Biliary Complications after Liver Transplantation: New Insights and Biomarkers

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Contents

General introduction and outline of the thesis

1

In Urdu the word for liver, jiggar (جگر), is used to refer to courage or strength as a figure of speech. The liver itself could indeed be classified as courageous or strong as it is burdened with a large variety of vital functions, including detoxification of the human body, disassembly and synthesis of proteins, carbohydrates and fats, and digestion, absorption and storage of nutrients, which are released as required. It is therefore an essential organ required for maintaining homeostasis. Unsurprisingly, chronic or acute, dysfunction of the liver, due to congenital, metabolic, infectious, or malignant disease, is incompatible with life and therefore requires substitute therapy, which can only be achieved by liver transplantation.

The first attempt at human liver transplantation was reported in 1963, by dr. Thomas E. Starzl, in a 3-year old boy suffering of congenital biliary atresia[1]. He died intraoperatively because of excessive hemorrhage. Preceding this unsuccessful attempt in a human being, auxiliary and orthotropic liver transplants had already been attempted in canines dating back to the 1950's by dr. C. Stuart Welch and dr. Francis D. Moore respectively[2, 3]. However, human liver transplants had not been attempted until introduction and successful application of the immunosuppressive cocktail consisting of Azathioprine and prednisone in kidney transplantation, by 1963[4].

In addition to the first unsuccessful human liver transplant, 8 additional human liver transplants were performed by 1967[1, 5-7], none of which were considered successful as all of the transplanted patients died within 23 days of transplantation mainly due to pulmonary embolism caused the venous shunts required intraoperatively.

On July 23rd 1967, Starzl performed the first successful human liver transplantation in a 16-months old girl suffering from a hepatoma[8, 9]. This patient survived for over one year. The consecutive 6 liver transplantations that followed, all survived at least 2 months postoperatively marking a new era in clinical liver transplantation[8].

Merely half a century after the first successful liver transplantation has passed and liver transplantation has now become the gold standard for treatment of end-stage liver disease. One and 5-year patient survival is currently reported in excess of 85% and 70% respectively, while over half of the recipients survive longer than 20 years after liver transplantation[10]. Over the decades, advances in surgical techniques[9, 11-14], liver preservation[15-20] and immunosuppression[21-25], have contributed to this excellent survival after liver transplantation.

However, with this extended survival, new problems arise, which limit further improved survival after liver transplantation. These include for instance recurrence of liver disease and development of de novo malignancies and renal dysfunction, caused by long-term side effects of immunosuppressive therapies[26-30]. One of the most important complications after liver transplantation however, is the development of diffuse biliary strictures, known as non-anastomotic strictures (NAS) or ischemic-type biliary lesions (ITBL).

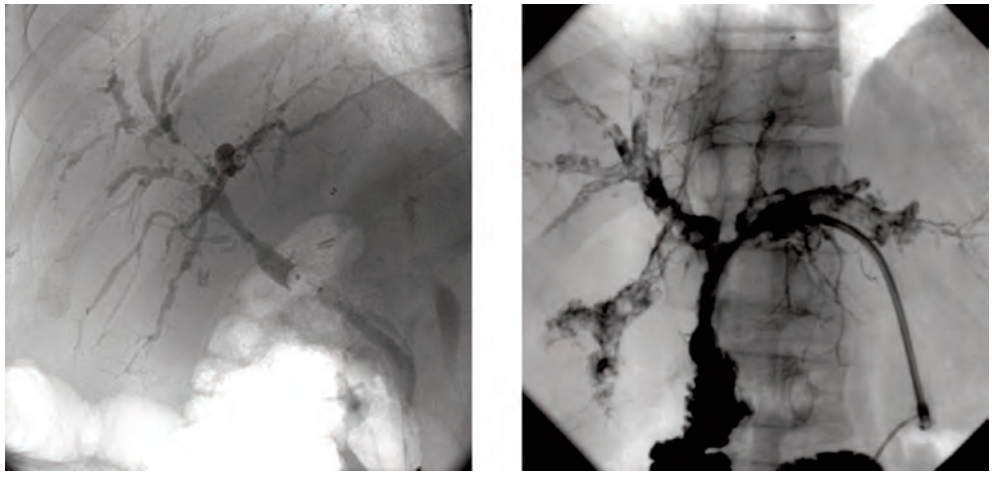

Figure 1. Two examples of cholangiograms showing severe ITBL after liver transplantation. Diffuse stricturing and prestenotic dilatations can be seen in the intrahepatic biliary tree.

ITBL or NAS is characterized by the destruction of biliary epithelium and development of consequent diffuse intra and/or extrahepatic biliary strictures and dilatations accompanied by cast formation in the bile ducts, in the presence of normal blood supply by the large hepatic arteries (Fig. 1)[31, 32]. Its incidence varies between 5-15%, but incidences of more than 50% have also been reported in early series[33]. ITBL usually develops within the first year after transplantation, but its prevalence continues to rise with time after transplantation and it can occur more than 10 years after liver transplantation[34]. These biliary complications are the third most common cause of hepatic retransplantation, and are considered the Achilles' heel of liver transplantation[35]. Up to 50% of the affected recipients require hepatic retransplantation[33]. It is thought that NAS or ITBL is a multifactorial problem. Although several risk factors have been identified, the exact pathogenesis of these complications remains unknown and therefore prediction and prevention of NAS or ITBL is cumbersome. Known risk factors are discussed in the following paragraphs.

DONOR CHARACTERISTICS

Throughout the years many donor characteristics have been scrutinized in order to find an explanation for the development of ITBL after liver transplantation. Only donor age has until now been identified as a significant risk factor for developing ITBL.

Donor age

Donor age has been identified by several studies as an independent risk factor for the development of ITBL[32, 36-42]. Higher donor age has been related to a higher incidence of ITBL. A recent study showed that donor livers developing ITBL after liver transplantation were on average 5 years older than allografts that did not develop ITBL after transplantation[32]. Although donor age has been confirmed as an independent risk factor the underlying mechanism is still unclear. It is hypothesized that the higher risk of developing ITBL in older donor livers could be related to their higher susceptibility to ischemia-reperfusion injury[43, 44].

ISCHEMIA-REPERFUSION INJURY

Ischemia-reperfusion injury has been implicated as the prime cause of ITBL from the beginning since the biliary lesions strongly resemble the ischemic biliary lesions found after hepatic artery thrombosis. Hence the name 'ischemic-type' biliary lesions. Mounting evidence indeed shows that ischemia-reperfusion injury is at least partially responsible for the incidence of ITBL.

Donation after cardio-circulatory death and warm ischemia time

Donation after cardio-circulatory death (DCD) has been introduced in liver transplantation in order to expand the organ pool and overcome the increasing problem of organ shortage[45]. However, the use of DCD organs introduces an additional period of warm ischemia, which has been shown to have negative impact on postoperative liver function and importantly lead to higher incidences of ITBL[46, 47]. Several other studies have also revealed the relation between DCD, warm ischemia and the development of ITBL[40, 48].

Additionally, it is argued that during the additional period of warm ischemia microthrombi are formed in the peribiliary plexus (PBP), surrounding the bile ducts and responsible for their oxygenation, which leads to inadequate perfusion and organ preservation during cold ischemia, subsequently leading to ITBL[49]. Animal studies have also shown that DCD leads to changes in bile composition, which in turn aggravates injury to the bile ducts resulting in ITBL[50].

On average overall biliary complications are almost twice as common in patients transplanted with organs from DCD donors, while the incidence of ITBL is 5 times higher when using organs from DCD donors[46, 47, 51-56]. Moreover, ITBL appears to occur in grafts from DCD donors earlier after transplantation as compared to donor organs from heart-beating donor[40].

Cold ischemia time

Besides warm ischemia time, cold ischemia time has been identified as an independent risk factor for developing ITBL[57]. A cold ischemia time of more than 14 hours has been associated with a two-fold increase in preservation injury, resulting in biliary strictures and decreased graft survival[57-59]. With a cold ischemia time less than 13 hours an incidence of ITBL was reported at 7%, whereas the percentage increased to 52% when the cold ischemia time exceeded 13 hours, and to 69% if cold ischemia time was over 15 hours. This suggests a high impact of cold ischemia time on the development of ITBL[32, 35]. Hence to minimize the risk of ITBL, cold ischemia times should be kept as short as possible, which was confirmed by a more recent study[32].

GRAFT PRESERVATION

Although various forms of machine perfusion are being researched in recent literature, static cold storage is currently the most applied method for preservation of the donor liver[60]. It is known that liver preservation techniques influence the graft's quality[61]. Therefore studies have been conducted to investigate the influence of preservation on postoperative development of ITBL.

Preservation solution

University of Wisconsin (UW) solution is generally utilized for flushing of the donor liver and its preservation[62]. However, in the past years it has increasingly received competition from histidine-tryptophan-ketoglutarate (HTK) preservation solution, especially in DCD liver transplantation[63-72]. Although UW solution is superior to HTK solution in protection of hepatocytes[73-75], its higher viscosity may hinder adequate flushing of, especially small, capillaries and cause suboptimal preservation. HTK solution on the other hand, has a viscosity similar to that of water and during perfusion sustains a 3 times higher flow compared to UW solution[66]. This results in quicker cooling (thus earlier lowering of base metabolism) of the donor organ and improved flushing of the small PBP (thus preventing stagnation of blood rests and consequent formation of microthrombi), thereby reducing ischemic biliary injury[41, 66, 76-78]. Indeed studies suggest that use of low-viscosity preservation solutions, including HTK, result in a lower incidence of ITBL postoperatively[32, 64, 79].

Pressurized arterial perfusion

Continuing in the line that improved perfusion leads to a lower incidence of ITBL, studies have demonstrated that improved perfusion through pressurized arterial perfusion with preservation solution is beneficial and leads to a significant lower incidence of ITBL after liver transplantation[32, 41].

Perfusion with thrombolytic drugs

Thrombolytic drugs have been added to preservation solutions to prevent the formation of microthrombi in the PBP. Only one clinical study has been conducted in the setting of liver transplantation, which shows that perfusion with urokinase during preservation lowers the incidence of ITBL by 4 times[80]. An experimental study showed that perfusion with streptokinase, another thrombolytic drug, attenuated parenchymal cell injury in a rat model of DCD graft procurement[81].

CYTOTOXIC INJURY

Cytotoxic injury by bile has been identified as an additional risk factor for the development of ITBL after liver transplantation. Intrahepatic cholestasis after liver transplantation is common[82-85]. It is mainly caused by ischemia-reperfusion injury due to warm and cold ischemia times[82, 86], and leads to increased exposure of cholangiocytes and hepatocytes to bile[87], due to cellular changes[83].

Toxic bile

In addition, ischemia-reperfusion injury can also lead to abnormal bile composition, with increased toxicity, due to its effects on bile transporters, which in turn is hazardous to the exposed cholangiocytes and hepatocytes[88-90].

The toxic bile after liver transplantation is characterized by a low biliary phospholipid/bile salt ratio and associated with histological signs of injury of the biliary tract in the liver[50, 91, 92]. More importantly, one study has indeed shown that toxic bile composition shortly after liver transplantation is associated with the development of ITBL later on[93].

Furthermore, it has been shown that ischemia-reperfusion injury may lead to disturbed secretion of HCO_3^-[94] and Mucins[95, 96], which are excreted by cholangiocytes to lubricate and protect themselves against a variety of injuries, including ones caused by cytotoxic bile[97]. As a result of changes in these protective measures ITBL might be favored[95].

A recent study demonstrates that adequate flushing of the biliary tract during perfusion reduces the effects of bile salt toxicity and resulted in diminished cold ischemic injury to the biliary epithelium[98]. It is therefore advisable to adequately flush the bile ducts during procurement, especially in DCD donors[99].

IMMUNE-MEDIATED INJURY

Several immunologic factors have also been associated with a higher incidence of ITBL after liver transplantation.

ABO incompatible transplantation and rejection

Although ABO incompatible liver transplantation has been discouraged in the past, some centers have reverted to using this form of transplantation due to the shortage of donor organs. However, the use of ABO incompatible donor grafts results in a significant higher incidence of ITBL[100-102]. It is thought that these biliary strictures are secondary due to immunological injury to the PBP and consequent thrombosis resulting in ischemia of the bile ducts.

In a similar fashion chronic allograft rejection has also been associated in some studies with a higher incidence of ITBL[33, 100, 102-105], which is again thought to be caused by inflammation, foam cell formation and obliterative arteritis with consequent biliary ischemia and stricture formation[82, 106].

Preexisting (autoimmune) disease

Preexisting (autoimmune) disease has also been associated with the development of postoperative ITBL.

Cytomegalo virus (CMV) infection is common in liver transplant recipients and is present in 30-50% of recipients[107, 108]. CMV is thought to cause ITBL due to its induced injury to the PBP and consequent ischemia and formation of ITBL[109, 110].

Studies have shown that autoimmune hepatitis (AIH) and primary sclerosing cholangitis (PSC) as the indication for liver transplantation are associated with higher incidences of ITBL[39, 40]. In the case of AIH it is unclear why this results in a higher incidence. PSC on the other hand is a pathological condition, which strongly resembles ITBL. The underlying mechanism causing it remains unclear and no biomarkers exist, which can sensitively and specifically differentiate between PSC and ITBL after liver transplantation. It is therefore unclear whether the higher incidence of ITBL is indeed to be classified as ITBL or recurrent PSC.

DIAGNOSIS AND TREATMENT OF ITBL

ITBL related symptoms usually arise within 1 year after transplantation and are typically unspecific. They may consist of fever, unspecific abdominal complaints and other symptoms related to cholestasis. In severe cases this is accompanied by jaundice and itchiness, although such extreme and late cases are rare at initial presentation.

Liver tests at this moment usually show elevation of gamma glutamyl transferase (γ-GT) and/or alkaline phosphatase (ALP) but are non-specific and do not lead to diagnosis[111].

When a patient is suspected of having developed ITBL, the first diagnostic step consists of non-invasive transabdominal ultrasonography (TAUS). But due to its low sensitivity for

small lesions it cannot be considered reliable for early detection. However TAUS can be helpful as a first step to generally evaluate the situation (e.g. vascular patency) and determine the seriousness and location of the lesions. Yet, a negative TAUS does not exclude the presence of ITBL.

For this reason direct visualization by endoscopic retrograde cholangio-pancreaticography (ERCP), percutaneous transhepatic cholangio-drainage (PTCD), or drain-cholangiography are necessary[33]. These methods of visualization are more sensitive and specific, and are considered as the golden standard for diagnosing ITBL. Furthermore, the advantage of these methods is that in addition to diagnosing ITBL, these are also suitable as methods for therapeutic access of the biliary tree[33, 35, 40, 41, 112-114]. Magnetic resonance cholangio-pancreaticography (MRCP) is becoming increasingly important as a diagnostic test with high positive and negative predicted values[33]. But despite this cholangiography remains the golden standard.

Once ITBL is diagnosed its treatment is symptomatic and consists of relieving symptoms by assuring adequate drainage of the bile ducts. This is usually accomplished by dilating the bile ducts during ERCP and utilizing stents to maintain adequate drainage. In severe cases percutaneous drainage is utilized or even partial hepatectomy is considered when the lesions are not generalized[34, 42, 111]. Despite these forms of treatment, up to 50% of the recipients developing ITBL require retransplantation[33].

In spite of the knowledge on risk factors mentioned, ITBL still remains an unpreventable problem in many cases of liver transplantation and is responsible for significant morbidity and mortality. Its exact pathogenesis still remains to be elucidated, although ischemia-reperfusion seems to play an important role. In addition, the unavailability of adequate biomarkers for early identification or prediction of ITBL hampers new research, as it hinders quantification of potential preventive or therapeutic strategies.

AIM AND OUTLINE OF THE CURRENT THESIS

The aim of this thesis is 1) to investigate the pathogenesis of ITBL and 2) to investigate the use of potential novel biomarkers for early identification of hepatic injury and ITBL. Accordingly, this thesis is divided into two sections.

Section 1 focuses on novel insights into the role of vasculature in the development of ITBL. In chapter 2 of this thesis an elaborate histological study in liver biopsies taken at time of transplantion is described. Data shows that changes occur in the intrahepatic portal vein branches between cold ischemia and reperfusion, namely that the portal vein branches seem to constrict in the ITBL group, suggesting a decreased portal flow and possible persisting biliary ischemia leading to ITBL. In chapter 3 the hypothesis that the portal vein is also responsible for flow in the PBP, and thereby oxygenation of the bile

ducts, is further investigated through laser Doppler flowmetry and reflectance spectrophotometry of the PBP during clamping of blood vessels. Data indeed shows that the portal vein is responsible for significant contribution to the blood flow through the extra hepatic PBP and could therefore be important in development of ITBL. Chapter 4 describes clinical evidence highly suggesting the role of the portal vein in development of ITBL after liver transplantation. It is shown that patients developing partial intrahepatic portal vein thrombosis develop ITBL specifically in the segments affected by the portal vein thrombosis. These findings in section I together suggest that the portal blood flow, in contrast to popular belief, can be of importance in the pathogenesis of ITBL.

Section 2 of the thesis focuses on the use of microRNAs as novel biomarkers for detection of liver injury in the setting of liver transplantation. In chapter 5 an overview of literature is given describing the use of (circulating) microRNAs as biomarkers in liver transplantation and disease. Furthermore the potential therapeutic use of microRNAs in the setting of liver transplantation is discussed. Chapter 6 demonstrates the use of stable, circulating microRNAs in serum as specific, sensitive and early markers of liver injury and acute rejection after liver transplantation, proving their potential as novel biomarkers of liver injury in liver transplantation. In chapter 7 the presence of microRNAs in bile is demonstrated. In addition it is shown through various in vivo and in vitro experiments that the excretion of these microRNAs is an active process, affected by liver injury and function, rather than general leakage. This makes microRNAs suitable not only for use as biomarkers but also for studying their biological, potentially therapeutic, effects. Chapter 8 describes the presence of microRNAs in perfusates used for flushing liver grafts and the diagnostic potential of these released microRNAs for sensitively and specifically identifying grafts later developing ITBL.

Finally in chapter 9, the results presented in this thesis are summarized and discussed.

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Section I

New insights in the role of the portal circulation

Relationship between the histological appearance of the portal vein and development of ischemic-type biliary lesions after liver transplantation

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ABSTRACT

Ischemic-type biliary lesions (ITBL) are a major cause of morbidity after liver transplantation (LT). Their assumed underlying pathophysiological mechanism is ischemia-reperfusion injury of the biliary tree, for which a role of the portal circulation has been proposed recently. The aim of this study was to investigate whether early histological, particularly portal venous, changes predispose for ITBL.

A case-control study was performed in 22 LT recipients, by retrospectively assessing more than 30 histological parameters, in 44 intraoperative liver biopsies taken after cold ischemia (t=0) and portal reperfusion (t=1). Eleven grafts developed ITBL requiring retransplantation (ITBL group) and 11 matched controls had normal functioning grafts on average 11 years after LT (non-ITBL group). Additionally, 11 liver biopsies, from hemihepatectomies performed for metastases of colorectal cancer, were assessed similarly (CRC group).

Analyses showed no significant histological differences at t=0 between the ITBL and the non-ITBL group. However, the t=1 biopsies of the ITBL group showed smaller portal vein branches (PVB) significantly more often compared to the non-ITBL group, which also showed persisting paraportal collateral vessels. Larger PVB and paraportal collateral vessels were also found in the CRC group. Morphometric analysis confirmed these findings, showing measurements of the PVB were significantly smaller in the ITBL group at t=1 compared to itself at t=0, the non-ITBL and CRC group (measuring largest in the CRC group). Thus dimensions of the PVB decreased in the ITBL group when compared to the t=0 biopsies and were significantly smaller at t=1 compared to the non-ITBL and CRC group.

In conclusion, smaller PVB lumen size in post-reperfusion biopsies of liver grafts, suggesting a relative decreased portal blood flow, is associated with a higher incidence of ITBL. These findings support recent clinical studies suggesting a possible pathophysiologic role of portal blood flow in the oxygenation of the biliary tree after LT.

INTRODUCTION

Biliary complications are one of the leading causes of morbidity and mortality after liver transplantation (LT) and are therefore often considered the Achilles' heel of LT[1-8]. In particular, ischemic-type biliary lesions (ITBL) represent the most troublesome biliary complication due to their resistance to therapy[5-10]. ITBL is a non-technical or surgical biliary complication usually occurring within the first year of LT and is characterized by diffuse hilar or intrahepatic non-anastomotic biliary strictures, dilatations and necrotic cast formations in the bile ducts without impairment of arterial blood flow[3, 11].

Incidences of ITBL of up to 26% have been reported in literature[3]. Even higher incidences of over 50% have been reported for grafts with cold ischemia times exceeding 13 hours and grafts donated after cardiac death (DCD grafts) suggesting an important role for pretransplant graft ischemia[2, 12]. Further evidence for the role of ischemia is provided by the fact that ITBL strongly resembles the ischemic biliary lesions found after hepatic artery thrombosis in LT patients. In addition to prolonged ischemia times, several other risk factors for the development of ITBL have been identified, including bile salt toxicity, inadequate flushing of the graft and the peribiliary plexus (PBP) during organ procurement, blood group incompatibility, and preexisting autoimmune disease in the recipient[2, 6, 7, 10, 12-16]. The real pathogenesis of ITBL however is still largely unknown[7].

Ischemia or ischemia-reperfusion injury (IRI) remains the major topic of interest since it appears to be the most important risk factor for the development of ITBL. Recent studies shed new light on the pathogenesis of ITBL. They suggest, in contrast to popular belief, that the portal venous blood supply to the biliary system, additional to hepatic arterial blood flow might also play a role in biliary oxygenation and thus in development of ITBL after LT[17, 18].

The aim of this study was to discover early histological changes, in particular in the hepatic vasculature, in liver graft biopsies predisposing to the development of ITBL after LT in order to shed more light on the pathogenesis of ITBL and the potential role of the portal circulation therein.

MATERIALS AND METHODS

Patients

During LT at our center, all grafts are biopsied at the end of cold ischemia (t=0 biopsy) and one hour after portal reperfusion (t=1 biopsy) in the form of a peripheral liver wedge biopsy. For this study twenty-two t=0 and twenty-two t=1 biopsies were examined from two groups of eleven case-control matched patients, which were identified using the hospital's general electronic and liver transplant databases in the period of 1997 until

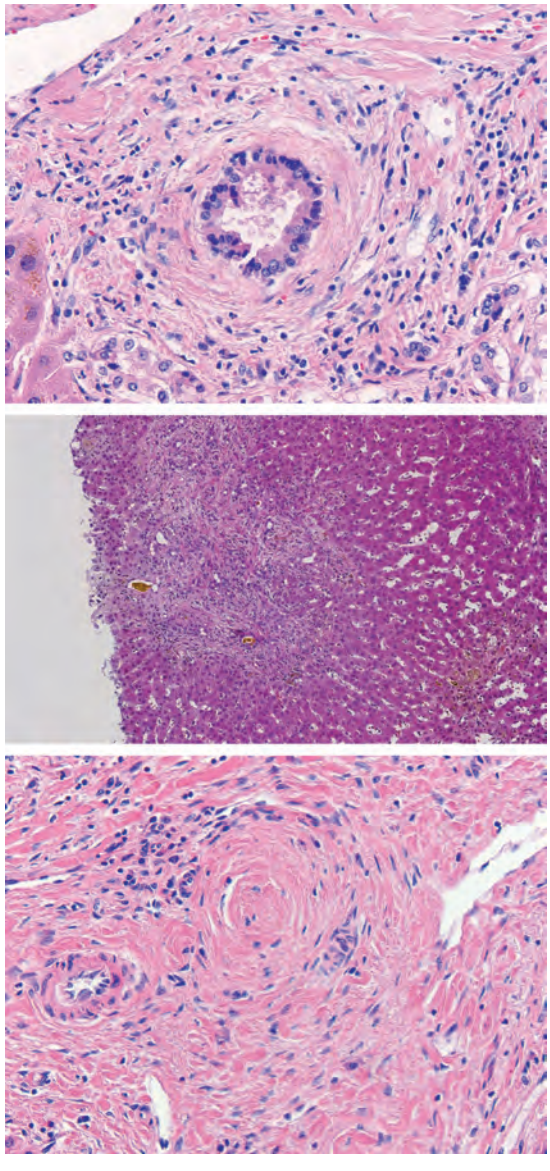

Figure 1. Explant biopsies are shown from explanted liver transplants developing ITBL. Although predominantly a clinical diagnosis, these biopsies show disruptment of biliary epithelium and detachment of it from the underlying layers (top), bile stasis, ductular reaction and inflammation (middle), and development of concentric fibrosis with even complete replacement of the bile ducts by fibrosis (bottom).

2005. Only grafts that developed ITBL assuredly and required retransplantation or were completely impeccable and complication-free on the basis of clinical, laboratory and radiological test were selected for this analysis. Eleven grafts were identified, which developed diffuse ITBL, on the basis of clinical and radiological findings, and consequently required retransplantation (ITBL group). After retransplantation the diagnosis of ITBL was reconfirmed by an experienced pathologist (P.E.Z.), who was unaware of the presumed diagnosis, through histological examination of the explanted allograft (Fig. 1).

Eleven other allografts (non-ITBL group), which did not develop ITBL, were from case-control matched patients and functioned impeccably and complication-free during the on average follow-up of 11 years (range 9 - 15 years). ITBL was defined as diffuse hilar or intrahepatic non-anastomotic biliary strictures with or without accompanying prestenotic biliary dilatations and necrotic cast formations in the absence of any other plausible cause of the biliary complications, such as recurrent biliary disease, hepatic artery thrombosis or chronic rejection. An attempt was made to match the non-ITBL group with the ITBL group for cold and warm ischemia time, donor and recipient age, blood group, gender, primary diagnosis, type of biliary reconstruction, and preservation solution used during organ procurement. An additional control group consisted of 11 patients who underwent right hemihepatectomy for liver metastasis of colorectal cancer (CRC group) after first-line chemotherapy for colorectal metastases, which consisted of a regimen of oxaliplatin and capecitabine (XELOX). Liver wedge biopsies were obtained from the normal part of the right hemi-liver specimen postoperatively. Those liver wedges biopsies were processed and analyzed in an identical fashion as the other wedges. The Medical Ethical Council of the Erasmus MC approved the use of human samples and all patients provided informed consent for the use of materials for medical research.

Histological analyses

All 55 biopsies were routinely fixed in formalin, parafinated, cut into 5 μm sections, and stained with Hematoxylin and Eosin. Blinded histological assessment was performed by the pathologist (P.E.Z.) and the first author (W.R.R.F.). Evaluation of the sections included assessment of the complete section and then specifically the liver parenchyma and central hepatic vein, as well as bile duct, hepatic artery and portal vein branches in the portal triad. In general for every biopsy inflammation, edema, necrosis, Kupffer cell activation, steatosis (Gr0: < 5%, Gr1: 6-33%, Gr2: 34-66%, Gr3: > 66%), Metavir fibrosis score (F0: none, F1: portal fibrosis without septa, F2: portal fibrosis with septa, F3: numerous septa without cirrhosis, and F4: cirrhosis)[19], vasculitis, luminal obstruction, and dilatation or structuring of different microscopic anatomical structures were assessed. Additionally, specific points were assessed for different anatomical regions, such as, the presence of perivenular fibrosis of the central vein, interface hepatitis between portal triads, cholangiocyte necrosis or detachment in the bile ducts, thickening of the hepatic artery wall, and presence of paraportal collateral vessels (defined as presence of primitive sinusoidal-like lumen originating from the portal vein and protruding into the paraportal tissue nearby) or dilatation of the portal vein branches (relative to the surrounding structures). Details of factors evaluated for the different anatomical regions of the liver are described in Table 1.

	Scoring Options				
Liver Parenchyma	0	1	2	3	4
General					
Inflammation	None	Mild	Moderate	Severe	
Edema	No	Yes			
Hepatocyte necrosis	None	Individual	Confluent	Combined	
Activated Kupffer cells	No	Yes			
Intracellular bile stasis	No	Yes			
Steatosis	Grade 0	Grade 1	Grade 2	Grade 3	
Fibrosis (Metavir)	F0	F1	F2	F3	F4
Central vein					
Vasculitis	No	Yes			
Luminal obstruction/thrombosis	No	Yes			
Dilated sinusoids	No	Yes			
Perivenular fibrosis	No	Yes			
Portal triad					
General					
Vasculitis	No	Yes			
Vascular necrosis	None	Mild	Moderate	Severe	
Interface hepatitis	None	Mild	Moderate	Severe	
Bile ducts					
Pericholangiolar edema	No	Yes			
Cholangitis	None	Mild	Moderate	Severe	
Cholangiocyte necrosis	No	Yes			
Detached cholangiocytes	No	Yes			
Pericholangiolar fibrosis	No	Yes			
Ductular reaction	No	Yes			
Ductopenia	No	Trend	Yes		
HABs					
Luminal obstruction/thrombosis	No	Yes			
Arterial wall thickening	No	Yes			
PVBs					
Luminal obstruction/thrombosis	No	Yes			
Paraportal collateral vessels	No	Yes			
Dilated branches	No	Yes			

Table 1. The details of all points assessed in the biopsies and their scoring options are listed in this table.

Morphometrical analyses

In addition to this assessment, blinded morphometrical measurements of the surface areas of the lumen of the bile duct branches (BDB), hepatic artery branches (HAB), and the portal vein branches (PVB) in the portal triad, and the total surface of the portal triads area (PTA) were conducted. Measurements were taken from three random locations for every section using Leica QWin image analysis software (Leica Microsystems, Cambridge, United Kingdom). The three measurements for each section were added to the database and linked to the corresponding sections of liver tissue. The mean BDB, HAB, PVB and PTA were calculated for every section and used for statistical analysis. Additionally, ratios between all measurements of every portal triad location were calculated and used for analysis in order to exclude findings due to coincidence and to correct for the difference in cutting angles of the biopsy slices and the structures within it.

Statistics

All acquired data from these assessments were standardized and entered into a database using SPSS version 15.01. Statistical analyses were carried out using version 15.0.1

of SPSS and GraphPad Prism 5.0. Analyses of the data obtained from the histological assessment were performed using Fisher's exact test. Morphometrical data were analyzed by Mann-Whitney U test. When analyzing the histological and morphometrical data in a three-group comparison, comparing the ITBL, non-ITBL and CRC groups at t=0 and t=1, the Chi-squared test (exact method) and the Kruskall-Wallis test were used. These test were followed up by a two-group Fisher's exact or Mann-Whitney U test to determine the exact nature of the difference between the three groups. Differences were considered statistically significant when P-values were less than 0.05.

RESULTS

Patient Demographics

No differences were noted between the ITBL and non-ITBL group with respect to indications for transplantation, donor and recipient gender, BMI, and blood group (Table 2). As for known risk factors for ITBL, there were no differences in the type of preservation solutions used, no blood group incompatible grafts were transplanted, cold and warm ischemia times were not significantly different between the two groups and types of biliary anastomosis used were similar. Also no difference in the incidence of primary sclerosing cholangitis or autoimmune hepatitis between the recipients of the two groups was noted. The age of the CRC group patients however was significantly higher than the recipient age. Additionally, despite efforts to match the groups, donor age in the non-

	ITBL Group	Non-ITBL Group	CRC Group	*P* Value
Recipient/CRC patient				
Age (years)	44 ± 4	51 ± 2	63 ± 3	<0.005
Sex: male/female (n/n)	10/1	5/6	8/4	NS
Body mass index (kg/m^2)	24.7 ± 2.0	25.3 ± 1.2	—	NS
Blood group: A/B/AB/O (n/n/n/n)	7/0/0/4	5/1/0/5	4/0/2/5	NS
Indication (n)				NS
Cholestatic	6	4		
Viral	2	3		
Cryptogenic	2	1		
Other	1	3		
Donor				
Age (years)	51 ± 2	34 ± 5	—	<0.05
Sex: male/female (n/n)	5/6	5/6	—	NS
Body mass index (kg/m^2)	23.6 ± 0.8	23.8 ± 0.9	—	NS
Blood group: A/B/AB/O (n/n/n/n)	7/0/0/4	5/1/0/5	—	NS
Graft				
Preservation solution: UW/HTK (n/n)	9/2	10/1	—	NS
Cold ischemia time (minutes)	516 ± 44	579 ± 61	—	NS
Warm ischemia time (minutes)	37 ± 7	50 ± 7	—	NS
Biliary anastomosis (n)				NS
Choledochocholedochostomy	7	9	—	
Choledochojejunostomy	4	2	—	

Table 2. Donor, recipient and graft characteristics of the ITBL and non-ITBL group are portrayed. Despite efforts to match the two groups as much as possible, grafts from significantly younger donors were transplanted in the non-ITBL group. In addition characteristics of the CRC group are also listed. Numerical data are presented as mean ± standard error of the mean for age, BMI, and cold and warm ischemia times.

ITBL group was significantly lower compared to the donors of the ITBL group (Table 2). It is important to note that none of the patients in either group showed any signs of hepatic artery, portal vein or hepatic vein thrombosis during Doppler ultrasonographic follow-up, which is part of the normal post-transplant clinical follow-up.

Histological Assessment

The diagnosis of ITBL was reconfirmed in the ITBL group by examining the affected liver grafts after explantation during retransplantation. Arterial, portal venous or hepatic venous thrombi were not found in any of the explants, thereby excluding the possibility of biliary complications due to circulatory impairment.

Analysis of t=0 biopsies taken at the end of cold ischemia time did not show any significant differences between the ITBL and non-ITBL group with respect to any of the assessed parameters in the liver parenchyma, central vein and portal triad areas. Mild inflammation and activation of Kupffer cells were observed in most t=0 biopsies in both the ITBL and non-ITBL group when assessing the general parenchyma. As were dilated sinusoids in the central vein area. In the portal vein area circulatory disturbances characterized by large PVB with accompanying paraportal collateral vessels were noted in most t=0 biopsies in the ITBL and non-ITBL group (Table 3). Only sporadic abnormalities of the other assessed parameters were found in some t=0 biopsies. All details of the assessment of the t=0 biopsies are summarized in Table 3.

As in the t=0 biopsies, no significant differences were found between the ITBL and non-ITBL group when assessing the general parenchyma in the t=1 biopsies, taken one hour after reperfusion. Again mild inflammation and activated Kupffer cells were also observed in most t=1 biopsies in both groups. Similarly, dilated sinusoids in the central vein area were again noted and not significantly different between the ITBL and non-ITBL group. In contrast however, histological evaluation of t=1 biopsies, showed a significant difference between ITBL and non-ITBL group. In the t=1 biopsies, large diameter PVBs (Fig. 2) accompanied by paraportal collateral vessels in the portal triad (Fig. 2C), persisted almost exclusively in the non-ITBL group, while they seemed to resolve in the ITBL group. In the non-ITBL group, 7 out of 11 t=1 biopsies presented with large PVBs. All 11 t=1 biopsies of patients in the non-ITBL group presented with paraportal collateral vessels, in at least one of the analyzed portal triads, against only one t=1 biopsy from the ITBL group ($P < 0.02$ and $P < 0.01$, respectively), which exhibited both enlarged PVBs and paraportal collateral vessels. Again, as in t=0 biopsies, other abnormalities were only noted sporadically in the t=1 biopsies (Table 3). All results of the histological assessment are detailed in Table 3.

The histological assessment of the CRC group showed a comparable pattern. Mild to moderate inflammation was seen in all samples, while activated Kupffer cells were

	Scoring for Time 0 ITBL/Non-ITBL Biopsy Samples						Scoring for Time 1 ITBL/Non-ITBL Biopsy Samples					
Liver Parenchyma	0	1	2	3	4	*P* Value	0	1	2	3	4	*P* Value
General												
Inflammation	0/1	11/8	0/1	0/1		0.21	0/0	8/7	3/2	0/2		0.64
Edema	10/8	1/3				0.59	10/9	1/2				>0.99
Hepatocyte necrosis	7/9	3/1	1/0	0/1		0.44	4/7	6/2	1/0	0/2		0.12
Activated Kupffer cells	0/0	11/11				—	0/0	11/11				—
Intracellular bile stasis	11/8	0/3				0.21	9/9	2/2				>0.99
Steatosis	7/5	2/5	1/0	1/1		0.66	3/2	6/7	2/2	0/0		>0.99
Fibrosis (Metavir)	9/11	2/0	0/0	0/0	0/0	0.48	10/11	1/0	0/0	0/0	0/0	>0.99
Central vein												
Vasculitis	9/10	2/1				>0.99	10/10	1/1				>0.99
Luminal obstruction/thrombosis	11/11	0/0				—	11/10	0/1				>0.99
Dilated sinusoids	3/3	8/8				>0.99	4/1	7/10				0.31
Perivenular fibrosis	10/9	1/2				>0.99	10/11	1/0				>0.99
Portal triad												
General												
Vasculitis	11/10	0/1				>0.99	11/11	0/0				—
Vascular necrosis	11/11	0/0	0/0	0/0		—	10/11	1/0	0/0	0/0		>0.99
Interface hepatitis	8/8	3/2	0/1	0/0		>0.99	11/7	0/4	0/0	0/0		0.90
Bile ducts												
Pericholangiolar edema	11/11	0/0				—	10/11	1/0				>0.99
Cholangitis	11/10	0/1	0/0	0/0		>0.99	8/11	3/0	0/0	0/0		0.21
Cholangiocyte necrosis	11/11	0/0				—	11/11	0/0				—
Detached cholangiocytes	11/9	0/2				0.48	10/11	1/0				>0.99
Pericholangiolar fibrosis	8/9	3/2				>0.99	8/10	3/1				0.59
Ductular reaction	11/11	0/0				—	10/11	1/0				>0.99
Ductopenia	11/11	0/0	0/0			—	11/11	0/0	0/0			—
HABs												
Luminal obstruction/thrombosis	11/11	0/0				—	11/11	0/0				—
Arterial wall thickening	10/10	1/1				>0.99	10/10	1/1				>0.99
PVBs												
Luminal obstruction/thrombosis	11/11	0/0				—	11/11	0/0				—
Paraportal collateral vessels	6/2	5/9				0.18	10/0	1/11				<0.01
Dilated branches	3/5	8/6				0.66	10/4	1/7				0.02

Table 3. Results of the biopsy assessment, according to the scoring system presented in Table 1, are summarized for the ITBL and non-ITBL groups. The number of biopsy samples for ITBL and non-ITBL groups is cited for every paramater, and the scoring options are presented for the time 0 and time 1 biopsy samples.

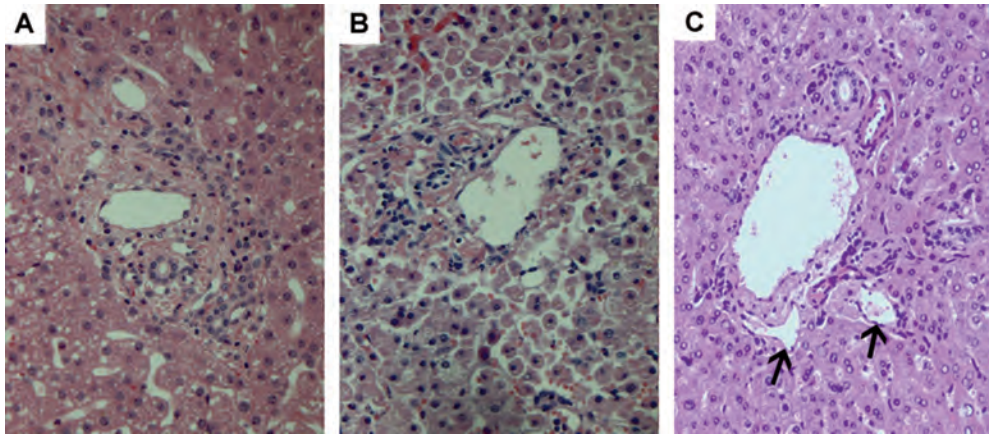

Figure 2. Two representative post-reperfusion allograft biopsies, one from the ITBL (A) and one from the non-ITBL group (B), are shown. The lumen of the portal vein branch of the non-ITBL group is larger compared to the biopsy from the group later developing ITBL. (C) Histology of one of the livers from the CRC group showing multiple paraportal collateral vessels (marked by arrows). These vessels are near, but clearly outside of the, portal boundaries and have a thin wall compared to the hepatic artery.

observed in all CRC biopsies. Similarly, dilated sinusoids in the central vein area were noted in 6 of the CRC biopsies. However, none of the analyzed parameters were significantly different from the ITBL and non-ITBL group at t=0 when conducting a three-group statistical analysis (Table 4). When comparing the CRC group with the ITBL and non-ITBL group, using a three-group statistical test, at t=1 there was a difference concerning interface hepatitis ($P = 0.04$). A follow up two-group test showed that mild interface hepatitis appeared to be more frequent in in the CRC group compared to the ITBL group at t=1 ($P < 0.05$; Table 4). However, a more apparent and statistical significant difference, in the three-group analysis, was the presence of paraportal collateral vessels and dilated PVB in the CRC group compared to the t=1 biopsies ($P < 0.005$). As in the non-ITBL group, the CRC group showed paraportal collateral vessels and dilated PVB. Seven and 9 out of 11 CRC biopsies showed paraportal collateral vessels and dilated PVB respectively, compared to only 1 out of 11 in the ITBL group at t=1 ($P < 0.05$ and $P < 0.005$, respectively).

