BIOMARKERS TO ASSESS GRAFT QUALITY IN LIVER TRANSPLANTATION

C.J. VERHOEVEN

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ABSTRACT

Liver transplantation (LT) has become the only curative treatment for end-stage liver disease since half a century. Patient survival has improved drastically over the years, but poor initial graft quality and complications following transplantation still limit patient and graft survival. Monitoring and evaluation of graft quality during follow-up is achieved by routine biomarker measurements in recipients' blood, starting directly following surgery and in the months and years thereafter. This allows clinicians to early detect complications following LT, like early allograft dysfunction and biliary complications. They are also used as a tool for deciding on further diagnostics or interventions. Classic biomarkers are able to assess liver injury (aspartate- and alanine aminotransferase, lactate dehydrogenase), biliary injury and obstruction (gamma-glutamyltransferase, alkaline phosphatase), and liver function (albumin, bilirubin, prothrombin time). Novel genetic markers such as microRNAs also show potential as more accurate or specific biomarker for various types of injury and functions. Some of these serum biomarkers were shown to be promising in predicting disease or severity of injury when measured in bile, though widespread implementation in clinical practice has not yet happened. Therefore, liver biopsy remains the gold standard for diagnosing acute cellular rejection, even with less invasive serum biomarkers that are currently available. Future applications of biomarkers should enable early assessment of marginal graft function when applied to preservation solution in both simple cold storage as well as during *ex situ* machine perfusion. These developments could help to increase the donor pool for LT by optimizing and allocating grafts based on favourable biomarker profiles from donors with unfavourable clinical characteristics.

DEFINITIONS OF WORDS AND TERMS

Primary sclerosing cholangitis (PSC): auto-immune disease with progressive fibrosis of the intra- and extrahepatic bile ducts.

Cholestasis: accumulation of bile due to obstruction of flow to the duodenum or altered bile composition.

Non-anastomotic strictures (NAS): benign tapering of the intrahepatic and/or (perihilar)-extrahepatic bile ducts following LT.

Anastomotic stricture (AS): isolated benign tapering of the biliary anastomosis following LT.

Early allograft dysfunction (EAD): poor graft function in the first week post LT, based on AST or ALT >2000 IU/L, or total bilirubin serum levels >10 µg/L on day 7 post LT, or INR >1.6 on day 7 post LT.

Cholangiocarcinoma (CCA): malignancy of the hepatic bile ducts and cholangiocytes.

Hepatocellular carcinoma (HCC): malignancy of liver parenchyma and hepatocytes.

Donation after circulatory death (DCD): procurement of donor organs after circulatory arrest of the donor. Associated with warm-ischemic injury of organs.

Donation after brain death (DBD): procurement of donor organs after disappearance of brain stem functions (brain death), while the circulation is still intact. Organs are usually of better quality compared to DCD.

MicroRNAs: small, non-coding RNAs involved in post-transcriptional gene regulation. Potential novel biomarkers.

Preservation: storage of organs at cold temperature and suitable fluids to prevent detoriation of the grafts, for optimal quality and functioning following transplantation.

INTRODUCTION

The liver is the largest visceral and most multifunctional organ of the human body. It produces and drains bile, which is responsible for digestion. Furthermore, the liver metabolizes glucose, proteins like albumin and coagulation factors, amino acids and lipids. Detoxification is achieved by the breakdown of hormones like insulin and drugs. Cells in the livers' reticuloendothelial system are responsible for immunological effects and protection against certain antigens[1]. This enumeration describes only part of all liver functions, but also illustrates the livers' diverse and essential role for the body. Under stable conditions, the liver has 60-70% overcapacity. This allows for resection in healthy individuals of up to 70% of liver volume[2]. After such surgery, the liver will regenerate to its normal volume within weeks. However, an absent liver function due to acute liver failure or chronic end-stage liver disease is not compatible with life and can only be cured by liver transplantation (LT).

It took four years for Thomas Starzl to perform the first successful LT in human in 1967, after several unsuccessful attempts since 1963, with most patients dying on the operation table[3, 4]. Still, the first LT series in human reported a one year survival rate of only 25%, illustrating the complex surgical technique and severe complications that could occur early following LT in those days. One of the major complications limiting patient and graft survival was acute rejection of the transplanted organ against the recipient. A decade later, survival rates of LT recipients improved drastically after Sir Roy Calne introduced cyclosporine, an immunosuppressant drug, into the clinic[5].

Nearly 50 years later, LT is regarded standard treatment for end-stage liver disease and performed worldwide in various populations suffering from different pathologies. Because of optimized surgical techniques and immunosuppressant regimens, graft survival can now reach beyond 20 years with excellent graft function in some recipients[6]. This has also led to an expansion of the designated indications for LT; on-going trials investigate the benefit of LT in selected patients with cholangiocarcinoma[7], hepatocellular carcinoma[8], and colorectal liver metastases[9]. However, while the list of patients awaiting LT is getting longer, the number of transplantable organs remains scarce. Moreover, the quality of transplantable organs is deteriorating due to increasing donor age, liver steatosis, viral hepatitis of the donor, and prolonged ischemia times following donation after circulatory death (DCD)[10]. All these factors can cause a wide range of complications threatening graft and patient survival following LT. Early complications mainly consist of infections, graft primary non-function (PNF), early allograft dysfunction (EAD), biliary complications (i.e. leakage and anastomotic and non-anastomotic biliary strictures), and acute rejection. Beside biliary complications, other complications at the intermediate and long-term usually consist of recurrence of liver disease that initially required LT (like hepatitis C viral infection and primary sclerosing cholangitis), the development of malignancies, chronic rejection and liver fibrosis[11].

In order to discover these complications in LT recipients timely, monitoring of graft function with suitable biomarkers is required. Routine monitoring of minimally- or non-invasive biomarkers enables early recognition of complications to which physicians can adapt their medical policy. Two examples are to obtain histology in case of suspicion of allograft rejection or to perform imaging/endoscopic treatment in case of suspicion of biliary complications. Therefore, LT recipients are subjected to protocol (blood) measurements depending on their clinical status during follow-up; varying from daily monitoring at the intensive care unit directly after surgery, to yearly routine measurements at the outpatient clinic. Different patients and underlying diseases require personalized or precision monitoring with established biomarkers in liver disease.

The following paragraphs provide an outline on the definition of biomarkers in the field of LT and the different types of biomarkers that are used in clinical practice for short and long-term monitoring of graft function. Finally, potentially interesting novel

biomarkers are discussed and recommendations are given regarding future applications of biomarkers in the context of LT.

DEFINITION OF BIOMARKERS IN LIVER TRANSPLANTATION

The term 'biomarker', an amalgamation of the words 'biological marker', was defined in 1998 by a working group of the National Institutes of Health, describing it as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention"[12]. Since that time, however, multiple other definitions have been introduced that further expanded the interpretation of the term biomarker. This was for instance done by a collaboration of the World Health Organization, the United Nations and the International Labor Organization, who defined a biomarker as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease."[13] Based on the descriptions above, one can conclude that biomarkers can be used to measure the effect of treatment as well as predict- or be related to a clinical endpoint. Biomarkers are also increasingly being used as a primary or secondary outcome measure in experimental or clinical studies, and therefore sometimes applied as a surrogate endpoint[14]. Especially in LT, definitions like EAD or PNF are mainly defined by persistently elevated transaminase levels in serum, often combined with perturbed coagulation function of the liver.

Furthermore, the previously described definitions on biomarkers allow to distinguish 'dynamic' markers from 'static' markers. In the context of LT, dynamic markers are usually molecular markers and liver enzymes that can be measured in serum and which levels fluctuate depending on the functional status or degree of injury of the liver graft. As an example, immediately after LT, ischemia-reperfusion injury of the graft causes elevation of serum aspartate- and alanine aminotransferase levels (AST, ALT) above 200 IU/L, while a more gradual rise in gamma-glutamyl transferase (GGT) or alkaline phosphatase (ALP) starts approximately 24-48h after LT (**Fig. 1**). When a patient has been transplanted because of viral hepatitis as underlying pathology, routine measurements of viral load during follow-up are part of regular clinical practice. This is because of the reasonable chance of recurrence of disease in the new liver graft[15]. Depending on the type of complication, treating the cause will ultimately result in normalization of serum levels of dynamic markers. Therefore, dynamic markers are variable markers that can be suitable for determining whether treatment or interventions are successful.

Static markers on the other hand are less subjected to change by the (patho-)physiological status of the liver graft. One could think of genetic polymorphisms like single nucleotide polymorphisms (SNP's) of either donors or recipients that are related with

certain outcomes following LT. Genetic markers or SNP's are more often fixed factors that do not fluctuate or change by graft injury. However, certain polymorphisms do make LT recipients more susceptible for certain complications; several SNP's involved in the innate immunity system have been correlated to a higher incidence of severe infections post LT[16]. Also in recipients that were transplanted for cholestatic diseases like primary sclerosing cholangitis (PSC), certain SNP's were identified that cause earlier recurrence of severe biliary injury after LT[17]. Because of the predicting capacity for outcome rather than their monitoring capacities, in literature, SNP's are more often referred to as 'risk factors' instead of biomarkers.

A separate category of markers are histological markers or markers measured in liver biopsies. Up to a decade ago, many transplant centres monitored graft injury and rejection by evaluating histological changes in by-protocol liver biopsies during follow-up. Most dynamic markers for liver injury in serum are related to histological changes of the liver parenchyma and bile ducts[18]. However, it usually takes more time to detect histological and morphological changes in liver tissue and puncture of the liver is not harmless. Therefore, taking liver biopsies is nowadays mainly indicated to confirm suspected graft rejection and recurrence of disease or malignancy based on changes in serum biomarkers and imaging.

The next chapters will focus mainly on dynamic markers in blood and serum and the most important histological markers associate with liver injury and function following LT.

DIFFERENT BIOMARKERS FOR DIFFERENT CELL TYPES

In liver disease, biomarkers are divided in predominantly hepatocellular- or cholestatic markers. Liver enzymes as AST and ALT are indicative of hepatocellular injury, while GGT and ALP reflect biliary injury or obstruction. Beside these two categories, markers of liver function are also of importance for the evaluation of graft quality, especially in the first days following LT. Very often, the liver enzymes AST and ALT are used to indirectly asses liver function. Strictly spoken they do not represent liver function but are more indicative of liver cell death. Thus, for this purpose it is more useful to analyse products that are normally metabolized or synthesized by the liver, like proteins such as albumin and certain coagulation markers. **Table 1** provides an overview of classic biomarkers per cell type, injury or function, which are discussed more extensively in the following paragraphs.

Table 1. Conventional biomarkers used in liver transplantation for graft monitoring.

Category	Biomarkers
Hepatocellular injury	AST, ALT, LDH
Cholangiocyte injury & cholestasis	GGT, ALP, Bilirubin
Liver function	Albumin, Bilirubin, PT, INR
Recurrence or new onset HCC	AFP
Recurrence or new onset CCA	CA 19-9

AST = aspartate aminotransferase, ALT= alanine aminotransferase, LDH = lactate dehydrogenase, GGT = gamma-glutamyl transferase, ALP = alkaline phosphatase, PT = prothrombin time, INR = international normalized ratio, HCC = hepatocellular carcinoma, AFP = alpha-fetoprotein, CCA = cholangiocarcinoma, CA 19-9 = cancer antigen 19-9.

Biomarkers for hepatocellular injury

Aspartate aminotransferase (AST)

AST is an enzyme involved in the production of proteins and catabolization of amino acids, allowing them to cross membranes and enter the citric acid cycle. In humans, AST is present in a descending concentration in the following tissues: heart, liver, skeletal muscle, kidney, pancreas, spleen, lungs, brain and erythrocytes. Current clinically applied techniques however do not trace tissue origin from which AST was released. Therefore, it is often necessary to involve other markers as well for the interpretation of serum AST in the clinical setting. AST can be measured in serum and plasma obtained through venipuncture, remaining stable for at least 24 hours at room temperature. Its halftime is approximately 12 hours. Two iso-enzymes of AST can be distinguished that occur in separate cellular compartments, namely in the cytoplasm (c-AST) and in the mitochondria (m-AST). Following mild tissue injury, particularly c-AST can be elevated in serum, while severe injury will also lead to a release of m-AST[19]. In adult healthy individuals, the range of AST varies between 31-35 U/l, but usually depends on sex and age[20].

Following LT, peak AST in serum is usually reached within the first 24-48 hours after surgery, sometimes being a 100-fold increased or higher. In particular when a liver graft is of poor quality, for instance due to increased warm ischemia time, high donor age, or liver steatosis, peak AST can reach extreme values during the first week post-LT (>1000 U/l). Although transaminase levels usually decrease quickly following LT, one should be careful with interpreting this as graft recovery. Massive hepatocellular necrosis can result in hepatic failure, which should be evaluated based on the capacity of the graft's coagulation function and bile production. Therefore, both markers for hepatocellular injury (AST, ALT) as well as cholestatic markers (ALP, GGT) and functional markers (PT, INR, albumin, bilirubin) should always be evaluated together directly following LT.

Alanine aminotransferase (ALT)

Alanine aminotransferase (ALT) catalyses the transfer of the amino group L-alanine to α-ketoglutarate, resulting in the production of pyruvate and L-glutamate. High concentrations occur in the hepatocyte cytoplasm, whereas only low concentrations are found in heart and kidney tissue[21]. Therefore, ALT is considered to be more liver specific compared to AST. However, because of their differences in intra-lobular distribution, elevation of AST levels is usually faster than ALT. Nevertheless, serum or plasma ALT has proven to be of value in the diagnostic process of various liver disease. For instance in acute viral hepatitis, serum ALT can quickly rise up to twenty-fold its normal range, while levels of AST remain lower or show only mild increase. At the same time the ALT/AST ratio, which is <1 in healthy individuals, becomes >1[22]. Chronic (viral) hepatitis results in milder elevations of AST and ALT. When levels of AST become higher then ALT, one should be aware of cellular necrosis.

Despite being markers of hepatocellular injury, biliary obstruction can also result in liver injury and therefore increased levels of AST and ALT. Furthermore, peak serum ALT levels in the first week following LT have been associated with the development of severe biliary complications[23]. A possible explanation for this finding could lay within the distribution of ALT in the liver acinus; the bile ducts and hepatic artery are located peri-portally (zone 1). Ischemic injury in this zone will cause release of ALT into the serum. Zone 3 on the other hand is located peri-centrally, is less oxygenated, and contains higher concentrations of AST[18]. It remains unclear whether serum levels of AST are also related to the development of biliary complications.

Just like AST, the reference value of ALT depends on sex and age, but normally does not rise above 50 U/l. Be aware that halftime of ALT in plasma or serum is however longer, approximately fifty hours.

Lactate dehydrogenase (LDH)

This enzyme catalyses the conversion of lactate into pyruvate and vice versa. Pyruvate, the product of glycolysis, is converted to lactate under anaerobic conditions. The inverse reaction takes place in the liver and results in gluconeogenesis. LDH is present in the cell cytoplasm of practically all organs in the human body, making it widely applicable but thereby also less attractive for diagnostic purposes. Also distinguishing between the five different isotypes of LDH, which differ in characteristics as halftime, does not seem to give additional diagnostic benefit. Furthermore, haemolysis can give an overestimation of LDH activity in serum. The normal range of LDH in healthy adults is below 225 U/l. [24].

Despite these apparent shortcomings, LDH is still used as a clinical biomarker in the follow-up of liver transplant recipients. Strong elevations of LDH in serum or plasma directly after liver transplantation are usually indicative for the severity of ischemia-reper-

fusion injury of the graft. When strong elevations of LDH prolong and are accompanied with high levels of other transaminases one should be aware of serious complications, like hepatic artery thrombosis[25]. But experimental studies also suggest the measurement of LDH in bile to assess the amount of biliary or cholangiocyte injury[26]. However, this application of LDH is currently not used in standard clinical practice.

Biomarkers for biliary obstruction or cholestasis

Gamma-glutamyl transferase (GGT)

The enzyme GGT is a carboxypeptidase located in cellular membranes. It transfers gamma-glutamyl gluthathione to acceptor aminoacids, peptides or water. Furthermore, it transfers amino acids across the cellular membrane. The hepatopancreatobiliary system is the largest contributor of GGT levels in serum, but high concentrations of GGT are also present in kidney tubular epithelium and prostate tissue. Lower tissues are found in spleen, brain and heart. The liver excretes GGT via the bile. Therefore, biliary obstructions can cause strong elevations of GGT in serum[27]. Together with alkaline phosphatase (ALP), GGT is useful to screen whether recipients have developed significant biliary complications following LT, in particular anastomotic and non-anastomotic strictures (AS and NAS respectively). In contrast to ALP, GGT is not elevated in bone disease[28]. Increased serum levels of cholestatic markers are an indication to perform further imaging to determine the cause of obstruction, generally via endoscopic retrograde cholangiopancreaticography (ERCP) or via magnetic resonance cholangiopancreaticography (MRCP).

In healthy adults, GGT serum levels are below 35-40 U/l. Directly following LT, levels of GGT are often not elevated, but start to rise within the first post-operative days. If a recipient develops AS, levels of GGT and ALP are expected to be high, up to 400 to 500 U/L. Stenting of the biliary anastomosis will give a rapid normalization of serum levels, as illustrated in the right panels of **Fig. 1**. When a liver graft is affected by NAS, levels of GGT and ALP can strongly fluctuate, but will increase over time, since these strictures are more stubborn to treat by stents or percutaneous drains. When a mild rise in cholestatic markers is accompanied by a rise in hepatocellular markers, one should also think of (recurrence of chronic) hepatitis[29].

A paradoxical finding confirmed by multiple researchers is that higher levels of GGT early following LT are associated with improved 90-day survival in recipients, while recipients who died before the 90th post-operative day had lower GGT serum levels[30]. After the first 90 days however, high levels of GGT are associated with impaired 5-year survival. It has been suggested that high levels of GGT early following surgery are the result of a proper systemic response to reactive oxygen species that are released after graft reperfusion. A different hypothesis states that the increase of GGT is correlated to regeneration of hepatocytes following LT[31]. Direct evidence for this hypothesis is however not available.

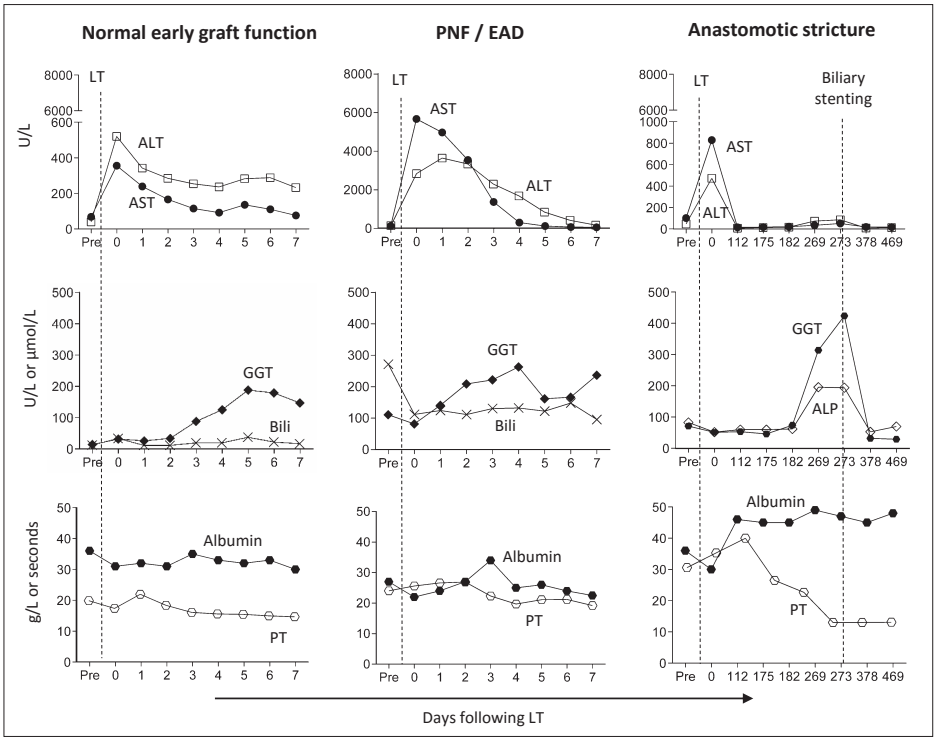

Figure 1. Biomarker dynamics in blood and serum following LT. The left panels show biomarker dynamics in recipients with normal early graft function, the median panels show elevated biomarker levels in serum during PNF/EAD, the right panels show increased cholestatic markers in AS. Data represent biomarker serum levels of individual patients following LT and were derived from the database of the Erasmus Medical Center Rotterdam, The Netherlands.

Alkaline phosphatase (ALP)

The enzyme ALP is responsible for dephosphorylation of multiple types of molecules. It is bound to plasma membrane lipoproteins of tissues throughout the entire body. Serum ALP is mostly derived from liver parenchyma, biliary epithelium (cholangiocytes), and bone osteoblasts. To a lesser extent, serum ALP can also originate from intestinal mucosa, placenta, and kidney tissue[32]. The isoenzymes of intestinal and placental ALP are different from ALP in other tissues. It is possible to distinguish between the different isoenzymes for instance by elektropheresis. In clinical practice, however, ALP is generally tested together with GGT to differentiate. Strong elevation of both ALP and GGT indicates biliary obstruction, whereas extrahepatic obstruction causes a stronger rise in ALP compared to intrahepatic obstruction. Other hepatic causes for elevation consist of alcoholic abuse, hepatitis and cholestatic disease. A sole elevation of ALP without rise in GGT levels indicates extrahepatic pathology, like bone disease or hyperthyroidism.

In adults, serum values of ALP are <125 U/l. The halftime of most ALP isoenzymes is 3-7 days, while the halftime of intestinal ALP is <8 hours.

Biomarkers to assess graft function

Albumin

Albumin is one of the most abundant proteins in human serum and plasma beside blood coagulation factors. It is involved in pH homeostasis, maintaining oncotic pressure, and the transportation of blood compounds, hormones and drugs. Synthesis takes place in the liver and therefore, serum albumin is considered to be an important marker for liver function. Over twenty structural variants of albumin exist, and its half time is approximately 20 days. In healthy adults, serum/plasma levels are usually between 35-55 g/l, but levels can be influenced by body fluid distribution, for instance by dehydration[33].

In particular hypoalbuminemia has been associated with liver disease and, following LT, with impaired graft function. A higher degree of graft injury, mirrored by high postoperative transaminase levels, often negatively affects liver graft function. However, the increased use of marginal grafts for LT has gained more interest for pure functional markers; because despite extensive injury, some marginal grafts manage to function well in recipients. Therefore, experimental studies with graft machine preservation focus on the assessment of liver function already prior to graft implantation in recipients[34]. But also following LT, early allograft dysfunction is estimated by a lack of markers that normally result from good liver function, like conjugated bilirubin and INR (coagulation). However, serum albumin is not included in this definition.[35] Though albumin could be of use for assessing graft function, one should also be aware for other causes of hypoalbuminemia, like inflammation, malnutrition/malabsorption, malignancies and hypothyroidism. Furthermore, albumin levels can remain in the normal range when patients suffer from biliary obstruction.

Bilirubin (indirect and direct)

Bilirubin is the yellow-coloured breakdown product of haemoglobin when erythrocytes are degraded. A vast majority of bilirubin is derived from aged erythrocytes (over 85%), but ineffective erythropoiesis by bone marrow and certain hepatic enzymes can also contribute to bilirubin formation. When heme is degradated by splenic macrophages, unconjugated bilirubin is formed, which is not soluble in water and cannot be excreted. Subsequently, unconjugated bilirubin is bound to albumin and is transported to the liver, where hepatocytes conjugate bilirubin with glucuronic acid (90% diglucuronic, 10% monoglucuronic). This step makes bilirubin soluble in water and suitable for excretion via the hepatobiliary system. Once transported to the intestine and colon, conjugated bilirubin is hydrolysed and reduced to urobilinogen by bacteria, and excreted via

the faeces. A small part of the urobilinogen (2-5%) is resorbed into the enterohepatic circulation and excreted via the urine[36].

Human plasma or serum contains four fractions of bilirubin; unconjugated bilirubin (30%), unconjugated bilirubin bound to albumin (35%), monoconjugated bilirubin (25%), and di-conjugated bilirubin (10%). 'Indirect' bilirubin consists of unconjugated bilirubin and the fraction of bilirubin not-covalently bound to albumin. 'Direct' bilirubin usually refers to fractions of conjugated bilirubin and bilirubin that is covalently bound to albumin. In clinical practice, total bilirubin and direct bilirubin are measurable in human serum or plasma. Total bilirubin consists of conjugated as well as unconjugated forms of bilirubin. Based on these measurements, the indirect bilirubin can be calculated with the formula: indirect bilirubin = total bilirubin – direct bilirubin. In healthy adults, total bilirubin levels are <20 µmol/l, and direct bilirubin levels are <5 µmol/l. Jaundice usually occurs when serum bilirubin exceeds 50 µmol/l[37].

Based on total and direct bilirubin, one can distinguish different causes for hyperbilirubinemia. Strong elevation of unconjugated bilirubin indicates pre-hepatic pathophysiology like hemolysis, or dysfunction of hepatocytes and conjugation at the hepatic level. However, most complications that can occur following LT will cause conjugated hyperbilirubinemia. At the hepatic level, hepatocyte injury due ischemia-reperfusion injury, EAD or PNF, is accompanied by a rise in bilirubin and liver transaminases. These changes can occur early after LT. In case of intrahepatic cholestasis, for instance due to biliary strictures, but also extrahepatic bile duct obstruction (post-hepatic level), hyperbilirubinemia is accompanied by a rise in ALP and GGT. Recurrence of (viral) hepatitis can elevate both conjugated and unconjugated serum bilirubin. Thus, by measuring conjugated and unconjugated hyperbilirubinemia and comparing serum levels with hepatocellular and cholestatic markers, one can distinguish between different complications following liver transplantation. When hepatocellular function is impaired, bilirubin levels also become measurable in urine and are per definition pathologic[38]. When possible, collection of bile following liver transplantation can also be used for determining biliary bilirubin levels, that can mirror hepatocyte function but also cholangiocyte injury[39].

Prothrombin time (PT) and international normalized ration (INR)

Synthesis of tissue factors for sufficient blood coagulation is an important function of the liver. A lack of tissue factors in blood plasma could indicate severe liver disease or, in case of transplantation, graft failure. To assess the degree of graft failure or graft (dys)function following LT one could measure individual coagulation factors, but instead, PT and INR are commonly used as general indicators;

Prothrombin time measures the time it takes for blood plasma to form a fibrin clot after adding tissue factor. In healthy individuals, PT is usually between 12-15 seconds, but it depends on the standards of the laboratory performing the analysis. A prolonged

PT could indicate a deficiency in the production of coagulation factors I (fibrinogen), II (prothrombin), V, VII and X, which are all part of the extrinsic coagulation cascade. Logically, the use of anticoagulant drugs should be taken into account when interpreting PT. Immediately after LT, PT is usually prolonged and can reach up to 100 seconds. When PT does not decrease or normalize in the first post-operative week, this could indicate severe graft dysfunction with risk of developing serious complications and impaired patient survival. Urgent re-transplantation can be lifesaving in these cases. As mentioned before, the analysis and subsequent interpretation of PT is very institutionally dependent[40].

Therefore, a standardized PT-ratio, also known as the international normalized ratio (INR), is used more often to determine early allograft dysfunction. Outside the context of LT, INR is often used as a tool to monitor patients on vitamin-K antagonists. The INR standardizes PT values of patients by calibrating reagents to an international sensitivity index (ISI) and by comparing patients' PT value with the mean PT of healthy individuals (normal), with the formula $INR=(PT_{patient}/PT_{normal})^{ISI}$[41]. At one week following LT, INR is used as one of the parameters to evaluate early allograft dysfunction; an INR ≥ 1.6 is considered to be a risk factor for shortened graft and recipient survival[35]. Importantly, the cut-off of 1.6 seems to be a predictor of graft failure for grafts that were obtained from brain death donors as well as those obtained from circulatory death donors. Therefore, it has been suggested to give more weight to INR as a predictor of graft failure following LT[42].

BIOMARKERS FOR RECURRENCE OF DISEASE FOLLOWING LIVER TRANSPLANTATION

Beside the threat of cellular damage due to severe ischemia-reperfusion injury, biliary injury, and rejection, the recurrence of disease for which recipients were transplanted is also an important factor for graft loss. In particular recurrence of PSC, HCC, and viral hepatitis-B and -C are notoriously recurring diseases in the transplanted graft[43]. Furthermore, over the last years patients with unresectable cholangiocarcinoma are transplanted, but survival rates are limited due to recurrent or metastatic disease[44]. Several biomarkers are clinically available to monitor recurrence of the above-mentioned diseases in LT recipients, which are described shortly in the following paragraphs.

Cholestatic markers in recurrence of PSC

Primary sclerosing cholangitis is an autoimmune related disorder that causes chronic inflammation and strictures of the (mainly intrahepatic) bile ducts. This progressive disease occurs more frequently in men compared to women and has been associated with ulcerative colitis[45]. Incidence is highest in the US and north European countries. The

time of onset until end-stage liver disease is approximately twelve years and currently, LT is the only curative treatment for PSC. Unfortunately, recurrence of disease occurs in up to 20% of the PSC recipients, sometimes requiring re-transplantation[46].

Clinical symptoms of recurrence of PSC consist of obstructive jaundice, bacterial cholangitis, fever, and fluctuating elevations of liver enzymes and cholestatic serum markers. Cholangiography shows typical intra- or extrahepatic strictures, beading and irregularities. Histological features consist of fibrous cholangitis or fibro-obliterative lesions. Because of the overlap in clinical presentation with NAS, one of the criteria of recurrent PSC prescribes this diagnosis should be excluded if it develops within the first 90 days following LT[47]. Beside recurrence, PSC patients also have an increased risk to develop CCA . Therefore, it could be plead to monitor these recipients for cancer antigen 19-9 (CA 19-9), a potential marker of CCA. **Table 2** illustrates expected serum levels of classic biomarkers in PSC.

Table 2. Serum biomarkers during different pathophysiological states of the liver graft following LT. Values represent which serum levels can be expected for the various outcomes or diagnoses following LT. Except for PNF and EAD, these values are an indication and can diverge between different LT recipients.

Biomarker	Healthy liver	PNF / EAD*	AS	NAS	ACR	Rec PSC	(Rec) HCC, CCA
AST (U/L)	<50	>2000 within 7 days post LT	=	50-400	100-1000	50-400	=↑
ALT (U/L)	<50	>2000 within 7 days post LT	=	50-200	100-1000	50-200	=↑
Total bili (µmol/L)	<20	≥170 on day 7 post LT	= ↑	↑, up to 300	↑, up to 300	↑, up to 300	=↑
Albumin (g/L)	35-55	↓	=	=↓	↓	=	=↓
GGT (U/L)	<40	= ↑	↑ up to 500	↑, up to 200	↑, up to 300	↑, up to 200	=↑
ALP (U/L)	<125	= ↑	↑ up to 400	↑, up to 200	↑, up to 300	↑, up to 200	=↑
INR or PT (sec)	12-15	≥1.6 on day 7 post LT	=	Prolonged	Prolonged	Prolonged	=↑
AFP (mcg/L)	<10-15	<10-15	<10-15	<10-15	<10-15	= ↑	↑
CA 19-9 (U/mL)	Neg	Neg	↑ prior to stenting	↑	Neg	If >100, higher risk of CCA	↑

*EAD (and PNF) are defined by serum biomarkers in the first week post LT and consist of one of the following: serum AST or ALT > 2000 U/L in the first postoperative week, *or* total bilirubin levels ≥10 mg/dL (=170 µmol/L) on day 7 post LT, *or* INR ≥1.6 on day 7 post LT.

Legend: PNF, primary non-function; EAD, early allograft dysfunction; AS, anastomotic stricture; NAS, non-anastomotic stricture; ACR, acute cellular rejection; Rec, recurrence; PSC, primary sclerosing cholangitis; HCC, hepatocellular carcinoma; CCA, cholangiocarcinoma; = normal levels, ↑ increased levels, ↓decreased levels.

Cancer antigen 19-9 (CA 19-9) in recurrence of CCA

Cholangiocarcinoma is a rare disease that accounts for less than three percent of all gastrointestinal malignancies, but which has a poor prognosis due to its aggressive nature. Transplant centres recently started exploring the success of LT for perihilar CCA, either with or without use of neoadjuvant chemo(radio)therapy[7]. In particular patients suffering from PSC have a 398-fold increased risk to develop CCA compared to the general population[48].

A potential serum marker to screen for (recurrent) CCA in PSC patients is CA 19-9. This carbohydrate structure is found in pancreatic tissue as well as on epithelial cells of the stomach and gallbladder. It can be secreted into serum by cancer cells. Beside cholangiocarcinoma, increased serum levels of CA 19-9 have been associated with pancreatic- and colon cancer, but also with benign causes of biliary obstruction. Therefore, when assessing the risk of malignancy based on CA 19-9 serum levels, one should take into account whether cholestasis or cholangitis is present (preferring a cut-off value of ≥300 U/mL) or absent (better discrimination with a cut-off of ≥37 U/mL)[49]. It is recommended to evaluate CA 19-9 levels after recovery of cholangitis. However, the optimal cut-off value for CA 19-9 remains inconclusive. A lower cut-off at 37 U/mL can be undesirable in terms of specificity, but higher cut-off values are at the expense of sensitivity[50]. Current guidelines recommend a cut-off between 100-127 U/mL. Another important limitation of CA 19-9 is that its biosynthesis depends on the activity of fucosyltransferase-2 and fucosyltransferase-3 (FUT2 and FUT3, respectively). Individuals with inactive FUT3 do not express CA 19-9 on their epithelial cells. In contrast, FUT2 inactivity increases CA 19-9 expression. These genetic variations in FUT2 and FUT3 are not uncommon and strongly influence the optimal cut-off level for CA 19-9 in individuals[51].

Finally, one could plea for use of CA 19-9 during follow-up after LT, since post-transplant CA 19-9 levels are predictive of recurrence of CCA (HR 1.8). This could influence the timing of adapted medical policy[44].

Alpha-fetoprotein (AFP) in recurrence of HCC

The glycoprotein AFP is mainly produced in the fetal liver and yolk sac during gestation. In the first months after birth, plasma levels of AFP decrease and become undetectable at the age of approximately one year. In healthy adults, AFP levels are usually <10-15 µg/L[52]. Experimental animal studies have shown a role of AFP in estradiol transport and preventing virilisation of female foetuses, but its function in humans remains largely unknown. After malignant degeneration, cells from various tissues are able to produce AFP. These cells can originate from the yolk sac, the gonads, hepatocytes and certain gastric cells[53].

In patients with HCC, pre-transplant levels of AFP were shown to be predictive of recurrence of HCC during follow-up. Therefore, it has been suggested to incorporate

pre-transplant AFP levels in the Milan criteria, which are currently used for screening of HCC patients to undergo LT[54]. A rise in AFP levels during follow-up has also been associated with the recurrence of disease[55, 56]. However, no clear correlation exists between AFP levels and tumor size, stage or prognosis. Current guidelines advise to measure AFP every three to six months for two years combined with imaging in patients transplanted for HCC. After that, annual monitoring is sufficient. If AFP levels show a strong elevation, further diagnostics for possible recurrence should be undertaken.

Patients with chronic HBV or HCV infection have an increased risk to develop HCC. Serum levels of AFP can be elevated without the presence of an intrahepatic malignant process. However, AFP levels >500 mcg/L increase the risk of HCC[57]. Half-life of AFP is five to seven days and are expected to decrease within 25 to 30 days after effective therapy.

BIOMARKER DYNAMICS IN VARIOUS COMPLICATIONS FOLLOWING LT

After discussing the specific markers for recurrent disease, the next paragraphs will provide an outline on biomarker dynamics that can be expected for common complications that can occur following LT (summarized in **Table 2**).

Graft primary non-function (PNF) and early allograft-dysfunction (EAD)

Incidence of PNF is 5-8% and despite being one of the most severe complications following LT, no formal definition of PNF exists. Usually, the diagnosis of PNF is ascertained by exclusion and in retrospect, the transplanted liver fails to start functioning in the first post-operative days and requires liver re-transplantation, or otherwise will inevitably result in the patients' death[58]. Risk factors of PNF can be for instance donor related (high donor age, steatosis, small for size) or procedure related (prolonged cold or warm-ischemia times, DCD, (hepatic artery)-thrombosis)[10, 59]. However, in up to 50% of the cases, the exact cause of PNF remains unknown. Complete failure of the graft in PNF results in extremely elevated liver enzymes in serum, impaired or absent bile production, encephalopathy and coagulopathy within the first 72 hours following LT.

A complication similar to PNF is early allograft dysfunction (EAD). In 2010, Olthoff et al. formulated and validated criteria in order to determine EAD based on one or more of the following serum biomarker levels in the first week post-transplant; bilirubin ≥10mg/dL on day 7, INR ≥1.6 on day 7, and ALT or AST levels >2000 IU/L within the first 7 days . Though EAD is a risk factor for impaired graft and patient survival, in contrast to PNF it will not inevitably result in liver re-transplantation or patient death. One could consider PNF as an excessive form of EAD and therefore it might be questioned whether the two definitions should be fused. Furthermore, liver grafts obtained by DCD usually have

poor immediate function and elevated serum biomarker levels compared to donation after brain death (DBD). It has been suggested to adjust the definition of EAD for this category of LT in order to better assess the risk for graft failure[42]. Especially since DCD is responsible for a significant contribution of the donor pool in many (particularly Western) countries, early prediction of EAD for this category could benefit graft and patient outcome. The median panels of **Fig. 1** shows examples of biomarker dynamics during the first post-operative week in LT recipients suffering from PNF and EAD. Such dynamics are usually accompanied with extensive ischemic necrosis at the histological level.

Acute cellular rejection (ACR)

As explained before, the introduction of cyclosporine significantly improved graft survival by lowering the degree of cellular rejection. Nevertheless, in individual patients it remains a challenge to lower immunosuppressant's use in order to avoid related complications on one hand, and to prevent ACR on the other hand. ACR is the result of a T-cell mediated immune response directed against tissue of the donor graft and mostly occurs within the first 90 days following LT (early ACR). However, low serum levels of immunosuppressant drugs have also been associated with ACR even years after transplantation[60]. Clinical symptoms in recipients consist of fever, abdominal pain, hepatomegaly and sometimes ascites. Laboratory test can show increased serum levels of hepatocellular and cholangiocyte-injury markers as well as bilirubin. The gold standard for diagnosing ACR however remains liver biopsy.

In 1995, experts formulated the so-called histological Banff-criteria to evaluate the degree of ACR in liver biopsies, also known as the rejection activity index[61]. This index, outlined in **Table 3**, scores the extend of inflammation and lymphocytic infiltration into (i) the portal triads, (ii) the bile ducts, and (iii) the venous endothelium. To date, this index is used as part of standard clinical practice. In the early days, tissue biopsies were taken frequently post-LT to monitor for ACR, but are now only indicated based on clinical symptoms.

Because of the low specificity of regular laboratory tests for ACR and the invasiveness of liver biopsies, many other surrogate-biomarkers have been investigated to monitor for ACR, among which interleukins, intercellular adhesion molecules, and many other. None have make it into clinical practice jet. A potential novel biomarker reported for ACR but also for other complications following LT are microRNAs (miRNAs), which will be discussed separately later.

Biliary complications

Biliary complications are very common after LT and can vary in nature, location and time of onset. The most common biliary complications consist of biliary leakage, anastomotic

Table 3. Banff scoring criteria or rejection activity index to evaluate histological graft rejection.

Category	Description	Score
Portal inflammation	Mostly lymphocyte involving, but not noticeably expanding, a minority of the triads.	1
	Expansion of most or all triads, by a mixed infiltrate containing lymphocytes with occasional blasts, neutrophils and eosinophils.	2
	Marked expansion of most or all triads by a mixed infiltrate containing numerous blasts and eosinophils with inflammatory spillover into the peripheral parenchyma.	3
Bile duct inflammation/ damage	A minority of the ducts are cuffed and infiltrated by inflammatory cells and show only mild reactive changes such as increased nuclear:cytoplasmic ratio of the epithelial cells	1
	Most or all of the ducts are infiltrated by inflammatory cells. More than an occasional duct shows degenerative changes such as nuclear pleomorphism, disordered polarity, and cytoplasmic vacuolization of the epithelium.	2
	As above for two, with most or all of the ducts showing degenerative changes or focal luminal disruption.	3
Venous endothelial inflammation	Subendothelial lymphocytic infiltration involving some, but not a majority of the portal and/or hepatic venules.	1
	Subendothelial infiltration involving most or all of the portal and/or hepatic venules	2
	Subendothelial infiltration involving most or all of the portal and/or hepatic venules as above for two, with moderate or severe perivenular inflammation that extends into the perivenular parenchyma and is associated with perivenular hepatocyte necrosis.	3

biliary strictures (AS) and non-anastomotic biliary strictures (NAS), which will all be discussed shortly.

Leakage of the biliary anastomosis usually occurs early following LT and the cause is either technical or because of insufficient blood supply to the biliary tree resulting in biliary necrosis. Suspicion for biliary leakage rises when patients have pain and feel ill due to irritation of the peritoneum. Abdominal free bile collections can be imaged by ultrasound but is more sensitive with ERCP, which is also useful for therapeutic stenting[62]. Biliary leakage is often accompanied by AS.

Benign local narrowing or tapering at the site of the biliary anastomosis, also known as AS, occurs in approximately 5-10% of LT recipients. Shortly after LT, the biliary anastomosis can be edematous due to surgical trauma and/or ischemia. The development of AS does not depend on the type of biliary anastomosis[63]. It is usually detected by elevated cholestatic markers in serum combined with clinical symptoms in recipients. Diagnosis and therapy of AS are accomplished by ERCP (**Fig. 2A**) and depending on the severity of the stricture, the bile duct can be cannulated by single or multiple stents (in case of duct-duct) or by percutaneous drains (in case of hepaticojejunostomy). If repeated attempts via the endoscopic or percutaneous route fail, AS can also be treated surgically[64]. Anastomotic strictures can occur early but also later following LT. Some

recipients have recurrence of AS for which they need progressive stenting[65]. Successful treatment of AS will result in a rapid decrease of cholestatic markers in serum and patients can recover without residual symptoms. An example of cholestatic biomarker dynamics in AS is provided in the right panels of **Fig 1**.

Beside the biliary anastomosis, some liver grafts develop strictures of the intrahepatic bile ducts or extrahepatic hilar region, which are called NAS[66]. The method of post-mortem donation strongly influences the risk for a liver graft to develop NAS; ~10% of DBD grafts vs. ~30% of DCD grafts[67, 68]. Furthermore, it is known that thrombosis of the hepatic artery, the major supplier of blood to biliary tree, will inevitably lead to NAS. Therefore, warm ischemia is thought to play a key-role in the pathophysiology of NAS. In contrast to AS, the (multiple) strictures in NAS and their anatomical localization are often less accessible for biliary stents or drains (**Fig. 2B**). Therefore, liver retransplantation is indicated in 10-15% of all LT recipients due to NAS[69]. Large HAT usually indicates immediate liver re-transplantation. In serum, NAS give elevation of cholestatic markers and only in few cases, normalization of biomarker levels to baseline is achieved. Eventually, NAS will lead to such severe cholestasis that patients will become ill and liver function will be affected.

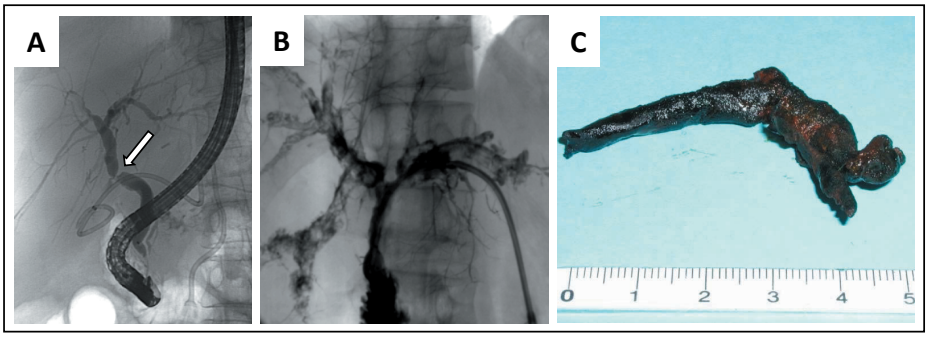

Figure 2. Visualization of biliary complications following LT. (A) ERCP showing an isolated stricture at the biliary anastomosis, pointed out by the white arrow, with dilatation of the common bile duct and slim intrahepatic bile ducts. (B) ERCP showing dilated intrahepatic bile ducts throughout the entire liver graft with loss of normal architecture due to NAS. (C) Biliary cast removed from the hilar region of a liver graft that was formed due to biliary obstruction and which is often seen in NAS. The length of the cast is displayed in cm. Pictures are derived from the database of the Erasmus Medical Center Rotterdam, The Netherlands.

NOVEL BIOMARKERS IN THE FIELD OF LIVER TRANSPLANTATION

MicroRNAs (miRNAs) as novel biomarker

In the last decade, miRNAs have gained interest in the field of biomarker research. MicroRNAs are short, hairpin-shaped RNAs with the potential to regulate gene-expression by inhibiting messenger RNA translation (**Fig. 3**). MiRNAs are highly cell-type abundant and can be release via active and passive mechanisms into the circulation and other body

fluids in which they remain stable up to 24 hours. These characteristics make miRNAs attractive candidate biomarkers for various disease. Beside their biomarker potential, the knowledge regarding miRNA induced gene-expression and regulation is increasing, though not yet fully understood[70].

For various liver diseases, particularly miR-122 has been related to hepatocellular liver injury. Serum levels of miR-122 increase earlier than conventional transaminase levels, which was shown in patients with viral hepatitis as well as in LT recipients who developed ACR[71, 72]. Therefore, hepatocyte-derived miRNAs (HDmiRs) might be suitable early markers for severe hepatocellular injury following LT, as is the case in grafts developing EAD or PNF. In contrast to liver transaminases, which are mainly injury markers, HDmiR-122 secretion into bile has also been correlated to good bilirubin excretion of

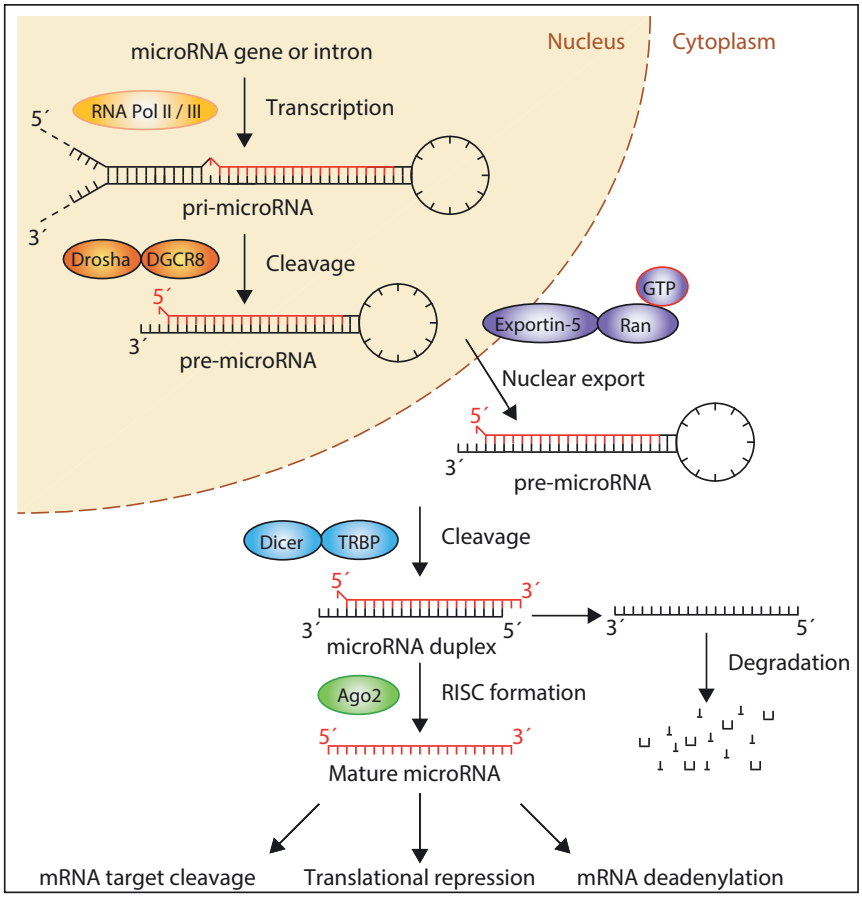

Figure 3. MicroRNA structure and biogenesis. Biogenesis of miRNAs by cells. Immature precursor miRNAs are formed inside the cell nucleus. In the cell cytoplasma, miRNAs reach their mature form and are able to inhibit mRNA translation, thereby regulating gene expression. Illustration from Winter et al.[80].

Key facts of microRNAs

- MicroRNAs (also called miRNAs or miRs) are 20-23 nucleotide long, hairpin-shaped RNA. Up to 30% of the human genes is regulated by miRNAs via inhibition of mRNA translation.
- A single miRNA is responsible for the regulation of multiple genes.
- The first reports on the presence of miRNAs in Caenorhabditis-elegans date from 2001 and since then, over a 1000 different miRNAs have been discovered in mammals.
- Various cell types express distinct sets of miRNAs that are related to metabolism, oncology, endocrinology, the vascular system, and infection.
- Tissue-abundant miRNAs are released from cells into the circulation and other body fluids under different (patho)physiological conditions via active and passive mechanisms.
- In contrast to mRNA, extracellular miRNA are protected from degradation in fluids, making them attractive for non-invasive biomarker research.

hepatocytes into bile[39]. Therefore, HDmiR-122 and perhaps other HDmiRs might also be suitable markers for graft function.

Cholangiocytes have a different expression of miRNAs compared to hepatocytes [73]. Therefore, cholangiocyte-derived miRNAs (CDmiRs) could be more sensitive or specific in the detection of biliary complications. Already at time of graft preservation, CDmiRs are released in response to ischemia-induced biliary injury that causes severe complications in LT recipients during follow-up[74]. Beside changes in expression, also the composition of miRNAs in bile is changed during biliary obstructions[75].

Despite the growing evidence of their utility, miRNAs as biomarker are currently not part of clinical practice in liver disease. Future research should focus on validation of sensitivity and specificity of previously identified CDmiRs and HDmiRs. Another challenge for implementing miRNAs as a routine laboratory test lays within the technical aspect of measuring miRNAs. This is now done by real-time quantitative polymerase-chain-reaction (RT-qPCR), which takes approximately three hours before miRNAs are isolated and analysed. This issue could be facilitated by improving accelerated PCR-techniques. Because of the highly sensitive analysis of qPCR, mild elevations of miRNA levels in blood or other body fluids can be determined quite accurately. Despite the fact that much is still unknown about miRNAs as therapeutic target, the first clinical series in human showed that inhibition of HDmiR-122 reduces viral load in HCV patients[76]. Whether CDmiRs are potentially interesting in (prevention of) cholestatic disease needs to be explored by future research.

POTENTIAL APPLICATION TO PROGNOSIS, OTHER DISEASES OR CONDITIONS

The previous paragraphs provided an overview of different types of biomarkers that are regularly used in liver disease and how these should be interpreted in the context of LT. Routine monitoring of graft quality based on biomarkers helps clinicians to decide whether or not to perform additional (mostly more invasive) tests like ERCP or liver biopsy. Biomarker levels can be reason to adjust therapy, for instance to increase immunosuppressant dosage when high transaminase levels indicate cellular rejection. But also as a definition of outcome, biomarkers play an important role in predicting prognosis early after LT.

Some important complications that can occur following LT, like EAD and biliary strictures, are often related to marginal quality of the liver graft already at time of transplantation. As mentioned before, grafts obtained by DCD have a higher risk to develop EAD and NAS. For this reason, DCD liver grafts from elderly donors (over 60 years of age) are often rejected for LT. However, some of the rejected DCD grafts might have functioned well in recipients. With the increasing number of marginal grafts for LT, there is a need to improve- and simultaneously to objectify graft quality in an earlier phase of LT. The prolonged time-window between graft procurement and graft implantation, known as the preservation period, is in particular useful for this purpose. Many studies showed that during static cold-storage, liver grafts can still release some injury markers that have been associated with outcome. A novel technique designed to preserve and improve graft quality is machine perfusion (MP)[77]. With MP, the liver graft is flushed *ex situ* on a pump that recirculates preservations solution (perfusates) before implantation into the recipient. Many different techniques of MP have been investigated with variations in solutions, temperature, oxygenation, single-portal or dual portal-hepatic artery perfusion, flow pressure and more. The first clinical studies with MP show promising results regarding prevention of hepatic and biliary injury[78]. However, during MP it remains a challenge to objectify that marginal grafts show enough recovery to be transplanted and which should still be rejected for LT. Multiple options are available to assess graft quality during MP with the use of biomarkers in graft perfusates and produced bile, depending on the applied technique[11].

