende conditie.
Ja, dat klopt ❑❑❑❑❑ Nee, dat klopt niet

Appendix

List of abbreviations

List of abbreviations

aHR	Adjusted Hazard Ratio
AIH	Auto-Immune Hepatitis
ALD	Alcoholic Liver Disease
ALT	Alanine Aminotransferase
AMR	Antibody-Mediated Rejection
ARMS	Amplification Refractory Mutation System
AS	Anastomotic Biliary Stricture
AST	Aspartate Aminotransferase
ATP	Adenosine Triphosphate
BMI	Body Mass Index
CI	Confidence Intervals
CIT	Cold Ischemia Time
CMV	Cytomegalovirus
DAMP	Damage-associated Molecular Pattern
DBD	Donation after Brain Death
DCD	Donation after Circulatory Death
DSA	Donor Specific Antibody
DWIT	Donor Warm Ischemia Time
ECD	Extended Criteria Donors
EQ-5D	Euroqol 5D Questionnaire
ERC(P)	Endoscopic Retrograde Cholangio(pancreatico)graphy
ET-DRI	Eurotransplant Donor Risk Index
GGT	Gamma Glutamyltransferase
HAT	Hepatic Artery Thrombosis
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HLA	Human Leukocyte Antigen
HMGB1	High Mobility Group Box 1 Protein
HMP	Hypothermic Oxygenated Machine Perfusion
HR	Hazard Ratio
HTK	Histidine-Tryptophan-Ketoflutarate Solution
IF	Immunofluorescence Staining
IRI	Ischemia Reperfusion Injury

IU	International Unit(s)
LBSC	Leiden Biliary Stricture Classification
LDSI	Liver Disease Symptom Index Questionnaire 2.0
LT	Liver Transplantation
LMX	Luminex Screening Assay
MELD	Model for End-Stage Liver Disease
MFI	Mean Fluorescence Intensity
MFI-20	Multidimensional Fatigue Index 20
MMP	Matrix Metalloproteinase
MP	Machine Perfusion
MRC(P)	Magnetic Resonance Cholangio(pancreatico)graphy
NAS	Non-anastomotic Biliary Strictures
NRP	Normothermic Regional Perfusion
OLT	Orthotopic Liver Transplantation
PBC	Primary Biliary Cirrhosis
PCR	Polymerase Chain Reaction
PSC	Primary Sclerosing Cholangitis
PTC(D)	Percutaneous Transhepatic Cholangiography (with Drainage)
QOL	Quality of Life
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
RNS	Reactive Nitrogen Species
ROC	Receiver Operating Characteristics
ROS	Reactive Oxygen Species
RWIT	Recipient Warm Ischemia Time
SF-36	Short Form 36 Questionnaire
SNP	Single Nucleotide Polymorphism
TE	Transient Elastography
TIMP	Tissue-Inhibitor Matrix Metalloproteinase
US	Ultrasound
UW	University of Wisconsin Solution
VAS	Visual Analogue Scale