Morphometrical Assessment

To further quantify and confirm the histological findings additional morphometrical measurements were conducted. Morphometrical analysis of raw data from the t=0 biopsies did not show any significant differences between the ITBL and non-ITBL group. The mean lumen surface area of the BDB was 77 $\mu m^2 \pm 7$ (mean ± SEM) vs. 64 $\mu m^2 \pm 10$ for the ITBL and non-ITBL group, respectively ($P = 0.06$). Similarly the mean lumen surface area of the HAB was 143 $\mu m^2 \pm 19$ vs. 139 $\mu m^2 \pm 29$ ($P = 0.67$). The mean surface lumen area of

	Scoring for CRC Biopsy Samples (n = 11)					Time 0			Time 1		
Liver Parenchyma	0	1	2	3	4	*P* Value	P^{ITBL} Value	$P^{non\text{-}ITBL}$ Value	*P* Value	P^{ITBL} Value	$P^{non\text{-}ITBL}$ Value
General											
Inflammation	0	10	1	0		0.45			0.38		
Edema	9	2				0.79			>0.99		
Hepatocyte necrosis	8	2	1	0		0.85			0.15		
Activated Kupffer cells	0	11				—			—		
Intracellular bile stasis	11	0				0.91			0.51		
Steatosis	2	6	3	0		0.11			>0.99		
Fibrosis (Metavir)	9	2	0	0	0	0.51			0.76		
Central vein											
Vasculitis	10	1				>0.99			>0.99		
Luminal obstruction/thrombosis	11	0				—			>0.99		
Dilated sinusoids	5	6				0.72			0.24		
Perivenular fibrosis	10	1				>0.99			>0.99		
Portal triad											
General											
Vasculitis	11	0				>0.99			—		
Vascular necrosis	11	0	0	0		—			>0.99		
Interface hepatitis	6	5	0	0		0.52			0.04	0.04	>0.99
Bile ducts											
Pericholangiolar edema	11	0				—			>0.99		
Cholangitis	11	0	0	0		>0.99			0.09		
Cholangiocyte necrosis	11	0				—			—		
Detached cholangiocytes	10	1				0.76			>0.99		
Pericholangiolar fibrosis	5	6				0.27			0.09		
Ductular reaction	11	0				—			>0.99		
Ductopenia	11	0	0			—			—		
HABs											
Luminal obstruction/thrombosis	11	0				—			—		
Arterial wall thickening	6	5				0.16			0.16		
PVBs											
Luminal obstruction/thrombosis	11	0				—			—		
Paraportal collateral vessels	4	7				0.27			<0.001	0.02	0.09
Dilated branches	2	9				0.52			0.002	<0.005	0.64

Table 4. Results of the biopsy assessment, according to the scoring system presented in Table 1, are summarized for the CRC group. The number of biopsy samples is cited for every parameter, and their scoring is presented. P values are presented first for the 3-group comparison with the time 0 and time 1 groups. If there is statical significance, additional P values are presented for the2-group analyses with the CRC group.

the PVB was 4535 μm^2 ± 499 vs. 4317 μm^2 ± 823 (P = 0.48). Finally the mean surface of the PTA measured 26091 μm^2 ± 3475 vs. 21603 μm^2 ± 4079 in the ITBL and non-ITBL group, respectively (P = 0.42; Fig. 3A).

Analysis of the raw data from the t=1 biopsies showed that the mean lumen surface area of the BDB was 81 μm^2 ± 6 (mean ± SEM) vs. 64 μm^2 ± 11 in the ITBL and non-ITBL group respectively (P = 0.06). The mean lumen surface area of the HAB in t=1 biopsies measured 121 μm^2 ± 7 vs. 128 μm^2 ± 35 (P = 0.25). There was no significant difference in these measurements between the ITBL and non-ITBL group. However, measurements of the PVB in the portal triad showed that the mean lumen surface area in the ITBL group was significantly smaller than that of the non-ITBL group. Mean surface area in the ITBL group measured 2822 μm^2 ± 381 while surface areas in the non-ITBL group measured 4619 μm^2 ± 447 (P = 0.004), thereby confirming earlier histological findings. Finally, the portal

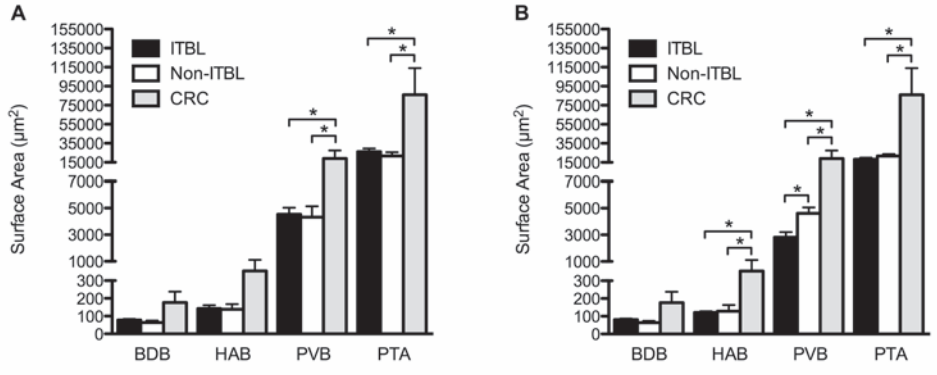

Figure 3. Raw surface area measurements (mean ± SEM) of bile duct branches (BDB), hepatic artery branches (HAB), portal vein branches (PVB), and total portal triad area (PTA) are shown for the (A) t=0 and (B) t=1 biopsies compared between the ITBL, non-ITBL group and the CRC group. Compared to the CRC group the lumen surface area of the PVB significantly smaller in the ITBL and non-ITBL group ($P < 0.01$ for both). Similarly, also the PTA was significantly larger in the CRC group compared to the ITBL and non-ITBL group ($P < 0.01$ and $P < 0.005$ respectively) at t=0. At t=1 the HAB surface lumen was significantly larger in in the CRC group compared to the ITBL and non-ITBL group ($P < 0.05$ for both). Also the PVB lumen surface was significantly larger in the CRC group compared to the ITBL and non-ITBL group ($P < 0.001$ and $P < 0.005$ respectively). In addition, the PTA was also significantly larger in the CRC group compared to the ITBL and non-ITBL group ($P < 0.001$ and $P < 0.005$ respectively). The only difference observed between the ITBL and non-ITBL group was a significantly smaller PVB in t=1 biopsies of patients later developing ITBL ($P < 0.005$).

triad surface area, measuring 18218 $\mu m^2 \pm 1731$ vs. 21672 $\mu m^2 \pm 2052$, was not significantly smaller in the ITBL group ($P = 0.12$; Fig. 3B).

In a three-group comparison, comparing the raw data of the ITBL and non-ITBL groups at t=0 with the CRC group, mean lumen surface area of the BDB and HAB was 176 $\mu m^2 \pm 62$ (mean ± SEM) and 702 $\mu m^2 \pm 409$ respectively in the CRC group, and this did not differ significantly from the ITBL or non-ITBL group ($P = 0.06$ and $P = 0.16$ for BDB and HAB, respectively). The mean lumen area of the PVB was 19090 $\mu m^2 \pm 8315$ and this differed significantly in the three-group analysis ($P < 0.01$). Additional two-group analysis showed that the mean PVB lumen surface was significantly larger in the CRC group than in both the ITBL ($P < 0.01$) and non-ITBL group ($P < 0.01$). Also the mean surface of the PTA, which measured 85984 $\mu m^2 \pm 28010$, was significantly different in the three-group analysis at t=0 ($P < 0.005$; Fig. 3A). Follow-up two-group analysis showed that the PTA surface area was significantly larger in the CRC group compared to both the ITBL ($P < 0.01$) and non-ITBL group ($P < 0.005$) at t=0.

When comparing the ITBL, non-ITBL and CRC groups at t=1, in a three-group analysis, it was found that the lumen surface area of the BDB did not differ significantly when comparing to the CRC group ($P = 0.051$; Fig. 3B). However, HAB lumen surface ($P < 0.05$), PVB lumen surface ($P < 0.001$) and PTA ($P < 0.001$) were all significantly different in the three-group analysis. Follow up two-group analysis showed that the difference for HAB

lumen was attributable to its significant larger size in the CRC group compared to both the ITBL ($P < 0.05$) and non-ITBL group ($P < 0.05$). Also for the PVB lumen surface the difference, in the three-group analysis, was attributable to both the ITBL ($P < 0.001$) and non-ITBL group ($P < 0.005$), which had both smaller lumen sizes compared to the CRC group. Similarly, also the difference for PTA could be attributed to both the ITBL ($P < 0.001$) and non-ITBL group ($P < 0.005$), which were significantly smaller than in the CRC group at t=1 (Fig. 3B).

Additional analyses conducted to confirm and correct the raw data for possible difference in slicing angles by using ratio's between different structures showed, that ratios between different hepatic structures in the ITBL and non-ITBL group were not significantly different in the t=0 biopsies. Ratios were, 197 ± 41 vs. 173 ± 26 for PVB/BDB (mean ± SEM; $P = 0.85$), 101 ± 18 vs. 83 ± 14 for PVB/HAB ($P = 0.81$) and 0.19 ± 0.01 vs. 0.21 ± 0.02 for PVB/PTA ($P = 0.81$) for the t=0 biopsies of the ITBL and non-ITBL group, respectively (Fig. 4).

Ratios for the t=1 biopsies, however, differed significantly between the ITBL and non-ITBL group, confirming that earlier findings were due to specific differences in size of the PVB lumen area and not due to differences in general size of the whole portal triad in the non-ITBL group. The ratio PVB/BDB was 59 ± 20 (mean ± SEM) vs. 136 ± 19 ($P = 0.001$) for the ITBL and non-ITBL group, respectively. Similar significant differences were also found for the PVB/HAB ratio, which measured 34 ± 7 vs. 88 ± 12 ($P = 0.002$) for the ITBL and non-ITBL group respectively. Finally the PVB/PTA ratio was also significantly smaller in the ITBL group and measured 0.18 ± 0.03 vs. 0.26 ± 0.03 ($P = 0.008$; Fig. 4).

Conducting a three-group statistical analysis, to compare the ratios between the ITBL, non-ITBL and CRC groups, showed there were no statistical significant differences for the

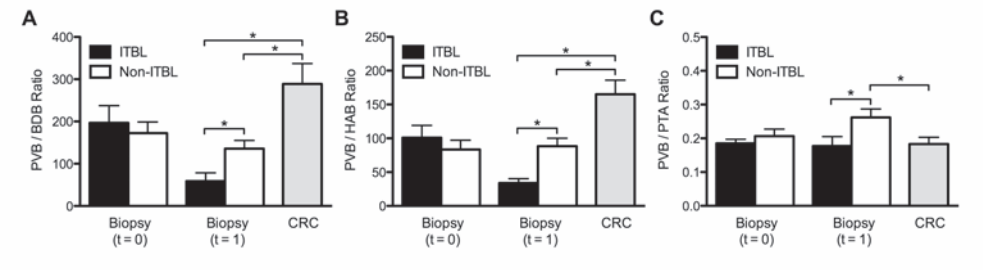

Figure 4. Ratios of (A) PVB/BDB, (B) PVB/HAB and (C) PVB/PTA are shown (mean ± SEM) to rule out that earlier findings were not due to coincidence and to normalize data for possible differences in slicing angles. As portrayed all three ratios were significantly decreased in the t=1 biopsies of the ITBL group ($P < 0.01$), confirming that the smaller PVB found in this group earlier were not caused by general size differences, but indeed by actual differences specifically in the size of the PVB. In addition the PVB/BDB and PVB/HAB ratios were larger in the CRC group compared to both the ITBL ($P < 0.001$ for both ratios) and non-ITBL group ($P < 0.01$ for both ratios) at t=1. Finally, the PVB/PTA ratio seemed to lower in the CRC group compared to the non-ITBL group only (marginally significant at $P < 0.05$). These data suggest a decrease in PVB size in both the ITBL and non-ITBL group compared to CRC. However, this decrease seems to be more prominent in the ITBL group.

PVB/BDB (P = 0.24), PVB/HAB (P = 0.053) and PVB/PTA (P = 0.77) ratios at t=0. A similar analysis with the t=1 biopsies showed that the PVB/BDB (P < 0.001), PVB/HAB (P < 0.001) and PVB/PTA (P < 0.05) ratios were significantly different between the three groups. Follow up two-group analysis showed that the PVB/BDB ratio was significantly larger compared to both the ITBL (P < 0.001) and non-ITBL group (P < 0.01) at t=1 (Fig. 4A). A similar analysis of the PVB/HAB ratio showed that the difference found in the three-group analysis of t=1 biopsies could again be attributed to a significant higher ratio in the CRC group compared to both the ITBL (P < 0.001) and the non-ITBL group (P < 0.01; Fig. 4B). Finally, analysis showed that the statistical significance found it the three-group test could be attributed only to a slightly significant lower PVB/PTA ratio in the CRC group compared to the non-ITBL group (P = 0.048; Fig. 4C).

None of these differences in surface lumen areas and surface ratios were associated with any other patient characteristics or other histologic parameters analyzed in this study, except the paraportal collateral vessels and large diameter PVB.

DISCUSSION

In the current study we show that liver graft biopsies taken intraoperatively exhibit significant histological differences between grafts that later do or do not develop ITBL. No histologic or morphometric differences were found between the biopsies of the ITBL and non-ITBL groups at the end of cold ischemia. However, smaller PVB lumen surface and absence of paraportal collateral vessels were noted one hour after reperfusion in grafts later developing ITBL. Compared to the CRC group, both the ITBL and non-ITBL group showed smaller PVB lumen size after reperfusion, however the size in the ITBL group was also significantly smaller than that of the non-ITBL group. The exact mechanism leading to these histopathological changes remains unclear. However, these findings could prove crucial in clarifying the pathogenesis and in preventing these high morbidity complications in future transplantations[1, 5, 8, 13, 20].

Recent studies suggest a pivotal role for the portal circulation in the development of biliary complications in at least a subgroup of patients after LT. They demonstrate significant contribution of the portal vein to the biliary microcirculation[17, 18]. Earlier studies support this role of the portal vein in biliary oxygenation by showing that the PBP communicates with portal branches through arterio-portal anastomoses, the so called internal roots, which deliver the same amount of oxygen as the hepatic artery to the biliary epithelium[21-23]. Additional support for the role of the portal vein in the pathogenesis of ITBL is implicated by a study demonstrating diminished enhancement of the bile duct wall in the portal phase of contrast-enhanced ultrasonography in grafts, which are affected by ITBL[24].

Large sized PVB and paraportal collateral vessels are known to occur in patients with cavernous portal transformation during acute and chronic portal hypertension[25-27]. Persisting cavernous transformation can lead to portal biliopathy resembling ITBL[26, 28, 29]. However, no incidences of arterial or hepatic venous thrombosis were observed in this series in the postoperative Doppler ultrasonography or in the histopathology of the explanted grafts affected by ITBL, which could have explained the larger PVB and paraportal collateral vessels observed. In addition, compared to the CRC group the lumen size of the PVB was actually smaller in both the ITBL and non-ITBL group after reperfusion, suggesting that the PVB, although visually appearing large, were actually strictured in the non-ITBL group and more so in the ITBL group compared to normal livers in the CRC group These strictured PVB could result in diminished portal flow and oxygenation of the liver and the biliary tree, leading to persistent ischemia or more profound ischemia-reperfusion injury and this could result in development of ITBL.

Alternatively, it could be hypothesized that the observed smaller PVB diameter represents the overall status of the liver graft and is accompanied by a similar decrease in size of the HAB and the PBP, resulting in the stagnation of flow, formation of micro thrombi and hypoxia, leading to persistent ischemia and subsequent development of ITBL. Evidence for this is provided by several studies demonstrating that enhanced clearance of the PBP using low viscosity preservation solutions[6, 8, 30-32], pressurized perfusion[3, 7] and infusion of fibrinolytic drugs during preservation[33] significantly reduces the incidence of ITBL. However, a difference in lumen size of the HAB was not observed between the ITBL and non-ITBL group, making this hypothesis less likely. Therefore we hypothesize that the strictured PVB in the ITBL group compared to the non-ITBL and CRC group leads to diminished portal flow and this consequently leads to development of ITBL.

The main limitation of the present study is the absence of true normal liver samples. Liver wedge biopsies in the CRC group were obtained from the normal part of the right hemi-liver specimen resected for liver metastasis of colorectal cancer. Although the analyzed wedge biopsies were taken at a distance from the tumor, and contained no tumor micro- or macroscopically, distant effects of the tumors and the chemotherapy regimen, especially on liver vasculature, cannot be excluded. However, for obvious medical-ethical reasons true normal livers could not biopsied for this study. Secondly, despite all efforts to match acceptor and donor characteristic, donor age in the non-ITBL group was significantly lower. High donor age is a known risk factor for the development of ITBL[3, 34, 35]. Although no relation has been described between the size of PVB and age differences in adults and no evidence in our study was found for such a relation, still this should be taken into consideration. Finally, the hypothesis that smaller PVB lead to diminished portal flow, and therefore results in ITBL, cannot be confirmed in

this study as no hemodynamic measurements were conducted. We therefore suggest detailed, possibly continuous, hemodynamic measurements of the portal, arterial and hepatic flow during and after transplantation as the next important step towards further unraveling the pathogenesis of ITBL.

In conclusion, our findings, although the underlying mechanism is not fully understood, suggest that smaller PVB lumen size in recirculation biopsies of liver grafts is associated with a higher incidence of ITBL. Our findings shed additional light on the possible role of the portal circulation in development of ITBL. Therefore, the portal circulation should be considered as a possible cause of biliary strictures when identifying patients with biliary complications of 'unknown' etiology. Furthermore, we argue that evaluation of reperfusion biopsies may serve as a criterion for a low-threshold policy during follow-up of patients to intervene at an earlier stage.

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Significant contribution of the portal vein to blood flow through the common bile duct

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3

ABSTRACT

Biliary complications are a common cause of graft loss after liver transplantation. The occurrence is, partly, attributed to hepatic artery thrombosis, which is considered to be the sole provider of blood flow to the bile ducts. However, the contribution of the portal vein and the gastroduodenal artery to the bile ducts is unknown.

Microvascular blood flow in the common bile duct (CBD) was determined in 15 patients who underwent a pancreaticoduodenectomy with a combination of laser Doppler flowmetry and reflectance spectrophotometry. Microvascular blood flow was measured at baseline, during clamping of the portal vein, during clamping of the hepatic artery, and during clamping of both. After transection of the CBD, these 4 measurements were repeated. The aim of this study was to determine the contribution of the hepatic artery, gastroduodenal artery, and portal vein to the microvascular blood flow in the CBD.

Compared with baseline measurements, the microvascular blood flow through the CBD decreased to 62% after clamping the portal vein, 51% after clamping the hepatic artery, and 31% after clamping both. After the CBD was transected, these 3 measurements were 60%, 31%, and 20%, respectively.

Historically, the hepatic artery has been considered mainly responsible for biliary blood flow. We show that after transection of the CBD, mimicking the situation after liver transplantation, the contribution of the portal vein to the microvascular blood flow through the CBD is 40%. This study emphasizes the importance of the portal vein, and disturbances in portal venous blood flow could contribute to the formation of biliary complications after liver transplantation.

INTRODUCTION

Biliary complications form a significant cause of morbidity and mortality in patients after liver transplantation[1–6]. A large proportion of these complications are due to biliary strictures, which can be found either at the anastomotic site or at other locations in the biliary tree of the graft[7]. Non-anastomotic strictures can occur after hepatic artery thrombosis[8,9] or with an open hepatic artery. Non-anastomotic strictures occurring with and without a patent hepatic artery share many radiological similarities and, thus, the latter have been called ischemic-type biliary lesions (ITBL). Donation after cardiac death (DCD) is a major risk factor for development of ITBL, probably due to increased ischemia/reperfusion injury[10] Ischemic-type biliary lesions may affect up to 25% of the recipients of a DCD graft, with significant graft loss and up to 50% retransplantation rate[11–14].

In recent years, there is increasing interest in impaired biliary microcirculation as a possible cause of ITBL[15, 16]. Intrahepatic and extrahepatic bile ducts have a unique anatomic feature in that they are provided with blood by a peribiliary capillary system, fed by both the portal vein and the hepatic artery from within the liver, and additionally by branches of the gastroduodenal artery in the hepatoduodenal ligament. Previous studies concluded that this peribiliary plexus (PBP) is exclusively provided with blood from the hepatic artery[17, 18]. However, some authors have raised questions regarding the role of the portal vein in the vascularization of the PBP[19–22]. Furthermore, these studies did not take into account the fact that the hepatoduodenal ligament is completely divided during liver transplantation leading to cessation of the contribution of the gastroduodenal artery to the PBP blood flow of the graft. In this situation, disturbances in the portal venous blood supply may indeed become important as a possible cause of the development of ITBL.

The aim of this study was to determine the contribution of the hepatic artery, gastroduodenal artery, and portal vein to the microvascular blood flow in the common bile duct (CBD).

METHODS

This research protocol was approved by the medical ethical committee of the Erasmus Medical Center, Rotterdam. Fifteen patients undergoing a standard pylorus preserving pancreaticoduodenectomy (PPPD) for cancer of the pancreatic head were included. During this operation, the CBD is transected as it is in liver transplantation, mimicking the lack of arterial supply through the hepatoduodenal ligament. Patients undergoing a PPPD for cholangiocarcinoma were excluded from this study. Flow measurements were

carried out after exploration of the hepatoduodenal ligament. The gastroduodenal artery, common hepatic artery, and portal vein were identified, isolated, and slinged, and the gall bladder was routinely removed before measurements. The gastroduodenal artery was not ligated in any patient before the flow measurements. None of the patients included in this study exhibited lymph node enlargement or tumor involvement of the hepatoduodenal ligament. In patients with aberrant arterial anatomy, all arterial branches were isolated and slinged separately to ensure complete arterial clamping.

Flow measurements

The microvascular blood flow in the CBD was determined using the O2C (Lea Medizin Technik, Giesen, Germany). This device combines 2 optical techniques in one optic fiber: laser Doppler flowmetry and reflectance spectrophotometry. In this study, a flat probe LF-1 was used, with a measurement depth of 4 to 6 mm. Using laser Doppler flowmetry, microvascular blood flow is determined by analysis of the power spectra from moving blood cells generated by Doppler frequencies of backscattered laser light (820 nm). The microvascular blood flow value is defined mathematically as the first moment of the Doppler power spectra, so it relates to the velocity of the erythrocytes multiplied by the number of moving erythrocytes. The blood flow is calculated, but not actually measured, as the surface area of the vessels is not known and is, therefore, expressed in arbitrary units (AU). Applying a flow probe on tissue may cause compression and alter the findings of flow measurement. The O2C (Lea Medizin Technik) probe is designed to correct for compression that can alter the findings of flow measurement by demonstrating the hemoglobin oxygen saturation (Hb saO_2) graphics during measurement. A typical 2-wave figure must be present at 540 and 580 nm, otherwise measurement could be influenced by external compression.

The probe was positioned on the proximal CBD, at the hilum of the liver, about 2 to 3 cm proximal from the transection surface of the CBD. The overlying peritoneum was opened up to the liver and surrounding tissue was dissected, but the CBD was not completely cleaned off. Microvascular blood flow of the CBD was measured under 8 predefined conditions. Measurements were performed at baseline with intact CBD. Subsequently, measurements were taken during temporary clamping of the portal vein, the common hepatic artery, or both. Microvascular blood flow was recorded for 30 seconds, yielding 15 values. After each measurement, the vascular clamp and probe were removed and a 2-minute rest period allowed reperfusion. Next, the CBD was transected, without any additional dissection, and 5 minutes were allowed to reach a new equilibrium. Subsequently, after transection of the CBD, the probe was placed on the proximal CBD, at the hilum of the liver, and a new baseline situation was measured. Measurements were performed while temporarily clamping the portal vein, the common

hepatic artery, or both. During microvascular blood flow measurements, mean arterial pressure, and heart rate were registered.

Statistics

For each patient the baseline situation was set at 100% and the relative change in microvascular blood flow with respect to baseline was calculated per time point (clamping of the portal vein, clamping of the hepatic artery, clamping of both, and after transection of the CBD). After transection of the CBD, the new baseline measurement of the CBD was set at 100% and relative changes with respect to this value were again calculated for the 3 time points during sequential clamping of the vessels mentioned earlier. Data are presented as means ± standard error of means (SEM). Statistical analysis of the difference between the hemodynamic parameters before and after transection of the CBD was performed with the Wilcoxon signed rank test. Statistical analysis of the differences in percent changes in microvascular blood flow between baseline and clamping of the portal vein, common hepatic artery, or both were calculated with a Mann-Whitney *U* test.

RESULTS

In the intact CBD, temporary clamping of the portal vein resulted in a decrease in microvascular blood flow to 62% of the baseline value. After arterial clamping, microvascular blood flow decreased to 51% of the baseline value. When both portal vein and hepatic artery were clamped simultaneously, microvascular blood flow decreased to 31% of the baseline value. All of these relative changes in blood flow compared with baseline measurements were statistically significant ($P < 0.05$; Fig. 1).

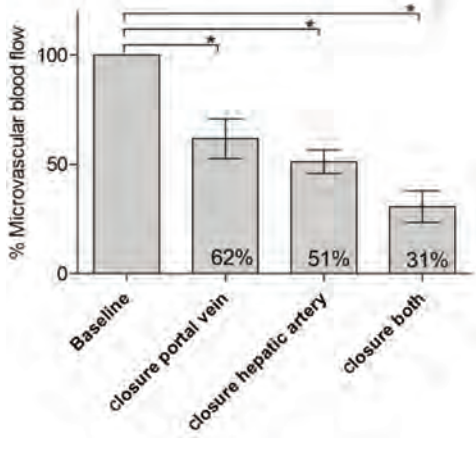

Figure 1. Changes in microvascular blood flow in the intact CBD: baseline value 100%; microvascular blood flow after closure of the portal vein: 62% ± 9.1 (P = 0.00); and after closure hepatic artery: 51% ± 5.4 (P = 0.00); after closure both: 31% ± 7.1 (P = 0.00). The asterisk (∗) indicates P < 0.05 for statistical testing of differences.

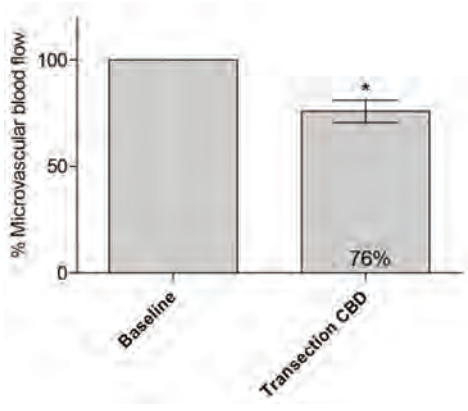

Figure 2. Change in microvascular blood flow after transection CBD: baseline value 100%; and after transection CBD: 76% ± 5.3 (P = 0.004). The asterisk (∗) indicates P < 0.05 for statistical testing of differences.

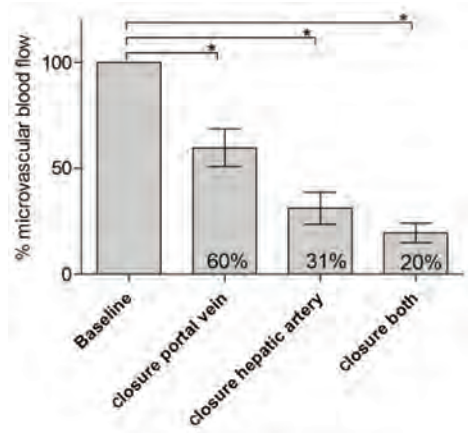

Figure 3. Changes in microvascular blood flow in the transected CBD: baseline value 100%; microvascular blood flow after closure of the portal vein: 60% ± 8.9 (P = 0.001); after closure hepatic artery: 31% ± 7.5 (P = 0.00); and after closure both: 20% ± 4.7 (P = 0.00). The asterisk (∗) indicates P < 0.05 for statistical testing of differences.

Transection of the CBD resulted in a decrease in microvascular blood flow to 76% compared with the initial baseline value (Fig. 2). For the next set of experiments, this 24% lower microvascular blood flow of the transected CBD was considered the new baseline value. Temporarily clamping of the portal vein after transection of the CBD resulted in a decrease in microvascular blood flow to 60% of this new baseline value. After arterial clamping, microvascular blood flow decreased to 31% of the new baseline value, and after clamping, both portal vein and hepatic artery microvascular blood flow decreased to 20%. Again all relative changes in blood flow compared to the new baseline values were statistically significant (P < 0.05; Fig. 3). There were no differences in mean arterial pressure and heart rate before and after transection of the CBD, as seen in Table 1.

	Intact CBD	Transected CBD	*P*
Mean arterial pressure	69 (64–77)	70.5 (63.25–79)	0.40
Heart rate	58 (50–79)	59 (49.5–66)	0.47

Table 1. Differences in hemodynamic parameters before and after transection of the CBD (Medians with interquartile ranges).

Figures 4 and 5 illustrate the variance in flow in AU between patients and between measurements. Figure 4 shows the mean with SEM microvascular blood flow in AU for the baseline measurement of all 15 patients. Figure 5 shows the mean with SEM microvascular blood flow in AU for all measurements.

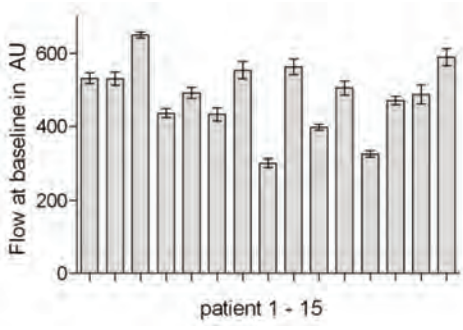

Figure 4. Values of microvascular blood flow in AU at baseline in all 15 patients.

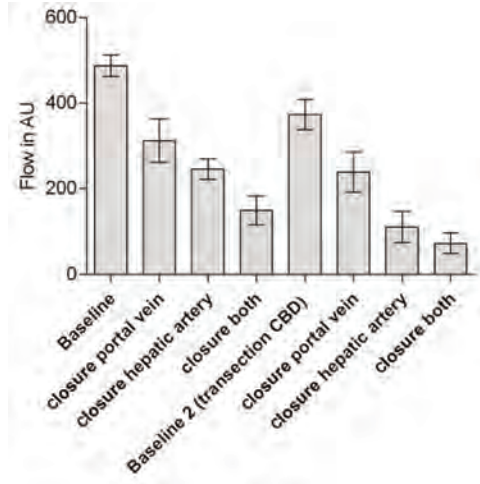

Figure 5. Values of microvascular blood flow in AU for all 8 measurements.

DISCUSSION

Non-anastomotic strictures of the biliary system after liver transplantation remain a serious complication that may affect up to 25% of patients[11,12, 23–25]. The most frequent causes of non-anastomotic strictures are hepatic artery thrombosis and ITBL. In ITBL, the hepatic artery is open; however, a paucifocal or plurifocal pattern of bile duct destruction and subsequent stricture formation can be observed, often leading to destruction of the graft's biliary tree and development of secondary liver failure. The majority of strictures occur within the first year of transplantation[23, 26]. Patients with ITBL have a significantly worse graft and overall survival rate, and it has become a leading indication of liver retransplantation[11, 24]. The cause of ITBL is still not completely understood, but its origin is thought to be multifactorial. Several studies have identified factors influencing the incidence of ITBL, including cold ischemia time[13, 23], ABO blood group incompatibility[5], the use of different preservation solutions[27], the use of DCD grafts[10], bile salt toxicity[28], and arterial back-table pressure perfusion[29]. In recent years, interest in impaired biliary microcirculation as a possible cause of ITBL has increased[15,16].

Despite the frequent biliary complications occurring after liver transplantation, controversies still remain concerning blood flow in the peribiliary plexus and publications on the subject differ in their conclusions[30]. Injection of dyes and vascular casting have demonstrated that the terminal branching of the hepatic artery opens into a peribiliary network and into the hepatic sinusoids[17, 18, 31, 32]. Therefore, the hepatic artery is traditionally considered the main provider of blood and responsible for the oxygenation of cholangiocytes[17, 18]. However, hepatic arterial-portal venous anastomoses are known

to exist at various sites in the hepatic vasculature and their role in the distribution of blood flow in this peribiliary plexus remains to be determined[19–22].

Mitra and coworkers[21] showed in an animal study that a large part of the arterial blood entered the portal vein through arterio-portal anastomoses in the peribiliary plexus, supplying the hepatic lobules with mixed arterial and portal venous blood. This was confirmed by Cho and coworkers[33], who also found extensive arterio-portal communications in the peribiliary plexus in a study of rabbits, permitting mixing of arterial and portal venous blood before entering the sinusoids. Restrepo and Warren[34] have reported that the volume of portal venous blood flow increases with hepatic arterial ligation. Tygstrup et al.[35] found that arterial contribution to total hepatic flow was 35%, with 50% of oxygen consumption from this source. With occlusion of the proper hepatic artery, extraction of oxygen from the portal venous blood increased. An animal study conducted by Tavoloni and coworkers[22] showed that hepatic artery ligation did not result in a decreased bile flow, portal venous oxygenation, or altered hepatic ultrastructure. Finally, supplemental information on distribution of hepatic flow is available through case reports of accidental hepatic artery. Brittain et al.[36] described 5 cases having undergone injury to the common hepatic or right hepatic artery, without signs of hepatic ischemia. Thus, despite current beliefs, the intrahepatic biliary epithelium seems to be adequately oxygenated in the absence of hepatic artery perfusion, at least in different animal models. Until now, no studies have been conducted quantifying the contribution of the hepatic artery, gastroduodenal artery, and portal vein to the microvascular blood flow in the CBD in man.

Recently, we encountered 3 cases of ITBL after solitary portal vein thrombosis after liver transplantation, with patent hepatic arteries[37]. One of these patients had a partial portal vein thrombosis leading to ITBL in the affected side of the transplanted liver and segmental cholangitis, which necessitated a partial hemihepatectomy. In another patient, there was complete portal vein thrombosis, with extensive ITBL, requiring retransplantation. Only a few studies have reported on the consequences of portal vein complications after liver transplantation[38, 39], one of which describes biliary complications after portal vein occlusion[38]. After we encountered this complication of liver transplantation, we hypothesized that the portal venous blood flow may contribute significantly to the vascularization of the bile ducts.

To unravel this intriguing question, we chose to study a cohort of patients undergoing PPPD. We think that this procedure mimics the situation of liver transplantation where the hepatoduodenal ligament is divided. Moreover, this clinical model is more physiological than liver transplant recipients who exhibit increased vascular resistance in the graft after transplantation due to uncontrollable graft-related factors and frequently present with portal hypertension, collaterals, and shunting.

In this study, a decrease in microvascular blood flow through the intact CBD to 51% was shown during closure of the common hepatic artery and to 62% during closure of the portal vein. After transection of the CBD, measurement of the microvascular blood flow through the CBD at the hilum of the liver, that is, in the liver transplantation-mimicking situation, revealed that the contribution of the portal vein remained highly consistent with a decrease in microvascular blood flow to 60%. The contribution of the hepatic artery seemed to become more important once the contribution of the gastroduodenal artery ceased; after transection of the CBD and closure of the hepatic artery, the blood flow decreased to 31%.

Transection of the CBD provokes a change in vascularization of the peribiliary plexus through the cessation of retrograde blood flow through the gastroduodenal artery. In this study, 76% of the original blood flow remained after transection of the CBD, implying that 24% of the blood flow was accounted for by retrograde perfusion provided by the gastroduodenal artery. The study conducted by Northover and coworkers[30] showed a higher contribution of the gastroduodenal artery, because they found in their cadaveric study that 60% of the arterial supply to the CBD originates from the gastroduodenal artery or its branches.

After closure of both hepatic artery and portal vein in the transected CBD, 20% of the blood flow still remained. Unlike in liver transplantation, during PPPD the liver attachments, such as the triangular ligaments, are not dissected, thus preserving collateral flow. Not much research has been done concerning collateral flow. Popper et al.[40] showed survival in dogs after ligation of the hepatic artery. However, most animals died if the phrenic arteries were also ligated. Furthermore, retrograde blood flow from the inferior vena cava and hepatic veins can contribute to hepatic perfusion. Charnsangavej et al.[41] conducted an angiographic classification of hepatic arterial collaterals in patients with hepatic malignancies treated by dearterialization, embolization, or surgical ligation. They found collaterals in the portal triads, the subcapsular area between the lobes of the liver, in the ligaments that suspend the liver in the peritoneal cavity, and through the structures that are closely attached to the liver. Kim et al.[42] described the extrahepatic collateral vessels they encountered in 860 hepatocellular carcinomas. The extrahepatic vessels originated from the inferior phrenic artery, omental branch, adrenal artery, intercostal artery, cystic artery, internal mammary artery, renal or renal subcapsular artery, superior mesenteric artery, gastric artery, or lumbar artery. It is important to realize both studies described earlier were performed in patients with hepatic neoplasia, influencing the growth of collaterals; however, it gives us information on the presence and location of hepatic collaterals, even though those will be smaller in importance in the physiologic situation. Nonetheless, these multiple findings could explain why after clamping both the portal vein and the hepatic artery, the blood flow in the CBD was not reduced to zero.

The limitation of this study is that measurements reflect an acute change in microvascular blood flow through the CBD. It is well possible that, in time, redistribution may occur within the microcirculation. Another factor that should be considered is that in this specific patient group, neovascularization or alterations in the region due to cancer of pancreatic head that affect microvascular blood flow in the hepatoduodenal ligament could have influenced results. To reduce this factor, we excluded patients with cholangiocarcinoma or macroscopic lymph node involvement in the hepatoduodenal region.

In this study, we found that the hepatic artery is the most important contributor to microvascular blood flow through the CBD. This is in agreement with the common observation that hepatic artery thrombosis is a major risk factor for non-anastomotic strictures of the bile ducts after liver transplantation. However, this is the first clinical study to show the importance of portal venous blood flow in biliary tract vascularization. This contribution may be of little influence in a physiologic situation; however, in already damaged bile duct epithelium after ischemia/reperfusion injury during liver transplantation, the contribution of the portal vein could become of increasing importance. Even with an open hepatic artery in the hilum of the liver, the arterioles surrounding the PBP can be obstructed on a microcirculatory level by incomplete flushing with high viscosity preservation solutions leading to microthrombi.

On the basis of the outcome of this study, disturbances in the portal venous blood flow after liver transplantation should be considered, like hepatic artery perfusion deficits, for intervention to prevent reduced peribiliary blood flow. This may prevent biliary ischemia and subsequent ITBL after liver transplantation.