Despite the potential of biomarkers to assess graft quality during preservation, their clinical application is still experimental and the decision to accept a graft for LT is mainly driven by clinical donor variables and the macroscopic aspect on inspection by the donor surgeon. Beside donor variables, some researchers plea for implementation of recipient variables as well in allocation algorithm's, since recipient factors as age, MELD score and gender can strongly influence survival[79]. Because of the limited number of performed LTs annually in transplant centres, many biomarker studies omit validation of potential biomarkers in multiple cohorts. This will however delay the implementation

of biomarkers to assess graft quality during preservation. Furthermore, criteria for EAD should be adapted for DCD liver grafts; despite their worse biomarker profile post-LT, multiple DCD grafts show good recovery during follow-up. The current criteria might be insufficient to distinguish grafts that will eventually function properly in recipients from the ones that actually cause PNF. This could also be the case for other types of donation, like living donor LT, for which other literature is recommended.

SUMMARY POINTS AND DISCUSSION

To conclude, this overview discussed routinely measured biomarkers and more novel ones for evaluation of graft injury and function in the follow-up of LT recipients and their dynamics at time of various complications and (recurrence of) disease. It is evident that biomarkers can indicate hepatocellular injury, biliary obstruction and liver function. Evaluation of biomarkers can play a key-role in the early recognition of complications and provide an objective tool to monitor graft quality after transplantation. As in recent years, many new potential biomarkers have been discovered. Therefore, this overview is incomplete and limited to established serum biomarkers. Furthermore, it should be emphasized that experienced clinical knowledge and imaging techniques of the liver are two other key factors in clinical decision making, and determining the need of intervention will rarely be based on biomarkers solely. Much likely, LT recipients will start with monitoring of graft function through biomarker measurements in the home situation as part of individualized medicine. Finally, novel application of biomarker measurements during graft preservation seems promising in early evaluation of graft quality that could help extend the donor pool for LT.

AIMS AND OUTLINE OF THIS THESIS

The aim of this thesis is to evaluate conventional and novel biomarkers on their performance to assess graft quality and predict outcome in LT. The rising incidence of severe complications like NAS, which is strongly related to DCD, urges for predictive biomarkers in an earlier phase of LT. For example during graft preservation and by identifying recipients or donors with an increased risk for complications.

To identify potentially relevant markers, **part I** of this thesis provides an overview of the literature on conventional and novel biomarkers. **Chapter 1** discusses the various definitions of biomarkers and which biomarkers are generally applied for graft monitoring during follow-up. Subsequently, **chapter 2** is a systemic review of the literature on which conventional and novel markers are useful to assess graft quality prior to graft

implantation during graft preservation by static cold storage or MP. This chapter also provides a brief overview on the different techniques of MP and the pros and cons of using different biomaterials as perfusates, biopsies and bile.

Part II focuses on biomarkers that can be useful to assess the risk of developing biliary complications, in particular NAS. Since many years, researchers hypothesize whether liver grafts obtained by DCD have an increased tendency to form microthrombi in their microvasculature at time of graft preservation that could explain the higher incidence of NAS. Therefore, some transplantation centres apply potentially harmful intraoperative fibrinolytic therapy that can cause major blood loss in recipients. In **chapter 3**, we investigate whether there is histological evidence to support the hypothesis of microthrombi formation in DCD that could justify fibrinolytic therapy. Beside diminished blood supply, immunological factors have also been associated with the development of NAS. This is particularly the case in patients who suffer from auto-immune diseases, like PSC. Because of the clinical similarities between PSC and NAS, a shared underlying pathophysiological mechanism has been suggested. A polymorphism of the fucosyltransferase-2 (FUT2) gene has been associated with PSC, by altering the glycosylation of cholangiocytes that enhances bacterial translocation. **Chapter 4** investigates the association between FUT2 polymorphism and the development of NAS in a large cohort of LT donors and recipients.

Part III entirely focuses on the discussion of aforementioned miRNAs in the setting of LT. First, the potential advantages and applications of extracellular miRNAs as dynamic markers in liver disease and transplantation are evaluated based on the literature in **chapter 5**. Most studies on extracellular miRNAs investigated release in serum, since this is an attractive and easily obtainable medium. However, the presence of miRNAs in bile could also be of importance in liver disease and interesting for biomarker purposes. In **chapter 6**, we therefore examine the release of hepatocyte and cholangiocyte-derived miRNAs, so-called HDmiRs and CDmiRs, into bile and serum of LT recipients to gain more insight into the mechanisms of release during different pathophysiological states of the liver. Extracellular HDmiRs and CDmiRs are also released during graft preservation, that are detectable in preservation solutions for flushing the graft. The features of these HDmiRs and CDmiRs in perfusates and their capacity to predict the development of NAS is subject of **chapter 7**. Although extracellular miRNAs are highly stable in biofluids, reliable detection can be challenged if samples are contaminated with components that are able to inhibit reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR), like heparin. In **chapter 8** we investigate whether perfusate samples contain traces of heparin that are used during organ retrieval. In addition, the effect of heparinase I to counteract the inhibition of RT-qPCR is studied, in order to optimize miRNA detection in perfusates. Since heparin is partly excreted by the kidneys, **chapter 9** investigates whether miRNA detection in urine samples from hospitalized transplant patients is inhibited and whether detection can be optimized by treating samples with

heparinase I. This is particularly relevant for future biomarker studies on urinary miRNAs, which are becoming increasingly popular because of their non-invasiveness.

Finally, the results of this thesis are summarized in **part IV - chapter 10**, including a general discussion with recommendations for future research.

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Chapter 2

Biomarkers to assess graft quality during conventional and machine preservation in liver transplantation

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SUMMARY

A global rising organ shortage necessitates the use of extended criteria donors (ECD) for liver transplantation (LT). However, poor preservation and extensive ischemic injury of ECD grafts has been recognized as an important factor associated with primary non-function, early allograft dysfunction, and biliary complications after LT. In order to prevent for these ischemia-related complications, machine perfusion (MP) has gained interest as a technique to optimize preservation of grafts and to provide the opportunity to assess graft quality by screening for extensive ischemic injury. For this purpose, however, objective surrogate biomarkers are required which can be easily determined at time of graft preservation and the various techniques of MP. This review provides an overview and evaluation of biomarkers that have been investigated for the assessment of graft quality and viability testing during different types of MP. Moreover, studies regarding conventional graft preservation by static cold storage (SCS) were screened to identify biomarkers that correlated with either allograft dysfunction or biliary complications after LT and which could potentially be applied as predictive markers during MP. The pros and cons of the different biomaterials that are available for biomarker research during graft preservation are discussed, accompanied with suggestions for future research. Though many studies are currently still in the experimental setting or of low evidence level due to small cohort sizes, the biomarkers presented in this review provide a useful handle to monitor recovery of ECD grafts during clinical MP in the near future.

INTRODUCTION

Graft quality at time of liver transplantation (LT) is a major determinant of early graft performance and thereby strongly influencing graft survival and morbidity during recipient follow-up[1]. Over the last decade, grafts from extended criteria donors (ECD) had to be used increasingly for LT due to organ shortage. The quality of these grafts has been shown to be variable[2, 3]. Although some ECD liver grafts turn out to function properly in recipients, their use has also been associated with impaired graft survival due to primary non function (PNF), early allograft dysfunction (EAD) and severe biliary complications like ischemic-type biliary lesions (ITBL, **Fig. 1**)[4, 5].

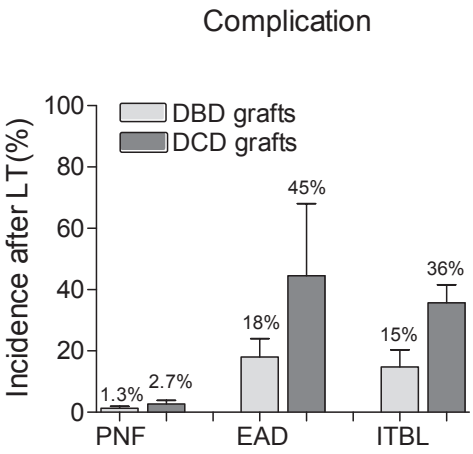

Figure 1. Incidence of ischemia/preservation related complications after LT. Estimation of the incidence of PNF, EAD and ITBL in separate DBD and DCD grafts, based on cohort- and case-matched studies[5, 9-11, 76, 86, 110]. Percentages represent the mean incidence ± standard error. Studies used to calculate the incidence of EAD maintained the criteria formulated by Olthoff et al.[9, 10]. Definitions of PNF and ITBL can be found in the supplementary information.

Though pathophysiology between PNF, EAD and biliary complications is assumed to differ, extensive ischemic- and preservation injury has been recognized as a shared risk factor in these entities[1, 6]. Primary non-function occurs in up to 5-8% of LT's and necessitates immediate re-transplantation in all cases. Though PNF may be caused by technical failure resulting in inadequate blood flow through the graft[7], the association between unfavourable donor risk factors and PNF suggests that its cause is likely multifactorial[8]. Early allograft dysfunction is typically characterized by increased serum transaminase levels in recipients during the first postoperative week[9], but unlike PNF, liver grafts showing EAD do not always need immediate re-transplantation[10]. The most common complication associated with ischemic- and preservation injury are biliary complications. Dependent on the type of graft (donation after brain death; DBD vs. donation

after circulatory death; DCD), up to 50% of recipients develop complications due to bile leakage, anastomotic strictures, ITBL, bile duct necrosis, and cast formation[11, 12]. The various times of onset and different nature of biliary complications suggest that they are caused by different underlying mechanisms, including surgical trauma, DCD, high donor age, prolonged ischemia time, cytotoxicity of bile salts and immune factors[6, 11].

Prediction models as the donor risk index were developed to estimate the risk of graft failure in recipients and to match high-risk grafts to suitable recipients[13]. Furthermore, earlier research on the topic of predicting graft function after LT has focussed mainly on clinical characteristics from donors and recipients, including the model for end-stage liver disease-score (MELD)[14-16]. However, models that are mainly based on such characteristics are unable to assess the degree of injury that is caused by the process of graft procurement, cold preservation and reperfusion. Moreover, the under-utilization of grafts with unfavourable donor characteristics like advanced donor age, DCD and African race, can lead to an undesirable diminution of the donor pool[17].

Therefore, machine perfusion (MP) is increasingly being investigated as a novel technique to improve graft preservation of particularly ECD grafts. Through MP, ischemia related complications like PNF, EAD or ITBL can be reduced or even prevented and potentially allow for expansion of the extended criteria donor pool to be utilized for LT. Other potentially beneficial features of MP consist of the possibility to add supplements during perfusion that could further benefit graft quality[18, 19], or even attempt for restoration of ischemic injury[20, 21]. Beside safety and technical feasibility of MP, investigators pronounce on the need of sensitive biomarkers that can distinguish poor quality grafts from those that will function properly after implantation[22, 23]. Next to other well-known risk factors for impaired graft quality as illustrated in **Fig. 2**, the time required for ex vivo MP provides the opportunity to monitor graft quality by measurement of biomarkers in perfusates and biopsies, which could be a helpful decision tool for improving the accuracy of selecting grafts for LT. This purpose however demands for objective surrogate biomarkers that are easily obtainable at time of graft preservation and is challenged by the various techniques of MP currently investigated.

In this review, we provide an overview of potentially useful biomarkers that were identified through a systematic search of the literature (**supplementary information**), in order to assess graft viability testing during various techniques of MP. Because of the limited experience with clinical MP in LT, biomarker studies regarding conventional graft preservation by static cold storage (SCS) that correlated with either PNF, EAD or biliary complications after LT and which could potentially be applied as predictive markers during MP were also included. Finally, the pros and cons of the different biomaterials are discussed, accompanied with suggestions for future research.

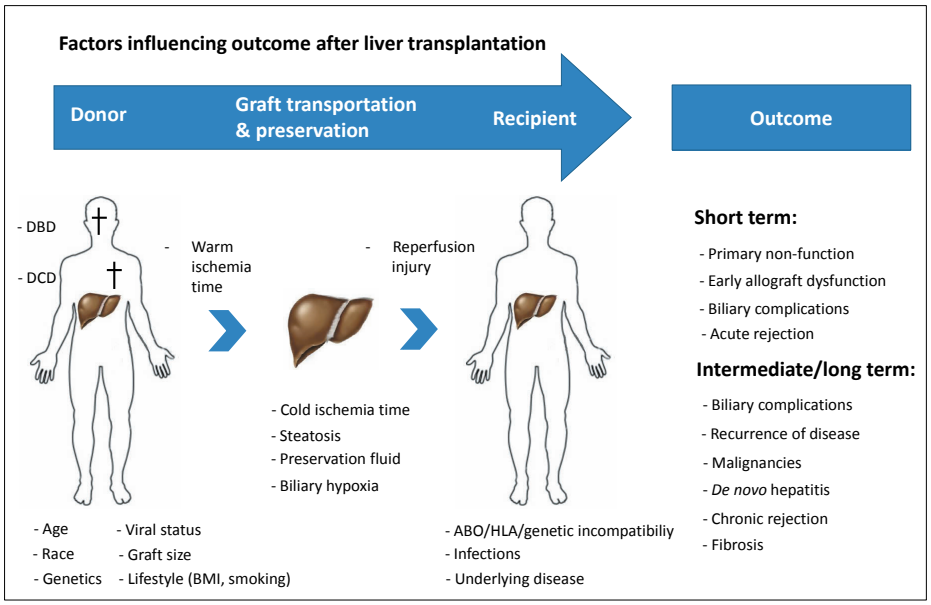

Figure 2. Risk factors for outcome following LT. Risk factors in donors, recipients and during the transplantation and transportation procedure influcening graft quality and graft/recipient outcome.

DIFFERENT MACHINE PRESERVATION STRATEGIES

Because of easier accessible logistics and lower costs, SCS has become the standard preservation technique in clinical practice of LT to date. The low temperature during SCS delays metabolic processes in order to restrict ischemic injury. However, especially ECD grafts seem more vulnerable for prolonged ischemia, increasing morbidity and mortality in recipients after LT. Therefore, during the last ten years, various techniques by MP have been investigated in pre-clincal and clinical setting in order to further optimize graft quality and thus improve outcome of ECD liver transplantation. The main differences in the setup of MP are determined by pumping-temperature, the route- and pressure of recirculating preservation solution, and whether oxygen is administered (**Fig. 3**). As summarized in **Table 1**, several studies already performed MP on human liver grafts. Hypothermic MP (HMP) without the administration of oxygen comes closest to conventional preservation by SCS, but is believed to improve preservation through continuous recirculation of solution to all segments of the liver and the removal of remnant metabolites from the graft (**Fig. 4**). Guarrera et al.[24] performed the first clinical series of non-oxygenated HMP in humans (n=20) using standard criteria donors. In this study, HMP was shown to be safe and analysis of perfusates and biopsies demonstrated an attenuation of ischemic injury markers during preservation[25-27]. Furthermore, the

Table 1. Studies on machine perfusion of human liver grafts.

Author	Year	MP temp	Oxygenated	Pressure (mmHg)		Size	Subject	Donor	Transp-lanted	Markers during MP for impaired viability	Biomaterial
				arterial	venous						
op den Dries et al.[37]	2013	37°C	Yes	50	11	4	Human	ECD	No	↑ Enzymes, lactate levels, ↓ bile production, bile composition (γGT, bilirubine, LDH), O_2, CO_2	Perfusate, tissue, bile
Dutkowski et al.[29]	2013	10°C	Yes	n.a.	<3	8	Human	ECD	Yes	n.r.	-
Guarrera et al.[24]	2010	4-8°C	No	6	3	20	Human	SCD	Yes	↑ AST, ALT, LDH	Perfusate
Guarrera et al.[25] *	2011	4-8°C	No	6	3	6	Human	SCD	Yes	↑ ICAM-1, IL-8, TNF-α	Perfusate, tissue
Henry et al.[26] *	2012	4-8°C	No	6	3	33	Human	SCD	Yes	↑ Inflammatory cytokines, adhesion molecules, oxidative markers, acute phase proteins, CD68	Tissue
Jomaa et al.[104]	2013	4-8°C	No	30	7	16	Human	ECD	No	n.r.	-
Monbaliu et al.[28]	2012	4-6°C	No	20-30	7	17	Human	ECD	No	↑ AST, ↑ LDH	Perfusate, tissue
Tulipan et al.[27] *	2011	4-8°C	No	6	3	n.r.	Human	SCD	Yes	↑ MCP-1, ↑ IL-1Rα	Perfusate, serum

Studies on biomarkers to monitor quality of grafts obtained from standard criteria donors (SCD) or extended-criteria donors (ECD) during MP. N.r.=not reported. N.a.=not applied. * These studies all derived from the trial by Guarrera in 2010. AST= aspartate aminotransferase, ALT=alanine aminotransferase, LDH=lactate dehydrogenase, ICAM-1=intracellular adhesion molecule 1, IL-8=interleukin 8, TNF- α=tumor necrosis factor alpha, MCP-1=monocyte-chemoattractant protein 1, IL-1R α=interleukin 1 receptor antagonist

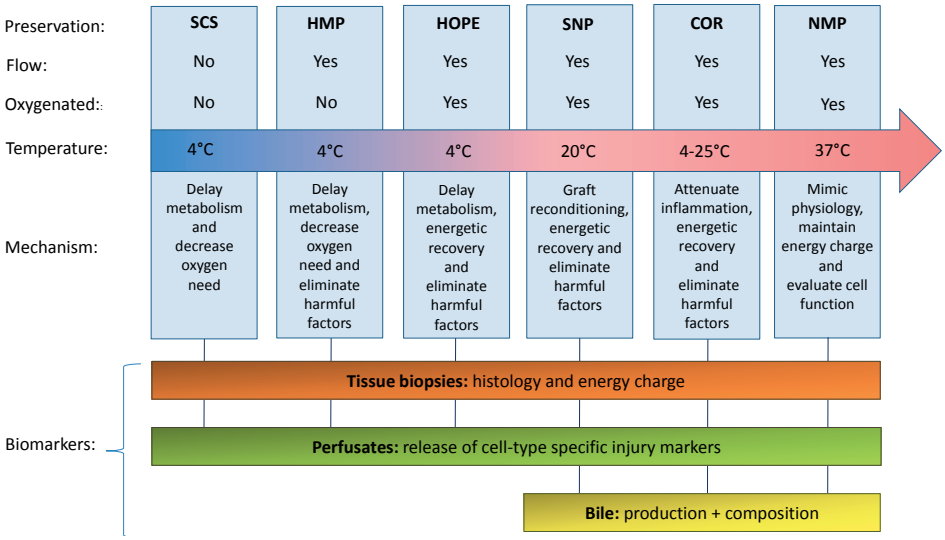

Figure 3. Mechanisms of various machine preservation strategies. Different techniques of graft preservation can be used to protect against ischemic injury, to recondition the graft before reperfusion or even to maintain physiology. The various techniques have different potentially protective underlying mechanisms. Via all techniques, graft quality could be evaluated through markers in tissue biopsies or perfusate analysis. The (sub)normothermic conditions also allow for the analysis and evaluation of bile. SCS = static cold storage, HMP = hypothermic machine perfusion, HOPE = hypothermic oxygenated machine perfusion, SNP = subnormothermic perfusion, COR = controlled oxygenated rewarming, NMP = normothermic machine perfusion.

authors suggest that HMP could have beneficial effect on the incidence of EAD and biliary complications in recipients after LT. The feasibility of HMP was also investigated by Monbaliu et al.[28], who used HMP as a screening-tool to distinguish transplantable from non-transplantable ECD human liver grafts that were rejected for LT. Beside Guarrera et al, the second reported clinical trial using MP prior to LT is from Dutkowski et al.[29]. In contrast to Guarrera et al., this study used hypothermic oxygenated MP (HOPE) for the preservation of ECD grafts. Previous experimental studies from this group showed beneficial effects of HOPE on biliary injury and endothelial damage[30, 31]. Protective mechanisms of HOPE seem to be based mainly on the down regulation of mitochondrial and nuclear activity prior to reperfusion. Moreover, the used low-pressure perfusion at 3 mmHg caused less endothelial injury compared to more physiological pressures around 8 mmHg. Notably, grafts were perfused solely through the portal vein due to practical considerations and to prevent further damaging of the usually fragile hepatic artery[32]. Reactive oxygen species that are generated during ischemia can induce injury to mitochondria, which effects appear to exacerbate after hypothermic conditions[33, 34]. Some researchers believe that reconditioning of the tissue by MP at higher temperatures can prevent this[35, 36]. Moreover, (sub)normothermic MP (SNP) is seen as a preferable

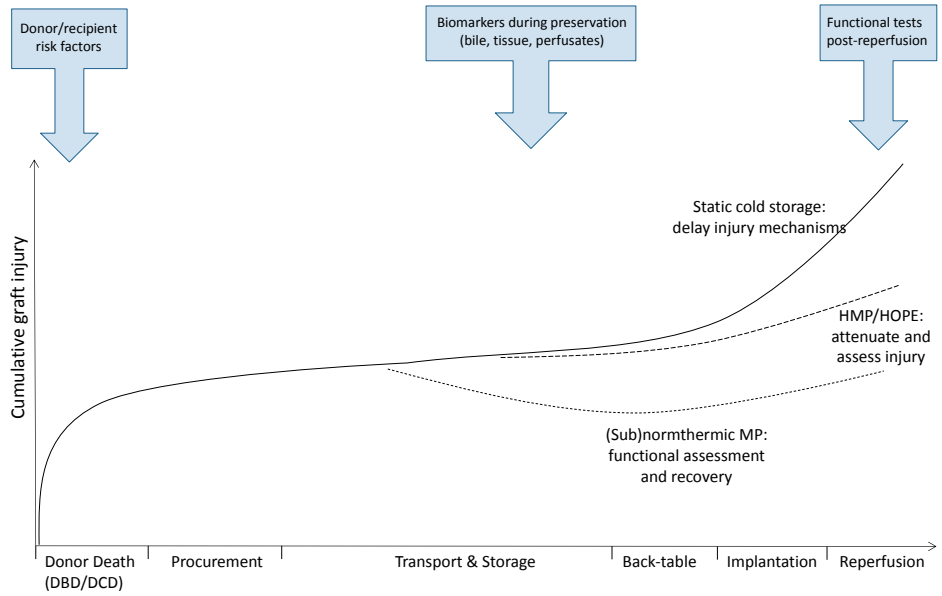

Figure 4. **Cumulative graft injury & evaluation points for graft quality.** Already in an early phase of LT, known risk factors in donors and recipients could be used for deciding to accept a graft for transplantation and for which recipient. In order to determine the degree of preservation injury, biomarkers can be measured in tissue, perfusates or bile during various preservation methods. The cumulative injury of grafts that are preserved by hypothermic MP (either oxygenated or non-oxygenated) is believed to be less compared to static cold stored grafts (SCS). Mimicking normal physiological functions through (sub)normothermic MP is hypothesized to even recover injury of ECD grafts, for instance steatosis, and to provide a better assessment of graft function. After graft reperfusion, other functional tests are available in order to monitor graft quality during follow-up.

model for viability testing because metabolic function can be judged, for instance through bile output during warm MP[37, 38]. Although not yet performed in clinical LT, op den Dries et al. performed a feasibility study of normothermic perfusion (NMP) on four discarded human donor livers, which showed no harmful effects on liver tissue after 6 hours of pumping[37]. Also in large animal models, graft NMP improved survival compared to SCS [35, 39]. Finally, an alternative for perfusion with constant temperature is controlled oxygenated rewarming (COR) of primarily cold stored liver grafts. Gradual increase of the MP temperature is thought to minimize re-oxygenation injury that is normally triggered by immediate rewarming of the graft, like in reperfusion and NMP. First results of COR in animal models indicate that post-reperfusion recovery is more successful in grafts that were subjected to COR compared to HMP, SNP and SCS[40]. Gradual rewarming in this study however did not exceed 20-25°C because of potentially toxic effects of the preservation solution at higher temperatures.

Many experimental studies have been performed on the different techniques of MP, of which some also attempted to identify biomarkers for graft quality assessment (**Table 2**).

Table 2. Studies on biomarkers that were measured during various types of MP prior to (mimicked) reperfusion in animal models.

Study	Year	MP temp	Oxygenated	Pressure (mmHg)		Size	Subject	Donor model	Markers during MP	Assay
				arterial	venous					
Boehnert et al.[23]	2013	38°C	Yes	60	7	30	Pig	DCD	ALT, necrosis, bile volume, pO_2, urea	Perfusate, tissue, bile
Brockmann et al.[35]	2009	39	Yes	n.d.	n.d.	38	Pig	DBD & DCD	Bile volume, base excess, AST, ALT, HA, portal pressure, portal venous resistance	Bile, perfusate
Fondevila et al.[39]	2011	36-37	Yes	40-60	8	18	Pig	DCD	AST, bilirubine, LDH, pH, PO2	Perfusate
Fondevila et al.[105]	2012	4	Yes	20-30	4	11	Pig	DCD	Venous/arterial flow, AST, pH, O_2, Na^+, K^+	Perfusate
Jamieson et al.[21]	2011	39	Yes	85-95	n.d.	8	Pig	Steatosis	Bile volume, base excess, albumin, AST, ALT, steatosis, glucose, urea	Bile, tissue, perfusate
Liu et al.[45]	2014	4-6	Yes	20	3	36	Pig	DCD	pH, AST, L-FABP, ATP, redox active iron, arterial resistance	Perfusate
Minor et al.[40]	2013	4-20	Yes	25	4	24	Pig	DBD	Energy charge potential, ATP, AST, ALT, lactate, LPO	Perfusate, tissue
Obara et al.[106]	2012	4-8	Yes	88	6	7	Pig	DCD	AST, ALT, LDH, arterial flow	Perfusate
Olschewski et al.[107]	2010	4-21	Yes	n.d.	n.d.	30	Rat	DCD	Portal venous resitance, bile volume, lactate, ALT	Bile, perfusate
Perk et al.[90]	2012	37	Yes	n.d.	7-9	19	Rat	DCD	Glucose, urea, lactate	Perfusate
Schlegel et al.[30]	2013	4	Yes	n.a.	3	46	Pig	DCD	NADH, pCO_2	Perfusate
Shigeta et al.[108]	2013	4-25	Yes	28	4	9	Pig	DCD	AST, LDH, HA	Perfusate
Vairetti et al.[36]	2008	4-37	Yes	n.a.	n.d.	30	Rat	DBD	AST, LDH, bile volume (LDH), γGT	Perfusate, bile
Vairetti et al.[71]	2009	20	Yes	n.a.	6-7		Rat	Steatosis	AST, LDH,	Perfusate
Xu et al.[38]	2012	39	Yes	70-80	5-8	12	Pig	DCD	ALT, bile volume, CO_2, ATP, necrosis, apoptosis	Bile, tissue, perfusate

N.d.=not defined. N.a.=not applied. ALT=alanine aminotransferase, HA=hyaluronic acid, LDH=lactate dehydrogenase, L-FABP=liver-type fatty acid binding protein, ATP=adenosine triphosphate, LPO=lipid peroxides, NADH=Nicotinamide adenine dinucleotide.

One would expect that these various MP techniques require different biomarkers for the assessment of graft quality. In the next paragraphs, we highlight on the most promising biomarkers for viability testing in MP of which some have been shown also to be predictive for early graft function after clinical LT (**Table 3**).

BIOMARKERS FOR VIABILITY ASSESSMENT DURING MACHINE PERFUSION

Production and composition of bile

Beside using bile output as a parameter for outcome after reperfusion[32], some studies also investigated whether bile production *during* MP is a useful indicator for graft viability and the secretory function of hepatocytes and cholangiocytes; Brockmann et al. identified bile outflow during NMP as a discriminative variable for early graft survival[35]. Op den Dries et al.[37] also demonstrated the production of bile by human liver grafts under normothermic conditions. Based on this small series, they conclude that bile production during NMP is the most important parameter for viability[41], although no strong correlations could be made since these grafts were not actually transplanted. Vairetti et al. demonstrated that bile is also produced during colder SNP[36]. More importantly, this study showed that bile outflow during MP was no guarantee for improved bile flow after graft reperfusion. Boehnert et al. emphasized that evaluation of solely bile flow during MP might be biased due to the secretion of serum-like fluids from the injured biliary mucosa, which could falsely increase bile volume[23, 42]. In order to correct for this bias, they measured lactate dehydrogenase (LDH) in bile as a marker for biliary epithelial injury and found its content in bile to be lower after NMP compared to SCS, while bilirubin and phospholipid concentrations were higher[23]. Impaired secretion of phospholipids gives a surplus of free bile salts which are toxic for cholangiocytes. A higher ratio of bile salts/phospholipids, rather than bile production solely, has been associated with the development of ITBL[43, 44]. Also the secretion of HCO_3^- into bile, involved in local pH regulation, has been described as a marker for cholangiocyte function. The evidence of bile outflow or -composition as a marker at temperatures below 20°C is however marginal. Since lower temperatures shut-down metabolic cellular processes, bile parameters are probably more informative under (sub)normothermic conditions.

Liver enzyme release as indicator of hepatocyte injury

Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and LDH are the most frequently studied biomarkers in liver disease. Both AST and LDH are enzymes that are mainly present in the cell cytoplasm of various tissues, including liver, and they are often used as general injury markers to monitor graft function after LT. For a more specific assessment of hepatocyte injury, ALT is often determined. In their clinical trial, Guarrera

et al. found perfusate levels of AST and ALT measured during HMP to strongly correlate with post-transplant peak AST and ALT serum levels in recipients. This suggests that injury that becomes apparent after graft implantation, can already be detected during HMP. Monbaliu et al. distuingished transplantable from non-transplantable grafts based on AST levels in perfusates during HMP[28]. But also during NMP, the release of AST and ALT were predictive for recipient survival in a large animal model[35]. Moreover, hepatic enzyme release during MP strongly correlated with donor warm-ischemia time, which in turn has been associated with poor graft quality[45]. The value of enzyme release into perfusates to predict PNF and EAD has also been confirmed by clinical LT studies with conventional SCS (**Table 3**)[46-48].

Energetic recovery status by adenine nucleotides

Cold temperatures and the absence of oxygen supply to tissue causes the shutdown of adenine nucleotide metabolism, which causes failure of ion transport by electron pumps on the cell membrane[49]. Therefore, Minor et al. investigated whether oxygenation during MP could recover energy status by measuring the energy charge potential and adenosine triphosphate (ATP) levels in tissue[40]. At the end of various MP methods and already before reperfusion, oxygenated tissue showed a higher energy charge potential and increased ATP levels compared to cold stored livers. This study furthermore demonstrated that hypothermic conditions hampered energetic recovery compared to (sub)normothermic conditions. In clinical LT, decreased ATP levels have been shown to increase the risk for graft PNF or EAD; Kamiike et al.[50] used expression of ATP and total adenine nucleotides in peri-transplant liver biopsies to predict graft viability, based on functional outcome within the first days after LT. Compared to other nucleotides, ATP was demonstrated to be most sensitive for ischemia, as its expression decreased faster. However, a reduction of total adenine nucleotide levels in liver biopsies was more predictive for PNF after LT than ATP levels solely. Following revascularization, good functioning grafts also showed a better recovery of ATP and total adenine nucleotide levels. These levels were inversely related to the period of warm ischemia during graft implantation. Similar studies performed by Lanir et al.[51] and Hamamoto et al.[52], confirmed lower (total) adenine nucleotide levels in biopsies that were obtained during respectively cold storage and post-reperfusion, which also correlated with the development of PNF. Moreover, Hamamoto et al. found increased levels of Xanthine in perfusates also to be associated with PNF. These findings suggest that assessing energetic recovery of grafts in tissue and perfusates might be a good predictor for graft viability during MP in both hypo- as (sub)normothermic conditions.

Table 3. Overview of research papers investigating biomarkers during clinical LT associated with PNF, EAD or biliary complications.

Study	Outcome	Incidence	Year	Size	Injury marker	Donor assay	Graft type	Evidence level
Abraham et al.[70]	PNF	29%	1996	38	↑ Hepatocyte swelling Apoptosis, hemorrhage, hepatocyte swelling and necrosis	Liver tissue	DBD*	3B
Hamamoto et al.[52]	PNF	6%	1994	68	↓ Adenine nucleotides ↑ Xanthine	Liver tissue Perfusate	DBD*	3B
Kamiike et al.[50]	PNF	20%	1988	30	↓ Adenine nucleotides ↓ Bile flow rate	Liver tissue Bile	DBD*	4
Lanir et al.[51]	PNF	20%	1988	25	↓ Adenine nucleotides (ATP <2nmoles/mg)	Liver tissue	DBD*	3B
Bronsther et al.[57]	PNF	9%	1993	70	↑ HA (>400 µg/L)	Perfusate	DBD*	3B
Rao et al.[58]	PNF	6%	1996	102	↑ HA (>400 µg/L)	Perfusate	DBD*	2B
Berberat et al.[67]	PNF & EAD	7% / 22%	2006	59	↑ CRP, ↓ CTGF, WWP2, CD274, VEGF, FLT1	Liver tissue	n.d.	3B
Khettry et al.[69]	PNF & EAD	8% / 16%	1991	50	10%-50% hemorrhage and/or necrosis	Galbladder tissue	DBD	3B
Lange et al.[48]	PNF & EAD	10% / 4%	1996	50	↑ AST, ALT, LDH	Perfusate	DBD*	4
Calmus et al.[68]	EAD	19%	1995	32	↑ Amino acids	Perfusates	DBD*	3B
Cywes et al.[109]	EAD	n.d.	1993	30	↑ platelet adhesion	Liver tissue	DBD*	3B
Devlin et al.[46]	EAD	19%	1995	53	↑ AST, LDH	Perfusate	DBD*	3B
Pacheco et al.[47]	EAD	21%	2010	47	↑ AST, ALT, LDH	Perfusate	n.d.	4
Suehiro et al.[65]	EAD	14%	1997	58	↑ TM (>20 FU/mL) ↑ Sinusoidal TM staining	Perfusate Liver tissue	DBD*	3B

Table 3. Overview of research papers investigating biomarkers during clinical LT associated with PNF, EAD or biliary complications. (continued)

Study	Outcome	Incidence	Year	Size	Injury marker	Donor assay	Graft type	Evidence level
Brunner et al.[12]	Biliary complications	n.d.	2013	79	>10% epithelial damage, disturbed tight junction protein architecture	Extrahepatic bile duct tissue	DBD	3B
op den Dries et al.	ITBL	16%	2014	128	Vascular injury with arteriolonecrosis, >50% loss of cells in deep peribiliary glands	Extrahepatic bile duct tissue	DBD & DCD	2B
Farid et al.[80]	ITBL	n.d.	2013	22	↓ Portal vein branch-size	Liver tissue	DBD	3B
Hansen et al.[75]	ITBL	19%	2012	93	Presence of arteriolonecrosis	Extrahepatic bile duct tissue	DBD	2B
Verhoeven et al.[86]	ITBL	35%	2013	56	↑ HDmiR/CDmiR ratio	Perfusate	DBD & DCD	2B

ATP=adenosine triphosphate, HA=hyaluronic acid, TM=thrombo modulin, AST= aspartate aminotransferase, ALT=alanine aminotransferase, LDH=lactate dehydrogenase, HDmiR=hepatocyte-derived miRNA, CDmiR=cholantiocyte-derived miRNA, DBD=donation after brain death, DCD=donation after circulatory death, n.d. = not defined. *= graft type assumed to be DBD, derived from the year of publication.

Endothelial injury markers: hyaluronic acid & thrombomodulin

The absence of blood and oxygen causes ischemic- and preservation injury to cells of the liver sinusoids[53]. Hyaluronic acid (HA) is a high-molecular weight glycosaminoglycan (4 - 8 million kD) formed by the cellular plasma membrane[54] and its uptake mainly occurs by sinusoidal endothelial cells of the liver[55]. In clinical LT, a disruption of the hepatic micro-vascular integrity by preservation injury was shown to reduce the uptake of HA from the circulation, causing levels of HA in the liver to rise, which subsequently lead to EAD[56]. Comparable studies by Bronsther et al.[57] and Rao et al.[58] provided stronger evidence for HA to be associated with PNF and diminished graft survival after LT; levels over 400 µg/L in the perfusate had a highly negative predictive value of 95%. Furthermore, these studies demonstrated a correlation between HA levels in perfusates and post-operative AST and ALT levels in recipients. In the setting of NMP, Brockmann et al. found HA levels during NMP as one of their most significant predictors for graft viability after LT in a large animal model[35]; the mean level of HA in perfusates of successful grafts was 108 ng/ml, while non-succesful grafts released much higher HA levels (6087 ng/ml).

Another endothelial cell marker is Thrombomodulin (TM), which has potential anticoagulant effects if it forms a complex with thrombin. When the vascular endothelium of liver sinusoids is injured for instance by graft preservation, TM is inactivated by cleavage into smaller fragments of so-called soluble thrombomodulin (sTM) and it is subsequently released from the cell surface[59-64]. Suehiro et al.[65] found TM levels over 20 FU/ml in perfusates to be sensitive for identifying grafts with PNF or EAD after LT. These grafts showed a higher expression of TM on liver sinusoidal endothelial cells at the end of cold storage. In a smaller study performed by Sido et al, intraoperative sTM levels were measured in blood to assess graft endothelial reperfusion injury[60]. After reperfusion, sTM levels correlated significantly with release of liver enzymes and increased adherence of leukocytes in liver tissue. In clinical LT, however, only one study investigated TM as a predictor for outcome and graft quality[65] and no data are known on the potential use of TM as a marker for viability testing in the setting of MP.

Inflammatory markers, kupffer cells and proteolytic enzymes

Graft ischemia induces an inflammatory cascade that attracts leukocytes and neutrophils to the site of tissue injury and subsequent leakage of proteolytic enzymes, causing breakdown of cells and surrounding tissue post-reperfusion[66]. In a retrospective study that derived from the first clinical trial applying HMP for LT, Henry et al. investigated the effect of HMP on the expression of several injury markers[26]. Oxidative stress markers as Hypoxia-inducible factor-1α and -1β were significantly decerased in biopsies that were taken at the end of HMP, compared to SCS grafts. Also the expression of inflammatory markers like tumor necrosis factor-α (TNF- α) were significantly lower in grafts

already at time of HMP. The authors hypothesize that these pro-inflammatory factors are eliminated through the diluting effects of HMP, thereby also reducing the production of down-stream chemokines and adhesion molecules like intercellular adhesion molecule-1 and P-selectin. This hypothesis was supported by the observation that at the end of HMP, less infiltrating kupffer cells (CD68 positive) were present in tissue compared to SCS biopsies. Berberat et al.[67] found several inflammatory genes in post-reperfusion biopsies predictive of graft outcomes; high expression of TNF-α was correlated with shortened graft survival, while high c-reactive protein expression correlated with the need of interventions after LT. A linear combination of five down regulated vascular genes was superior in forecasting graft related complications, with a positive predictive value of 72% and negative predictive value of 96%. Calmus et al.[68] also demonstrated a strong correlation between ongoing proteolysis during SCS and EAD; increased levels of free amino acids that were released from the liver into perfusates showed good positive- and negative-predictive value (respectively 100% and 95%) for EAD in the first postoperative week. As Henry et al. and Calmus et al. show, it is feasible to measure inflammatory markers and proteolytic enzymes during cold graft preservation prior to reperfusion. However, the strongest effect on these markers usually becomes apparent after revascularization of the graft[25] and therefore it would be highly interesting to observe the predictive value of these markers in normothermic conditions. Up to now, many MP studies only investigate such markers after reperfusion[25, 32].

Tissue hemorrhage and cell necrosis

The degree in which tissue is affected by graft ischemia varies and is usually reflected by histopathological changes. Xu et al. investigated these histological changes during NMP of porcine liver grafts[38]. A remarkable finding was that the degree of necrosis and apoptosis in biopsies taken after warm ischemia and subsequent cold storage, appeared to be reversed after 4h of NMP. This not only suggests that histological evaluation at time of NMP might be a useful indicator for graft viability, it also indicates that NMP has the potential to recover ischemic damage. This has also been suggested by other NMP studies that performed histological evaluation after reperfusion[23, 39]. The prognostic value of necrosis and apoptosis occurring during SCS was also evaluated in different tissues from clinical studies; Khettry et al. demonstrated extensive hemorrhage and/or necrosis of 10-50% in the donor gallbladder mucosa to have a high positive- and negative predictive value for PNF and impaired graft survival, whereas vascular congestion was present in all donor gallbladders[69]. In addition, Abraham et al. identified apoptotic cells and zone 3 hemorrhage in post-reperfusion liver tissue to have good discriminative power for PNF (AUC=0.90 and 0.77 respectively)[70].

Degree of graft steatosis

Beside DCD, steatotic livers form another important source within the category of ECD grafts that could benefit from improved preservation and subsequent graft outcome by MP. Bessems et al.[20] found improved functional parameters in steatotic rat livers after HMP compared to normal preservation by SCS. Similar beneficial effects were observed by Vairetti et al, who concluded that subnormothermic temperatrues are preferred over colder temperatures for the recovery of steatotic grafts[71]. Despite exciting results on MP for optimizing the quality of steatotic grafts, these studies were not informative on potential biomarkers prior to reperfusion. However, a more recent study by Jamieson et al. measured a decrease in lipid deposits during NMP of rat livers which correlated with a reduction in the degree of steatosis[21]. Previous clinical studies showed the value of histological macro vesicular steatosis to predict graft PNF, which has been extensively reviewed earlier[72]. Dutkowski et al.[73] integrated the degree of steatosis in a balance of risk score with other risk factors for graft failure, consisting of recipient age, MELD-score, re-transplantation, cold ischemia, and donor age. This score indicates that one should be reluctant with the use of moderate to severe steatotic liver grafts (>30%) in recipients with a balance of risk-score ≥9, but microvesicular steatosis has not been related to poorer outcome. Though histological scoring in steatotic grafts seems promising in the setting of both MP and SCS, in general, one should be aware for the risks of intra- and inter observer variability that hampers a standardized histological evaluation.[74]

MARKERS FOR BILIARY INJURY

As previously explained, bile ducts of particularly ECD grafts have been shown to be vulnerable for ischemic injury and are responsible for a high percentage of graft loss (**Fig. 1**). Therefore, biliary complications are also an important outcome for several MP studies. Up to now, MP studies on human liver grafts (**Table 1**) have shown that MP is not harmful for bile ducts, but most studies are too small to demonstrate whether a significant benefit actually exists[24, 29, 37]. Schlegel et al. recently demonstrated beneficial effects of HOPE on biliary fibrosis, but no markers were investigated during HOPE on their predictive capacity for biliary injury[31]. Several clinical studies however identified markers in tissue and perfusates during SCS that were able to predict biliary complications.

Peribiliary epithelial damage and vascular injury

Brunner et al. developed a bile duct damage-score based on the degree of injury in the epithelium of the extrahepatic bile duct and diminished epithelial barrier integrity

measured by tight junction proteins[12]. Samples of common bile duct tissue showing more than 10% of destructed epithelium or/and subepithelial connective tissue at the beginning of cold preservation were predisposed to develop major biliary complications and diminished graft survival. Also Hansen et al.[75] scored extrahepatic bile duct specimens and found arteriolonecrosis causing mural necrosis to be the most prominent risk factor for ITBL. Similar observations were recently reported in a larger cohort studied by op den Dries et al.[76]. Additionally, the investigators found that grafts that would develop ITBL, lost over 50% of cells within deep peribiliary glands that are located along the common bile duct and which are involved in cholangiocyte proliferation in response to injury[77, 78]. Based on their findings, the authors formulated the hypothesis that ITBL results from an insufficient regenerative capacity of injured cholangiocytes by peribilary glands, caused by arteriolonecrosis in the bile duct wall, rather than being the result of extensive epithelial injury alone[79]. Remarkably, the degree of injury in peribiliary glands did not differ between DBD and DCD grafts. Beside changes in the arterial vasculature of the peribiliary plexus, a case-control study by Farid et al. showed changes in the luminal size of the portal vein branch (PVB) in liver tissue specimens to be more pronounced after reperfusion[80]; a smaller PVB size was seen in grafts that later developed ITBL. This supports earlier findings on the importance of portal blood flow, which is responsible for approximately 40% of the blood supply in the common bile duct, for the risk to develop ITBL[81, 82]. Unfortunately, differences in PVB size became apparent only after reperfusion.

Cholangiocyte-derived microRNAs

MicroRNAs (miRNAs) are small regulatory RNAs with high cell-type specificity and their resistance against RNAse mediated degradation in different media and conditions makes them an attractive candidate for biomarker research[83-85]. Hepatocyte-derived miRNAs (HDmiRs) were identified as sensitive markers in serum for acute graft rejection and more recently, our group reported that lower levels of cholangiocyte-derived miRNAs (CDmiRs) in perfusates during SCS are predictive of ITBL in both DBD and DCD grafts[86, 87]. In this study, miRNAs remained stable in University of Wisconson (UW) and histidine-tryptophan-ketoglutarate (HTK) perfusates, also after incubation at room temperature. Preliminary data show that miRNAs can also be measured during MP (data not shown). Furthermore, HDmiRs and CDmiRs are also released into bile[88]. Interestingly, a very recent study shows that recipients developing ITBL have an altered miRNA composition in bile[89].

DISCUSSION

As dynamic preservation is now entering the clinic, researchers emphasize on the need of predictive and sensitive biomarkers that are able to objectively assess graft quality during MP. Biomarkers could help to enlarge the donor pool by objectively screen liver grafts that initially would be discarded based on their predisposing characteristics. Several experimental studies already demonstrated that a combination of biomarkers measured during MP could be used as a damage index for ECD grafts[45, 90]. However, since the clinical application of MP is still in its infancy, the introduction of such damage scores based on surrogate biomarkers should be studied in larger cohorts. Prospective randomized clinical trials on MP would offer the best opportunity for unbiased evaluation of potential biomarkers, provided that sampling of materials during MP is executed accurately. Moreover, such trials could also definitely answer the question which MP strategy is most capable of optimizing ECD graft quality.

The requirements for a biomarker to make it into clinical practice are that its measurement should be easy and relatively fast, with a high sensitivity and specificity for outcome. Moreover, biomarkers should be measurable in biomaterials that are available at time of graft preservation, so its discriminative capacity could be used in graft screening and allocation[91]. Biopsies from liver or extrahepatic bile duct specimens can be collected during preservation and are suitable for histological evaluation and quantification of injury based on (low) expressed biomarkers. It should however be emphasized that biopsies are obtained invasively and only represent a small part of the liver or bile duct, which could lead to incorrect interpretation when injury is unequally distributed throughout the tissue (**Table 4**). Moreover, inter- and intra-observer variability can hamper a standardized evaluation of histological markers. The collection of perfusates form an attractive non-invasive alternative for a variety of markers during conventional preservation and MP. Another advantage of using perfusates over tissue biopsies is that larger volumes can be collected and markers released into perfusates are believed to represent the condition of the entire liver parenchyma rather than only a small part of the liver. Limitations consist of difficulties in the normalization of markers; most MP systems use a recirculating perfusion system, in which biomarkers can accumulate. Therefore, perfusate levels of conventional biomarkers like AST could differ from standards in clinical practice. This also applies to perfusion temperature; hypothermic conditions will cause a delayed metabolism of the liver and requires an adjusted evaluation of biomarkers and cut-off values compared to normothermic, physiologic conditions .

Table 4. Materials for biomarker measurement during graft preservation. Summary on the advantages and disadvantages between the different biomaterials that can be used to asses graft quality at time of MP or during SCS prior to LT.

Biomaterial	Advantages	Disadvantages
Tissue	Histological evidence for graft quality Large amount of cells	Invasive Only local representation Risk of inter- or intra obser variability
Perfusate	Non-invasive Larger quantities available Suitable application for various types of MP	Timing; short before implantation No standardized workup between LT centers
Bile	Non-invasive Indicative for hepatocyte and cholangiocyte function Suitable application for MP	Less informative during hypothermic conditions smaller quantities available

A limitation for many biomarkers in general is that their quantification can be labour intensive and time consuming. Some techniques, for instance polymerase chain reaction, are however progressing in terms of accelerated measurements, which makes them applicable in the prolonged time-window created by MP[92, 93].

In general, biomarkers can be used either to determine graft injury or graft function. Up to now, most biomarkers concern markers for injury, while bile production currently is the only marker for liver function. Robust markers of function rather than injury are however of importance, because severe ischemic injury not necessarily means that a graft will not function properly following LT. Additional markers of function could consist of subtrates which do not naturally occur in the body, but are cleared by the liver. For instance the plasma disappearance rate of intravenously administered indocyanine green (PDR-ICG) or ^{13}C-labeled methacetin (LiMAx test), which are predictive of PNF, EAD and hepatic artery thrombosis after LT [94-97]. However, results of such tests are influenced by perfusion flow rates[98, 99]. Moreover, functional markers require a metabolically active liver, which can only be achieved under (sub)normothermic conditions (**Fig. 4**).

Beside biomarkers for injury and function, it is evident that donor- and recipient risk factors can influence outcome after LT (**Fig. 2**). Genetic polymorphisms in both donors and recipients have been identified that increase the risk for recipients to develop ITBL or bacterial infections after LT[100-102]. Therefore, genetic profiling could be helpful in matching donors to equivalent recipients[91]. Moreover, information on donor and recipient risk factors are usually available in an early stage of LT[103].

Key points

- The increased use of extended criteria grafts demands for more objective and sensitive biomarkers to evaluate the large discrepancy of graft quality in LT.
- Measurement of prudent biomarkers during machine preservation (MP) could be helpful in the prediction of early graft performance after LT.
- During MP, surrogate biomarkers for graft quality could help select the most optimal preservation technique before implantation.
- Research shows discriminative potential of a variety of biomarkers for graft injury and function, but requires robust validation in larger cohorts before applicable in the clinic.
- Non-invasive evaluation of biomarkers released into perfusates during MP is an attractive alternative for invasively obtained tissue biopsies.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The limited experience of MP in clinical LT hampers the evaluation on which MP strategy is most optimal for graft quality and the evaluation of potential biomarkers for quality assessment. Another factor that hampers evaluation of biomarkers is the inconclusiveness between studies on outcome definitions; investigators maintain different criteria for comparing cohorts, making it impossible to perform a reliable meta-analysis on outcomes describing corresponding markers. More clear international guidelines on outcome definitions are therefore recommended, as was previously initiated by Olthoff et al.[9]. Comparing biomarkers during MP and conventional SCS, we can however conclude that non-invasive measurement of injury markers into perfusates and the assessment of liver function based on the production of bile are well-possible in MP. For all markers, however, one should take into account the baseline differences that can exist between donors, liver grafts and MP techniques that influence biomarker measurements and pleas for custom criteria and cut-off values in the evaluation of biomarkers[10]. This review forms a starting point for future studies on quality assessment by biomarkers and graft screening in the changing setting of graft preservation and MP in clinical LT in the coming years.

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SUPPLEMENTARY INFORMATION

METHODS

Literature search

Two systematic literature searches in Embase, Ovid Medline and Cochrane were performed to identify potentially relevant studies regarding biomarkers in machine perfusion (MP) and in conventional static cold-storage LT (SCS) that were published until January 2014, as described in **Supplementary Table 1** and **Table 2**. The search regarding biomarkers in MP consisted of the following key elements: liver transplantation, biological markers and machine perfusion/preservation. The search regarding biomarkers in conventional LT consisted of: liver transplantation, biological markers and preservation. All elements were searched using the corresponding thesaurus terms (EMTREE or MeSH) or words in title or abstract.

Supplementary Table 1. search terms used for identifying biomarkers using MP.

Database	Search terms
Embase, Medline and Cochrane	('liver perfusion'/de OR ((liver/exp OR (liver OR hepatic):ab,ti) AND ((machine* OR hypotherm* OR cold OR normoterm* OR warm* OR subnormotherm* OR oxygenated) NEAR/3 (perfusion* OR preservation*)):ab,ti)) AND (marker/de OR 'biological marker'/de OR 'molecular marker'/de OR 'biochemical marker'/de OR 'genetic marker'/exp OR 'pharmacological biomarker'/de OR biomarker*:ab,ti OR marker*:ab,ti)

For studies regarding biomarkers in liver MP, we mainly focussed on studies using human liver grafts. Because of the low incidence of clinical MP, we also included experimental studies on liver MP that have been performed in animal models. To amplify and validate whether biomarkers in MP are useful, we also searched for comparable data in clinical LT studies using conventional SCS for graft preservation. Inclusion criteria were as follows: (i) Biomarkers should be measured in donor graft material, and (ii) should be obtained during graft preservation, and (iii) they should be associated with impaired graft quality resulting in impaired graft viability (in case of MP) or be predictive for PNF, EAD, or the occurrence of biliary complications after static cold storage in LT (see definitions). Studies describing steatosis as a marker for early graft dysfunction were excluded, since this has been reviewed extensively earlier. The Oxford Centre for Evidence-based Medicine Level of Evidence scale was used to assess methodological quality of potentially relevant publications.

Supplementary Table 2. search terms used for identifying biomarkers during graft SCS.

Database	Search terms
Embase	('liver transplantation'/exp OR ((liver* OR hepatic*) NEAR/3 (Transplant* OR graft*)):ab,ti) AND (marker/de OR 'biological marker'/de OR 'molecular marker'/de OR 'biochemical marker'/de OR 'genetic marker'/exp OR 'pharmacological biomarker'/de OR biomarker*:ab,ti OR marker*:ab,ti) AND ('preoperative period'/exp OR 'perioperative period'/de OR 'intraoperative period'/de OR prediction/de OR 'predictive value'/de OR (((pre OR before OR per OR prior OR intra OR during OR peri) NEAR/6 (transplant* OR surg* OR donat* OR operat*)) OR pretransplant* OR pertransplant* OR predonat* OR perdonat* OR presurg* OR persurg* OR intrasurg* OR preoperat* OR peroperat* OR perioperat* OR intraoperat* OR predict* OR (early NEXT/1 marker*)):ab,ti) NOT ([Animals]/lim NOT [humans]/lim)
Medline	(liver transplantation/ OR ((liver* OR hepatic*) ADJ3 (Transplant* OR graft*)).ab,ti.) AND (exp Biological Markers/ OR biomarker*.ab,ti. OR marker*.ab,ti.) AND ("preoperative period"/ OR "perioperative period"/ OR "intraoperative period"/OR (((pre OR before OR per OR prior OR intra OR during OR peri) ADJ6 (transplant* OR surg* OR donat* OR operat*)) OR pretransplant* OR pertransplant* OR predonat* OR perdonat* OR presurg* OR persurg* OR intrasurg* OR preoperat* OR peroperat* OR perioperat* OR intraoperat* OR predict* OR early marker*).ab,ti.) NOT (exp Animals/ NOT humans/)
Cochrane	(((liver* OR hepatic*) NEAR/3 (Transplant* OR graft*)):ab,ti) AND (biomarker*:ab,ti OR marker*:ab,ti) AND ((((pre OR before OR per OR prior OR intra OR during OR peri) NEAR/6 (transplant* OR surg* OR donat* OR operat*)) OR pretransplant* OR pertransplant* OR predonat* OR perdonat* OR presurg* OR persurg* OR intrasurg* OR preoperat* OR peroperat* OR perioperat* OR intraoperat* OR predict* OR early marker*):ab,ti)
Pubmed	(((liver*[tiab] OR hepatic*[tiab]) AND (Transplant*[tiab] OR graft*[tiab]))) AND (biomarker*[tiab] OR marker*[tiab]) AND ((((pre[tiab] OR before[tiab] OR per[tiab] OR prior[tiab] OR intra[tiab] OR during[tiab] OR peri[tiab]) AND (transplant*[tiab] OR surgery[tiab] OR surgical[tiab] OR donat*[tiab] OR operat*[tiab])) OR pretransplant*[tiab] OR predonat*[tiab] OR presurg*[tiab] OR persurg*[tiab] OR intrasurg*[tiab] OR preoperat*[tiab] OR peroperat*[tiab] OR perioperat*[tiab] OR intraoperat*[tiab] OR predict*[tiab] OR early marker*[tiab])) AND publisher[sb]

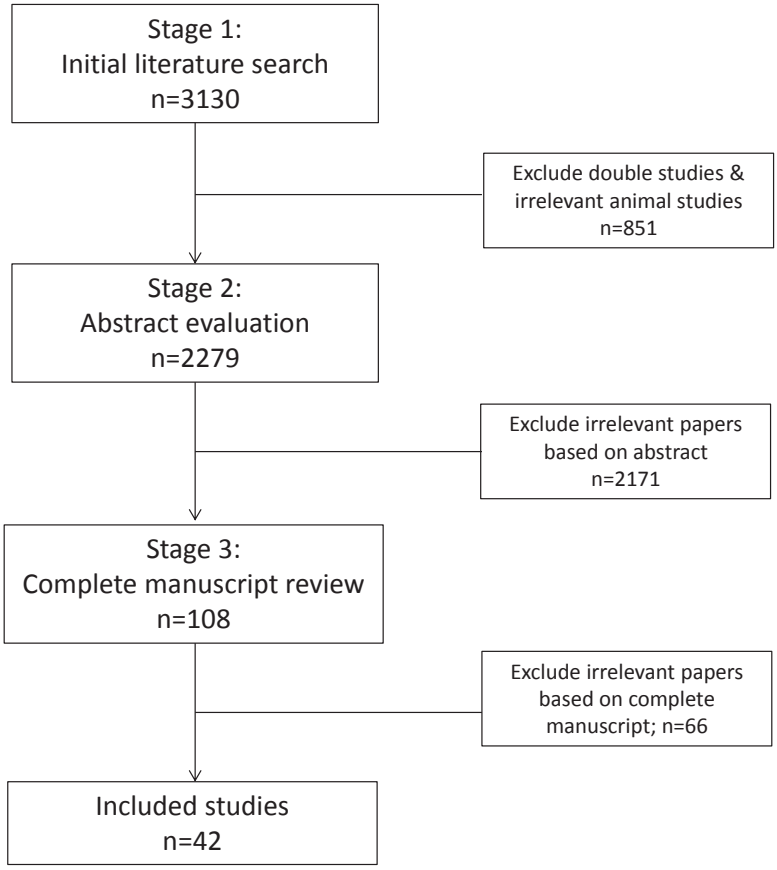
Definitions

Primary non-function was defined as the occurrence of liver re-transplantation or patient death within the first week after LT. For EAD, most studies maintained different criteria, but usually consisted of the following parameters: elevation of AST and/or ALT above a defined cut-off value (1500-2000) within the first week after LT, elevated international normalized ratio on day 7 after LT, elevated bilirubin levels on day 7 after LT, or graft loss within the first month after LT. Biliary complications following LT included bile duct leakage, necrosis, anastomotic strictures and ITBL. Ischemic-type biliary lesions were defined as (i) symptomatic, non-anastomotic strictures and associated dilatations of the intrahepatic or extrahepatic hilar bile duct(s) after LT, which (ii) were confirmed

by cholangiography and in the presence of a patent hepatic artery as demonstrated by Doppler ultrasonography, and (iii) requiring endoscopic or percutaneous interventions of the biliary system or liver retransplantation in recipients.

Literature screening

The selection of relevant publications was performed by three stages of evaluation performed by two authors independently (CV and WF) and a final random check by two others (GK and LL). At first, studies were excluded if they concerned duplicate studies in the three databases, animal research (except for MP studies), reviews and conference abstracts (**Supplementary Fig. 1**, stage 1). At the second stage, manuscript abstracts were evaluated for their relevance; research papers describing organ transplantation other than deceased donor LT, or not using biomarkers at time of graft preservation were excluded. The remaining studies were completely reviewed at stage 3.



Supplementary Figure 1. Inclusion scheme for relevant publications.

Methodological quality of clinical studies was assessed by using the Oxford Centre for Evidence-based Medicine Level of Evidence scale (CV and GK).

Part II

Risk factors for biliary complications

Chapter 3

Liver grafts procured from donors after circulatory death have no increased risk of microthrombi formation

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ABSTRACT

Microthrombi formation provoked by warm ischemia and vascular stasis is thought to increase the risk of non-anastomotic biliary strictures (NAS) in liver grafts obtained by donation after circulatory death (DCD). Therefore, potentially harmful intraoperative thrombolytic therapy has been suggested as a preventive strategy against NAS. Here, we investigated whether there is histological evidence of microthrombi formation during graft preservation or directly post-reperfusion in DCD livers and the development of NAS. Liver biopsies collected at different time points during graft preservation and post-reperfusion were stained *in triplo* with hematoxylin-eosin (HE), Von Willebrand Factor VIII (VWF), and Fibrin Lendrum (FL) to evaluate the presence of microthrombi. In a first series of 282 sections obtained from multiple liver segments of discarded DCD grafts, microthrombi were only present in 1-3% of the VWF stainings, without evidence of thrombus formation in paired HE and FL-stainings. Additionally, analysis of 132 sections obtained from matched, transplanted brain death (DBD) and DCD grafts showed no difference in microthrombi formation (11.3% vs. 3.3% respectively, P=0.082), and no relation to the development of NAS (P=0.729). Furthermore, no microthrombi were present in perioperative biopsies in recipients who developed early hepatic artery thrombosis. Finally, the presence of microthrombi did not differ before or after additional flushing of the graft with preservation solution. **Conclusion:** The results of our study derogate from the hypothesis that DCD livers have an increased tendency to form microthrombi. It weakens the explanation that microthrombi formation is a main causal factor in the development of NAS in DCD, and that recipients could benefit from intraoperative thrombolytic therapy to prevent NAS following liver transplantation.

INTRODUCTION

Biliary complications following liver transplantation (LT) are associated with high morbidity and mortality rates in recipients[1, 2]. In particular non-anastomotic strictures (NAS) located in the hilar and intrahepatic regions of the liver, which appear in 15-30% of the LT recipients, have been associated with shortened graft survival[3]. In severe cases, the distribution of NAS makes them less accessible for endoscopic or percutaneous intervention and often requires a more extensive treatment[4, 5]. Eventually, re-LT due to NAS is necessary in up to 15% of LT recipients, underscoring the severity of this condition[6].

Several risk factors have been identified for the development of NAS, but its pathogenesis is still not fully understood. Immune-mediated risk factors such as ABO-incompatibility[7], CMV infection[8], and primary sclerosing cholangitis as underlying disease[9, 10] increase the risk of NAS. A second category of risk factors include bile salt toxicity against cholangiocytes[11-13]. However, as the incidence of NAS is significantly higher in grafts donated after circulatory death (DCD) compared to those donated after brain death (DBD), a leading role has been attributed to ischemia-reperfusion injury[2, 3, 6, 9]. Possible mechanisms for NAS in DCD livers could be the detrimental effects of insufficient flushing of the graft on the biliary epithelium or to the microcirculation of the peribiliary plexus (PBP)[14, 15].

The involvement of vascular obstruction, in particular the hepatic artery that provides the majority of blood supply to the PBP, and the development of NAS has been demonstrated by multiple studies. Hepatic artery thrombosis (HAT) was shown to be a major risk factor for severe NAS, often requiring re-LT in recipients due to graft failure[16, 17]. Although conflicting data exists on the occurrence and pathophysiology of HAT[18], some studies suggest that their incidence is higher in DCD grafts[19]. Experimental studies showed a positive correlation between the length of warm ischemia time and bile duct injury due to occlusion of the PBP, induced by post-transplant hepatic arterial ischemia[20, 21]. Beside the hepatic artery, 40% of the microvascular blood flow originates from the portal vein, which when obstructed can also cause severe NAS[22-24].

In contrast to DBD grafts, DCD grafts experience additional warm ischemia time with no-flow status in the period between cardiac arrest and in situ cold perfusion. Due to the diffuse presentation of NAS and its relation with compromised blood flow, it is hypothesized that DCD grafts have an increased tendency to form microthrombi in the microcirculation of the liver during warm ischemia time[25]. This could subsequently lead to inadequate flushing of the capillary network with suboptimal cold preservation and exacerbation of ischemic injury following reperfusion[26]. In order to improve the flush of the microcirculation, an additional arterial back-table pressure perfusion has been recommended[14]. Additionally, some transplant centers advocate the use of thrombolytic agents in preservation solutions to prevent formation of microthrombi[27]. Several

studies suggest that arterial perfusion of DCD livers with urokinase or tissue plasminogen activator prior to implantation may reduce the incidence of NAS[25, 28]. However, such management could result in severe complications, such as perioperative bleeding, making many centers reluctant to use thrombolytic agents in the transplantation setting.

To date, only a limited number of studies have performed a histological evaluation of microthrombi formation in DCD livers, with the primary emphasis on extrahepatic bile duct tissue[15, 29]. Therefore, it remains questionable whether the aforementioned hypothesis on the role of intrahepatic formation of microthrombi in NAS is correct and whether it justifies intraoperative treatment with aggressive thrombolysis. To address this question, we performed a comprehensive histological study on the presence of microthrombi formation at time of graft preservation and following reperfusion in liver tissue biopsies from; (i) a series of extended criteria DCD liver grafts that were discarded for human LT, and (ii) a series of transplanted DCD and DBD liver grafts that developed NAS or HAT following LT.

MATERIALS AND METHODS

Study design and donor livers

For the current study, we retrospectively analyzed two series of prospectively collected liver biopsies. The first series of biopsies were obtained from extended criteria DCD donors that were discarded for human LT between July 2012 and December 2013 (see **supplementary information** for further inclusion details). As these grafts were discarded for human LT, a more extensive sampling of biopsies was possible. In order to correlate the formation of microthrombi with the development of NAS, a second series of biopsies obtained from liver grafts transplanted between January 2007 and December 2012 were analyzed as well. Microthrombi formation was compared between DCD and DBD grafts and between grafts that developed NAS or remained free of biliary complications. Biopsies were selected from LT procedures with comparable donor-, recipient- and procedural characteristics, so matched groups would only differ based on graft type and outcome, namely DBD – no NAS, DBD – NAS, DCD – no NAS, and DCD – NAS. Finally, biopsies from explanted liver grafts that developed HAT and required re-transplantation were analyzed as a control group. An illustration of the inclusion of liver grafts and the study design is provided in **Fig. 1A**.

Graft procurement, graft preservation, and collection of tissue biopsies

All liver grafts in this study were procured via standard procedure and conformed to Dutch organ retrieval protocols. Details regarding organ retrieval and liver perfusion are described in the supplementary information. **Fig. 1B** provides an outline of the time

points for collecting the first series of biopsies that were obtained from central and peripheral segments of the left and right liver lobes of discarded DCD grafts by an 18G Tru-Cut® biopsy needle. Biopsies were retrospectively selected for histological evaluation if they were available from similar liver segments at a minimum of two different time points per discarded DCD graft. **Fig. 1C** provides an outline of the collection time points for the second series of biopsies from transplanted DBD and DCD grafts, which were wedges of liver tissue from the left lateral segment. All collected biopsies from

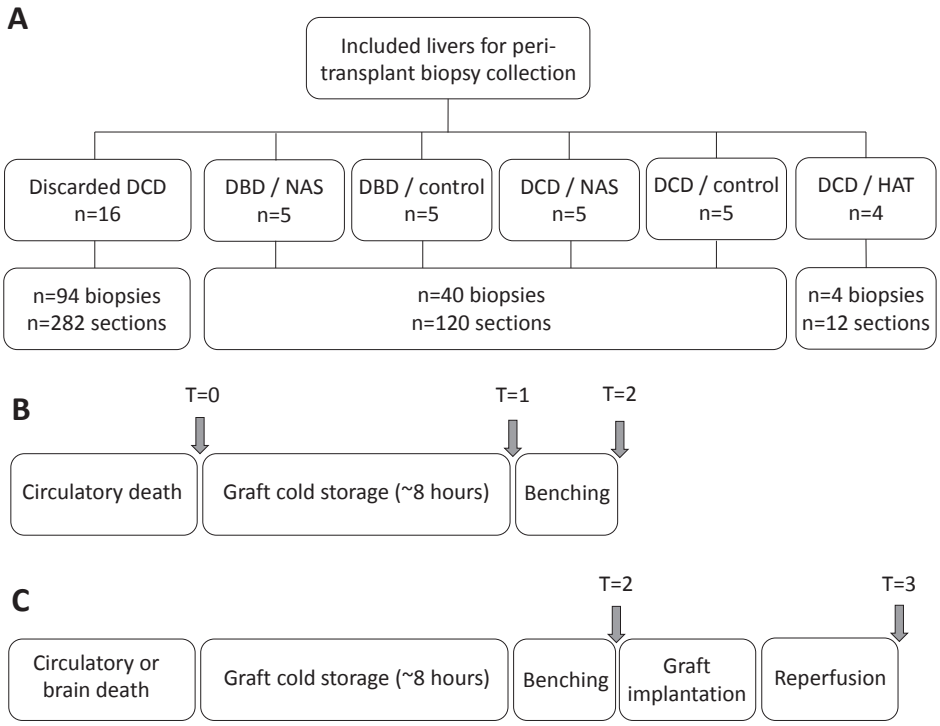


Figure 1. Study design. (A) Inclusion scheme of liver grafts for the collection of peri-transplant liver tissue biopsies. (B) Schematic illustration of time points for collection of the first series of liver biopsies obtained from DCD grafts discarded for human LT. Directly following organ retrieval, needle biopsies were taken (t=0) with an 18G Tru-Cut® biopsy needle from left and right-sided liver segments before grafts were transported to our center. During transportation, grafts remained cold stored for at least 6-8 hours and subsequently, biopsies were taken from similar segments as at time of organ retrieval (t=1). In order to mimic the normal transplantation procedure, after cold storage, the liver was additionally flushed *ex situ* with 1000 ml UW or HTK solution, depending on the preservation fluid initially used during procurement. After this second flush, the final series of biopsies were taken (t=2). (C) Comparable to discarded grafts, transplanted livers also received an additional *ex situ* perfusion with 1000 ml of UW or HTK upon arrival at the operating room. This was followed by flushing with 600ml of human albumin solution (Albuman human albumin 40g/l, Sanquin, The Netherlands). Next, a wedge of liver tissue from the anterior side of the left lateral segment was taken at the end of cold ischemia (comparable to t=2 in discarded DCD livers). After implantation, a paired wedge of liver tissue was collected following graft arterial and venous reperfusion (t=3). Explant biopsies from four DCD grafts that developed HAT following LT were used as a control group.

both discarded as well as transplanted liver grafts were directly fixated in 4% formaldehyde for a minimal duration of 24 hours and subsequently embedded in paraffin blocks (FFPE-blocks). All FFPE-blocks were stored at the department of Pathology in our center according to the latest regulations of the International Organization for Standardization (ISO) for optimal preservation of the blocks with less risk of protein degradation for histochemical staining and immunohistochemistry. For more detailed information regarding the collection of biopsies, see the **legend of Fig.1**.

Stainings and histological scoring for microthrombi

All biopsies were cut into 4µm sections and evaluated on the presence of microthrombi using three different staining protocols; hematoxylin-eosin (HE), Fibrin Lendrum (FL) histochemical staining and immunohistochemistry for von Willebrand Factor VIII (VWF), performed with anti-Factor 8 polyclonal antibody (Dako, Glostrup, Denmark). Placental tissue and endothelial tissue of maximally two to three years old were used as positive controls for FL and VWF staining's, respectively. This is comparable to the age of FFPE-blocks used for the second series of biopsies from transplanted DBD and DCD grafts.

For the VWF staining, deparaffinization and staining of sections was processed automated by a Ventana BenchMark ULTRA Stainer (Ventana, Tucson Arizona, USA) according to manufacturers' instructions at the department of Pathology. Peroxidase-coupled antibodies were detected using 3,39 - diaminobenzidine as a substrate and the slides were counterstained with haematoxylin. This staining method has been validated for use in patient care, and human placenta was used as a positive control for every section.

For the FL staining, sections were deparaffinized and rehydrated by graded alcohol series. Subsequently, sections were stained at room temperature with the following solutions: Weigert's Iron Haematoxylin for core staining (10 min.), 1% acid alcohol for differentiation (30 sec.), Martius Yellow for erythrocyte staining (3 min.), Crystal Ponceau solution for Fibrin staining (5 min.), Phospotungstic acid solution for collagen (2 times 5 min.), and Methyl blue solution for connective tissue staining (5 min.). Between each staining step, sections were thoroughly rinsed with tap water. After staining, sections were rapidly dehydrated through ascending ethanol series, cleared in xylene substitute, and covered with pertex. Endothelial tissue was used as a positive control.

For the HE staining, sequentially sliced sections were cut, deparaffinized and stained using a fully automated Ventana Discovery Stainer (Ventana, Tucson Arizona, USA) according to the manufacturers' instructions for HE staining. All sections were evaluated for the presence of microthrombi by two experienced liver transplant pathologists (MD and KB) who were blinded to the clinical data.

Definitions

Definitions of HAT and NAS are provided in the supplementary information.

Statistical analysis

Statistical analysis was performed using SPSS statistics 20 (SPSS Inc, Chicago, IL, USA). Group comparisons were performed using Mann-Whitney U or Kruskal-Wallis-test for continuous data and X^2-test for categorical data. P-values < 0.05 were considered statistically significant.

RESULTS

Donor characteristics of discarded, extended criteria DCD liver grafts

For the analysis of the first series of biopsies, 16 extended criteria DCD liver grafts, discarded for human LT, were included in this study. Donor demographics and procedural variables are summarized in **Table 1**. The primary reasons for discarding the liver graft for transplantation were donor age >60 years (62.5%; median of 64 years), followed by high donor BMI >28 (12.5%). The majority of the livers were obtained from male donors (62.5%) and UW was more frequently used for preservation than HTK (62.5%). Clinical blood values of the donors were comparable to those in transplanted liver grafts (listed in **Table 2**). Median first warm ischemia time from circulatory arrest until cold perfusion was 17 minutes, while the functional first warm ischemia time from saturation <70% or systolic blood pressure <50 mmHg until cold perfusion was 29 minutes.

No histological evidence of microthrombi formation during preservation of extended criteria DCD grafts

From 16 DCD grafts, 94 liver biopsies were collected at three different time points during graft preservation (**Fig. 1A-B**). With a standard of three sections per biopsy, a total of 282 sections were available to analyse for the presence of microthrombi; 63 sections at t=0, 111 sections at t=1 and 108 sections at t=2. All included liver biopsies contained a median of 23 portal triads (IQR 14-33) suitable for histological evaluation. A more extensive collection of biopsies was possible after graft arrival at the hospital, explaining the larger number of biopsies at t=1 and t=2.

In sections stained with HE and FL, no microthrombi could be identified by both pathologists (**Fig. 2A-C**). Only 4 out of 282 sections (1.4%) showed positive staining for VWF in arterial vessels within the portal triad (**Fig. 2E**). However, paired HE and FL sections did not provide convincing evidence of thrombus formation (**Fig. 2D and F**). Two positive stainings were found at t=0, prior to graft transportation and cold storage (**Fig. 2J**). Additional positive stainings were found at the end of cold storage (t=2) and after additional flushing at the back table (t=3). The formation of microthrombi showed no preference for central, peripheral, left or right liver segments.

Table 1. Clinical variables of extended criteria DCD grafts that were discarded for human LT.

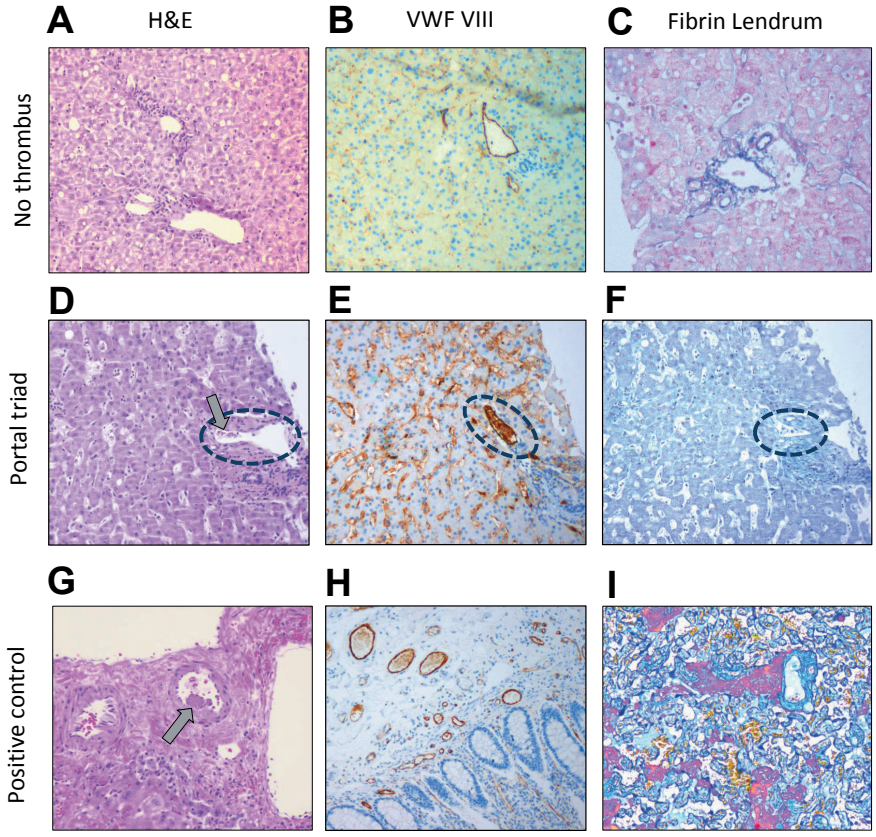
	Number (n=16)
Donor characteristics	
Age (yr)	64 (54-70)
Male/Female	10/6
Body mass index	25 (22-33)
Cause of death	
Cardiovasculair event	2 (12.5%)
Cerebrovasculair accident	5 (31.3%)
Trauma	4 (25%)
Other	5 (31.3%)
Clinical blood values at time of donation	
AST (U/L)	49 (29-90)
ALT (U/L)	35 (18-65)
GGT (U/L)	55 (25-111)
Total bilirubin (μmol/L)	7 (5-8)
Procedure variables	
Preservation solution and volume (cc)	
UW (n=10)	10000 (-)
HTK (n=6)	8000 (7250-9000)
First warm ischemia time (min)[1]	17 (14-18)
Functional 1st warm ischemia time (min)[2]	29 (21-35)
Reasons for discarding	
Donor age >60 yr	10 (62.5%)
Donor BMI >28	2 (12.5%)
Elevated donor transaminases	1 (6.3%)
No cardiac arrest 60 min following switch-off	1 (6.3%)
Hypoperfusion	1 (6.3%)
Steatosis	1 (6.3%)

[1] Time from circulatory arrest until cold perfusion

[2] Time from saturation <70% or systolic blood pressure <50 mmHg until cold perfusion (min)

AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transferase; UW, University of Wisconsin solution; HTK, histidine-tryptophan-ketoglutarate solution; BMI, body mass index. For numerical variables, values represent the median (interquartile range), for categorical variables, values represent the number (percentage).

Fig.2H and 2I show stainings in endothelial and placental tissue, which served as positive controls. As a positive control for HE staining, a biopsy obtained from an explanted graft that developed HAT after initial LT was analyzed. **Fig. 2G** shows a positive control for HE staining, with an arterial thrombus in the hilar region of the liver graft at day six following LT. However, analysis of wedge biopsies from the same graft at the end of cold ischemia and post-reperfusion did not reveal any microthrombi formation.



J

Staining	T=0 (n=63)	T=1 (n=111)	T=2 (n=108)	Total (n=282)
H&E	0 (0%)	0 (0%)	0 (0%)	0 (0%)
VWF VIII	2 (3%)	1 (1%)	1 (1%)	4 (1%)
Fibrin Lendrum	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Figure 2. Histological grading of liver biopsies from discarded DCD liver grafts for the presence of microthrombi. **Panel A-C**: portal triad in a liver biopsy with no signs for thrombus formation in none of the *in triplo* stained sections. **Panel D-F**: portal triad in a liver biopsy with a small amount of fibrin in the portal vein (D), doubtful thrombus formation in VWF immunostaining (E) and negative in FL (F). Magnifications x10. **Panel G-I**: positive control for HE with thrombus formation in the branch of a hepatic artery in the hilar region of a liver that developed HAT following LT (G). Positive controls for thrombus detection with VWF staining in endothelial tissue (H) and FL staining in placental tissue. (I) Magnifications x20. **Panel J**: number (%) of sections with positive staining for microthrombi per time point and per staining in discarded DCD grafts.

Donor and recipient characteristics of transplanted liver grafts

To validate our findings from discarded grafts in the clinical setting and test the hypothesis of microthrombi formation causing NAS, a second series of biopsies obtained from twenty liver grafts was also evaluated. Biopsies from ten grafts that developed NAS were compared with biopsies from ten grafts that remained free of biliary complications. Groups were matched for graft type to analyze the occurrence of microthrombi in DCD compared to DBD grafts. Out of ten recipients who developed NAS, re-transplantation was required in seven. Three other recipients needed frequent endoscopic treatment with stent placement and biliary dilatation or surgical revision. As a positive control group, biopsies of four explanted DCD livers from recipients who developed early HAT were included, in which we expected to find microthrombi in the liver microvasculature. These recipients had to undergo re-transplantation within the first 2-6 days following primary LT.

As shown by **Table 2**, groups were matched with respect to donor variables (graft type, age, donor risk index), recipient variables (age, MELD score) and procedural variables (ischemia times, preservation solution, intraoperative blood loss). Indications for LT did not differ between groups (p=0.665) and consisted of acute liver failure (n=3), hepatitis C virus (n=8), cholestatic liver disease (n=3), alcoholic steatohepatitis (n=3), non-alcoholic steatohepatitis (n=4), Wilson disease (n=1), polycystic liver disease (n=1) and porphyria (n=1).

Interestingly, the international normalized ratio (INR), a marker for standardized prothrombin time or coagulation activity in blood, was not different between groups (**Table 2**, P=0.687). Also intra-operative blood loss and the volume of blood transfusions did not significantly differ (P≥0.290). However, serum of levels of AST and ALT were significantly increased directly post-surgery in recipients who developed NAS and HAT (P=0.017 and P=0.036, respectively). Injury markers at time of donation were not different between groups.

No histological evidence of microthrombi formation during preservation and post-reperfusion in liver grafts that developed NAS

Of each transplanted liver, a wedge of liver tissue was collected at two perioperative time points (**Fig. 1C**); one biopsy at the end of cold ischemia (t=2) and one post-reperfusion (t=3). Just like the first series, sections from this second series of biopsies were stained *in triplo*, providing a total of 120 sections for histological evaluation of microthrombi.

Fig. 3B shows the number of sections that were positive for (partial) microthrombi formation, stratified for graft type (DBD vs. DCD) and outcome (development of NAS vs. controls). Out of 120 sections, seven showed partial microthrombi formation (5.8%); four in VWF sections, of which three also showed had a positive HE staining. Again, all FL stainings were negative for microthrombi. Microthrombi formation was not increased in DCD grafts compared to DBD grafts (**Fig. 3C**, 3.3% vs. 11.3%, respectively, P=0.083). Furthermore, the formation of microthrombi did not differ between grafts that remained free of biliary complications and those that developed NAS (8.3% in controls vs. 6.6% in NAS, P=0.729).

Table 2. Clinical variables of grafts that developed NAS, HAT, or remained free of biliary complications. Groups were matched for donor, recipient and procedural variables.

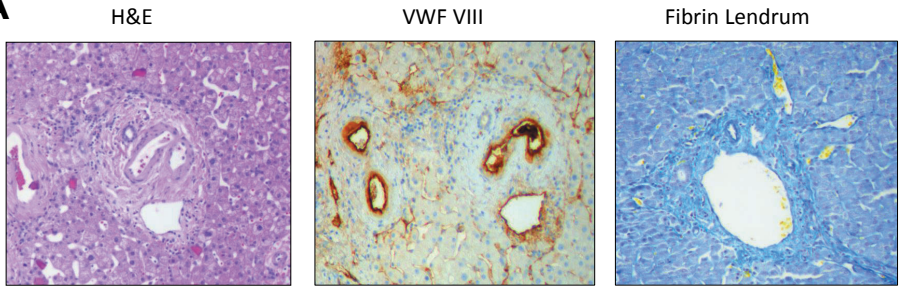
	Control (n=10)	NAS (n=10)	HAT (n=4)	P-value
Donor characteristics				
Donor risk index	2.1 (1.6-2.4)	2.0 (1.8-2.5)	2.0 (1.8-2.4)	0.99
Age (yr)	55 (46-69)	57 (51-62)	45 (41-61)	0.31
Body mass index	24 (23-28)	26 (24-26)	23 (22-24)	0.13
DBD/DCD	5/5	5/5	0/4	0.18
Male/female	5/5	4/6	2/2	0.93
Cause of death				0.42
Cardiovascular event	0	1	0	
Cerebrovascular accident	5	8	3	
Trauma	3	0	1	
Other	2	1	0	
Clinical blood values at time of donation				
AST (U/L)	43 (28-127)	46 (32-79)	41 (16-87)	0.65
ALT (U/L)	38 (11-61)	34 (23-62)	30 (10-52)	0.62
GGT (U/L)	30 (21-34)	52 (23-85)	91 (40-143)	0.09
ALP (U/L)	58 (46-68)	60 (46-122)	58 (45-69)	0.80
Total bilirubin (μmol/L)	7 (6-10)	10 (7-14)	7 (5-7)	0.20
Recipient characteristics				
Age (yr)	52 (36-61)	54 (41-57)	51 (44-61)	0.96
Male/female	5/5	5/5	3/1	0.66
MELD score	23 (20-26)	22 (20-31)	22 (18-28)	0.82
Clinical blood values directly post LT				
AST (U/L)	754 (558-1089)	2474 (1458-5133)	2165 (1554-7589)	**0.02**
ALT (U/L)	572 (457-810)	1742 (812-3585)	1466 (733-4370)	**0.04**
GGT (U/L)	50 (33-128)	117 (54-150)	149 (86-304)	0.24
ALP (U/L)	71 (48-90)	72 (57-133)	75 (62-161)	0.67
Total bilirubin (μmol/L)	110 (54-147)	74 (41-132)	57 (13-115)	0.41
INR	1.8 (1.7-2.7)	2.1 (1.7-3.0)	2.2 (1.9-2.8)	0.69
Procedure variables				
Cold ischemia time	345 (284-388)	367 (343-445)	360 (339-444)	0.45
First WIT (min)[1]	12 (10-19)	18 (16-26)	17 (12-19)	0.18
Functional 1st WIT (min)[2]	28 (17-32)	30 (22-36)	23 (15-26)	0.35
Second WIT (min)	28 (25-32)	31 (26-37)	30 (23-42)	0.56
Preservation UW/HTK	3/7	6/4	0/4	0.09
Preservation volume (cc)	8500 (5750-15500)	8000 (5000-10625)	12500 (7750-15000)	0.40
Blood loss (cc)	3050 (1575-5563)	3500 (1950-6050)	4800 (3525-8400)	0.40
Blood transfusion (cc)	4047 (1554-5381)	4675 (1995-9388)	6333 (4621-6994)	0.29

[1] Time from circulatory arrest until cold perfusion.

[2] Time from saturation <70% or systolic blood pressure <50 mm Hg until circulatory arrest (min).

For numerical variables, values represent the median (interquartile range). For categorical variables, values represent the number (percentage).

A



B

Staining (nr. sections)	DBD - NAS (n=30)	DBD - control (n=30)	DCD - NAS (n=30)	DCD - control (n=30)
H&E	1 (3%)	2 (6%)	1 (3%)	0 (0%)
VWF VIII	1 (3%)	3 (10%)	1 (3%)	0 (0%)
Fibrin Lendrum	0 (0%)	0 (0%)	0 (0%)	0 (0%)

C

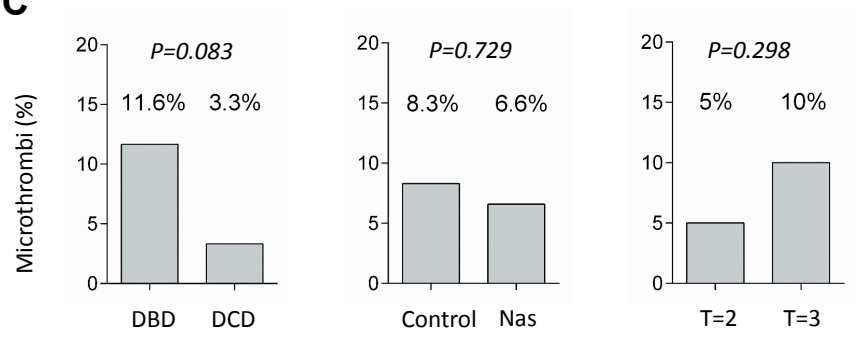


Figure 3. Histological grading of liver biopsies obtained from transplanted liver grafts that developed NAS or remained free of biliary complications, which were matched for clinical variables such as graft type. (A) The panels represent the *in triplo* staining of a biopsy for HE, VWF and FL. (B) Shown are the number (%) of positive staining for microthrombi per staining, per graft type and per outcome in transplanted livers. (C) Microthrombi formation was not significantly increased in DCD grafts (P=0.083). The number of positive stainings was similar between grafts that remained free of biliary complications and those that developed NAS (P=0.729). Finally, formation of microthrombi was not significantly increased following reperfusion (P=0.298).

Comparable to the first series, the formation of microthrombi was not significantly different between various time points of biopsy collection (5% at t=2 vs. 10% at t=3; P=0.298).

Finally, we also evaluated the formation of microthrombi in the microvasculature of four DCD liver grafts (n=12 sections) which developed early HAT following LT. Macroscopic investigation of the explanted liver grafts and microscopic evaluation of hilar sections showed clear formation of partially obstructive thrombi in the arterial vessels, resembling the positive control in the first series. In biopsies outside the hilar region, however, none of the three stainings showed any formation of microthrombi in the microvasculature of grafts

that developed HAT. Moreover, biopsies from the implantation procedure at the end of cold ischemia and post-reperfusion did not show any signs of microthrombi formation as well.

DISCUSSION

This study shows that neither preservation with standard flushing, nor vascular stasis during warm ischemia increases the risk of microthrombi formation in DCD grafts during LT. Previous studies hypothesized that prolonged warm-ischemia with no flow-status in the period between circulatory arrest and in situ cold perfusion causes the formation of microthrombi in the microcirculation of DCD livers. Additionally, suboptimal preservation due to an inadequate flush of the liver microcirculation could exacerbate ischemic injury and cause NAS in LT recipients. This caused several transplant centers to perform additional flushing of the hepatic artery with thrombolytic agents. However, the results presented here show no histological evidence of microthrombi formation in DCD livers that would justify the use of potentially harmful, intraoperative fibrinolytic therapy during LT. Furthermore, in DBD nor DCD grafts could we correlate the presence of microthrombi to the development of NAS.

Based on the results of four clinical studies that have investigated the administration of tissue plasminogen activator (tPA) and urokinase during LT, it still remains inconclusive whether such treatment prevents the development of NAS in recipients[25, 27, 28, 30]. The first and largest study by Lang et al.[27] performed a double flush of the hepatic artery with urokinase in DCD grafts during the benching procedure. They found a significantly decreased incidence of intrahepatic NAS (1.4%) in grafts pre-treated with urokinase (n=140) compared to untreated grafts (5.9%, n=220). A more recent study by Seal et al.[28] also found a lower incidence of intrahepatic NAS in 85 DCD grafts which received tPA just before anastomosis and reperfusion of the portal vein and hepatic artery (3.5% vs. 21.2% in 33 controls). The benefit of tPA in this study was demonstrated in two different transplant centers. In contrast, two other studies could not demonstrate beneficial effects of tPA and urokinase. Pietersen et al. recently described similar incidences of NAS in DBD and DCD grafts that were treated with arterial urokinase administration immediately before implantation (18%)[30]. Three studies did not experience increased risk of excessive postoperative bleeding in recipients. This complication was however present in 64% of the recipients in a study by Hashimoto et al.[25], who investigated the administration of tPA into the hepatic artery during the back-table procedure in a small series of 22 DCD grafts. Even after tPA treatment, six grafts developed biliary complications, of which two developed NAS. This underlines the potential danger of using anti-coagulative therapy during LT, even after researchers attempted to wash-out remaining thrombolytic agents by additional flushing with perfusate solution before graft

implantation. Nonetheless, unfavorable donor factors and previous laparotomy could also contribute to bleeding complications as argued by Seal et al. The main limitations of the aforementioned studies were the retrospective character, lack of randomization and short follow-up in treatment groups. The use of historical controls could bias the outcome of the tPA and urokinase intervention studies, because there is evidence of an overall improvement in DCD outcomes regardless of whether tPA is used. Therefore, the potential benefit demonstrated by these studies might be based on factors as center experience, procurement technique and donor-recipient selection rather than tPA or urokinase treatment. A decisive answer on the usefulness of thrombolytic therapy would require a randomized study, but this still will not provide direct evidence for the hypothesis of increased microthrombi formation in DCD grafts.

To our knowledge, the current study is the first that performed a comprehensive histological analysis of microthrombi formation in intrahepatic liver tissue biopsies with two additional stainings specific for coagulation activity. Biopsies were obtained from multiple liver segments of extended criteria DCD grafts discarded for LT and DCD grafts that developed NAS. Taking into account that even though NAS is more prevalent in the extrahepatic bile duct, it particularly causes severe problems when occurring in the intrahepatic bile ducts[31]. Previous studies on the topic of histological parameters and NAS mainly focused on biopsies from the extrahepatic bile ducts. Two studies analyzing the microcirculation of the extrahepatic bile ducts demonstrated that no microthrombi were found in 31,3% and 26,3% of the microcirculation of the extrahepatic bile ducts after reperfusion, respectively[15, 29]. However, arteriolonecrosis and the loss of peribiliary gland mass, but not the formation of microthrombi, were associated with the development of NAS. In addition, Op den Dries et al. found that the formation of microthrombi in the peribiliary plexus did not differ between DBD and DCD liver grafts[15, 32]. Altoghether, these findings lead to a new hypothesis, stating that arteriolonecrosis of the common bile duct disables cholangiocyte regeneration by peribiliary glands. The histological injury found at the distal end of the extrahepatic bile duct was representative for proximal, intrahepatic large bile ducts[33]. Also, a study performed by Brunner et al. found severe epithelial injury and diminished epithelial barrier integrity in bile ducts from DBD grafts that developed biliary complications following LT[34]. Beside the current study, Farid et al. investigated perioperative intrahepatic liver histology and NAS. They reported a smaller luminal size of the portal vein branch in post-reperfusion biopsies from DBD livers that developed NAS, supporting an earlier observation that diminished portal flow is also involved in biliary blood supply[24]. However, this study also found no thrombi in the intrahepatic arterial or venous vasculature. All the aforementioned studies observed a negligible degree of microthrombi formation at time of graft preservation that was not related to the development of NAS, supporting the results of the current study.

To determine whether microthrombi formation is indeed more prevalent in DCD grafts, biopsies from matched DBD grafts were also evaluated. This confirmed our finding from the first series that DCD grafts do not appear to have an increased tendency to form microthrombi during preservation. It furthermore weakened the hypothesis that microthrombi formation is the main underlying cause of NAS. It seems unlikely that the microthrombi that we found in a minority of cases of particularly DBD grafts, which only covered part of the vascular lumen, were of clinical significance. Recipients from these DBD grafts, that remained free of biliary complications, had significantly lower serum levels of AST and ALT post-operatively compared to the NAS group, suggesting that the present microthrombi did not cause additional injury. Higher postoperative transaminase levels in recipients who developed NAS, however, is in concordance with other studies[35, 36]. This further supports the thought that injury, and not increased coagulation, is the leading factor in the pathophysiology of NAS.

Until now, most clinical studies investigating the presence of microthrombi used HE-staining as the gold standard for histological scoring[15, 29]. Although HE provides insight on the degree of tissue and epithelial disruption, as well as morphological features of cells, it might be less specific for detecting the presence of coagulation factors[37, 38]. In order to minimize the chance of missing microthrombi formation, we also applied additional stainings with Von Willebrand Factor VIII and Fibrin Lendrum. At the tissue level, Von Willebrand Factor VIII marks endothelial cell activation, which occurs earlier in the coagulation cascade, whereas Fibrin deposits occur at the end of the cascade. The fact that all biopsies were negative for FL staining suggests that no older microthrombi, that could have formed during graft procurement, are present after cold storage or even following reperfusion. However, the fact that we mainly observed microthrombi in the VWF staining's demonstrates possible increased endothelial activity earlier in the coagulation cascade. It is unlikely that these more fresh microthrombi will transform into fibrin clots in a later phase, since the VWF positive staining's in our study were not related to the development of NAS.

The current study contains several limitations, most importantly the possibility of sampling bias as biopsies only provide a local representation of the liver tissue. In particular for the scoring of microthrombi, certain areas of the liver might be overlooked when analyzing biopsies. Also, the included number of discarded livers was limited. Furthermore, there might be inter- or intra-observer variability of histological findings. We tried to deal with these issues by collecting a large number of biopsies from multiple liver segments, by staining sections with various coagulation-specific markers, and have evaluation performed by two independent pathologist. The large amount of biopsies taken from both extended criteria as well as transplanted DCD grafts showed a uniform absence of microthrombi in the microcirculation of all liver segments. Whether additional staining of the larger intra- and extrahepatic bile ducts with FL and VWF could

provide more insight into microthrombi formation in the peribiliary vascular plexus should be investigated by future research. Beside histological evaluation, Vendrell et al. investigated coagulation profiles in the blood of DCDs by thromboelastometry assays[39]. They found hyperfibrinolysis induced by all DCDs, suggesting no increased tendency of microthrombi formation. Although we did not perform thromboelastography, postoperative INR values in recipients from our study suggest no increased coagulation activity in DCD grafts that developed NAS. Another limitation is that the collection of intrahepatic liver biopsies from multiple segments, like the first series of DCD grafts, is invasive and disruption of the tissue could cause severe perioperative complications[40]. Therefore, it is not justified to collect such intrahepatic biopsies in grafts that are suitable for clinical LT. However, recent studies already initiated a shift in methods to assess graft quality away from histology by measuring early and non-invasive biomarkers during graft preservation)[35, 40].

In conclusion, the results of our study suggest that DCD livers do not appear to have an increased tendency to form microthrombi during graft preservation or following graft reperfusion. Also, formation of microthrombi was not associated with the development of NAS or HAT. Thus, in our opinion, the absence of histological evidence for microthrombi formation contradicts with the use of intraoperative thrombolytic therapy that could contribute to additional, unnecessary risks for complications in recipients after LT.

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SUPPLEMENTARY INFORMATION

MATERIALS AND METHODS

Study design and donor livers

For the first series, discarded DCD livers were procured and included in this study between July 2012 and December 2013, when the following criteria were fulfilled; (i) if donor age was between 18-80 years, (ii) if the deceased donor was officially registered as an organ donor or, in case of unknown registration, if the next of kin gave permission for organ donation, (iii) if organs other than the liver were procured for transplantation, (iv) if the liver was not accepted for transplantation due to medical reasons, and (v) if the next of kin gave informed consent to use the donor liver for scientific research. The criteria for using discarded livers from deceased donors for scientific research are extensively described in a study protocol that was accepted by the medical ethical committee in our center, as well as by the Dutch Committee on Organ Donation and the Dutch Transplantation Foundation. The donor livers included in this study were procured in the same way as livers used for clinical LT.

In a second series, we investigated microthrombi formation and the development of NAS in transplanted liver grafts, by retrospectively analyzing perioperative biopsies which were collected as part of standard clinical practice during LT at our center. Biopsies were selected from both DBD and DCD grafts that were matched for clinical parameters and were transplanted between January 2007 and December 2012. The use of donor materials was approved by the Medical Ethical Committee and all patients provided informed consent for the use of clinical information for medical research.

Organ retrieval and liver perfusion

During each multi-organ retrieval, a standard *in situ* pressurized perfusion of the liver was performed via the aorta with University of Wisconsin solution (UW; Viaspan, Duramed Pharm Inc, Pomona, NY) or histidine-tryptophan-ketoglutarate solution (HTK; Custodiol HTK, Essential Pharmaceuticals, LLC, Pennsylvania, USA) in order to remove remnant blood. Heparin was added to the first bag of preservation solution in a dose of 25,000 to 35,000 IU. An additional *ex situ* perfusion via the portal vein was performed during the back-table procedure with 1000 ml of UW or HTK depending on the preservation fluid that was initially used during retrieval. In transplanted livers, this step was followed by flushing with 600 ml of human albumin solution (Albuman human albumin 40g/l, Sanquin, The Netherlands) just prior to implantation of the graft. Surgical implantation procedures were identical between DBD and DCD grafts, with initial portal vein reconstruction and reperfusion.

Definitions

Non-anastomotic biliary strictures (NAS) were defined as (i) symptomatic strictures and associated dilatation of the intrahepatic or hilar bile duct(s) after LT, 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 re-transplantation in recipients. Transplant recipients without biliary complications during follow-up were defined as controls.

Hepatic artery thrombosis (HAT) was defined as the (complete) absence of blood flow through the arterial vessels of the donor liver from the site of the common hepatic artery of the donor's site, proven by duplex ultra-sound and/or CT-angiography. Donor and recipient characteristics and clinical parameters were obtained from the LT database of the institution.

Chapter 4

Fucosyltransferase-2 polymorphism is a risk factor for non-anastomotic biliary strictures after liver transplantation in recipients with primary sclerosing cholangitis

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ABSTRACT

Background. Non-anastomotic biliary strictures (NAS) are a common cause of graft loss after liver transplantation (LT). Similarities in clinical manifestation between NAS and primary sclerosing cholangitis (PSC) suggest a shared pathophysiology. The aim of this study was to investigate whether a polymorphism affecting fucosyltransferase-2 (FUT2) secretor status, a recently discovered risk factor for PSC and inflammatory bowel disease, is associated with the development of NAS after LT.

Materials and methods. In DNA obtained from 418 LT procedures, genotyping for FUT2 was performed in 255 donors, 356 recipients and 193 paired donors-recipients.

Results. As expected, FUT2 non-secretor status was more prevalent in patients with underlying PSC compared to non-PSC patients (33% vs. 22%, P=0.034). FUT2 non-secretor status in donors and non-PSC recipients was not associated with NAS. In patients with PSC, however, recipient FUT2 non-secretor status was the most important independent risk factor for NAS (cox-regression HR: 2.34, P=0.034), with an incidence of 56% already during the first five years after LT (log-rank P=0.002). Analysis of paired donor-recipient genotypes showed that transplantation of a graft from a FUT2-secretor donor into a FUT2 non-secretor PSC recipient further increased the risk for developing NAS (P=0.002). Finally, FUT2 non-secretor status was higher among patients with ulcerative colitis, but coexistence of ulcerative colitis with PSC did not increase the risk of NAS.

Conclusion. Donor FUT2 non-secretor status has no effect on the risk of NAS. However, FUT2 non-secretor status of PSC recipients is an independent risk factor for NAS after LT. This is possibly related to an increased bacterial translocation or an aggravated immune response induced by FUT2 mismatching between donors and recipients.

INTRODUCTION

Non-anastomotic biliary strictures (NAS) are a common complication after liver transplantation (LT) and have a detrimental effect on patient morbidity and graft survival. Narrowing of the bile ducts typical for NAS can be centrally located in the (sub)hilar region, but it can also occur more diffusely throughout the graft[1]. Therefore, NAS often present themselves through severe obstructive jaundice in LT recipients, requiring expensive and physically invasive endoscopic treatment, or even necessitate re-transplantation in 15% of the cases[2, 3].

Although the exact pathophysiology of NAS is not fully understood, previous studies identified several risk factors. Most importantly, grafts obtained by donation after circulatory death (DCD) have an up to three-fold increased risk to develop NAS compared to grafts obtained by donation after brain death (DBD)[4]. It is hypothesized that prolonged warm ischemia of the graft, typically associated with DCD, damages biliary epithelium and peribiliary glands. Subsequently, this damage is said to cause insufficient regenerative capacity of bile ducts[5]. Longer after LT, also immunological factors contribute to deformations of the bile ducts. These immunological factors include various genetic polymorphisms and underlying autoimmune diseases in recipients.[6, 7] In particular recipients who are transplanted because of primary sclerosing cholangitis (PSC) are prone to develop NAS both early as well as late after LT[8, 9].

The high incidence of PSC in Nordic countries makes this disease the leading indication for LT in the North European region[10]. PSC is characterized by irregular sclerosis of the intrahepatic bile ducts, it has been associated with inflammatory bowel disease (IBD) such as ulcerative colitis[11], and it affects males more often than females (3:2). Just as with NAS, patients suffering from PSC are often treated for biliary obstructive jaundice through endoscopic retrograde pancreatico-cholangiography (ERCP) or percutaneous transhepatic cholangiography (PTC).

Genome-wide association studies identified various single nucleotide polymorphisms (SNPs) associated with the development of PSC[12]. In particular a mutation in the fucosyltransferase-2 (FUT2) gene, responsible for the glycosylation of proteins, accelerates the disease course in PSC and reduces the transplantion-free survival of these patients[13, 14]. This rs608133 mutation (G>A) causes a truncated, dysfunctional FUT2 protein on the surface of epithelial cells throughout the body. It is thought that these so called FUT2 non-secretors have a perturbed composition of their cellular glycocalyx and, therefore, are less resistant against the translocation of bacteria and other pathogens from the intestinal epithelium to extra-intestinal sites such as the liver and bile ducts. Subsequently, translocation of bacteria to the liver may result in inflammatory responses and fibrosis[15, 16].