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The importance of portal venous blood flow in ischemic-type biliary lesions after liver transplantation

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ABSTRACT

Ischemic-type biliary lesions (ITBL) are the most frequent cause of non-anastomotic biliary strictures after liver transplantation. This complication develops in up to 25% of patients, with a 50% retransplantation rate in affected patients. Traditionally, ischemia-reperfusion injury of the biliary system is considered to be the major risk factor for ITBL. Several other risk factors for ITBL have been identified, including the use of livers grafts donated after cardiac death, prolonged cold and warm ischemic times and use of University of Wisconsin preservation solution. In recent years however, impaired microcirculation of the peribiliary plexus (PBP) has been implicated as a possible risk factor. It is widely accepted that the PBP is exclusively provided by blood from the hepatic artery and therefore, the role of the portal venous blood supply has not been considered as a possible cause for the development of ITBL.

In this short report, we present three patients with segmental portal vein thrombosis and subsequent development of ITBL in the affected segments in the presence of normal arterial blood flow. This suggests that portal blood flow may have an important contribution to the biliary microcirculation and that a compromised portal venous blood supply can predispose to the development of ITBL.

INTRODUCTION

Biliary complications are a significant cause of morbidity and even mortality in patients after liver transplantation[1]. Early biliary complications, either leakage or stenosis at the anastomotic site, are usually caused by surgical failure and occur within the first weeks after liver transplantation. Non-anastomotic strictures can occur in the context of hepatic artery thrombosis[2, 3], or with an open hepatic artery. Non-anastomotic strictures occurring with and without a patent hepatic artery share many radiological similarities and thus the latter have been called ischemic-type biliary lesions (ITBL). They are characterized by bile duct destruction, subsequent stricture formation and sequestration. Several studies have identified factors influencing the incidence of ITBL, including cold ischemia time[4], ABO incompatibility[5], the use of different preservation solutions[6], the use of grafts donated after cardiac death (DCD)[7], bile salt toxicity[1], and arterial back-table pressure perfusion[8]. In recent years, interest in impaired biliary microcirculation as a possible cause of ITBL has increased[9, 10]. Previous studies have concluded that the peribiliary plexus (PBP), which is responsible for the blood supply to the bile ducts, is exclusively provided by blood from the hepatic artery[11, 12]. Therefore, the role of the portal venous blood supply has not been considered as a possible cause for the development of ITBL. In this study we describe three patients with partial portal vein thrombosis and intact arterial blood supply who develop isolated ITBL in the segments affected by portal vein thrombosis. The findings in these patients strongly suggest that portal blood flow has an important contribution to the biliary microcirculation and that a compromised portal venous blood supply can predispose to the development of ITBL.

CASE 1

A 57-year-old male, diagnosed with non-alcoholic steatohepatitis, was transplanted for cirrhosis and three intrahepatic hepatocellular carcinomas smaller than 2 cm in diameter. A blood group compatible liver from a 51-year old heart-beating donor was transplanted. Cold storage was in University of Wisconsin (UW) preservation solution (ViaSpan, Bristol-Myers Squibb, Belgium). The surgical procedure was uncomplicated. The liver was reperfused after the side-to-side cavo-cavostomy and portal vein anastomosis resulting in a cold ischemia time of 510 minutes and a warm ischemia time of 23 minutes. The arterial anastomosis was performed after portal reperfusion in 19 minutes. An end-to-end biliary anastomosis, without the use of a T-tube, was performed. Total blood loss was estimated at 2300 ml but no packed red blood cells were administered. Intra- and direct post-operative Doppler color ultrasonography showed intact arterial, hepatic and portal venous blood flow. On the first post-operative day however, routine Doppler

ultrasound examination showed a turbulent flow in the right portal vein branch, without abnormalities in arterial flow. Liver function was improving quickly, measured by decreasing (from 400 IU/L) transaminases, recovering coagulation, and decreasing serum bilirubine and γ-GT. Therefore surgical intervention was considered unnecessary. Ultrasound imaging in the following days showed persistent turbulent flow in the right portal vein branch but normal hepatic artery flow. Post-operative recovery was complicated by a wound infection, which was treated by draining the wound. Immunosuppressive therapy consisted of tacrolimus (Prograft, Astellas Pharma, The Netherlands) and a low-dose prednisone withdrawal scheme. The patient was discharged from the hospital on the 24th postoperative day. However, over a course of several months he suffered from recurrent cholangitis with bilirubine levels up to 150 μmol/L (normal < 16 μmol/L). On subsequent magnetic resonance cholangiopancreatography (MRCP) studies and endoscopic retrograde cholangiopancreatographic (ERCP) interventions, multiple biliary strictures in the right anterior and posterior biliary tree were seen, while bile ducts in the left liver half remained unaffected (Fig. 1). Portal venous flow was uncompromised in the main left and right

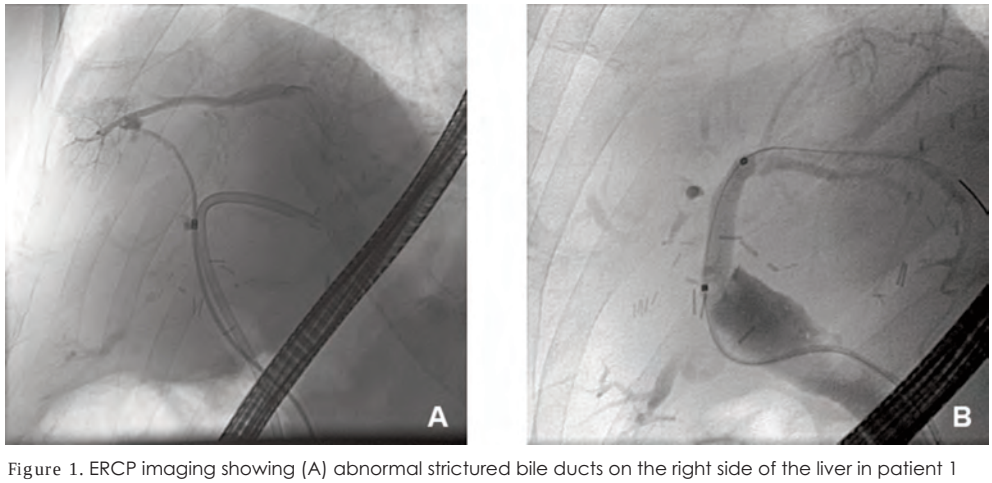

Figure 1. ERCP imaging showing (A) abnormal strictured bile ducts on the right side of the liver in patient 1 with isolated right-sided portal vein thrombosis and (B) normal bile ducts in the same patient in the left hemiliver.

portal branches, but showed tapering at the right anterior and posterior portal bifurcation and diminished portal flow to the complete right hemiliver. Several procedures of biliary dilatation and stenting were only successful for short periods of time. Additional treatment with ursodeoxycholic acid did not resolve symptoms. Eventually, 15 months after the initial transplantation, a right hemihepatectomy was performed. Histopathological examination of the explanted right hemiliver showed an intact arterial circulation, but extensive circulatory impairment and thrombosis of the portal system (Fig. 2A). It

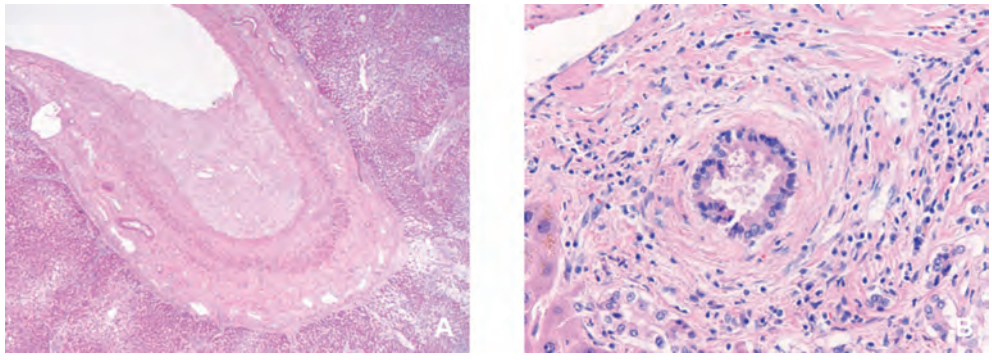

Figure 2. Hematoxyline and eosin stained biopsy of the resected right hemiliver of patient 1 showing (A) extensive thrombosis of a large portal vein branch in the liver and (B) irregular biliary epithelium with concentric fibrosis and separation from the outer wall as typically seen in ITBL.

furthermore showed typical microscopic characteristics of ITBL, including extensive damage and irregularity of the biliary epithelium accompanied by concentric fibrosis, ductular reaction and complete ductopenia in several portal tracts (Fig. 2B). The patient recovered uneventfully from surgery and is currently asymptomatic with normal bilirubine levels and otherwise intact liver function.

CASE 2

A 53-year old female diagnosed with primary sclerosing cholangitis (PSC) was transplanted for severe cholestasis and recurrent cholangitis despite years of endoscopic stenting and dilatations. For her, a 66-year old, blood group compatible, liver from a heart-beating donor became available. Cold storage was in UW preservation solution. The surgical procedure was uncomplicated, with cold and warm ischemia times of 541 and 28 minutes respectively. Arterial anastomosis was performed in 25 minutes and bile flow was restored with a Roux-en-Y hepaticojejunostomy. Total blood loss was estimated at 2000 ml and 3 units of packed red blood cells were administered. Arterial, hepatic and portal venous blood flow was intact intra- and postoperatively measured by Doppler color ultrasonography. Routine Doppler ultrasound the following day showed normal perfusion patterns. On the third postoperative day however, liver transaminases were highly elevated (AST 3682 IU/L and ALT 1880 IU/L) and subsequent ultrasonography showed the absence of portal flow in the left portal vein branch while a normal triphasic arterial flow was present in all parts of the liver. Contrast enhanced three-phase CT confirmed these findings. A relaparotomy was performed due to fever and abdominal pain and the suspicion of bile leakage, during which the perfusion defect of the left liver segments was confirmed, showing a perfusion defect of the left liver segments with intact right portal

flow and normal flow in the left and right hepatic arteries. Concomitantly, a small bile leak, which was diagnosed on the anterior side of the hepaticojejunostomy, was sutured. The patient was put on intravenous heparin and could be discharged 30 days after transplantation after an otherwise uneventful postoperative course. Immunosuppressive therapy consisted of cyclosporine (Neoral, Novartis Pharma, The Netherlands) and a low-dose withdrawal scheme of corticosteroid. Within 2 months the patient's condition deteriorated due to recurrent periods of cholangitis with bilirubine levels constantly exceeding 100 µmol/L. CT imaging showed atrophy and diffuse biliary strictures limited to the left hemiliver (Fig. 3). However the patient was not considered eligible for a left hemihepatectomy because of uncertainty of extension of ITBL into the right liver lobe. Finally she was retransplanted 3 months after the initial transplantation and symptoms alleviated after this second transplantation. Examination of the first graft showed extensive damage and irregularity of the biliary epithelium with concentric fibrosis and complete loss of bile ducts in several portal tracts, characteristic for ITBL, confined to the left lobe (Fig. 4). In this part of the liver the portal vein was obstructed while the artery was patent as in the previous patient.

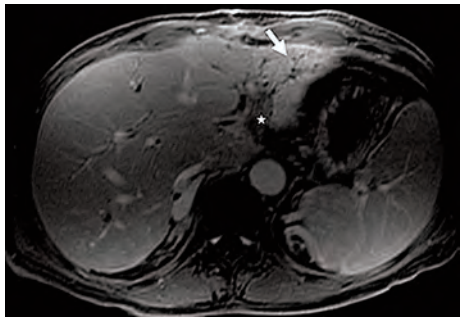

Figure 3. T1-weighted post-contrast MR image showing atrophy of the left liver lobe with focal irregular dilatation of bile ducts and local arterial compensatory hyperperfusion (arrow) in patient 2. Thrombosis of the left portal vein can be appreciated (star).

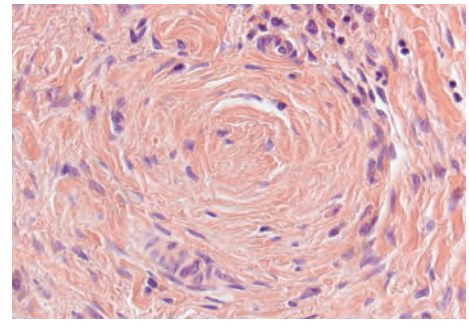

Figure 4. Hematoxyline and eosin stained biopsy from the affected part of the explanted liver showing complete replacement of biliary epithelium by concentric fibrosis in a portal tract typical for severe ITBL pathology in patient 2.

CASE 3

The third patient, a 44-year old female, was transplanted with a liver from a blood group compatible heart-beating donor, for primary biliary cirrhosis. Cold storage of the liver graft was in UW preservation solution. Reperfusion was performed after the side-to-side cavo-cavostomy and portal vein anastomosis. Cold and warm ischemia times measured 506 and 27 minutes respectively, and the hepatic artery anastomosis was performed in 25 minutes. The bile duct was anastomosed end-to end and due to

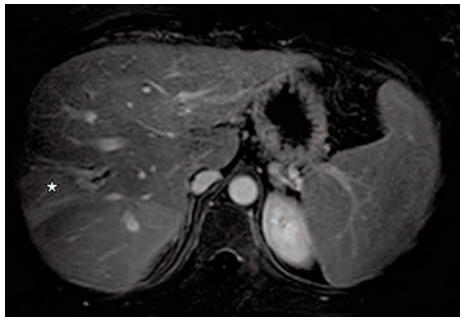

Figure 5. Thrombosis of the segmental portal vein of 5/8 (not shown) with segmental dilatation of the bile ducts and local compensatory arterial hyperperfusion (star) in patient 3.

blood loss 4 units of packed red cells were administered. Intra- and post-operative ultrasonography showed normal hepatic and portal flow patterns. Post-operative course was complicated by the formation of a biloma, which needed drainage. The patient was discharged in good health 15 days post-operatively with a mycophenolate mofetil (CellCept, Roche Pharma, The Netherlands) and low-dose Tacrolimus immunosuppressive regime. During a routine Doppler ultrasound examination six months after transplantation however, a partial portal vein thrombosis was found at the bifurcation in the right portal vein and portal flow to segments 5 and 8 was partially compromised. Portal flow to segments 6 and 7 remained uncompromised and arterial flow was normal in the entire liver. The patient at that moment presented with slightly elevated transaminase levels (up to 150 IU/L) and was treated with phenprocoumon (Marcoumar, Roche Pharma, The Netherlands). However, three weeks later a complete thrombosis of the right anterior portal branch was diagnosed and anticoagulant therapy was discontinued. Currently the patient is in good clinical condition, however liver function tests show persistent elevated levels of bilirubine (30 µmol/L), alkaline phosphatase (> 200 IU/L) and γ-GT (> 400 IU/L), while diffuse segmental biliary dilatation can be noted on imaging studies (Fig. 5).

DISCUSSION

Ischemic-type biliary lesions is a serious complication that generally occurs at 1 to 12 months after liver transplantation and can affect up to 25% of patients[1, 8, 9, 13, 14]. Patients developing ITBL have a significantly worse graft and overall survival and ITBL has become a leading cause of liver retransplantation[8, 13]. Reported risk factors for the development of ITBL include: prolonged ischemia times, bile toxicity, inadequate flushing of the graft and PBP, blood group incompatibility, grafts donated after cardiac death and PSC in the recipient[1, 4-8, 13, 15]. The real pathogenesis of ITBL however, remains to be elucidated.

Recently, a retrospective study in over 1750 transplants identified 6 risk factors for

ITBL, but in the multivariate analysis only cold ischemia time and organ retrieval without high pressure hepatic artery perfusion remained significant[14]. Ischemia-reperfusion injury has further been suggested, as it has been shown that the length of the cold ischemia time correlates with the magnitude of ischemia-reperfusion injury[16]. Cold ischemia times in excess of 13 hours have been reported to cause a 50% incidence ITBL in patients[4]. Damage to the endothelium of peribiliary arterioles could lead to ischemic damage to biliary epithelium. This is in line with the findings that ITBL closely resembles the biliary pathology occurring after hepatic artery thrombosis and that additional arterial back table perfusion could significantly reduce the rate of ITBL[8]. Gravity perfusion through the hepatic artery may not completely rinse the PBP, which causes blood to stagnate locally in the small, incompletely flushed vessels, leading to formation of microthrombi in these arterioles. Consequently, local ischemia may occur, resulting in biliary fibrosis and strictures, as seen in ITBL. In contrast, flushing the hepatic artery with fibrinolytic drugs has been reported to decrease the incidence of ITBL[9]. An incomplete rinsing of the PBP due to the high viscosity of UW might provide an explanation why ITBL develops more often after UW preservation than after preservation with the much less viscous histidine-tryptophan-ketoglutarate (HTK) preservation solution[6].

In our three patients, risk factors for ITBL were also present: cold storage with UW in all liver grafts, cold ischemia times approaching 10 hours and two recipients with a history of immune-mediated end-stage liver disease. Patient 2 and 3 were transplanted for PSC and PBC respectively. In literature it is known that immune-mediated liver disease as the cause of liver transplantation is related to a higher incidence of ITBL. Patients transplanted for PSC and auto-immune hepatitis (AIH) were found to have a 3.7 and 3.0-fold increased risk respectively for developing ITBL vs. PBC, in a report of 749 patients and 842 liver grafts[17]. In this same report, the incidence of ITBL did not differ significantly between patients transplanted for PBC or transplanted for alcoholic, viral, cryptogenic or other liver disease. Similarly, an earlier report describes a higher percentage of non-anastomotic strictures in patients transplanted for PSC compared to non-PSC recipients (27 vs. 13% for intrahepatic and 6 vs. 2% for extrahepatic strictures)[18]. In addition, Feller et al. have reported that PSC was more often the indication for transplantation in patients developing non-anastomotic strictures compared to controls (31 vs. 9%)[19]. Similar results were also published more recently by Buis et al.[20]. The latter study again did not find a correlation between ITBL and PBC as the indication for liver transplantation. Based on literature it can be concluded that patients with PSC, and AIH, but not PBC, are at greater risk of developing ITBL. However, none of the original manuscripts and reviews on ITBL in PSC and AIH disease report such a segmental ITBL pattern, as noticed in our three patients. In cases 2 and 3,

generalized immune-mediated disease was found in the native explant livers, but in our case 2, the second explant liver did not show any signs of biliary disease in the non-affected lobes, making ITBL more likely than recurrent immune-mediated biliary injury.

Regarding the blood flow in the PBP, traditionally the hepatic artery is considered the sole provider of blood and responsible for oxygenation of the cholangiocytes[11, 12]. In all three patients, the hepatic artery blood flow was proven to be preserved by Doppler US and yet ITBL developed in segments of the liver that were deprived of portal venous blood. In patients 3 and 2 additional studies in the form of contrast enhanced MRI and CT confirmed the patency of the hepatic artery. Based on literature the latter should serve as the golden standard for detecting vascular complications after liver transplantation[21]. Doppler US and contrast enhanced MRI have a reported sensitivity and specificity of up to 91.3% and 100%[22, 23], and ranging between 67 to 100% and 90 to 100% respectively[23, 24], for detecting hepatic artery thrombosis following liver transplantation. However these studies do not consider small arterial lesions, which one can contemplate, could be undetectable on the conducted imaging studies as well as CT-angiography performed in this study. Therefore a possibility exists that such undetectable small arterial lesions caused the biliary complications seen in the presented cases, which could have been present in the three patients in conjunction with the portal vein thrombosis.

The portal vein thrombi in the presented patients were diagnosed using Doppler US, which is routinely conducted upon arrival at the ICU, day 1 and day 7 after liver transplantation at our center and is reported to have a sensitivity and specificity of 100% in diagnosing generalized portal vein thrombosis after liver transplantation[22]. The conducted study does not apply to segmental portal vein thrombosis, thus one can contemplate that the sensitivity for diagnosing segmental portal vein thrombosis might be lower. However, all ultrasonographies in this study were performed or supervised by two dedicated hepatologists, both with over 10-years of experience on liver ultrasonography in liver transplantation, and additional imaging studies discussed earlier also confirmed the partial portal vein thrombi in cases 2 and 3.

In literature, 1.7% of the patients receiving a whole liver graft develop total portal thrombosis after liver transplantation. In this published series however, the development of ITBL was not described[25]. In contrast to the presented patients though, (immediate) restoration of the portal circulation was attempted in these reports. In our patients no risk factors for portal vein thrombosis, such as preexisting portal vein thrombosis, earlier splenectomy, use of venous conduits, and small portal vein size[25], were present. Other post-operative complications capable of causing portal vein thrombosis as well as biliary strictures such as periportal edema, cholangitis, hepatitis, abscess formation,

hepatic artery thrombosis, and hepatic vein outflow obstruction, were ruled out on repeated imaging and laboratory studies.

In conclusion, our case presentation indicates that portal circulation may play an important role in the integrity of the biliary tree and especially in development of ITBL after liver transplantation. Our findings suggest that in the case of partial intrahepatic portal vein thrombosis, even with sufficient liver function, a thrombectomy or thrombolysis should be considered to prevent late biliary complications.

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Section II

MicroRNAs as novel biomarkers in liver transplantation

The ins and outs of microRNAs as biomarkers in liver disease and transplantation

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ABSTRACT

Ongoing research is being conducted in the field of transplantation to discover novel non-invasive biomarkers for assessment of graft quality before transplantation and monitoring of graft injury after transplantation. MicroRNAs (miRNAs) are among the most promising in this field. MiRNAs are small non-coding RNAs that function as important regulators of gene expression in response to cellular stress and disease. An advantage that makes miRNAs attractive candidates for biomarker research is their fast release from cells in response to stress and injury, which can occur via different routes. In the context of liver transplantation (LT), non-invasive measurement and stability of extracellular miRNAs in blood, bile and graft perfusates has been linked to cell-type specific injury and early graft outcome following LT. Furthermore, specific intrahepatic miRNA expression patterns have been associated with graft survival and recurrent disease, like hepatitis C virus related fibrosis and hepatocellular carcinoma. Therefore, miRNAs with strong predictive value and high sensitivity and specificity might be successfully applied to assess hepatic injury and to diagnose (recurrent) liver disease before, during and after LT. In this review, the current features and future prospects of miRNAs as biomarkers in and out of the liver are discussed.

INTRODUCTION

Liver transplantation (LT) remains the only curative treatment for end-stage liver disease. Both short and long-term patient and graft survival however remain far from satisfactory, despite substantial advances in immunosuppressive therapy and surgical techniques[1, 2]. The increased heed for use of marginal donors due to allograft shortage, transplantation of recipients with increasingly higher MELD scores, and recurrence of liver disease are major factors that are negatively influencing outcome following LT[2]. Ongoing research is conducted in the field of transplantation to discover novel, non-invasive biomarkers for assessment of graft quality before transplantation and monitoring graft injury after transplantation. MicroRNAs (miRNAs) are among the more promising in this field.

MiRNAs are a class of newly discovered small non-coding RNAs, which serve as important regulators of post-transcriptional gene expression and as such control many cellular processes[3]. They exert down-regulating effects by preventing translation of messenger RNA (mRNA) into functional proteins. Increasing evidence establishes the important role of miRNA expression in physiological as well as pathophysiological processes, including tissue injury and repair[4-11].

Although the gene regulating function of miRNAs is complex and far from fully unraveled, their unique features make them attractive candidate biomarkers for prognostic and diagnostic purposes in liver disease and LT. Profiles of miRNAs that are expressed by various cell types, like hepatocytes, cholangiocytes and endothelial cells, allow for the study of cell-type specific injury or stress[12-14]. Moreover, in response to injury, cell-type specific miRNAs can be released into the circulation and other body fluids via different routes, which has been demonstrated by multiple studies[13-18]. Surprisingly, these extracellular miRNAs remain fairly stable, despite the abundance of RNA degrading enzymes[7, 19-23].

For LT, both miRNA expression patterns in tissue (the Ins) as well as miRNA release into serum, bile and graft preservation solutions (the Outs) have been linked with complications that form major threats for patient and graft survival. These include severe ischemia-reperfusion injury (IRI), acute rejection, hepatitis C virus (HCV) re-infection, and recurrence of hepatocellular carcinoma. MiRNAs with strong predictive value and high sensitivity and specificity might be successfully applied to assess graft quality and monitor graft function during different phases of clinical LT. Moreover, they could be valuable contributors to existing decision-making models like the donor risk index and the Milan criteria, which are currently used for the selection of respectively suitable donors and recipients in order to optimize graft and patient survival.

In this review, we discuss recent literature with special attention towards the use of miRNAs as biomarkers to assess graft quality in LT, to monitor graft function shortly after LT

and for diagnosis of recurrent disease after LT. Emphasis is put on the biological relevance of miRNAs in response to cell stress and the associated release of miRNAs from cells.

MICRORNAS AS MASTER REGULATORS OF CELLULAR STRESS

Approximately 30% of all human genes are believed to be regulated by miRNAs, of which over 1000 types have been identified to be expressed by different cells. A distinct set of miRNAs was found to be expressed by hepatocytes and cholangiocytes in the liver, including miR-30a[1], miR-30c, miR-30e, miR-122, miR-133a, miR-148a, miR-191, miR-192, miR-194, miR-198, miR-200c, miR-222, miR-296, miR-710, and miR-711[15, 24-28]. The most abundantly expressed miRNA in liver tissue is miR-122[12, 28]. This miRNA has been shown to be an important regulator of cholesterol metabolism[29], iron homeostasis[30] and as a crucial host factor for hepatitis C virus (HCV) infection and replication[31, 32].

General miRNA-induced gene regulation is a two-way process that is able to respond rapidly to specific cellular needs, especially under circumstances of cellular stress where they play a central role[33]. Not only do miRNAs regulate gene expression, they are sometimes also regulated themselves by stress signals such as NF-kB and p53 during for instance inflammation and DNA damage[34]. Furthermore, miRNAs have been shown as important mediators of metabolic stress for example during hypoxia, hyperglycemia, hypertriglyceridemia and hypercholesterolemia, and caloric restriction[35]. More importantly, this regulation due to repression by miRNAs is a reversible process. For instance, the mRNA for cationic amino acid transporter 1 (CAT-1), which is normally repressed by miRNA-122, is relieved from repression during cellular stress (amino acid deprivation) to allow increased CAT-1 protein formation by translation of preexisting mRNA[33], suggesting an important role for miRNAs as regulators of cellular stress.

CIRCULATING MICRORNAS, THEIR RELEASE AND THEIR EXTRACELLULAR STABILITY

The presence of tissue-specific, extracellular miRNAs in the circulation has made them an important subject for non-invasive biomarker research. Already in the early 1970's it was reported that, beyond expectation, intact free stable RNA could be found in the blood circulation, suggesting that such RNAs had to be relatively resistant to degradation by RNases[36]. More recently, circulating miRNAs have also been demonstrated to exert unexpected stability. Even after prolonged times at room temperature and after

[1] Under a standard nomenclature system, names are assigned to experimentally confirmed miRNAs before publication of their discovery. The prefix "miR" is followed by a dash and a number, the latter often indicating order of naming. For example, mir-123 was named and likely discovered prior to mir-456. Species of origin is designated with a three-letter prefix, e.g., hsa-miR-123 is a human (Homo sapiens). MiRNAs with nearly identical sequences except for one or two nucleotides are annotated with an additional lower case letter. For example, miR-123a would be closely related to miR-123b.

repeated cycles of freezing and thawing, miRNAs in serum, plasma and graft perfusate samples remained insensitive to degradation[7, 13, 14, 19]. But miRNAs can also be detected in other body fluids, including amniotic fluid, breast milk and colostrum, bronchial lavage, cerebrospinal and peritoneal fluid, bile, saliva, tears, urine, pleural fluid, and seminal fluid[17, 18, 37], suggesting protection against degradation.

The general observation is that stability of circulating miRNAs exceeds the stability of circulating mRNA. This has been attributed to either packing of miRNAs in small particles or their association with (lipo)protein complexes, protecting miRNAs from RNase activity. According to literature, the largest portion of circulating miRNAs in plasma or serum is present in a protein-bound form. They have been shown to bind to the Ago2-protein in particular, which is a catalyzing component in the RNA-induced silencing complex (RISC)[22, 38]. The involvement of proteins in stabilizing extracellular miRNAs has been demonstrated in serum samples treated with proteinase K, which results in degradation of proteins and subsequently diminished stability of extracellular miRNAs[22]. The exact mechanism by which miRNA-protein complexes are formed and excreted in the setting of LT is unknown. Furthermore, it is currently unknown what the faith of liver-derived miRNAs is once they have been released from cells. One interesting hypothesis is that released miRNAs can be taken up by cells inside or outside the liver and thereby remotely regulate gene expression in recipient cells[39]. However, this hypothesis requires further research in order to demonstrate a biological role of extracellular miRNAs.

In addition to the protein-bound form, a smaller portion of miRNAs is transported in small particles like exosomes, microvesicles and apoptotic bodies[20-23]. All these particles contain a lipid layer surrounding the miRNAs cargo to protect their content. Apoptotic bodies are released by cells during programmed cell death and are relatively large in size compared to microvesicles and exosomes. Microvesicles again are larger in their size compared to exosomes and are released from living cells by bleb formation of the lipid layer. The smallest particles known to carry miRNAs are exosomes. These small particles are produced in endosomes and are released from cells by fusing with the lipid cell membrane[20-23]. Recent studies have already shown that genetic exchange, and even transmission of HCV, through exosomes is possible[20, 39]. Hypothetically, these small vesicles could be involved in signal transduction and intercellular communication mediated by miRNA exchange.

Finally, stable forms of extracellular miRNAs have recently been found in association with high-density lipoproteins (HDL) and low-density lipoproteins (LDL)[23, 40]. The exact method of binding between miRNAs and lipoproteins is not understood. Some studies suggest that this association occurs within the circulation, where miRNAs are picked up by lipoproteins, rather than packaged in HDL and LDL particles in the cell[23, 40]. A summary of all routes of cellular miRNA release is illustrated in Figure 1.

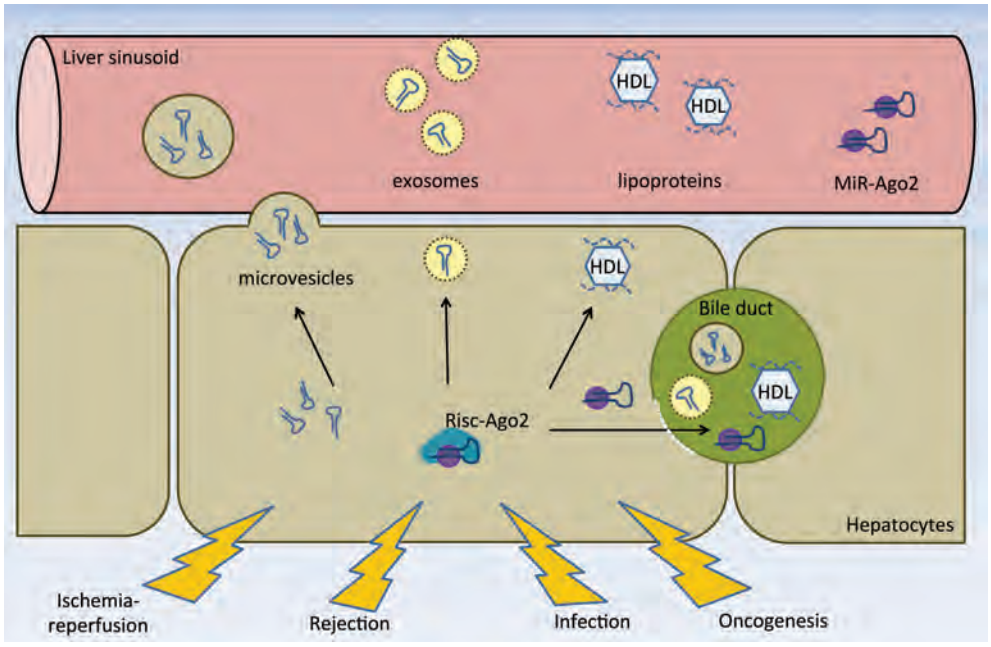

Figure 1. Mechanisms of miRNA release from (injured) cells. Mature miRNAs inside the cell cytoplasm are bound to the RISC-argonaute2 complex. Cell stress induced by for instance ischemia-reperfusion, infection, rejection and oncogenesis, can cause active release or secretion of miRNAs into the circulation. Extracellular circulating miRNAs have been found in vesicles and smaller exosomes, or bound to lipoproteins (HDL and LDL) and argonaute2. Recently, microRNAs were also described to be released from the liver into bile (Not included in this figure: miRNA release through apoptotic bodies).

For most miRNAs found in circulation, it appears that excretion is caused by a selective and active mechanism of controlled release rather than a passive or coincidental leakage[22]. In vitro studies show differences in ratios of intracellular miRNAs and their release through small particles; some miRNAs were effectively excreted, while others were retained completely by the same cells, suggesting selective packaging and excretion mechanisms[20, 41, 42]. In case of lipoprotein-associated miRNAs, it was shown that levels varied in certain diseases, underlining their potential as biomarkers[23, 43]. This specific controlled release further strengthens the hypothesis that released miRNAs are involved in regulatory, pathophysiologic mechanisms.

CIRCULATING MICRORNAS AS NON-INVASIVE BIOMARKERS FOR LIVER INJURY IN A NON-TRANSPLANT SETTING

Current research concerning circulating miRNAs as biomarkers has mainly focused on liver disease and liver-failure prior to transplantation. This has encouraged further investigation of miRNAs as biomarkers in the setting of LT, though the total number of

Manuscript	Medium	miRNAs	Description
van der Meer et al.[44]	Serum	miR-122 miR-192	Sensitive detection of liver injury by miRNAs even when transaminases are low in HCV infected patients
Cermelli et al.[45]	Serum	miR-16 miR-34a miR-122	Increased levels in patients with HCV infection and NAFLD Positive correlation of miR-122 and miR-34a with liver enzyme levels and histological fibrosis stage and inflammation activity
Roderburg et al.[46]	Serum	miR-29	Lower circulating levels in patients with liver fibrosis
Roderburg et al.[47]	Serum	miR-571	Levels closely correlated with disease stages during alcoholic or HCV induced liver cirrhosis
Gui et al.[48]	Serum	miR-885-5p	Increased levels in patients with HBV, HCC and liver cirrhosis
Xu et al.[49]	Serum	miR-21 miR-122 miR-223	Elevated levels in patients with HCC but also in patients with chronic hepatitis
Li et al.[50]	Serum	let-7f miR-25 miR-375	Differentiation between HBV infected patients with concurrent HCC and healthy controls and patients with only HBV or HCV infection Specificity of 96% and sensitivity of 100% for predicting HCC with miR-375
Zhou et al.[51]	Serum	miR-21 miR-26a miR-27a miR122 miR-192 miR-801	Combined miRNA profile with high diagnostic accuracy for predicting HCC in HBV infected patients
Li et al.[52]	Serum	miR-221	Elevated levels correlated with HCC tumor size, cirrhosis, tumor stage, and significantly diminished patient survival by 2.5 times

Table 1. A summary of literature is given of identified miRNAs and their potential as biomarkers of liver injury in a non-transplant setting.

published studies for this field is still limited. Markers for liver disease however, could be relevant for predicting or diagnosing recurrent disease after LT. Therefore, this paragraph discusses relevant studies regarding circulating miRNAs in a non-transplant setting (Table 1).

Globally, viral hepatitis is one of the most important indications for LT. A study by van der Meer et al. demonstrated that serum levels of previously described hepatocyte-abundant miR-122 and miR-192 are elevated in HCV infected patients. Interestingly, these miRNAs were also able to identify patients with normal transaminase levels during active HCV infection[44]. In patients with HCV infection and non-alcoholic fatty liver disease (NAFLD), not only miR-122, but also miR-34a and miR-16 were found to be elevated in serum compared to controls[45]. These levels of miR-122 and miR-34a correlated with liver enzyme levels and histological fibrosis stage and inflammation activity in both HCV and NAFLD patient groups. Roderburg et al. showed lower serum levels of miR-29 in mice and humans with liver fibrosis compared to healthy controls[46] and that serum levels of miR-571 were closely correlated with the stage of disease during alcoholic or HCV induced liver cirrhosis[47]. The findings from these studies indicate a higher sensitivity of serum miRNAs compared to conventional transaminases in screening liver injury, and the potential of miRNAs as biomarkers for monitoring fibrosis and severity of cirrhosis.

Studies by other groups show that miR-885-5p[2] is significantly increased in serum of

[2] When two mature microRNAs originate from opposite arms of the same pre-miRNA, they are denoted with a -3p or -5p suffix. In the past, this distinction was also made with 's' (sense) and 'as' (antisense). When relative expression levels are known, an asterisk following the name indicates an miRNA expressed at low levels relative to the miRNA in the opposite arm of a hairpin. For example, miR-123 and miR-123* would share a pre-miRNA hairpin, but more miR-123 would be found in the cell.

patients with hepatocellular carcinoma (HCC), liver cirrhosis, and hepatitis B virus (HBV) infection compared to controls, but does not differentiate between the different types of liver disease[48]. Similarly, levels of miR-21, miR-122, and miR-223, which are commonly deregulated in HCC tissue, were elevated in serum of patients with HCC compared to healthy controls, but also in patients suffering from chronic viral hepatitis without known HCC[49]. This illustrates the problem that some serum miRNAs can only differentiate patients with liver injury from healthy controls, but not specify for the nature of the injury.

In contrast, a different study shows that serum miRNAs could specifically identify HBV infection. Serum levels of miR-25, miR-375, and let-7f clearly differentiated between patients with combined HBV infection and concurrent HCC from healthy controls and patients with only HBV or HCV infection. Serum levels of miR-375 achieved specificity and sensitivity of respectively 96% and 100% for predicting HCC, making it a useful marker for HCC in HBV infected patients[50]. A comparable study in three independent cohorts identified a different set of circulating miRNAs (miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a, and miR-801) that provided high diagnostic accuracy for predicting of HCC in HBV infected patients[51]. Li et al. found that increased serum levels of miR-221 correlated with HCC tumor size, cirrhosis, tumor stage, and diminished patient survival by 2.5 times, suggesting its prognostic usefulness[52].

In general, these studies demonstrate the potential of miRNAs as predictive, diagnostic, and prognostic biomarkers in liver diseases, which are common indications for LT, with higher sensitivity and specificity compared to transaminases. However, small sample sizes and the lack of prospective studies renders most current miRNA biomarkers still premature. This is indicative of the limitations of current biomarker discovery research. Whether miRNAs as biomarker could be utilized in the clinical setting of LT therefore remains to be determined. However, the strong correlation of specific miRNAs with the degree of histological inflammation, fibrosis and cirrhosis suggests that they could prove useful for various purposes in LT, such as screening for (and thus early treatment of) recurrent disease, identification of specific post-transplant complications and safe tapering of immunosuppressive drugs to minimize side effects.

CIRCULATING MICRORNAS AS NON-INVASIVE BIOMARKERS IN LIVER TRANSPLANTATION

As mentioned earlier, outcome after LT has improved considerably over the last decades, but patient and graft survival and quality of life could still be improved[14, 53-56]. Outcome after LT is often compromised as a result of various causes such as inadequate graft selection and consequent primary non-function or delayed graft function, recurrence of disease, ischemic cholangiopathy, and life-long usage of immunosuppressive drugs and

Manuscript	Medium	miRNAs	Description
Farid et al.[13]	Peritransplant liver tissue and posttransplant serum	miR-122 miR-148a miR-194	Reduction of miR-122 and miR-148a in liver tissue negatively correlated with length of ischemia time Correlation of serum miR-122, miR-148a and miR-194 levels with transaminases Early detection and quick response of miR-122 and miR-148a during acute rejection and its treatment
Verhoeven et al.[14]	Pretransplant graft perfusates	miR-30e miR-122 miR-148a miR-222 miR-296	Profiles of combined cholangiocyte and hepatocyte-derived miRNAs predictive for development of post-transplant ischemic cholangiopathy
Hu et al.[59]	Plasma and portal lymphocytes	miR-122 miR-146a miR-192	Increased plasma levels of all miRNAs during acute rejection Specific higher expression of miR-146a in portal lymphocytes
Lankisch et al.[61]	Posttransplant bile	miR-517a miR-892a miR-106a*	Elevated in bile after development of ischemic cholangiopathy after liver transplantation

Table 2. A summary of literature is given of identified miRNAs and their potential as biomarkers of liver injury in a transplant setting.

its complications[2, 55, 57, 58]. The need for non-invasive biomarkers to monitor graft quality before, during and after LT therefore remains. Despite this need, only a limited number of studies have been conducted so far in the field of LT, which results are summarized in Table 2.