Similarities in the clinical manifestation between PSC and NAS suggest a potential overlap in pathophysiological mechanisms. Since FUT2 non-secretor status is associated with PSC, this raised the hypothesis that the rs608133 polymorphism might also be associated with the development of NAS. Therefore, in this study we investigated the association between FUT2 non-secretor status and the development of NAS in a large LT cohort by genotyping both donors and recipients.

MATERIALS AND METHODS

Study design

In this longitudinal cohort study, DNA samples were collected prospectively during consecutive, adult LTs between February 1990 and December 2011 at the Erasmus University Medical Center, Rotterdam. Available DNA samples from donors and recipients were retrospectively genotyped for the FUT2 mutation rs608133. The primary outcome measure of this study was the time to diagnosis of NAS following LT, which was compared between different categories based on FUT2 status of donors, or recipients, or of paired donors and recipients. Secondary outcome measures were graft and recipient survival. Besides the association between FUT2 status and development of NAS in the entire cohort, a sub analysis was performed in PSC and non-PSC recipients, since pathophysiologic effects of mutations in FUT2 have been described particularly in the context of PSC. Cases of hepatic artery thrombosis (HAT) or primary non-function (PNF) directly after LT were excluded. Partial- or split liver LTs were excluded as well because of their small number and the unique surgical procedure that is different from whole LT. Donor and recipient characteristics and clinical parameters were obtained from the LT database of the institution. The Medical Ethical Committee of the Erasmus MC approved the use of donor and recipient DNA and all patients provided informed consent for the use of clinical information and their materials for medical research.

Definitions

Non-anastomotic biliary strictures were defined as described previously[17], namely as (i) symptomatic tapering or narrowing with associated dilatation of the intrahepatic or (sub)hilar bile ducts after LT, (ii) confirmed by cholangiography or MRCP 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 re-transplantation in recipients. The 'no NAS' group consisted of transplant recipients without biliary complications during follow-up and recipients who had biliary complications other than NAS, such as isolated anastomotic strictures and bile leakage.

Time to event was calculated from the date of LT until the date of intervention associated with NAS (i.e. biliary stenting by ERCP, bile drainage by PTC or surgical intervention). Graft survival was calculated from the date of LT until the date of re-LT or recipient death. Patient survival was calculated from the day of LT until the date of death. The final date for collection of follow-up was 20th February 2016, 4.2 years after inclusion of the last LT patient.

Sample collection and genotyping

Donor DNA was obtained from splenocytes that were sampled together with the liver after graft procurement. Recipient DNA was derived from peripheral blood collected from patients at time of hospital admission just prior to surgery. DNA was isolated using the Wiyard Genomic DNA Purification Kit (Promega Corp., Madison, Wisconsin, USA), according to the manufacturers' instructions and stored at -20°C until further use. SNP genotyping for rs601338 was performed by LGC Genomics, Teddington, UK (http://www.lgcgroup.com/genotyping), using a PCR based KASP genotyping technology and as previously described by De Mare-Bredemeijer et al.[18].

Statistical analyses

Results of continuous data are expressed as medians (interquartile range; IQR) unless stated otherwise. Statistical analysis was performed using SPSS statistics 20 (SPSS Inc, Chicago, IL, USA). Analyses on both ungrouped as well as grouped FUT2 genotypes were performed. For the grouped analysis, donors or recipients with a homozygous mutation (=AA) were considered to be FUT2 non-secretors, while donors or recipients with either GA or GG genotype were considered as FUT2 secretors. Group comparisons were performed using Mann-Whitney U tests for continuous data and two-sided chi-square test for categorical data. Associations with the time to diagnosis of NAS were conducted by Kaplan-Meier analysis and log-rank test. Prediction analyses were constructed through backward stepwise Cox proportional hazards regression analysis. P-values smaller than 0.05 were considered as statistically significant.

RESULTS

Donor and recipient characteristics

Between November 1989 and December 2011, 721 adult LTs were performed in our center. All transplantation procedures were ABO-compatible. From 453 transplantations, DNA from either donor, recipient or from both donor and recipient was available for FUT2 genotyping, as illustrated by the inclusion scheme in **Fig. 1**. After exclusion of

cases of HAT (n=26), PNF (n=6) and split LTs (n=3), 418 LT procedures were included for final analysis, consisting of 385 primary LTs and 33 re-LTs.

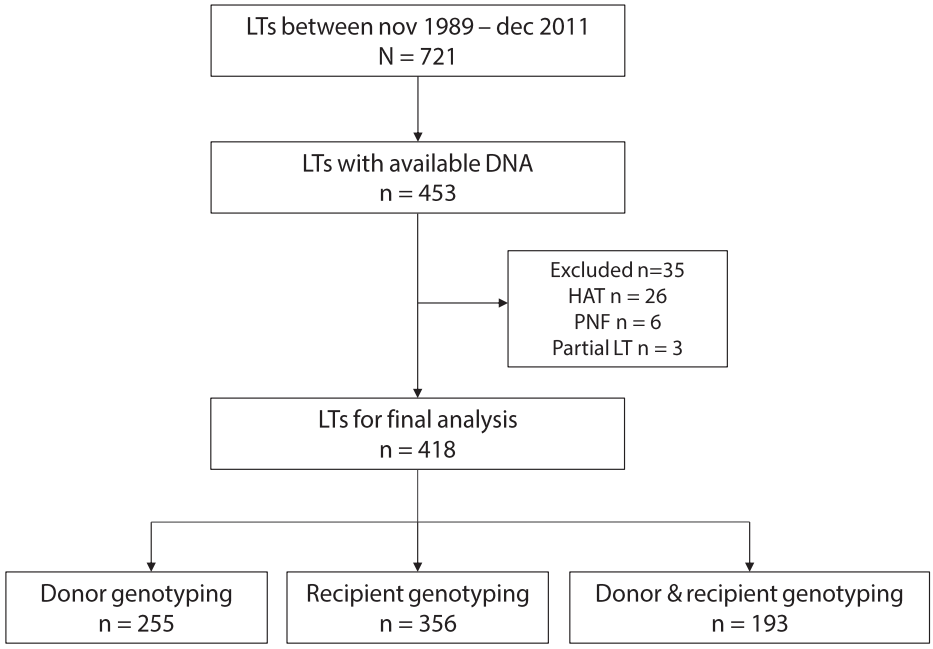

Figure 1. Inclusion scheme for genotyping of donor and recipient DNA. From transplantations performed between November 1989 and December 2011, DNA from either donors, recipients or both was available from 453 LT. Thirty-five cases were excluded due to HAT, PNF or partial LT. For final analysis, FUT2 genotype was available of 255 donors, 356 recipients and 193 paired donors and recipients, in a total number of 418 LT's.

DoLTs., recipient and procedural variables are listed in **Table 1**. The median duration of follow-up of the entire study cohort in years was 9.2 (5.0-14.7). Out of 418 transplants, 73 resulted in the development of NAS (17.5%), with a median time to diagnosis of 380 days (102-1345) after transplantation. In primary LTs, viral hepatitis was the main indication for LT in the no NAS group, while PSC was the main indication in the NAS group. NAS was the main indication for re-LT (19 out of 33 re-LTs), and the incidence of recurrence of NAS was significantly higher in this group (8 out of 19 re-LTs for NAS, P=0.002). Graft survival was significantly lower in the NAS group than in the no NAS group (5.3 versus 9.1 years), though recipient survival was similar. In the NAS group, biliary anastomosis was more often performed via hepaticojejunostomy (P<0.001). Also, NAS occurred more frequently in DCD grafts, which in most cases were preserved using HKT solution (P=0.004). Male to male transplantation occurred more frequently in the NAS group (P<0.001).

Table 1. Donor and recipient characteristics and procedural variables between recipients who developed NAS and those who did not develop NAS following LT.

	No NAS (n=345)	NAS (n=73)	P-value
Donor characteristics			
Age (yr)	46 (33-55)	47 (35-57)	0.391
Men/women	166/179	43/30	0.094
Rhesus (neg/pos)	54/291	8/65	0.305
CMV (neg/pos)*	164/180	42/30	0.125
Recipient characteristics			
Age (yr)	50 (41-58)	47 (36-55)	0.056
Men/women	207/138	49/24	0.256
Rhesus (neg/pos)	46/299	13/60	0.318
CMV (neg/pos)*	154/190	36/36	0.345
Primary LT (n=385), indication			
Viral hepatitis	90 (26.1%)	7 (9.6%)	**0.003**
PSC	56 (16.2%)	31 (42.5%)	**<0.001**
Alcoholic	50 (14.5%)	9 (12.3%)	0.717
Cryptogenic	29 (8.4%)	2 (2.7%)	0.106
PBC	22 (6.4%)	4 (5.5%)	0.833
ALF	19 (5.5%)	6 (8.2%)	0.326
AIH	16 (4.6%)	3 (4.1%)	0.896
Other	38 (11.0%)	3 (4.1%)	-
Re-LT (n=33), indication			
NAS	11 (3.2%)	8 (11.0%)	**0.002**
Other	14 (4.1%)	0 (0%)	-
Graft failure ending in re-LT (%)	9 (2.6%)	27 (37%)	**<0.001**
Graft survival (yr)	9.1 (4.8-14.9)	5.3 (2.2-10.0)	**<0.001**
Recipient survival (yr)	9.4 (5.0-15.1)	8.7 (4.8-12.3)	0.331
Procedural variables			
Graft type (DBD/DCD)	318/27	58/15	**<0.001**
Preservation solution			**0.004**
UW	289	49	
HTK	54	23	
Other	2	1	
cold ischemia time (min)	451 (359-590)	449 (396-555)	0.759
2nd warm ischemia time (min)	36 (26-59)	32 (26-44)	0.150
Biliary anastomosis			
Duct-to-duct/Roux-Y	281/64	43/30	**<0.001**
Recipient/donor			
Sex match			**<0.001**
male/male	99 (28.7%)	37 (50.7%)	
male/female	108 (31.3%)	12 (16.4%)	
female/male	67 (19.4%)	6 (8.2%)	
female/female	71 (20.6%)	18 (24.7%)	
Rhesus match			0.493
neg/neg	8 (2.3%)	1 (1.4%)	
neg/pos	38 (11%)	12 (16.4%)	
pos/neg	46 (13.3%)	7 (9.6%)	
pos/pos	253 (73.3%)	53 (72.6%)	

Table 1. Donor and recipient characteristics and procedural variables between recipients who developed NAS and those who did not develop NAS following LT. (continued)

	No NAS (n=345)	NAS (n=73)	P-value
CMV match			0.314
neg/neg	71 (20.6%)	21 (28.8%)	
neg/pos	83 (24.1%)	15 (20.5%)	
pos/neg	92 (26.7%)	21 (28.8%)	
pos/pos	97 (28.1%)	15 (20.5%)	

***** In both the No NAS and the NAS group, one donor and recipient CMV status was missing.
CMV, cytomegalovirus; PSC, primary sclerosing cholangitis; PBC, primary biliary cirrhosis; ALF, acute liver failure; AIH, auto-immune hepatitis; re-LT, liver retransplantation; NAS, non-anastomotic biliary strictures; DBD, donation after brain death; DCD, donation after circulatory death; UW, University of Wisconsin solution; HTK, histidine-tryptophan-ketoglutarate solution.
Continuous data are presented as medians (IQR), categorical data are presented as numbers (%).

FUT2 non-secretor status is more common in PSC recipients

From 418 included LTs, FUT2 genotype was determined in 255 donors, 356 recipients and 193 paired donors-recipient. The distribution of FUT2 genotype in donors and recipients is displayed in **Fig. 2A**. The proportion of donors with homozygous FUT2 non-secretor status (AA-genotype) was 17.6% and allele frequency in donors was in Hardy Weinberg equilibrium (P=0.979). In recipients, however, the proportion of homozygous FUT2 non-secretors was higher (24.4%) and the Hardy Weinberg equilibrium was violated (P=0.006). The high incidence of patients suffering from PSC in our cohort (20.8%, **Table 1**) could contribute to this observation. Indeed, the proportion of homozygous recipient FUT2 non-secretor status was higher among PSC patients than in non-PSC patients (**Fig. 2B**, 33.3% vs. 21.8%, respectively, P=0.034).

PSC recipients with FUT2 non-secretor status have a higher incidence of NAS following transplantation

Mutation of the FUT2 gene is common in the general population and thus, the impact of FUT2 secretor status in donors as well as recipients on the development of NAS could be studied. When analyzing the entire cohort, FUT2 genotype in donors (n=255) was not associated with the development of NAS following LT (**supplementary Table 1 and Supplementary Fig.1A**, P=0.993). In recipients, occurrence of NAS was most frequent in homozygous non-secretors (AA-genotype), but did not significantly differ from the other genotypes (**Supplementary Table 1 and Supplementary Fig.1B**, P=0.152). Incidence of NAS was however comparable between recipients heterozygous for FUT2 mutation and those without the mutation. Therefore, all cases of GA and GG-genotypes were clustered as 'FUT2 secretors' for following analyses, while AA-genotypes were clustered as 'FUT2 non-secretors'.

We subsequently analyzed the influence of FUT2 non-secretor status on the development of NAS for separate PSC and non-PSC recipients. As shown by **Fig. 3A and B**, grafts obtained from donors with FUT2 non-secretor status did not increase the risk of developing NAS in both non-PSC and PSC recipients (P=0.696 and P=0.379, respectively). However, when analyzing recipient genotype (**Fig. 3C and D**), NAS developed already within the first 5 years following LT in up to 50% of the patients with PSC and FUT2 non-secretor status, against 10% of the PSC recipients with FUT2 secretor status and the non-PSC recipients in general (P=0.002). In the PSC group, 86% of the cases of NAS developed after the first 90 days following LT, which is usually set as a cut-off for diagnosis of recurrence of PSC[19]. These results suggest that NAS observed in PSC recipients might in fact reflect recurrence of disease.

Next, the effect of NAS and recipient FUT2 status on graft and patient survival was analyzed for both cohorts. The development of NAS was associated with significantly impaired graft survival in both non-PSC and PSC patients (**Supplementary Fig. 2**, log-rank P<0.001 and P=0.015, respectively). FUT2 non-secretor status in the recipient, however, did not significantly reduce graft survival (log-rank P=0.076 in non-PSC and P=0.306 in PSC). Recipient survival was similar for the NAS and the no NAS group and was not affected by recipient FUT2 non-secretor status (log-rank P=0.096 in non-PSC and P=0.843 in PSC).

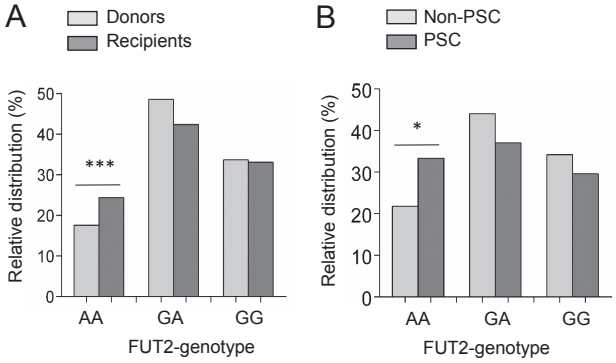

Figure 2. Distribution of FUT2 genotype in donors and recipients with different underlying disease. (A) Distribution of donor FUT2 genotype was in Hardy-Weinberg equilibrium. In recipients, however, the number of FUT2 non-secretors (AA-genotype) was higher and the Hardy-Weinberg equilibrium was violated (P=0.006). (B) Distribution of recipient FUT2 status in non-PSC and PSC patients. The incidence of FUT2 non-secretor status was significantly higher in PSC patients (P=0.034), probably explaining the violation of the Hardy-Weinberg equilibrium in recipients.

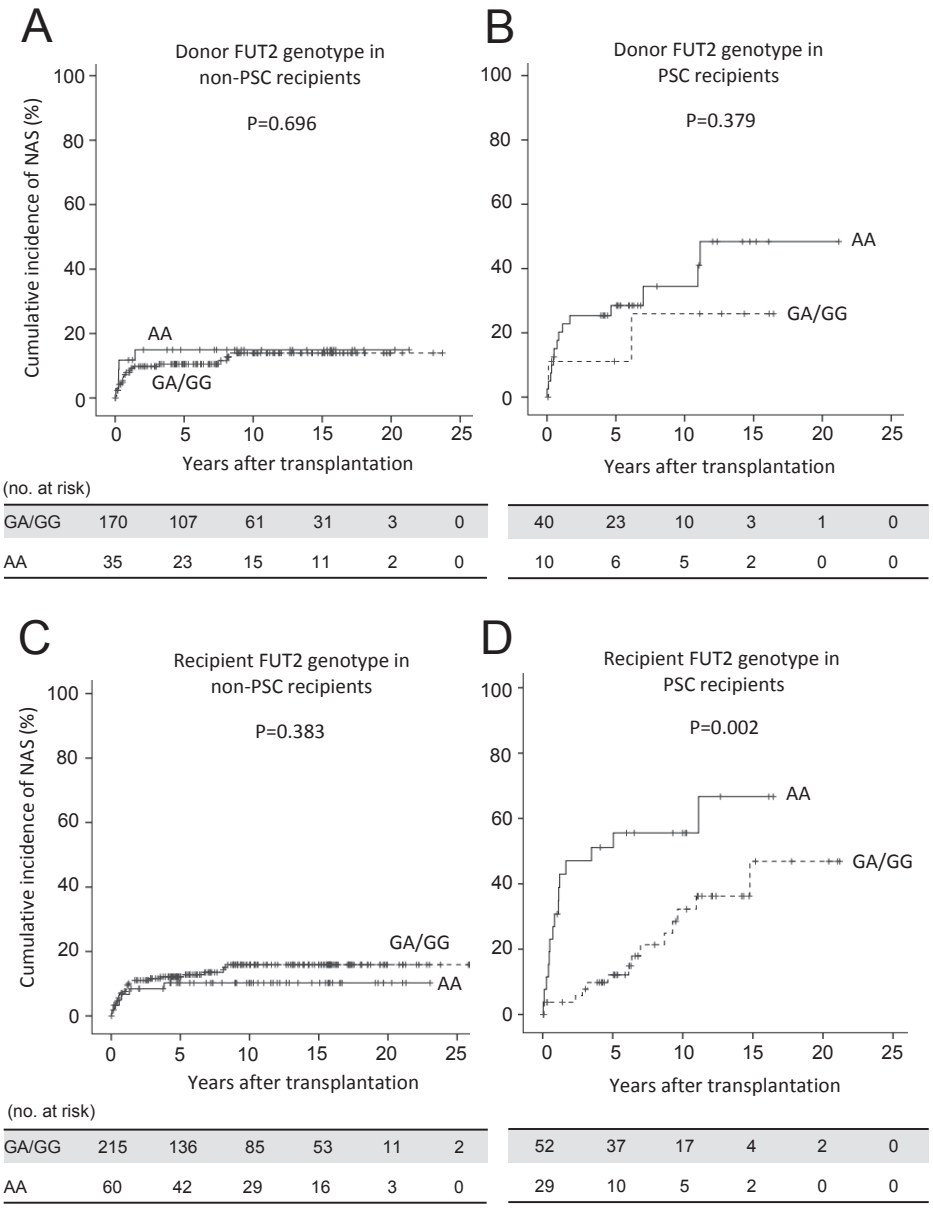

Figure 3. Cumulative incidence of NAS between FUT2 secretors versus FUT2 non-secretors. (A and B) Transplantation of a graft obtained from a FUT2 non-secretor donor did not lead to a higher incidence of NAS following LT in non-PSC patients, nor did it in PSC patients. (C) In non-PSC patients, recipient FUT2 non-secretor status was also not associated with a higher occurrence of NAS. (D) In PSC patients, however, the incidence of NAS was 60% and significantly faster in the first five years following LT in FUT2 non-secretors (AA), while it was 10% in FUT2 secretors (GA or GG).

Recipient FUT2 non-secretor status is an independent risk factor for NAS in PSC recipients

In order to identify other risk factors for NAS, we compared demographic data based on recipient FUT2 genotype for separate non-PSC and PSC recipients (**Table 2**). Variables with a P-value ≤0.2 were selected for univariate Cox regression analysis. Variables that were significant with univariate analysis were subsequently included for multivariate analysis.

As shown by **Table 3**, FUT2 non-secretor status was the strongest independent risk factor for the development of NAS in PSC recipients (HR: 2.34, P=0.034). We also foud a tendency for an increased risk of NAS when PSC recipients were transplanted with a DCD graft, though this was not statistically significant. Remarkably, sex mismatch between donor and recipient was a protective factor against NAS in PSC recipients (HR: 0.23, P=0.02), while the risk was highest in male-to-male transplantation (**Table 2**). In non-PSC recipients, transplantation with a DCD graft was the only independent risk factor the development of NAS (HR: 2.51, P=0.048). In univariate analysis, viral hepatitis as indication for LT seemed to lower the risk for the development of NAS in non-PSC patients (HR: 0.39, P=0.022), while NAS on itself as indication for re-LT was predisposing for recurrence of NAS in this group (HR: 3.37, P=0.049). These factors however lost significance in multivariate analysis.

Finally, a separate analysis was performed on re-LTs. Out of 30 re-LTs with available recipient FUT2 status, n=8 recipients developed NAS (26.7%). Overall, recipient FUT2 non-secretor status did not increase the risk of (recurrent) NAS after re-LT (P=0.234). When analyzing separate PSC recipients (n=11), three out of four recipients who developed NAS were FUT2 non-secretor. This however remained insignificant due to the small number of cases in this group (P=0.554).

FUT2 mismatch between donors and recipients is an additional risk factor to develop NAS

In addition to separate donor and recipient FUT2 status, the association between paired donor-recipient FUT2 genotypes and the occurrence of NAS was analyzed. Of the 193 paired donor-recipient genotypes, 149 pairs were in the non-PSC group and 44 pairs were in the PSC group. For both groups, the incidence of NAS for different combinations of FUT2 secretor status between donors and recipients is displayed in **Fig. 4**. In non-PSC recipients, there was no association between the incidence of NAS and (mis) match in FUT2 secretor status (**Fig. 4A**). In PSC recipients, however, incidence of NAS was significantly higher when a graft from a FUT2 secretor donor was transplanted into a FUT2 non-secretor recipient (D+R- group); 80% versus ≤21.4% in the other donor-recipient combinations (P=0.002**, Fig. 4B**). In addition, the median time to develop NAS in recipients with this unfavorable FUT2 combination was only 1 year, compared to 6.4 years in other FUT2 combinations (log-rank <0.001). The hazard to develop NAS with the D+R- combination was 8.7 (95% CI: 2.9-26.5, P<0.001). However, the limited number of data made it impossible to perform a reliable multivariate analysis.

Table 2. Donor, recipient and procedural variables for the development of NAS for separate non-PSC recipients and PSC recipients. Data are based on FUT2 secretor status in recipients.

	Non-PSC recipients			PSC recipients		
	No NAS (n=239)	NAS (n=36)	P-value	No NAS (n=52)	NAS (n=29)	P-value
Donor characteristics						
Age (yr)	46 (33-54)	47 (35-55)	0.453	47 (36-56)	46 (34-58)	0.768
Men/women	107/132	17/19	0.783	29/23	20/9	0.244
Rhesus (neg/pos)	40/199	4/32	0.391	7/45	2/27	0.367
CMV (neg/pos)*	110/128	19/17	0.707	28/24	21/7	0.074
Recipient characteristics						
Age (yr)	50 (41-58)	51 (36-59)	0.642	48 (39-58)	44 (32-49)	0.056
Men/women	142/97	21/15	0.902	35/17	24/5	0.134
Rhesus (neg/pos)	36/203	8/28	0.275	4/48	5/24	0.190
CMV (neg/pos)*	94/145	18/18	0.224	35/17	14/14	0.129
FUT2-genotype						
GA and GG vs. AA	185/54	30/6	0.422	40/12	14/15	**0.009**
Viral hepatitis (no/yes)	157/82	30/6	**0.034**			
Previous NAS	6 (2.5%)	4 (11%)	**0.010**	3 (5.8%)	4 (13.4%)	0.218
Procedural variables						
Graft type (DBD/DCD)	219/20	29/7	**0.037**	50/2	24/5	**0.040**
Preservation solution			0.195			0.150
UW	204	27		41	18	
HTK	33	9		11	10	
Unknown	2	0		0	1	
cold ischemia (min)	459 (360-610)	445 (393-534)	0.649	455 (347-566)	454 (401-638)	0.317
2nd warm ischemia (min)	38 (28-63)	35 (28-45)	0.292	29 (23-52)	32 (25-40)	0.828
Biliary anastomosis						
Duct-to-duct/Roux-Y	230/9	33/3	0.211	6/46	3/26	0.870
Recipient/donor						
Sex match			0.333			0.001
male/male	65	13		17	20	
male/female	77	8		18	4	
female/male	42	4		12	0	
female/female	55	11		5	5	
Rhesus match			0.395			0.324
neg/neg	5	0		2	1	
neg/pos	31	8		2	4	
pos/neg	35	4		5	1	
pos/pos	168	24		43	23	
CMV match			0.570			0.080
neg/neg	43	9		21	11	
neg/pos	51	9		14	3	
pos/neg	67	10		7	10	
pos/pos	77	8		10	4	

***** In non-PSC recipients, one donor CMV status was missing. In PSC-recipients, one donor and recipient CMV status was missing.

PSC, primary sclerosing cholangitis; NAS, non-anastomotic biliary strictures; CMV, cytomegalovirus; FUT2, fucosyltransferase-2; DBD, donation after brain death; DCD, donation after circulatory death; UW, University of Wisconsin solution; HTK, histidine-tryptophan-ketoglutarate solution.

Table 3. Cox regression analysis of potential risk-factors for the development of NAS in non-PSC recipients and PSC recipients. Factors from univariate analysis with a P-value <0.05 were included for multivariate analysis.

	Univariate		Multivariate	
	HR	P-value (95% CI)	HR	P-value (95% CI)
Variables in PSC recipients				
FUT2 non-secretor status (AA)	3.05	**0.003** (1.47-6.35)	2.34	**0.034** (1.08-5.08)
Graft type (DCD)	4.58	**0.009** (1.73-12.1)	2.74	0.079 (0.98-7.67)
Gender mismatch	0.21	**0.001** (0.07-0.62)	0.23	**0.002** (0.08-0.67)
Recipient sex (male)	2.38	0.058 (0.90-6.29)		
CMV status donor (positive)	0.56	0.175 (0.24-1.34)		
CMV status recipient (positive)	1.70	0.167 (0.81-3.58)		
Recipient/donor CMV mismatch	1.12	0.770 (0.53-2.36)		
Recipient age	0.98	0.146 (0.95-1.01)		
Preservation solution (HTK)	2.08	0.079 (0.95-4.56)		
Variables in non-PSC recipients				
Indication viral hepatitis	0.39	**0.022** (0.16-0.94)	0.45	0.060 (0.19-1.10)
Indication NAS	3.37	**0.049** (1.19-9.55)	3.19	0.063 (1.09-9.32)
Graft type (DCD)	2.68	**0.036** (1.17-6.14)	2.81	**0.030** (1.21-6.53)
Preservation solution (HTK)	2.07	0.077 (0.97-4.41)		

FUT2, fucosyltransferase-2; DCD, donation after circulatory death; CMV, cytomegalovirus; HTK, histidine-tryptophan-ketoglutarate solution; NAS, non-anastomotic biliary strictures.

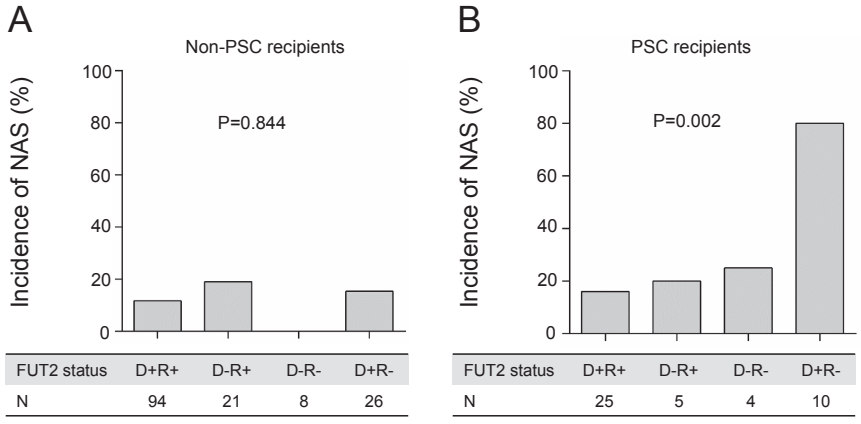

Figure 4. Paired donor-recipient FUT2 status and incidence of NAS. (A) In non-PSC patients there was no unfavourable donor-recipient combination of FUT2 genotypes causing a higher incidence of NAS. (B) In PSC patients, particularly the transplantation of a graft with donor FUT2 secretor status into a recipient with FUT2 non-secretor status was associated with a higher incidence of NAS, up to 80%. The tables below the graphs show the total number of paired donors-recipients per group. Abbreviations: D = donor, R = recipient, + = FUT2 secretor (GA or GG genotype), - = FUT2 non-secretor (AA genotype).

Relation between inflammatory bowel disease, PSC and FUT2 status

Previous studies showed an association between PSC and inflammatory bowel disease (IBD). Moreover, FUT2 non-secretor status is a known genetic risk factor for IBD and might be associated with a "leaky gut". This led to the hypothesis that increased bacterial translocation could be involved in the pathophysiology of PSC. As shown in **Fig. 5A,** also in our study cohort, a strong correlation existed between IBD and PSC: in particular, ulcerative colitis was common among PSC recipients (56%) while it was almost absent in non-PSC recipients (0.7%, P<0.001). And as shown by **Fig. 5B**, in the entire cohort a higher incidence of FUT2 non-secretors was observed in patients with ulcerative colitis

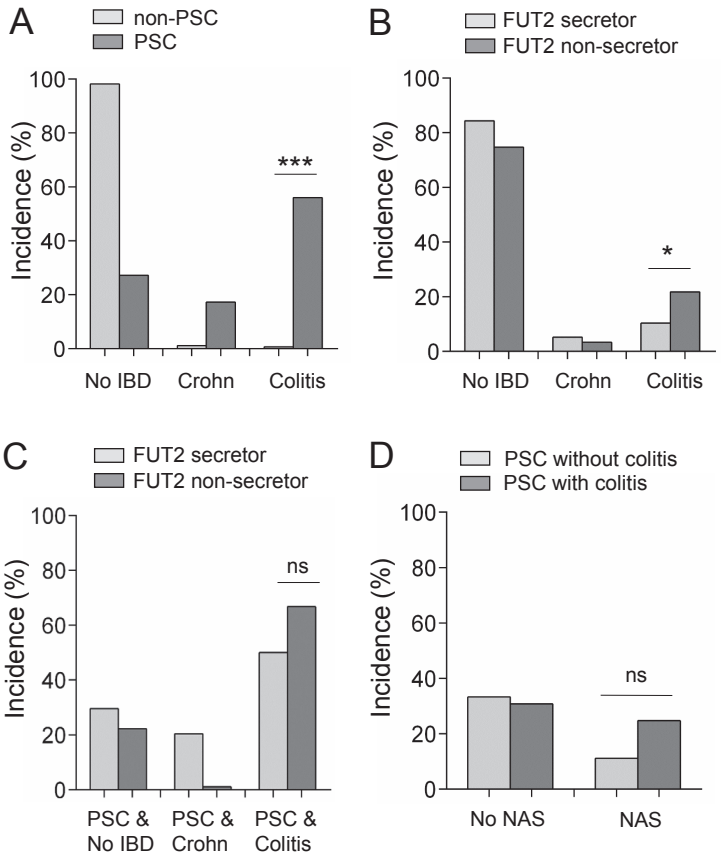

Figure 5. Association between PSC, IBD and NAS. (A) Incidence of PSC without coexisting IBD or with Crohn's disease or ulcerative colitis (n=81). In particular ulcerative colitis was associated with PSC, occurring in 56% of PSC patients (P<0.001). (B) Association between IBD and FUT2 non-secretor status for the entire cohort (n=356). The percentage of FUT2 non-secretors was higher in patients with ulcerative colitis than in those with Crohn's or without IBD (p=0.021). (C) Association between combined PSC and IBD and FUT2 non-secretor status (n=81). The percentage of FUT2 non-secretors was not significantly higher in PSC recipients with coexisting ulcerative colitis (P=0.155). (D) Comparable incidence of NAS between PSC recipients with or without coexisting ulcerative colitis (P=0.070).

than in patients with Crohn's disease or without IBD. In PSC recipients with coexistent ulcerative colitis, the number of FUT2 non-secretors was however similar to FUT2 secretors (**Fig. 5C**), and the incidence of NAS was comparable between PSC patients with or without coexisting ulcerative colitis (**Fig. 5D**, P=0.070).

It has been suggested that colectomy in PSC patients is protective for the recurrence of disease following LT[20]. Since many cases of NAS in PSC patients are possibly caused by recurrence of disease, we also investigated whether colectomy was protective against development of NAS. Out of 81 PSC recipients, 16 patients were subjected to colectomy; in 9 patients prior to development of NAS and in 7 patients after development of NAS. Two out of nine PSC recipients that had undergone colectomy developed NAS, while seven did not develop NAS (P=0.487).

DISCUSSION

This study shows that patients suffering from PSC with a homozygous polymorphism in the FUT2 gene have a higher risk to develop NAS following LT.. This so-called FUT2 non-secretor status was more common among PSC patients, in whom it was an independent risk factor for the development of NAS particularly during the first five post-operative years. Moreover, analysis of paired donor-recipient genotypes showed that FUT2 mismatch further increased the risk of NAS, namely up to 80% when PSC recipients with FUT2 non-secretor status received a graft from a donor with FUT2 secretor status. In non-PSC recipients, transplantation with a DCD graft was the most important risk factor for development of NAS, as demonstrated by others before[21]. The results of our study not only confirm the pathophysiological role of altered glycosylation in PSC as identified by previous studies, it is the first that shows FUT2 genotype is an independent risk factor for outcome following LT in PSC.

Fucosyltransferase-2 is an enzyme that catalyzes transfer of fucose into glycoproteins, which are expressed on the surface of epithelial cells throughout the body. The first studies on the biological role of FUT2 mainly concerned its function in the biosynthesis of blood group antigens[22]. But in the more recent years, the focus has moved to dysfunction of FUT2; the non-sense mutation caused by rs608133 creates a truncated FUT2 protein. Approximately 20% of the general population is FUT2 non-secretor, with inactivity of both alleles[23]. A strong correlation has been found between Crohn's disease and FUT2 non-secretors[24]. Possible effects of FUT2 dysfunction in Crohn's disease are altered host-microbe interactions due to a lack of fucosylation that causes epithelial barrier dysfunction[16]. Moreover, FUT2 non-secretors have a different, less diverse bacterial composition in their intestine[25]. These factors may lead to increased bacterial translocation to extra-intestinal sites which may explain the involvement of

FUT2 dysfunction in other diseases such as chronic pancreatitis[26] and auto-immune disorders as psoriasis[27, 28] and Behcet's disease[29]. Remarkably, the correlation between FUT2 dysfunction and ulcerative colitis is less clear, with contradictory results in various studies[24, 30]. While in the current study, we observed a higher frequency of FUT2 non-secretors in LT recipients also suffering from ulcerative colitis than recipients with Crohn's disease or patients without IBD. This observation could however be biased by the transplant population, which mainly consisted of PSC recipients with ulcerative colitis.

Beside the intestine, FUT2 non-secretion also affects the bacterial composition of bile, as demonstrated by Folseraas et al.[13]. This finding supports the hypothesis that in patients with PSC, adhesion of specific bacteria to the cholangiocyte epithelium could provoke (recurrent episodes of) cholangitis. Since the intestinal epithelium is also affected by the mutation, bacteria from the intestinal site could also translocate to the liver via the portal vein. The detection of *Enterococcus*, *Escheria coli* and *Candida* in bile of LT recipients with post-transplant biliary complications has been associated with shortened retransplantation-free survival[31]. A recent study by Rupp et al. showed an increased frequency of cholangitis by biliary Candida infection in PSC patients with heterozygous FUT2 carriers[14]. Dominant stenosis occurred more frequently in homozygous-mutated patients. To investigate whether bacterial translocation from the intestine also played a role in our cohort of PSC recipients, we tried to correlate NAS with the presence of IBD. The incidence of NAS was however comparable between PSC patients with and without coexisting IBD. Also, the preventive effect of colectomy on the recurrence of NAS that was shown by previous studies, was not proven to be effective against NAS in our study [20]. This could however be due to the limited number of PSC patients with colectomy in our study cohort.

A different mechanism that could explain the biliary injury in FUT2 non-secretors is the increased vulnerability of cholangiocytes for bile salt toxicity. In a physiologic situation, cholangiocytes are protected against the potential toxicity of bile acids by the excretion of HCO_3^-, also known as the bicarbonate umbrella[32]. Together with a glycocalyx layer on the apical membrane of cholangiocytes, the alkaline environment in the biliary lumen is maintained[33]. It is believed that the absence of FUT2, as is the case in non-secretors, can perturb the integrity of the biliary glycocalyx and bicarbonate umbrella and thereby increase the toxic effects on the biliary epithelium by bile salts. Multiple studies in both humans and animals demonstrated the relation between bile salt toxicity and bile duct injury in the context of LT[34, 35]. Whether absence of FUT2 further exposes cholangiocytes to bile acids remains unknown and should be confirmed by future research.

Another hypothesis on the pathophysiology behind FUT2 and NAS raised after analysis of paired donor-recipient FUT2 genotypes. In particular the combination of

donor FUT2 secretor with recipient FUT2 non-secretor showed a strong effect on the development of NAS. This raises the question whether FUT2 mismatch aggravates an immune response by the provision of fucose-positive antigens to recipients who have not been exposed to such antigens before. Strikingly, an experimental study by Gock et al. demonstrated that skin and heart grafts from transgenic mice with overexpression of human α1,2-fucosyltransferase were rapidly rejected by wildtype recipients[36]. This in contrast to mice that were matched for fucosyltransferase expression and that showed a much better graft survival. Histological evaluation of rejected grafts indicated that altered glycosylation patterns can trigger the innate as well as the adaptive immune system. This study supports our hypothesis that FUT2 mismatch could lead to an aggravated immune-response. In addition, the possibility of popularization of the donor liver with recipients cells should also be considered. Hove et al. demonstrated chimerism of recipient cholangiocytes in the donor bile duct after transplantation[37]. Although this migration of recipient cells is supposed to induce tolerance, the displacement of fucosylated donor cholangiocytes by the non-fucosylated ones of the recipient could also activate immune responses, leading to NAS or recurrence of PSC.

This brings up an important point of the discussion; are the NAS observed in our cohort of PSC recipients in fact recurrence of the underlying disease? Already in 1999, Graziadei et al. formulated a definition for the diagnosis of recurrence of PSC after LT[19]. All PSC recipients in our cohort had a confirmed diagnosis of PSC prior to transplantation and the vast majority developed NAS at least after 90 days of follow-up. This, together with the fact that we found a correlation between NAS and FUT2 in patients with PSC only, strongly suggests that NAS in our cohort of PSC recipients was in fact caused by a recurrence of disease. Nevertheless, few cases occurred before the limit of 90 days post LT. But if recurrence of PSC is triggered by a powerful immune response, as hypothesized in the previous paragraph, perhaps the cut-off in days for the definition of recurrence of PSC should be revised.

The most important limitation of our study is the small cohort size. Although the study included donor and recipient DNA from a total of 418 LTs, paired donor-recipient samples were only available in 193 cases. Also, the number of patients in the PSC cohort was relatively small with 81 recipients. Despite this, we found a clear correlation between PSC, NAS and FUT2 non-secretor status. It did however hamper the performance of a reliable multivariate analysis for the matched donor-recipient genotype analysis. Finally, our study does not contain an independent validation cohort. But the data presented here do confirm the findings of previous large GWAS-studies in the context of LT, which makes this study a validation study on itself[13].

In conclusion, this study demonstrates that FUT2 non-secretor status is a risk factor for the development of NAS or recurrence of disease in patients with PSC during the first years following LT. This is potentially related to an aggravated immune response due to FUT2 mismatch between donors and recipients.

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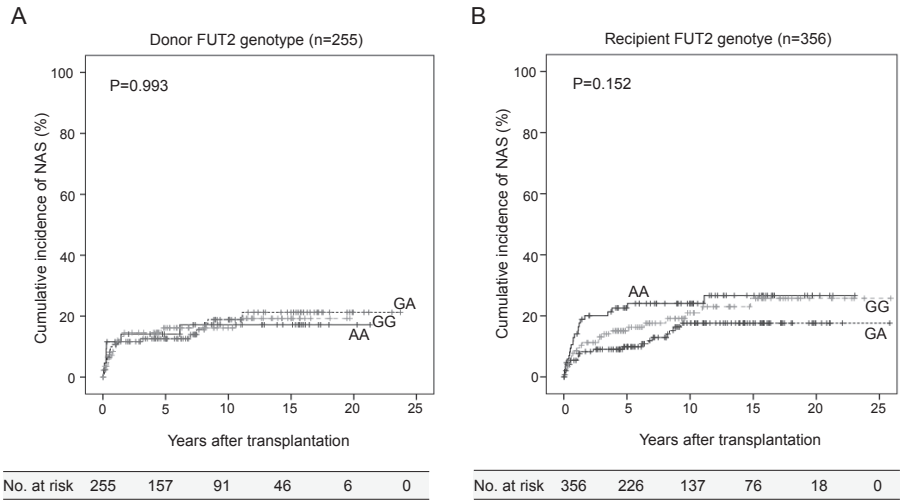
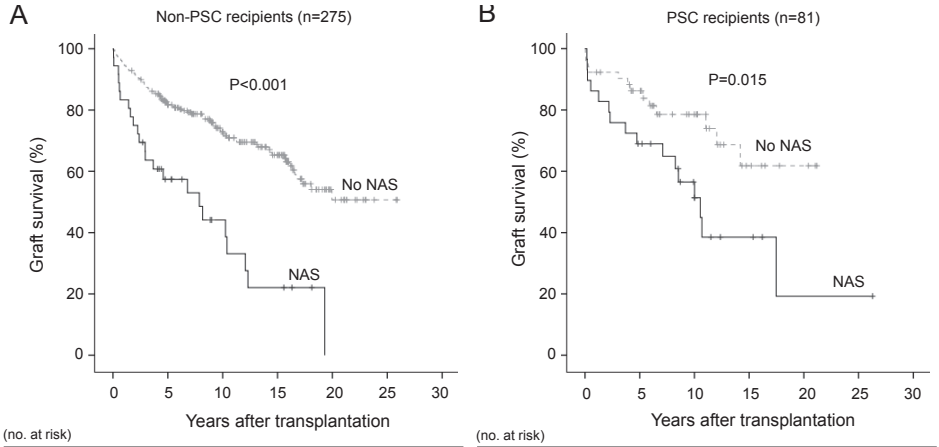
SUPPLEMENTARY TABLES AND FIGURES

Supplementary Table 1. Association of donor or recipient FUT2 genotype with NAS.

Donor FUT2 genotype	No NAS (n=214)	NAS (n=41)	
AA	38	7	
GA	104	20	
GG	72	14	
			P=0.994
Recipient FUT2 genotype	**No NAS (n=291)**	**NAS (n=65)**	
AA	66	21	
GA	130	21	
GG	95	23	
			P=0.132



Supplementary Figure S1. Association between FUT2 genotype in donors and recipients and time to diagnosis of NAS.



(no. at risk)							
No NAS	239	173	114	70	15	1	0
NAS	36	16	8	4	0	0	0

(no. at risk)							
No NAS	52	38	21	7	3	0	0
NAS	29	19	10	4	1	0	0

Supplementary Figure S2. Association between NAS and graft survival in non-PSC and PSC recipients.

Part III

MicroRNAs as novel biomarkers in liver transplantation

Chapter 5

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 more 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 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 need 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.

MicroRNAs 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. MicroRNAs 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 of 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 & hypercholesterolemia, and caloric restriction[35]. Furthermore, 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 MIRNAS, 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

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.

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 repeated cycles of freezing and thawing, miRNAs in serum, plasma and graft perfusate samples remained insensitive from 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 **Fig. 1.**

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

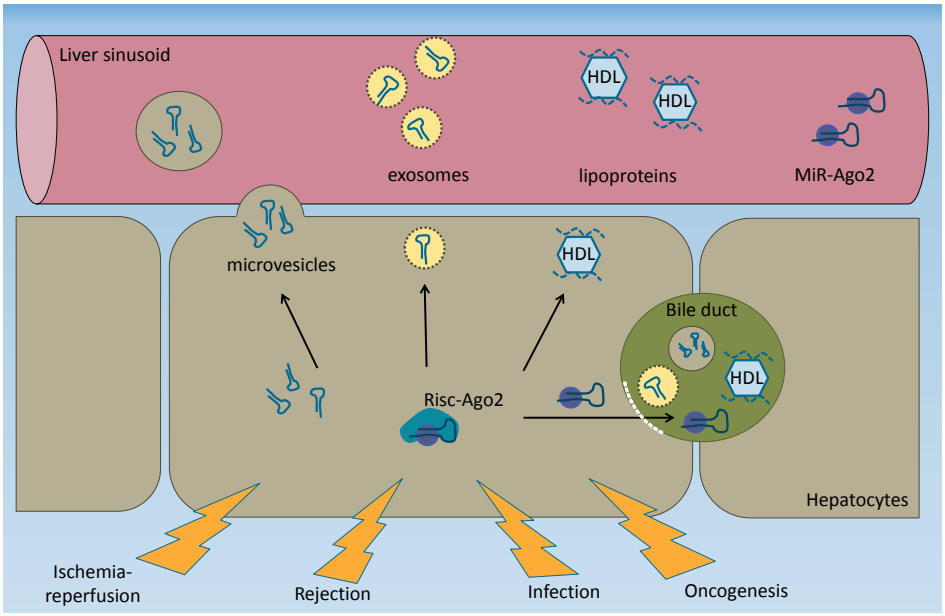

Figure 1. Mechanisms of miRNA release from (injured) cells. Mature miRNAs inside the cell cytoplasm are bound to the RISC-argonaute2 complex. Cell stress induced by for instance ischemia-reperfusion, infection, rejection and oncogenesis, can cause active release or secretion of miRNAs into the circulation.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

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 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**).

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

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

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 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 make most current miRNA biomark-

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.

ers 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 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 bio-

markers already before graft implantation to predict graft quality could be a valuable feature for the selection of allografts in the future[14, 60].

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

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

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 for post-transplantation follow-up, such as monitoring for recurrent disease.

TISSUE MIRNA 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. 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 microRNAs 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 did not identify 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 into 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) recurrence of HCV.

TISSUE MIRNA 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

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 could also be useful. However, in our search of literature currently no studies were found concerning the use of microRNAs 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 body fluids and graft perfusion fluid can be cumbersome due to lack of generally accepted protocols for isolation, detection and normalization, and the lack 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 into 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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Chapter 6

Polarized release of hepatic microRNAs into bile and serum in response to cellular injury and impaired liver function

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ABSTRACT

Background and aims. Extracellular microRNAs (miRNAs) in serum and bile are currently under intense investigation for biomarker purposes in liver disease. However, the directions and pathways by which miRNAs are released from hepatic cells remains largely unknown. Here, we investigated the release of hepatocyte and cholangiocyte-derived miRNAs (HDmiRs and CDmiRs) into blood and bile during various (patho)physiological hepatic conditions.

Methods. MiRNA release was analyzed using longitudinally collected tissue and paired bile and serum samples (n=124) that were obtained from liver transplant recipients during follow-up.

Results. Cell-type specificity of HDmiRs and CDmiRs was confirmed in liver and common bile duct biopsies ($P<0.001$). Analysis of paired bile and serum samples showed up to 20-times higher miRNA-levels in bile compared to serum ($P<0.0001$). Fractionation of bile showed the majority of miRNAs being present in the unpelletable supernatant, where protein-conjunctions protect miRNAs against degradation ($P<0.0001$). During episodes of liver injury and histologically proven rejection in liver transplant recipients, relative HDmiR-levels in bile decreased while its levels in serum increased ($P\leq0.015$). Simultaneously, relative CDmiR-levels in bile significantly increased, while their levels in serum decreased. Related to liver excretory function, a strong positive correlation was observed between HDmiR-122 levels and bilirubin excretion into bile ($R=0.694$, $P<0.0001$), whereas CDmiRs showed an inverse correlation ($P<0.05$).

Conclusion. During impaired excretory function and injury, the liver shows polarized release of extracellular HDmiRs and CDmiRs. This sheds new light on the biology of hepatic miRNA release which is relevant for the interpretation of hepatic miRNAs as biomarkers.

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 cell-type abundant makes them attractive for biomarker research[7]. 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[8, 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.

Because of their cell-type abundancy, extracellular miRNAs are increasingly being investigated as non-invasive biomarkers in serum for various liver disease[7, 15]. Recently, in concordance with other human and animal studies[8, 9, 16], our team has demonstrated the specific release of hepatocyte-derived miRNAs (HDmiRs) in blood during liver injury, chronic hepatitis C infection and acute rejection after liver transplantation (LT)[17, 18]. 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[19]. Moreover, CDmiRs were shown to be predictive of severe biliary injury already at time of graft preservation in LT[20]; in contrast to HDmiRs, grafts that developed biliary complications following LT contained lower levels of CDmiRs in perfusates. These findings suggest that during injury, there is a polarized release of HDmiRs and CDmiRs by hepatocytes and cholangiocytes[21].

The hypothesis that dynamics in miRNA release are different between hepatocytes and cholangiocytes was recently suggested by Lankisch et al., who found that during injury, cholangiocytes release miRNAs to bile rather than to blood[22]. The first report on miRNAs in bile by Shigehara et al. found miR-9 to be a potential biomarker for biliary tract cancer and showed that miRNAs were protected against RNAse activity in bile[23]. In addition, Li et al. used miRNAs in extracellular vesicles in bile for the identification of diagnostic miRNAs in cholangiocarcinoma[24]. Yet, these studies did not address the dynamics behind miRNA release into both bile and serum. Thus, the question remains whether polarized cells like hepatocytes and cholangiocytes can control miRNA release into both serum and bile. This knowledge is of importance for the interpretation of miRNAs as injury markers in the circulation or, as suggested by previous studies, a role of miRNAs as gene regulators in the entero-hepatic circulation and biliary homeostasis[25-27].

For this purpose, we investigated the following aspects of extracellular miRNAs in bile and serum: (1) the presence and stability of HDmiRs and CDmiRs in fresh human bile samples, and (2) the directional or polarized release of HDmiRs and CDmiRs into bile and serum during impaired liver excretory function and liver injury.

Key points

- Release of hepatocyte and cholangiocyte-derived miRNAs can be used to distinguish between biliary injury and hepatocyte injury.
- In bile, the majority of extracellular miRNAs are in the unpelletable fraction and protected against degradation through conjunctions with proteins.
- Different hepatic (patho)physiologic conditions, like impaired hepatic excretory function, histologically proven acute rejection, and biliary injury, affect the directional release of hepatocyte and cholangiocyte-derived miRNAs into serum and bile.
- These new insights into the dynamics of miRNA release are of importance for the interpretation of miRNA-based biomarkers for liver injury and disease.

MATERIALS AND METHODS

Tissue, blood and bile sampling

First, abundance of HDmiRs and CDmiRs was evaluated by comparing expression of these miRNAs in liver biopsies (n=10) and common bile duct specimens (CBD, n=8) 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 polarized release of HDmiRs and CDmiRs during different (patho)physiological conditions, we analyzed paired serum and bile samples (n=62 each, n=124 in total) that were collected from ten LT recipients during the first three weeks of follow-up after transplantation. Serum was withdrawn by venipuncture, while bile was collected from a T-tube that was inserted routinely into the common bile duct during LT. Samples were processed within two hours of withdrawal to prevent any degradation or contamination and were stored at -80˚C. Standard serum liver tests (AST, ALT, alkaline phosphatase, gamma-GT and bilirubin) were obtained. Bilirubin levels were also determined in bile for the assessment of liver excretory function.

Fresh bile samples were obtained from donor gallbladders during the benching procedure for centrifugal fractionation and protease treatment (n=7) and miRNA stability assays (n=4). Directly after collection, large components were removed from bile by a two-step centrifugation protocol; samples were centrifuged for 10 min at 453g at 4°C, followed by 15 min of centrifugation at 3220g at 4°C. Bile samples were stored at -20°C until further use.

During recipient follow-up, liver 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[28] by an experienced pathologist as part of regular clinical practice. 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.

Fractionation, protease and RNAse-treatment of bile samples

For fractionation of bile, 4 ml of cell-free bile was diluted with 8 ml of sterile PBS and a baseline sample was taken. More detailed information regarding centrifugation steps of bile and protease-K and diethylpyrocarbonate (DEPC) treatment are available in the **legend of Fig.1** and the **Supplementary Information**.

RNA isolation, reverse transcription and Real-Time quantitative Polymerase Chain Reaction (RT-PCR)

More detailed information on RNA isolation and RT-qPCR is provided in the supplementary data.

Statistical analyses

Statistics for non-parametric correlations were generated by Spearman's Rank Correlation test, using relative miRNA levels that were calculated by threshold cycle values (2^{-CT}). Because of the lack of well-validated reference miRNAs in both bile and serum, for comparative statistics (Kruskal-Wallis, Mann-Whitney U and Wilcoxon matched pair test), we used relative miRNA levels that were normalized by setting their total in each sample at 100% to correct for any differences in bile and serum concentration[20]. In tissue biopsies, relative miRNA levels were normalized using a previously described reference gene[18, 20], RNU43 ($2^{-\Delta CT}$), which was equally expressed between CBD and liver tissue (**Supplementary Fig. S1A**). *P*-values <0.05 were considered significant. Figures represent the median ± interquartile range (IQR). 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

Levels of HDmiRs and CDmiRs in liver and bile duct tissue

The cellular origin of miRNAs in serum or bile is of importance to discriminate between injury of different cell types. Therefore, we first validated the abundance of HDmiRs and CDmiRs, as identified by previous studies[19], in liver biopsies (n=10) and CBD specimens (n=8) that were collected during LT. MiR-122 and -148a were previously reported as hepatocyte-abundant, while miR-30e, -200c and -222 were reported as cholangiocyte abundant. As illustrated by **Supplementary Fig. S1B**, expression of miR-122 was over a 1000-fold higher in liver tissue compared to CBD (median ± IQR; 128 ± 20 vs. 0.02 ± 0.8, P=0.0021). Although less abundant than miR-122, also the expression of miR-148a was significantly higher in liver tissue (5.6 ± 0.9 vs. 1.3 ± 0.2, P=0.0003). In bile duct tissue, miR-222 was expressed highest and showed up to 17-fold higher levels compared to liver

tissue (0.5 ± 0.5 vs. 8.5 ± 1.6, P=0.0002). The discrepancy in expression of miR-200c was even higher, up to 70-fold; 0.04 ± 1.6 in liver vs. 2.8 ± 0.5 in CBD. The results demonstrate that miR-122 and miR-148a are more abundant in hepatocytes, while miR-200c and miR-222 are more specific for cholangiocytes (**Supplementary Fig. S1B**). The expression of miR-30e did not significantly differ between liver and CBD tissue.

Fractioning and stability of HDmiRs and CDmiRs in bile

To investigate the direction of HDmiR and CDmiR release into bile and blood *in vivo,* n=62 paired serum and bile samples (n=124 in total) were analyzed that were collected from ten recipients during the first three weeks after LT. Donor and recipient characteristics are listed in **Supplementary Table 1**. As shown in **Fig. 1A**, HDmiR-122 was the most abundant miRNA in serum as well as in bile. Of the three tested CDmiRs, CDmiR-222 was the most abundant in bile. Only a small portion was accounted for by miR-148a, while miR-200c was virtually absent in serum. Overall, relative levels of all HDmiRs and CDmiRs were significantly higher in bile compared to serum.

In order to determine the subcellular localization of HDmiRs and CDmiRs, bile samples obtained from human donor gallbladders (n=7) were fractionated. In short, cell-free bile samples (sample S0) were sequentially centrifuged at 20,000*g* to obtain a pellet enriched with mitochondria, lysosomes and peroxisomes (sample P1). Subsequently, the supernatant was centrifuged at 100,000*g* to obtain a pellet enriched with microsomes and membrane fragments (sample P2). Finally, the supernatant was centrifuged at 140,000*g* to obtain a pellet with exosomes, ribosomes and viruses (sample P3). As shown in **Fig. 1B**, all pellets contained only a small percentage of HDmiRs and CDmiRs compared to the unfractioned baseline sample (S0); 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 were found in the non-pelletable supernatant after the final spinning step (sample S3). These percentages were similar between all tested CDmiRs and HDmiRs. The stability of extracellular miRNAs has been linked to the formation of miRNA-protein complexes[20, 29]. In order to test whether miRNAs in bile are indeed bound to proteins, samples of unpelletable bile supernatant (S3) were treated with Proteinase-K for protein degradation (ProtK treated S3). As shown within the dotted square in **Fig. 1B**, up to 89% of the miRNAs became undetectable after degradation of proteins in bile (P<0.0001).

To further test the stability of biliary miRNAs, bile samples spiked with synthetic *C.elegans* cel-miR-39 and cel-miR-238 were incubated at room temperature up to 24h (n=4). As shown by **Fig. 1C**, 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 were almost completely degraded within the first five minutes after incubation, consistent with previous studies[20, 23]. To investigate whether degradation was caused by RNase activity, stability of miRNAs was also determined in an RNase-free environment by treat-

ing bile with diethylpyrocarbonate (DEPC). As shown by **Fig. 1D**, HDmiRs and CDmiRs remained more stable (up to 24 hours) in bile when samples were treated with DEPC. These findings suggests that the majority of biliary miRNAs is present in the unpelletable biliary fraction, where they remain stable and protected against RNAse activity through protein conjunctions.

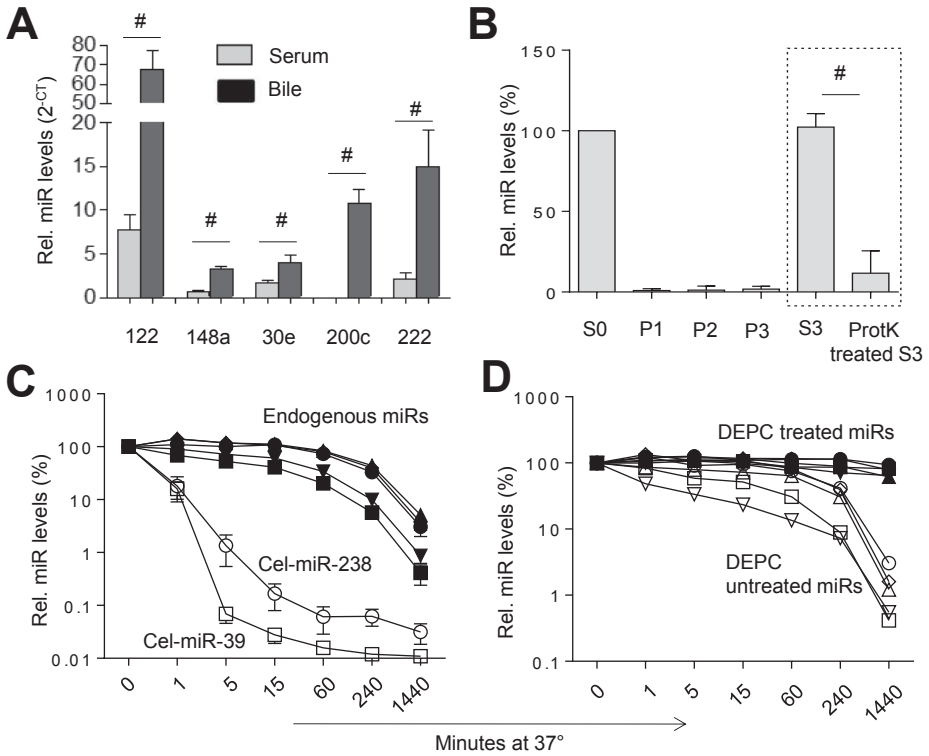

Figure 1. (A) Distribution of HDmiRs and CDmiRs in n=62 paired serum and bile samples. In general, miRNA levels in bile were higher compared to miRNA levels in paired serum samples. (B) Cell-free bile samples (sample S0, n=7) were fractionated by centrifugation at increasing speeds of 20,000*g*, 100,000*g* and 140,000*g*, in order to obtain pellets enriched with mitochondria, lysosomes and peroxisomes in pellet 1 (sample P1), microsomes and membrane (fragment)s in pellet 2 (sample P2) and exosomes, ribosomes and viruses in pellet 3 (sample P3). The supernatant after the final centrifugation step (sample S3) contained soluble proteins and protein complexes. The fractionation of bile showed that all pellets only contained a very small percentage of the tested HDmiRs and CDmiRs compared to the baseline sample (S0), while the majority of miRNAs is present in the supernatant (S3). When proteins in biliary supernatant were degraded by Prot-K treatment, up to 89% of miRNAs become undetectable (ProtK treated S3). This suggests that most miRNAs are bound to proteins for protection against RNAse activity and degradation. (C) Stability of miRNAs in bile (n=4). Levels of HDmiRs and CDmiRs in bile remained relatively stable up to 4 hours (closed symbols), while exogenously spiked-in cel-miR-39 (open squares) and cel-miR-238 (open circles) degraded within 5 minutes after incubation at 37°C. (D) Eliminating RNAse activity in bile samples by DEPC treatment resulted for all tested miRs in stability for at least 24 hours in bile (closed symbols), whereas untreated bile showed a gradual decrease for all miRNAs tested (open symbols). Figures represent the median ± IQR. **P<0.01, ***P<0.001, #P<0.0001.

Release of miRNAs into bile and serum during impaired liver function

To investigate the effect of impaired liver function on the release of HDmiRs and CDmiRs, paired bile and serum samples were compared between grafts with good bilirubin excretion into bile versus grafts with impaired bilirubin excretion (**Table 1**). Bile samples containing low concentrations of bilirubin (≤1000 IU/L, n=14) were considered to represent poor liver excretory function, while bile samples with bilirubin concentrations >1000 IU/L (n=36) indicate good excretory function[30]. As shown in **Fig. 2A**, livers with good bilirubin excretion had higher levels of HDmiR-122 in bile (P<0.0001). When liver excretory function was impaired, however, levels of HDmiR-122 in bile drastically lowered. Simultaneously, CDmiR-levels in bile increased. Interestingly, out the five tested miRNAs, three of them showed no differences in miRNA levels in paired serum samples (**Table 1, Fig. 2B**). This could indicate that miRNAs in bile rather than serum better reflect the functional status of the liver. In particular levels of HDmiR-122 in bile were strongly correlated with bilirubin secretion to bile (P<0.001, R=0.695). This correlation is displayed in more detail by **Fig. 2C**, showing highly similar dynamics of HDmiR-122 and bilirubin levels in bile in six individual patients. The opposite dynamics between HDmiR and CDmiR secretion to bile suggest that their release is polarized, dependent on liver excretory function.

Table 1. MicroRNA levels in paired bile and serum samples during impaired liver function and liver injury.

	Relative biliary miRNA levels (%) (median ± IQR)			**Relative serum miRNA levels (%) (median ± IQR)**		
Impaired excretrory function						
MicroRNA	Bile bili >1000 µmol/L (n=36)	Bile bili <1000 µmol/L (n=14)	P-value	Bile bili >1000 µmol/L (n=36)	Bile bili <1000 µmol/L (n=14)	P-value
HDmiR-122	81 (73-87)	2 (1-44)	<0.0001	56 (42-69)	62 (46-77)	0.319
HDmiR-148a	3 (3-4)	5 (3-6)	0.006	8 (5-10)	4 (2-7)	0.022
CDmiR-30e	3 (2-3)	8 (5-11)	<0.0001	18 (9-23)	10 (6-20)	0.106
CDmiR-200c	7 (4-11)	24 (13-30)	<0.0001	0.2 (0.1-0.4)	0.0 (0.0-0.1)	0.006
CDmiR-222	6 (4-9)	42 (15-64)	<0.0001	19 (12-29)	19 (11-33)	0.825
Cellular liver injury						
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
HDmiR-122	83 (80-88)	75 (42-84)	0.015	42 (27-57)	58 (51-76)	<0.0001
HDmiR-148a	3 (3-4)	3 (2-4)	0.557	10 (7-12)	11 (6-20)	0.001
CDmiR-30e	2 (2-3)	3 (2-5)	0.315	21 (18-25)	11 (6-20)	<0.0001
CDmiR-200c	7 (4-9)	10 (5-19)	0.035	0.2 (0.0-0.4)	0.1 (0.0-0.2)	0.024
CDmiR-222	4 (3-6)	7 (4-18)	0.011	26 (14-36)	16 (12-27)	0.045

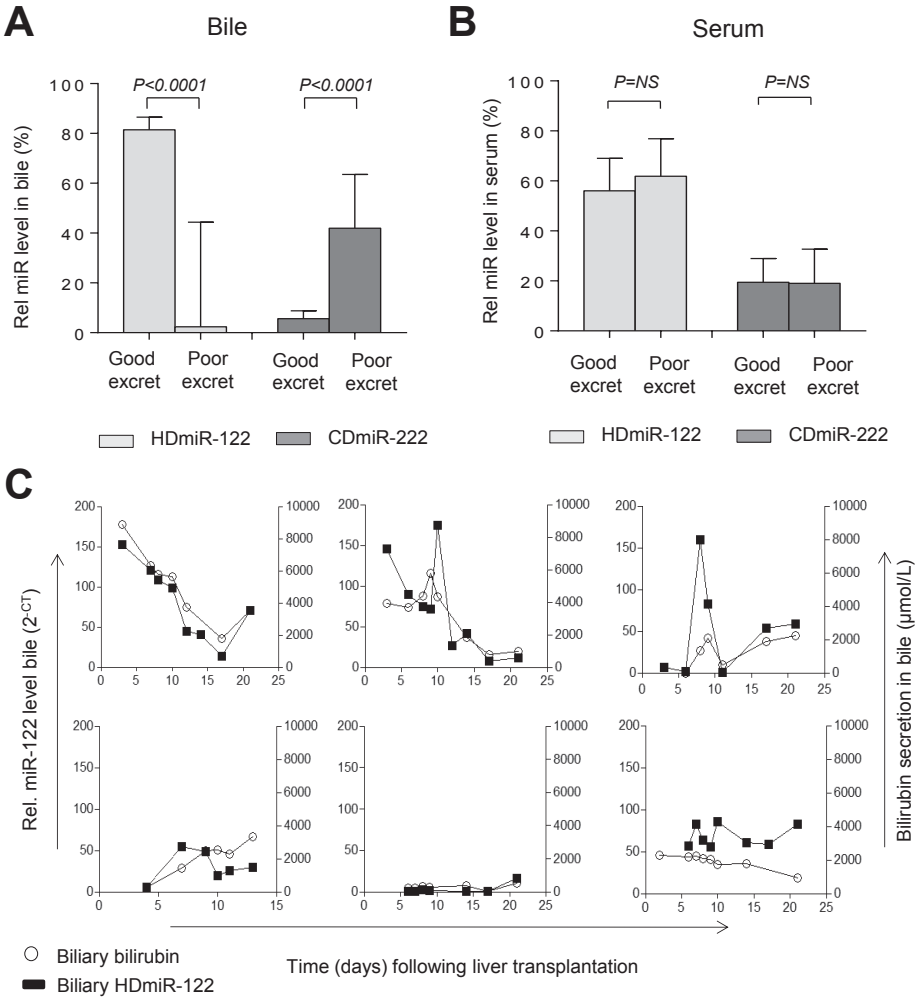

Figure 2. (A) Directional release of HDmiRs and CDmiRs into bile and (B) into serum during good versus impaired bilirubin excretion. Livers that excreted ≤1000 IU/L bilirubin into bile were considered to have poor excretory function (n=14), while livers that excreted >1000 IU/L bilirubin into bile were considered to have a proper excretory function (n=36 paired samples). Figures represent the median ± IQR of relative miRNA levels. (C) Correlation between HDmiR-122 and bilirubin levels in bile. Dynamics between bilirubin levels in bile and HDmiR-122 levels in bile were similar, suggesting a relation between HDmiR-122 and hepatocyte excretory function (P<0.001, R=0.694). Each graph represents values in an individual recipient during the first three weeks of follow-up after liver transplantation.

HDmiR and CDmiR release into bile and serum during liver injury

To analyze the direction of miRNA release during liver injury, pairs of bile and serum samples from LT recipients were analyzed at time of liver injury with elevated serum transaminase levels (serum AST>50 IU/L, n=41) and compared to samples at time of limited liver injury (serum AST<50 IU/L, n=21). The median miRNA levels of HDmiRs and

CDmiRs in paired serum and bile samples are summarized in **Table 1**. As illustrated by **Fig. 3A**, during injury there was a polarized release of HDmiR-122 and CDmiR-222 into bile (P≤0.015). This inverse relation was also observed in serum (**Fig. 3B**), showing an increase in HDmiR-122 (P=0.0002) while CDmiR-222 was decreased (P=0.045). In contrast to excretory function, miRNAs in serum rather than bile showed the biggest discrepancy at time of liver injury (**Table 1**).

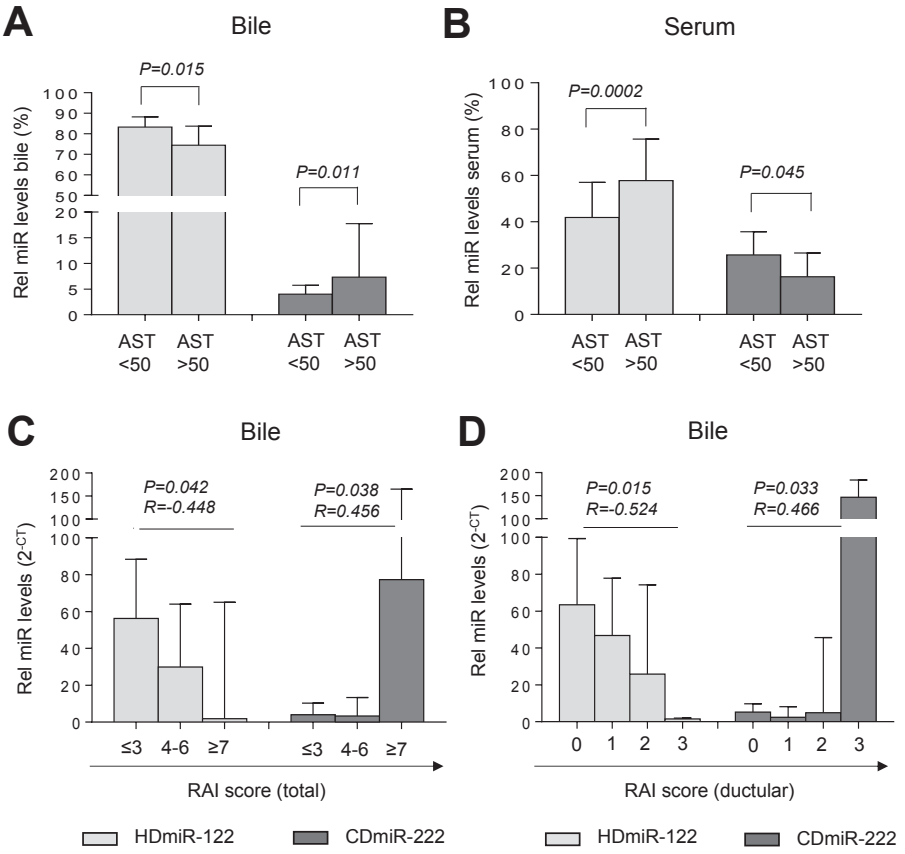

Figure 3. (A) Directional release of HDmiRs and CDmiRs into bile and (B) into serum during early (cellular) liver injury. Samples were divided into two groups: the low transaminase group with serum AST ≤50 IU/L (n=21) and the high transaminases group with serum AST >50 IU/L (n = 41). (C, D) During the first three weeks of follow-up, approximately three liver biopsies were taken per patient for histological evaluation. Biopsies were routinely stained for HE and CK19 and scored by the rejection activity index (RAI-score). 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 levels of CDmiR-222 increased (C). This correlation became even more apparent when looking at the ductular component of the RAI-score (D). All figures represent the median ± IQR.

To further investigate the release of HDmiRs and CDmiRs during injury, levels of these miRNAs were correlated with the degree of rejection. From the ten recipients in our study, a total of n=21 biopsies were evaluated within the first three weeks following LT by an experienced pathologists, according to the BANFF scoring criteria. Based on histology and clinical parameters, recipients received adjusted immunosuppressive treatment in order to clear cellular rejection. As shown by **Fig. 3C**, levels of HDmiR-122 in bile showed a modest though significant inverse correlation with the total rejection activity index (RAI) score (P=0.042, R=-0.448). Oppositely, higher levels of biliary CD-miR-222 were positively associated with the RAI score (P=0.038, R=0.456). This contrast between HDmiR and CDmiR release into bile became even more apparent when we correlated their levels to the degree of ductular rejection (**Fig. 3D**, P=0.015, R=-0.524 for HDmiR-122 and P=0.033, R=0.466 for CDmiR-222). These correlations were absent in paired serum samples (data not shown). Interestingly, biliary levels of CDmiR-222 mildly correlated to serum levels of gamma-GT (P=0.045, R=0.258). Conventional markers as serum gamma-GT and alkaline phosphatase, however, did not correlate to histological ductular injury (data not shown). **Supplementary Fig. S2** shows an example of a liver biopsy taken at two different time points during follow-up, and which was evaluated based on H&E and CK19 staining.

DISCUSSION

This is the first study showing that release of HDmiRs and CDmiRs into bile and blood is polarized in response to stress, impaired excretory liver function and during mild or severe liver cell injury. In recipients early after LT, good bilirubin excretion into bile was accompanied with high HDmiR-122 excretion into bile, while this was impaired when excretory function was poor. At the same time, opposite dynamics were observed for CDmiRs. Also during episodes of cellular liver injury and rejection, inverse dynamics in HDmiR-122 and CDmiR-222 release into both bile and serum were found. The correlation between bilirubin and HDmiR-122 excretion suggest that besides being a marker for injury, HDmiR-122 might also be involved in the conjugation and exocrine function of hepatocytes. Moreover, the polarized release of HDmiRs and CDmiRs into bile and serum suggests active rather than passive underlying release mechanisms and this provides us with new information on the dynamics of extracellular miRNAs in liver disease. In **Fig. 4**, we illustrated proposed mechanisms of miRNA release during various (patho) physiologic conditions of the liver that are supported by our findings.

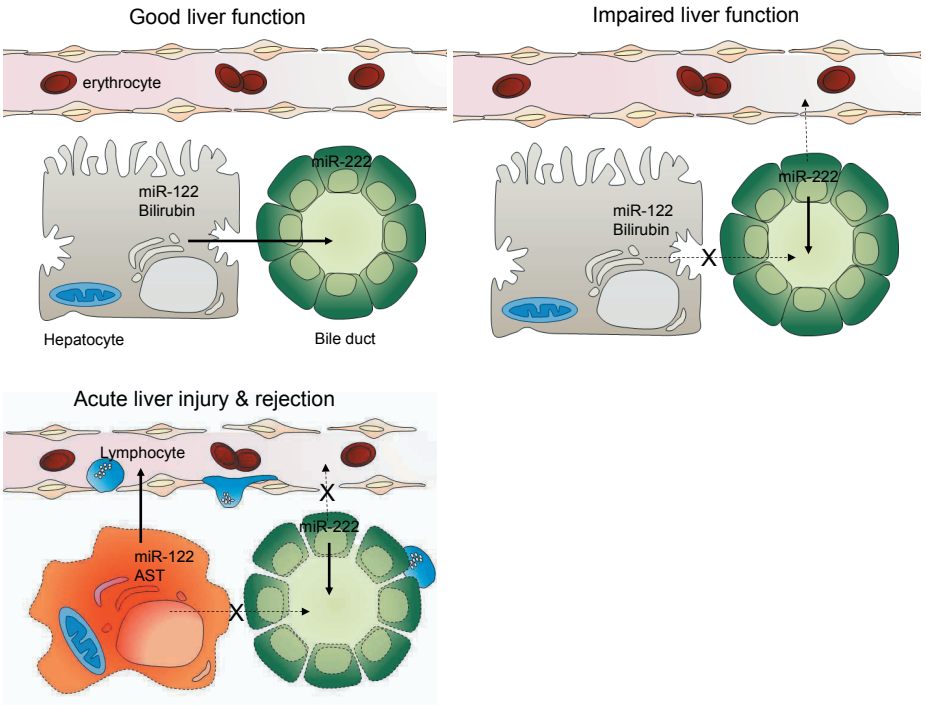

Figure 4. Illustration of suggested mechanisms and routes of miRNA release from hepatocytes and cholangiocytes in different (patho)physiological conditions. (A) During normal graft function, there is good excretion of HDmiR-122 and bilirubin by hepatocytes into bile. Levels of CDmiRs, like miR-222, remain low in bile when liver function is sufficient. (B) When excretory function is impaired, the excretion of HDmiR-122 and bilirubin into the bile is inhibited. There is, however, no release of HDmiR-122 and CDmiR-222 into the circulation. (C) During cellular injury and rejection, a similar mechanism of miRNA release is seen as during impaired function. The polarized release of HDmiRs and CDmiRs is however also directed into serum. Release of HDmiRs is more pronounced into serum while CDmiRs are mainly released into bile.

Previous studies reported on miRNAs in bile solely. The first report on biliary miRNAs identified miR-9 as a potential biomarker for biliary tract cancer[23]. The investigators also verified the presence of HDmiR-122, CDmiR-200c and CDmiR-222 in bile, which were identified earlier in liver and bile duct tissue[19]. Also, despite the RNA hostile environment of human bile, miRNAs were found to be highly stable and protected from degradation. The current study not only confirmed that miRNAs can remain stable in bile for a certain period of time, but also that this is probably due to the fact that most miRNAs are bound to proteins in the unpelletable biliary supernatant. This is in accordance with earlier findings regarding stability of extracellular miRNAs in plasma and serum[29]. The most well-studied protein responsible for miRNA stability is Argonaute-2 (Ago2)[29, 31]. In the cell cytoplasm, miRNAs are loaded onto Ago2 and together they form the RNA-induced silencing complex (RISC). Subsequently, this RISC is guided to

target mRNA by specific miRNAs. Being the active component of the RISC, Ago2 is able to inhibit messenger RNA (mRNA) translation through cleavage[32]. Other proteins that have been associated with miRNA stability are nucleoplasmin and high-density lipoproteins[33, 34]. It, however, remains unclear which protein is mainly responsible for miRNA stability in bile. Furthermore, in our study, approximately 10% of the initial biliary miRNAs remained detectable after protein degradation, suggesting protection against degradation in a different manner. Perhaps a minority of miRNAs is bound to complexes that have not yet been discovered. Nevertheless, it cannot be ruled out that fragments as vesicles, exosomes, or apoptotic bodies were incompletely removed from the biliary supernatant even after centrifugation at 140,000*g*.

Besides biliary miRNAs in the unpelletable fraction, a recent paper by Li et al. reported on biliary miRNAs located in extracellular vesicles as potential diagnostic markers for cholangiocarcinoma[24]. The investigators plea for the analysis of miRNAs present in extracellular vesicles rather than those in whole bile, in order to better discriminate between cholangiopathologies. 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 shown here, the percentage of miRNAs present in vesicles like exosomes appeared to be very low[23]. By only looking at miRNAs in the vesicle fraction, over 90% of the miRNA signal in bile would be overlooked and ignored for analysis[35].

In addition to characterizing of biliary miRNAs, the studies from Shigehara at al. and Li et al. also confirmed that CDmiR-222 is a potentially relevant marker for cholangiopathy[23, 24]. 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 LT[20]. Based on this observation, earlier we hypothesized that cholangiocytes release their miRNA content into bile rather than into blood. The results of the current study support this hypothesis by the polarized release of HDmiRs and CDmiRs into bile during impaired function and graft rejection.

Furthermore, the observation that HDmiR-122 is released into serum at time of injury is in accordance with other studies[17, 18, 36]. The finding that HDmiR-122 is also excreted into bile and strongly correlates with cellular excretory function, however, is new. This suggests a role of HDmiR-122 in biliary hemostasis via the entero-hepatic circulation. Other miRNAs that have been linked to biliary homeostasis are miR-506, miR-222 and miR-199a-3p; miR-506 was found upregulated in cholangiocytes of patients with primary biliary cirrhosis, causing impaired biliary secretory function by diminished anion exchanger 2 activity[27]. In esophageal adenocarcinoma cells, CDmiR-222 expression and farnesoid-X receptor (FXR) activity were affected by bile acids[26]. FXR has also been linked to protection of hepatocytes by repressing miR-199a-3p[25]. On the other hand, some miRNAs were reported to regulate expression of FXR, thereby increasing the risk

for cholangiocarcinoma[37]. The exact mechanism by which HDmiR-122 is involved in the context of biliary homeostasis should be explored by future research.

The current study contains several limitations. First, we did not confirm the results from our human data in an *in vitro* model. Investigating polarized miRNA release appears unsuitable in hepatocyte or cholangiocyte cell-lines. Thereby, it also remains difficult to judge whether miRNAs are either passively or actively released to bile. For future research, the design of 3D organ-like structures could serve this purpose for specific liver pathologies[38]. In particular organoids appear to have a ductular phenotype during their differentiation, that could be of interest for in vitro molecular research of bile ducts and directional release of miRNAs[39]. Finally, the bile and serum samples used in this study were obtained from only ten LT recipients. However, the longitudinal collection of multiple samples (n=124) was particularly useful for studying the biology of extracellular miRNAs in terms of dynamics and directions of release.

The results from this study are important for understanding miRNA biology and subsequent development and interpretation of new diagnostic assays. We observed that injury to cholangiocytes causes a release of CDmiRs into bile rather than into serum. So, counterintuitively, serum of patients with a higher degree of cholangiocyte injury contained lower levels of CDmiRs. Moreover, bile could be considered as a novel diagnostic medium or so-called liquid biopsy for biomarkers. For instance when patients suffering from cholangiocarcinoma need interventions via the endoscopic or percutaneous route, the collection of bile is accessible and perhaps less sensitive for sampling bias, as can be the case with brush cytology[40]. But also in the context of LT, novel organ preservation techniques like machine perfusion create a prolonged time window in which graft quality could be evaluated more objectively; the production of bile during machine perfusion is potentially useful for assessing liver graft viability[41]. In addition, measurement of miRNAs in bile or perfusates during machine perfusion might be informative on graft function or the degree of biliary injury, which are the main causes of graft failure after LT[42, 43].

In conclusion, this study demonstrates the polarized release of hepatocyte and cholangiocyte abundant miRNAs into bile and serum during impaired graft function and liver injury following LT. These findings shed new light on the underlying biology as well as the interpretation of extracellular miRNAs as biomarkers for hepatic injury and function.

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SUPPLEMENTARY INFORMATION

Fractionation and RNAse and protease treatment of bile samples

For fractionation of bile, 4 ml of cell-free, -20°C stored bile extracted from human organ donor gallbladders was diluted with 8 ml of sterile PBS and a baseline sample was taken (sample S0). In short, larger components, ie. mitochondria, lysosomes and peroxisomes, were pelleted by centrifugation at 20.000*g* for 20 min at 4°C (sample P1). The supernatant was then transferred to a new tube and centrifuged at 100.000*g* for 1 hour at 4°C in order to obtain a pellet enriched with microsomes and membrane fragments (sample P2). Finally, the remnant supernatant was centrifuged a third time for two hours at 140.000*g* at 4°C to obtain a pellet containing exosomes, ribosomes and viruses (sample P3). The pellets were 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. 400 µl of the remaining, unpelletable, supernatant was mixed with 1400 µl of Qiazol and stored at -80°C until further processing (sample S3).

Protein degradation was tested in samples of biliary supernatant after centrifugation (sample S3). Samples of 400 µl of bile supernatant 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.

For RNAse inactivation, cell-free, -20°C stored bile samples were 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.

RNA isolation

Total RNA was extracted using the miRNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol with minor modifications. For the isolation of miRNAs from tissue, 750µl of Qiazol lysis was added to approximately 10mg of snap frozen liver and bile duct specimens and homogenized by extensive vortexing with glass beads. For the isolation of miRNAs from serum and bile, 1.5ml 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, 1400 µl of Qiazol lysis agent was added to 400 µl of bile (S0), supernatant (S3), or pellet (P1, P2, P3) as mentioned earlier. After 5 minutes of resting at room temperature, 200µl or 280 µl of chloroform was added respectively, 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

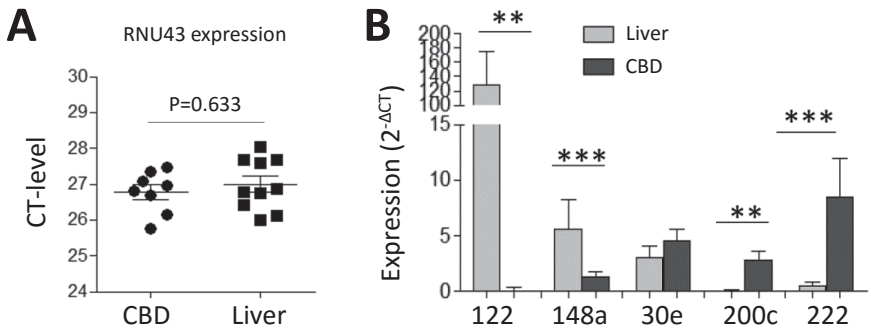
further processed according to the manufacturer's protocol (Qiagen). RNA content was quantified, handled and stored as described previously[1].

Reverse transcription and Real-Time quantitative Polymerase Chain Reaction (RT-PCR)

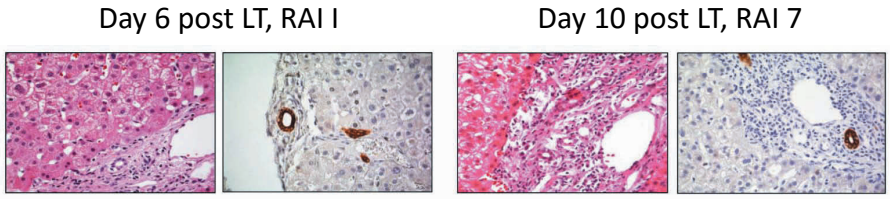
RNA samples were analyzed for HDmiRs and CDmiRs as previously reported[2-4]. As HDmiRs, miR-122 and miR-148a were determined and for CDmiRs, miR-30e, miR-200c and miR-222 were analyzed.

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[4]. 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[1].



Supplementary Figure S1. (A) RNU43 was equally expressed in common bile duct (CBD, n=8) and liver tissue (n=10) and was used as a reference gene for comparing hepatocyte and cholangiocyte-derived miRNAs in these two types of tissue. (B) Confirmation of hepatocyte- and cholangiocyte abundance of HDmiR-122, HDmiR-148a, CDmiR-30e, CDmiR-200c and CDmiR-222 in liver biopsies (n=10) and common bile duct tissue (CBD; n=8). RNU43 levels were used for normalization. HDmiR-122 was the most abundant in liver tissue, while CDmiR-222 expression was highest in tissue of CBD.** P<0.01, ***P<0.001.



Supplementary Figure S2. For each LT recipient, liver biopsies were taken at different time points during follow-up. Displayed are two biopsies taken at day 6 and day 10 following LT. The rejection activity index (RAI) was evaluated based on H&E and CK19 staining.

Supplementary Table 1. Characteristics of LT donors and recipients.

Variable	Number
Donor characteristics	
Age (mean ± SD)	44.4 ± 8.6
Sex (m/f)	5/5
BMI (mean ± SD)	23.3 ± 1.9
Donor lab (mean ± SD)	
AST (IU/L)	43.8 ± 30.4
ALT (IU/L)	20.6 ± 12.0
Gamma-GT (IU/L)	26.2 ± 35.9
Bilirubin (µmol/L)	27.0 ± 42.1
Natrium (mmol/L)	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 in min (mean ± SD)	519.0 ± 137.1
Recipient characteristics	
Age (mean ± SD)	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
RAI first 21 days post LT (mean ± SD)	4.2 ± 2.5

Abbreviations: BMI; body mass index - DBD; donation after brain death – DCD; donation after circulatory death – CVA; cerebral vascular accident – UW; university of Wisconsin solution – HTK; histidine tryptophan ketoglutarate – HCV; hepatitis C virus – HBV; hepatitis B virus – PSC; primary sclerosing cholangitis – PBC primary biliary cirrhosis – AIH; auto-immune hepatitis – PNF; primary non-function – RAI; rejection activity index.

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Chapter 7

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

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ABSTRACT

Background & aims. 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 of the development of ITBL after liver transplantation.

Methods. Graft preservation solutions (perfusates) and paired liver biopsies collected at the end of cold ischemia were analysed 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.

Results. 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 (DCD; $P\leq0.016$) and was superior to expression in liver biopsies ($C=0.77$ in perfusates vs. $C<0.50$ in biopsies).

Conclusion. This study demonstrates that differential release of CDmiRs during graft preservation is predictive of 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[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 peri-biliary 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 favourable 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 un-

known. 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 after transplantation, and whether they have the potential to serve as accurate and stable biomarkers.

MATERIALS AND METHODS

Explanations on study design, definition of ITBL, sample collection & processing, RNA isolation, RT-qPCR, stability assay and statistical analyses are listed in the **Supplementary information**. Detailed information on the fractionation assay is also provided in the **legend of Fig. 2a.**

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 perfus-

Table 1. MicroRNA primer sequences.

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

ates 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

Table 2. Recipient and donor characteristics.

	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

n.s., not significant

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.

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 **Fig. 1C**, HDmiRs and CDmiRs in cell-free perfusates remained stable up to 24 hours after storage at room temperature. In contrast, an exogenously added Caenorhabditis 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 **Fig. 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 (**Supplementary Fig. 1**). 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.

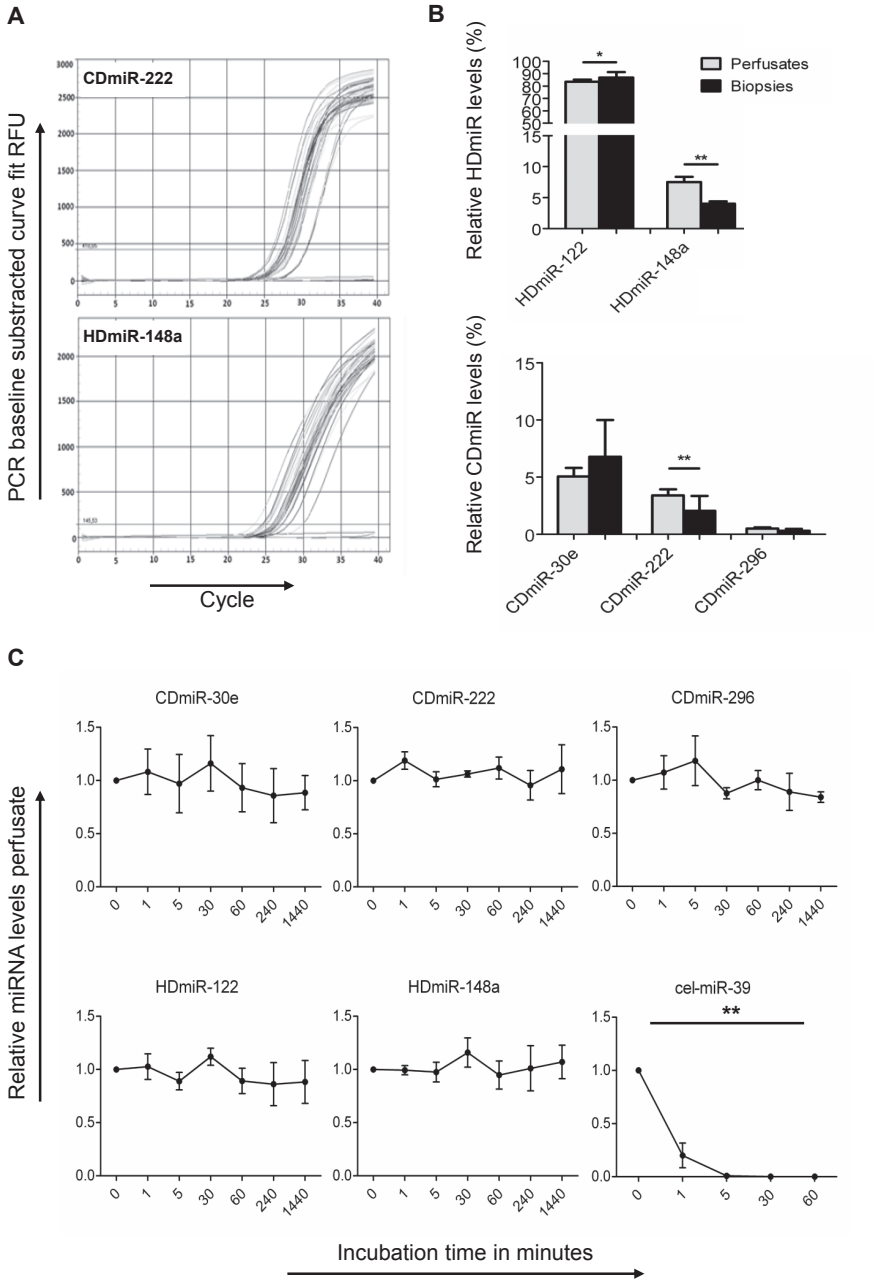

Figure 1. Relative HDmiR and CDmiR levels in perfusates and tissue biopsies. (A) RT-qPCR results for CDmiR-222 and HDmiR-148a in n=29 perfusates. (B) Relative levels of HDmiR-122 (P=0.033), HDmiR-148a (P=0.008) and CDmiR-222 (P=0.010) were significantly different between 33 paired perfusate- and biopsy 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 of incubation. The synthetic exogenously spiked-in cel-miR-39 however was degraded within five minutes (*P<0.05, **P<0.01). All figures demonstrate the mean ± SEM.

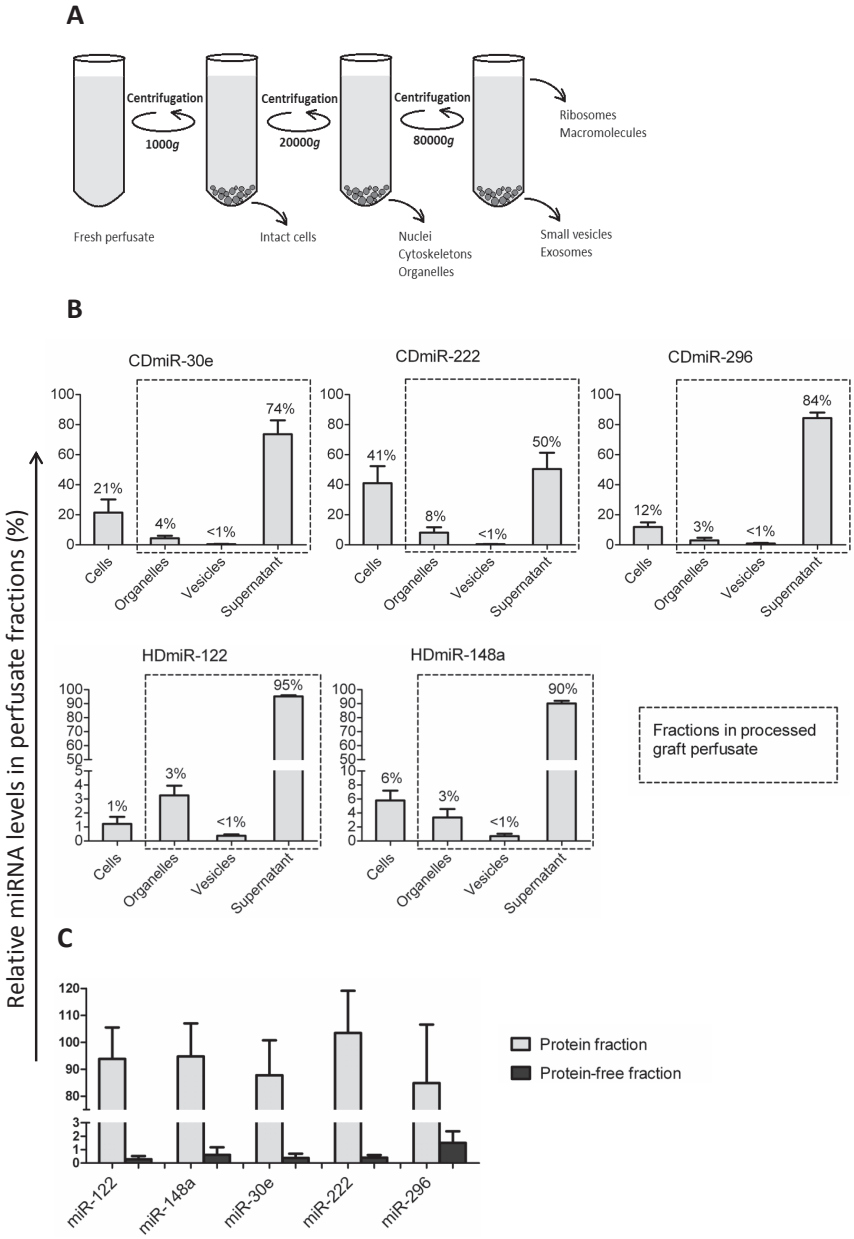

Figure 2. MicroRNA fractions in perfusates. To investigate in which different fractions miRNAs are present in perfusate, separate cell and subcellular fractions were prepared by different centrifugations steps (A); fresh perfusates were centrifuged at low speed (1,000*g* for 10 min.) to pellet intact 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. (C) After the final spin, acetone precipitation of the supernatantshowed that over 90% of miRNAs was bound to proteins (n=4). Shown are the mean ± SEM.

Ratios between HDmiRs and CDmiRs in perfusate are predictive for the 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 **Fig. 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, -222 and -296 as independent risk factors for ITBL (**Table 3**). This model also demonstrated discriminative power of miRNAs in perfusate, calculated by C-statistics, 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%.

Table 3. Cox regression analysis and C-statistics on HDmiR/CDmiR ratio's in perfusate and the development of ITBL.

	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)

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.

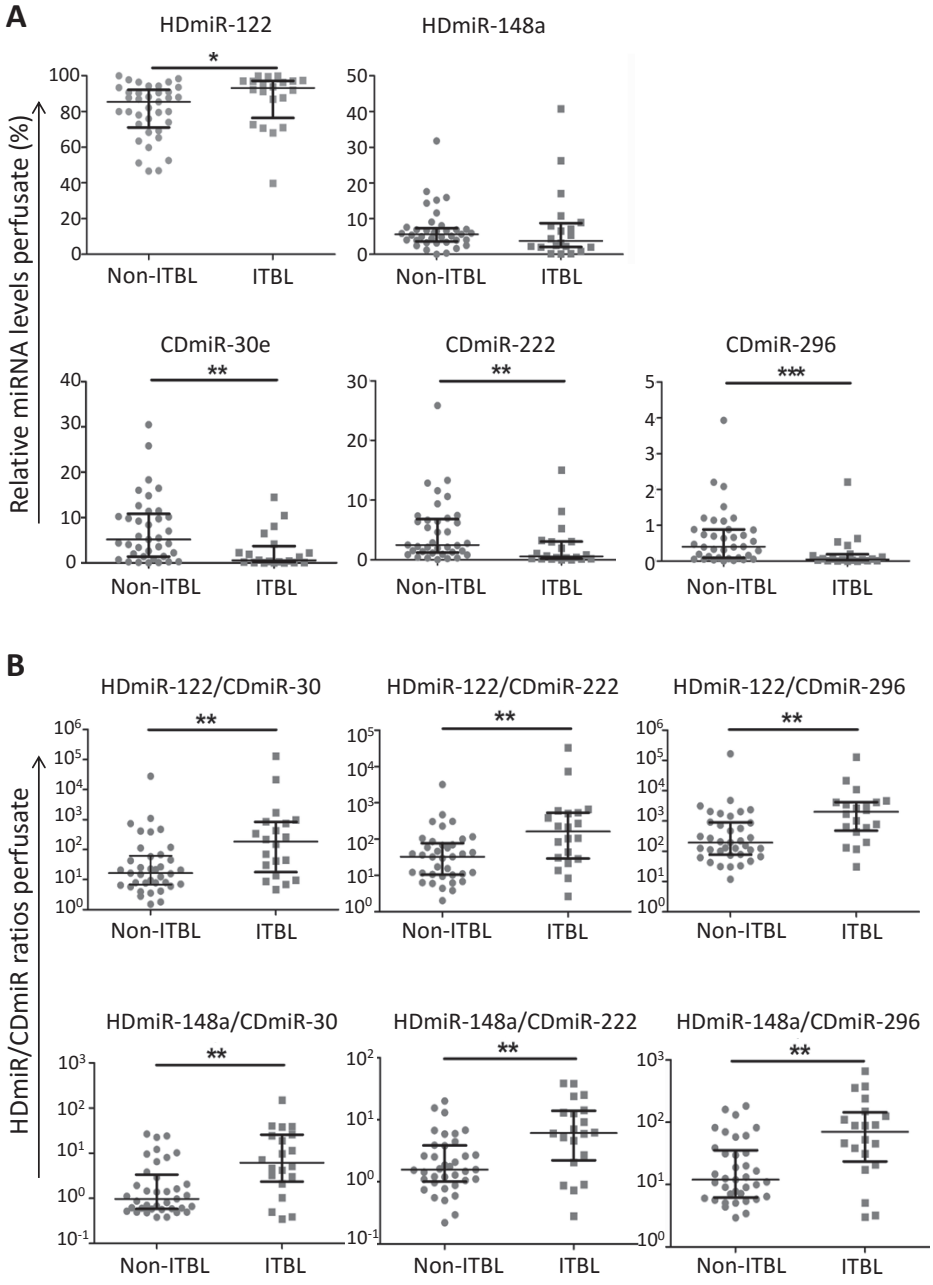

Figure 3. Distribution of relative HDmiR and CDmiR-levels and ratios between HDmiRs/CDmiRs in perfusates. (A) Comparison of relative HDmiR- and CDmiR-levels in perfusates obtained from grafts that developed ITBL (n=20) and grafts that did not develop biliary strictures (non-ITBL group, n=37). (B) Due 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.

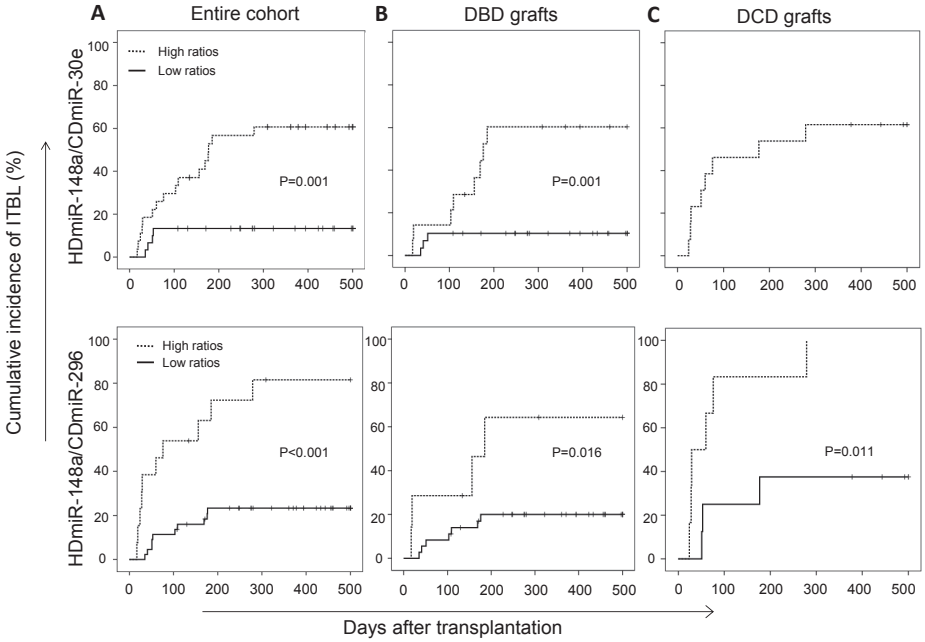

Figure 4. Increased incidence of ITBL in grafts with high HDmiR/CDmiR ratios in perfusates. Through a grid search on HDmiR/CDmiR values within the inter-quartile range, different cut-off values could be obtained to distinguish grafts with an increased incidence of ITBL from grafts without biliary complications 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).

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 (**Supplementary Fig. 2A and 2B**). 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 of 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 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 microvesicle 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 isolations 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 **Fig. 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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SUPPLEMENTARY INFORMATION

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[1]. Through RT-qPCR, we confirmed that expression of CDmiRs are up to eight-fold higher in choledochus 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.