In an earlier study by our group, a diminished expression of hepatocyte-abundant miR-122 and miR-148a in allograft tissue during LT was shown to significantly correlate with the length of graft ischemia time. At the same time, serum levels of these miRNAs increased and correlated with traditional markers of liver injury after LT. Furthermore, during episodes of histologically proven acute cellular rejection, miRNAs rised earlier compared to transaminases during injury and normalized more rapidly after treatment, showing that miRNAs are promising candidates for very early detection of liver injury after transplantation[13]. More recently these findings were confirmed in a rat model, showing plasma levels of miR-122, miR-146a and miR-192 to be significantly increased during acute rejection. Interestingly, the researchers suggest miR-146a to be more specific in detecting of acute rejection because this miRNAs was higher abundant in portal lymphocytes within the liver, compared to levels of miR-122 and miR-192 that were assumed to represent more general hepatic injury[59].

More recent work from our team shows that pre-transplant perfusates, that are used for cold storage of liver allografts, contain stable extracellular miRNAs originating from hepatocytes (miR-122, miR-148a) as well as cholangiocytes (miR-30e, miR-222, miR-296). Profiles of these miRNAs were independent predictors for the development of ischemic cholangiopathy after LT. The proof of concept that miRNAs could be used as early biomarkers already before graft implantation to predict graft quality could be a valuable feature for the selection of allografts in the future[14, 60].

Not only blood or perfusates, but also measurement of miRNAs in bile can be of use after LT. Very recently, Lankish et al. showed that bile levels of miR-517a, miR-892a and

miR-106a* were elevated in patients with ischemic cholangiopathy and could distinguish between ischemic cholangiopathy and other causes for biliary obstructions[61]. In particular for biliary complications, miRNA composition in bile rather than serum might better reflect ongoing injury of cholangiocytes[62].

Although miRNA biomarkers clearly have potential for clinical application in the setting of LT, the number of studies on this topic should be expanded as their numbers are limited. During transplantation, miRNAs in perfusates can be used for diagnostic and in the future also maybe for therapeutic, purposes. Hence, not only selection of good quality grafts might benefit, but also alleviating ischemia-reperfusion injury might be an option once the biology of miRNAs has been unraveled. Furthermore, detection of circulating miRNAs in bile and serum can be equally useful during post-transplantation follow-up, such as monitoring for recurrent disease.

TISSUE MICRORNA EXPRESSION PATTERNS AND HEPATITIS C RECURRENCE AFTER LIVER TRANSPLANTATION

Recurrence of disease is the most important cause of graft loss after LT and its prevention could lead to decreased need of re-LT and significant improvement of outcome after LT. One major determinant for patient and graft survival after LT is the recurrence of HCV infection of the graft. Several studies investigated whether miRNA profiles in liver tissue in recipients can be used to predict the severity and time to develop fibrosis caused by recurrence of HCV and whether miRNAs can monitor response to antiviral therapy.

One study investigated slow vs. fast progressing fibrosis in recipients with recurrent HCV after LT. Recipients with slow progression of liver fibrosis at 12 months after LT (Ishak score <F2) showed up-regulated expression of miR-146a, miR-19a, miR-20a and let-7e in graft liver biopsies compared to recipients with fast progression (Ishak score ≥F2 at 12 months)[63]. In addition, the investigators were also able to distinguish fast progressing HCV re-infection from acute cellular rejection using miRNAs, which can usually be clinically challenging after LT but is essential as therapies for both conditions differ significantly.

A similar study compared miRNA expression between non-progressors (Knodell fibrosis score F0-F1) and progressors (F3-F4) in liver allograft tissue biopsies that were collected during clinical recurrence of HCV. In a training set of 27 recipients, a profile of 9 differentially expressed miRNAs was identified of which 7 could be validated successfully in an independent set of recipients. In particular miR-155 and miR-30c were respectively up- and down regulated in progressors and were described as key-regulator miRNAs for the development of fibrosis through ingenuity pathway analysis[64]. Why these two comparable studies do not identified common miRNAs is unclear.

Another study investigated which miRNAs target HCV receptors and relate to HCV

infection and response to antiviral therapy after LT. Different from the previous two papers, the investigators did not use gene-array analysis for identification of potentially relevant miRNAs, but miRNAs were selected by target prediction software. High viral load at time of HCV recurrence was significantly associated with increased expression of miR-122. Furthermore, in patients with sustained virological response, miR-122 expression significantly increased when recipients responded to antiviral therapy, next to five other miRNAs. Pretreatment profiles in tissue were however not predictive for success of antiviral therapy[65].

These identified miRNAs could serve as diagnostic methods, but more importantly, their biological function should be further investigated as this can give vital insight in the process of recurrence of HCV after LT and why its clinical course can differ considerably between recipients. These insights in biological functions will inevitably be useful in recipient and graft matching and the development of novel therapeutic strategies in order to minimize (the effects of) recurrent HCV.

TISSUE MICRORNA PROFILES AND RECURRENCE OF HEPATOCELLULAR CARCINOMA AFTER LIVER TRANSPLANTATION

Another important recurrent disease associated with diminished patient survival after LT is HCC. The Milan criteria, often used for the selection of patients suffering from HCC in need of a LT, have been shown to be only moderately successful in the reduction of recurrence of HCC in recipients following LT[66]. Therefore, studies have been conducted to investigate the predictive or prognostic value of miRNAs for HCC recurrence after LT.

In a study by Han et al., miRNA gene-array analysis in primary HCC liver samples identified 18 miRNAs that were expressed differentially in recipients who developed HCC recurrence (n=5) and recipients who did not (n=5). Six miRNAs with the strongest fold-change, miR-19a, miR-886-5p, miR-126, miR-223, miR-24, and miR-147 were successfully validated in 105 primary HCC samples of the same center and in 50 patients from another transplant center. Especially the combination of all six miRNAs showed high sensitivity and specificity and was demonstrated to be an independent predictor for HCC recurrence in patients transplanted within the Milan criteria as well as outside of the Milan criteria[67]. Based on this multiple-miRNA based profile, recipients could be divided into having a low-risk signature with a better recurrence-free and overall survival compared to recipients with a high-risk signature. In addition, in another study, high levels of miR-155 in HCC tissue were demonstrated to promote cell invasion resulting in poor overall en recurrence-free survival[68].

The same research group performed further clinical and experimental studies on the correlation between miR-126 and HCC recurrence. A lower expression of miR-126 in

primary HCC was associated with an increased incidence of HCC recurrence and impaired patient survival[69]. Moreover, in vitro and in vivo experiments showed that over-expression of miR-126 could inhibit HCC cell migration and invasion, thereby suppressing HCC metastasis. The involvement of several miRNAs, including miR-96, miR-139-5p, miR-126*, and miR-142-3p in HCC recurrence was demonstrated by Sato et al. in an elaborate study[70]. Patients in this study were all operated within the Milan criteria but received resection as therapy for HCC instead of LT.

Based on these findings, stricter clinical and radiological follow-up can be granted in recipients identified as high-risk patients for recurrence of HCC, so that early identification of recurrence will result in earlier therapeutic intervention probably resulting in higher quality of life and longer survival.

MICRORNAS AND RECURRENCE OF OTHER HEPATIC PATHOLOGY AFTER LIVER TRANSPLANTATION

MiRNAs could also be useful for detecting recurrence of other liver diseases after LT, such as primary sclerosing cholangitis (PSC), primary biliary cirrhosis (PBC), non-alcoholic fatty liver disease (NAFLD), non-HCV viral hepatitis, auto-immune hepatitis (AIH), and a variety of metabolic diseases. However, in our search of literature currently no studies were found concerning the use of miRNAs after LT in other hepatic diseases, and thus we are unable to report on this topic in this review.

CURRENT CHALLENGES AND FUTURE APPLICATIONS

As discussed earlier, the analysis of miRNAs for biomarker purposes can be performed in many different biomaterials and at different stages of LT. Ideally, liver biopsies should be avoided, as they impose a risk to the patient due to their invasive nature. Much of the earlier described research however has used liver biopsies for identification of miRNAs as they are easier to detect in tissue. Detection of miRNAs in bodily fluids and graft perfusion fluid can be cumbersome due to lack of generally accepted protocols for isolation, detection and normalization, and of adequate reference genes. Further investigation on technical standards in detecting miRNAs in fluids is thus needed for discovery of new biomarkers. But most importantly also the verification of earlier identified markers is crucial. Validation of biomarkers is critical before translation of non-invasive or minimally-invasive form can be applied in the clinic and can replace existing suboptimal and/or invasive markers. Therefore, it is not expected that in short-term, non-invasive diagnostics will replace liver biopsies taken for the purpose of histologic assessment.

Invasive diagnostic methods however, do not necessarily always pose a risk. Sometimes, invasively acquired material is already conveniently available due to the nature of the therapy, like tumor tissue that was collected from liver resection specimens[67, 68, 71]. Though invasive diagnostics in these cases do not pose an additional risk, non-invasive biomarkers could still be useful as the expected prognosis could be known beforehand, and patients be followed-up easier and non-invasively. Another complicating factor of using biopsies as a source for miRNA identification is the fact that biopsies only represent local expression instead of systematic changes. Therefore, using this technique, many interesting miRNAs could be overlooked and this might also explain the limited overlap in identified miRNAs by the different studies. Moreover, in diseases that tend to have patchy distribution, such as ischemic cholangiopathy, the chances of a sample error are high.

As mentioned earlier, the absence of generally accepted protocols and technologies specifically designed to analyze large amounts of circulating miRNAs at once have significantly hampered research. However, novel technologies now available allow quantification of hundreds of circulating miRNAs at once in a more standardized fashion and have already lead to the discovery of many biomarkers[72-78], thereby opening new possibilities in the setting of transplantation.

Novel non-invasive biomarkers could be used for earlier detection and treatment of disease possibly preventing the need for transplantation or used for quantifying the response of novel therapies for diseases. During transplantation, biomarkers will aid in selecting appropriate good quality allografts[14]. Whereas after transplantation, they could be utilized for individually tailoring the need of immunosuppression, allowing a better balance between effects (prevention of graft rejection) and side effects (long-term nephrotoxicity, infection and malignancy)[58], or be utilized for early detection of recurrent disease.

The miRNAs discussed in the present study can not only serve as biomarkers but could also give more insight in mechanisms of several clinical entities, such as recurrence of disease or ischemia-reperfusion injury and its repair. This however remains difficult, as target prediction of miRNAs is achieved by in silico algorithms on the basis of (partial) complementarity and one unique miRNA usually has many hundreds of potential targets. These targets need to be confirmed through in vitro studies, as many predicted targets do not show any regulation by the miRNA expected to regulate[79]. No technique is currently available for mass target verification, which is time consuming, and thus usually a small number of targets are selected on the basis of hypotheses. This inevitably leads to a selection bias in studies and does not give a complete picture of the biology in a certain situation. This currently makes it difficult to quickly relate a certain miRNA to a certain biological function elucidating the pathogenesis.

Another potential role for miRNAs could be their therapeutic appliance. Recent literature implicates that released miRNAs serve as a way of cell-to-cell communication and that they can trigger remote (regenerative) responses following injury and disease[21, 80-85]. Studies have already demonstrated the use of anti-sense, anti-miRNA, technology with surprising therapeutic results[11, 86]. This application of miRNAs could be used not only for treatment of (recurrent) disease, but possibly also for optimizing allograft quality by treatment of grafts after organ retrieval but prior to transplantation. However, as discussed, actual regulation of targets by miRNAs cannot be calculated reliably and one should therefore be careful that many other unwanted targets are not affected when applying the miRNAs therapeutically, which can lead to severe side effects.

Finally, when applying miRNAs for diagnostic utility, besides plasma and serum, many other non-invasively obtainable substrates, as mentioned earlier, contain miRNAs, but they have not been investigated thoroughly[14, 17, 18, 37]. All these substrates present possible sources of non-invasive diagnostic possibilities and should be researched. All in all, miRNAs represent a very promising field not only for diagnostic but also future therapeutic possibilities and therefore extensive research on miRNAs as biomarkers, their role in regulation and pathogenesis, and finally therapeutic appliance is justified and warranted.

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Hepatocyte-derived microRNAs as serum biomarkers of hepatic injury and rejection after liver transplantation

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ABSTRACT

Recent animal and human studies highlight the potential of hepatocyte-derived microRNAs (HDmiRs) in serum as early, stable, sensitive, and specific biomarkers of liver injury. Their usefulness in human liver transplantation, however, has not been addressed. Aim of this study is to investigate serum HDmiRs as markers for hepatic injury and rejection in liver transplantation.

Serum samples of healthy controls and liver transplant recipients (n = 107), and peri-transplant liver allograft biopsies (n = 45) were analyzed by RT-PCR quantification of HDmiRs, miR-122, miR-148a and miR-194.

The expression of miR-122 and miR-148a in liver tissue was significantly reduced with prolonged graft warm ischemia times. Conversely, serum levels of these HDmiRs were elevated in patients with liver injury and positively correlated with transaminase levels. HDmiRs appears to be very sensitive, as patients with normal transaminase values (below 50 IU/L) had 6 to 17-fold higher HDmiRs levels as healthy controls ($P < 0.005$). During an episode of acute rejection, serum HDmiRs were elevated up to 20-fold and appear to rise earlier than transaminase levels. HDmiRs proved stable during repeated freezing and thawing of serum.

In conclusion, this study shows that liver injury is associated with release of HDmiRs into the circulation. HDmiRs represent promising candidates as early, stable and sensitive biomarkers for rejection and hepatic injury after liver transplantation.

INTRODUCTION

MicroRNAs (miRNAs), a class of small non-coding RNAs, are important regulators of gene expression and they control many cellular processes by post-transcriptional suppression of gene expression[1, 2]. Altered tissue expression levels of miRNAs have lately been linked to various pathologic conditions in humans, including malignant, infectious, metabolic, autoimmune, and cardiovascular diseases[3-9]. These findings have lead to increased interest in miRNAs as potential diagnostic markers as well as targets for therapeutic interventions.

Hepatocytes express a distinct set of miRNAs of which miR-122 is most abundant[10]. MiR-122 was found to be an important regulator of cholesterol metabolism[11], iron homeostasis[12] and a crucial host factor for hepatitis C virus infection and replication[13, 14]. In addition to these important cellular functions, recent studies in rodents have demonstrated that miR-122, as well as other hepatocyte-abundant miRNAs, are released from cells during drug-induced liver injury[15, 16]. These hepatocyte-derived miRNAs (HDmiRs) were detectable in serum or plasma and levels increased dependent on the dose and duration of drug exposure. HDmiRs were found to correlate with serum transaminases, aspartate transaminase (AST) and alanine transaminase (ALT), as well as liver histology. Importantly, the rise in serum miRNA in these animals appeared earlier than the rise in transaminases. In addition to the diagnostic potential of miRNA, experimental animal studies have shown that miRNAs are a feasible target for therapeutic intervention to minimize and even reverse severe tissue injury caused by ischemic insults[17]. In humans, it has recently been shown that the HDmiR miR-122 can also be detected in serum and was found to be elevated in patients with hepatocyte injury caused by viral, alcoholic or chemical-related hepatotoxicity[18, 19]. Also in these patients, serum and plasma miR-122 showed a close correlation with transaminases and liver histology. However, this has not been evaluated in the setting of liver transplantation.

Liver transplantation has developed from a risky experimental procedure to a life-saving and effective treatment of end-stage liver failure. However, despite this success, transplant recipients can suffer from serious side effects of long-term immune suppression and remain at risk of de novo malignancies[20] or lose their allograft due to rejection, recurrent disease or biliary complications[21, 22]. The potential benefit of tapering immunosuppressive medication in patients to reduce toxicity is countered by the potential risk of losing the graft by immune mediated rejection. Therefore, there is an urgent need for better biomarkers that could provide earlier and more sensitive signs of rejection or liver graft dysfunction in a non-invasive fashion. Given their cell-type specific distribution, their biological stability and sensitivity of detection, HDmiRs could represent promising candidates for this. Indeed, several recent studies in the setting of kidney

transplantation have highlighted the potential of mRNA and miRNA as biomarkers for assessing renal allograft status[23-26]. Current protein-based markers for liver injury, AST and ALT, are also expressed outside the liver in muscle tissue and they can cause false elevations during muscle injury[27]. Therefore, assessment of liver allograft status often still requires tissue biopsies for more definite proof of hepatic injury. Particularly after liver transplantation, taking trough-cut biopsies is a relative perilous procedure associated with pain, bleeding and infections[28-31]. Alternatively, more sensitive, specific and non-invasive methods for monitoring graft injury are needed to minimize the need for liver biopsies and allow safer weaning-off of immunosuppressive medication.

The aim of the current study was to investigate the utility of serum HDmiRs as markers for hepatic injury and acute rejection after liver transplantation. We found that the expression of miR-122 and miR-148a in liver tissue was significantly diminished with prolonged graft warm ischemia times and, conversely, was elevated in serum during ischemia and reperfusion injury and acute rejection. HDmiRs were found to represent promising candidates as biomarkers for assessing allograft status after liver transplantation.

MATERIALS AND METHODS

Patient samples

All liver transplantations were performed at Erasmus Medical Center, Rotterdam, The Netherlands. Liver graft biopsies (n = 45) were obtained during transplantation 60 minutes after portal reperfusion and directly snap frozen for storage. Serum samples were taken from 12 healthy controls and 43 recipients at different times after liver transplantation and included 13 patients with histologically proven acute rejection. All blood samples were collected using a standardized protocol and serum was processed within 2 hours and quickly stored in -80 °C. Serum samples with signs of red blood cell lysis were not used. Patient demographics and clinical variables were extracted from a prospectively filled database and summarized in Table 1. The intrinsic stability of HDmiRs in serum was determined by subjecting four individual serum samples from liver transplant recipients to five freezing and thawing cycles (-80 °C / +20 °C). The Medical Ethical Council of the Erasmus MC approved the use of human samples and all patients provided informed consent for the use of materials for medical research.

Serum levels of AST and ALT < 50 UI/L were considered normal. Acute cellular rejection was defined by the presence of all three following criteria: a transient rise in AST and ALT levels above the upper limit of normal, a rejection activity index (RAI) of 6 or more in the consequent needle biopsy at histological examination and a decrease in transaminase levels upon treatment with methylprednisolone[32].

Characteristic	Healthy Controls	After Transplantation	
		Nonrejectors	Rejectors
Serum samples (n)	12	33	62
Mean AST (IU/L)	—*	369 ± 83	135 ± 37
Mean ALT (IU/L)	—*	386 ± 60	144 ± 29
Subjects/patients (n)	12	30	13
Age (years)	42 ± 3	45 ± 3	39 ± 6
Sex: male/female (n/n)	7/5	18/12	6/7
Underlying disease (n)	—		
Viral	—	14	6
Cholestatic	—	5	3
Alcoholic	—	4	2
Other	—	7	2

Table 1. Characteristics of patients and healthy controls (*ND = not determined).

RNA isolation

Total RNA was extracted from approximately 10 mg of liver tissue using the miRNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). A modified protocol was used to isolate total RNA from serum. For this, 1.5 ml of Qiazol Lysis Reagent was added to 200 µl of serum and extensively mixed by vortexing. Chloroform (300 µl) was added and after centrifugation (15 minutes, 16.000 RCF), 800 µl of an aqueous RNA-containing layer was obtained, which was further processed according to the manufacturer's protocol (Qiagen). RNA extracted from liver tissue was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and normalized to a concentration of 50 ng/7.5 µl. RNA extracted from serum could not be quantified due to its low concentration and was normalized only for initial serum input.

Reverse transcription and real-time polymerase chain reaction (RT-PCR)

The TaqMan microRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) was used to prepare cDNA, for multiple miRNAs in one reaction, using a modified protocol. Every multiplex cDNA reaction consisted of 0.4 µl dNTP mix, 1.35 µl Multiscribe RT enzyme, 2.0 µl 10x RT Buffer, 0.25 µl RNase Inhibitor, 1.0 µl of each RT primer, and 7.5 µl of diluted template RNA. The total reaction volume was adjusted to 20 µl with nuclease free water. Based on literature, 15 miRNAs were initially tested, namely miR-30a, miR-30c, miR-30e, miR122, miR-133a, miR-148a, miR-191, miR-192, miR-194, miR-198, miR-200c, miR-222, miR-296, miR-710 and miR-711[15, 33-36]. Three highly expressed hepatocyte-rich miRNAs, miR-122, miR-148a and miR-194, were selected and further used. For analysis of liver biopsies, additional cDNA was prepared for a small nuclear RNA, RNU43, which served as reference gene for normalization of RNA input. For serum samples two additional non-liver-abundant miRNAs, miR-133a (muscle-abundant) and miR-191 (blood-abundant), served as controls. All cDNA reactions were performed according to the manufacturer's instructions. PCR reactions were carried out in duplicate and according to the manufacturer's instructions. Each reaction consisted of 10 µl TaqMan Universal PCR Master

Mix, 0.5 µl microRNA-specific PCR primer (Applied Biosystems) and 5.0 µl of the previously diluted (1:10 dilution) cDNA. The final volume of every PCR reaction was adjusted to 20 µl with nuclease-free water.

Statistical analyses

Statistics for correlation were generated using Spearman's Rank Correlation test. Comparative statistics between groups were tested using the Mann-Whitney U and the Wilcoxon matched pairs test by GraphPad Prism software (GraphPad Software Inc., San Diego, USA). P-values < 0.05 were considered significant.

RESULTS

Reduced hepatic miRNA levels in liver grafts with long warm ischemic times

To investigate changes in intrahepatic miRNA expression in response to ischemia-reperfusion injury, 45 biopsies taken from liver grafts one hour after reperfusion were analyzed. Average cold ischemia time was 484 ± 25 minutes (mean ± SEM) and the mean warm ischemia time was 35 ± 2 minutes. As shown in Figure 1A, there was a significant positive correlation between the levels of hepatocyte-abundant miRNAs. Levels of miR-122 strongly correlated with miR-148a and miR-194 ($R \geq 0.85$, $P < 0.001$), but were approximately 20-fold higher than those of miR-148a and miR-194. As shown in Figure 1B, the levels of miR-122 and miR-148a, but not miR-194, in these liver graft biopsies showed a significant reverse correlation with the length of warm ischemia time ($R = -0.307$, $P = 0.038$ and $R = -0.404$, $P = 0.005$ respectively). No significant correlation of miRNA levels and cold ischemia times was observed (data not shown). These findings suggest that graft injury associated with longer warm ischemia times reduced levels of specific hepatocyte-abundant miRNAs, possibly by the release of miRNAs from injured cells.

Serum HDmiRs are associated with peri-transplant ischemic liver injury

Serum samples from healthy individuals and liver allograft recipients within 2 weeks of transplantation were analyzed for the presence of HDmiRs. All three HDmiRs, miR-122, miR-148a and miR-194, and both control miRNAs, miR-133a and miR-191, were detectable in the serum from healthy individuals and patients. As shown in Figure 2, the levels of HDmiRs were significantly elevated in patients after liver transplantation as compared to healthy controls. In serum samples with high transaminase levels (AST or ALT > 50 UI/L), the levels of miR-122 were respectively 124-fold and 102-fold elevated with respect to average levels in healthy controls ($P < 0.0001$). When compared to healthy controls, levels of miR-148a and miR-194 were respectively 30-fold and 40-fold higher in

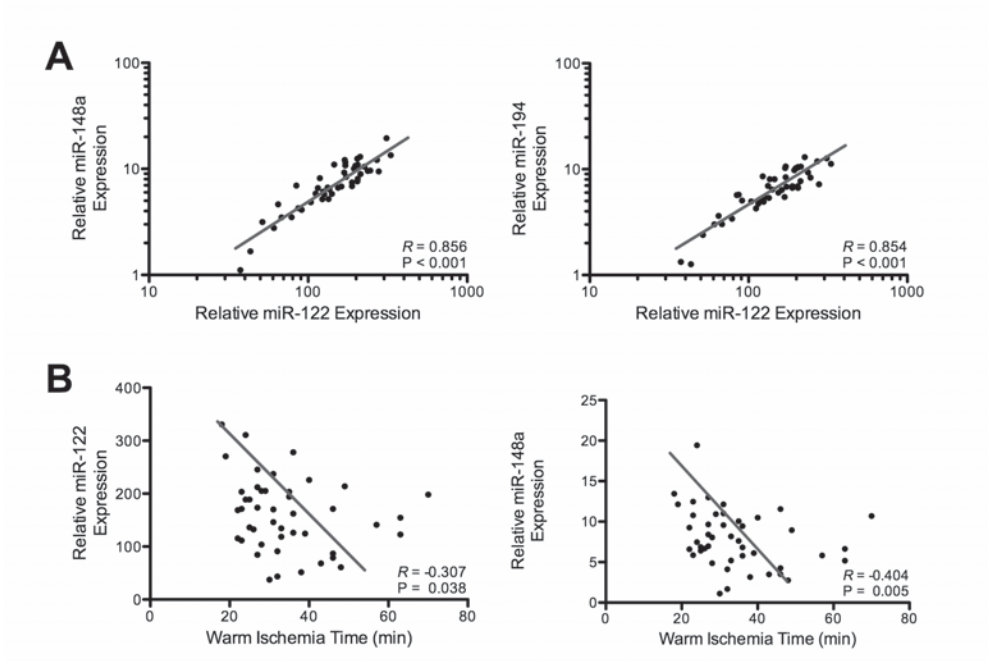

Figure 1. Decreased levels of hepatic miRNAs in liver grafts with extended warm ischemia times. Liver graft tissue biopsies (n = 45) were analyzed for the hepatocyte-abundant miRNAs, miR-122, miR-148a and miR-194, by quantitative RT-PCR. MiRNA levels were normalized to small nuclear RNA RNU43, which served as a reference gene. (A) Relative expression levels of miR-122 correlated significantly with miR-148a and miR-194 in the liver grafts ($R \geq 0.85$, $P < 0.001$). MiR-122 levels were approximately 20-fold higher than miR-148a and miR-194. (B) Decreased levels of miR-122 and miR-148a in liver graft biopsies correlated significantly with length of the warm ischemia time to which the graft had been exposed during liver transplantation ($P < 0.05$).

the high transaminase groups ($P < 0.0001$). Levels of all HDmiRs were significantly higher in the high AST and ALT groups compared to the low AST and ALT groups ($P < 0.005$, Fig. 2) with the exception of miR-194 in the high ALT group, which was only 2-fold elevated and not statistically significant. Levels of the control miRNAs, miR-133a and miR-191, were not significantly different between any of the groups (Fig. 2). The HDmiRs appeared to be sensitive, as patients with normal transaminase values had significantly elevated levels of miR-122, miR-148a and miR-194 compared to healthy controls (respectively 11, 7, and 9-fold higher in the low AST group and respectively 8, 6 and 17-fold higher in the low ALT group, $P < 0.005$). As shown in Figure 3, a positive correlation was observed between serum HDmiRs levels and transaminases in patients. The correlation with AST and ALT resulted in a coefficient R of respectively 0.80 and 0.77 for miR-122, while for miR-148a the coefficient R was 0.60 for both AST and ALT ($P < 0.0001$). No significant correlations were found for miR-194 ($R < 0.30$, $P > 0.05$).

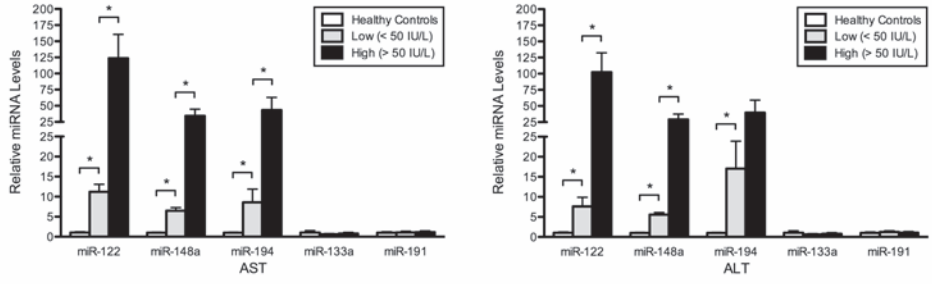

Figure 2. Hepatocyte-derived miRNAs (HDmiRs) are elevated in serum during peri-transplant ischemic liver injury. HDmiRs miR-122, miR-148a and miR-194, were quantified using RT-PCR in 92 serum samples obtained from liver transplant recipients (n = 40) and healthy controls (n = 12). Compared to healthy controls, levels of miR-122, miR-148a and miR-194 were significantly elevated in serum samples of patients with low AST and ALT levels by 11-, 7-, 9-, and 8-, 6- and 17-times, respectively. Levels were further elevated in serum of patients with transaminase levels above the clinical diagnostic threshold of 50 UI/L. For the high AST group miR-122, miR-148a and miR-194 were 11-, 5- and 5-fold higher and for the high ALT group 13-, 5- and 2-fold higher compared to the low AST and ALT groups. Levels of control miRNAs, miR-133a and miR-191, were not significantly elevated in any of the serum samples compared to healthy controls. * P < 0.005.

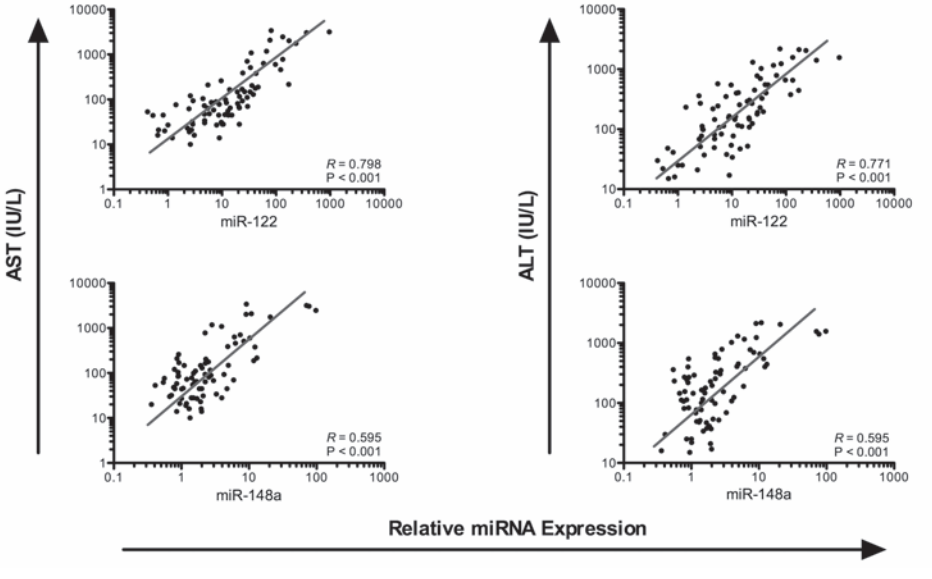

Figure 3. Levels of serum HDmiRs in liver transplant recipients correlate with AST and ALT. HDmiRs, miR-122 and miR-148a, were quantified using RT-PCR in eighty serum samples obtained from liver transplant recipients. Serum levels of miR-122 and miR-148a correlated significantly with levels of AST and ALT in the same samples.

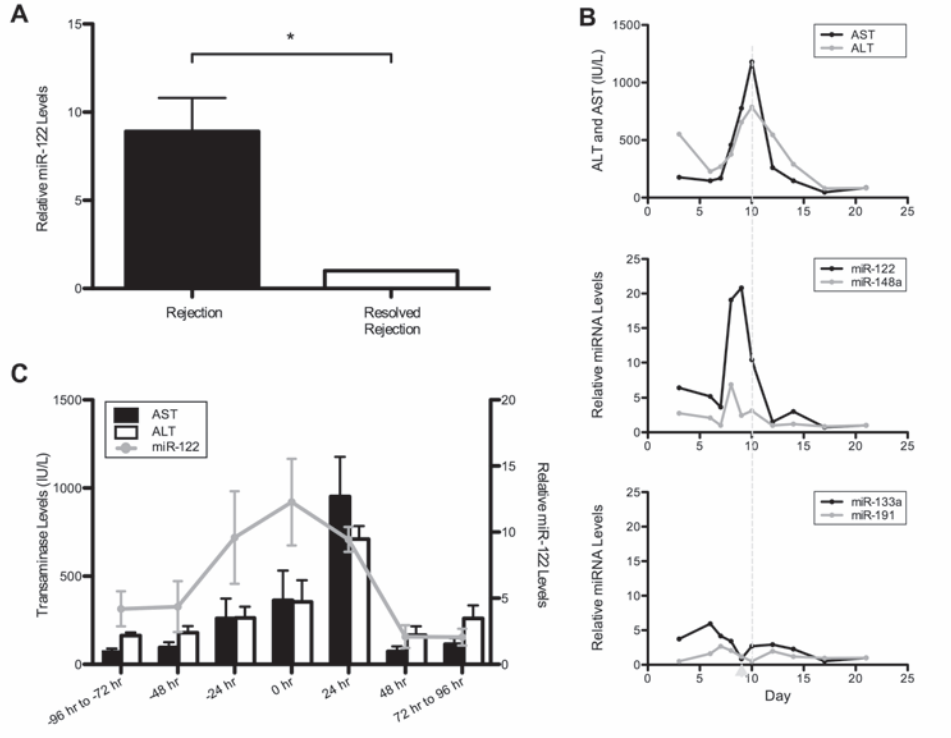

Figure 4. Changes in serum HDmiRs during acute rejection. Serum samples from 13 liver transplant recipients experiencing one or more episodes of biopsy-proven acute rejection were analyzed. (A) Levels of serum miR-122 were significantly elevated during acute rejection by approximately 9-fold compared to levels in the same recipients 6 months after rejection was resolved (n = 13, $P < 0.005$). (B) From five of these patients a longitudinal series of serum samples, taken at daily intervals, was analyzed. Representative results from one patient are shown. Serum levels of miR-122 and miR-148a increased up to 20-fold during acute rejection (middle panel) and showed similar kinetics to those of AST and ALT (top panel). The peak of HDmiRs appears to precede the peak of transaminases (indicated with dashed line) and quickly normalized after starting treatment with intravenous methylprednisolone (arrow on axis). Levels of control miRNAs, miR-133a and miR-191, did not show an increase during acute rejection (lower panel). (C) Levels of serum transaminases and miR-122 of the 5 patients at the histologic diagnosis and start of methylprednisolone treatment (t = 0 hr) and up to 96 hrs before and after are shown. Levels of miR-122 reached a maximum level at the start of treatment and quickly decreased after treatment whereas transaminase levels still continued to rise 24 hrs later.

Additional experiments to test the stability of HDmiRs in serum showed that levels of miR-122, miR-148a and miR-194 in serum were not significantly affected after five cycles of freezing (-80 °C) and thawing to room temperature (mean 120% ± 11 SEM, 100% ± 6 and 99% ± 19 of untreated baseline values, respectively).

Elevated serum HDmiRs during acute rejection

Serum HDmiRs were analyzed in liver transplant recipients experiencing an episode of acute rejection. As shown in Figure 4A, serum HDmiR miR-122, was significantly elevated during rejection. An average 9-fold increase was observed at the time of rejection compared to levels 6 months after resolving rejection ($P < 0.005$). For five patients a longitudinal series of serum samples before, during and after acute rejection was analyzed. One representative patient is shown in Figure 4B. Serum levels of miR-122 and miR-148a showed kinetics similar to those of AST and ALT and increased up to 20-fold during acute rejection. Levels of the control miRNAs, miR-133a and miR-191, did not increase during acute rejection (Fig. 4B). Although miR-122 showed similar kinetics, it appeared to rise and drop one or two days earlier than transaminase levels (Fig. 4B). As shown in Figure 4C, in pooled data of five patients a similar trend was observed. At the moment of diagnosis and start of treatment of the acute rejection (0 hr) miR-122 was already elevated to its maximum level. Levels of miR-122 dropped quickly after the start of intravenous methylprednisolone treatment, while levels of AST and ALT continued to rise even after the start of treatment and took longer to normalize.

DISCUSSION

Small non-coding RNAs, in particular miRNAs, have emerged as important genetic regulators of cellular processes, including tissue injury and repair responses[17]. Recent studies in small animal models as well as humans have demonstrated that HDmiRs are highly stable and sensitive serum biomarkers of liver injury[15, 16, 18, 19]. In both humans and rodents, HDmiRs appeared to increase earlier and more rapidly in serum than AST and ALT. In particular miR-122 was significantly elevated even in subjects with transaminases below the threshold of 50 IU/l[15, 16, 18, 19]. In the current study we provide evidence that the concept of miRNAs as biomarkers of hepatic injury is also feasible in the setting of liver transplantation. Serum levels of HDmiRs were elevated in patients with liver injury after liver transplantation (Fig. 2) and during acute rejection (Fig. 4). Conversely, hepatic miRNA levels in liver graft biopsies exhibited diminished expression with prolonged warm ischemic times (Fig. 1). During acute rejection, serum HDmiRs showed similar kinetics, however, miRNA levels increased and decreased earlier than transaminases (Fig. 4B and C). As in previous studies[15, 18], miRNAs showed higher sensitivity than transaminases and miRNA stability was confirmed as proposed by earlier studies[6, 9, 37-40].

HDmiRs could provide a solution for the urgent need for better non-invasive biomarkers that could serve as earlier and more sensitive signs of rejection or liver graft dysfunction. Better markers would greatly help the management of liver transplant recipients and could allow the safer reduction of immunosuppressive medication to achieve a better

balance between effects (prevention of graft rejection) and side effects (toxicity, infection and malignancy). Long-term complications of immunosuppressive drugs, such as nephrotoxicity and de novo cancer, are becoming a bigger problem due to the long survival of liver transplant recipients[20]. Currently, the potential benefit of tapering immunosuppressive medication in patients is countered by the potential risk of losing the graft by immune mediated rejection. Serum ALT and AST are often insufficient for the early and definitive diagnosis of acute rejection, necessitating the use of liver biopsies. Particularly in the setting of liver transplantation, liver biopsies pose a significant risk for complications such as pain, bleeding and infections[28-31]. Feasibility of the concept of minimally invasive diagnosis of acute rejection, based on the detection of messenger RNA, has been demonstrated for kidney transplants[24, 25].

Currently, little is known about the mechanism and biology of release of hepatocyte-abundant miRNAs in response to liver injury. Ideally an unbiased genome-wide approach would be preferred to study release, but it is very challenging to perform gene-array analysis on serum samples because of the low yields of RNA and the relative high amounts required. In our initial analyses we tested 15 different types of hepatocyte and cholangiocyte abundant and control miRNAs selected from other studies[15, 33-36]. These included miR-30a, miR-30c, miR-30e, miR122, miR-133a, miR-148a, miR-191, miR-192, miR-194, miR-198, miR-200c, miR-222, miR-296, miR-710 and miR-711, but only the three HDmiRs were found to be significantly elevated during acute rejection. Likely, many other miRNAs expressed in hepatocytes and other liver cells are released during hepatic injury, but only the most abundant and liver-specific miRNAs will be detectable in serum. Nevertheless, the hepatocyte-abundant miRNA miR-194, with expression levels in liver tissue significantly correlating with miR-122 (Fig. 1A), did not correlate with transaminase levels (data not shown). This suggests that there may be sequence specificity or selectivity regarding the release of miRNAs, rather than just a general leakage of all miRNAs from the injured cell. This hypothesis is supported by the observation that cellular miRNAs can be released from cells by secretion of microvesicles including exosomes and that only distinct sets of miRNAs are selectively packaged into microvesicles[40, 41].