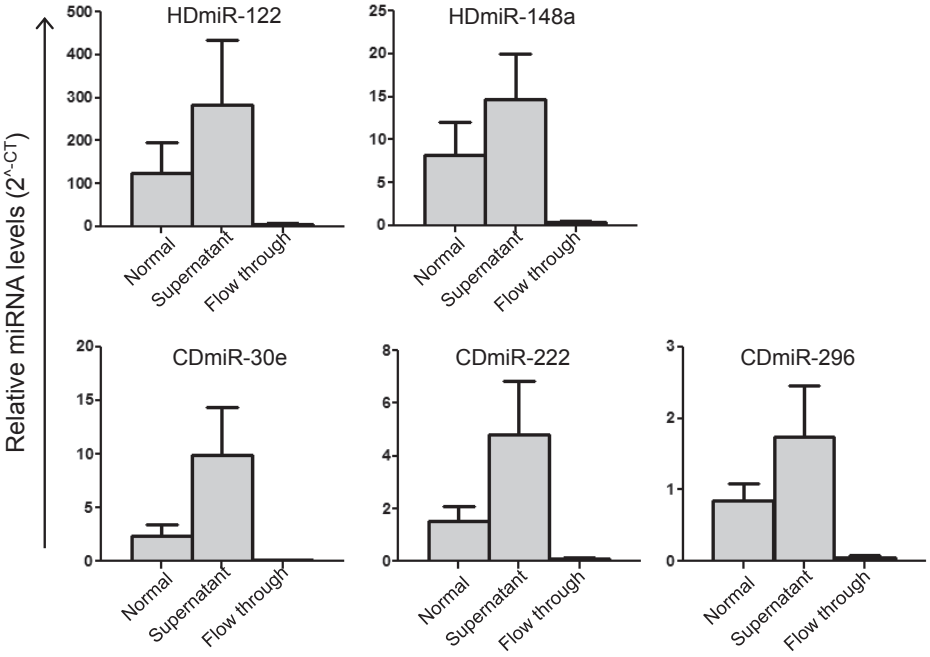
RNA isolation

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 min. In order to optimize signaling for RT-qPCR on lower abundant miRNAs, cell-free perfusate samples were concentrated with a 100 kD Amicon filter (**Supplementary Fig. 1**); Of cell-free perfusates, 3 mL 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 ($2^{-\Delta Ct}$).

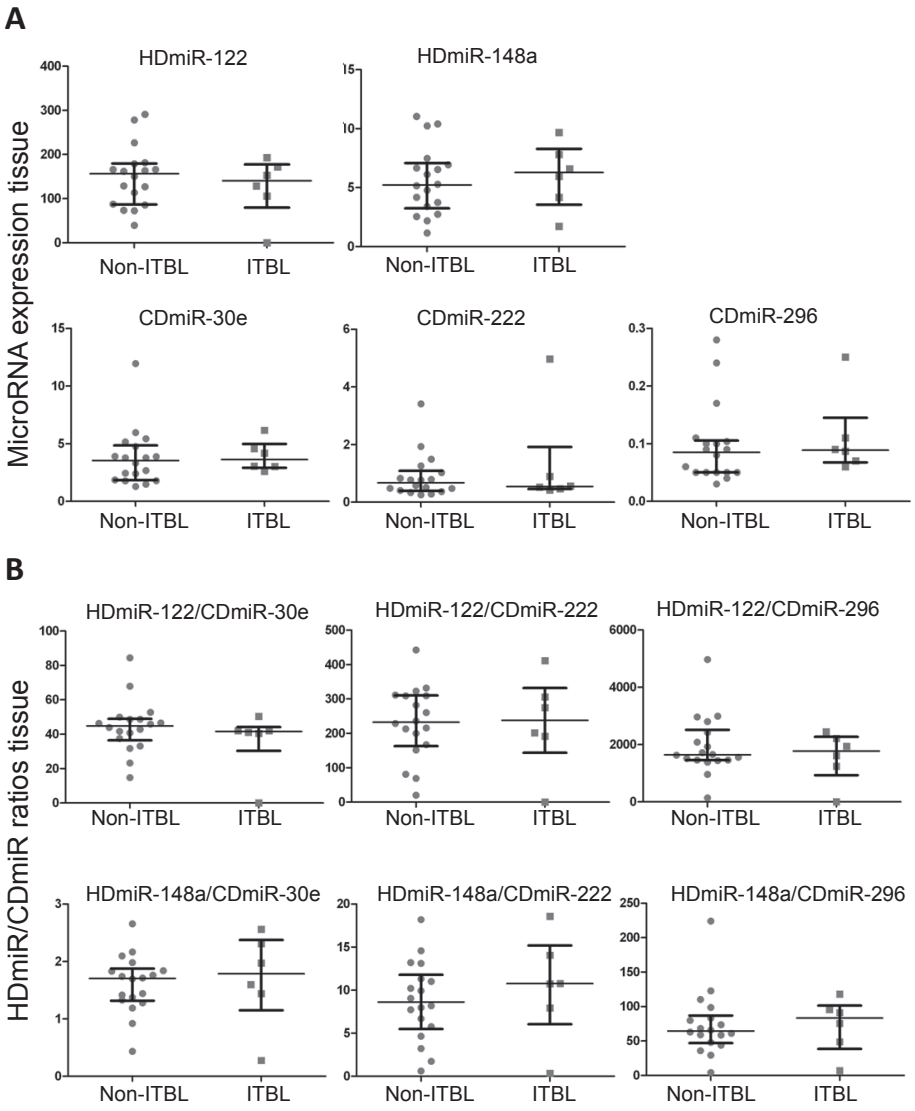


Supplementary figure 1. Concentration of perfusates improves miRNA detection. Perfusate samples obtained from n=3 different liver transplantations were cleared from cells by centrifugation. Of the cell-free perfusate, 3 mL was concentrated by 100 kD Amicon Ultra filters. After concentration, 100 µL of the supernatant and 100 µL of the flow-through were used for miRNA isolation and to test for respectively increased signaling in the supernatant and the amount of miRNAs passing the 100 kD filter. Compared to paired non-concentrated perfusate samples, miRNA detection was two to four-fold higher after concentration. The fraction of miRNAs in the flow-through that passed the filter was less than 5%, suggesting that most miRNAs in perfusates are bound to (protein) complexes ≥100 kD.

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 de-

scribed 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 (**Fig. 2A**). For this, 50 ml fresh unpro-



Supplementary figure 2. Expression of HDmiRs and CDmiRs in liver biopsies. Comparison of HDmiRs, CDmiRs, and HDmiR/CDmiR ratios in liver biopsies of grafts that did (n=6) or did not (n=18) develop ITBL. Tissue miRNA levels were determent by RT-qPCR and normalized to the RNU43 reference RNA. (A) Both HDmiRs and CDmiRs showed no significant differences in expression in biopsies of ITBL or non-ITBL grafts. (B) HDmiR/CDmiR ratios were also not significantly different between ITBL or non-ITBL grafts. Shown are the median ± interquartile ranges of normalized miRNA expression.

cessed (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.

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Chapter 8

Optimizing detection of microRNAs in graft preservation solution as a biomarker for ischemic-type biliary lesions

CJ Verhoeven, JW Selten, H Roest, HJ Metselaar, JNM IJzermans, LJW van der Laan

To the editor,

Ischemic-type biliary lesions (ITBL) are a major complication after liver transplantation (LT), which can lead to severe obstructive jaundice in recipients and impaired graft survival. Previously, we showed the potential of extracellular microRNA (miRNA) profiles in flush-outs of preservation solutions, so called perfusates, to predict the development of ITBL after LT[1]. We demonstrated that particularly the ratio of hepatocyte and cholangiocyte-derived miRNAs (HDmiRs/CDmiRs) was predictive for the development of ITBL. As a follow-up to our previous study, here we would shortly like to elucidate on new means to further optimize detection of miRNAs in perfusates and improve their accuracy as biomarkers.

Recently, Plieskatt et al.[2] showed the inhibitory effects of heparin on the quantification of circulating miRNAs by reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR). These inhibitory effects could be best negated by treating RNA samples with heparinase I during the RT-step. In the setting of liver transplantation, in our center graft procurement in the donor is performed with a standard amount of approximately 30,000 IU heparin. Directly following organ retrieval, the graft is thoroughly flushed with preservation solution, which is repeated at the end of cold storage. Because of the strong connection with endothelial cells[3], traces of heparin might still be present in the liver even after multiple flushes with preservation solution. Therefore, it remained unknown whether perfusates at the end of cold storage contain heparin and whether the amount of heparin would be sufficient to interfere with RT-qPCR detection of miRNAs. To investigate this, we tested the presence of heparin by re-evaluating our earlier analysis of miRNA profiles in graft perfusates after treating samples with heparinase I. To do so, specific cDNA for HDmiR-122, HDmiR-148a, CDmiR-222, and CDmiR-296 was synthesized in the presence of 6 IU of heparinase I. A synthetic *C.elegans* miRNA (Cel-miR-39) was added as an internal control for sample variation during the RT step. Relative expression levels were calculated and normalized for Cel-miR-39 levels.

Heparinase I treatment improved detection of miRNAs in all perfusates (n=57). As shown by **Fig. 1A**, the median decrease in Ct-value by qPCR was 1.15 for HDmiR-122 (IQR: 0.31-2.46), 2.65 for HDmiR-148a (IQR: 2.13-4.90), 1.28 for CDmiR-222 (IQR: 0.82-2.07) and 1.50 for CDmiR-296 (IQR: 0.58-3.38). The decrease in Ct-values after heparinase I treatment was significantly stronger for HDmiR-148a compared to the other miRNAs. This could be explained by the low detection levels of HDmiR-148a before heparinase I treatment, which was even >35 Ct in a small number of samples. Heparinase I treatment also effected the ratios between HDmiR-148a/CDmiR-222 and HDmiR-148a/CDmiR-296, which significantly increased after heparinase I treatment (**Fig. 1B**).

Out of the 57 patients included for this study, n=22 developed ITBL and n=35 remained free from biliary complications. After heparinase I treatment, the ratio between

HDmiRs/CDmiRs remained higher in perfusates obtained from grafts that developed ITBL compared to the non-ITBL group (**Fig. 1C**, P≤0.012). This distribution of perfusate HDmiR/CDmiR ratios between ITBL and non-ITBL grafts was almost identical to that observed without heparinase I treatment. However, the re-evaluation of the cox-regression analysis on the heparinase I treated samples showed that ratios of HDmiRs/CDmiRs became a stronger predictor for ITBL (**Fig. 1D**). Furthermore, ratios between HDmiR-122 and CDmiRs also appeared to be independent predictors, which was not the case in our already published results. The incidence of ITBL was up to six-fold higher in grafts with high HDmiR/CDmiR ratios (**Fig. 1E-G**). In grafts donated after brain death (DBD), HDmiR-148a/CDmiR-296 had a high negative predictive value of 88%. In grafts donated after circulatory death (DCD), in particular HDmiR-122/CDmiR-296 had high positive predictive value (90%).

These findings show that some perfusate samples can be contaminated with traces of heparin that are sufficient to inhibit miRNA detection. This inhibitory effect can however effectively be counteracted by treating isolates of perfusate RNA with heparinase I during the RT-step in qPCR analysis. Therefore, we would like to emphasize that in PCR-based biomarker research, one should always take into account the possibility of inhibitory components like heparin. For future applications of miRNAs as biomarker, HDmiRs and CDmiRs may provide valuable predictive information. Thus, with the growing interest in the use of machine perfusion and assessment of graft quality, counteracting the interference of heparin in perfusate must be reckoned with[4, 5].

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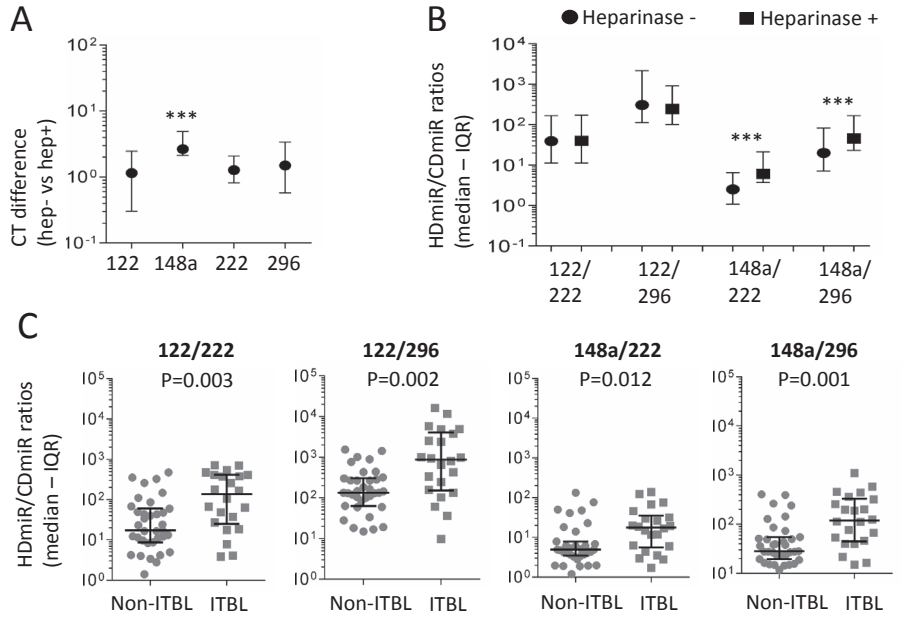

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	Univariate			Multivariate		
HDmiR/CDmiR ratio	HR	p value	(95% CI)	HR	p value	(95% CI)
HDmiR-122/CDmiR-222	2.79	0.002	(1.46-5.34)	2.39	0.031	(1.04-5.50)
HDmiR-122/CDmiR-296	2.84	<0.001	(1.60-5.04)	2.49	0.028	(1.06-5.83)
HDmiR-148a/CDmiR-222	2.89	0.005	(1.38-6.07)	4.17	0.011	(1.41-12.32)
HDmiR-148a/CDmiR-296	4.71	<0.001	(2.00-11.11)	6.19	0.005	(1.72-22.31)

In the multivariate model, separate HDmiR/CDmiR ratios were adjusted for graft type (DBD vs. DCD) and the type of solution used for graft preservation (UW vs. HTK).

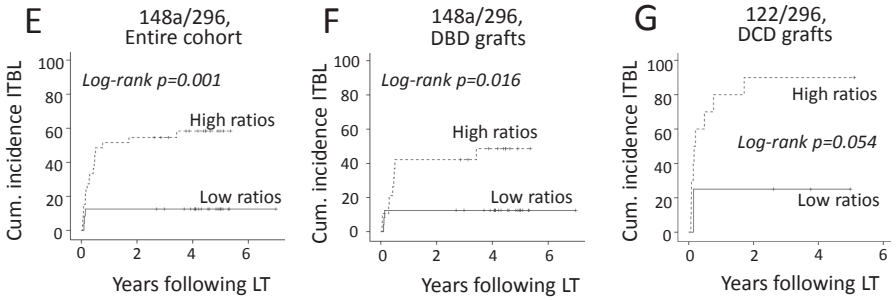

Figure 1. (A) After heparinase I treatment, the median decrease in Ct-level was stronger in HDmiR-148a compared to other miRNAs. (B) Ratios of HDmiR-122/CDmiR-222 and HDmiR-122/CDmiR-296 were not significantly changed by heparinase I treatment. However, due to the stronger effect of heparinase I on HDmiR-148a, ratios of HDmiR-148a/CDmiR-222 and HDmiR-148a/CDmiR-296 increased. (C) Increased ratios of HDmiRs/CDmiRs in perfusates from grafts that developed ITBL compared to those that remained free from biliary complications after heparinase I treatment. The distribution between the two groups was almost identical to samples that were non-heparinase treated samples (data not shown). (D) Heparinase I treatment further strengthened miRNA ratios as independent predictor of ITBL. (E-G) Increased incidence of ITBL in grafts with high HDmiR/CDmiR ratios in perfusate. *** P<0.001.

Chapter 9

Improving accuracy of urinary microRNA quantification in heparinized patients using heparinase I digestion

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ABSTRACT

MicroRNAs (miRNAs) have emerged as promising biomarkers due to their association with cell stress and diseases and their easy detection and stability in many body fluids. Because of the sensitivity, the method of choice to detect miRNAs is quantitative reverse transcribed quantitative polymerase chain reaction (RT-qPCR). Therapeutics, in particular circulating anti-coagulants, are notorious for their inhibitory effect on RT-qPCR-based measurements. The effect of heparin contamination on inhibition of RT-qPCR on miRNAs isolated from urine has, however, never been investigated. We obtained urine samples from healthy controls and from heparinized patients undergoing major surgery (live kidney donation or liver transplantation) (n=27). Samples were spiked with synthetic miRNAs to monitor RNA loss during work-up and levels of endogenous and spiked-in miRNAs were quantified by RT-qPCR. We showed that endogenous miRNAs in urine were protected from degradation but levels differed substantially within surgery groups. Variability in detection levels of spiked-in miRNAs was low in non-hospitalized controls, but was high in both surgery groups, and the difference in miRNA levels correlated well with the heparin concentration in urinary samples. Treatment of urinary RNA with heparinase I during RT-qPCR strongly reduced this variation in a dose dependent manner. Heparinase I should therefore be considered as standard step for detection of miRNA in urine from hospitalized individuals.

INTRODUCTION

MicroRNAs (miRNAs) are small RNA molecules of approximately 19-23 nucleotides in length that play an important role in the regulation of gene expression.[1] As an estimated 30-60% of all genes are post-transcriptionally regulated by these molecules and the possibility of sensitive quantification, miRNAs have a favorable position as biomarker for many diseases and physiological processes.[2] Two important prerequisites for a molecule to be considered as a biomarker for routine applications, being (i) ease of attainment, and (ii) stability during sample isolation, storage, and subsequent processing for analysis, apply to miRNAs. Although the majority of miRNAs are located intracellularly, a significant amount of miRNAs are released from cells into body fluids like blood, lymph, saliva, bile and urine.[3] These extracellular miRNAs also remain stable in environments like bile and graft preservation solution,[4, 5] despite high RNAse and protease activity present in these liquids. Due to non-invasive sampling and stability, urinary miRNAs can also be considered an excellent source for biomarker identification.[6] This has been acknowledged for various medical fields like urology, nephrology, and oncology of the urinary tract. Recent data show that urinary miRNAs are also discriminative in systemic diseases and malignancies, expanding the potential applications of urinary miRNAs as biomarker.[7, 8]

Polymerase chain reaction (PCR) and reverse transcription (RT) are among the most powerful tools in molecular biology and frequently used for detection of diagnostic and prognostic DNA and RNA biomarkers. Though sequence specific amplification through PCR improves miRNA detection, some factors can actually inhibit this molecular approach and thus interfere with detection. The existence of RT-qPCR inhibiting components have been described decades before the invention of PCR and this technique became a common practice in RNA quantification and DNA amplification.[9, 10] The currently known RT-qPCR inhibitors represent a very diverse group of chemical substances, both organic and inorganic, with different modes of action. They can interfere with sample processing, nucleotide extraction, substrate and enzyme degradation, or polymerase inhibition.[11]

In the clinical setting, therapeutics are considered one of the main concerns for PCR inhibition. Particularly anticoagulants often administered during hospital stay or used in plasma sampling are notorious PCR inhibitors.[12-14] Recently, Boeckel et al. described the inhibitory effect of heparin on the amplification of miRNA in human blood samples to be dose dependent with complete inhibition occurring at levels as low as $2x10^{-5}$ IU/µl.[15] Heparin is a highly sulfated disaccharide polymer ranging in weight from 3-30 kDa. Two distinct mechanisms are known for eliminating heparins from the circulation, which depends on the size and concentration of molecules: i) The reticuloendothelial system for high doses of unfractionated heparin (UH) and ii) renal clearance for lower

doses of UH and low molecular weight heparins (LMWH).[16] Urinary excretion of UH and LMWH after systemic administration is well documented and the remaining anticoagulant activity well characterized.[17-19] However, to what extend these degradation products can interfere in the non-invasive measurement of biomarkers such as miRNAs has not been documented.

The aim of the current study was to investigate the excretion of LMWH and UH into urine and determine their effects on miRNA detection levels by RT-qPCR. Subsequently, we examined the effectiveness of heparinase I digestion to restore detection of urinary miRNA.

MATERIALS AND METHODS

Drugs and reagents

All reagents used were laboratory grade and pharmaceuticals were in-house manufactured by the hospital pharmacy (UH 500 IU/mL; protamine-sulfate 1000 IU/mL) or clinical grade, commercially available, LMWH (Fraxiparine® (GlaxoSmithKline, Zeist, The Netherlands)).

Patient and healthy control characteristics

Urine samples were obtained from the following groups: Post-surgically from liver transplant recipients that were heparinized prior to (heparinized, n=10) or after urine collection (non-heparinized, n=9) and who were operated between March 1st 2014 and March 1st 2015; Pre-surgically from healthy, heparinized kidney donors prior to surgery between June 1st 2009 and July 1st 2010 (n=10); From volunteering healthy controls (n=6). Seven combined urine and kidney biopsy samples were obtained between 21st January and February 19th of 2015 as part of the PROTECT Trial (NTR3663). Collection and use of urine and biopsy material from hospitalized individuals was approved by the Medical Ethical Council of the Erasmus MC and all participants provided informed consent for the use of these materials for medical research. Baseline characteristics are presented in **Table 1**.

Sample preparation and storage

Urine was obtained from liver transplant recipients within 24 hours after transplantation while on the intensive care unit, directly from a urinary catheter in a vacutainer without additives (Becton-Dickenson, Breda, the Netherlands). Urine from live kidney donors was collected the morning prior to kidney donation. Urine from healthy individuals was first-morning urine. Samples were cleared from large fragments and cells by centrifugation for 15 min 3,200*g* at 4°C and subsequently stored at -20 °C or -80°C until further use.

Table 1. Baseline characteristics.

	Healthy controls		Liver transplant recipients		
	No heparin (n=6)	Heparinized (n=10)	No heparin (n=9)	Heparinized (n=10)	*p* value*
Median age (IQR)	45.9 (35.5-48.7)	58.8 (42.8-62.6)	56.4 (51.9-58.8)	47.5 (30.0-59.2)	n.s.
Sex					
Male (%)	3 (50)	7 (70)	8 (89)	7 (70)	n.s.
Female (%)	3 (50)	3 (30)	1 (11)	3 (30)	
heparinized individuals#					
LMWH	0	10	0	7	
UH	0	0	0	5	
liver function					
median Alk. Phos. (IQR)	ND	75.5 (68.75-94.5)	54 (37-143)	104 (67.75-157.25)	n.s.
median γGT (IQR)	ND	22 (20.25-31.25)	59 (23.35-113.25)	68 (47.75-163.25)	0.0098
median AST (IQR)	ND	23.5 (18.5-32.25)	304 (232.5-697.75)	757.5 (411-895.75)	<0.0001
median ALT (IQR)	ND	20.5 (17.5-22.5)	350 (264.5-545.25)	640 (354-915.25)	<0.0001
median PT-INR (IQR)	ND	ND	1.6 (1.6-1.9)	1.95 (1.8-2.88)	n.s.
renal function					
median serum urea (IQR)	ND	5.4 (4.73-5.83)	10.8 (8.9-13)	13.25 (8-17.85)	0.0001
median serum creat. (IQR)	ND	75.5 (66.75-81.5)	110 (66-136)	97.5 (67.5-117.5)	n.s.

*:*p*-values are determined by a 1-way ANOVA between 3 or more groups. For PT-INR a non-parametric t-test was used

#: 2 Liver transplant recipients received both LMWH and UH prior to surgery

ND: Not Measured. N.s.: not significant

RNA isolation

Total RNA was isolated from urine using the Qiagen miRNeasy kit as described by the manufacturer with some minor modifications (Qiagen, Venlo, The Netherlands). In short, 200 µL cell-free urine was lysed using 1400 µL Qiazol. After adding 280 µL chloroform, samples were centrifuged for 15 min at 12000xg at 4°C. Subsequently, 800 µL of the aqueous layer was used for further processing. RNA was eluted from the column with 30 µL RNAse-free water and stored at -80°C. Urine samples were spiked with 200 amol of artificial C*aenorhabditis elegans* miR-39 (Cel-miR-39) and miR-238 (Cel-miR-238) (Sigma Aldrich, Zwijndrecht, The Netherlands) during the lysis procedure or Cel-miR-54 during

cDNA synthesis from biopsy material. When required, RNA concentrations were determined using a BioAnalyzer 2100 (Agilent Technologies, Middelburg, the Netherlands).

Heparinase I treatment

5 µL of isolated total RNA was added to a reaction mixture consisting of 6 IU Heparinase I (New England Biolabs, Ipswich, MA) in 2.5 µL with RNAse-free water. Heparinase I was co-incubated during the RT step for cDNA synthesis as described hereafter.

Reverse transcriptase and quantitative real-time polymerase chain reaction

cDNA was synthesized using the Taqman microRNA Reverse Transcription Kit (Applied Biosystems/Life technologies, Carlsbad, CA) as described previously[5], using 5 µL of isolated total RNA. cDNA was diluted to 100 µL with water and stored at -20°C. PCR reactions were conducted on a Applied Biosystems StepONE plus real-time PCR machine (Applied Biosystems) according to the manufacturer's guidelines with 45 cycles of amplification. Reactions consist of 6 µL Taqman Universal PCR Master mix (Life technologies), 0.5 µL miRNA specific primer, 0.5 µL sterile milliQ water and 5 µL of diluted cDNA. Threshold levels were manually set at 0.25 for all microRNA assays, and the upper Cq limit for reliable detection was set at 35 cycles.

Colorimetric heparine assay

UH and LMWH content in urine was determined using a chromogenic anti Xa-activity assay[20] according to the manufacturer's instructions (HemosIL™ liquid heparin test kit, Instrumentation Laboratory, Breda, The Netherlands) on a Sysmex CS5100 coagulation analyzer (Siemens, the Hague, the Netherlands) with minor adjustments. The presence of antithrombin and plasma proteins was assured by diluting the cell-free urine with an equal volume of normal plasma prior to the assay.

Statistical analysis

Statistical analysis was performed using Graphpad Prism 5.0 (Graphpad Software, San Diego, CA). Spearman's Rank Correlation Test was used to generate correlation results. Group comparisons were analyzed using the Wilcoxon matched pair test or Mann-Whitney U-test for non-paired samples. p-values <0.05 were considered significant.

RESULTS

Stability of miRNAs in urine

Since stability during storage and isolation is an essential prerequisite for a good biomarker, we set out to determine whether extracellular miRNAs in urine remained present and were

not degraded. Urine samples from three healthy controls with adequate detection levels of miRNA-30e were spiked with 0.2 fmol of synthetic Cel-miR-39, and incubated at 37°C for 0, 7, 15, 60, 240 and 1440 minutes. After isolation, total RNA samples were analyzed for the variation in detection levels of endogenous miRNA miR-30e and the synthetic, spiked-in miRNA Cel-miR-39. As shown in **Fig. 1**, the endogenous miR-30e remained stable with levels of approximately 60% compared to baseline still detectable after 24 hours. The spiked-in Cel-miR-39, on the other hand, showed a strong decrease with levels dropping to less than 1% compared to baseline already after 7 minutes of incubation.

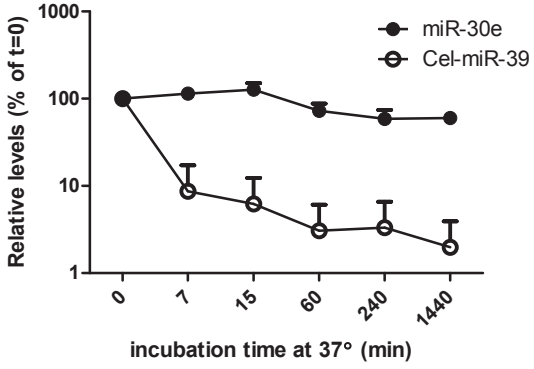

Figure 1. Stability of endogenous miRNAs in urine. Mean stability of endogenous miRNA miR-30e (closed circles) and spiked-in Cel-miR-39 (open circles) of healthy control samples (n=3) after increasing incubation times at 37°C.

Detection of endogenous and spiked-in miRNAs in urine of heparinized and non-heparinized individuals

Five different miRNAs that were described in literature as being present in urine of healthy individuals as well as patients with acute kidney injury (miR-18a, -30e, -92a, -155, and -637), were selected for further analysis in our study cohorts. These miRNAs showed less than five-fold difference between healthy controls and patients, Cq values did not exceed 32, and no false melting curves were present in any of the samples.[21] Out of these five miRNAs, miR-30e and miR-92a were selected because only these two miRNAs were detectable in most of the urine samples used in this study. As shown in **Fig. 2A**, Median (IQR) Cq values in urine were 29.53 (28.84-30.65) and 31.51 (31.03-32.8) for miR-30e and miR-92a, respectively, but were significantly higher in the heparinized group as compared to the non-heparinized group ($p \leq 0.001$) with median values (IQR) of 32.32 (31.01-34.11) and 34.70 (32.88-36.19). This observation was further supported and substantiated when the same urine samples were spiked with synthetic Cel-miR-39 and miR-238. RT-qPCR for these synthetic miRNAs resulted in median Cq values (IQR) in the non-heparinized group of 24.88 (24.13-25.11) and 26.6 (25.06-26.75) for Cel-miR-39 and Cel-miR-238, respectively but in the heparinized group results were 28.43 (26.49-28.90) and 30.16 (27.01-30.09) ($p<0.001$, **Fig. 2B**).

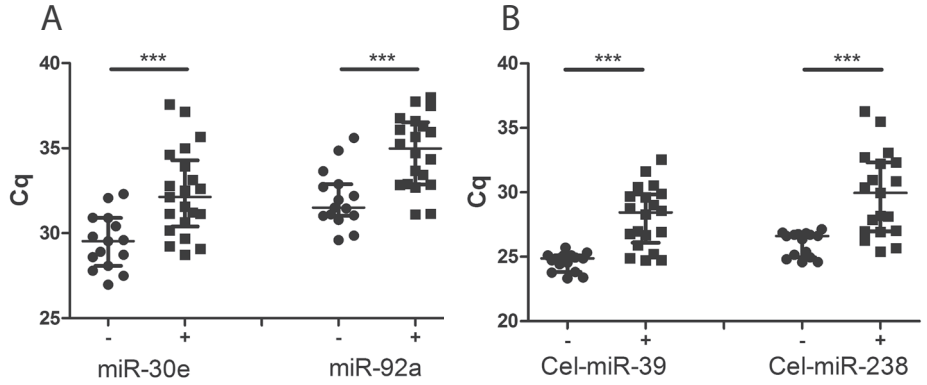

Figure 2. Variation in spike-in and endogenous miRNA detection. RT-qPCR results of endogenous miR-30e and miR-92a (A) and of spiked-in Cel-miR-39 and miR-238 (B) presented as Cq values in non-heparinized individuals (closed circles), and heparinized individuals (closed squares). Bars indicate median with interquartile range. *** $p<0.001$.

Heparin is a potent inhibitor of RT-qPCR analysis in urinary RNA samples and can be counteracted using heparinase I

To confirm that heparin is indeed able to inhibit RT-qPCR analysis of urine samples, we added increasing amounts of UH and LMWH to urine of a healthy, not-heparinized individual, as well as 50 amol Cel-miR-39 during lysis. Isolated RNA was analyzed for both spiked-in miRNA Cel-miR-39. PCR levels without anticoagulants were set at 100%. Concentrations of 0.01 IU UH/ml urine or higher were sufficient to completely inhibit RT-qPCR (**Fig. 3A**).

Heparinase I is a bacterial enzyme able to degrade approximately 80% of highly-sulfated disaccharides polymers. This enzyme has recently been re-established as a potential treatment to counteract RT-qPCR inhibition in the analysis of miRNA in plasma.[15, 22] To develop more general standards and optimize incubation conditions for RNA isolated from urine, heparinase I was added in different concentrations during cDNA synthesis. The effect of heparinase I treatment was assessed using the PCR results from spiked-in miRNA Cel-miR-39 and the endogenous miRNAs miR-30e and miR-92a. Five µl of total RNA, isolated from urine of two individuals that displayed high levels of RT-PCR inhibition, were co-incubated with variable amounts of heparinase I, and Cq values were converted to percentages relative to the co-incubation with 12 IU of heparinase I. Twelve IU of heparinase I is considered the optimal amount during co-incubation in RNA isolated from plasma.[22] However, non-linear fitting of the data showed that for RNA isolated from urine, co-incubation of 6 IU of heparinase I is already sufficient to reduce the inhibiting effect of heparin in RT-qPCR analysis of urinary RNA (**Fig. 3B**).

Variation in urinary miRNA detection of heparinized individuals is strongly reduced after heparinase I treatment

To validate the effect of heparinase I treatment in our cohorts, RNA samples from heparinized (n=20) and non-heparinized individuals (n=15) were co-incubated with or without 6 IU of heparinase I during cDNA. Subsequently, qPCR was performed for Cel-miR-39, miR-30e and miR-92a (**Fig. 3C**). For Cel-miR-39, detection levels in the non-heparinized group did not improve after heparinase I treatment (**Fig. 3C, left panel**). Similar results were observed for miR-30e and miR-92a in the non-heparinized individuals (**Fig. 3C, middle and right panel**, respectively). For the group of combined heparinized individuals, however, levels of detection improved significantly after treatment with heparinase I and the range in Cq values was strongly decreased with approximately 4 to 7 cycles (**Fig. 3C, left panel**). For the endogenous miRNAs miR-30e and miR-92a, the Cq range was not so much reduced using heparinase I, but overall Cq values were lowered significantly. As a result these samples ended well within the more reliable detection range (**Fig. 3C, middle and right panel**, respectively). When not treated with heparinase I, 8 samples gave results for miR-92a that were above the upper limit of detection that we considered as reliable (cut-off at Cq≤35). After heparinase I treatment, only 2 out of these 7 remained above the limit of detection (**Fig. 3C, right panel**). Treatment of RNA samples with 6 IU of heparinase I for 1 hr at 30°C prior to cDNA synthesis did not further improve PCR results (data not shown).

Diagnostic measurement of heparin in urine and correlation with RT-qPCR inhibition

Depending on the molecular size and dosage of the molecule, heparin is removed from the body via two distinct mechanisms, of which renal clearance is the most prominent route.[16] To confirm the presence of UH and LMWH in urine, a chromogenic assay, developed by Larsen et al. to monitor heparin in plasma,[23] was modified and used to measure heparin in urine of 11 hospitalized individuals. The urine samples were ranked, based on the heparin concentration (**Fig. 3D**, **black bars**) indicating that this assay shows high potential to detect heparin in other fluids then plasma. When compared with the inhibition of RT-qPCR in three different miRNA assays (**Fig. 3D**, **white bars**) we show that the concentration of LMWH in urine correlated well with the degree of inhibition of miRNA levels by RT-qPCR (**Fig. 3E**). These results demonstrate the relation between presence of heparin in urine and its inhibitory effects on RT-qPCR.

Protamine-sulfate is not applicable as a heparin antagonist for *in vitro* studies

As sample contamination with heparin clearly obscures RT-qPCR results, the use of a heparin antidote during analysis is essential. Heparinase I is only able to cleave a specific substrate, the2-*O*-sulfo-α-L-iduronic acid/2-deoxy-2-sulfamido-α-D-glucopyranosyl-6-*O*-

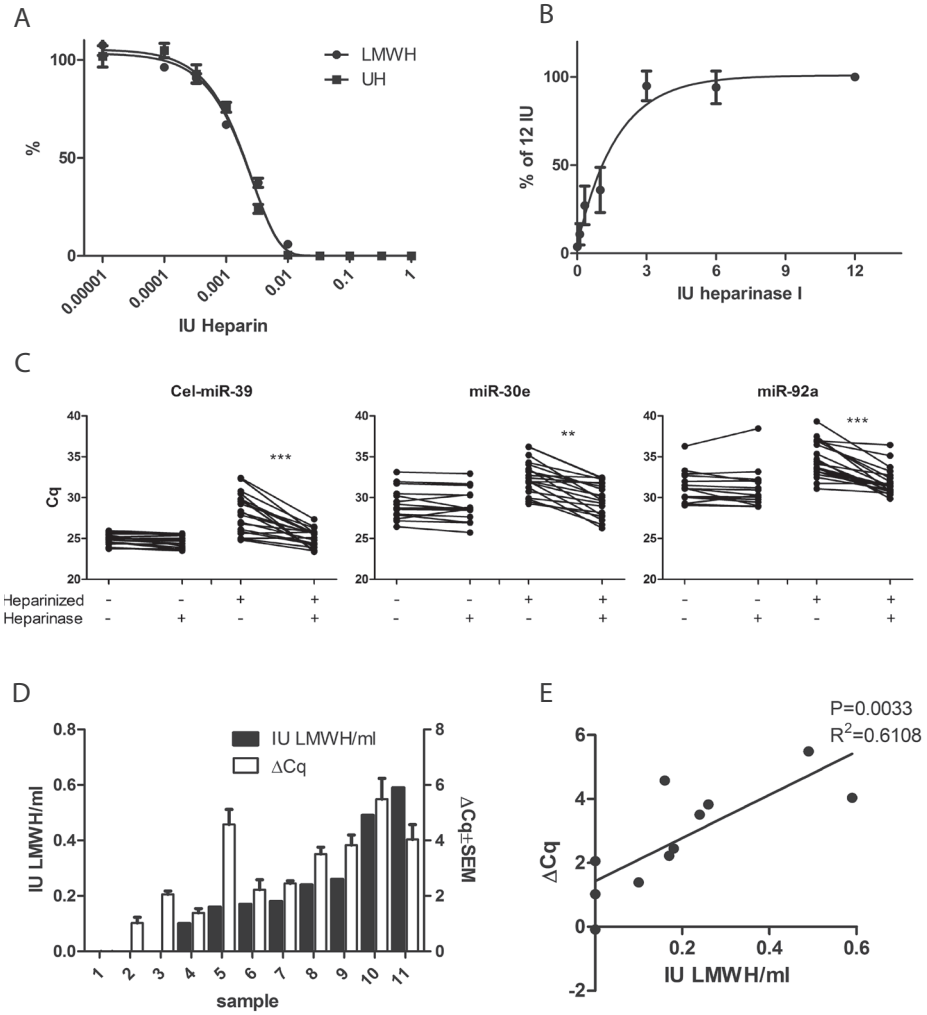

Figure 3. Heparin inhibits RT-qPCR analysis in urinary miRNA samples in a dose-dependent manner and can be neutralized with 6 IU heparinase I. (A) RT-qPCR inhibition of 50 amol synthetic Cel-miR-39 at increasing amounts of unfractionated heparin (UH) and nadroparin (LMWH). PCR levels without anticoagulants were set at 100%. Values represent the mean ± SD. (B) Minimally required amount of heparinase I to reach a plateau of RT-qPCR improvement. Cq levels were converted to miRNA expression levels for Cel-miR-39, miR-30e, and miR-92a. Data were obtained from two LMWH contaminated urinary RNA samples. Results obtained with 12 IU heparinase I were set to 100%. Data points represent the mean ± SD . (C) Effect of co-incubation with (+) or without (-) 6 IU heparinase I during cDNA synthesis in the non-heparinized group (n=15) and heparinized group (n=20). Left panel shows the effect of heparinase I on detection of the spiked-in Cel-miR-39, middle panel the effect on endogenous miR-30e, and the right panel on miR-92a. ** p<0.01, *** p<0.001. (D) LMWH concentration (IU LMWH/ml) (closed bars) and average RT-PCR inhibition (open bars) in eleven urine samples (1-11). (E) Correlation analysis between LMWH concentration and the average ΔCq. ΔCq was calculated as the difference in Cq values with or without treatment with 6 IU heparinase I.

sulfate disaccharide bond leaving other bonds unaffected. Protamine-sulfate is clinically available to counteract the (adverse) effects of anticoagulants. To investigate the application of protamine-sulfate *in vitro*, the effect on LMWH-induced RT-qPCR inhibition was determined for the spiked-in synthetic Cel-miR-238. Protamine-sulfate treatment was either applied prior to the RNA isolation procedure, (**Fig. 4, A-D**), or directly mixed with the synthetic Cel-miR-238 and LMWH to test the application in isolated RNA (**Fig. 4, E-H**). For the samples A-D, 50 amol of Cel-miR-238 was mixed with the indicated amounts of LMWH and protamine sulfate, and the miRNAs were subsequently isolated using the miRNeasy isolation kit. The sample without the addition of LMWH and protamine (**Fig. 4, A**), displayed a normal detection of the Cel-miR-238, whereas Cel-miR-238 was not detectable in samples that contained LMWH (**Fig. 4, B-C**). The results of this experiment showed that protamine-sulfate is not able to neutralize LMWH prior to RNA isolation, even when present in excess amount (**Fig. 4, C**). Protamine-sulfate alone, present prior to isolation of RNA, had no effect on the detection of Cel-miR-238 (**Fig. 4, D**). However, protamine-sulfate is a highly basic, low molecular weight protein and therefore, lack of heparin inhibition could also be caused by reversing the ion pair formation of protamine with LMWH during the RNA isolation procedure. To determine if protamine-sulfate is useful in neutralizing LMWH during the actual RT-qPCR procedure, 50 amol of Cel-miR-238 was reverse transcribed and amplified in the presence or absence of LMWH and protamine-sulfate (**Fig. 4, E-F**). Only when present in equal amount of units, protamine-sulfate was capable of neutralizing the inhibitory effect of LMWH (**Fig. 4, E**). Non-equal amounts (**Fig. 4, G**), but also the presence of only protamine-sulfate, completely inhibits the detection of Cel-miR-238 (**Fig. 4, H**).

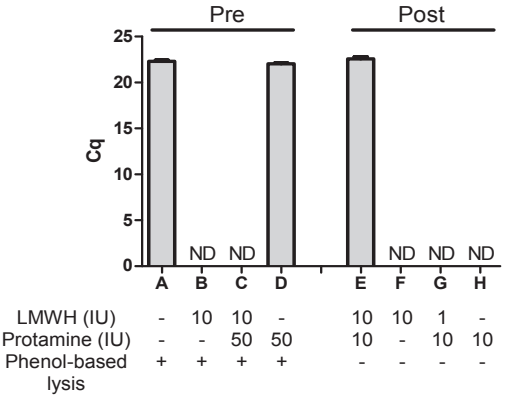

Figure 4. Effect of protamine treatment on RT-PCR results. Urine samples of healthy controls were contaminated with 10 IU LMWH where indicated and either counteracted with 50 IU protamine sulfate prior to RNA isolation using the miRNeasy kit (A-D), or RNA samples were contaminated with the indicated amount of LWMH and protamine sulfate prior to RT (E-H). Results are presented in Cq. ND: Not Detected

Heparin treatment affects RT-qPCR in urine, but not in paired kidney biopsies

The detection and analysis of miRNAs for biomarker purposes in the context of kidney transplantation and organ survival has also gained considerable attention.[24] Our observation that urine can be contaminated with heparin and thereby interfere with miRNA detection raised the question whether this will also affect miRNA detection in kidney biopsies. To answer this question, RNA from 7 paired urine samples and kidney biopsies was analyzed for the effect of 6 IU heparinase I during RT. Urinary RNA samples were analyzed for the endogenous microRNAs miR-30e, and miR-92a and the spiked-in Cel-miR-39 and Cel-miR-238 as described before. RNA from biopsies was analyzed for the spike-in Cel-miR-54, and the endogenous miRNAs miR-21 and miR-192. The latter two being highly expressed miRNAs in kidney.[25, 26] Small nucleolar RNA from SNORD43 (indicated as RNU43) was selected as the reference RNA for biopsy material. Levels of synthetic Cel-miRs and endogenous miRNAs were converted to relative expression levels as described previously.[5] Ratios were obtained by dividing results of relative expression levels of untreated samples with that of levels of the same sample after heparinase I treatment. Urinary miRNAs (**Fig. 5A**) clearly showed a ratio <1, indicative for LMWH contamination, although to various extend. **Fig. 5B** shows the ratios of the concomitant biopsies were all around 1 for RNU43, Cel-miR-54, miR-21, and miR-192 with small inner quartile ranges (0.87±0.1, 1.05±0.16, 0.93±0.26, 0.91±0.11, respectively), indicative for very low or even complete absence of RT-qPCR inhibiting compounds.

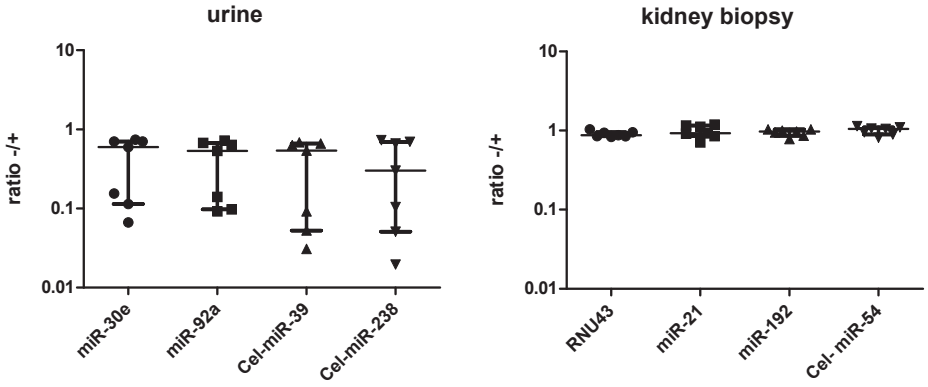

Figure 5. Measurement of miRNA expression in kidney biopsies is not affected by the presence of heparin in urine. Paired urine (A) and kidney biopsies (B) were tested by RT-qPCR for the presence of heparin. The y-axis indicates the ratio of the RT-qPCR results of every sample with or without the incubation with 6 IU heparinase I. Bars indicate median with interquartile range.

DISCUSSION

In this study we showed that endogenous miRNAs in urine are protected from degradation, but that these samples can be contaminated with compounds that inhibit RT-qPCR, and in that way can influence the analysis of biomarkers like miRNAs. This is especially evident for hospitalized individuals who systemically receive doses of heparin, either with UH or LMWH. UH and LMWH can be directly measured in urine, and clearly correlates with the inhibition of detection levels of miRNA. The presence of UH and LMWH is a result of co-isolation with, but independent of the presence of, RNA. This inhibition could not be counteracted using the clinically used antidote Protamine sulfate, neither prior to isolation nor at the level of RT-qPCR. Heparinase I, on the other hand, ameliorated detection of microRNAs almost to levels detected in non-contaminated samples.

LMWH is routinely administered to hospitalized individuals in our center who have no contra-indications for the use of anticoagulants. Most of the LMWH fraction is excreted via the urine and therefore, upon analysis of this fluid, caution should be taken in the interpretation of RT-qPCR results. A heparin flush, however, is also performed prior to organ procurement.[27] With the rapid binding of heparin to endothelial cells and macrophages, especially at higher dosages, the inhibitory effect of this compound on RT-qPCR might also be observed in the analysis of blood and urine from the recipient as well as in other transplantation-related fluids.[28]

As the inhibitory effect of heparin on viral replication and reverse transcription of RNA is already known for decades,[9, 10, 29] the development of PCR and its application as a highly sensitive method for analysis of polynucleotides also requires that more attention is paid to inhibitory components that can obscure results obtained using this method. Recent studies have shown that heparin, in addition to the quantification of mRNA from tissue[12] and plasma,[30] also affects the quantification of miRNAs in human blood samples.[15, 30] It was shown that heparinase I treatment of RNA samples isolated from plasma significantly increased the detection of both spiked-in Cel-miR-39 and cholangiocarcinoma associated, endogenous, miRNAs.[22]

As there are no well-established reference transcripts identified for normalization of miRNA in fluids up to this moment,[31, 32] the only available option to compare samples for specific miRNAs is by correcting for input volume and the use of synthetic miRNAs, absent in mammalian species, to monitor RT-qPCR efficiency.[33] Although PCR inhibition is significantly reduced upon treatment with heparinase I, small variations in the Cq levels of the spiked-in Cel-miR-39 remained present. This suggests that other inhibitory compounds might be present as well.

PCR inhibitors come in many forms and classes. Both organic or inorganic substances can affect PCR outcome and they are found in a variety of biological fluids, environmental materials and food (reviewed in [11]). Heparin is a mixture of highly sulfated, repeating

disaccharide units, that vary in weight from 3-30 kDa. The most common disaccharide unit is the the 2-*O*-sulfo-α-L-iduronic acid/ 2-deoxy-2-sulfamido-α-D-glucopyranosyl-6-*O*-sulfate disaccharide. This is the primary substrate for *Bacteroides eggerthii* Heparinase I. Other disaccharide moleclules, however, are insensitive to degradation by Heparinase I. This possible incomplete degradation of heparin, however, might be a factor responsible for the remaining PCR inhibition. This effect, referred to as the "heparin effect", can be a result of the disease state of the patient.[34, 35] Especially in liver diseases and liver transplantation coagulation is affected, and it is postulated that heparin-like components can also be of endogenous origin. The heparin effect was already observed in the 90's, when coagulation was inhibited in a pig model for liver transplantation without the use of heparin.[36]

In conclusion, our study shows that heparin is excreted to urine, where it impairs miRNA detection by RT-qPCR in a dose-dependent manner. Heparin levels in urine correlated very well with the inhibitory effect. Protamine-sulfate, the clinical antidote for heparin, is not suitable for use in the *in vitro* situation, but heparinase I treatment of RNA samples during RT-qPCR provides an excellent alternative to counteract the inhibitory effect of heparin. Our results, in combination with already published data, clearly emphasizes the need for a thorough examination of the (more recent) medical and pharmaceutical history of patients and controls when using RT-PCR for identification or validation of biomarkers. However, it is also well imaginable that the same characteristics that are responsible for the binding of RNA to the column matrix during the isolation procedure are probably also responsible for the binding of heparin. Detection of synthetic miRNAs that are spiked into a sample that consists only of water with heparin during the miRNeasy isolation procedure is also strongly inhibited (data not shown). Choosing an RNA isolation procedure that is devoid of nucleotide-binding columns might therefor also be considered to reduce RT-qPCR inhibition. It is therefore eminent that, in the development of a biomarker, not only standardization of sampling time, work-up and storage are monitored but, for molecular analysis of samples, the isolation of the molecule of interest and the medication history of the patient also requires the necessary attention.

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Part IV

Summary and discussion

Chapter 10

Summary, discussion and future perspectives

Parts of this chapter are published in 'Barking up the wrong tree: microRNAs in bile as markers for biliary complications', CJ Verhoeven, HJ Metselaar, LJW van der Laan, Liver Transpl 2014;20:637-639 and 'MicroRNAs in bile vesicles: finding a trade-off for biomarker discovery' Roest HP, Verhoeven CJ, van der Laan LJ, Hepatology, 2015 Mar;61(3):1094-5.

Liver transplantation (LT) has become victim of its own success. In the last decades, the number of patients on the waiting list for transplantation has expanded due to a growing demand and the limited availability of transplantable donor organs. In the Netherlands, this has led to the increased use of grafts obtained from circulatory death donors (DCD), despite the fact that these grafts are of inferior quality compared to those donated after brain death (DBD). Prolonged warm ischemia during graft procurement causes extensive ischemia-reperfusion injury to hepatocytes and cholangiocytes in DCD liver grafts. Therefore, these grafts have a higher risk to develop serious complications already early after liver transplantation, like primary non-function (PNF), early allograft dysfunction (EAD) and non-anastomotic biliary strictures (NAS). The latter is also known as ischemic-type biliary lesions (ITBL) or ischemic cholangiopathy. However, grafts of impaired quality like DCD are necessary to deal with the rising organ shortage and currently, potentially transplantable organs are rejected based on clinical characteristics (such as high donor age) and evaluation by the procuring surgeon. This problem is highlighted in **Fig. 1**, which shows the number of organs used for LT in the Netherlands in 2015, based on numbers from the Dutch transplantation registry. In 2015, 130 post mortal donors were DBD, which were effectuated for transplantation in general in 97% and specifically for LT in 79% (**Fig. 1A**). Although the number of DCDs was almost two-fold higher (n=218), the percentage of effectuated donors was much less (64%) and the liver was transplanted in only 22% of the cases (**Fig. 1A**). Thus, from the total number of liver transplantations executed in the Netherlands in 2015, 32% were performed with a DCD graft (**Fig. 1B**). Based on the numbers from **Fig. 1A**, the contribution of DCD grafts

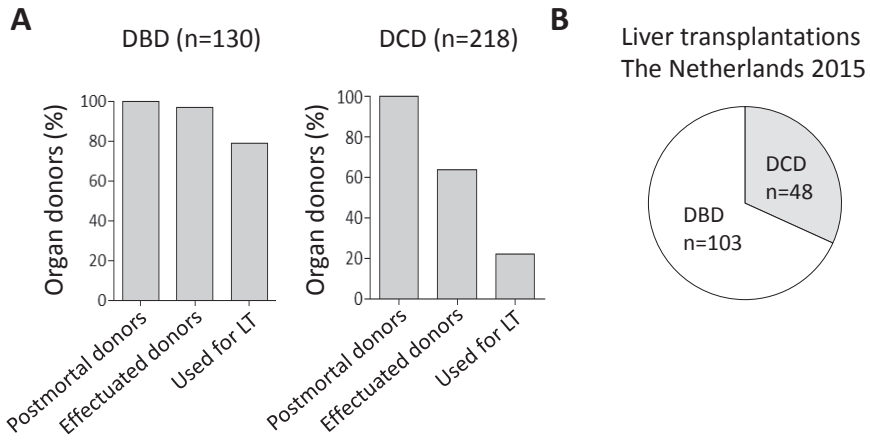

Figure 1. (A) Number of post mortal donors and effectuated transplantations and LT's in The Netherlands in 2015. Of all potential DBD organ donors (n=130), the liver was transplanted in 79% of the cases. Of all potential DCD organ donors (n=218), the liver was transplanted in only 22%. (B) In 2015, 152 liver transplantations were performed. Approximately 32% of the transplantations concerned a DCD graft. Data originate from the Dutch Transplantation Foundation registry.

in LT could however be much higher. If effective biomarkers would be available, this could contribute to a more objective assessment of graft quality and potentially expand the donor pool. The research in this thesis was performed to evaluate conventional and novel biomarkers on their performance to assess graft quality and to predict outcome in LT. A short overview of all chapters is provided in **Table 1**.