This specificity in release and the distinct repertoires of miRNAs expressed by various cell types in the liver may allow in the future distinguishing between different causes and types of liver injury, like cholangiocyte injury in bile ducts and endothelial cell injury in veins and arteries. Preliminary data from our research group indeed suggests that tissue levels of specific miRNAs expressed by biliary epithelial cells could be used to quantify biliary injury and can predict the development of long-term biliary complications and graft loss after liver transplantation[42]. In addition, miRNA-based diagnostics could facilitate allograft selection, particularly of marginal donors, and potentially enlarge the pool of grafts. For example, several experimental studies demonstrated a role of hepatic miRNAs,

including miR-122, in regulation of cell proliferation during liver regeneration after partial hepatectomy[43-48]. Although the exact biology is not clear, it is conceivable that the decrease in miR-122 expression during graft storage may be related to hepatic cell cycle progression in response to ischemic injury. It is tempting to speculate that manipulation of miRNAs using anti-sense, anti-miRNA technology[11], could allow therapeutic intervention for rescue of marginal grafts or allow the use of smaller size split grafts by minimizing injury and stimulating cell proliferation[17].

In summary, we demonstrate that circulating HDmiRs, miR-122, miR-148a and miR-194, are stable and detectable during hepatic injury in patients after liver transplantation. The levels of two of these HDmiRs closely correlate with AST and ALT during post-transplant liver injury and acute rejection. These data support the potential of miRNA-based diagnostic tools for various types of liver injury in liver transplant recipients.

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Bidirectional release of microRNAs into bile and blood after liver cell injury, during impaired graft function and rejection following liver transplantation

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7

ABSTRACT

Hepatocyte and cholangiocyte-derived microRNAs (HDmiRs and CDmiRs respectively) in blood and bile have proven to be useful biomarkers for various hepatic pathologies. However, the exact mechanism of miRNA release in response to injury after liver transplantation (LT) remains largely unknown. The aim of this study was to determine the release of HDmiRs and CDmiRs by cells during ischemia, and determine release into blood and bile during liver injury or impaired liver function in human recipients after LT as well as in a large animal model.

Cellular specificity of HDmiRs and CDmiRs was confirmed in liver (n=10) and extrahepatic bile duct biopsies (n=9). *In vitro*, cellular ischemia caused a significant release of HDmiRs and CDmiRs from respectively hepatoma and cholangiocarcinoma cell lines. In human bile, most HDmiRs and CDmiRs were present in non-pelletable fragments, and they were differently protected against RNase degradation through protein-conjunctions. Analysis of paired bile and serum samples (n=62) from LT recipients showed a strong positive correlation between miR-122 levels in bile and graft function (R = 0.694, P < 0.0001), while an inverse correlation was found with CDmiRs (P < 0.05). In contrast, early graft injury and histology-proven rejection inhibited the release of HDmiR-122 into bile and caused a significant increase in biliary CDmiR levels (P < 0.001). Finally, the induction of (severe) hepatic ischemia-reperfusion was measured *in vivo*; hepatic warm ischemia induced in pigs caused an increase in miRNA levels to both bile and circulating perfusate (P ≤ 0.005, R ≥ 0.471).

In conclusion depending on the type of liver injury, HDmiRs and CDmiRs are directionally released into blood and bile. Biliary HDmiRs and CDmiRs seem a promising marker for both hepatocyte and cholangiocyte injury and cellular function.

INTRODUCTION

MicroRNAs (miRNAs) are important regulators of post-transcriptional gene expression and as such control many cellular processes[1]. Previous studies have shown the involvement of miRNAs in physiological as well as pathophysiological processes[2-6]. In addition, the fact that certain miRNAs are highly cell-type abundant makes them attractive for biomarker research. Recent studies have investigated the release of such specific miRNAs into the circulation and proposed their use as highly sensitive and specific markers for cellular injury[7-9]. Moreover, some studies suggest that miRNAs released upon injury might serve as a danger signal that can trigger remote regenerative responses[10-14]. Despite the increasing knowledge on miRNAs as potential biomarkers, though, our understanding on the underlying mechanisms of miRNA release in response to injury remains incomplete, especially in the field of liver transplantation (LT).

The presence of (extracellular) miRNAs has been demonstrated in many bodily fluids, including amniotic fluid, breast milk, bronchial lavage, cerebrospinal and peritoneal fluid, plasma, saliva, tears, urine, pleural fluid, colostrum, and seminal fluid[15]. Recently, in concordance with other human and animal studies[8, 9, 16], our research group has demonstrated the specific release of hepatocyte-derived miRNAs (HDmiRs) in blood during liver injury, chronic hepatitis C infection and acute rejection after LT[7, 17]. These miRNAs were shown to be stable, early, and sensitive markers of liver injury. In addition to HDmiRs, miRNAs derived from cholangiocytes (CDmiRs) were diagnostic in patients with cholangiocarcinoma[18]. Moreover, CDmiRs were shown to sensitively identify grafts with severe biliary injury already at time of graft preservation in LT[19]. Whereas HDmiRs seem to increase in serum during episodes of injury, simultaneously, a decreased expression in tissue was observed. In contrast, lower levels of CDmiRs were observed in graft perfusates at time of transplantation in grafts that later developed biliary complications. This supports the current view that miRNA release is a selective and active process rather than passive and non-specific leakage from apoptotic or damaged cells. Furthermore, it could indicate that HDmiRs and CDmiRs are released in a different direction during injury; as earlier suggested by Lankisch et al. Due to the anatomical organization of cells in the liver, it seems more likely that cholangiocytes will release miRNAs to bile rather than to blood[20]. Shigehara et al. were the first group reporting on miRNAs in bile. They found miR-9 to be a potential biomarker for biliary tract cancer, and showed miRNAs were protected against RNase activity[21]. In addition, Li et al. used miRNAs in extracellular vesicles in bile for the identification of diagnostic miRNAs in cholangiocarcinoma[22]. Yet, these studies did not address the relation between miRNA levels in both bile and serum. Thus, the question remains whether polarized cells like hepatocytes and cholangiocytes can control miRNA release bidirectionally into both blood and bile.

In the current study, we aimed to validate the use of extracellular miRNAs as markers for liver injury and liver function in the setting of LT and to gain a better understanding on underlying mechanisms and routes of miRNA release. Therefore, we investigated miRNA release from (i) hepatoma and cholangiocarcinoma cells following ischemia, (ii) release into blood and bile during normal graft function and during early graft injury and rejection in human LT recipients, and (iii) release into bile in response to severe warm ischemia in a large animal model.

MATERIALS AND METHODS

Donor and recipient samples

First, abundance of HDmiRs and CDmiRs was evaluated by comparing expression of selected miRNAs in liver biopsies (n=10) and common bile duct (CBD) specimens (n=9), which were collected from donor livers at the end of cold ischemia during human LT. Tissue samples were snap frozen and stored at -80 °C until further use. In order to investigate directional HDmiR and CDmiR release during different (patho)physiological conditions, paired serum and bile samples (n=62 each) from 10 recipients were collected at different time points during the first three weeks following LT. Serum was withdrawn by venipuncture while bile was collected from a T-tube that was inserted routinely into the CBD during LT. Samples were processed within two hours of withdrawal to prevent any degradation or contamination and were stored at -80 °C. Standard liver function tests (AST, ALT, ALP, γ-GT and bilirubin) were obtained from serum. Bilirubin levels were also determined in bile for the assessment of liver function.

During the benching procedure, additional bile samples were obtained from donor gallbladders for centrifugal fractionation (n=4) and miRNA stability testing (n=8). Large components were removed from these bile samples in a two-step centrifugation protocol. Samples were centrifuged for 10 minutes at 453 g at 4 °C followed by 15 minutes of centrifugation at 3220 g at 4 °C. Bile samples were stored at -20 °C until further use.

During recipient follow-up, protocol fine-needle biopsies were taken for histological evaluation. Samples were stained for Hematoxylin-eosin (HE) and cytokeratin 19 (CK19) and scored for rejection in correspondence with the rejection activity index[23] by an independent, blinded, pathologist. The use of all human samples was approved by the Medical Ethical Council of the Erasmus MC and all patients provided informed consent for the use of materials for medical research.

Localization of miRNAs in bile fractions

For fractionation of stored bile, 4 ml of bile extracted from donor gallbladders was diluted with 8 ml of sterile PBS and a baseline sample was taken (sample S0). Large components

were pelleted by centrifugation at 20.000 g for 20 minutes at 4 °C (sample P1). The pellet was resuspended in 400 µl sterile PBS and mixed with 1400 µl Qiazol lysis agent (Qiagen, Hilden, Germany) and stored at -80 °C until further use. The supernatant was transferred to a new tube and centrifuged at 100.000 g for 1 hour at 4 °C. Pellets were again prepared as described above (sample P2). The supernatant was then centrifuged for a third time for 2 hours at 140.000 g at 4 °C and the resulting pellet (sample P3) was dissolved and stored as mentioned. 400 µl of remaining supernatant was mixed with 1400 µl of Qiazol and stored at -80 °C until further processing (sample S3).

DEPC treatment and protein degradation of stored bile

Stored bile was treated with diethylpyrocarbonate (DEPC; Sigma-Aldrich, Zwijndrecht, The Netherlands) in a final concentration of 0.02% for 3 hours at room temperature. To remove traces of DEPC completely, samples were boiled for 15 minutes, and aliquots were stored at -20 °C until further use. Immediately prior to incubation at 37 °C, samples were spiked with 2 fmol each of artificial C. elegans miR-39 (cel-miR-39) and miR-238. To degrade stabilizing proteins in stored bile samples, 400 µl samples were incubated for 1 hour at 37 °C with Proteinase K (Roche Diagnostics, Almere, The Netherlands) in a final concentration of 0.1 mg/ml.

In vitro miRNA release assay

The human hepatoma cell line Huh7 and cholangiocarcinoma cell line TFK1 were used to investigate miRNA release following cellular ischemia. Monolayers of Huh7 and TFK1 cells were cultured in serum-free medium at 37 °C. Ischemia was induced by incubating cells with mineral oil for a median of 3 hours. After removal of the oil, the cells were again incubated with serum-free medium for approximately 14 hours. Afterwards, the supernatant was centrifuged for 15 minutes at 12.000 g in order to obtain cell-free supernatant for miRNA analysis. The cells were harvested and analyzed for viability by MTT-assay and surface expression of Annexin V and 7 AAD.

Porcine model for hepatic ischemia-reperfusion injury

In collaboration with the University of Leuven, the release of HDmiRs and CDmiRs during severe hepatic ischemia-reperfusion injury was investigated in a porcine model for warm ischemia as previously described[24]. Inbred female Landrace pigs (25-40kg) were subjected to incremental times of hepatic warm ischemia by clamping the portal vein, the thoracic aorta, and caval vein (n=6 per group). After harvesting, liver grafts were flushed extensively with histidine tryptophan ketoglutarate (HTK, 5L) and connected to a hypothermic machine preservation device for four hours (Organ Recovery Systems, Chicago, IL). Subsequently, a three hour isolated, oxygenated normothermic liver

perfusion at 37 °C was performed to mimic graft reperfusion after LT, using Sanguineous AQIX RS-I solution. During the entire normothermic perfusion time, graft bile production was registered and collected at the end of perfusion, just as samples of recirculating perfusate. After processing, samples were stored at -80 °C until further use. Liver biopsies taken at the end of isolated normothermic perfusion were scored on morphology as developed by Imber et al. and consisted of the following parameters; dilatation, anoxic vacuoles, enlarged space of disse, los of cellular cohesion, parenchymal cell loss, presence of neutrophils, and congestion. This resulted in a score between 0 and 47 points[24, 25]. Biopsies were scored by two independent, experienced, pathologists.

RNA isolation

Total RNA was extracted using the miRNeasy mini kit (Qiagen, Hilden, Germany) using the manufacturer's protocol with minor modifications. For the isolation of miRNAs from tissue, 750µl of Qiazol lysis reagent was added to approximately 10mg of snap frozen liver and CBD specimens, which were homogenized by extensive vortexing with glass beads. For the isolation of miRNAs from serum and bile, 1500µl of Qiazol lysis reagent was added to 200 µl of serum or bile and mixed extensively by vortexing. In case of supernatant or pellet from the fractioning experiment or the Proteinase K treatment, 1400 µl of Qiazol lysis agent was added to 400 µl of stored bile (S0), supernatant (S3), or pellet (P1, P2, P3) as mentioned earlier. After 5 minutes of resting at room temperature, respectively 200µl or 280 µl of chloroform was added and the samples were again mixed vigorously by vortexing. After centrifugation (15 minutes, 16.000 RCF at 4˚C), 700 µl of aqueous RNA containing layer was obtained, which was further processed according to the manufacturer's protocol (Qiagen). RNA content was quantified, handled and stored as described previously[19].

Reverse transcription and real-time polymerase chain reaction (RT-PCR)

RNA samples were analyzed for HDmiRs and CDmiRs as previously reported[8, 18, 26]. As HDmiRs, miR-122 and miR-148a were determined and for CDmiRs, miR-30e, miR-200c and miR-222 were analyzed.

The TaqMan microRNA reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA) was used to prepare cDNA for multiple miRNAs in one reaction, using a modified protocol as reported previously[7]. In short, for every multiplex cDNA reaction 0.4 µl dNTP mix, 1.35 µl Multiscribe RT enzyme, 2.0 µl 10x RT Buffer, 0.25 µl RNase Inhibitor, 1.0 µl of each RT primer and 7.5 µl of template RNA were used. The total reaction volume was adjusted to 20 µl with nuclease-free water. All cDNA reactions were performed according to the manufacturer's instructions (Applied Biosystems).

For the analysis of paired serum and bile samples from LT recipients, PCR reactions were carried out in duplo on 384 wells plates to prevent inter-plate variability and consisted of 5 µl TaqMan Universal PCR Master Mix, 0.25 µl microRNA-specific PCR primer (Applied Biosystems) and 2,5 µl of the previously prepared cDNA (1:10 dilution). The final volume of every PCR reaction was adjusted to 10 µl with nuclease-free water and the PCR reactions were run according to the manufacturer's instructions for 45 cycles. For stability and protein degradation experiments and the analysis of cell line and porcine bile and perfusate samples, PCR reactions were performed as described previously[19].

Statistical analyses

Statistics for non-parametric correlations were generated using the Spearman's Rank Correlation test. Comparative statistics were generated using the Kruskal-Wallis, Mann-Whitney U and Wilcoxon matched pair tests. P-values < 0.05 were considered significant, as were coefficient R's ≥ 0.70 or ≤ -0.70. Analyses were conducted using IBM SPSS Statistics for Windows, Version 20.0 (Armonk, NY: IBM Corp.) and GraphPad Prism version 6.0 (GraphPad Software, San Diego, California, USA).

RESULTS

Which miRNAs are abundant in hepatocytes and cholangiocytes?

Previous studies demonstrated high cell-type specificity of several miRNAs. The cellular origin of miRNAs is of importance for biomarker purposes in order to discriminate between different types of injury. Therefore, we first validated the abundance of HDmiRs and CDmiRs, as such identified by previous studies[18], in liver biopsies (n=10) and CBD specimens (n=9) that were collected during LT. HDmiR-122 and miR-148a were chosen to test for hepatocyte injury and/or function, while CDmiR-30e, miR-200c and miR-222 were tested for cholangiocyte injury/function. As illustrated by Figure 1A, median expression of HDmiR-122 was over a 1000-fold higher in liver tissue compared to CBD tissue (128 ± 20 vs. 0.02 ± 0.80, P = 0.0021, median ± Interquartile range). Although less abundant than HDmiR-122, also the expression of HDmiR-148a was significantly higher in liver tissue (5.63 ± 0.93 vs. 1.29 ± 0.22, P = 0.0003). In CBD tissue, CDmiR-222 was expressed highest and up to 17-fold increased compared to liver tissue (0.52 ± 0.45 vs. 8.47 ± 1.56, P = 0.0002). The discrepancy in expression of CDmiR-200c was even higher, up to 70-fold; 0.04 ± 1.56 in liver vs. 2.78 ± 0.50 in the CBD. The results from this experiment suggest that miR-122 and miR-148a are indeed significantly present in hepatocytes, while miR-200c and miR-222 are more specific for cholangiocytes (Fig. 1A).

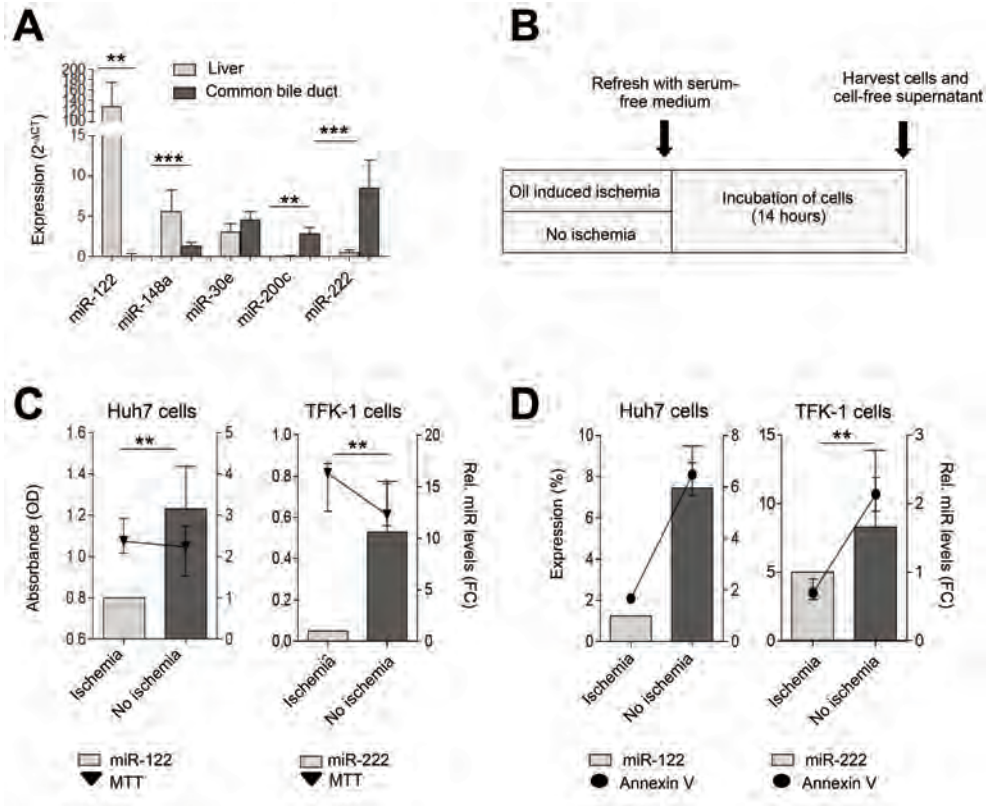

Figure 1. (A) Confirmation of hepatocyte and cholangiocyte abundance of HDmiR-122, HDmiR-148a, CDmiR-30e, CDmiR-200c and CDmiR-222 in liver biopsies (n=10) and CBD tissue (n=9). RNU43 levels were used for normalization. HDmiR-122 was highest abundant in liver tissue, while CDmiR-222 expression was highest in tissue of the CBD. (B) Experimental set-up of an in vitro assay of miRNA release from cell lines. Ischemia was induced into Huh7 and TFK-1 cell lines by mineral oil (1h to 6h). Next, serum-free medium was refreshed and cells were incubated for approximately 14h to mimic an in vitro reperfusion period. Afterwards, cells were harvested for viability assays and the cell-free supernatant was analyzed on levels of (released) miRNAs. HDmiR-122 was tested in Huh7 cell lines, while CDmiR-222 was tested in TFK-1 cell lines. (C) miRNA release and cell viability after ischemia in Huh7 cells (n=9) and TFK-1 cells (n=9). An increase in extracellular HDmiR-122 and CDmiR-222 respectively was observed in the supernatant, while cellular viability assessed by MTT was not affected. (D) Repeating the experiment in Huh7 cells (n=3), a non-significant increase was found in both HDmiR-122 levels in the supernatant and the expression of Annexin V, a marker for cellular apoptosis (P = 0.25). A similar, significant result was observed in TFK-1 cells (P = 0.0039). In both cell lines, the expression of 7AAD remained <1% of the cells, suggesting that necrosis is absent. Figures represent the median ± Interquartile range. **P <0.01, ***P <0.001.

Are HDmiRs and CDmiRs released during cellular stress?

Following our findings from the first experiment, we were interested whether HDmiRs and CDmiRs are released by cells that experience stress. Therefore, Huh7 cell lines (representing hepatoma cells) and TFK1 cells (representing cholangiocarcinoma cells) were subjected to oil-induced ischemia (Fig. 1B). Subsequently, levels of extracellular

HDmiR-122 and respectively CDmiR-222 were determined in the extracellular supernatant and correlated with different markers for cell viability. As shown by Figure 1C, cell viability measured by MTT assay (n=9) was not reduced after induction of ischemia, while levels of extracellular HDmiRs and CDmiRs in the supernatant increased ($P < 0.01$). This suggests that miRNAs are released by cells before their viability is negatively affected. To test this hypothesis further, the experiment was repeated and cells were evaluated after intervention on the expression of Annexin V and 7AAD receptors, which are markers for cellular apoptosis and necrosis respectively. In both Huh7 cells (n=3) as TFK-1 cells (n=9), the percentage of cells expressing 7AAD on their surface was very low (<1%), indicating a marginal degree of cellular cell necrosis. However, the expression of Annexin V on the cell surface as well as the release of miRNAs into the supernatant strongly increased after induction of ischemia (Fig. 1D; $P = 0.25$ for Huh7 cells and miR-122, $P = 0.0039$ for TKF1 cells and miR-222). These findings suggest that ischemia induces cellular stress and the release of miRNAs from apoptotic cells, even though the degree of cellular necrosis is low.

How are HDmiRs and CDmiRs distributed in bile and protected against degradation?

To investigate the in vivo mechanism of HDmiR and CDmiR release to either apical or basolateral direction, 62 paired serum and bile samples that were simultaneously collected from patients in the first three weeks after liver transplantation were analyzed. Donor and recipient characteristics are listed in Table 1. As shown in Fig 2A, HDmiR-122

Donor characteristics	
Age (mean ± SD)	44.4 ± 8.6
Sex (m/f)	5/5
BMI (mean ± SD)	23.3 ± 1.9
Donor lab	
AST	43.8 ± 30.4
ALT	20.6 ± 12.0
Gamma-GT	26.2 ± 35.9
Bilirubin	27.0 ± 42.1
Sodium	146.9 ± 5.8
Graft type (DBD/DCD)	10/0
Cause of death	
CVA	6
Trauma	3
Suicide	1
Graft preservation (UW vs. HTK)	10/0
Graft cold ischemia time (min)	519.0 ± 137.1
Recipient characteristics	
Age	46.2 ± 9.9
Sex (m/f)	7/3
Indication for LT	
HCV	2
HBV	2
Auto-immune (PSC, PBC, AIH)	3
Alcoholic	2
PNF	1
Mean RAI first 21 days post LT	4.2 ± 2.5

Table 1. Donor and recipient characteristics.

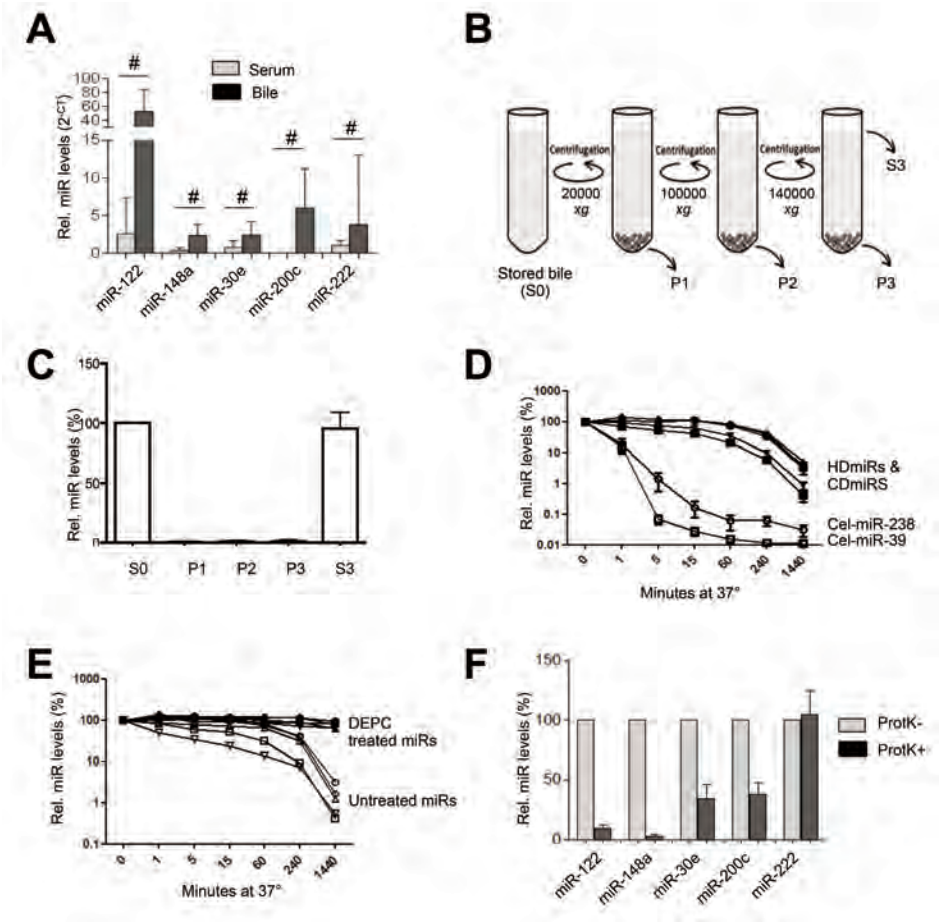

Figure 2. (A) Distribution of HDmiRs and CDmiRs in 62 paired serum and bile samples. HDmiR-122 was the most abundant miRNA in serum, followed by CDmiR-222 and CDmiR-30e. Only a small portion was accounted for by miR-148a, while miR-200c was virtually absent in serum. Also in bile, HDmiR-122 remained the most prominent of miRNA. In general, miRNA levels in bile were higher compared to miRNA levels in paired serum samples. (B) Centrifugation procedure for fractionation of stored bile samples (S0). Pellets were enriched with mitochondria, lysosomes and peroxisomes in pellet 1 (P1), microsomes and membrane (fragments) in pellet 2 (P2) and exosomes, ribosomes and viruses in pellet 3 (P3). The supernatant after the final centrifugation step (S3) contained soluble proteins and protein complexes. (C) The fractionation of bile (n=8) showed that, after differential centrifugation steps at 20.000, 100.000 and 140.000 g, all respective pellets (P1, P2 and P3) only contained a very small percentage of the tested HDmiRs and CDmiRs compared to the baseline sample (S0), suggesting that the majority of the tested miRNAs in bile are protein-bound and not pelletable using the procedure presented in Figure 2B. (D) Levels of HDmiRs, and CDmiRs in bile remained stable up to 4 hours (closed symbols), while exogenously spiked-in cel-miR-39 (open squares) and cel-miR-238 (open circles) already degraded within 5 minutes in bile after incubation at 37 °C. (E) When RNase activity in bile was inhibited by DEPC treatment, all tested miRNAs remained stable for at least 24 hours in bile (closed symbols) whereas untreated HDmiRs and CDmiRs showed a strong decrease (open symbols). (F) When proteins in bile were degraded by ProtK treatment, over 90% of the HDmiRs in bile supernatant were degraded as well. In contrast, CDmiRs were less affected by ProtK treatment, suggesting that CDmiRs in bile have less conjunction with proteinase K sensitive bile components than HDmiRs. Figures represent the median ± Interquartile range. #P <0.0001

Table 2. MicroRNA levels, portrayed as Median (SD), in paired bile and serum samples during impaired graft function following LT.

	Biliary miRNA levels			Serum miRNA levels		
MicroRNA	Bile bili >1000 (n=36)	Bile bili <1000 (n=14)	P-value	Bile bili >1000 (n=36)	Bile bili <1000 (n=14)	P-value
miR-122	71.2 (76.3)	2.41 (23.9)	<0.0001	1.82 (10.6)	7.07 (21.2)	0.06
miR-148a	2.48 (2.36)	2.92 (3.58)	0.36	0.39 (0.93)	0.42 (1.07)	0.65
miR-30e	2.32 (2.72)	4.90 (10.8)	0.02	0.81 (2.35)	1.12 (4.02)	0.35
miR-200c	5.63 (13.3)	12.5 (14.8)	0.05	0.007 (0.01)	0.007 (0.01)	0.40
miR-222	3.88 (6.43)	21.8 (57.0)	0.03	1.02 (1.89)	1.75 (11.5)	<0.01

was the most abundant miRNA in serum as well as in bile. Levels of CDmiR-200c in serum were significantly lower compared to other CDmiRs. CDmiR-222 was the highest abundant CDmiR in bile. Overall, relative levels of all HDmiRs and CDmiRs were significantly higher in bile compared to serum.

Fresh bile samples obtained from human donor livers (n=8) were fractioned, to determine the subcellular fraction in which HDmiRs and CDmiRs are released in bile. As shown in Figure 2B and 2C, fresh bile samples were sequentially centrifuged at 20.000, 100.000, and 140.000 g to obtain pellets with a different composition (samples P1, P2 and P3). All pellets contained only a small percentage of the tested HDmiRs and CDmiRs compared to the baseline sample (S0). As shown in Figure 2C, approximately 0.9% of the miRNAs was present in P1, 1.2% in P2 and 1.9% in P3. In contrast, over 96.4% of the miRNAs was found in the non-pelletable fraction (S3). These percentages were similar between all tested CDmiRs and HDmiRs and suggests that both HDmiRs and CDmiRs in bile are mainly present in the non-pelletable supernatant.

In order to test the stability of miRNAs, stored bile samples spiked with artificial C. elegans cel-miR-39 and cel-miR-238, were incubated at room temperature up to 24h. As shown by Figure 2D, both HDmiRs and CDmiRs remained stable for at least one to four hours in bile. However, spiked-in control miRNAs cel-miR-39a and cel-miR-238 degraded within the first five minutes after incubation, as was also shown by other studies[19, 21]. To confirm that the degradation is caused by RNase activity, we also investigated stability of miRNAs in an RNase-free environment by treating bile supernatant with diethylpyrocarbonate (DEPC). As shown by Figure 2E, HDmiRs and CDmiRs remained stable up to 24 hours in bile when the bile supernatent was treated with DEPC. The higher stability of HDmiRs and CDmiRs in normal bile supernatant despite the presence of RNases, compared to exogenously spiked-in miRNAs, has been linked to the formation of miRNA-protein complexes[19, 27]. In order to confirm this hypothesis, an additional experiment was performed by treating the bile supernatant with Proteinase-K (ProtK) to analyze how the degradation of proteins influences the stability of separate HDmiRs and CDmiRs. As shown in Figure 2F, HDmiR-122 and HDmiR-148a were almost completely degraded after ProtK treatment of bile, while this degradation was less for CDmiR-30e and CDmiR-200c. In particular CDmiR-222 seemed insensitive for treatment with ProtK.

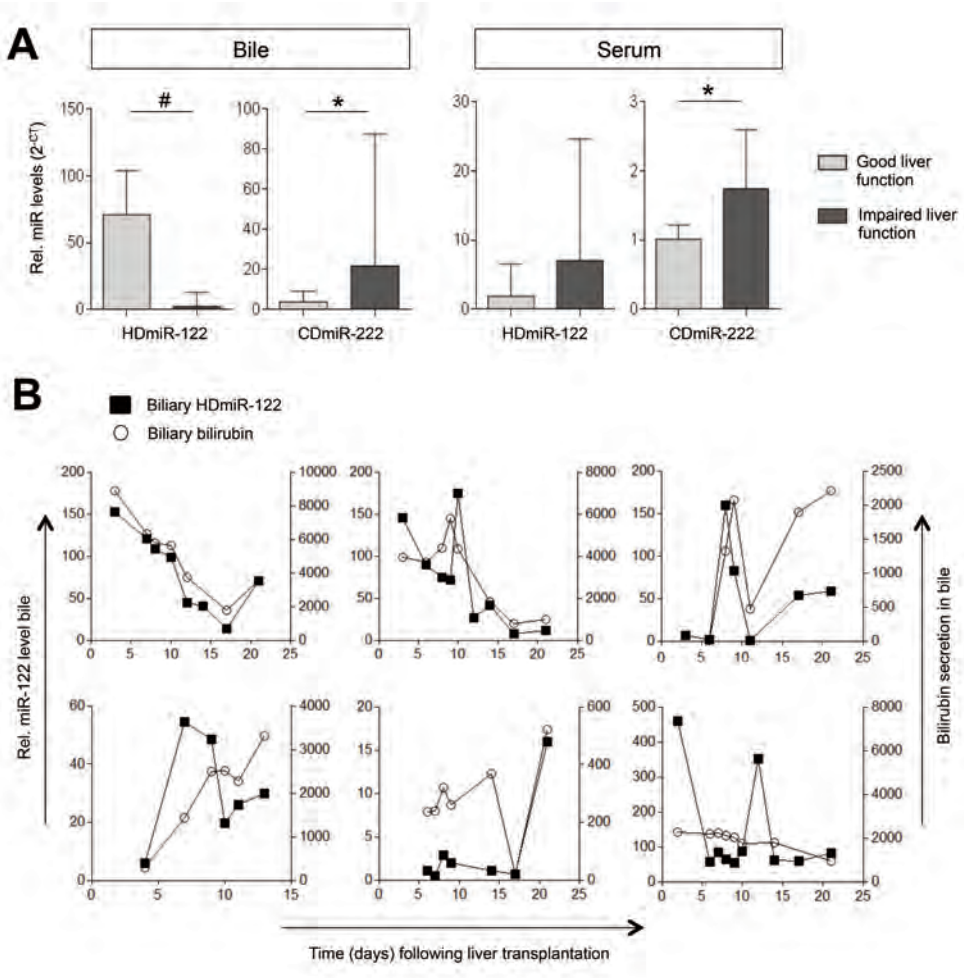

Figure 3. Directional release of HDmiRs and CDmiRs to bile and blood during good vs. impaired liver function. (A) Bile with concentrations of bilirubin ≤ 1000 IU/L (n=14 paired samples) were considered poor functioning grafts, while bile samples with bilirubin concentrations > 1000IU/L (n=36 paired samples) were considered as good functioning grafts as it meant that conjugation, and thus excretion of bilirubin by the hepatocytes was taking place. When graft function was good, excretion of HDmiR-122 to bile was high. When graft function was however impaired, the excretion of HDmiR-122 into bile was inhibited. Inversely, CDmiR levels, for instance miR-222, increased in bile during poor graft function. Simultaneously, there was a slight, but significant, increase of CDmiR-222 in serum. Figures represent the median ± Interquartile range. #P <0.0001, *P <0.05. (B) Correlation between HDmiR-122 and bilirubin levels in bile during the first weeks of recipient follow-up after liver transplantation. Dynamics between bilirubin levels in bile and HDmiR-122 levels in bile were similar, suggesting a relation between HDmiR-122 and hepatocyte cellular function (P <0.001, R = 0.694). Each graph represents values during the follow-up of one patient.

These findings suggest that a substantial fraction of CDmiRs (up to 100% for CDmiR-222) are protected in a different manner against RNase activity compared to HDmiRs, which are mainly protected through formation of protein complexes.

How are miRNAs released to bile and serum during impaired graft function following LT?
To investigate the effect of impaired liver function on the release of HDmiRs and CDmiRs, groups were stratified by their bilirubin concentration in bile (Table 2). Bile with low concentrations of bilirubin (≤ 1000 IU/L, n=14) were considered poor functioning grafts, while bile samples with bilirubin concentrations > 1000IU/L (n=36) were considered good functioning grafts as it meant that conjugation, and thus excretion, of bilirubin in the hepatocytes was taking place. As shown by Figure 3A, grafts with a good liver function had high levels of HDmiR-122 in their bile, suggesting a good excretion of this miRNA by hepatocytes ($P < 0.0001$). However, when liver function was impaired, excretion of HDmiR-122 to bile was inhibited and levels of HDmiRs-122 in serum tended to increase. Interestingly, the excretion of CDmiRs to bile showed an opposite pattern in good vs. poor function grafts; levels of CDmiR-222 in bile were increased when bilirubin secretion was impaired. At the same time, CDmiR-222 also increased in serum when cellular function was impaired. As shown by Figure 3B, a strong correlation existed between HDmiR-122 and bilirubin levels in bile ($P < 0.001$, $R = 0.694$); dynamics of bile HDmiR-122 levels were similar to bilirubin levels in bile. These results suggest that HDmiRs and CDmiRs are released bidirectionally, dependent on liver graft function.

How are miRNAs released to bile and serum during graft injury and rejection following LT?
To analyze the effect of liver injury on the levels of liver-derived miRNAs in bile and serum, samples were analyzed by dividing them into two groups: the low transaminases group with serum AST ≤ 50 IU/L (n=21) and the group with transaminases > 50 IU/L (n=41). The median miRNA levels of HDmiRs and CDmiRs in paired serum and bile samples for these groups are summarized in Table 3. As illustrated by Figure 4A, levels of miR-122 were significantly higher in serum during liver injury ($P = 0.031$), while its relative levels did not significantly differ in bile. This difference was not observed for the less abundant HDmiR-148a ($P = 0.732$). Remarkably, all CDmiR levels were increased in bile ($P \leq 0.01$) during injury (serum AST > 50 IU), but not in serum.

To further investigate the mechanism of HDmiR and CDmiR release during injury, levels of these miRNAs were correlated with the degree of rejection during recipient follow-up.

Table 3. MicroRNA levels, portrayed as Median (SD), in paired bile and serum samples during injury following LT.

	Biliary miRNA levels			Serum miRNA levels		
MicroRNA	Serum AST <50 U/L (n=21)	Serum AST >50 U/L (n=41)	P-value	Serum AST <50 U/L (n=21)	Serum AST >50 U/L (n=41)	P-value
miR-122	56.2 (38.9)	52.0 (90.4)	0.97	1.65 (2.00)	4.16 (16.0)	0.03
miR-148a	1.86 (1.26)	2.49 (2.97)	0.07	0.41 (0.27)	0.41 (1.05)	0.73
miR-30e	1.46 (1.09)	2.96 (7.15)	0.01	0.90 (0.48)	0.83 (3.17)	0.80
miR-200c	3.95 (3.26)	7.18 (14.8)	<0.01	0.008 (0.006)	0.006 (0.011)	0.11
miR-222	3.05 (3.39)	5.30 (38.1)	<0.01	1.03 (0.52)	1.09 (7.02)	0.77

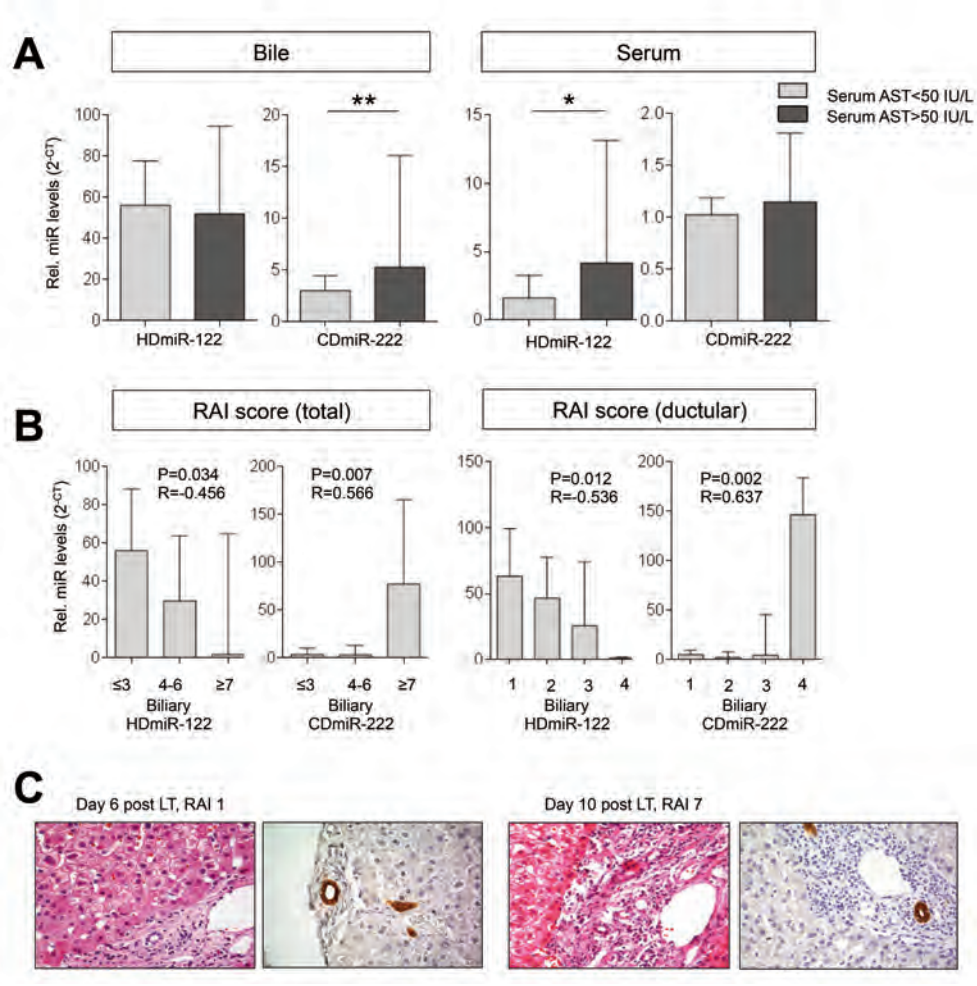

Figure 4. Directional release of HDmiRs and CDmiRs to bile and blood during early graft injury and cellular rejection. (A) Samples were stratified into two groups: the low transaminase group with serum AST ≤ 50 IU/L, (n=21 paired samples) and the high transaminases group with serum AST > 50 IU/L (n=41 paired samples). During injury, levels of HDmiR-122 were significantly increased into serum but not into bile. Oppositely, levels of all CDmiRs were significantly higher in bile during injury. (B, C) During the first three weeks of follow-up, approximately two to three protocol fine-needle biopsies were taken for histological evaluation. Biopsies were standard stained for HE and CK19 and scored for rejection activity (RAI score/ BANFF criteria). Paired analysis of biopsies and bile samples (n=21) showed that levels of HDmiR-122 in bile significantly diminished when the rejection activity was higher, while the levels of CDmiR-222 increased. This correlation became even more apparent when looking at the ductular component of the RAI score. Based on the rejection activity, recipients' immunosuppression was adjusted to diminish the acute cellular rejection. Figures represent the median ± Interquartile range. *P <0.05, **P <0.01

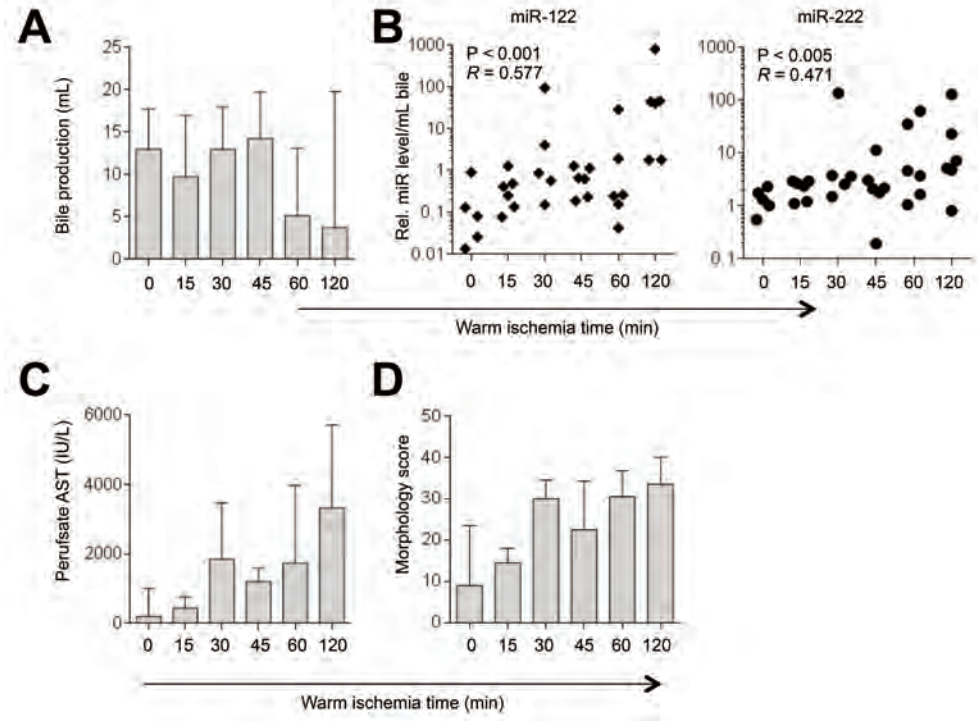

Figure 5. Release of HDmiRs and CDmiRs into bile and circulating perfusates in a porcine model for severe ischemia-reperfusion injury. Pigs were subjected to incremental durations of hepatic warm ischemia, after which isolated normothermic perfusion was performed to mimic graft reperfusion (n=6 per group). (A) Bile production of the liver graft during isolated normothermic machine perfusion. The production of bile tended to decrease when graft warm ischemia was longer, suggesting poor function and/or increased injury of these grafts. (B) A significant correlation was found between release of HDmiRs and CDmiRs into bile and the duration of graft warm ischemia (shown are the relative miRNA levels per mL of bile production). In contrast to the release during rejection, in this model, HDmiRs and CDmiRs were both increased during ischemia-reperfusion injury. The massive release of these miRNAs in bile (up to 1000 fold) and in recirculating perfusates (data not shown), showed a strong correlation with AST levels in perfusates (C) and morphological changes in the tissue (D). These morphological changes indicate an increased degree of cellular necrosis, which could cause leakage to both bile and the circulation. Figures represent the median ± Interquartile range.

From the ten recipients in our study, a total of n=21 protocol biopsies were evaluated within the first three weeks following LT by experienced (blinded) pathologists, according to the BANFF scoring criteria. Based on histology and clinical parameters, recipients received adjusted immunosuppressive treatment in order to clear rejection. As shown by Figure 4B, levels of HDmiR-122 in bile seemed to diminish when the rejection activity index (RAI) score was higher. Inversely, levels of biliary CDmiR-222 seemed to increase. This opposite effect between HDmiR and CDmiR release to bile became even more apparent when we correlated their levels to the ductular rejection score ($P \leq 0.012$). These correlations were however less clear in paired serum samples. These results confirm that

also in the setting of early graft injury following LT, release of HDmiRs and CDmiRs to bile behave oppositely. Moreover, during injury, hepatocytes seem to prefer HDmiR-122 release to blood while cholangiocytes release CDmiRs mainly to bile.

How are miRNAs released to bile and serum during severe ischemia-reperfusion injury?
The previous paragraphs illustrated the routes of HDmiR and CDmiR release during cellular stress, impaired cellular function and (reversible) acute cellular rejection in LT recipients. Finally, we were interested whether the induction of severe injury induced by ischemia-reperfusion would cause similar release of HDmiRs and CDmiRs. Therefore, we analyzed miRNA levels in paired bile and perfusate levels from liver grafts during isolated normothermic perfusion, that were obtained from pigs subjected to incremental durations of warm ischemia (n=6 per group). As shown in Figure 5A, the production of bile by the graft during isolated perfusion diminished when the duration of warm ischemia time was longer, indicating an impaired cellular function and/or increased degree of injury in these grafts. The concentration of HDmiRs and CDmiRs in these bile samples showed a strong positive correlation with the duration of warm ischemia (Fig. 5B; $P \leq 0.005$, $R \geq 0.471$). Also in recirculating perfusates, the release of miRNAs corresponded with the duration of warm ischemia (data not shown). So in contrast to our findings in human LT recipients, this model did not show an inverse release of HDmiRs and CDmiRs in bile. However, an increase of HDmiR and CDmiR levels in bile strongly correlated with AST levels in perfusates (Fig. 5C; $P < 0.0001$, $R \geq 0.6697$) and an increased degree of injury determined by morphological parameters (Fig. 5D; $P \leq 0.0042$, $R \geq 0.4781$). In grafts subjected to longer warm ischemia time, the tissue showed a larger degree of cellular dilatation, anoxic vacuoles, loss of parenchyma and cohesion, and congestion. Therefore, cellular leakage caused by necrosis rather then regulated active release, could explain the increase of both HDmiR and CDmiR levels into bile and recirculating perfusates in this porcine model.

DISCUSSION

In this study, we showed various mechanisms of release from hepatocytes and cholangiocytes into bile and blood in response to stress, impaired graft function and during mild or severe graft injury. In vitro, we showed that miRNAs are released by cells during stress and apoptosis. In vivo, we found that proper functioning grafts excreted HDmiR-122 and bilirubin to bile sufficiently, while this was inhibited in poor functioning grafts. Also during episodes of early acute cellular rejection in LT recipients, we found diminished levels of HDmiR-122 in bile when the rejection activity was higher. Inversely, CDmiRs were increasingly released to the bile during graft injury. This not only suggests

that HDmiR-122 could be a useful marker for hepatocyte function, but also that this miRNA might be involved in the conjugation and exocrine function of hepatocytes. Moreover, it shows a bidirectional release of miRNAs, suggesting active rather than passive underlying release mechanisms. However, when severe injury causes cellular necrosis, like in the porcine model from our study, miRNAs appear to (passively) leak from cells. Beside these different mechanisms and directions of release, also the protection of HDmiRs and CDmiRs against RNase activity seems to differ; while HDmiRs in bile supernatant were degraded by ProtK-treatment, CDmiR levels remained detectable, ranging from 25% (CDmiR-30e) to 100% (CDmiR-222), suggesting that these miRNAs, at least in part, have less conjunctions with protein complexes and are protected in a different manner.

Previous studies confirmed that serum levels of HDmiR-122 are sensitive for the detection of liver injury[7, 17, 28]. The finding that HDmiR-122 is also secreted to bile and the strongly correlates with cellular function however is new. The first report on biliary miRNAs identified miR-9 as a potential biomarker for biliary tract cancer. Moreover, the investigators of this study already verified the presence of HDmiR-122, CDmiR-200c and CDmiR-222 in bile. Despite the RNA hostile environment of human bile, in general, biliary miRNAs were found to be highly stable and protected from degradation[21]. A recent paper by Li et al. reports on biliary miRNAs located in extracellular vesicles as potential diagnostic markers for cholangiocarcinoma[22]. The results of this study suggest that patients suffering from cholangiocarcinoma have higher miRNA contents in extracellular bile vesicles compared to patients with non-malignant biliary obstructions. The investigators plea for the analysis of miRNAs present in extracellular vesicles rather than those in whole bile, in order to have a better discrimination between pathologies. Evidence that using whole bile is inferior for designing biomarker assays was however not provided. Furthermore, based on the results from previous studies as well as in the current one, the percentage of miRNAs present in vesicles like exosomes appears to be very low[21]. By only looking at miRNAs in the vesicle fraction, over 90% of the miRNA signal in bile would be overlooked and ignored for analysis[29].

The studies from Shigehara at el. and Li et al. both report on CDmiR-222 as one of the enriched miRNAs in cholangiocarcinoma[21, 22], confirming that this miRNA is a potentially relevant marker for various cholangiopathies. Earlier work from our group showed CDmiR-222 release to be lower in preservation solutions that were used to flush grafts which later developed ischemic-type biliary lesions after liver transplantation[19]. Based on this observation, we hypothesized that cholangiocytes release their miRNA content to the bile rather than to the blood. The results from the current study further support this hypothesis by the inverse release between HDmiRs and CDmiRs to bile and blood.

A remarkable finding from our study was the difference in ProtK-treated sensitivity

between HDmiRs and CDmiRs. As mentioned before, CDmiR levels were less influenced when protein was degraded, while HDmiR levels drastically decreased. This further supports our observation that cholangiocytes release their miRNAs to the bile differently from hepatocytes. Previous studies suggested that miRNAs can be released and bound to lipoproteins as HDL[33, 34], which perhaps could explain the protein-independence of CDmiRs, though no evidence for this hypothesis is provided in this study.

Importantly, the direction of miRNA release appears to differ when injury is more severe. In Figure 6, we illustrate proposed mechanisms of miRNA release during various (patho)physiologic conditions of the liver. In particular, we observed the largest differences in miRNA release into bile when we compared acute cellular rejection in humans and warm ischemia-reperfusion injury in pigs. Likely, this difference can be explained by the type of cell injury; during rejection, the tissue was mainly affected by lymphocytic or mixed infiltrate, whereas warm ischemia resulted in more cellular dilatation, loss of parenchyma and cellular cohesion, and increased congestion. Therefore, after prolonged warm ischemia, cells probably lose their miRNA content through necrotic cell leakage, rather than through active release. This finding is important for the evaluation of extracellular miRNAs as biomarkers, as one should take into account that this might affect detection levels in bile. Therefore, future studies should focus on determining cut-off values of HDmiR and CDmiR detection levels in bile and serum between healthy individuals or recipients with impaired function, rejection or severe injury.

Our study contains several limitations. First, we used hepatoma and cholangiocarcinoma cell lines for measurements of miRNA release after cellular stress. It can be questioned whether such oncological cell lines adequately represent release in normal physiological cells. The levels of miRNAs in the supernatant correlated with the degree of apoptotic markers in cells. Therefore, cellular leakage cannot be ruled out, even though necrosis appeared to be absent. Furthermore, the increase in miRNA levels in Huh7 cells did not reach statistical significance due to the small sample size (n=3). Therefore, our current data should be validated in a larger experimental setup. Finally, as discussed earlier, our data provide more insight on the role of protein conjunctions as a factor for stability of extracellular HDmiRs and CDmiRs in bile. However, to thoroughly investigate the mechanism of miRNA release into bile and blood following cellular injury real-time imaging at a molecular level is required.

The results from this study not only confirm that HDmiR-122 could be a suitable injury marker in serum, but also that its levels in bile are strongly correlated with hepatocyte function. This provides insight in the mechanism and direction of miRNA, which is relevant for the development of diagnostic assays. Moreover, miRNA composition in different fragments of the bile could be helpful in distinguishing different cholangiopathies, as previously described by Shigehara et al. and Li et al.[21, 22]. But also in the setting of liver

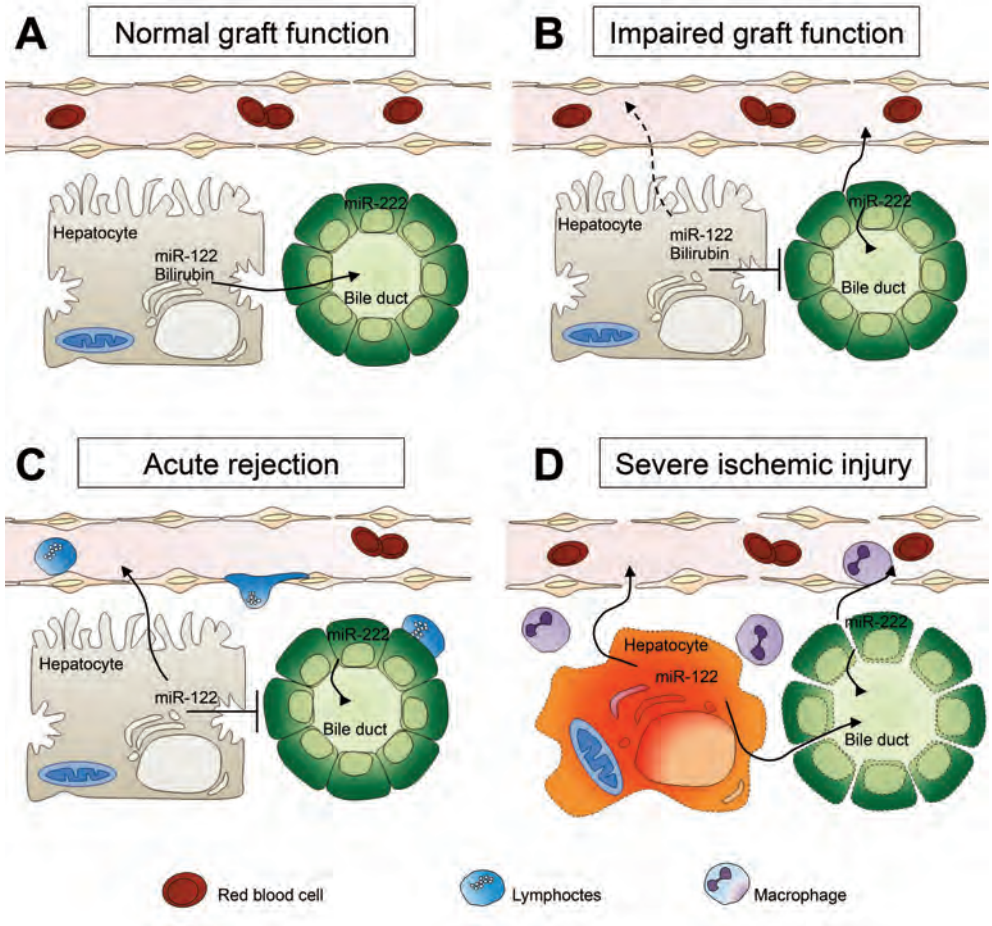

Figure 6. Illustration of suggested various mechanisms and routes of miRNA release from hepatocytes and cholangiocytes in different (patho)physiological conditions. (A) During normal graft function, there is a sufficient excretion of HDmiR-122 and bilirubin by hepatocytes into the bile. Levels of CDmiRs, like miR-222, remain low in bile when graft function is sufficient. (B) When graft function is impaired, the excretion of HDmiR-122 and bilirubin to the bile is inhibited. Simultaneously, hepatocytes and cholangiocytes start to release miRNAs to the circulation. (C) During acute cellular rejection (which is still reversible), a similar mechanism of miRNA release is seen as during impaired function. The release of HDmiRs to the circulation is however more pronounced, while CDmiRs are mainly released to the bile. (D) Severe ischemia-reperfusion injury causes an increased degree of hepatocyte necrosis and detachment of cholangiocytes. Therefore, HDmiRs and CDmiRs are leaked into bile as well as the circulation.

transplantation, Lankisch et al. reported on miRNA profiles in bile that could successfully distinguish ischemic-type biliary lesions from other, less severe, biliary complications following LT[20]. These studies all evaluated miRNAs during patient or recipient follow-up. However, novel organ preservation techniques like machine perfusion make it also attractive to evaluate miRNAs as a biomarker for graft quality prior to transplantation[35].

These novel preservation techniques create a prolonged time window in which researches could objectively evaluate graft quality; the measurement of miRNAs in bile or perfusates during machine perfusion might be informative on graft function or the degree of biliary injury, which is currently the second cause of graft failure after liver transplantation[36].

In conclusion, this study demonstrates the directional release of hepatocyte and cholangiocyte abundant miRNAs into bile and blood during impaired graft function and liver injury following LT. The difference in HDmiR and CDmiR release is further underlined by the disparity in protein (in)dependent stability in bile. Extracellular miRNAs in bile are potential markers for liver function and biliary injury in liver transplantation and cholestatic diseases.

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MicroRNA profiles in graft preservation solution are predictive of ischemic-type biliary lesions after liver transplantation

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ABSTRACT

Ischemic-type biliary lesions (ITBL) are the second most common cause of graft loss after liver transplantation. Though exact pathophysiology of ITBL is unknown, bile duct injury during graft preservation is considered to be a major cause. Here, we investigated whether the release of cholangiocyte-derived microRNAs (CDmiRs) during graft preservation is predictive for the development of ITBL after liver transplantation.

Graft preservation solutions (perfusates) and paired liver biopsies collected at the end of cold ischemia were analyzed by RT-qPCR for CDmiR-30e, CDmiR-222 and CDmiR-296 and hepatocyte-derived miRNAs (HDmiRs) HDmiR-122 and HDmiR-148a. MicroRNAs in perfusates were evaluated on their stability by incubation and fractionation experiments. MicroRNA profiles in perfusates from grafts that developed ITBL (n = 20) and grafts without biliary strictures (n = 37) were compared.

MicroRNAs in perfusates were proven to be stable and protected against degradation by interacting proteins. Ratios between HDmiRs/CDmiRs were significantly higher in perfusates obtained from grafts that developed ITBL ($P < 0.01$) and were identified as an independent risk factor by multivariate analysis ($P < 0.01$, HR = 6.89). The discriminative power of HDmiRs/CDmiRs in perfusates was validated by analysis of separate brain death- (DBD) and cardiac death donors (DBD; $P \leq 0.016$) and was superior to expression in liver biopsies (C = 0.77 in perfusates vs. C < 0.50 in biopsies).

This study demonstrates that differential release of CDmiRs during graft preservation is predictive for the development of ITBL after liver transplantation. This provides new evidence for the link between graft-related bile duct injury and the risk for later development of ITBL.

INTRODUCTION

Biliary strictures after liver transplantation – in particular non-anastomotic strictures, which are more diffusely distributed throughout the liver graft – can cause considerable morbidity, graft loss, and mortality[1, 2]. Hepatic artery thrombosis (HAT) after liver transplantation can result in such non-anastomotic strictures after liver transplantation[3, 4]. However, similar patterns of diffuse biliary strictures and dilatations may occur in the presence of normal arterial circulation, which are often referred to as ischemic-type biliary lesions (ITBL)[3, 5]. Up to 31% of liver transplant recipients suffer from ITBL[6]. In contrast to isolated strictures at the site of the biliary anastomosis, treatment of ITBL by biliary stenting through the percutaneous or endoscopic route is often ineffective and retransplantation is necessary in up to 15% of liver transplant recipients[7]. This renders ITBL the second most common cause of graft loss after liver transplantation[7, 8].

Previous studies report on various factors to be associated with ITBL, including primary sclerosing cholangitis (PSC) as indication for liver transplantation[9], blood type incompatibility between donor and recipient[10], concomitant cytomegalovirus infection[11], grafts donated after cardiac death (DCD)[7], prolonged cold ischemia time[7], and insufficient flushing of the peribiliary capillary plexus during graft preservation[12, 13]. However, these risk factors for ITBL and other factors related to graft quality lack specificity and are unable to predict outcome of individual grafts prior to transplantation. The increased use of marginal donors due to relative organ shortage[7] however emphasizes the need for biomarkers to forecast ITBL, since many of these marginal grafts, in particular grafts from DCD donors, are more likely to develop ITBL. Conversely, marginal grafts that are currently rejected for transplantation because of presumed high chances of developing ITBL could be used successfully in the future if they are diagnosed with a favorable biomarker profile.

MicroRNAs (miRNAs) have recently emerged as promising candidates for biomarker research[14]. This class of small non-coding RNAs can regulate gene expression by repressing messenger-RNA translation; specific miRNA profiles have been associated with a variety of pathologic conditions in humans, such as malignant, metabolic and autoimmune diseases[15-17]. In addition to their altered expression in tissues, gene-array studies have identified cell-type abundant miRNAs excreted in serum, plasma, urine and other body fluids, which were proven to be detectable and stable[18-22]. Moreover, their feature as early and sensitive marker was demonstrated in mice with drug-induced liver injury, in which hepatocyte-derived miRNAs (HDmiRs) in serum increased earlier than conventional transaminase markers[23]. This has been confirmed in acute and chronic hepatitis patients[24, 25]. In liver transplant recipients, HDmiRs in serum were found to be an early and sensitive marker of acute rejection after transplantation[26].

Despite this relationship between hepatocyte injury and HDmiR release, the role of cholangiocyte-derived miRNAs (CDmiRs) and their release during biliary injury is unknown. Since ITBL are thought to be related to ischemic injury of the bile ducts, we hypothesized this may lead to the release of CDmiRs during cold storage that can be detected in graft preservation solution or so called perfusates. Grafts are flushed at the back-table just prior to implantation to remove unwanted products accumulated in the graft during cold storage. These flushes or perfusates are believed to represent the condition of the entire liver parenchyma rather than only a small part of the liver, as is typically the case with liver biopsies. The fact that they contain biological material from the donor exclusively renders perfusate an attractive medium to assess graft quality prior to transplantation without the influence of recipient factors[27, 28].

The aim of our study was to determine whether it is feasible to detect CDmiRs and HDmiRs in graft perfusates, whether their levels are associated with the development of ITBL, and whether they have the potential to serve as accurate and stable biomarkers.

MATERIALS AND METHODS

Study design

In this longitudinal cohort study, perfusates and paired liver tissue biopsies were collected prospectively during liver transplantations from adult recipients between April 2010 and March 2012 at the Erasmus University Medical Center, Rotterdam. Available perfusates and biopsies were retrospectively analyzed for the presence of two HDmiRs (HDmiR-122 and HDmiR-148a) and three CDmiRs (CDmiR-30e, CDmiR-222 and CDmiR296). The selection of these particular miRNAs was based on microarray data from literature[29]. Through RT-qPCR, we confirmed that expression of CDmiRs are up to eight-fold higher in common bile duct tissue compared to expression in liver tissue, while HDmiR levels are almost zero (preliminary results, data not shown). Levels of miRNAs in graft perfusates from grafts that developed ITBL and grafts that did not develop biliary strictures after liver transplantation were compared.

Definition of ITBL

Ischemic-type biliary lesions were defined as (i) symptomatic strictures and associated dilatation of the intrahepatic or hilar bile duct(s) after liver transplantation, which (ii) were confirmed by cholangiography and in the presence of a patent hepatic artery as demonstrated by Doppler ultrasonography, and (iii) which required endoscopic or percutaneous interventions of the biliary system or liver retransplantation in recipients. Imaging was reviewed by both a transplant hepatologist (HJM) and a transplant surgeon (GK) who were blinded to the presence of miRNAs in perfusates. Transplant recipients

without biliary complications during follow-up were defined as non-ITBL. Time to event was calculated from the date of liver transplantation until the date of intervention associated with ITBL (i.e. biliary stenting by ERCP and/or bile drainage by PTC). Donor and recipient characteristics and clinical parameters were obtained from the liver transplantation database of the institution. The Medical Ethical Committee of the Erasmus MC approved the use of donor materials and all patients provided informed consent for the use of clinical information for medical research.

Sample collection and processing

Perfusate samples were obtained during the back-table procedure. After a standard in situ perfusion of the liver with University of Wisconsin solution (Viaspan, Duramed Pharm Inc, Pomona, NY) or Histidine-tryptophan-ketoglutarate (Custodiol HTK, Essential Pharmaceuticals, LLC, Pennsylvania, USA), liver grafts were procured and transported to our center. Upon arrival at the operating room, an additional ex situ perfusion of the portal venous system of the graft was performed with 1000 ml of UW or HTK depending on the preservation fluid initially used during harvesting. This was followed by flushing with 500 ml of human albumin solution (Albuman human albumin 40g/l, Sanquin, The Netherlands) just prior to implantation of the graft. Flushing was performed under normal hydrostatic pressure. Perfusates were collected directly after the second flush with albumin and cold stored at -4 °C until further processing. Paired biopsies, consisting of wedges of liver tissue obtained from the anterior side of the left lateral segment of the liver graft, were taken at the end of the cold ischemia time and directly snap-frozen for storage at -80 °C.

RNA isolation

Graft perfusates were cleared of cells and cell debris by a first centrifugation (445 g at 4 °C for 15 minutes) and a second centrifugation of 10 ml supernatant at 3.166 g at 4 °C for 15 minutes. In order to optimize signaling for RT-qPCR on lower abundant miRNAs, cell-free perfusate samples were concentrated with a 100 kD Amicon filter; 3 mL of the cell-free perfusates was centrifuged at 3.166 g at 4 °C for 30 minutes, obtaining a volume of supernatant of approximately 750 µl. Total RNA was extracted from 100 µl concentrated supernatant perfusate by adding 1.5 ml of Qiazol Lysis reagent to homogenize samples. Chloroform (300 µl) was added, and after centrifugation (15 minutes at 20.817 g) an aqueous RNA-containing layer of 700 µl was obtained, which was further processed according to the manufacturer's protocol (Qiagen, Hilden, Germany). Extraction of total RNA from liver biopsies (approximately 10 mg of tissue per biopsy) was performed following the manufacturer's instructions and normalized to a concentration of 50 ng/7.5 µl, using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, Ma, USA).

Reverse transcription and real-time polymerase chain reaction (RT-PCR)

After RNA isolation, sample-specific cDNA was prepared using the Taqman microRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). In a modified protocol, every multiplex cDNA reaction consisted of 0.4 µl dNTP mix, 1.35 µl Multiscribe RT enzyme, 2.0 µl 10x RT Buffer, 0.25 µl RNase inhibitor, 1.0 µl of each RT primer, and 7.5 µl of diluted template RNA. The total reaction volume was adjusted to 20 µl with nuclease free water. The sequences of the primers used for RT-PCR are summarized in Table 1. All cDNA and PCR reactions were performed according to the manufacturer's instructions and carried out in duplicate. Each PCR reaction consisted of 10 µl TaqMan Universal PCR Master Mix, 0.5 µl microRNA-specific PCR primer (Applied Biosystems) and 5.0 µl of the previously 1:10 diluted cDNA. The final volume of every PCR reaction was adjusted to 20 µl with nuclease-free water.

Because of the lack of a detectable conventional reference gene, relative perfusate miRNA levels were calculated by threshold cycle values (2^{-Ct}) and normalized by setting their total at 100% to correct for any differences in perfusate concentration. Subsequently, ratios of HDmiRs/CDmiRs in graft perfusate were determined. In tissue biopsies, relative miRNA levels were normalized by a reference gene, RNU43 ($2^{-\Delta Ct}$).

MicroRNA	Mature microRNA sequence
Hsa-miR-122	UGGAGUGUGACAAUGGUGUUUG
Hsa-miR-148a	UCAGUGCACUACAGAACUUUGU
Hsa-miR-30e	UGUAAACAUCCUUGACUGGAAG
Hsa-miR-222	AGCUACAUCUGGCUACUGGGU
Hsa-miR-296	AGGGCCCCCCCUCAAUCCUGU

Table 1. MicroRNA primer sequences.

MicroRNA stability and fractionation

MicroRNA stability was assessed by measuring miRNA degradation in graft perfusates over time. From three liver transplantations, samples of 400 µl cell-free graft perfusate were incubated at room temperature and total RNA was extracted at scheduled time points after incubation, varying from 0 to 24 hours. Samples were spiked with 40 µl of synthetic Caenorhabditis elegans miR-39 (cel-miR-39) to investigate nuclease activity and to test for exogenous miRNA stability. After isolating RNA using previously described methods, relative levels of HDmiRs and CDmiRs were determined. To investigate the location of released miRNAs in graft perfusate, separate cell and subcellular fractions were prepared by sequential centrifugation steps. For this, 50 ml fresh unprocessed (and non-concentrated) perfusate was centrifuged for 10 minutes at 1.000 g to pellet intact cells. A second centrifugation step of 20.000 g for 20 minutes was performed to obtain a pellet with nuclei, cytoskeletons, and other organelles. A final centrifugation for 1 hour at

80.000 g was performed to spin down small vesicles and exosomes. The remaining supernatant contained miRNA fractions associated with protein complexes, ribosomes and large macromolecules. RNA was extracted from each pellet fraction and 400 µl of the final supernatant; miRNA levels were quantified as a percentage of the total of HDmiRs and CDmiRs in a sample. The percentage of miRNAs bound to protein complexes was determined by adding 1600 µl acetone to 400 µl supernatant of perfusate. After one hour at -20 °C, the supernatant was centrifuged for 10 minutes at 15.000 g at 4 °C. The remaining pellet was air-dried and dissolved in 700 µl Qiazol. The acetone was evaporated from the solution and further dissolved in 700 µl Qiazol. MicroRNAs in these protein fractions were isolated and measured using previously described methods.

Statistical Analysis

Statistical analysis was performed using SPSS statistics 20 (SPSS Inc, Chicago, IL, USA) and SAS 9.2 PROC GENMOD (SAS institute, Cary, NC). Correlations were estimated using Spearman's Rank correlation test. Group comparisons were performed using Mann-Whitney U tests for continuous data and log-rank tests for categorical data. C-statistics were calculated to test for discriminative power. Prediction analyses were constructed through Cox proportional hazards regression analysis. P-values smaller than 0.05 were considered significant.

RESULTS

Recipient and donor characteristics

Between April 2010 and March 2012, perfusates from 75 consecutive liver transplantations were collected at the end of cold ischemia time. Samples were retrospectively analyzed for the presence of two HDmiRs (HDmiR-122 and HDmiR-148a) and three CDmiRs (CDmiR-30e, CDmiR-222 and CDmiR296). The selection of these particular miRNAs was based on microarray data from literature[29] (Table 1). Levels of miRNAs in graft perfusates from grafts that developed ITBL and grafts that did not develop biliary strictures after liver transplantation were compared.

Recipient and donor characteristics are shown in Table 2. Out of 75 liver transplantations, 20 recipients developed ITBL (26,7%) with a median time to event of 57 days after transplantation (= ITBL group). Thirty-seven recipients remained free of biliary strictures and associated interventions during follow-up (= non-ITBL group). Median follow-up of the entire study cohort was 487 days. Subjects who were never at risk for ITBL due to immediate re-transplantation (HAT n = 6; primary non-function n = 4) or with biliary interventions due to causes other than ITBL (anastomotic strictures n = 4; recurrent disease n = 2; rejection n = 2) were excluded for analysis.

	Non-ITBL (n=37)	ITBL (n=20)	Total (n=57)	P-value
Recipient characteristics				
Demographics				
median (SD) age (y)	52.0 (11.2)	52.5 (10.4)	52.0 (10.8)	*ns*
male/female	24/13	11/9	35/22	*ns*
PSC (%)	9 (24)	2 (10)	11 (19)	*ns*
Clinical blood values 24 hours post-surgery				
median (SD) AST	843 (1673)	2005 (2465)	1271 (2038)	*0.006*
median (SD) ALT	713 (1202)	1398 (1656)	978 (1431)	*0.012*
median (SD) AF	84.0 (68.1)	78.0 (85.7)	80.5 (74.6)	*ns*
median (SD) yGT	73.0 (73.1)	89.0 (108.6)	81.0 (86.4)	*ns*
median (SD) Bili	57.0 (101.0)	56.0 (107.3)	56.5 (102.3)	*ns*
Anastomosis				
Duct to duct/Roux-Y	29/8	18/2	47/10	*ns*
Median days of follow-up(SD)	526 (218)	408 (368)	487 (218)	*ns*
Median days to event (SD)		57 (74)		
Donor characteristics				
Demographics				
median (SD) age (years)	52.0 (15.7)	51.0 (17.8)	51.0 (16.3)	*ns*
male/female	17/20	9/11	26/31	*ns*
median BMI	23.0 (3.7)	22.0 (2.8)	22.6 (3.4)	*ns*
Graft type				
DCD / DBD	5/32	9/11	14/43	*0.006*
Graft preservation				
HTK / UW	11/26	11/9	22/35	*0.052*
median (SD) cold ischemia time (min)	389.0 (115.3)	405.0 (86.7)	392.0 (106.9)	*ns*
Laboratory results at time of donation				
median (SD) AST	52.5 (27.9)	36.5 (62.6)	44.0 (43.1)	*0.042*
median (SD) ALT	31.0 (44.0)	24.0 (49.7)	30.0 (45.8)	*ns*
median (SD) AF	67.5 (60.2)	57.0 (18.1)	62.5 (50.1)	*ns*
median (SD) yGT	30.0 (104.7)	28.5 (42.6)	30.0 (88.1)	*ns*
median (SD) Bili	9.0 (18.3)	8.0 (7.6)	9.0 (15.5)	*ns*

Table 2. Recipient and donor characteristics. n.s., not significant.

Recipients who developed ITBL received a DCD graft more often than recipients without biliary strictures (9 out of 20 in the ITBL group vs. 5 out of 37 in the non-ITBL group, P = 0.006) and graft preservation tended to be performed more frequently with HTK (11 out of 20 in the ITBL group vs. 11 out of 37 in the non-ITBL group, P = 0.052). Donors' serum AST levels were higher in the non-ITBL group (P = 0.042) and 24-hour post-operative serum AST and ALT levels were increased in recipients who eventually developed ITBL (P ≤ 0.012).

Detection and stability of miRNAs in perfusates

To study the release of miRNAs during graft preservation specifically, perfusate samples cleared of cells were used for analysis. It was feasible to detect levels of both HDmiRs

and CDmiRs in cell-free perfusates through quantitative RT-PCR (Fig. 1A). Comparing relative miRNA levels, HDmiR-122 levels were higher in liver tissue than in perfusates (P = 0.033). This in contrast to levels of HDmiR-148a (P = 0.008) and CDmiR-222 (P = 0.010), which were lower in liver tissue compared to perfusates (Fig. 1B). Levels of HDmiRs and CDmiRs in perfusates did not correlate to expression in paired biopsy samples. These differences between perfusates and liver tissue could indicate selectivity in the release of miRNAs from both hepatocytes and cholangiocytes.

As shown in Figure 1C, HDmiRs and CDmiRs in cell-free perfusates remained stable up to 24 hours after storage at room temperature. In contrast, an exogenously added Caenhorhabditis elegans miRNA (cel-miR-39) degraded within 5 minutes of incubation (P < 0.01), suggesting that HDmiRs and CDmiRs are protected against RNase activity in perfusates. To further investigate the stability and fractionation of released miRNAs in perfusates, separate cell and subcellular fractions were prepared by sequential centrifugation steps (Fig. 2A). As shown in Figure 2B, the fraction of HDmiRs and CDmiRs present in cell debris and vesicles was only small. The largest fraction of miRNAs however was found in the remaining perfusate supernatant, containing protein complexes, ribosomes and macromolecules. Acetone precipitation showed that over 90% of miRNAs in the supernatant is attached to protein complexes (Fig. 2C) of which the majority is larger than 100 kD, as was demonstrated by perfusate concentration. The conjunction between miRNAs and proteins, rather than their embedding in small vesicles, could explain miRNA stability and their protection against RNase activity in perfusates.

Ratios between HDmiRs and CDmiRs in perfusate are predictive for development of ITBL

Relative levels of HDmiRs and CDmiRs in perfusates were compared between grafts that developed ITBL (n = 20) and grafts without biliary strictures (n = 37). Since we did not have a reliable reference miRNA that could be measured consistently in perfusates, we instead used the ratio of HDmiRs/CDmiRs to normalize data and calculate relative CDmiR levels. As shown in Figure 3A, levels of HDmiR-122 but not HDmiR-148a were higher in perfusates from grafts that developed ITBL after liver transplantation (P = 0.032). Levels of CDmiRs however were all significantly lower in these perfusates (P ≤ 0.006). These low CDmiR levels resulted in high ratios of HDmiRs/CDmiRs and CDmiRs in perfusates obtained from grafts that developed ITBL, (P ≤ 0.004, Fig. 3B).