Table 1. Overview of thesis chapters.

Ch.	Main findings	Strengths	Limitations	Clinical relevance	Suggestions future research
2	Overview of biomarkers to assess graft quality during conventional and machine preservation.	Systematic review. Both human and experimental studies. Markers prior to graft implantation.	No meta-analysis due to incomparable studies and differences in outcome definitions. Most studies miss validation, low evidence.	Early assessment of graft quality. Objective. Expanding the donor pool for LT by selecting grafts with favourable biomarker profiles.	More validation studies. Meta-analysis on performance of separate biomarkers. Better definitions for outcome analysis.
3	No increased risk of microthrombi formation in DCD grafts during preservation or development of NAS.	Large number of biopsies collected at different time points during preservation. *In triplo* staining for microthrombi. Both extended criteria DCD grafts and transplanted livers included.	Small cohort size and number of included livers. Possible sampling bias in transplanted livers. No biopsies from all time points available.	Undermines the hypothesis of microthrombi formation in development of NAS. No histological evidence for fibrinolytic therapy during LT.	Include larger number of transplanted livers. Comparison with paired bile duct specimens. Perform a RCT for preventive use of fibrinolytic therapy.
4	FUT2 non-secretor status is a risk factor for NAS in patients with PSC. Donor-recipient mismatch gives additional risk for NAS.	Large cohort size. Validation of earlier findings in non-transplanted PSC patients. Combined donor-recipients data.	Relative low percentage of PSC patients and combined donor-recipient data. Possible overlap NAS and recurrence PSC.	Provides insight in underlying mechanism of NAS in PSC patients. Potential graft allocation based on genetic profile in PSC patients.	Validate findings in PSC patients. Perform additional basic research to check for bacterial translocation and aggravated immune response.
5	Overview of extracellular miRNAs as biomarker in liver disease and transplantation.	Broad topic focused on extracellular miRNAs in the field of hepatology. Human studies.	No systematic search. Limited amount of publications regarding extracellular miRNAs as biomarker in LT.	Evaluates usefulness of miRNAs as biomarker in clinical setting.	Expand miRNA research in the setting of LT. Perform a systematic review or meta-analysis.

PART I: BIOMARKERS IN LIVER TRANSPLANTATION

The various definitions of biomarkers and the potential of conventional markers in the context of LT are highlighted in the general introduction, **chapter 1.** In particular liver enzymes like aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are commonly used dynamic markers in serum for monitoring hepatocellular injury following LT with increased levels at time of complications such as PNF, EAD and rejection.

Table 1. Overview of thesis chapters. (continued)

Ch.	Main findings	Strengths	Limitations	Clinical relevance	Suggestions future research
6	Impaired graft excretory function and graft injury causes polarized release of HDmiRs and CDmiRs into bile and blood.	Longitudinal analysis of large number of paired bile and blood samples during follow-up after LT. Validation of previous findings in serum .	Small cohort size, less suitable for analysing biomarker performance. No *in vitro* 3D-model for polarized release.	Provides insight in the biology of miRNA release. Shows the potential of bile as biofluid to measure biomarkers.	Further investigate polarized release in a 3D model or organoid. Investigate biliary miRNA profiles in cholangiopathies like NAS.
7	High levels of perfusate HDmiR/CDmiR ratios at time of graft preservation are predictive of NAS.	Non-invasive measurement. Independent predictor. Large quantities available. Potential cholangiocyte-specific markers.	Limited number of miRNAs analysed. Small cohort size. No validation. No measurement performed on conventional markers in perfusate.	Early prediction of NAS. Cholangiocyte-specific marker. Demonstrates the use of perfusates as biofluid for biomarker studies.	Perform microarray analysis on graft perfusates. Validate findings in separate cohort. Apply measurements in machine perfusion.
8	Sample contamination with heparin potentially inhibits perfusate miRNA measurement.	Re-evaluation of previous findings in ch. 7. Optimized detection of miRNAs in perfusate. No effect on predictive capacity of HDmiR/CDmiR ratios.	Small difference with previous study. No direct evidence for presence of heparin.	Create awareness for inhibitory effects and false results when samples are contaminated with heparin.	Correct for inhibitory effects in samples with heparinase I.
9	Excretion of heparin into urine inhibits miRNA detection by RT-qPCR. Sample treatment with heparinase I counteracts inhibition.	Comparison between heparinized and non-heparinized patients, availability of healthy controls. Different concentrations of heparinase I tested.	No data on co-isolation of heparin and possible inhibition by other RNA isolation techniques.	Optimize miRNA detection in body fluids like urine. Better understanding of heparin excretion via urine.	Investigate whether inhibition of RT-qPCR by heparin is a factor in other RNA isolation protocols.

For cholestatic injury, gamma-glutamyl transferase (GGT) and alkaline phosphatase (ALP) are serum markers that indicate biliary obstruction caused by anastomotic biliary strictures (AS) or NAS. Finally, markers as bilirubin, albumin and prothrombin-time are usually indicative for graft function. All these markers concern dynamic biomarkers, which measurement levels fluctuate depending on the pathophysiological state of the liver graft. Beside dynamic markers in blood and serum, histological characteristics in tissue are used to determine the degree of rejection, like the Banff score (also known as the rejection activity index). The disadvantage of histology is the invasive obtainment of liver tissue and the local representation, while serum markers are more informative on the entire status of the graft. Moreover, the increase of serum markers is usually faster than changes in histology. Therefore, histology in general is a less dynamic marker. Markers such as GGT and ALP have a delayed increase following liver transplantation and are therefore less useful as early markers for severe biliary injury such as NAS. MicroRNAs (miRNAs) were identified as potential early markers in the context of biliary complications, as further discussed in part III of this chapter. Finally, the option to determine genetic profiles in donors and recipients that could be predictive for outcome are discussed. Genetic polymorphisms are static factors that do not change during the course of disease or after LT and therefore are considered as risk factors rather than biomarkers.

Due to the increased use of extended criteria grafts and rising incidence of associated complications, transplant centres throughout the world investigate different strategies to improve graft quality and outcome in LT recipients. A biomarker-based evaluation of graft quality prior to implantation helps to identify grafts with an increased risk for PNF, EAD, NAS, or other complications. A novel strategy to optimize graft preservation and performance is machine perfusion. **Chapter 2** provides a systemic review on studies that investigated biomarkers to assess graft quality during conventional and machine preservation in LT. Since 2010, various techniques of machine perfusion have made their way into the clinic and the first results in human seem promising. Another advantage of machine perfusion is that it creates a time window for measuring biomarkers. Beside comparing different techniques of machine perfusion, **chapter 2** discusses the pros and cons of different biomaterials. Biopsies provide histological evidence of graft quality (mainly cellular injury) and a large amount of cells for analysis of biomarker expression. The disadvantages, however, are the invasive collection with tissue disruption, the local representation and the possibility of inter and intra-observer variability. In contrast, preservation solutions that are used to flush the liver graft, so-called perfusates, are available in larger amounts during different techniques of preservation. Perfusate collection is non-invasive and it gives a better representation of the entire graft. Machine perfusion at higher temperatures also allows the collection of bile. The production and composition of bile is especially interesting to assess graft function. Unfortunately, preservation at lower temperatures does not trigger liver grafts to produce bile and quantity can be

low. An important conclusion of the study was that although many studies, including ours, show discriminative potential of a variety of biomarkers for graft injury and function, robust validation in larger cohorts are missing. First, cohort sizes are small due to the limited number of annually performed LTs. Also, prospective collection and storage of biomaterials can be time consuming, logistically challenging and the use of novel measurement techniques can be expensive. All these factors contribute to the lack of validation in biomarker studies, while this is actually a crucial step before biomarkers can be applied in the clinic. Importantly, most studies investigated biomarker performance in DBD grafts, which usually have lower biomarker levels at baseline compared to DCD grafts. Therefore, the cut-off values for biomarker levels to predict complications in DBD grafts are often different from DCD grafts[1, 2]. Finally, the variations in outcome definitions make it difficult to perform a reliable meta-analysis. Introducing an international guideline on the definition of common complications in LT could improve the comparability between study populations[3].

PART II: RISK FACTORS FOR BILIARY COMPLICATIONS

Biomarkers can serve multiple purposes. Outcomes can be defined by biomarkers crossing beyond a predetermined cut-off level, which is for instance the case with EAD. Less dynamic markers, such as histology or genetic markers like polymorphisms, can however be interpreted as risk factors for outcome. In Part II of this thesis, we investigated two factors which could be possibly useful as a risk factor to predict the development of NAS following LT.

Formation of microthrombi as an underlying cause for NAS: fact or fable?

In **chapter 3**, we investigated whether DCD grafts have an increased tendency to form microthrombi in their microvascular circulation during graft preservation. In the last decade, researchers have hypothesized whether the additional first warm ischemia and vascular stasis in DCD grafts causes microthrombi formation in the peribiliary vascular plexus, which could subsequently lead to the development of NAS. According to Virchow's triad, three factors are mainly responsible for the formation of thrombi; hypercoagulability, endothelial cell injury and disturbed blood flow. These events can induce a coagulation cascade with activation of platelets via two independent pathways. In the collagen pathway, exposure of sub-endothelial collagen initiates platelet activation. In the tissue factor pathway, thrombin is generated by tissue factor derived from the vessel wall or from flowing blood[4]. The formation of a thrombus in LT, in particular hepatic artery thrombosis (HAT), has been associated with the number of arterial anastomoses, aberrant arterial anatomy, low donor and recipient body weight, previous abdominal

surgery, CMV-mismatch, re-LT and prolonged operation time[5, 6]. Insufficient flushing of the graft during organ retrieval and prior to implantation is believed to further increase this risk. In order to prevent the formation of microthrombi, some transplant centres therefore apply potentially harmful intraoperative fibrinolytic therapy that could cause major bleedings in transplant recipients.

The rationale for our study was to determine whether there is actual evidence of microthrombi formation in liver tissue that could justify intraoperative fibrinolytic therapy. Beside conventional staining with hematoxylin-eosin, we also stained for Von Willebrand Factor VIII, a marker for endothelial cell activation earlier in the coagulation cascade, and Fibrin Lendrum which occurs at the end of the cascade. Our study did not show convincing evidence of microthrombi formation at different time points during graft preservation, despite thorough histological evaluation of intrahepatic biopsies obtained from multiple liver segments from extended criteria DCD grafts that were rejected for human LT. In transplanted livers, the presence of microthrombi was not increased compared to matched DBD grafts, nor was their number higher after graft reperfusion or in grafts that developed NAS. Although some biopsies showed partial positive staining for Von Willebrand Factor 8, paired staining's with Fibrin Lendrum and hematoxylin-eosin remained negative. This suggests that DCD grafts do not appear to have a significantly increased tendency for microthrombi formation at time of graft preservation. However, it does not rule out the possibility of microthrombi formation at a later stage during follow-up.

An important limitation of our study consisted of the possibility of sampling bias - in particular in transplanted livers, where we could only collect wedged shaped biopsies from the left lateral segment. And despite the large number of sections analysed on the presence of microthrombi, biopsies were collected from a small number of liver grafts. Nevertheless, our findings are in concordance with other histological studies that mainly evaluated microthrombi formation and cholangiocyte injury of extra- and intrahepatic bile ducts[7-10]. Moreover, a study by Vendrell et al. showed that DCD induces hyperfibrinolysis, further undermining the hypothesis that DCD grafts would have an increased tendency to form microthrombi[11].

With two studies showing potential benefit [12, 13] and two other studies that could not demonstrate any effect[14, 15], the results of interventional studies remain inconclusive in demonstrating beneficial effect of intraoperative fibrinolytic therapy on the development of NAS. All these studies were however based on retrospective analyses, while a randomized controlled trial should be performed to provide the strongest evidence on whether fibrinolytic therapy is successful in preventing microthrombi and associated NAS. For now, we conclude that the histological evidence for microthrombi is marginal and together with the conflicting results in clinical studies, we discourage the use of intraoperative fibrinolytic therapy during LT.

Injury to the peribiliary glands as a possible cause for NAS

Although we did not investigate the involvement of the peribiliary glands (PBGs), the findings from other research groups make this topic worthwhile in the discussion of factors causing NAS. Already in 1994, Nakanuma et al. described how PBGs might be a niche containing stem cells for regeneration of cholangiocytes[16]. An elegant study from DiPaola et al. using confocal microscopy in mice, demonstrated that PBGs populate the submucosal compartment of the entire extrahepatic biliary tract, with exception of the gallbladder[17]. The PBGs form intramural epithelial networks between several segments of the large bile ducts and are connected with the luminal surface through small canals. Furthermore, this study found an increase in cellular proliferation in PBGs in response to bile duct ligation. Recently, Carpino et al. found hyperplasia of the PBGs expanding up to the surface epithelium in bile ducts from patients suffering from severe primary sclerosing cholangitis (PSC) with biliary fibrosis[18]. These PBGs expressed hedgehog pathway components and markers of senescence and autophagy. Expression of the cystic-fibrosis transmembrane receptor was decreased, making cholangiocytes less protected against toxic bile salts via the so-called 'bicarbonate umbrella', which will be discussed later. Importantly, there was epithelial-to-mesenchymal transition, a risk factor for potential malignant degeneration in PSC.

These findings are in concordance with an earlier study from the group of Porte from Groningen, the Netherlands[19]. Beside loss of the epithelial lining of the luminal bile duct surface, this group found increased expression of Ki-67 and keratin-19 in the PBGs of explanted donor livers obtained from recipients with severe NAS. In patients who received surgical intervention for cholangitis, the expression of Ki-67 and keratin-19 was more pronounced in cholangiocytes. The authors suggest that in case of mild biliary injury, a first-line regenerative mechanism is induced by cholangiocytes, but in case of extensive injury, a second-line of regeneration is activated by PBGs. This lead to the formulation of a new paradigm within the pathophysiologic mechanisms of NAS; namely that of insufficient regenerative capacity of cholangiocytes due to injury of the PBGs located in the larger bile ducts[20]. Subsequently, Op den Dries et al. investigated injury to PBGs in a large series of extrahepatic bile duct specimens from transplanted DBD and DCD grafts[7]. They found a higher rate of vascular injury, mural stroma necrosis, and deep PBG injury in bile ducts from grafts which later developed NAS compared to those that did not develop NAS. Interestingly, not deep PBG injury, but vascular injury was significantly higher in DCD grafts. This suggests that solely PBG injury does not explain the higher incidence of NAS in DCD grafts, but that vascular injury and diminished blood supply could add to the risk in DCDs. Notably, histological injury at the distal end of the extrahepatic bile duct was representative for injury of the larger intrahepatic bile ducts[21].

Fucosyltransferase-2 dysfunction in the pathophysiology of biliary disease

Genetic polymorphisms are often used as a biomarker to predict outcome. In **Chapter 4,** we investigated whether a single-nucleotide polymorphism (SNP) in the fucosyltransferase-2 gene (FUT2) is a risk factor for the development of NAS. The mutation rs608133 (G>A), identified through a genome-wide association study, is a prevalent polymorphism that occurs in approximately 20% of the general population but which has also been associated with PSC[22, 23]. The mutation causes a dysfunctional FUT2 protein, resulting in reduced glycosylation of surface epithelial cells. It is believed that this reduced glycosylation impairs the barrier function of cells against pathogens[24]. Because of the clinical similarities that exist between NAS and PSC, the question raised if the highly prevalent rs608133 mutation of the FUT2 gene in LT donors or recipients could be a risk factor for NAS. We therefore retrospectively genotyped donor and recipient DNA samples from a total of 418 LT procedures on FUT2 secretor status. Donor FUT2 status was not associated with the development of NAS. In PSC patients, however, recipient FUT2 non-secretor status (AA-genotype) was the strongest independent risk factor for NAS (HR 2.34, P=0.034), with the biggest effect during the first five years of follow-up. FUT2 status was not a risk factor in LT recipients without PSC as underlying disease.

FUT2 dysfunction has also been correlated to inflammatory bowel diseases (IBD) like Crohn's disease and ulcerative colitis, which can coexist with PSC[25, 26]. It is thought that the impaired barrier function and less diverse bacterial composition of the intestinal epithelium in FUT2 non-secretors causes a 'leaky gut', with passage of bacteria and pathogens into the circulation[27-29]. To investigate the role of bacterial translocation, we analysed the coexistence of IBD in our cohort of recipients with underlying PSC and the relation with FUT2 secretor status. With an incidence of 56%, there was a strong correlation between PSC and ulcerative colitis. We could however not demonstrate that coexistent PSC and IBD occurred more frequently in FUT2 non-secretors, nor was the incidence of NAS higher in this group. This could be due to the limited number of patients with coexisting PSC and IBD in our study cohort. This made it also hard to investigate whether colectomy had a preventive effect on the occurrence of NAS, as was suggested by another study concerning PSC[30].

The results from **chapter 4** could also indicate that an aggravated immune response was triggered by FUT2 mismatching. Analyses of combined donor-recipient genotypes revealed that incidence of NAS was highest (80%) among PSC recipients with FUT2 non-secretor status (AA) who received a liver graft from a FUT2 secretor donor (GG or GA). An experimental study in transgenic mice supports this hypothesis, demonstrating an increased immune response and impaired graft survival of skin and hearts with overexpression of fucosyltransferase into wildtype recipients[31]. Furthermore, our results do not exclude that cholangiocytes from the recipient possibly repopulate the donor bile

duct. This type of chimerism has been demonstrated before, but not in the context of NAS[32].

Aside from the intestinal epithelium, FUT2 non-secretors also have an altered glycosylation of cholangiocytes and bacterial composition of bile, which could both contribute to the development of NAS[22]. The altered glycocalyx layer affects the alkaline environment of cholangiocytes that is normally sustained by the bicarbonate umbrella and makes them more vulnerable for toxic bile salts[33, 34]. In addition, altered bile composition has been linked to a higher incidence of cholangitis, biliary complications and shortened transplantation-free survival[35-37]. Our study does not provide direct evidence for increased bile salt toxicity in FUT2 non-secretors, but it remains an interesting topic for future research.

Finally, the detection of biomarkers which biosynthesis normally depends on FUT2 enzyme activity can be influenced by FUT2 genotype. This was shown by Wannhoff et al. for cancer antigen 19-9 (CA 19-9), a biomarker that is often used for the screening of cholangiocarcinoma in PSC patients[38]. It appears that PSC patients with FUT2 non-secretor status have higher serum levels of CA 19-9 compared to FUT2 secretors. In order to gain a better biomarker performance in terms of sensitivity, specificity and positive/negative predictive value, cut-off values for CA 19-9 should be higher in FUT2 non-secretors. So researchers should be aware of genotype-dependent biomarker levels in future studies.

Other polymorphisms associated with NAS and PSC following liver transplantation

Besides FUT2, other SNPs have also been investigated in the context of NAS following LT. Ten Hove et al. showed that a mutation in the gene coding for matrix metalloproteinase-2 (MMP-2) of either donors or recipients increased the risk to develop NAS[39]. MMP-2 is a tissue-remodelling enzyme that regulates matrix degradation and has been associated with liver fibrosis and cirrhosis. A polymorphism present in both donor and recipient had a synergistic effect and further increased the risk of NAS (HR 3.48). Serum levels of MMP-2 were however not affected by the SNP. A subsequent study from the same research group showed that the polymorphism was associated with worse outcome in PSC recipients in terms of the need of LT or higher mortality[40].

Another SNP associated with NAS is that of chemokine receptor CCR5 (CCR5-Δ32 mutation)[41]. CCR5 is expressed on various cells of the innate as well as the adaptive immune system. For instance, homo- and heterozygote carriers of the mutation have impaired chemotaxis of regulatory T-cells. CCR5-Δ32 in recipients but not in donors increases the risk to develop NAS with 4-fold. The mutation in recipients was shown to be an independent risk factor for NAS, which was strongest in patients with PSC.

Unfortunately, no clear stratified analysis was performed on the effects of CCR5- Δ32 in non-PSC recipients.

All these studies on genetic polymorphisms and the development of NAS described above, including our own, showed a strong association with PSC. This leads to the discussion whether the occurrence of NAS in these cohorts are in fact recurrence of the underlying disease. In fact, multiple studies have identified PSC as a risk factor for the development of NAS[42, 43]. The criteria for diagnosing recurrent PSC, which were formulated in 1999, consist of (i) a confirmed diagnosis of PSC prior to LT, with (ii) intra and/or extrahepatic biliary strictures, beading and irregularity after 90 days of follow-up demonstrated with cholangiography, *or* fibrous cholangitis and/or fibro-obliterative lesions with or without ductopenia, biliary fibrosis or biliary cirrhosis demonstrated by histology[44, 45]. Established ductopenic rejection, HAT, single anastomotic strictures, NAS before post-operative day 90, and ABO-incompatibility are described as factors excluding recurrent PSC. Despite this clear description, the diagnosis of recurrent PSC is not always obvious in clinical practice. First of all, in a minority of cases, the diagnosis of PSC becomes clear after histological evaluation on the explant liver by the pathologist. Furthermore, the typical beading seen on cholangiography is often a result of progressive recurrent disease, but more isolated intrahepatic strictures and associated dilatations are seen in earlier stages. Sometimes, the first symptoms of recurrent disease actually present before post-operative day 90. And also through histological examination of for instance needle biopsies, distinguishing recurrent PSC from other conditions like chronic rejection can be challenging[46, 47].

Because of the difficulties in differentiating recurrent PSC from NAS, it deserves recommendation to perform a stratified analysis for separate PSC and non-PSC recipients in transplantation cohorts, next to the usually performed overall analysis. Certain SNPs associated with recurrent PSC or NAS can be used for risk profiling in patients and provide more insight into the underlying mechanism or pathophysiology of complications following LT. Furthermore, the knowledge that certain graft-recipient matches (or mismatches) can lead to unfavourable outcomes could possibly influence future allocation of donor organs, which is currently mainly based on recipient MELD-score and blood type.

Risk factors for biliary complications put in perspective

The paragraphs above illustrate a coherent set of risk factors that appear to reinforce each other's impact on the development of NAS. In short, bile duct injury starts with prolonged graft ischemia, explaining the higher incidence of NAS in DCD grafts[48]. Bile duct vitality strongly depends on blood supply through the hepatic artery, but diminished blood flow through the portal vein has also been associated with NAS[10, 49, 50]. Reperfusion worsens injury to the biliary epithelium and PBGs, not only disturbing

cholangiocyte regeneration but also the integrity of the biliary glycocalyx and bicarbonate umbrella which normally protect against toxic bile salts. Patients suffering from PSC more often have a perturbed biliary glycocalyx, which is permeable for pathogens like bacteria. This can cause enhanced bacterial translocation from the intestine to the liver, but also an aggravated immune response when a FUT2 secretor graft is implanted into a FUT2 non-secretor recipient.

PART III: MICRORNAS AS NOVEL BIOMARKER IN LIVER TRANSPLANTATION

Part III of this thesis focused on the use of microRNAs (miRNAs or miRs) as biomarkers for graft function and injury in the setting of LT. As an introduction to this part, **chapter 5** provides an overview of the literature regarding miRNAs in liver disease and transplantation, with special attention for extracellular miRNAs. After the discovery of miRNAs in 2001, an explosive amount of research has been published regarding their capacities as biomarker, but also their involvement in different pathologies. MiRNAs are 20-23 nucleotide long non-coding RNAs that can regulate gene expression at a post-transcriptional level. The fact that cell-type abundant miRNAs are present in the circulation and other body fluids, wherein they remain fairly stable against degradation, made them a popular subject for non-invasive biomarker research in the last decade. A possible explanation for the stability of miRNAs in fluids is that they are embedded in- or attached to other cellular components. For example, in response to injury, extracellular miRNAs were found in vesicles and smaller exosomes, lipoproteins, or bound to argonaute-2. In the field of hepatology, serum miRNA levels are indicative for liver injury in patients with viral hepatitis, non-alcoholic fatty liver disease, liver fibrosis, malignancy, and more. In the context of LT, however, the amount of literature on extracellular miRNAs as biomarker is more scarce.

MicroRNAs as biomarker for hepatocyte and cholangiocyte injury post-transplantation

Hepatocytes and cholangiocytes have different miRNA expression patterns. An elegant study by Chen et al. used laser capture microdissection to select cholangiocytes and hepatocytes from liver tissue for separate genome-wide microArray analysis on miRNAs[51]. Based on the results of this study, our group was one of the first to investigate the release of hepatocyte-derived miRNAs (HDmiRs) into serum at time of liver injury an acute rejection after liver transplantation[52]. The main findings of this study were that expression of HDmiR-122 and HDmiR-148a was significantly reduced in post-reperfusion biopsies when warm ischemia was prolonged. Inversely, serum samples collected during the first month of recipient follow-up showed increased levels of HDmiR-122 and

HDmiR-148a at time of injury and strongly correlated with AST and ALT values. In fact, serum HDmiR levels were already significantly elevated when transaminase levels were still <50 IU/L, and peak HDmiR levels were 24 hours ahead of peak serum HDmiR levels in recipients with histology proven acute rejection. These results suggests that HDmiRs are an earlier and perhaps more sensitive marker for liver injury then the classic serum transaminases, which are currently the golden standard as a biomarker to assess liver injury.

In order to gain more insight in the biology of miRNA dynamics during various (patho) physiological hepatic conditions after LT, we further investigated miRNA release into bile and serum in **chapter 6**. For this study, we not only tested HDmiRs (HDmiR-122 and -148a), but we also selected cholangiocyte-derived miRNAs (CDmiRs) that were previously identified by Chen et al. (CDmiR-30e, -200c and -222)[51]. The tissue-abundancy of these miRNAs was confirmed in liver biopsies and bile duct specimens that were collected during LT. HDmiR-122 was most abundant in liver tissue, while CDmiR-222 had the highest expression in common bile duct tissue. Overall, miRNA levels were higher in bile then in paired serum samples. Fractionation experiments demonstrated that the majority of extracellular miRNAs in bile were present in an unpelletable fraction and protected against degradation for at least 1-4 hours through protein-conjunctions. A strong correlation was found between excretion of HDmiR-122 and bilirubin into bile, with high levels of biliary HDmiR-122 during good excretory function. Biliary levels of HDmiR-122 however drastically lowered if graft excretory function was impaired. Interestingly, inverse dynamics were observed for CDmiR-222, with low biliary levels at time of good excretory function that increased when excretory function was impaired. Changes in excretory function were mainly reflected in biliary miRNA levels, without significant changes in paired serum samples. In case of liver injury and acute rejection, however, changes in serum were predominant; at time of injury, biliary HDmiR-122 levels decreased with simultaneously an increase in serum levels. Again, CDmiR-222 showed opposite dynamics.

Based on these results, it appears that release of HDmiRs and CDmiRs into bile and blood is polarized, depending on graft function and the degree of injury. Furthermore, the opposite dynamics between HDmiRs and CDmiRs suggests an active release mechanism rather than simple leakage from injured cells. The excretion of miRNAs into bile during proper function indicates a role in biliary homeostasis; previous studies showed that expression of miR-506, miR-222 and miR-199a-3p for instance influence the activity of anion exchanger 2 and farnesoid-X receptor[53, 54]. Furthermore, our study shows the potential use of bile as a biomaterial and biliary miRNAs to assess graft function in the setting of LT. Limitations of our study consisted of the small number of transplant recipients from whom bile and serum samples were collected (n=10) and the absence of a 3D experimental model to validate the hypothesis that release of HDmiRs and CDmiRs

is polarized. With the ongoing developments in organ tissue engineering, this goal can hopefully be realized in the near future[55, 56].

Regarding involvement of miRNA in cholangiopathies, cholangiocarcinoma is the most extensively studied disease[57]. In these studies, bile is the most frequently used biofluid for miRNA analysis, followed by serum. At the gene-regulatory level, miRNA expression in tissue has been linked with target genes involved with fibrosis, apoptosis, proliferation, invasion, inflammation, migration, tumour growth, oncogenesis, chemo-resistance and other pathophysiological factors[57]. Remarkably, much less is known about miRNAs in cholangiopathies as primary biliary cirrhosis (PBC), PSC and biliary atresia. In particular knowledge on miRNA regulation in the pathophysiology of PSC could be informative for NAS, seen their overlap in clinical presentation. In the context of PSC, miR-7a has been associated with cholangiocyte proliferation[58]. Other research mainly describes miRNA patterns in bile and serum to distinguish PSC from cholangiocarcinoma, which can be difficult based on brush cytology [59, 60]. By further unravelling the regulatory aspects of CDmiRs, these miRNAs could form the basis of novel therapeutic targets in NAS or other cholangiopathies, which was successful for HDmiRs in viral hepatitis[61, 62].

MicroRNAs as a biomarker for cholangiocyte injury before transplantation

Markers for cholangiocyte injury and NAS are scarce. Most studies concern histological bile duct specimens, which were extensively described earlier. Because of the surgical trauma and local representation associated with biopsy sampling, we were seeking for a cholangiocyte-specific biomarker that could be measured non-invasively prior to graft implantation. Therefore, the study from **chapter 7** was initiated to determine if extracellular miRNAs are released into perfusates that are used to flush the graft and whether these miRNAs could be an early marker for NAS. Levels of HDmiRs (miR-122 and -148a) and CDmiRs (miR-30e, -222 and -296) in perfusate supernatant from grafts that developed ITBL (n=20) were compared with those that did not develop biliary complications (n=37). In order to correct for any possible differences in perfusate concentrations, ratios of HDmiRs/CDmiRs were used for analyses. Recipients from a graft with high HDmiR/CDmiR ratios in the perfusate, mainly due to low CDmiR levels, had a 3 to 6-fold increased risk to develop ITBL during follow-up. The discriminative value of HDmiR/CDmiR ratios was fairly good with C-statistics ranging between 0.74-0.77, although a substantial overlap in biomarker levels existed between the ITBL and non-ITBL group.

A possible explanation for the lower CDmiR levels in perfusate from the ITBL group is the loss of cholangiocytes[7]. However, paired tissue biopsies (n=24), which performed less well as a predictive biomarker, did not show a lower expression of CDmiRs. This observation could be explained by possible sampling bias of the biopsies, which were wedges of liver tissue from the left lateral segment. Perhaps CDmiR levels in perfusate correlate better with their expression in bile duct tissue, which should be investigated in

future research. Furthermore, because of the high abundancy, small changes in miRNA expression in tissue might remain insignificant, while the change in perfusate is much bigger.

Increased CDmiR release into bile during injury, as demonstrated in **chapter 6**, could also explain the lower levels of CDmiRs in perfusate. The rationale that CDmiRs are released to the biliary lumen formed the basis of one other study investigating miRNA patterns in the context of ITBL; Lankisch et al. reported on miRNA profiles in bile obtained from patients with different types of biliary complications, with specific attention for patients who developed ITBL[63]. They suggest that modifications in the biliary epithelium as in ITBL are best reflected in the composition of bile, whereas choledocholithiasis mainly causes obstruction of bile. The investigators screened for relevant miRNAs by profiling whole bile samples that were obtained by endoscopic retrograde cholangiography (ERC) from LT recipients with varying severity in ITBL (n=4) versus recipients with solely anastomotic strictures (n=4). Out of 905 miRNAs, they identified seven miRNAs of interest which were validated in a larger cohort of patients (ITBL n=37, anastomotic strictures n=39 and patients with bile duct stones n=12). This way, three miRNAs, miR-517a, miR-892a and miR-106a*, were identified that were significantly different in bile from recipients with ITBL compared to recipients with bile obstruction caused by anastomotic strictures or bile duct stones. However, these miRNAs could not distinguish for severity of ITBL. This study was limited by the relative low abundance of miRNAs (high CT values), as well as a low sensitivity and specificity for diagnosing ITBL. Furthermore, due to the absence of a multivariate analysis, it remains to be determined whether the identified miRNAs in bile are independent of other patients characteristics.

Fractions of extracellular miRNAs; more than an elaborate way to detect injury?

Reasoning that injury of hepatocytes and cholangiocytes causes active or passive miRNA release from cells, we used biliary and perfusate supernatant to analyse extracellular miRNAs in **chapter 6 and 7.** MiRNAs were however also present in other cell-free fractions, like organelles and small vesicles as exosomes. The percentage of miRNAs in these fractions was low, with less than 1% of miRNAs residing in small vesicles.

There is much discussion on which fraction of bile, perfusate or other biofluids should be analysed for studying extracellular miRNAs. Lankisch et al. used unfractionated, whole bile samples for miRNA analysis. Consequently, it remained unclear whether the potentially relevant miRNAs identified in bile were present in cells or in the liquid fraction. Shigehara et al., who were the first to report on diagnostic miRNA profiles in bile for cholangiocarcinoma, showed that a majority of detected miRNAs were in fact present in cells, whereas much lower miRNA levels were found in biliary fractions containing microvesicles[64]. Previous studies reporting on microvesicles and exosomes in bile showed that these fractions are loaded with distinct miRNAs. For instance, Li et al. designed a

diagnostic miRNA assay for cholangiocarcinoma using isolated biliary microvesicles[65]. Analysis of miRNAs in biliary exosomes can be informative on cholangiocyte-regulatory mechanisms and transmissions of disease[66-68]. Most likely, there is no right or wrong in the decision to analyse extracellular miRNAs in exosomes or supernatant, depending on the research question. But researchers should be aware that the choice for a certain fraction could lead to a different performance of miRNAs as biomarkers; certain miRNAs will have predictive value in exosomes but not in supernatant and vice versa[69]. Moreover, the presence of miRNAs might differ between distinct populations of exosomes[70]. Our decision to analyse biliary and perfusate supernatant was mainly driven by the fact that exosomal miRNAs only represented a minority and that such a design carries the risk of overlooking more abundant though significant miRNAs[71].

The question remains whether extracellular miRNAs are not just an elaborate way of detecting cellular leakage, that could also be determined by conventional markers like AST and ALT? As discussed in **chapter 2**, perfusate levels of AST and ALT are predictive of EAD and PNF following LT. Moreover, Den Dulk et al. showed that peak levels of serum ALT >1300 IU/L following LT were predictive of NAS in DCD grafts.[2] So why perform expensive and labour intensive measurements on miRNAs? An argument in favour of miRNAs consist of the rapid elevation in biofluids that is more sensitive than liver transaminases[52, 72]. Furthermore, CDmiRs are more specific for cholangiocyte injury compared to transaminases, which are mainly present in hepatocytes. The correlation between peak serum ALT and NAS probably results from severe ischemic injury that affected both hepatocytes and cholangiocytes. Release of CDmiRs could however also relate to other factors associated with cholangiocyte injury, which should be investigated in future research. Finally, given the fact that miRNAs are present in other extracellular fragments make them suitable for studying underlying mechanisms of injury. The optimized and accelerated detection of miRNAs, which will be discussed in the following paragraphs, makes them more attractive for clinical use in LT.

Effect of heparin on microRNA detection

One of the major advantages of miRNAs over messenger RNA as a biomarker is their stability at room temperature, shown in **chapter 6 and 7,** and after repeated cycles of freezing and thawing[52]. Disadvantages concerning the measurement of miRNAs are the time consuming RNA isolation procedure and reverse-transcriptase quantitative PCR (RT-qPCR). Although RT-qPCR is a sensitive technique that is capable of measuring very small amounts of miRNAs and other genes, factors have been described that can inhibit qPCR and obtain false results. **Chapter 8 and 9** of this thesis focus on such PCR inhibitor, namely heparin, and the effects of sample contamination on miRNA detection. **Chapter 8** is a re-analysis of our study described in **chapter 7**, after we suspected that perfusate samples contained traces of heparin that was administered during graft

procurement. Heparin makes a strong connection with endothelial cells and therefore, it might still be present in the liver even after multiple flushes with preservation solution[73]. We repeated qPCR on miRNAs after performing the RT-step with heparinase I, an enzyme that counteracts the inhibitory effects of heparin[74]. This slightly improved the detection of miRNAs in perfusates, suggesting the presence of small amounts of heparin that affected the measurement of miRNAs in a similar degree. However, the effect on the detection of HDmiR-148a was significantly stronger; this could be explained by some samples that showed Ct values above the reliable cut-off level of 35 cycles prior to heparinase I treatment, but which improved drastically for a minority of samples. In addition, the possible contamination with heparin did not affect the predictive capacity of miRNAs as reported earlier in **chapter 7**, because HDmiR/CDmiR ratios did not significantly change within and between samples. This encourages the use of miRNA ratios rather than single miRNAs to avoid the risk of false results whenever there is doubt on the presence of heparin in samples.

More technical aspects on the inhibitory effects of heparin and counteracting heparinase I were studied in **chapter 9**. Beside bile and blood, other perhaps more easily obtainable body fluids like urine are gaining interest for non-invasive biomarker measurement. Urinary miRNAs were proven stable biomarkers in kidney transplantation and hepatopancreatobiliary malignancies[75-78]. Most studies investigating urinary miRNAs collected samples from hospitalized patients, who often receive prophylactic anticoagulant therapy such as unfractionated or low molecular weight heparin (UH and LMWH, respectively). Heparin is partly excreted by the kidneys into urine, but its potential inhibitory effect on miRNA measurement in this medium has not been investigated before. For our study, we investigated urinary supernatant that was collected from healthy kidney donors and liver transplant recipients, with heparinized and non-heparinized individuals in both groups. The detection of urinary miRNAs through RT-qPCR was inhibited in a dose-dependent manner in samples from heparinized individuals. This caused strong variation in urinary miRNA levels that could significantly influence outcome analysis in biomarker studies. This variation was however strongly reduced after treating samples with heparinase I, which almost completely negates the inhibitory effects from heparin. Measurement of miRNA expression in tissue was not affected by systemic heparin administration. Inhibition of RT-qPCR results from co-isolation of heparin with RNA, which could differ between various RNA isolation methods. In our study, RNA isolation was based on molecular size, allowing other molecules like LMWH to pass the column and end up in the isolate. Protocols that use a different approach to isolate miRNAs, like electrochemical magnetic beads, might experience less trouble from heparin contamination[79]. The results from this study indicate that in hospitalized patients who receive systemic administration of anticoagulants, the possibility of

sample contamination with heparin should always be investigated when performing biomarker research.

Optimizing microRNA detection and validation

Optimized miRNA detection and validation depend on various other factors that deserve mentioning. Challenges already present themselves in the discovery phase; on which material should one perform a miRNA Array for the discovery of potentially interesting miRNAs? Many studies choose to perform an Array on tissue samples from small identification cohorts (usually 4 to 7 patients per group) and to validate the most significantly up or downregulated miRNAs in a larger validation cohort. Performing an Array on tissue in order to validate in body fluids like serum and bile can however give disappointing results; some tissue miRNAs are barely detectable in body fluids. Furthermore, tissue samples can contain a variety of cells, which can lead to inequalities within groups and lead to insignificant results in validation cohorts, even when using similar biomaterial[80]. This problem can be circumvented for instance by selecting cell types through laser capture microdissection, instead of using whole biopsies for miRNA isolation[51].

Second, the work-up of tissue and biofluids can differ between transplant centres, making it harder to compare results of biomarker performance. This is particularly the case for the collection of perfusates. Not only the type of preservation solution, but also the volume and ways to flush the graft differs between hospitals. Moreover, the choice of biomaterial could have consequences for the quality of isolated RNA; formalin-fixed paraffin embedded (FFPE) tissue biopsies were considered inferior compared to snap-frozen biopsies for RNA isolation. However, in the last years it has become clear that FFPE biopsies are also a valuable source for miRNA research[81-83].

Finally, an increasing amount of research is invested in accelerating and simplifying the isolation and detection of miRNAs. With normal RT-qPCR, which is currently the golden standard for miRNA detection, it is unattractive for physicians to apply miRNAs in clinical liver transplantation due to the time consuming and labour intensive technical procedure. But novel techniques seem promising in accelerating miRNA detection. For example, Liu et al. recently developed a biosensor which can directly detect miRNAs in a label-free and real-time manner, through the measurement of the light phase change caused by the formation of the double-stranded structure between the complementary DNA capture probe and the target miRNA. This way, the detection of urinary miRNAs took approximately 15 minutes[84]. This is just one example of many innovative developments to fasten and optimize miRNA detection. With the upcoming technology of machine preservation to optimize graft quality, early detection of biliary injury via accelerated miRNA measurement in recirculating perfusates would be of great interest to evaluate graft performance.

CONCLUSIONS AND FUTURE PERSPECTIVES

The studies described in this thesis contribute to a better evaluation of graft quality and the potential of various biomarkers for accurately assessing the risk to develop serious complications following LT. The introduction of machine perfusion creates a time window in which perfusate biomarkers can be measured to evaluate graft performance of marginal grafts. But also the production and composition of bile are informative on graft function. It appears that DCD grafts do not have an increased tendency to form microthrombi during graft preservation and the intraoperative administration of fibrinolytic therapy in order to prevent NAS therefore seems unjustified. Genetic predisposition is an additional risk factor for NAS in LT recipients who also suffer from PSC as underlying disease. Recipient-donor mismatch in FUT2 secretor status could further increase the risk of early development of NAS. Therefore, adapted allocation based on genetic screening of donors and PSC recipients could optimize graft performance in this group. With the increasing use of extended criteria grafts, more objective and sensitive biomarkers are demanded to predict severe biliary complications like NAS. MicroRNAs, in particular CDmiRs, showed potential to early predict the development of NAS during graft preservation. The application of miRNAs into clinical practice will however depend on optimizing and accelerating measurement techniques. Novel developments in this field seem promising and miRNAs in biofluids can become a valuable addition to current classic biomarkers. With the use of objective biomarkers, we aim to expand the donor pool and increase the number of effectuated DCD in the very near future.

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Chapter 11

Nederlandse samenvatting, discussie en toekomstperspectieven

Levertransplantatie is het slachtoffer geworden van zijn eigen succes. De afgelopen tien jaar is het aantal patiënten op de wachtlijst steeds verder gestegen door het groeiende tekort aan en de toenemende vraag naar transplanteerbare organen. In Nederland heeft dit heeft geleid tot een toename in het gebruik van organen verkregen via donatie na circulatiestilstand (donation after circulatory death (DCD)), ondanks dat deze organen van slechtere kwaliteit zijn vergeleken met organen verkregen bij donatie na hersendood (donation after brain death (DBD)). Door de langere, warme ischemietijd tijdens de orgaanuitname procedure ontstaat er uitgebreidere ischemie-reperfusie schade aan de hepatocyten en cholangiocyten van DCD organen. Daarom hebben ontvangers van dergelijke levers een hoger risico op het ontwikkelen van ernstige complicaties vlak na levertransplantatie, zoals primaire non-functie (PNF), vroege transplantaat dysfunctie (early allograft dysfunction (EAD)) en non-anastomotische galwegstricturen (NAS). Deze laatste staan ook wel bekend als ischemie-achtige galweg laesies (ischemic-type biliary lesions (ITBL)) of ischemische cholangiopathie.

Het transplanteren van levers van minder goede kwaliteit, zoals bij DCD, is echter onvermijdelijk vanwege het toenemende orgaantekort. Momenteel worden dergelijke levers vaak en wellicht onterecht afgewezen voor levertransplantatie op basis van ongunstige klinische karakteristieken, zoals een hoge donorleeftijd of op basis van de empirische beoordeling door de chirurg. Dit probleem blijkt uit de cijfers van de Nederlandse Transplantatie Stichting. In 2015 waren er 130 potentiële DBD donoren, waarvan 97% bruikbaar waren voor transplantatie in het algemeen en 79% voor levertransplantatie. Hoewel het aantal DCD donoren bijna tweemaal zo hoog was (218), was slechts 64% van deze donoren geschikt voor orgaantransplantatie en werd in slechts 22% van de gevallen de lever getransplanteerd. Kortom, van het totale aantal levertransplantaties uitgevoerd in Nederland in 2015 (n=152) werd dat in 32% gedaan met een DCD lever. Dit percentage zou op basis van bovenstaande getallen echter veel hoger kunnen zijn. Een objectievere beoordeling van de kwaliteit van het levertransplantaat met behulp van biomarkers zou kunnen bijdragen aan het vergroten van het donorpotentieel. Het onderzoek beschreven in dit proefschrift had daarom tot doel om conventionele en nieuwe biomarkers te evalueren op hun vermogen om de kwaliteit van het transplantaat en de uitkomst na levertransplantatie te voorspellen.

DEEL I: BIOMARKERS IN LEVERTRANSPLANTATIE

In **hoofdstuk 1** worden de verschillende definities van de term biomarker besproken en wordt een overzicht gegeven van de gangbare markers in de context van levertransplantatie. Leverenzymen zoals aspartaat aminotransferase (AST) en alanine aminotransferase (ALT) worden vaak gebruikt als dynamische markers in serum om hepatocellulaire

schade na levertransplantatie te monitoren. Deze markers zijn verhoogd in serum ten tijde van complicaties als PNF, EAD en rejectie. Bij schade door cholestase zijn gamma-glutamyl transferase (GGT) en alkalisch fosfatase (ALP) serummarkers die kunnen wijzen op galwegobstructie, bijvoorbeeld ten gevolge van anastomotische galwegstricturen of NAS. Tenslotte zijn er markers die iets zeggen over de functie van het transplantaat, zoals bilirubine, albumine en protrombinetijd. Al deze markers zijn *dynamische* markers, waarvan de meetwaardes fluctueren naar gelang de pathofysiologische toestand van het levertransplantaat. Behalve dynamische markers in bloed en serum kunnen ook histologische karakteristieken gebruikt worden om de mate van rejectie te bepalen, zoals de Banff-score of de rejection activity index. Nadelen van het gebruik van histologie zijn echter dat het verkrijgen van het biopt gepaard gaat met weefselinvasie en dat een biopt slechts een lokale representatie geeft van de toestand van de lever, terwijl serummarkers meer zeggen over de toestand van de lever als geheel. Bovendien stijgen serummarkers normaliter sneller dan dat er veranderingen in histologie kunnen worden waargenomen. Daarom is histologie over het algemeen een minder dynamische marker.

Surrogaat markers voor cholangiocyt schade zoals GGT en ALP hebben een vertraagde stijging na levertransplantatie en zijn daarom minder geschikt als vroege marker voor ernstige galwegschade zoals NAS. De toepassing van MicroRNAs (miRNAs) als potentiële vroege markers voor galwegcomplicaties wordt apart besproken in deel III van dit hoofdstuk.

Tenslotte wordt in **hoofdstuk 1** ingegaan op genetische profielen in donoren en ontvangers waarmee de uitkomst na levertransplantatie beter voorspeld kan worden. Genetische polymorfismen zijn statische factoren die niet veranderen gedurende het beloop van een ziekte of na levertransplantatie, en kunnen dus eigenlijk niet als biomarkers maar meer als risicofactoren worden beschouwd.

Door het toenemend gebruik van donoren met uitgebreidere criteria en de daarmee gepaard gaande complicaties, worden wereldwijd door transplantatiecentra strategieën onderzocht om de kwaliteit van het transplantaat en de uitkomst in ontvangers te verbeteren. Een op biomarkers gebaseerde evaluatie van de kwaliteit van het transplantaat voorafgaand aan implantatie zou donorlevers kunnen opsporen met een verhoogd risico op PNF, EAD, NAS, of andere complicaties. Een andere moderne strategie om preservatie van het transplantaat te optimaliseren is machineperfusie. **Hoofdstuk 2** is een systematisch review van studies die biomarkers hebben onderzocht om de kwaliteit van het transplantaat te bepalen gedurende conventionele- en machinepreservatie in levertransplantatie. Sinds 2010 zijn verschillende technieken van machineperfusie geïntroduceerd in de klinische praktijk en de eerste resultaten in humane levertransplantatie zijn veelbelovend. Een bijkomend voordeel van het gebruik van machineperfusie is dat het extra tijd creëert voor het meten van biomarkers om de kwaliteit van het transplantaat objectief te kunnen beoordelen. Behalve een vergelijking van de verschillende

machineperfusie technieken worden in **hoofdstuk 2** tevens de voor- en nadelen van het gebruik van verschillende biomaterialen voor het meten van leverschade tegen elkaar afgewogen. Met behulp van biopten kan histologisch bewijs worden verkregen van voornamelijk cellulaire schade, en kan een groot aantal cellen verkregen worden voor de analyse van de expressie van biomarkers. Nadelen bestaan echter uit de invasieve aard waarmee de biopten worden verkregen, de lokale representatie en de kans op inter- en intra-individuele variabiliteit van de beoordeling. Dit in tegenstelling tot preservatievloeistoffen die gebruikt worden om het transplantaat mee te spoelen, zogenaamde perfusaten. Perfusaten zijn in grote volumina aanwezig tijdens verschillende preservatietechnieken, kunnen non-invasief verzameld worden en geven een betere representatie van het gehele transplantaat. Daarnaast biedt machineperfusie op hogere temperaturen de mogelijkheid om gal te verzamelen. De productie en samenstelling van gal is in het bijzonder interessant om de functie van het transplantaat te bepalen. Helaas produceert de lever vrijwel geen gal bij preservatie op lagere temperaturen. Een belangrijke conclusie van **hoofdstuk 2** is dat hoewel veel studies, evenals de studies beschreven in dit proefschrift, een discriminerend vermogen aantonen van verschillende biomarkers, robuuste validatie in grotere cohorten vaak achterwege blijft. Allereerst zijn de onderzochte studiecohorten vaak klein door het lage aantal levertransplantaties dat op jaarbasis wordt uitgevoerd. Daarnaast is het prospectief verzamelen en opslaan van biomaterialen tijdrovend, logistiek uitdagend en het toepassen van nieuwe meettechnieken vaak kostbaar. Al deze factoren dragen bij aan het gebrek aan validatie bij biomarker studies, hoewel dit cruciaal is voordat biomarkers daadwerkelijk toegepast kunnen worden in de klinische praktijk. Een ander belangrijk punt betreft het feit dat veel studies enkel biomarkers hebben onderzocht in DBD levertransplantaties. De grenswaardes voor biomarkers om complicaties in DBD levertransplantaties te voorspellen zijn echter niet zomaar toepasbaar in DCD levertransplantaties[1, 2]. Tenslotte maakt de variatie in uitkomstdefinities tussen verschillende studies het moeilijk om een betrouwbare meta-analyse uit te voeren. Het introduceren van internationale richtlijnen voor uitkomstdefinities van veel voorkomende complicaties bij levertransplantaties zou de vergelijkbaarheid van studies ten goede kunnen komen[3].

DEEL II: RISICOFACTOR VOOR GALWEGCOMPLICATIES

Biomarkers kunnen meerdere doelen dienen. De uitkomst van een transplantatie kan gedefinieerd worden door biomarkers die boven een vooraf gestelde grenswaarde stijgen, zoals het geval is bij EAD. Minder dynamische biomarkers, zoals histologie of genetische polymorfismen, kunnen daarentegen geïnterpreteerd worden als risicofactor

voor een bepaalde uitkomst. In deel II van dit proefschrift zijn twee markers onderzocht die een mogelijke risicofactor zijn voor het ontwikkelen van NAS na levertransplantatie.

De vorming van microtrombi als onderliggende oorzaak van NAS: feit of fabel?

In **hoofdstuk 3** is onderzocht of DCD levers een verhoogde neiging hebben om microtrombi te vormen in hun microvasculatuur ten tijde van preservatie. In de laatste 10 jaar is door meerdere groepen onderzocht of de extra warme ischemietijd en vasculaire stase in DCD levers leidt tot het ontstaan van microtrombi in de peribiliaire vasculaire plexus van de galwegen. Dit zou vervolgens leiden tot het ontstaan van NAS. Volgens de triade van Virchow zijn drie factoren verantwoordelijk voor de vorming van een trombus; hypercoagulatie, schade aan endotheelcellen en een verstoorde bloedstroom. Deze gebeurtenissen kunnen een coagulatie cascade induceren waarbij bloedplaatjes geactiveerd worden via twee onafhankelijke mechanismen. Bij de collageen route initieert de blootstelling van sub-endotheliaal collageen de activatie van bloedplaatjes. Bij de weefselfactor route wordt trombine gegenereerd door weefselfactoren afkomstig uit de vaatwand of het bloed[4]. Het risico op de vorming van een trombus bij levertransplantatie, met name van de arteria hepatica (HAT), is gerelateerd aan het aantal arteriële anastomosen, afwijkende arteriële anatomie, een laag gewicht van de donor of ontvanger, voorgaande buikchirurgie, CMV-mismatch, re-transplantatie en verlengde operatietijd[5, 6]. Er wordt gedacht dat onvoldoende spoelen van het transplantaat tijdens orgaanuitname en voorafgaand aan implantatie het risico op microtrombi verder verhoogt. Om de vorming van microtrombi te voorkomen gebruiken sommige transplantatiecentra potentieel gevaarlijke intra-operatieve fibrinolytica, die grote bloedingen kunnen veroorzaken in patiënten.