Univariate analysis (Table 3) revealed that high HDmiR/CDmiR ratios (HR ≤ 4.98) and the type of donor (HR = 3.21, P = 0.01) were possible risk factors to develop ITBL. The type of preservation fluid used during graft procurement also tended to increase the risk of ITBL (HR = 2.24, P = 0.059). Multivariate analysis identified ratios of HDmiR-148a/CDmiR-30e, miR-222 and miR-296 as independent risk factors for ITBL (Table 3). This model also demonstrated discriminative power of miRNAs in perfusate, calculated by C-statistics,

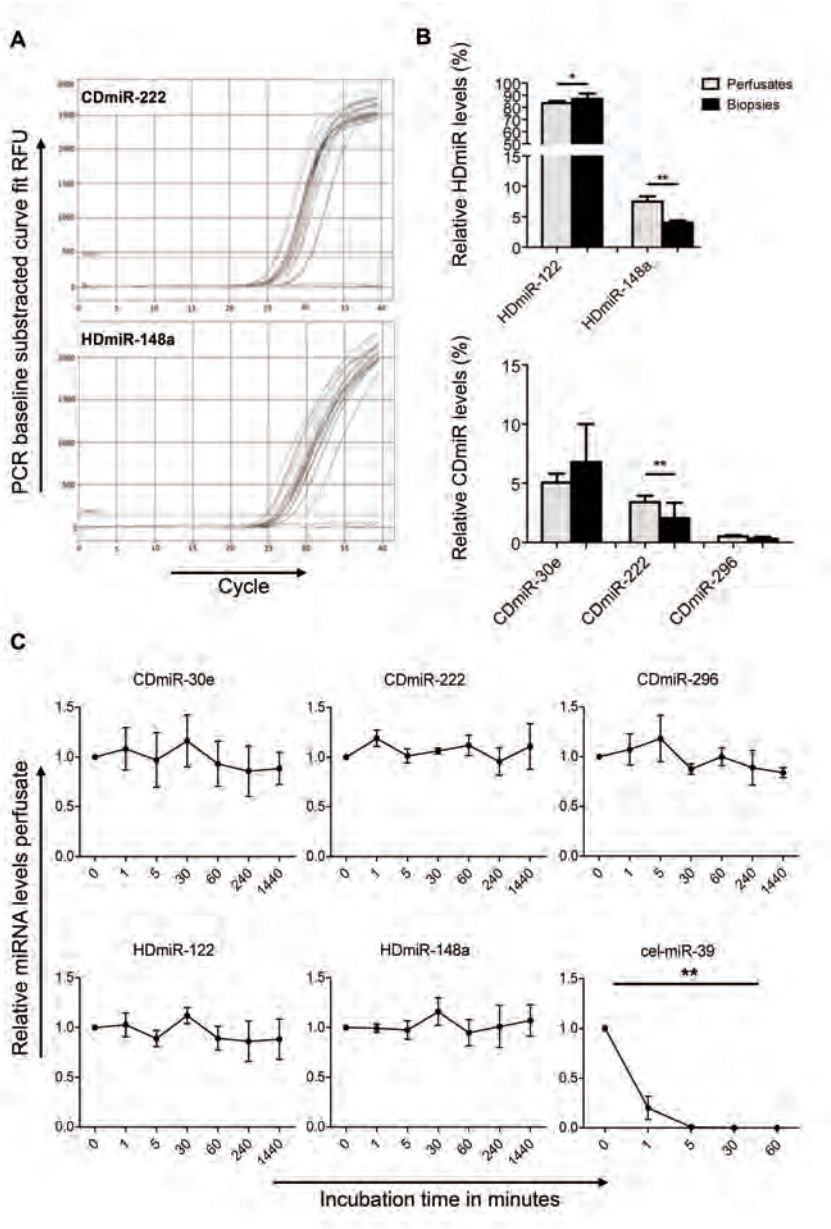

Figure 1. Relative HDmiR and CDmiR levels in perfusates and tissue biopsies. (A) RT-qPCR results for CDmiR-222 and HDmiR-148a in n=29 perfusates. (B) Relative levels of HDmiR-122 (P = 0.033), HDmiR-148a (P = 0.008) and CDmiR-222 (P = 0.010) were significantly different between 33 paired perfusate and biopsy samples, indicating selectivity in miRNA release. (C) Stability of HDmiRs and CDmiRs in perfusates after incubating samples at room temperature for different time points (n=3). Both HDmiRs and CDmiRs in perfusates remained stable up to 24 hours after incubation. The synthetic exogenously spiked-in cel-miR-39 however was degraded within five minutes (**P < 0.01). All figures demonstrate the mean ± SEM.

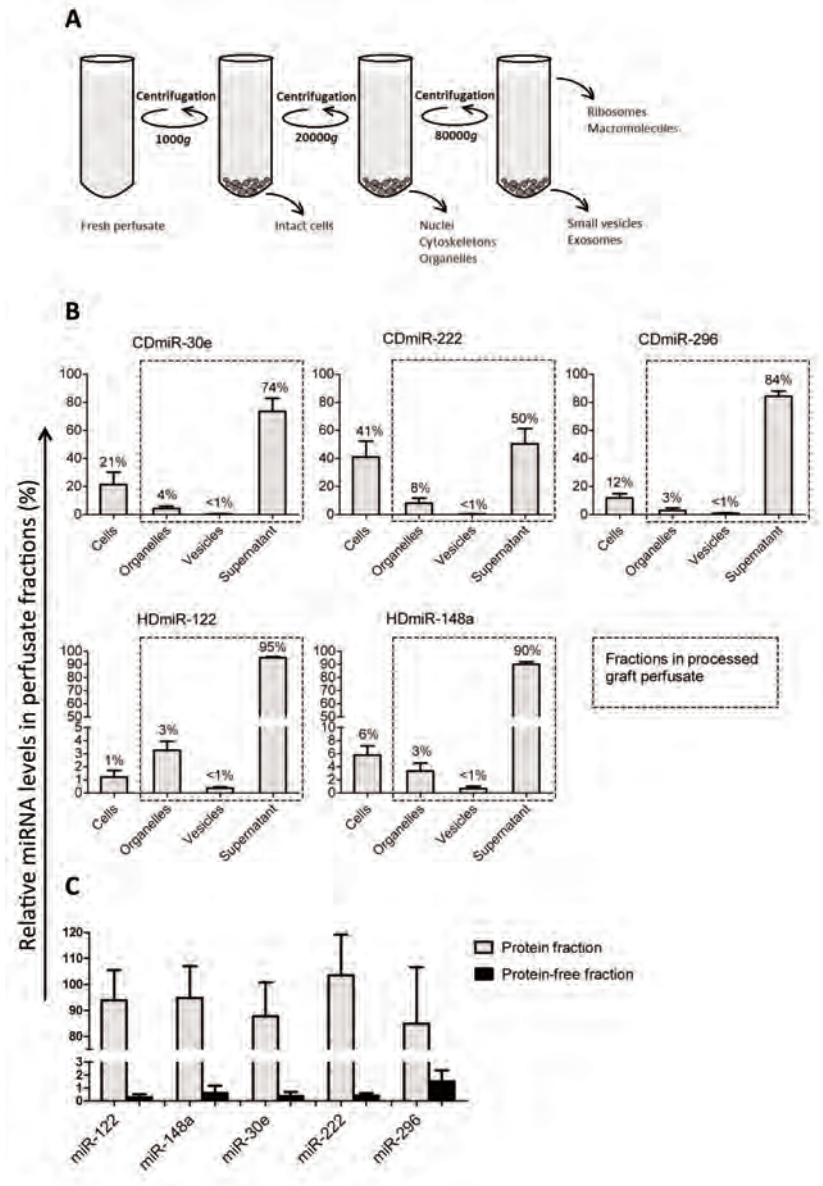

Figure 2. MicroRNA fractions in perfusates. To investigate in which different fractions miRNAs are present in perfusate, separate cell and subcellular fractions were prepared by different centrifugations steps (A); fresh perfusates were centrifuged at low speed (1.000 g for 10 min.) to pellet intact cells. Second, a centrifugation step at medium speed (20.000 g for 20 min.) was performed to pellet nuclei, cytoskeletons and other organelles. Finally, centrifugation at high speed (80.000 g for one hour) was performed to pellet small vesicles and exosomes. The remaining supernatant contained proteins, ribosomes, viruses and large macromolecules. (B) Percentages of HDmiRs and CDmiRs in different perfusate fractions (n=7). The dotted line delineates fractions in perfusates after standardized workup, as was done for the cohort study. (D) After the final spin, acetone precipitation of the supernatant showed that over 90% of miRNAs was bound to proteins (n=4). Shown are the mean ± SEM.

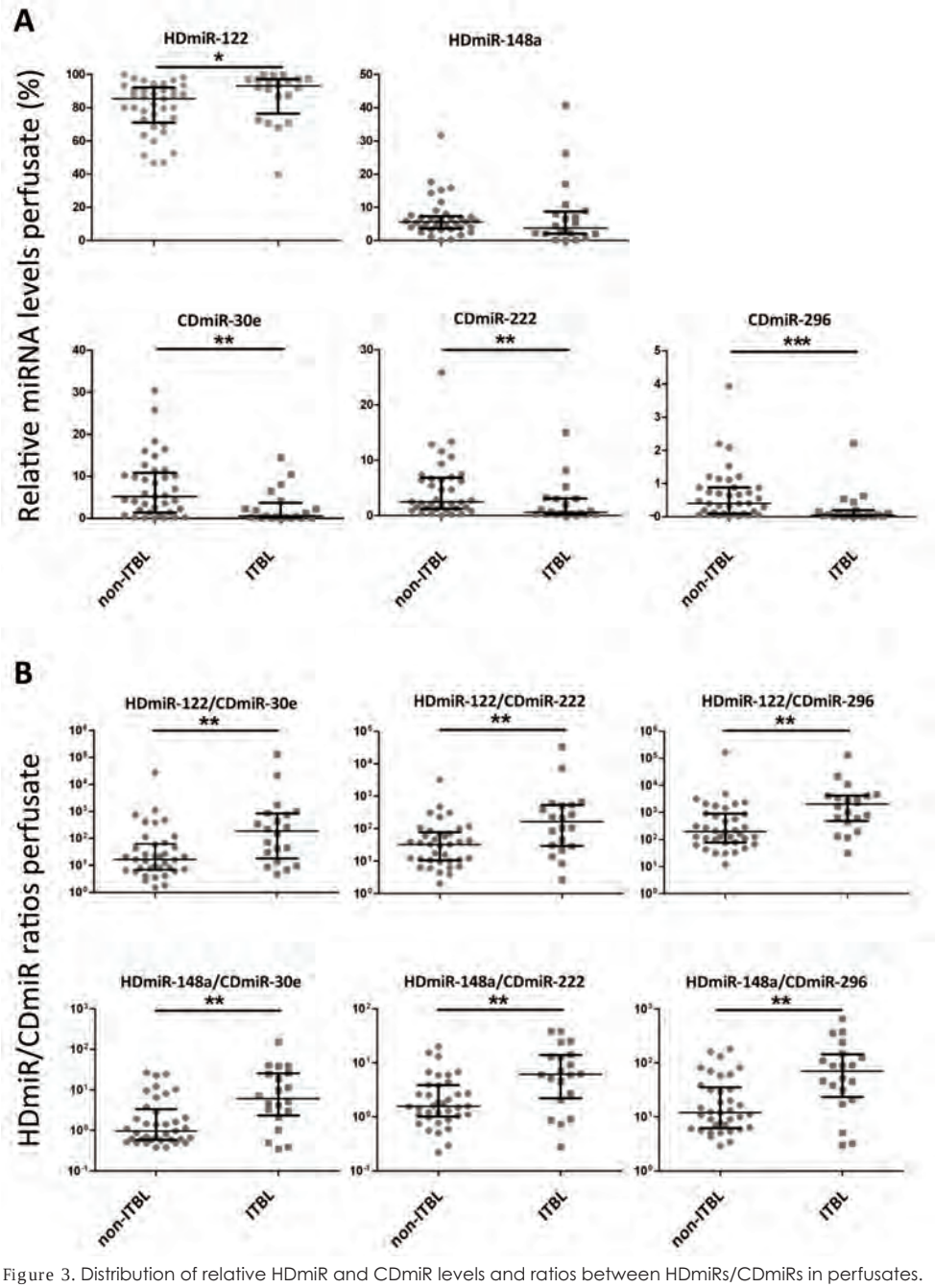

Figure 3. Distribution of relative HDmiR and CDmiR levels and ratios between HDmiRs/CDmiRs in perfusates. (A) Comparison of relative HDmiR- and CDmiR-levels in perfusates obtained from grafts that developed ITBL (n = 20) and grafts that did not develop biliary strictures (non-ITBL group, n = 37). (B) Due to the lack of reliable reference RNAs in solutions, CDmiR levels were normalized to HDmiR levels using a ratio. The HDmiR/CDmiR ratios of all perfusate samples were significantly different between ITBL and non-ITBL grafts. Figures demonstrate the median ± interquartile ranges. *P < 0.05, **P < 0.01, ***P < 0.001.

	Univariate		Multivariate		
HDmiR/CDmiR ratio	**HR**	**P-value (95%CI)**	**HR**	**P-value (95%CI)**	**C (low. limit - up. limit)**
HDmiR-122/CDmiR-30e	1.67	0.004 (1.18-2.35)	1.39	ns	-
HDmiR-122/CDmiR-222	2.04	0.002 (1.29-3.22)	1.62	ns	-
HDmiR-122/CDmiR-296	1.94	0.002 (1.27-2.96)	1.65	ns	-
HDmiR-148a/CDmiR-30e	3.22	0.001 (1.64-6.43)	6.89	0.003 (1.97-25.06)	0.77 (0.64-0.89)
HDmiR-148a/CDmiR-222	4.98	0.001 (1.91-12.95)	3.38	0.025 (1.28-11.11)	0.76 (0.63-0.88)
HDmiR-148a/CDmiR-296	3.45	0.001 (1.65-7.20)	4.03	0.025 (1.19-13.62)	0.74 (0.62-0.87)

Table 3. Cox regression analysis and C-statistics on HDmiR/CDmiR ratios in perfusate and the development of ITBL. In the multivariate statistical model, separate HDmiR/CDmiR ratios were adjusted for graft type (DBD vs. DCD), the type of solution used for graft preservation (UW vs. HTK) and the interaction between variables. The adjusted models were also used for the calculation of discriminative power by C-statistics. n.s., not significant.

since values reached up to 0.89 and were not below 0.60. To validate our findings, incidence of ITBL in the entire study cohort and in separate DBD and DCD grafts was compared between grafts with high HDmiR/CDmiR ratios in perfusate vs. grafts with low HDmiR/CDmiR ratios (Fig. 4). This revealed that in DBD grafts, ratios between HDmiR-148a/CDmiR-30e had strong discriminative power (P = 0.001) with high negative predictive value (90%) whereas in DCD grafts, ratios of HDmiR-148a/CDmiR-296 showed the strongest separability (P = 0.011) with a positive predictive value of 100%.

No correlation between HDmiR and CDmiR levels in graft perfusate and their expression levels in liver tissue

From the first 24 transplantations, paired liver biopsies were available and tested for miRNA expression. Six grafts developed ITBL and 18 remained free of biliary strictures. No differences were found in HDmiR and CDmiR expression, nor in ratios of HDmiRs/CDmiRs between ITBL and non-ITBL groups. In contrast to graft perfusates, HDmiR/CDmiR ratios expressed in liver tissue had no discriminative power (C < 0.5) and univariate cox regression analysis showed no significantly increased risk for the development of ITBL after transplantation (HR = 1.06, P = 0.487). Also donor type (DBD vs. DCD) did not significantly affect expression levels of HDmiRs or CDmiRs (data not shown). Accordingly, it is known that hypothermic conditions preserves mRNA patterns and prevents changes in gene expression.

DISCUSSION

This study shows that CDmiRs are released during graft preservation and that their profiles in perfusates are predictive for ITBL. High ratios of HDmiRs/CDmiRs in perfusate increased the risk for a graft to develop ITBL within a year after transplantation up to four fold, which was validated in a separate analysis of DBD and DCD grafts. Furthermore, we showed

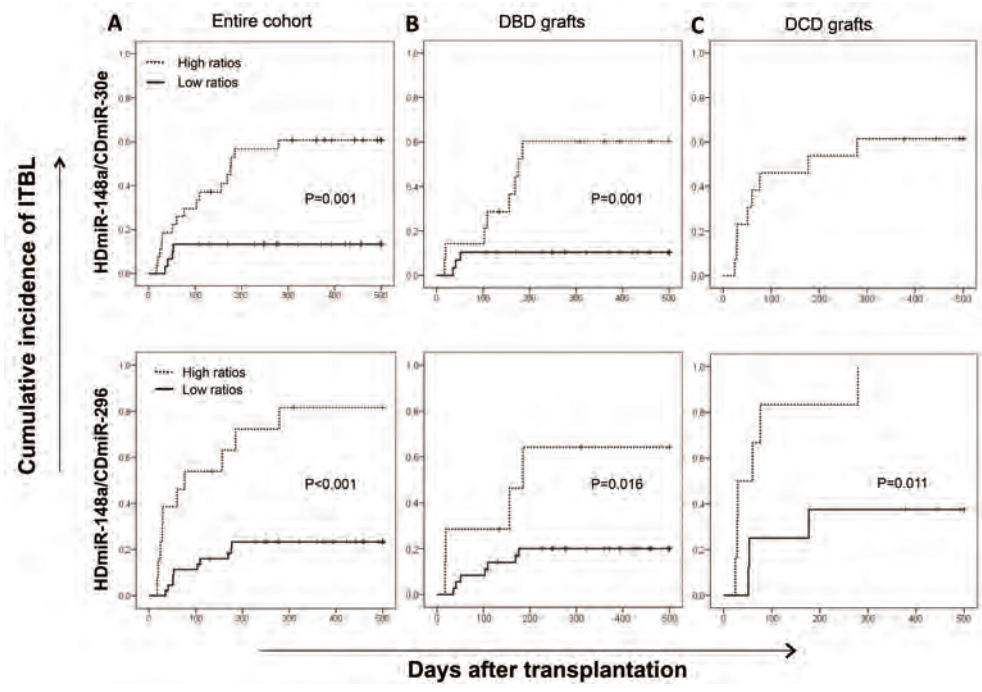

Figure 4. . Increased incidence of ITBL in grafts with high HDmiR/CDmiR ratios in perfusates. Through a grid search on HDmiR/CDmiR values within the inter-quartile range, different cut-off values could be obtained to distinguish grafts with an increased incidence of ITBL from grafts without biliary complications in the entire study cohort and for separate DBD and DCD grafts; (A) In the entire study cohort (n = 57), incidence of ITBL was threefold higher in grafts that released high ratios of HDmiR-148a/CDmiR-30e and fourfold higher in grafts that released high ratios of HDmiR-148a/CDmiR-296. (B) In DBD grafts (n = 43), incidence of ITBL was significantly higher in grafts with high levels of HDmiR-148a/CDmiR-30e (P = 0.001) and HDmiR-148a/CDmiR-296 (P = 0.016). (C) In DCD grafts (n = 14), all grafts except one developed ITBL when perfusates contained high ratios of HDmiR-148a/CDmiR-30e. Grafts releasing high HDmiR-148a/CDmiR-296 levels showed significant higher incidence of ITBL during follow-up (P = 0.011).

that miRNAs remained stable in perfusates for at least 24 hours. Given the stability of miRNAs in perfusates and their superior discriminative power to expression in tissue biopsies, our data indicate that they could be used as novel biomarker to assess bile duct integrity during cold storage prior to liver transplantation.

To our knowledge, we are the first to report on a potential marker that is able to assess graft biliary injury and predict the development of ITBL prior to liver transplantation. One advantage of miRNAs as injury markers is that many are expressed in a cell type specific fashion. In the current study, we used miRNAs that were reported to be highly abundant in cholangiocytes[29]. Distinctive cell-type abundant miRNAs have been identified that were able to diagnose graft rejection and ischemia-reperfusion injury[18, 30, 31]. In liver transplant recipients, HDmiR-122 and HDmiR-148a were found to be released into the circulation and were elevated in patient serum during episodes of graft rejection, prior

to the elevation of AST and ALT in serum[26]. Furthermore, CDmiR-222 and CDmiR-296 have been associated with injury to vascular endothelial cells and protection against ischemia-reperfusion injury in kidney transplantation[32, 33]. As diagnostic markers, miRNA profiles have been shown to be able to distinguish normal cholangiocytes from cholangiocarcinoma cells, and tumor derived miRNAs in bile were proven to be more sensitive in diagnosing cholangiocarcinoma than carcinoembryonic antigen[29, 34].

There are several benefits to use perfusates for diagnostic assays. As perfusates are non-invasively obtained from total vascular perfusion during cold ischemia, they represent injury events of the whole liver and therefore lack the sampling bias that is associated with tissue biopsies. Furthermore, perfusates can represent graft quality prior to transplantation without any influencing recipient factors in an early phase of liver transplantation[27, 28]. Since liver biopsies only provide information about that specific part of the liver, it may be less useful for the detection of bile duct injury as ITBL, which is known to occur focally and often does not affect the entire graft evenly. Therefore, it can be argued that perfusates provide more accurate information about conditions predisposing to ITBL than biopsies. Previous studies have reported the successful use of perfusates to predict graft survival and graft dysfunction by measuring hyaluronate- and aminotransferases[35-37] and recent experimental studies describe the identification of perfusate markers in hypothermic machine perfusion of marginal grafts[38, 39]. These markers however concerned hepatocyte injury and associated graft primary non-function, but failed to detect the degree of biliary injury in liver grafts.

The current study supports the notion that the release of miRNAs from cells is an active and selective process. For instance, the relative levels of CDmiRs and HDmiRs in liver tissue were significantly different from their levels in perfusates (Fig. 1B), suggesting that the release of miRNAs from cells is selective and does not just reflect the relative abundance of miRNA observed in the cells. This selectivity is consistent with the observed release of HDmiRs into serum, reported earlier[26]. Furthermore, concentrations of different miRNAs in perfusates did not correlate with donor or recipient serum transaminases or the length of cold ischemia time, which makes the hypothesis that miRNAs in perfusates represent leakage after cell damage less likely. Though one would expect increased levels of miRNAs following injury, the opposite was the case in the current study; relative levels of CDmiRs were found to be significantly lower in perfusates obtained from grafts that developed ITBL. This finding leads to several underlying hypotheses: the number of cholangiocytes could be lower already during cold preservation in grafts that will develop ITBL. This however seems unlikely, since no difference in CDmiRs expression was found in liver biopsies taken at the end of cold ischemia between ITBL and non-ITBL groups. Based on recently obtained insights on polarized release of miRNAs by cells[40], a more plausible explanation would be a shift of miRNA release into bile influenced by

cholangiocyte-injury. Furthermore, measurement of immature pre-miRNAs or apoptotic cholangiocyte markers, such as CK19, could be an alternative method to investigate gene regulation and cholangiocyte deregulations in tissue biopsies. However, markers like CK19 are also highly expressed by liver-derived mesenchymal stem cells and therefore could lack specificity[41]. These hypotheses were not further explored in the current study and need to be investigated through future research.

A majority of HDmiRs and CDmiRs in perfusates were associated with protein complexes. Of the cell-free fractions, an average of 7% of miRNAs were found in the organelle or micro vesicle fractions, whereas the remaining 93% of miRNAs were present in the unpelletable supernatant fraction (Fig. 2B) which were predominantly associated with proteins (Fig. 2C). This could explain miRNA stability in the RNase-rich environment of graft perfusates even hours after incubation at room temperature. Studies on the characterization of extracellular miRNAs revealed that proteins like Argonaute2 are mainly responsible for the stability of circulating miRNAs[21, 22]. Targeted release of miRNAs from cells through selective microvesicle and exosome secretion seems less likely, since only a minority of miRNAs was present in these fractions in the current study (Fig. 2B). It is however important to emphasize that different methods are available for the isolation of exosomes and microvesicles. Particularly for the quantification of miRNAs, modified exosome precipitation methods appear to be more suitable than conventional ultracentrifugation up to 200.000 g[42]. In the current study, centrifugation steps did not exceed 80.000 g and therefore it cannot be ruled out whether a fraction of the protein-bound miRNAs in the perfusate supernatant was derived from exosomes.

Several other limitations should be considered in the present study. Firstly, the number of liver transplantations performed by our center annually hampers the validation of miRNA performance to predict ITBL in an independent cohort in short term. The sample size of our study was however sufficient to validate our findings in a sub analysis of DCD and DBD grafts. Secondly, the median recipient follow-up for 17 months is relatively short, since other groups have described different clinical presentations of ITBL varying from early onset to late onset up to 18 years after transplantation[3, 6]. The vast majority of ITBL however occur within the first year after transplantation[6, 7, 43], which was also the case in the present study. Thirdly, wedge biopsies that were used in this study were taken from the periphery of the liver and were used to compare expression of miRNAs in tissue with their levels in perfusates. It can be argued that biopsies do not reflect ischemic bile duct injury adequately, since ITBL is usually more prominent in the center of the liver[43]. As argued earlier, we therefore strongly believe that particularly for ITBL, perfusates are more representative than random biopsies, taken from either the center of the liver or from the periphery. It should be emphasized that for the present study, analysis was performed on perfusates that were obtained after the second flush of the graft with human albumin

solution, during the back-table procedure. The use of these second flush perfusates implies that a considerable amount of miRNAs that were released during graft preservation might be lost for analysis during the first flush. Therefore, miRNA levels in the perfusate after the second flush might only represent the release of miRNAs at the end of cold storage, rather than their release during the entire preservation procedure. However, analysis on miRNA levels in perfusates obtained from the first flush provided similar trends in HDmiR/CDmiR ratios between ITBL and non-ITBL groups, though their discriminative power was less pronounced (data not shown). Finally, our study included only a limited number of CDmiRs and thereby possibly overlooks other miRNAs, which could indicate biliary injury or predict ITBL even more sensitively. As shown in Figure 3, extensive overlap in HDmiR/CDmiR levels exists between ITBL and non-ITBL groups, which might be detrimental for assessing graft quality on an individual level. More sensitive and specific CDmiRs could be identified by MicroRNA GeneArray analysis. Sclerosing and tapering of the biliary tree in the pathophysiology of ITBL however complicates the application of accurate techniques for miRNA isolation, like laser capture micro dissection from bile duct tissue. Therefore, we are currently attempting genome-wide miRNA gene array analysis on perfusate samples, though relative low RNA yields render this procedure technically challenging. Preliminary results from our ongoing research however do confirm that the CDmiRs investigated in this study are up to eight-fold higher in common bile duct tissue compared to liver tissue, while expression of HDmiRs is almost zero (data not shown).

In conclusion, this study demonstrates that differential release of CDmiRs during graft preservation is associated with biliary injury and predictive for the development of ITBL after liver transplantation. Our findings provide new possibilities to assess graft quality prior to transplantation, though future research is warranted to unravel the true merits of miRNAs in predicting or preventing the development of ITBL after liver transplantation.

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General discussion and future prospects

9

Liver transplantation is the only curative treatment for end-stage liver disease. Its first successful application was in the late 1960s after the introduction of adequate immunosuppressives. The improvements in surgical techniques, graft preservation and immunosuppressive drugs over time have made liver transplantation into a widespread and successful treatment. However, despite these improvements liver transplantation is still hurdled by postoperative complications, among them biliary complications, mainly ITBL. As a matter of fact, bile ducts and the biliary tree are considered the Achilles' heel of liver transplantation. Biliary complications and ITBL remain an important cause of significant morbidity and mortality[1-3]. Several risk factors have been identified related to donor characteristics, ischemia-reperfusion injury, inadequate preservation, cytotoxic injury, and immune-mediated injury. Despite the knowledge of these risk factors, ITBL still remains an unpreventable problem in many cases of liver transplantation[1-4]. Latest histological evidence indicates that ischemic injury, during graft procurement and preservation, leads to loss of intra and extrahepatic biliary epithelium as a consequence of detaching cholangiocytes, which are predictive for the development of ITBL[5-7]. This could be of importance in the pathogenesis of ITBL and further research should verify the importance of this early biliary injury. Despite this new finding, the exact pathogenesis of ITBL still remains to be elucidated, although ischemic injury seems to be the prime initiator. In this thesis the role of the portal vein in the blood supply of the biliary tree and the pathogenesis of ITBL was investigated (part I). Furthermore the potential of microRNAs as novel biomarkers for early identification of hepatocyte and cholangiocyte injury was investigated during and after liver transplantation and in relation to the development of ITBL (part II).

THE COMPROMISED PORTAL CIRCULATION AS A CAUSE OF ITBL

In chapter 2 of this thesis, we describe that intraoperatively taken liver graft biopsies exhibit significant histological differences between grafts that later develop ITBL and grafts that do not. Smaller portal vein branch (PVB) lumen surface areas and an absence of paraportal collateral vessels were noted 1 hour after reperfusion in grafts later developing ITBL. Compared to the control group, both the ITBL group and the non-ITBL group showed smaller PVB lumen sizes after reperfusion; however, the size in the ITBL group was significantly smaller than the size in the non-ITBL group. These results suggest a role for the portal circulation in the pathogenesis of post-operative ITBL.

In order to further study the potential role of the portal vein in chapter 3 we investigated the microvascular blood flow through the intact and transected common bile duct (CBD). A decrease in microvascular blood flow through the intact CBD to 51% was shown during closure of the common hepatic artery and to 62% during closure of the portal

vein. After transection of the CBD, measurement of the microvascular blood flow through the CBD at the hilum of the liver revealed that the contribution of the portal vein remained highly consistent with a decrease in microvascular blood flow to 60%. The contribution of the hepatic artery seemed to become more important once the contribution of the gastroduodenal artery ceased; after transection of the CBD and closure of the hepatic artery, the blood flow decreased to 31%. This study further reinforces the hypothesis that indeed the portal circulation is partially responsible for flow through the peribiliary plexus and therefore could play an important role in development of ITBL after liver transplantation.

Finally in chapter 4, further evidence is given for this hypothesis by discussing three cases of patients after liver transplantation, who develop a segmental portal vein thrombosis after liver transplantation and consequently develop segmental ITBL in the specific segments affected by the portal vein thrombosis. This provides circumstantial evidence that the portal circulation in at least a subgroup of patients is vital and plays a role in development of ITBL.

Injections of dyes and vascular castings have demonstrated that the terminal branching of the hepatic artery opens into a peribiliary network and into the hepatic sinusoids[8-11]. Therefore, the hepatic artery is traditionally considered the main provider of blood and responsible for the oxygenation of cholangiocytes[8, 10].

However, earlier studies have also suggested a role for the portal vein in biliary oxygenation by showing that the PBP communicates with portal branches through arterio-portal anastomoses, the so-called internal roots, which deliver the same amount of oxygen as the hepatic artery to the biliary epithelium[12-14]. Additional support for the role of the portal vein in the pathogenesis of ITBL is suggested by a study demonstrating diminished enhancement of the bile duct wall in the portal phase of contrast-enhanced ultrasonography of grafts affected by ITBL[15].

Also several experimental studies in animals support the role of the portal vein in biliary oxygenation. Mitra et al. showed in an animal study that a large part of the arterial blood entered the portal vein through arterio-portal anastomoses in the peribiliary plexus, supplying the hepatic lobules with mixed arterial and portal venous blood[16]. This was confirmed by Cho et al. who also found extensive arterio-portal communications in the peribiliary plexus in a study of rabbits, permitting mixing of arterial and portal venous blood before entering the sinusoids[17]. Restrepo and Warren have reported that the volume of portal venous blood flow increases with hepatic arterial ligation[18]. An animal study conducted by Tavoloni et al. showed that hepatic artery ligation did not result in a decreased bile flow, portal venous oxygenation, or altered hepatic ultrastructure[19].

Also studies from humans provide support for the hypothesis. Tygstrup et al. found that arterial contribution to total hepatic flow was 35%, with 50% of oxygen consumption from

this source. With occlusion of the proper hepatic artery, extraction of oxygen from the portal venous blood increased[20]. Furthermore, supplemental information on distribution of hepatic flow is available through case reports of accidental hepatic artery ligation. Brittain et al. described 5 cases having undergone injury to the common hepatic or right hepatic artery, without signs of hepatic ischemia[21]. Thus, despite current beliefs, the intrahepatic biliary epithelium seems to be adequately oxygenated in the absence of hepatic artery perfusion in some cases. However in contradiction, clinical experience shows that in most cases after hepatic artery thrombosis the bile ducts are affected by ischemic cholangiopathy.

In conclusion the studies presented in this thesis and previous clinical and animal studies propose a role for the portal circulation in oxygenation of the biliary tree and its compromise is therefore proposed as a cause of development of ITBL after liver transplantation in some patients. To further substantiate the role of the portal circulation additional clinical studies should be conducted. These could for instance consist of studies applying additional flushes of the portal circulation. In particular machine perfusion can play an important role in further investigating the role of vasculature as it allows for controlled reproducible graft perfusion during graft preservation. Early studies already show promising results in this field as DCD grafts preserved using hypothermic oxygenated machine perfusion show comparable or better short-term results than matched brain-death donor grafts[22]. Interestingly, more recent studies show that machine perfusion in an experimental DCD rat model only through the portal vein protects against biliary injury[23]. Therefore as also suggested by our studies the portal vein should be considered as an important cause of ITBL in clinical liver preservation and transplantation.

MICRORNAS AS NOVEL BIOMARKERS FOR DETECTION OF LIVER INJURY AND ITBL

In chapter 5 of this thesis we discuss the use of microRNAs (miRNAs) as promising biomarkers in the setting of liver disease and transplantation. Based on the literature we conclude that miRNAs are not only promising biomarkers in liver transplantation and disease but also posses the potential for future therapeutic utilization and should therefore be researched. In chapter 6 we provide evidence that the concept of miRNAs as biomarkers of hepatic injury is feasible in the setting of liver transplantation. We show that serum levels of hepatocyte-derived microRNAs (HDmiRs) are elevated in patients with liver injury after liver transplantation and during acute cellular rejection. Conversely, hepatic miRNA levels in liver graft biopsy samples exhibit diminished expression with prolonged warm ischemia times. During acute rejection, serum HDmiRs show similar kinetics as transaminases; however, miRNA levels appear to increase and decrease earlier than aminotransferase levels. This study demonstrates that miRNAs are sensitive

and stable biomarkers for detection of liver injury after liver transplantation and that they could be used for pathologies where biomarkers are still lacking. In chapter 7 the release of hepatic miRNAs is investigated in matched human bile and serum samples thereby providing insight in the method of release of miRNAs. It is concluded that the release of miRNAs is an active, specific and polarized form excretion, which has possible remote regenerative responses. Results were further confirmed in cell lins and an animal model of warm ischemia. This showed that during injury a shift occurs in the release of miRNAs and specific HDmiRs and CDmiRs are released in different compartments. Similarly, the release of these miRNAs is also influenced by liver function, which shows changes in release, as some tend to be released more into blood while others into bile. This further strengthens the hypothesis that the release of miRNAs is a controlled and active process with potential remote effects and could be utilized to trigger specific targets for therapeutic purposes. In chapter 9 we demonstrate that detection of miRNAs in perfusates used for flushing donor livers is feasible. Furthermore we demonstrate that these miRNAs are stable and more importantly that specific profiles of hepatic miRNAs in perfusates at the time of transplantation are predictive for identification of grafts later developing ITBL.

MiRNAs have proven to possess high potential and could provide a solution for the urgent need for better non-invasive biomarkers, which can serve as earlier and more sensitive signs of injury and graft dysfunction in liver transplantation. Better markers will greatly aid in the management of liver transplant recipients and can allow for better graft selection, early detection of recurrent disease and safer reductions of immunosuppressive medications to achieve a better balance between effects (the prevention of graft rejection) and side effects (toxicity, infection, and malignancy). Long-term complications of immunosuppressive drugs, such as nephrotoxicity and de novo cancers, are becoming bigger problems because of the long survival of liver transplant recipients[24]. Currently, the potential benefits from tapering immunosuppressive medications in patients are countered by the potential risk of losing the graft to immune-mediated rejection. Serum ALT and AST are often insufficient for the early and definitive diagnosis of acute rejection, and liver biopsy is necessitated. Particularly in the setting of liver transplantation, liver biopsy poses a significant risk for complications such as pain, bleeding, and infections[25-28]. The feasibility of this concept of a minimally invasive diagnosis of acute rejection based on the detection of messenger RNA has already been demonstrated for kidney transplants[29, 30].

FUTURE PROSPECTS AND RECOMMENDATIONS

In this thesis we have demonstrated the important role of the portal vascularization in the development of ITBL after liver transplantation. Not many studies in this field have been conducted in humans. To further substantiate the role of the portal circulation additional clinical studies should be conducted. These could for instance consist of studies applying additional flushes of the portal circulation. Machine preservation will play an important role in further investigating the role of vasculature as it allows for controlled reproducible circumstances during graft preservation. One thing that remains is that ischemia-reperfusion seems to be an important factor in the development of ITBL. Therefore, the risk factors known in literature should be addressed adequately during liver transplantation.

In the second part of this thesis we demonstrate the use of miRNAs as sensitive and specific non-invasive biomarkers of liver injury during and after liver transplantation. These miRNAs possess huge potential and were able to identify various forms of liver injury, including forecasting the development of ITBL later on. Although it is now clear that these miRNAs could serve as biomarkers their potential biological role remains unclear. Follow up studies should be conducted to verify the identified biomarkers and into the biology of these miRNAs to investigate whether they are related to targets, which could be of influence on known risk factors of ITBL. In addition, once these targets have been identified in vitro and later in vivo studies should be conducted wherein under and overexpression of the identified miRNAs and their effect on development of ITBL is researched. All in all the field of miRNAs is becoming increasingly important and it possesses huge potential for the field of transplantation with many possibilities for exploration.

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Nederlandse samenvatting

10

Een levertransplantatie is de enige levensreddende behandeling voor eindstadium leverfalen. Na een tijdperk van mislukte pogingen werd de eerste succesvolle levertransplantatie uitgevoerd in 1967 door dr. Thomas E. Starzl in de Verenigde Staten. De patiënt overleefde langer dan een jaar wat in die periode uitzonderlijk lang was na een levertransplantatie.

Een kleine 50 jaar later is levertransplantatie de gouden standaard voor behandeling van eindstadium leverfalen. De 1- en 5-jaarsoverleving overstijgt respectievelijk 85% en 70%. Ruim de helft van de getransplanteerden leeft langer dan 20 jaar. Deze resultaten zijn vooral te danken aan de vooruitgang van chirurgische technieken, een betere preservatie van het transplantaat en de betere immunosuppressieve medicatie.

Echter, met de toegenomen overleving komen nieuwe problemen aan het licht die verdere verbetering van resultaten beperken. Een van de belangrijkste complicaties na levertransplantatie is de ontwikkeling van diffuse galwegstricturen, beter bekend als niet-anastomose stricturen (NAS) of 'ischemic-type biliary lesions' (ITBL), welke over het algemeen voor komen bij 5-15% van de getransplanteerden. ITBL is de op twee na belangrijkste oorzaak van transplantaatverlies waardoor een volgende levertransplantatie vereist wordt. Tot 50 % van de ontvangers die ITBL ontwikkelen moeten op termijn een retransplantatie ondergaan. Hoewel verschillende risicofactoren zijn geïdentificeerd voor het ontwikkelen van ITBL blijft de exacte pathogenese van ITBL vooralsnog onbekend. Hierdoor is voorspellen en voorkomen van ITBL moeizaam. De bekende risicofactoren voor het ontwikkelen van ITBL worden besproken in de volgende paragrafen.

DONOR KARAKTERISTIEKEN

Door de jaren heen zijn vele donor karakteristieken onderzocht om een oorzaak voor het ontstaan van ITBL te identificeren. Slechts één donor kenmerk is tot op heden geïdentificeerd als een belangrijke risicofactor voor het ontwikkelen van ITBL, namelijk donorleeftijd.

Donorleeftijd is in verschillende studies geïdentificeerd als een onafhankelijke risicofactor voor het ontwikkelen van ITBL na levertransplantatie. Een hogere donorleeftijd is gerelateerd aan een hogere incidentie van ITBL. Een recente studie toont dat donorlevers welke ITBL ontwikkelen gemiddeld van 5 jaar oudere donoren afkomstig zijn dan transplantaten die geen ITBL ontwikkelen. Hoewel donorleeftijd een onafhankelijke risicofactor lijkt te zijn voor het ontwikkelen van ITBL is het onderliggende mechanisme nog onduidelijk. Er wordt verondersteld dat het hogere risico op het ontwikkelen van ITBL in oudere donorlevers is gerelateerd aan de hogere gevoeligheid voor ischemie-reperfusieschade.

ISCHEMIE-REPERFUSIESCHADE

Ischemie-reperfusieschade wordt als primaire oorzaak geacht van ITBL omdat ITBL sterke overeenkomsten vertoont met de ischemische galweglaesies die gevonden worden na trombosering van de leverslagader. Deze gelijkenis heeft ook geleid tot de naam 'ischemisch-type' biliary lesions. Uit steeds meer aanwijzingen lijkt dat ischemie-reperfusieschade een belangrijke risicofactor is voor het ontstaan van ITBL.

Donatie na hart-dood en warme ischemietijd

Donatie na hart-dood (DCD) is in de levertransplantatie geïntroduceerd om het groeiende probleem van tekort aan organen deels te reduceren. Het gebruik van DCD organen brengt een additionele periode van warme ischemie met zich mee. Van warme ischemie is aangetoond dat dit een negatieve invloed heeft op de postoperatieve leverfunctie en kan leiden tot een hogere incidentie van ITBL.

Daarnaast wordt gedacht dat gedurende de extra periode van warme ischemie microtrombi gevormd kunnen worden in de peribiliaire plexus (PBP) welke verantwoordelijk is voor de doorbloeding van de galwegen. Deze microtrombi kunnen leiden tot onvoldoende zuurstofaanbod en aanhoudende ischemie wat zou kunnen leiden tot ITBL.

Ook hebben experimentele dierenstudies aangetoond dat donatie na hart-dood leidt tot veranderingen in de galsamenstelling wat de opgetreden schade aan de galwegen verergert en zou kunnen bijdragen aan het ontwikkelen van ITBL.

Gemiddeld komen alle galwegproblemen twee keer zo vaak voor bij patiënten die een DCD levertransplantaat ontvangen. De incidentie van ITBL echter, is 5 maal hoger bij gebruik van DCD organen. Bovendien lijkt ITBL in het geval van DCD donoren eerder na transplantatie op te treden in vergelijking met donororganen van een hersen-dode donor.

Koude ischemietijd

Buiten de warme ischemietijd is koude ischemietijd door verscheidene studies geïdentificeerd als een onafhankelijke risicofactor voor het ontwikkelen van ITBL. Een koude ischemietijd van meer dan 14 uur is geassocieerd met een tweevoudige toename van preservatieschade resulterend in galwegstricturen en een verminderde transplantaatoverleving. Bij een koude ischemietijd van minder dan 13 uur wordt een incidentie van ITBL gerapporteerd van 7%, terwijl de incidentie stijgt tot 52 % wanneer de koude ischemietijd meer dan 13 uur bedraagt. Indien de koude ischemietijd langer dan 15 uur is loopt de incidentie van ITBL zelfs op tot 69%, wat suggereert dat koude ischemietijd een belangrijke risicofactor is voor het ontwikkelen van ITBL. Derhalve dient de koude ischemietijd zo kort mogelijk gehouden te worden om het risico op het ontwikkelen van ITBL te minimaliseren.

TRANSPLANTAAT PRESERVATIE

Het is bekend dat preservatietechnieken invloed hebben op de kwaliteit van het donororgaan. Hoewel de laatste jaren diverse vormen van machine perfusie met veel interesse worden onderzocht, is statische koude preservatie momenteel de enige klinisch goedgekeurde methode voor het behoud van het levertransplantaat. Studies naar verschillende factoren tijdens de preservatie en hun invloed op het ontwikkelen van ITBL zijn verricht en hebben een aantal risicofactoren geïdentificeerd.

Type preservatievloeistof

De University of Wisconsin (UW) oplossing wordt het meest gebruikt voor het spoelen en preserveren van de donorlever. In de afgelopen jaren is het gebruik van de concurrerende histidine-tryptofaan-ketoglutaraat (HTK) preservatievloeistof, vooral binnen de DCD levertransplantatie, toegenomen. Hoewel UW oplossing superieur is ten opzichte van HTK oplossing bij de bescherming van hepatocyten, kan de hogere viscositeit van de UW oplossing het adequaat doorspoelen van de donorlever in de weg staan. Met name de kleine haarvaten, zoals de PBP, zouden hieronder kunnen lijden door suboptimale conservering. HTK oplossing heeft een viscositeit vergelijkbaar met die van water en kan hierdoor tijdens het doorspoelen een 3 maal hogere stroomsnelheid bereiken in vergelijking met de UW oplossing. Dit resulteert in een snellere koeling van het donororgaan naar de gewenste temperatuur en een verbeterde spoeling van de kleine PBP waardoor theoretisch ischemische galwegproblemen voorkomen zouden kunnen worden. Dit laatste wordt bevestigd in een lagere incidentie van ITBL bij het gebruik van laag viskeuze preservatievloeistoffen, zoals HTK.

Hogedruk-perfusie en preservatie

Met dezelfde gedachte, dat verbeterde en snellere perfusie de vorming van microthrombi voorkomt en de gewenste orgaantemperatuur sneller wordt bereikt, is er getracht om donororganen door middel van hogedruk-perfusie te preserveren. Studies tonen aan dat een verbeterde perfusie door het arteriële vaatsysteem onder druk te perfunderen leidt tot een significant lagere incidentie van ITBL na levertransplantatie.

Perfusie met trombolytica

Om te onderzoeken of de formatie van microthrombi in de PBP tijdens preservatie een risicofactor is voor het ontwikkelen van ITBL zijn studies uitgevoerd waarbij het donororgaan wordt geperfundeerd met trombolytica. Een studie toont aan dat perfusie met het trombolyticum urokinase van het levertransplantaat tijdens preservatie resulteert in een viervoud lagere incidentie van ITBL. Een experimentele studie in ratten laat ook

zien dat perfusie met streptokinase, een ander trombolyticum, de ischemische schade significant vermindert. Het voorkomen van microthrombi en een adequate perfusie van het donororgaan lijken derhalve belangrijk voor het voorkomen van ITBL.

CYTOTOXISCHE SCHADE

Cytotoxische schade door gal lijkt ook een belangrijke rol te spelen bij de ontwikkeling van ITBL na levertransplantatie. Intrahepatische cholestase na levertransplantatie is gebruikelijk, met name als gevolg van ischemie-reperfusieschade. De cellulaire veranderingen die hierbij optreden resulteren in een toegenomen blootstelling van cholangiocyten en hepatocyten aan het toxische gal.

Naast de toegenomen blootstelling aan gal resulteert ischemie-reperfusieschade tot een abnormale galsamenstelling met verhoogde toxiciteit door effecten op de galtransporteurs. De toxiciteit van gal na levertransplantatie wordt gekenmerkt door een laag fosfolipide / galzout ratio welke blootgestelde cholangiocyten en hepatocyten beschadigt. Een laag fosfolipide / galzout ratio is geassocieerd met histologisch aantoonbare beschadiging van de galwegen en de ontwikkeling van ITBL.

Bovendien is aangetoond dat ischemie-reperfusieschade kan leiden tot verstoorde uitscheiding van waterstofcarbonaat en mucinen, welke normaliter worden uitgescheiden door cholangiocyten en die een barrière vormen ter bescherming tegen gal. Als gevolg van veranderingen in deze beschermende maatregelen neemt de kans op het ontwikkelen van ITBL toe.

Recent onderzoek toont aan dat adequate spoeling van de galwegen tijdens perfusie de effecten van galtoxiciteit vermindert en resulteert in verminderde schade aan het galwegepitheel. Daarom is het raadzaam om de galwegen adequaat te perfunderen tijdens levertransplantatie, en in het bijzonder in DCD donoren omdat in deze donoren meer toxiciteit van gal optreed in vergelijking met hersen-dode donoren.

IMMUUN-GEMEDIEERDE SCHADE

Verschillende studies laten zien dat immunologische factoren ook geassocieerd zijn met een hogere incidentie van ITBL na levertransplantatie en dus beschouwd dienen te worden als risicofactoren.

ABO incompatibele donoren en afstoting

Hoewel ABO incompatibele donatie in het verleden is afgeraden gebruiken sommige centra ABO incompatibele donoren vanwege het tekort aan donororganen. Het gebruik van ABO incompatibele donor organen resulteert in een significant hogere incidentie van

ITBL. Gedacht wordt dat de galwegstricturen secundair optreden door immunologische schade aan de PBP, met als gevolg trombose en ischemie van de galwegen.

Ook chronische afstoting, waarbij ook de bloedvaten aangedaan zijn, lijkt in sommige studies geassocieerd te zijn met een hogere incidentie van ITBL . De gedachte hierbij is dat dit ook een secundair gevolg is van ontsteking, schuimcelvorming, arteritis en trombosering van de kleine vaten, waaronder die van de PBP, met daaropvolgend ischemie en strictuurvorming in de galwegen.

Preexistente (auto-immuun) ziekten

Preexistente (auto-immuun) ziekten worden ook in verband gebracht met de ontwikkeling van ITBL na levertransplantatie.

Een cytomegalovirus (CMV) infectie is vaak aanwezig in ontvangers van levertransplantaten. 30-50% van de ontvangers is reeds geïnfecteerd met dit virus. De gedachte is dat CMV infectie van de vaatwand ITBL veroorzaakt vanwege schade aan de PBP en daaropvolgende ischemie en vorming van ITBL.

Ook hebben studies aangetoond dat preexistente auto-immuun hepatitis (AIH) of primaire scleroserende cholangitis (PSC) als indicatie voor de levertransplantatie geassocieerd zijn met een hogere incidentie van ITBL. In het geval van AIH is het nog onduidelijk waarom dit resulteert in een hogere incidentie van ITBL. PSC is een pathologische aandoening die sterke gelijkenissen vertoont met ITBL. Na transplantatie kan er opnieuw PSC optreden in het transplantaat, maar kan ook ITBL ontwikkelen. Het onderliggende mechanisme waardoor PSC leidt tot ITBL is onduidelijk en blijft lastig te onderzoeken aangezien er geen biomarkers bestaan die onderscheid kunnen maken tussen terugkeer van PSC in het transplantaat en ITBL na levertransplantatie. Het is daarom onduidelijk of de hogere incidentie van ITBL inderdaad moet worden aangemerkt als ITBL of terugkerende PSC.

DIAGNOSE EN BEHANDELING VAN ITBL

De eerste symptomen gerelateerd aan ITBL ontstaan meestal binnen 1 jaar na levertransplantatie en zijn meestal niet specifiek. Deze kunnen bestaan uit koorts, aspecifieke buikklachten en andere symptomen die verband houden met cholestase. In ernstige gevallen kan sprake zijn van geelzucht en jeuk, hoewel dergelijke extreme en late gevallen zeldzaam zijn als eerste presentatie.

Leverenzymen in het bloed zijn op meestal verhoogd. Met name een verhoging van gamma glutamyltransferase (γ-GT) en / of alkalische fosfatase (ALP) is dan aanwezig. Echter zijn deze niet specifiek voor de diagnose van ITBL. Na verloop van tijd is ook vaak het bilirubine verhoogd.

Wanneer er een verdenking is op ITBL bestaat de eerste stap in de diagnostiek uit niet-invasieve transabdominale echografie (TAUS). Vanwege de lage gevoeligheid voor kleine letsels, is TAUS niet betrouwbaar voor vroege detectie. TAUS kan echter nuttig zijn als een eerste stap om de situatie (bijv. vasculaire doorgankelijkheid) in het algemeen te evalueren. Een niet afwijkende TAUS sluit het ontstaan van ITBL dus niet uit.

Om deze reden wordt vaak gekozen voor een directe visualisatie van de galwegen door endoscopische retrograde cholangio-pancreaticografie (ERCP), percutane transhepatische cholangio-drainage (PTCD), of drain-cholangiografie. Deze beeldvorming heeft de voorkeur boven TAUS voor het diagnosticeren van ITBL en blijft de gouden standaard. Een bijkomend voordeel van deze methoden van beeldvorming is dat deze ook geschikt zijn om eventuele galwegstricturen meteen te behandelen. Naast bovengenoemde methoden wordt magnetische resonantie cholangio-pancreaticographie (MRCP) steeds belangrijker als diagnostische beeldvorming van de galwegen door de hoge positief en negatief voorspellende waarden. Desondanks blijft cholangiografie de gouden standaard.

Behandeling van ITBL bestaat uit het verlichten van de symptomen door adequate drainage van de galwegen te faciliteren. Dit wordt meestal bereikt door het dilateren van de galwegen tijdens ERCP waarbij stents worden geplaatst om adequate drainage te handhaven. In ernstige gevallen kan percutane drainage worden gebruikt of zelfs gedeeltelijke hepatectomie wanneer de letsels zich gelokaliseerd bevinden. Ondanks deze vormen van therapie bestaat bij tot 50% van de ontvangers die ITBL ontwikkelen op termijn de noodzaak voor een nieuwe levertransplantatie.

Ondanks de huidige kennis betreffende de risicofactoren blijft ITBL een veel voorkomend probleem. ITBL is nog steeds niet te voorkomen en is verantwoordelijk voor aanzienlijke morbiditeit en mortaliteit. De exacte pathogenese is nog onbekend, hoewel ischemie-reperfusie een belangrijke rol lijkt te spelen. Het ontbreken van geschikte biomarkers welke het ontstaan van ITBL kunnen voorspellen is een groot gemis. Hierdoor is het niet goed mogelijk om risicovolle organen te excluderen voor transplantatie. Naast het vroeg voorspellen en identificeren van ITBL zijn biomarkers ook van belang voor het kwantificeren van effecten van mogelijke preventieve of therapeutische strategieën. Hierdoor blijft het lastig om in experimentele setting strategieën te ontwikkelen om ITBL te voorkomen of te genezen.

DOEL EN DE HOOFDLIJNEN VAN HET HUIDIGE PROEFSCHRIFT

Het doel van dit proefschrift is 1) de pathogenese van ITBL verder te ontrafelen en 2) om potentiële nieuwe biomarkers voor de vroege detectie van leverschade en ITBL te onderzoeken. Het proefschrift is verdeeld in 2 delen.

Deel 1 richt zich op de nieuwe inzichten in de rol van het vaatstelsel in de ontwikkeling van ITBL. In hoofdstuk 2 worden de resultaten van een uitgebreide histologische studie in leverbiopsieën, genomen ten tijde van levertransplantatie, getoond. Hieruit blijkt dat veranderingen in de intrahepatische takken van de poortader in de periode tussen de koude ischemie en reperfusie, namelijk constrictie van de takken, voorspellend is voor het later ontstaan van ITBL. Dit suggereert dat een verminderde doorbloeding door de poortader mogelijk kan leiden tot ITBL. In hoofdstuk 3 wordt de hypothese dat de poortader ook verantwoordelijk is voor bloedvoorziening in de PBP, en daardoor oxygenatie van de galwegen, verder onderzocht door middel van laser Doppler flowmetrie en spectrofotometrie. Resultaten laten zien dat inderdaad de poortader een aanzienlijke bijdrage levert aan de bloedstroom door de galwegen en derhalve dus van belang kan zijn bij het ontstaan van ITBL. Tot slot worden in hoofdstuk 4 enkele patiënten besproken waarbij partiële trombosering van de poortader leidt tot partiële ITBL in de leversegmenten aangedaan door de trombosering. Deze bevindingen samen suggereren dat de portale bloedvoorziening, in tegenstelling tot het algemeen geaccepteerde concept, van belang kan zijn in de pathogenese van ITBL.

Deel 2 van dit proefschrift richt zich op het gebruik van microRNAs als nieuwe biomarkers voor detectie van leverschade in de setting van levertransplantatie. In hoofdstuk 5 word een overzicht gegeven van de literatuur met betrekking tot het gebruik van (circulerende) microRNAs als biomarkers in levertransplantatie en leverziekten. Daarnaast wordt het mogelijke therapeutische potentieel van microRNAs in de levertransplantatie besproken. Hoofdstuk 6 toont aan dat circulerende microRNAs in serum als stabiele, gevoelige en vroege markers van leverschade na levertransplantatie en tijdens acute afstoting kunnen dienen. Hiermee wordt het potentieel van microRNAs als nieuwe biomarkers in de levertransplantatie aangetoond. In hoofdstuk 7 word de aanwezigheid van microRNAs in gal aangetoond. Bovendien wordt via verschillende in vivo en in vitro experimenten aangetoond dat de uitscheiding van deze microRNAs een actief proces betreft welke beinvloed wordt door leverschade en -functie. Hierdoor zijn microRNAs niet alleen geschikt voor gebruik als biomarkers maar ook voor het bestuderen van hun biologische potentie en mogelijk therapeutische inzetbaarheid. Hoofdstuk 8 beschrijft de aanwezigheid van microRNAs in perfusaten gebruikt voor het perfunderen en preserveren van het levertransplantaat en de diagnostische mogelijkheden van deze microRNAs voor het sensitief en specifiek identificeren van levertransplantaten welke later ITBL ontwikkelen.

Tenslotte worden in hoofdstuk 9 de gepresenteerde resultaten in dit proefschrift samengevat en bediscussieerd.

Appendix

11

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Een kritische evaluatie van wetenschappelijk onderzoek is essentieel. Daarom wil ik ook de heren professoren IJzermans, Porte, Beuers en Looijenga oprecht bedanken uit het diepste van mijn hart voor hun bereidheid om zitting te nemen in de promotiecommissie en deze belangrijke taak op zich te nemen.

Natuurlijk zijn er veel meer mensen betrokken geweest bij het onderzoek waardoor dit eindresultaat uiteindelijk mogelijk is geweest. Hierbij doel ik uiteraard op de mede-onderzoekers, analisten en collegae die ondersteunende werkzaamheden verrichten.

Het chirurgische lab onderin de faculteit, waar ik in het eerste jaar heb gezeten, Sandra, Mariëlle en Henk, ik heb de eerste stappen onder jullie begeleiding mogen zetten. Mijn eerste ondernemingen waren regelmatig lachwekkend van aard waardoor ik (met name met jou Sandra) vaak in de slappe lach heb gelegen. Maar uiteindelijk heb ik toch veel geleerd en is het allemaal goed gekomen. Henk, jou ben ik later ook nog op het MDL lab tegen gekomen waar we plezierig samen hebben kunnen werken. Mijn dank aan jullie allen is groot.

Het MDL lab in de L-vleugel, waar ik de overige jaren heb mogen doorbrengen bevat zo veel behulpzame collegae dat ik niet weet waar ik moet beginnen.
Petra, jij bent eigenlijk de figuurlijke moeder geweest voor mij op het lab. Ongeveer elke techniek die gebruikt is voor de experimenten in dit proefschrift heb ik van jou mogen leren. Je bent een uitstekende leermeester. Jij maakte altijd tijd om mij uit de brand te helpen. Mijn dank daarvoor, en hopelijk heb je snel meer tijd om je eigen boekje te gaan schrijven.

Our "Van der Laan research group" (also known as 'The Daltons'), Abdullah, Renée, Veda, and Eelke, I'll do this in English so everyone understands. I think we had the best research group, not only when it comes to science but also the atmosphere. All of you have taught me something in the lab. It was an honor to be part of you guys, and I really mean that. Abdullah and Veda, thanks for teaching me all the little techniques and twists which I could use in my research. Renée en Eelke, aan jullie beiden heb ik de door mij opgedane kennis voor een deel weer mogen overdragen. Jullie zijn momenteel beiden in het buitenland hard bezig om het microRNA onderzoek verder uit te breiden. Renée, je bent een topper. In deze laatste fase ben jij essentieel geweest terwijl ik in de kliniek zat. Waarvoor mijn oprechte dank. Eelke en Renée, beiden heel veel succes in de toekomst en het was mij een waar genoegen om met jullie samen te kunnen werken.

De diagnostiek, Jan, Buddy en Martine, ik was regelmatig op jullie kamer aanwezig ook al had ik er vrij weinig te zoeken. Maar de gezelligheid en jullie oprechte interesse en zorg waren voor mij redenen om elke dag met nog meer plezier naar mijn werk te komen. Bedankt voor de leuke tijd.

André, Ron, Andrea, Hanneke, Jaap, Pauline, Alexander, Anthonie, Gertine, Özlem, Patrick, Paula, Shanta, Viviana, Aria, Werner, Ad, Arjan, Evelyn, Elvira, Gwenny, Elmer, Wendy, Emmeloes, Rik, Kiran, Jasper en de velen die ik hier nu waarschijnlijk vergeet. Met veel plezier heb ik met jullie gewerkt. De gezellige tijden tijdens de uitjes en borrels waren geweldig en zullen me altijd bijblijven. Bedankt voor deze fantastische tijd.

Dan nog een aantal mensen van het MDL lab die ik toch ook nog even apart wil noemen. Raymond en Leonie, jullie waren altijd aanspreekbaar voor de gekste problemen en het lukte jullie keer op keer om deze ook op te lossen zodat ik gewoon bezig kon zijn met het onderzoek. Ook zonder jullie was het allemaal niet gelukt. Waarvoor mijn dank.

Als laatste (but certainly not least) wil van het MDL lab nog noemen, Greta, velen zullen jou vergeten te bedanken in hun proefschrift, maar op de achtergrond ben jij essentieel. Doordat jij zorgde dat er altijd schoon materiaal klaar stond en alle rotzooi (die stiekem toch achter gelaten werd) werd opgeruimd kon ik door mijn experimenten heen racen. Heel veel dank daarvoor.

Ook gaat mijn hartelijke dank uit aan de alle chirurgische en anesthesiologische teams welke voor elke levertransplantatie bereid waren om de zoveelste biopten, perfusaten, ischemietijden etc. etc. af te nemen, noteren of door te geven. Zonder jullie inspanning zou veel van het onderzoek niet mogelijk zijn, waaronder de mijne.

Belangrijk niet te vergeten voor mij is het secretariaat van de levertransplantatie, Mirjam, Lara, Elly, Fatma, Catelijne, Sylvia, en Miranda. Er was altijd wel tijd om even rustig op de bank te zitten en onder het genot van een bak koffie wat vragen te stellen of voor de zoveelste keer gegevens van een studiepatiënt op te vragen. Ook zonder jullie was het niet gelukt.

Lieve Ellen, ook jou kan ik natuurlijk niet vergeten. Hoewel je secretaresse was van Geert, was je altijd bereid om ook dingen voor mij te regelen als ik er niet uit kwam. Er stond altijd een lekkere bak koffie klaar en voor een lastige bespreking stak je mij nog een hart onder de riem. Het is toch anders zonder jou. Bedankt voor al die keren dat je klaar stond.

Carola, ook jij bent essentieel geweest in het tot stand komen van deze promotie. Alle formulieren en al het administratieve geregel heb jij voor het grootste gedeelte mij uit de hand kunnen nemen zodat ik mij kon concentreren op mijn boekje. Heel veel dank hiervoor.

Mijn lieve paranimfen, Angela en Suomi, met jullie heb ik eigenlijk vanaf het begin op het MDL lab opgetrokken. Niet alleen tijdens het werk maar ook in de privé sfeer hebben jullie mij altijd gesteund. Al onze gezamenlijke congressen en alle planningen, oefensessies, noem maar op. Een voor een leuke momenten waardoor wij een hechte groep zijn geworden. We gaan allemaal een beetje onze eigen kant op maar blijven elkaar uiteraard zien. Bedankt voor al jullie steun en hulp in het lab en ook nu bij al het geregel van deze dag. En Angela, zet hem op met de laatste loodjes.

Ik wil eindigen met het bedanken van mijn familie. Allereerst mijn ouders. Lieve mommy en daddy, jullie zijn er altijd geweest voor mij en hebben mij altijd alle tijd gegund om aan mijn promotie te kunnen werken ondanks dat jullie vrijwel geen idee hadden van wat het inhield. Altijd was alles geregeld en gedaan. Ik hoefde geen vinger uit te steken thuis en kon zo door aan mijn promotie. Bedankt voor jullie onvoorwaardelijke steun en liefde. Want zonder jullie steun en liefde was deze promotie nooit tot stand gekomen.

Mijn schoonouders, lieve pap en mam, ook jullie hebben mij onvoorwaardelijk gesteund in deze laatste fase wat ik heel erg waardeer. Pap, regelmatig zette jij je werk opzij en kon ik met jou hele dagen aan het boekje werken. Mam, jij zorgde dat Inaaya de hele tijd vermaakt werd en niet in de weg ‘liep’. Bedankt voor al deze momenten.

Als laatste, de twee dames in mijn leven, lieve Rhiana en Inaaya, het leven is zo anders, zo compleet met jullie. Ondanks alle stress en dead lines kwam ik thuis altijd tot rust en kon ik weer aan het werk. Rhiana, ondanks alle drukte van je eigen promotie ben jij regelmatig in de weer geweest met mijn stukken, om zo mij uit de brand te helpen. Bedankt lieverd, en nu kun je eindelijk al je energie in je eigen promotie steken.

Met het einde van dit dankwoord komt ook een einde van mijn promotie. Ik ben vast en zeker mensen vergeten te bedanken die verder ook belangrijk zijn geweest in het tot stand komen van deze promotie. Maar mijn waardering ook voor jullie hulp is niet minder.

Appendix

PHD PORTFOLIO

Name	Waqar R.R. Farid
Department	Surgery
PhD period	September 2008 – October 2014
Promotors	Prof. dr. H.W. Tilanus & Prof. dr. G. Kazemier
Copromotor	Dr. L.J.W. van der Laan

General courses

2009	Laboratory animal science (proefdiercursus art. 9)
2009	'Liver transplantation for dummies' course

Presentations at national conferences

- Dilated Portal Tract Veins are Associated with a Lower Incidence of Ischemic Type Biliary Lesions after Liver Transplantation. (Oral)
  W.R.R. Farid, A. Demirkiran, J. de Jonge et al. Najaarsvergadering 2007, Nederlandse Vereniging voor Gastroenterologie, Veldhoven
- Larger Portal Tract Veins in Reperfusion Biopsies are Associated with a Lower Incidence of Ischemic Type Biliary Lesions. (Oral)
  W.R.R. Farid, A. Demirkiran, J. de Jonge et al. Bootcongres 2008, Nederlandse Transplantatie Vereniging, Zeewolde
- Lower Incidence of Ischemic Type Biliary Lesions with Portal Vein Dilatation after Reperfusion. (Poster)
  W.R.R. Farid, A. Demirkiran, J. de Jonge et al. Chirurgendagen 2008, Nederlandse Vereniging voor Heelkunde, Veldhoven
- Biliary Complications after Liver Transplantation: Clinical Treatment and Pathophysiology. (Oral)
  W.R.R. Farid & G. Kazemier. Liver Transplantation for Beginners 2009, Erasmus MC, Rotterdam
- Liver-specific MicroRNA-122 is a Serum Biomarker for Hepatic Injury. (Poster)
  W.R.R. Farid, Q. Pan, J. Kwekkeboom et al. Voorjaarsvergadering 2010, Nederlandse Vereniging voor Gastroenterologie, Veldhoven
- Liver-Specific MicroRNAs are Serum Biomarkers for Hepatic Injury and Acute Rejection. (Oral)
  W.R.R. Farid, Q. Pan, P.E. de Ruiter et al. Bootcongres, 2010, Nederlandse Transplantatie Vereniging, Rotterdam

- The Portal Vein Plays a Significant Role in the Perfusion of the Common Bile Duct. (Oral)
  W.R.R. Farid, J.C. Slieker, J. de Jonge et al. Najaarsvergadering 2010, Nederlandse Vereniging voor Heelkunde, Veldhoven
- Liver-specific MicroRNAs in Human Blood are Markers for Liver Injury in Liver Disease and Transplantation. (Oral)
  W.R.R. Farid, Q. Pan, P.E. de Ruiter et al. 23ste SEOHS Symposium 2010, Erasmus MC, Rotterdam
- Liver-derived MicroRNAs in Human Blood as Early Markers for Hepatic Injury in Liver Disease and Transplantation. (Oral)
  W.R.R. Farid, Q. Pan, P.E. de Ruiter et al. Stafdag Heelkunde 2010, Erasmus MC, Rotterdam
- Significant Contribution of the Portal Vein to Blood Flow Through the Common Bile Duct. (Oral)
  W.R.R. Farid, J.C. Slieker, J. de Jonge et al. Stafdag Heelkunde 2010, Erasmus MC, Rotterdam
- Significant Contribution of the Portal Vein to Blood Flow Through the Common Bile Duct. (Oral)
  W.R.R. Farid, J.C. Slieker, J. de Jonge et al. Voorjaarsvergadering 2011, Nederlandse Vereniging voor Gastroenterologie, Veldhoven
- Hepatocyte-derived MicroRNAs in Human Serum are Sensitive Markers for Hepatic Injury in Liver Transplantation. (Oral)
  W.R.R. Farid, Q. Pan, A.J.P. van der Meer et al. Voorjaarsvergadering 2011, Nederlandse Vereniging voor Gastroenterologie, Veldhoven
- Hepatocyte-derived MicroRNAs in Human Serum are Sensitive Markers for Hepatic Injury in Liver Transplantation. (Oral)
  W.R.R. Farid, Q. Pan, A.J.P. van der Meer et al. Bootcongres 2011, Nederlandse Transplantatie Vereniging, Amsterdam
- Expression of Hepatocyte- and Cholangiocyte-abundant MicroRNAs is Predictive for Graft and Patient Survival after Liver Transplantation. (Poster)
  W.R.R. Farid, C.J. Verhoeven, J. de Jonge et al. Chirurgendagen 2011, Nederlandse Vereniging voor Heelkunde, Veldhoven
- Liver-derived MicroRNAs in Human Blood as Markers for Hepatic Injury in Liver Transplantation. (Invited speaker)
  W.R.R. Farid, Q. Pan, P.E. de Ruiter et al. Dutch Highlights at E-AHPBA Symposium 2011, Werkgroep Leverchirurgie/Pancreatitis Werkgroep Nederland/Dutch Pancreatic Cancer Group, Zeist

- Diagnostic and Therapeutic Utility of MicroRNAs for Ischemic Type Biliary Lesions. (Poster)
  W.R.R. Farid, J. De Jonge, P.E. de Ruiter et al. 24ste SEOHS Symposium 2011, AMC, Amsterdam
- MicroRNA-based Assesment of Allograft Quality during Liver Transplantation. (Invited speaker)
  W.R.R. Farid, C.J. Verhoeven, J. de Jonge et al. Bootcongres 2012, Nederlandse Transplantatie Vereniging, Maastricht

Presentations at international conferences

- Larger Portal Tract Veins are Associated with a Lower Incidence of Ischemic Type Biliary Lesions after Liver Transplantation. (Poster)
  W.R.R. Farid, J. de Jonge, A. Demirkiran et al. 14th Annual International Congress 2008, International Liver Transplantation Society, Paris, France
- Liver-derived MicroRNAs in Human Serum and Blood Mononuclear Cells as Early Markers for Hepatic Injury. (Poster)
  W.R.R. Farid, Q. Pan, P.E. de Ruiter et al. 16th Annual International Congress 2010, International Liver Transplantation Society, Hong Kong, China
- Liver-derived MicroRNAs in Human Blood as Early Markers for Hepatic Injury in Liver Transplantation. (Oral)
  W.R.R. Farid, Q. Pan, A.J.P. van der Meer et al. 61th Annual Meeting 2010, American Association for the Study of Liver Diseases, Boston, USA
- Liver-derived MicroRNAs in Human Blood as Markers for Hepatic Injury in Liver Transplantation. (Oral)
  W.R.R. Farid, Q. Pan, P.E. de Ruiter et al. 9th European congress 2011, International Hepato-Pancreato- Biliary Association, Cape Town, South Africa
- Significant Contribution of the Portal Vein to Blood Flow Through the Common Bile Duct. (Poster)
  W.R.R. Farid, J.C. Slieker, J. de Jonge et al. American Transplant Congress 2011, American Society of Transplant Surgeons, Philadelphia, USA
- Hepatocyte-derived MicroRNAs in Human Serum are Sensitive Markers for Hepatic Injury in Liver Transplantation. (Poster)
  W.R.R. Farid, Q. Pan, A.J.P. van der Meer et al. American Transplant Congress 2011, American Society of Transplant Surgeons, Philadelphia, USA
- Levels of Cholangiocyte-abundant MicroRNAs in Liver Grafts Prior to Transplantation are Predictive for Long- term Graft Survival. (Oral)
  W.R.R. Farid, C.J. Verhoeven, J. de Jonge et al. American Transplant Congress 2011, American Society of Transplant Surgeons, Philadelphia, USA

- Levels of Cholangiocyte-abundant MicroRNAs in Liver Grafts Prior to Transplantation are Predictive for Long- term Graft Survival. (Oral)
  W.R.R. Farid, C.J. Verhoeven, J. Kwekkeboom et al. 46th Annual Congress 2011, European Society of Surgical Research, Aachen, Germany
- Hepatocyte-derived MicroRNAs in Human Serum are Sensitive Markers for Hepatic Injury in Liver Transplantation. (Oral)
  W.R.R. Farid, Q. Pan, J. Kwekkeboom et al. 46th Annual Congress 2011, European Society of Surgical Research, Aachen, Germany
- Hepatocyte-Derived microRNAs in Human Serum Are Sensitive Markers for Hepatic Injury in Liver Transplantation. (Oral)
  W.R.R. Farid, Q. Pan, A.J.P. van der Meer et al. 17th Annual International Congress 2011, International Liver Transplantation Society, Valencia, Spain
- Hepatocyte and Cholangiocyte MicroRNA Expression in Liver Graft Biopsies Predicts Graft and Patient Survival after Liver Transplantation. (Poster)
  W.R.R. Farid, C.J. Verhoeven, J. de Jonge et al. 17th Annual International Congress 2011, International Liver Transplantation Society, Valencia, Spain
- Levels of Cholangiocyte-Abundant MicroRNA in Liver Grafts Prior to Transplantation are Predictive for Long- Term Graft Survival. (Oral)
  W.R.R. Farid, J. Kwekkeboom, C.J. Verhoeven et al. Annual International Congress 2011, European Society of Organ Transplantation, Glasgow, United Kingdom
- MicroRNA-based Assesment of Allograft Quality during Liver Transplantation. (Invited speaker)
  W.R.R. Farid, C.J. Verhoeven, J. de Jonge et al. 40th Annual Meeting 2012, Surgical Research Society of Southern Africa, Stellenbosch, South Africa
- Polarized Release of MicroRNAs from Hepatocytes to Bile and Blood: Relation with Liver Injury and Bilirubin Secretion. (Oral)
  W.R.R. Farid, C.J. Verhoeven, P.E. de Ruiter et al. 19th Annual International Congress 2013, International Liver Transplantation Society, Sydney, Australia

Teaching & supervising

2009	Co-organisor and tutor at 'Liver Transplantation for Dummies' course
2010	Co-organisor of 'Bootcongres' of the Dutch Transplantation Society
2010	Tutor of minor students in subject of 'Clinical Transplantation'
2011	Tutor of minor students in subject of 'Clinical Transplantation'
2011-2012	Co-supervisor of NIHES master-student in subject of 'Clincal Research'

Appendix

Academic awards & funding

- ILTS: Young Investigator Award ($1.000,- & $1.000,-)
- CSL Behring Pharmaceuticals: Research Funding (€75.000,-)
- ESOT: Mini Oral Presentation Award (€250,-)
- ESSR: British Journal of Surgery Award (€1.000,-)
- Novartis Pharmaceuticals: Transplantation Advisory Board Grant (€2.000,-)
- NTV: Astellas Pharmaceuticals Trans(p)la(n)t(at)ionele Research Prijs (€5.000,-)
- NVGE: Travel Grants (€900,- & 700,-)
- Erasmus MC: Travel Grants (€650,- ; €750,- & €550,-)
- ILTS: Poster of Distinction
- NTV: Genzyme Speaker's Award (€100,-)

Memberships

Nederlandse Vereniging voor Heelkunde (NVvH)
Nederlandse Vereniging voor Gastro-Intestinale Chirurgie (NVGIC)
Nederlandse Transplantatie Vereniging (NTV)
Nederlandse Vereniging voor Gastro-Enterologie (NVGE)

LIST OF PUBLICATIONS

1 J. Dubbeld, H. Hoekstra, W. R. Farid et al. *"Similar liver transplantation survival with selected cardiac death donors and brain death donors."* Br J Surg, 2010. 97(5): 744-753.

2 W. R. Farid, J. de Jonge, J. C. Slieker et al. *"The importance of portal venous blood flow in ischemic-type biliary lesions after liver transplantation."* Am J Transplant, 2011. 11(4): 857-862.

3 W. R. Farid, Q. Pan, A. J. van der Meer et al. *"Hepatocyte-derived microRNAs as serum biomarkers of hepatic injury and rejection after liver transplantation."* Liver Transpl, 2012. 18(3): 290-297.

4 S. M. Fouraschen, Q. Pan, P. E. de Ruiter, W. R. Farid et al. *"Secreted factors of human liver-derived mesenchymal stem cells promote liver regeneration early after partial hepatectomy."* Stem Cells Dev, 2012. 21(13): 2410-2419.

5 J. C. Slieker, W. R. Farid, C. H. van Eijck et al. *"Significant contribution of the portal vein to blood flow through the common bile duct."* Ann Surg, 2012. 255(3): 523-527.

6 W. R. Farid, J. de Jonge, P. E. Zondervan et al. *"Relationship between the histological appearance of the portal vein and development of ischemic-type biliary lesions after liver transplantation."* Liver Transpl, 2013. 19(10): 1088-1098.

7 A. J. van der Meer, W. R. Farid, M. J. Sonneveld et al. *"Sensitive detection of hepatocellular injury in chronic hepatitis C patients with circulating hepatocyte-derived microRNA-122."* J Viral Hepat, 2013. 20(3): 158-166.

8 C. J. Verhoeven, W. R. Farid, P. E. de Ruiter et al. *"MicroRNA profiles in graft preservation solution are predictive of ischemic-type biliary lesions after liver transplantation."* J Hepatol, 2014. 59(6): 1231-1238.

9 W. R. Farid, C. J. Verhoeven, J. de Jonge et al. *"The ins and outs of microRNAs as biomarkers in liver disease and transplantation."* Transpl Int, 2014. In press.

10 C. J. Verhoeven, W. R. Farid, J. de Jonge et al. *"Biomarkers to assess graft quality during conventional and machine preservation in liver transplantation."* J Hepatol, 2014. In press.

11 C.J. Verhoeven, W.R.R. Farid, V. Ramakrishnaiah et al. *"Bidirectional release of microRNAs into bile and blood after liver cell injury, during impaired graft function and rejection following liver transplantation."* Under preparation.

CURRICULUM VITAE AUCTORIS

Waqar Farid werd op 17 maart 1984 geboren te Rotterdam. Hij groeide hier op als oudste zoon van eerste generatie Pakistaans-Nederlandse ouders. Deels Engelstalig opgevoed wist hij al dat hij chirurg wilde worden nog voor hij de Nederlandse vertaling van het word " surgeon" kende. In 2003 rondde hij het, deels tweetalige, voorbereidend wetenschappelijk onderwijs aan het OSG Wolfert van Borselen te Rotterdam af. In datzelfde jaar is hij gestart met zijn studie geneeskunde aan de Erasmus Universiteit in Rotterdam. De eerste stappen van zijn wetenschappelijke carrière zette hij tijdens de studie met zijn keuzeonderzoek naar "Ischemic-Type Biliary Lesions after Liver Transplantation". Na dit onderzoek is hij begonnen met zijn coschappen, welke hij in 2008 heeft onderbroken om zich volledig op zijn promotie binnen de levertransplantie te kunnen richten op het laboratorium van de heelkunde. In deze periode was hij tevens coördinator van het studententeam levertransplantaties en redacteur voor de nieuwsbrief LTx Nieuws. In 2011 heeft hij zijn coschappen hervat in de regio Rotterdam om in 2012 af te studeren. In het Maasstad Ziekenhuis te Rotterdam deed hij zijn eerste ervaring op als assistent chirurgie en dit bevestigde zijn ambitie om chirurg te worden.

Momenteel is hij druk als trotse vader van zijn eerste dochter maar vastbesloten om zijn carrière voort te zetten binnen de chirurgie. Geschoold in het Rotterdamse is hij nu op zoek naar een andere regio om meer ervaring op te doen en zijn blikveld op de kliniek en de wetenschap te verbreden.