De gedachte achter onze studie was om te bepalen of er eigenlijk wel bewijs is voor de vorming van microtrombi in leverweefsel die de toepassing van fibrinolytica zou kunnen rechtvaardigen. Voor de studie in **hoofdstuk 3** werden levercoupes, naast een conventionele kleuring met hematoxyline-eosine, ook gekleurd voor Von Willebrand Factor VIII (een marker voor endotheel activatie vroeg in de coagulatie cascade), en Fibrine Lendrum dat zich aan het eind van deze cascade bevindt. Onze studie leverde geen overtuigend bewijs voor de vorming van microtrombi gedurende verschillende tijdspunten van preservatie. Dit ondanks grondige evaluatie van intrahepatische biopten die waren verkregen uit verschillende leversegmenten van uitgebreide criteria DCD levers die waren afgewezen voor levertransplantatie. Maar ook in getransplanteerde DCD levers was het aantal microtrombi niet verhoogd ten opzichte van vergelijkbare DBD levers, noch was het aantal microtrombi verhoogd na reperfusie of in levers die NAS ontwikkelden. Hoewel in sommige coupes een deel van het vasculaire lumen positief kleurde voor Von Willebrand Factor VIII, bleven gepaarde coupes negatief voor Fibrine Lendrum en waren geen trombi waarneembaar met hematoxyline-eosine. DCD levers

lijken dus geen verhoogde neiging te hebben om microtrombi te vormen ten tijde van preservatie, die de verhoogde incidentie van NAS in deze organen zou verklaren. De vorming van microtrombi op een later moment tijdens de follow-up is echter niet uitgesloten.

Een belangrijke limitatie van onze studie is de mogelijkheid van sampling bias, met name in getransplanteerde levers waar enkel biopten van het linker laterale segment werden afgenomen. En ondanks het grote aantal coupes dat geanalyseerd is op de aanwezigheid van microtrombi, zijn deze van slechts een klein aantal levers verzameld. Desalniettemin komen onze resultaten overeen met andere histologische studies die de vorming van microtrombi en cholangiocyt schade van intra- en extrahepatische galwegen evalueerden[7-10]. Een onderzoek van Vendrell et al. laat bovendien zien dat DCD levertransplantatie hyperfibrinolyse veroorzaakt, wat de hypothese van verhoogde vorming van microtrombi en hypercoagulatie in DCD verder ondermijnt[11].

Met twee studies die een mogelijke voordelig effect aantonen[12, 13] en twee andere studies waarin geen effect wordt gezien[14, 15], lijkt er geen overtuigend bewijs te zijn voor een preventief effect van intra-operatieve fibrinolytica op NAS in interventiestudies. Alle studies betreffen echter retrospectieve analyses, terwijl een gerandomiseerde trial uitgevoerd zou moeten worden om het sterkste bewijs te leveren. Voor nu concluderen we dat het histologisch bewijs voor de vorming van microtrombi marginaal is. Dit, in combinatie met de tegenstrijdige resultaten in klinische studies, maakt dat we het gebruik van intra-operatieve fibrinolytica tijdens levertransplantatie afraden.

Schade aan de peribiliaire klieren als mogelijke oorzaak van NAS

Hoewel de betrokkenheid van de peribiliaire klieren (peribiliary glands (PBG)) bij de ontwikkeling van NAS niet is onderzocht in dit proefschrift, maken de resultaten van andere onderzoeksgroepen dit onderwerp wel de moeite van discussie waard. Al in 1994 beschreven Nakanuma et al. dat PBGs een niche zouden zijn die stamcellen bevat voor de regeneratie van cholangiocyten[16]. Een elegante studie van DiPaola et al., welke gebruik maakte van confocale microscopie in muizen, laat zien dat PBGs zich bevinden in het submucosale compartiment van de gehele extrahepatische galweg, met uitzondering van de galblaas[17]. De PBGs vormen intramurale epitheliale netwerken tussen verschillende segmenten van de grote galwegen en staan in verbinding met het galweglumen via kleine kanalen. Verder laat deze studie zien dat de proliferatie van cellen in PBGs toeneemt na ligatie van de galweg. Onlangs vonden Carpino et al. hyperplasie van PBGs die zich uitbreidde tot het oppervlakte epitheel van de galwegen in patiënten met primaire scleroserende cholangitis (PSC) en biliaire fibrose[18]. Deze PBGs brachten componenten van de hedgehog route en markers van senescence en autofagie tot expressie. De expressie van de cystische-fibrose transmembraan receptor was echter verminderd, waardoor de cholangiocyten minder beschermd waren tegen

toxische galzouten door middel van de zogenaamde 'bicarbonate umbrella', welke later in dit hoofdstuk nog aan bod zal komen. Belangrijk was de aanwezigheid van epitheliale naar mesenchymale transitie, een risicofactor voor maligne degeneratie in PSC.

Deze bevindingen zijn in overeenstemming met een eerdere studie door de groep van Porte uit Groningen, Nederland[19]. Behalve verlies van het luminale galwegepitheel vond deze groep een verhoogde expressie van Ki-67 en keratine-19 in PBGs van geëxplanteerde donorlevers van patiënten met ernstige NAS. In patiënten die chirurgische interventie nodig hadden vanwege cholangitis was de expressie van Ki-67 en keratine-19 meer uitgesproken in cholangiocyten. Op basis van deze bevindingen suggereren de auteurs dat in geval van milde galwegschade een eerstelijns regeneratie plaatsvindt door cholangiocyten, maar dat in geval van uitgebreidere schade een tweedelijns regeneratie geactiveerd wordt door PBGs. Dit heeft geleid tot een nieuw paradigma binnen de levertransplantatie, namelijk dat insufficiënte regeneratie van cholangiocyten door schade aan de PBGs in de grotere galwegen ten grondslag ligt aan NAS[20]. Vervolgens bestudeerden Op den Dries et al. de schade aan PBGs in een grote serie extrahepatische galwegbiopten van getransplanteerde DBD en DCD levers[7]. Zij vonden een hogere mate van vasculaire schade, necrose van het murale stroma en schade aan de dieper gelegen PBGs in levers welke later NAS ontwikkelden. Interessant genoeg bleek niet de schade aan PBGs, maar de vasculaire schade significant verhoogd in DCD levers. Dit suggereert dat de hogere incidentie van NAS in DCD levers toch zou kunnen voortkomen uit de vasculaire schade en verminderde bloedvoorziening. De histologische schade die werd gezien in de distale extrahepatische galweg bleek bovendien representatief voor de grotere intrahepatische galwegen[21].

Fucosyltransferase-2 dysfunctie in de pathofysiologie van galwegziekten

Genetische polymorfismen worden vaak toegepast als biomarker om de uitkomst van een levertransplantatie te voorspellen. In **hoofdstuk 4** hebben we onderzocht of een single-nucleotide polymorfisme (SNP) in het fucosyltransferase-2 gen (FUT2) een risicofactor is voor het ontwikkelen van NAS. De mutatie rs608133 (G>A), die gevonden is bij een genoomwijde associatie studie, is een prevalent polymorfisme dat voorkomt onder 20% van de algemene populatie, maar die ook in verband is gebracht met PSC[22, 23]. De mutatie veroorzaakt een dysfunctioneel FUT2 eiwit, wat resulteert in verminderde glycosylering van oppervlakte epitheliale cellen. Men denkt dat deze verminderde glycosylering de barrière functie van cellen tegen pathogenen verlaagt[24]. Vanwege de klinische overeenkomsten tussen NAS en PSC rees de vraag of deze mutatie van het FUT2 gen in levertransplantatiedonoren en ontvangers een risicofactor zou kunnen zijn voor NAS. Daarop hebben we retrospectief een genotypering van de FUT2 secretor status uitgevoerd op het DNA van donoren en ontvangers van in totaal 418 levertransplantatie procedures. De donor FUT2 status was niet geassocieerd met de ontwikkeling van NAS.

In patiënten met PSC bleek echter dat een FUT2 non-secretor status van de ontvanger (AA-genotype) een onafhankelijke risicofactor was voor NAS (HR 2.34, P=0.034), waarbij het sterkste effect werd gezien in de eerste vijf jaar na transplantatie. FUT2 status was geen risicofactor voor levertransplantatiepatiënten die een andere onderliggende ziekte dan PSC hadden.

FUT2 dysfunctie is ook gecorreleerd aan inflammatoire darmziekten (IBD), zoals de ziekte van Crohn en colitis ulcerosa, die kunnen voorkomen in combinatie met PSC[25, 26]. Er wordt gedacht dat de verlaagde barrièrefunctie en de minder diverse bacteriële samenstelling van het darmepitheel leiden tot een 'lekkende darm' in FUT2 non-secretors, waardoor bacteriën en pathogenen door de darmwand kunnen passeren naar de circulatie[27-29]. Om de rol van bacteriële translocatie te onderzoeken, hebben we het gelijktijdig voorkomen van IBD geanalyseerd in ons cohort van patiënten met onderliggend PSC en de relatie met FUT2 secretor status. Er was een sterke correlatie tussen PSC en het gelijktijdig bestaan van colitis ulcerosa, met een incidentie van 56%. We konden echter niet aantonen dat FUT2 non-secretor status vaker voorkwam bij patiënten met gecombineerd PSC en IBD, noch was de incidentie van NAS verhoogd in deze groep. Mogelijk was het aantal patiënten met gelijktijdig voorkomen van PSC en IBD in ons cohort te klein om dit verband goed te onderzoeken. Dit belemmerde tevens een betrouwbare analyse van het preventieve effect van colectomie op het ontwikkelen van NAS, wat aangetoond is in voorgaande studies betreffende PSC[30].

De resultaten van **hoofdstuk 4** zouden ook kunnen duiden op een verhoogde immuunrespons, veroorzaakt door ongunstige FUT2 combinaties. Analyse van gecombineerde donor- en ontvangergenotypen liet zien dat de incidentie van NAS het hoogste was (80%) onder PSC patiënten met een FUT2 non-secretor status (AA), die een donor lever ontvingen van een FUT2 secretor donor (GG of GA). Een experimentele studie in transgene muizen ondersteunt deze hypothese. Hierbij werd door een verhoogde immuunrespons een verkorte transplantaatoverleving aangetoond van huid en hartweefsel met overexpressie van fucosyltransferase, welke getransplanteerd werden naar wild-type ontvangers[31]. Daarnaast sluiten de resultaten van onze studie niet uit dat cholangiocyten uit de resterende galweg van de ontvanger de nieuwe donor galweg repopuleren. Dit type chimerisme is al eens eerder aangetoond, maar niet in de context van NAS[32].

Behalve het darmepitheel hebben FUT2 non-secretors ook een veranderde glycosylering van cholangiocyten en een veranderede bacteriële samenstelling van gal, welke beiden kunnen bijdragen aan het ontwikkelen van NAS[22]. Een veranderde samenstelling van de glycocalyx beïnvloedt het alkaline milieu van cholangiocyten, welke normaliter in stand wordt gehouden door de 'bicarbonate umbrella'. Dit maakt cholangiocyten meer kwetsbaar voor toxische galzouten[33, 34]. Bovendien is een veranderde samenstelling van de gal in verband gebracht met een hogere incidentie van cholangitis,

galwegcomplicaties en een verkorte transplantatievrije overleving[35-37]. Onze studie levert echter geen direct bewijs voor een verhoogde toxiciteit door galzouten in FUT2 non-secretors, hoewel dit een interessant onderwerp blijft voor toekomstig onderzoek.

Tenslotte blijkt dat de detectie van biomarkers die afhankelijk zijn van synthese door FUT2 enzymactiviteit, beïnvloed kan worden door het FUT2 genotype. Dit is aangetoond door Wannhoff et al. voor kanker antigen 19-9 (CA 19-9), welke vaak toegepast wordt als biomarker bij de screening op cholangiocarcinoom in patiënten met PSC[38]. Het blijkt dat PSC patiënten met een FUT2 non-secretor status hogere serumwaardes hebben van CA 19-9 vergeleken met FUT2 secretors. Om een betere voorspelbaarheid van deze biomarker te verkrijgen in termen van sensitiviteit, specificiteit en postief- of negatief voorspellende waarde, zou de grenswaarde van CA 19-9 hoger moeten liggen in FUT2 non-secretors. Dus onderzoekers moeten zich bewust zijn van mogelijke genotype-afhankelijkheid van biomarker waardes in toekomstige studies.

Andere polymorfismen geassocieerd met NAS en PSC in levertransplantatie

Naast FUT2 zijn ook andere SNPs onderzocht in de context van NAS na levertransplantatie. Ten Hove et al. lieten zien dat een mutatie in het gen coderend voor matrix metalloproteinase-2 (MMP-2) in zowel donoren als ontvangers een verhoogd risico geeft op het ontwikkelen van NAS[39]. MMP-2 is een enzym dat matrixdegradatie in weefsels reguleert en in verband is gebracht met leverfibrose en cirrose. Een polymorfisme aanwezig in zowel de donor als de ontvanger heeft een synergistisch effect en vergroot het risico op NAS (HR 3.48). Serumwaardes van MMP-2 veranderden echter niet door de SNP. Een vervolgstudie van dezelfde onderzoeksgroep liet zien dat het polymorfisme ook geassocieerd is met slechtere uitkomsten in termen van transplantatie-vrije overleving en mortaliteit in PSC patiënten[40].

Een andere SNP gerelateerd aan NAS is die van chemokine receptor CCR5 (CCR5-Δ32 mutatie)[41]. CCR5 wordt tot expressie gebracht op verschillende cellen van zowel het aangeboren als het verworven immuunsysteem. Homo- en heterozygote dragers van de mutatie hebben bijvoorbeeld een verminderde chemotaxis van regulatoire T-cellen. CCR5-Δ32 in ontvangers, maar niet in donoren, geeft een viervoudig verhoogd risico op het ontwikkelen van NAS. De mutatie in ontvangers is een onafhankelijke risicofactor voor NAS, met name in patiënten met PSC. Helaas is er geen duidelijke gestratificeerde analyse uitgevoerd op het effect van de mutatie in niet-PSC patiënten.

Al deze studies betreffende genetische polymorfismen en de ontwikkeling van NAS, inclusief onze eigen studie, tonen een sterke associatie aan met PSC. Dit leidt tot de discussie of de presentatie van NAS in deze studiecohorten in feite een terugkeer van de onderliggende ziekte is. Meerdere studies hebben aangetoond dat PSC een risicofactor is voor het ontwikkelen van NAS[42, 43]. De criteria voor recidief PSC, welke zijn geformuleerd in 1999, bestaan uit; (i) een bestaande diagnose van PSC voorafgaand aan

levertransplantatie, met (ii) intra- en/of extrahepatische galweg stricturen, kralensnoer aspect of irregulariteiten, optredend na tenminste 90 dagen follow-up en aangetoond met cholangiografie, *of* fibreuze cholangitis en/of fibro-obliteratieve laesies met of zonder ductopenie, met histologische aangetoonde biliaire fibrose of biliaire cirrose[44, 45]. Criteria die recidief PSC excluderen bestaan uit vastgestelde ductopene rejectie, HAT, enkelvoudige anastomotische stricturen, NAS optredend voor dag 90 na levertransplantatie en ABO-incompatibiliteit. Ondanks deze duidelijke beschrijving blijkt de diagnose van recidief PSC in de praktijk niet altijd even gemakkelijk. Ten eerste wordt de primaire diagnose van PSC in een minderheid van de gevallen pas duidelijk na een grondige histologische evaluatie van het leverexplantaat door de patholoog. Daarnaast wordt het typische kralensnoer aspect van PSC, zoals te zien is met cholangiografie, vaak pas duidelijk in een verder gevorderd stadium van de ziekte, terwijl meer geïsoleerde intrahepatische stricturen en dilataties vaker voorkomen in een vroeger stadium. Soms worden de eerste symptomen van de ziekte al duidelijk voor de 90^{e} postoperatieve dag. En op basis van histologisch onderzoek op naaldbiopten kan het lastig zijn om recidief PSC te onderscheiden van andere aandoeningen zoals chronische rejectie[46, 47].

Vanwege het moeilijke onderscheid tussen recidief PSC en NAS verdient het de aanbeveling om, indien mogelijk, altijd een gestratificeerde analyse uit te voeren voor apart PSC en niet-PSC patiënten in transplantatie cohorten. Bepaalde SNPs geassocieerd met recidief PSC en NAS kunnen gebruikt worden voor het in kaart brengen van risicoprofielen in patiënten. Bovendien verschaffen dergelijke SNPs meer inzicht in de onderliggende mechanismen en pathofysiologie van complicaties na levertransplantatie. De kennis dat bepaalde donor-ontvanger combinaties kunnen leiden tot ongunstige uitkomsten kan in de toekomst van invloed zijn op de toewijzing van donor organen, die momenteel vooral wordt bepaald door de MELD-score en de bloedgroep.

Risicofactoren voor galwegcomplicaties in perspectief

De bovenstaande paragrafen beschrijven een samenhangend geheel van risicofactoren die elkaars invloed op de ontwikkeling van NAS lijken te versterken. Samengevat lijkt galwegschade te ontstaan door verlengde ischemie van het transplantaat, wat de hogere incidentie van NAS verklaart in DCD levers[48]. De vitaliteit van de galweg is sterk afhankelijk van bloedvoorziening door de arteria hepatica, maar verminderde bloedtoevoer vanuit de vena porta is ook in verband gebracht met NAS[10, 49, 50]. De hierop volgende reperfusie vergroot de schade aan het galwegepitheel en de PBGs. Dit tast niet alleen de regeneratie van cholangiocyten aan, maar ook de integriteit van de biliaire glycocalyx en 'bicarbonate-umbrella', die normaliter cholangiocyten beschermt tegen het toxische effect van galzouten. Patiënten met PSC hebben vaker een verstoorde biliaire glycocalyx, die daardoor makkelijker doordringbaar wordt voor pathogenen zoals bacteriën. Dit kan vervolgens een verhoogde translocatie veroorzaken van bacteriën

vanuit de darm naar de lever, maar kan ook leiden tot een versterkte immuunrespons als een lever van een FUT2 secretor donor geïmplanteerd wordt in een FUT2 non-secretor ontvanger.

DEEL III: MICRORNAS ALS NIEUWE BIOMARKERS IN LEVERTRANSPLANTATIE

Deel III van dit proefschrift richt zich op de toepassing van microRNAs (miRNAs of miRs) als biomarkers voor transplantaatfunctie en schade bij levertransplantatie. Als introductie op dit onderwerp wordt in **hoofdstuk 5** een overzicht gegeven van de literatuur betreffende miRNAs in leverziekten en levertransplantatie, met speciale aandacht voor extracellulaire miRNAs. Na hun ontdekking in 2001 is het aantal publicaties over miRNAs als biomarkers explosief toegenomen, maar ook over de betrokkenheid bij andere pathologieën. MiRNAs zijn 20-23 nucleotiden lange, niet-coderende RNAs die post-transcriptioneel genexpressie kunnen reguleren. In het bloed, maar ook in andere lichaamsvloeistoffen, bevinden zich cel-specifieke miRNAs die stabiel zijn en beschermd blijven tegen degradatie. Dit maakt miRNAs een populair onderwerp voor non-invasief biomarker onderzoek. Een mogelijke verklaring voor de stabiliteit van miRNAs in lichaamsvloeistoffen is dat ze ingebed zijn in, of gebonden zijn aan, andere cellulaire componenten. Extracellulaire miRNAs zijn bijvoorbeeld gevonden in vesikels en kleinere exosomen, gebonden aan lipoproteïnen, of gebonden aan het eiwit argonaute-2 in reactie op schadelijke processen. Binnen de hepatologie geven serumwaardes van miRNAs informatie over leverschade in patiënten met virale hepatitis, non-alcoholische leververvetting, leverfibrose, maligniteit en meer. In de context van levertransplantatie is de hoeveelheid literatuur over extracellulaire miRNAs als biomarkers echter schaars.

MicroRNAs als markers voor schade aan hepatocyten en cholangiocyten post-levertransplantatie

Hepatocyten en cholangiocyten brengen verschillende miRNAs tot expressie. Een elegante studie uitgevoerd door Chen et al. gebruikte laser-capture microdissectie om cholangiocyten en hepatocyten te isoleren uit leverweefsel, waarna voor deze cellen afzonderlijk met behulp van genoomwijde microarray analyse werd bepaald welke miRNAs aanwezig waren[51]. Gebaseerd op de resultaten van Chen et al. heeft onze groep als eerste het vrijkomen van hepatocyt-afgeleide miRNAs (HDmiRs) in serum onderzocht op het moment van leverschade en acute rejectie na levertransplantatie[52]. De belangrijkste bevindingen van deze studie waren dat expressie van HDmiR-122 en HDmiR-148a significant verlaagd was in post-reperfusie biopten wanneer de warme-ischemie tijd verlengd was. Omgekeerd bleken de waardes van deze miRNAs te stijgen in serum en sterk te correleren met AST en ALT waardes gedurende de eerste maand na

levertransplantatie. Sterker, de waardes van HDmiRs waren al significant verhoogd in serum wanneer de transaminaselevels nog <50 IU/L waren. De piekwaardes van HDmiRs in serum liepen 24 uur voor op de piekwaardes van serum transaminases in patiënten met histologisch bewezen acute rejectie. Deze resultaten suggereren dat serum HDmiRs een vroegere en misschien zelfs sensitievere marker voor leverschade zijn dan de klassieke serum transaminases, welke momenteel nog de gouden standaard zijn als biomarker voor leverschade.

Om meer inzicht te verkrijgen in de biologie en dynamiek van miRNAs, hebben we in **hoofdstuk 6** het vrijkomen van miRNAs in gal en serum onderzocht gedurende verschillende (patho)fysiologische condities van de lever na transplantatie. In deze studie zijn behalve HDmiR-122 en -148a ook cholangiocyt-afgeleide miRNAs (CDmiRs) getest, welke tevens zijn geïdentificeerd door de eerder genoemde studie van Chen et al. (CDmiR-30e, -200c en -222)[51]. De weefsel-specificiteit van deze miRNAs werd bevestigd in leverbiopten en galwegweefsels die verzameld werden gedurende levertransplantatie. In leverweefsel was HDmiR-122 het meest overvloedig, terwijl in galwegweefsel CDmiR-222 het hoogst tot expressie kwam. Over het algemeen waren miRNA waardes in gal hoger dan in gepaarde serummonsters. Fractioneringsproeven lieten zien dat de meerderheid van extracellulaire miRNAs in gal aanwezig waren in de niet te pelleteren fractie, en dat ze hierin gedurende tenminste 1-4 uur beschermd werden tegen degradatie door verbindingen met proteïnes. Er werd een sterke correlatie gevonden tussen de excretie van HDmiR-122 en bilirubine naar gal, met hoge niveaus van HDmiR-122 als de lever een goede excretiefunctie had. De waardes van HDmiR-122 in gal daalden drastisch wanneer de excretiefunctie van de lever verminderde. Interessant was de observatie dat CDmiR-222 waardes in gal een omgekeerde dynamiek vertoonden, met lagere waardes ten tijde van goede excretiefunctie en een stijging als de excretiefunctie verminderde. Veranderingen in excretiefunctie werden het duidelijkst weerspiegeld in miRNA waardes in gal, zonder significante veranderingen van miRNAs in gepaarde serummonsters. Leverschade en acute rejectie werden echter het duidelijkst weerspiegeld door serum miRNAs. Ten tijde van schade stegen waardes van HDmiR-122 in gal en tegelijkertijd nog sterker in serum. Opnieuw vertoonde CDmiR-222 een tegenovergestelde dynamiek.

Op basis van deze resultaten lijkt het erop dat het vrijkomen van HDmiRs en CDmiRs in gal en bloed gepolariseerd is, afhankelijk van de transplantaatfunctie en de mate van schade. De tegenovergestelde dynamiek tussen HDmiRs en CDmiRs lijkt bovendien meer te berusten op een actief mechanisme van uitscheiding dan op slechts passieve lekkage vanuit beschadigde cellen. De excretie van miRNAs naar gal gedurende goede transplantaatfunctie kan wijzen op een rol van miRNAs in de biliaire homeostase. Voorgaande studies hebben bijvoorbeeld laten zien dat expressie van miR-506, miR-222 en miR-199a-3p invloed hebben op de activiteit van anion exchanger 2 en de farnesoid-x

receptor[53, 54]. Verder laat onze studie de potentie van het gebruik van gal zien als medium om met biliaire miRNAs de functie van het transplantaat te bepalen. Beperkingen van onze studie bestonden uit het kleine aantal patiënten van wie gal en serummonsters werden verzameld (n=10) en de afwezigheid van een 3D experimenteel model om het gepolariseerd vrijkomen van HDmiRs en CDmiRs te valideren. Met de voortdurende recente ontwikkelingen wat betreft het opbouwen en groeien van orgaanweefsel in het laboratorium kan dit laatste doel hopelijk in de nabije toekomst gerealiseerd worden [55, 56].

De betrokkenheid van miRNAs in cholangiopathieën is voornamelijk onderzocht bij cholangiocarcinomen[57]. In deze studies is gal de meest gebruikte bron voor miRNA analyse, gevolgd door serum. De expressie van miRNAs in weefsel is vooral gerelateerd aan de regulatie van doelwitgenen die betrokken zijn bij fibrose, apoptose, proliferatie, invasie, inflammatie, migratie, tumor groei, oncogenese, chemo-resistentie en andere pathofysiologische factoren. Opmerkelijk genoeg is er minder bekend over miRNAs in andere cholagiopathieën zoals primaire biliaire cirrose, PSC en biliaire atresie. De kennis over miRNA regulatie in de pathofysiologie van PSC zou in het bijzonder van belang kunnen zijn voor een beter begrip van NAS, gezien de overeenkomsten in klinische presentatie. In de context van PSC is miR-7a gerelateerd aan cholangiocytproliferatie[58]. Andere studies beschrijven vooral miRNA profielen in gal en serum om PSC te onderscheiden van cholangiocarcinoom, wat lastig kan zijn op basis van slechts borstelcytologie[59, 60]. Door de regulerende aspecten van CDmiRs verder te ontrafelen kunnen deze miRNAs de basis vormen van nieuwe therapeutische doelwitten in NAS of andere cholangiopathieën, wat eerder al succesvol is gebleken voor HDmiRs in virale hepatitis[61, 62].

MicroRNAs als potentiële markers voor cholangiocytschade voorafgaand aan levertransplantatie

Markers voor schade aan cholangiocyten en het voorspellen van NAS zijn schaars. De meeste studies betreffen onderzoek naar galwegweefsels, welke hiervoor al uitvoerig beschreven zijn. Vanwege het chirurgische trauma en de lokale representativiteit geassocieerd met het verzamelen van biopten, zochten we naar een cholangiocytspecifieke biomarker die non-invasief gemeten kon worden voorafgaand aan implantatie van de donorlever. De studie in **hoofdstuk 7** is geïnitieerd om te bepalen of extracellulaire miRNAs vrijkomen in perfusaten die gebruikt worden om het transplantaat te spoelen en of deze miRNAs een vroege marker zouden kunnen zijn voor NAS. Waardes van HDmiRs (miR-122 en -148a) en CDmiRs (miR-30e, -222 en -296) in het supernatant van perfusaten verkregen van levers die ITBL ontwikkelden (n=20) werden vergeleken met die van levers die geen galwegcomplicaties ontwikkelden (n=37). Om te corrigeren voor mogelijke verschillen in concentratie tussen perfusaten, werden ratio's van HD-

miRs/CDmiRs gebruikt voor analyse. Ontvangers van een donorlever die hoge HDmiR/CDmiR ratio's bevatte in het perfusaat, met name door lage CDmiR waardes, hadden een drie- tot zesmaal verhoogd risico op het ontwikkelen van ITBL tijdens follow-up. Het onderscheidend vermogen van HDmiR/CDmiR ratio's was redelijk, met C-statistiek waardes tussen de 0.74-0.77. Er bestond echter wel een substantiële overlap in biomarker waardes tussen de ITBL en de niet-ITBL groep.

Een mogelijke verklaring voor de lagere CDmiR waardes in perfusaten van de ITBL groep zou het verlies aan cholangiocyten kunnen zijn[7]. Analyse van gepaarde leverbiopten (n=24), welke overigens minder goed presteerden als voorspellende biomarker, liet echter geen verminderde weefselexpressie zien van CDmiRs. Deze observatie is op zijn beurt weer te verklaren door de manier waarop de biopten zijn verzameld; dit waren namelijk wigbiopten van leverweefsel afkomstig van het linker laterale segment. Wellicht dat CDmiRs in perfusaat beter correleren met de expressie in galwegweefsel, dat meer cholangiocyten bevat. Dit moet toekomstig onderzoek nog uitwijzen. Verder zou het kunnen dat door hun overvloedigheid in weefsel kleine veranderingen in miRNA expressie niet significant zijn, terwijl de effecten van kleine veranderingen duidelijker naar voren komen in cel-armere perfusaten. Daarnaast zou het vrijkomen van CDmiRs in gal gedurende galwegschade, zoals aangetoond in **hoofdstuk 6**, de lagere waardes van CDmiRs in perfusaten kunnen verklaren. De redenering dat CDmiRs vrijkomen in het galweglumen vormde de basis van een andere studie die profielen van miRNAs onderzocht in de context van ITBL. Lankisch et al. hebben miRNA profielen in gal beschreven bij patiënten met verschillende soorten galwegcomplicaties, met specifieke aandacht voor ITBL[63]. Zij opperden dat veranderingen in het galwegepitheel zoals bij ITBL het best weerspiegeld worden in de samenstelling van de gal, terwijl complicaties als choledocholithiasis voornamelijk voor een obstructie van gal zorgen. De onderzoekers screenden op relevante miRNAs in ongefractioneerde galmonsters die waren verkregen door middel van endoscopische retrograde cholangiografie (ERC) bij levertransplantatiepatiënten met ernstige ITBL (n=4) versus patiënten met enkele anastomotische stricturen (n=4). Uit een pool van 905 miRNAs identificeerden zij zeven potentiële miRNAs van belang, die vervolgens gevalideerd werden in een groter patiëntencohort (ITBL n=37, anastomotische stricturen n=39 en patiënten met galstenen n=12). Zo resteerden drie relevante miRNAs, miR-517a, miR-892a en miR-106a*, welke significant verschillende waardes hadden in gal van patiënten met ITBL ten opzichte van patiënten met anastomotische stricturen en galstenen. Deze miRNAs waren echter niet in staat om onderscheid te maken in de ernst of de mate van ITBL. Limitaties van de studie betreffen de relatief lage niveaus van miRNAs, als ook de lage sensitiviteit en specificiteit van miRNAs bij het voorspellen van ITBL. En door het achterwege laten van een multivariate analyse blijft het onbekend of de geïdentificeerde miRNAs in gal voorspellers zijn voor ITBL, onafhankelijk van andere patiëntkarakteristieken.

Fracties extracellulaire miRNAs: meer dan een ingewikkelde manier om schade te meten?

Redenerend dat schade aan hepatocyten en cholangiocyten leidt tot het actief of passief vrijkomen van miRNAs uit cellen, hebben we in **hoofdstuk 6 en 7** de aanwezigheid van extracellulaire miRNAs geanalyseerd in het supernatant van gal en perfusaten. MiRNAs waren echter ook aanwezig in andere cel-vrije fracties, zoals organellen en kleinere vesikels zoals exosomen. Het percentage miRNAs dat zich in deze fracties bevond was echter laag, met minder dan 1% van de miRNAs aanwezig in kleinere vesikels.

Er is veel discussie gaande over de vraag welke fractie van gal, perfusaat of andere biovloeistoffen geanalyseerd dient te worden bij de bestudering van extracellulaire miRNAs. Lankisch et al. gebruikten ongefractioneerde galmonsters voor miRNA analyse. Daarom blijft het onduidelijk welke van de door hen geïdentificeerde miRNAs in gal aanwezig waren in cellen of in de vloeibare fractie. Shigehara et al., die als eerste diagnostische miRNA profielen in gal beschreven voor cholangiocarcinoom, vonden een meerderheid van de gedetecteerde miRNAs in cellen, terwijl een veel lagere hoeveelheid teruggevonden werd in de fractie met microvesikels[64]. Voorgaande studies betreffende microvesikels en exosomen in gal laten zien dat deze fracties wel degelijk verschillende miRNAs kunnen bevatten. Li et al. hebben bijvoorbeeld een diagnostische miRNA assay ontworpen voor cholangiocarcinoom, gebruikmakend van geïsoleerde microvesikels in gal[65]. Analyse van miRNAs in exosomen in gal kan waarschijnlijk meer informatie opleveren over cholangiocyt-regulerende mechanismen en transmissie van ziekte[66-68]. Waarschijnlijk is er geen goed of fout in het besluit om exosomen of supernatant te analyseren op extracellulaire miRNAs, afhankelijk van de onderzoeksvraag. Maar onderzoekers dienen er rekening mee te houden dat de keuze om een bepaalde fractie te analyseren, kan leiden tot verschillen in de prestatie van miRNAs als biomarkers. Sommige miRNAs zullen namelijk voorspellende waarde hebben in exosomen maar niet in supernatant en vice versa[69]. Bovendien kan de aanwezigheid van miRNAs afwijken tussen verschillende populaties exosomen[70]. Ons besluit om het supernatant van gal en perfusaten te analyseren werd voornamelijk gedreven door het feit dat slechts een minderheid van de miRNAs zich in exosomen bevond en dat een dergelijke studieopzet het risico met zich meebrengt dat andere belangrijke miRNAs over het hoofd zouden worden gezien[71].

De vraag blijft of extracellulaire miRNAs een ingewikkelde manier zijn om simpele cellekkage te detecteren, die ook gemakkelijker bepaald zou kunnen worden met conventionele markers als AST en ALT. Zoals besproken in **hoofdstuk 2** zijn waardes van AST en ALT in perfusaten immers voorspellend voor het optreden van EAD en PNF na levertransplantatie. Bovendien laten Den Dulk et al. zien dat piekwaardes van serum ALT

>1300 IU/L voorspellend zijn voor NAS na DCD levertransplantatie[2]. Dus waarom zou men dan een dure en arbeidsintensieve meting verrichten naar miRNAs? Een argument in het voordeel van miRNAs is de vroege stijging in biovloeistoffen, die meer sensitief lijkt te zijn dan die van levertransaminasen[52, 72]. Daarnaast zijn CDmiRs meer specifiek voor schade aan cholangiocyten dan transaminasen die voornamelijk aanwezig zijn in hepatocyten. De correlatie tussen piek serum ALT en NAS komt dan ook waarschijnlijk voort uit ernstige ischemische schade die zowel hepatocyten als cholangiocyten aantast. Het vrijkomen van CDmiRs kan echter ook gerelateerd zijn aan andere factoren die samenhangen met cholangiocyt schade, wat moet blijken uit toekomstig onderzoek. Tenslotte, het feit dat miRNAs ook aanwezig zijn in andere extracellulaire fracties maakt ze meer geschikt voor het bestuderen van onderliggende mechanismen van schade. Een versnelde en geoptimaliseerde detectie van miRNAs, welke in een latere paragraaf nog aan bod zal komen, maakt ze aantrekkelijker en beter toepasbaar bij klinische levertransplantatie.

Het effect van heparine op de detectie van microRNAs

Een van de grote voordelen van miRNAs boven messenger RNA als biomarker is hun stabiliteit op kamertemperatuur, zoals aangetoond in **hoofdstuk 6 en 7**, en hun stabiliteit na herhaalde cycli van vriezen en ontdooien. Nadelen zijn de tijdsintensieve procedures van RNA isolatie en reverse-transcriptase kwantitatieve PCR (RT-qPCR). Hoewel RT-qPCR een sensitieve techniek is die zeer kleine hoeveelheiden miRNAs en andere genen kan meten, zijn er factoren beschreven die qPCR kunnen inhiberen en zodoende foutieve resultaten opleveren. **Hoofdstuk 8 en 9** van dit proefschrift focussen op een dergelijke PCR-inhiberende component, namelijk heparine, en het effect ervan op miRNA detectie. **Hoofdstuk 8** is een re-analyse van onze studie beschreven in **hoofdstuk 7**, nadat we vermoedens kregen dat perfusaten sporen konden bevatten van heparine die was toegediend gedurende de orgaanuitname procedure. Heparine gaat een sterke verbinding aan met endotheelcellen en kan daarom nog steeds aanwezig zijn in de donorlever, zelfs na veelvuldig spoelen met preservatie vloeistof[73]. We analyseerden onze monsters opnieuw met qPCR, maar ditmaal nadat de RT-stap was uitgevoerd in aanwezigheid van heparinase I, een enzym dat heparine afbreekt[74]. Dit gaf een lichte verbetering van de detectie van vrijwel alle miRNAs in perfusaten, wat er op duidt dat kleine hoeveelheden heparine aanwezig waren die de eerste meting van verschillende miRNAs in eenzelfde mate hebben beïnvloed. Het effect was echter sterker bij de detectie van HDmiR-148a. Dit kan verklaard worden doordat de niveaus van HDmiR-148a in sommige monsters, voorafgaand aan de behandeling met heparinase I, boven de detectielimiet was, maar waarvan de detectie in een aantal monsters na behandeling met Heparinase I drastisch verbeterde. Een mogelijke vervuiling met heparine had echter geen effect op de voorspellende waarde van miRNAs zoals beschreven in **hoofdstuk 7**, want HDmiR/CDmiR ratio's veranderden niet significant tussen de monsters. Het verdient dus aanbeveling om bij de analyse van miRNA ratio's te bepalen in plaats van enkelvoudige miRNA's

om het risico op foutieve resultaten te vermijden wanneer er mogelijk heparine in de monsters aanwezig is.

In **hoofdstuk 9** zijn meer technische aspecten met betrekking tot de inhiberende effecten van heparine en het tegengaan hiervan met heparinase I bestudeerd. Naast gal en bloed is er steeds meer interesse voor het bepalen van non-invasieve biomarkers in andere, wellicht nog gemakkelijker te verkrijgen lichaamsvloeistoffen. MiRNAs in urine blijken stabiele biomarkers te zijn bij niertransplantatie en hepato-pancreato-biliaire maligniteiten[75-78]. De meeste studies die miRNAs in urine onderzochten, verzamelden monsters van gehospitaliseerde patiënten die vaak profylactisch anticoagulantia ontvingen, zoals ongefractioneerde- of laag-moleculair-gewicht heparine (UH en LMWH, respectievelijk). Heparine wordt deels uitgescheiden naar de urine door de nieren, maar potentiële inhiberende effecten op de detectie van miRNAs in urine zijn nog niet eerder onderzocht. Voor onze studie hebben we het supernatant van urinemonsters onderzocht die waren verkregen van gezonde nierdonoren en levertransplantatie ontvangers, met geheparineerde en niet-geheparineerde individuen in beide groepen. De detectie van miRNAs in urine met RT-qPCR was sterk geïnhibeerd en dosis afhankelijk in monsters van geheparineerde individuen. Dit veroorzaakte een sterke variatie in de waardes van miRNAs in urine en zou dus kunnen leiden tot vertekende uitkomsten bij biomarker studies. De variatie nam echter sterk af wanneer monsters tijdens de RT-stap behandeld werden met heparinase I, waardoor de inhiberende effecten van heparine bijna volledig teniet werden gedaan. De detectie van miRNAs in weefsel werd niet beïnvloed door systemische toediening van heparine. De inhibitie van de RT-qPCR reactie komt voort uit de co-isolatie van heparine met het RNA. De mate van heparine co-isolatie varieert waarschijnlijk tussen verschillende isolatie methoden. In onze studie is RNA geïsoleerd op basis van molecuul grootte, waarbij ook andere moleculen zoals LMWH de isolatie kolom kunnen passeren en terecht kunnen komen in het geïsoleerde materiaal. Protocollen die een andere aanpak hebben voor de isolatie van miRNAs, zoals elektrochemische magnetische bolletjes, ondervinden mogelijk minder problemen van heparine vervuiling in RNA monsters[79]. De resultaten uit onze studie wijzen er echter op dat in gehospitaliseerde patiënten die systemisch anticoagulantia toegediend krijgen, monsters gebruikt wordendie bij biomarker onderzoek gecontamineerd kunnen zijn met heparine, met mogelijke effecten op de uitkomst van het onderzoek.

Optimalisatie en validatie van miRNA detectie

Geoptimaliseerde detectie en validatie van miRNAs is tevens afhankelijk van verschillende andere factoren die aandacht verdienen. Een van de vragen die opkomt is op welk materiaal een miRNA array uitgevoerd dient te worden voor het identificeren van potentieel interessante miRNAs. In veel studies wordt gekozen om een array uit te voeren op weefsel van een klein identificatie cohort (zo'n vier tot zeven patiënten per

groep) en om de meest significante miRNAs te valideren in een groter validatie cohort. Het uitvoeren van een array op weefsel om later te valideren in lichaamsvloeistoffen zoals serum en gal kan echter teleurstellende resultaten opleveren. Sommige miRNAs in weefsels zijn nauwelijks detecteerbaar in lichaamsvloeistoffen. Daarnaast bevat weefsel vaak een grote variatie aan celtypen, wat kan leiden tot ongelijkheid binnen groepen en niet significante resultaten in validatie cohorten, zelfs bij gebruik van vergelijkbare biomaterialen[80]. Dit probleem kan wellicht vermeden worden door bijvoorbeeld celtypen te selecteren door middel van laser capture microdissectie, in plaats van het gebruik van hele biopten voor de isolatie van miRNAs[51].

Ten tweede kan het opwerken van weefsels en biovloeistoffen verschillen tussen transplantatiecentra, wat het vergelijken van de bruikbaarheid van biomarkers bemoeilijkt. Dit is vooral het geval bij het verzamelen van perfusaten. Niet alleen het type preservatie vloeistof, maar ook het volume en de manier waarop het transplantaat wordt gespoeld verschilt tussen ziekenhuizen. Bovendien kan de keuze van het biomateriaal gevolgen hebben voor de kwaliteit van het geïsoleerde RNA. Formaline-gefixeerde biopten ingebed in paraffine (FFPE) worden als inferieur beschouwd voor de isolatie van RNA vergeleken met direct bevroren biopten. De laatste jaren is echter duidelijk geworden dat ook FFPE biopten een waardevolle en betrouwbare bron vormen voor miRNA onderzoek[81-83].

Tenslotte richt steeds meer onderzoek zich op het versnellen en vereenvoudigen van de isolatie en detectie van miRNAs. De normale RT-qPCR techniek, momenteel de gouden standaard voor miRNA detectie, maakt het voor clinici onaantrekkelijk om miRNAs toe te passen tijdens levertransplantatie vanwege de tijdrovende en arbeidsintensieve procedure. Maar nieuwere technieken om miRNA detectie te versnellen lijken veelbelovend. Liu et al. hebben bijvoorbeeld onlangs een biosensor ontwikkeld die direct miRNAs kan detecteren door middel van lichtfase veranderingen tussen complementaire DNA probes en doelwit miRNA. Op deze manier duurt de detectie van miRNAs in urine ongeveer 15 minuten[84]. Dit is slechts een voorbeeld van de vele innovatieve ontwikkelingen om miRNA detectie te versnellen en optimaliseren. Met de opmars van machine preservatie om de kwaliteit van het transplantaat te optimaliseren, wordt vroeg-detectie van galwegschade met behulp van versnelde miRNA metingen in recirculerende perfusaten extra interessant.

Conclusies en toekomstperspectieven

De studies beschreven in dit proefschrift dragen bij aan een betere evaluatie van de kwaliteit van het transplantaat en de prestatie van potentiële biomarkers om accuraat het risico op complicaties na levertransplantatie te voorspellen. De introductie van machineperfusie creëert extra tijd waarin biomarkers in perfusaat gemeten kunnen worden voor de evaluatie van levers afkomstig uit kwalitatief marginale donoren. Maar

ook de productie en samenstelling van gal geven informatie over transplantaatfunctie. DCD levers lijken geen verhoogde neiging te hebben op het vormen van microtrombi gedurende transplantaat preservatie. Daarom lijkt het intra-operatief toedienen van fibrinolytica ter preventie van NAS niet gerechtvaardigd. Genetische predispositie is een extra risicofactor op het ontwikkelen van NAS in levertransplantatie patiënten die tevens PSC als onderliggend lijden hebben. Donor-ontvanger mismatch in FUT2 secretor status verhoogt het risico op vroege ontwikkeling van NAS nog sterker. Een aangepaste toewijzing van donorlevers op basis van genetische screening van donoren en PSC patiënten zou daarom kunnen bijdragen aan betere uitkomsten in deze groep. Met de toenemende hoeveelheid levers afkomstig van uitgebreide criteria donoren zijn meer objectieve en sensitieve biomarkers noodzakelijk om ernstige galwegcomplicaties als NAS te voorspellen. MiRNAs, in het bijzonder CDmiR, hebben potentie als vroege voorspellers van NAS al ten tijde van transplantaat preservatie. De toepassing van miRNAs in de klinische praktijk is echter afhankelijk van het optimaliseren en versnellen van detectie technieken. Nieuwe ontwikkelingen in dit veld lijken veelbelovend en miRNAs in biovloeistoffen kunnen een waardevolle toevoeging zijn naast de klassieke biomarkers. Met objectieve biomarkers kan de donorvoorraad en het aantal geëffectueerde DCD donoren voor levertransplantatie in de nabije toekomst hopelijk worden vergroot.

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Appendices

LIST OF PUBLICATIONS

1. **CJ Verhoeven**, WRR Farid, PE de Ruiter, BE Hansen, HP Roest, J de Jonge, J Kwekkeboom, HJ Metselaar, HW Tilanus, G Kazemier, LJW van der Laan, MicroRNA profiles in graft preservation solution are predictive of ischemic-type biliary lesions after liver transplantation, *J Hepatol 2013 Dec;59(6):1231-8*
2. **CJ Verhoeven**, WRR Farid, J de Jonge, HJ Metselaar, G Kazemier, LJW van der Laan, Biomarkers to assess graft quality during conventional and machine preservation in liver transplantation, *J Hepatol 2014 Sep;61(3):672-84*
3. **CJ Verhoeven**, HJ Metselaar, LJW van der Laan, Barking up the wrong tree: microRNAs as biomarkers for biliary complications, *Liver Transpl 2014 Jun;20(6):637-9*
4. WRR Farid, **CJ Verhoeven**, J de Jonge, HJ Metselaar, G Kazemier, LJW van der Laan, The ins and outs of microRNAs as biomarkers in liver disease and transplantation, *Transpl Int 2014 Dec;27(12):1222-32*
5. HP Roest, **CJ Verhoeven**, LJW van der Laan, MicroRNAs in bile vesicles: finding a trade-off for biomarker discovery, *Hepatology 2015 Mar;61(3):1094-5*
6. P Dutkowski, WG Polak, P Muiesan, A Schlegel, **CJ Verhoeven**, I Scalera, ML DeOliveira, P Kron, PA Clavien, First comparison of hypothermic oxygenated perfusion (HOPE) vs. static cold storage of human DCD liver transplants – an international matched case analysis, *Ann Surg 2015 Nov;262(5):764-70*
7. **CJ Verhoeven**, WRR Farid, HP Roest, V Ramakrishnaiah, PE de Ruiter, J de Jonge, J Kwekkeboom, HJ Metselaar, HW Tilanus, G Kazemier, JNM IJzermans, LJW van der Laan, Polarized release of hepatic microRNAs into bile and serum in response to cellular injury and impaired liver function, *Liver Int 2016 Jun;36(6):883-92*
8. **CJ Verhoeven**, TC Simon, J de Jonge, M Doukas, K Biermann, HJ Metselaar, JNM IJzermans, WG Polak, Liver grafts procured from donors after circulatory death have no increased risk of microthrombi formation, *Accepted for publication in Liver Transplantation*
9. HP Roest, **CJ Verhoeven**, JE de Haan, J de Jonge, JNM IJzermans, LJW van der Laan, Improving accuracy of urinary microRNA quantification in heparinized patients by heparinase I digestion, *Accepted for publication in the Journal of Molecular Diagnostics*
10. **CJ Verhoeven**, LJW van der Laan, J de Jonge, HJ Metselaar, Biomarkers to monitor graft function following liver transplantation, *Accepted for publication in 'Biomarkers in Disease: Methods, Discoveries and Applications', 2017*

PhD PORTFOLIO

Name PhD student	Cornelia Johanna Verhoeven
Erasmus MC departments	Surgery, Gastroenterology & Hepatology
PhD period	September 2012 – September 2014
Promotors	Prof. dr. J.N.M. IJzermans, Prof. dr. H.J. Metselaar

Academic Education

2007-2012	Doctorate in Medicine, Erasmus MC, Rotterdam, the Netherlands
2009-2012	Research Master Clinical Research, Netherlands Institute for Health Sciences (NIHES), Erasmus MC, Rotterdam, the Netherlands *Including a summer programme at Harvard School of Public Health, Harvard University, Boston Massachusetts, USA.*
2005-2007	Dutch Law, University of Tilburg, Tilburg, the Netherlands

Activities

Courses

2013	Laboratory Animal Science (art. 9)
2013	Basic course on regulations in clinical research ('BROK')
2012	Course on basic didactics

International oral presentations

2015	21st congress of the international liver transplantation society (ILTS), Chicago, USA (two oral presentations)
2015	1st joint congress of the Dutch and British Transplantation Society (NTV/BTS), Bournemouth, UK
2014	World Transplant congress (WTC), San Francisco, USA
2014	20th congress of the ILTS, London, UK (two oral presentations)
2013	17th congress of the European Society for Organ Transplantation (ESOT) Vienna, Austria
2013	4th International conference on transplantomics and biomarkers, Cambridge, UK
2012	18th congress of the ILTS, San Francisco, USA
2011	15th congress of the ESOT, Glasgow, UK
2011	17th congress of the ILTS congress, Valencia, Spain

National oral presentations

2014	Chirurgendagen, Nederlandse vereniging voor Heelkunde (NVvH), Veldhoven
2014	Bootcongres NTV, Leiden (three oral presentations)
2014	Voorjaarsvergadering Nederlandse Vereniging voor Gastro-Enterologie (NVGE), Veldhoven
2014	Molecular Medicine Day Erasmus MC, Rotterdam
2013	Dutch liver retreat, NVH, Spier
2013	Voorjaarsvergadering NVGE, Veldhoven
2013	Bootcongres NTV, Nijmegen
2012	Dutch liver retreat, NVH, Spier
2012	Bootcongres NTV, Maastricht
2012	Voorjaarsvergadering NVGE, Veldhoven, Nederland
2011	24e Symposium Experimenteel Onderzoek Heelkundige Specialismen (SEOHS), Amsterdam

National conferences, participation

2015	Chirurgendagen, Nederlandse vereniging voor Heelkunde (NVvH), Veldhoven
2013	Chirurgendagen, Nederlandse vereniging voor Heelkunde (NVvH), Veldhoven
2012	25e Symposium Experimenteel Onderzoek Heelkundige Specialismen (SEOHS), Amsterdam

Teaching activities

2014	Supervisor Clinical review writing in transplantation, Medicine, Erasmus MC
2013	Supervisor Clinical review writing in transplantation, Medicine, Erasmus MC
2013	Lecturer, gastroenterology- and hepatology minor, Medicine, Erasmus MC
2013	Internship supervisor of a third year laboratory student, Erasmus MC
2012	Research supervisor of a minor student in Medicine, Erasmus MC

Scientific awards and grants

2015	Travel grant (€1000) KNAW Van Walree beurs
	Travel grant (€500), Erasmus Trustfonds
	Travel grant (€500) Gerrit Jan Mulder stichtin.
	Travel grant (€500), NVGE
2014	Travel Scholarship ($10.000), ILTS
	Rising star award ($1500), ILTS
	Novartis Transplantation Award (€1500), NTV
2013	Travel grant (€500), Erasmus Trustfonds
	Genzyme Speakers award, NTV
2012	Best research idea (€3500), NVH
	Best Master Thesis award (€500), NIHES
	Young Investigator's Award ($1000), ILTS
2011	Young Investigator's Award ($1000), ILTS

Other academic activities

2014-current	Member of the Basic Science Committee of the ILTS
2012-current	Invited reviewer for scientific journals (Liver Transplantation, Liver International, Transplant International)
2011-2014	Student-member of the committee of (bio)medical sciences of the Royal Dutch Academy of Sciences (KNAW)

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hebben we het onderzoek afgerond, ik ben trots dat ik je paranimf mocht zijn! Veel succes met je carrière als SEH-arts.

During my PhD, I was given the opportunity to perform research at the laboratory for hepatobiliary surgery in Zürich. **Highly learned mr. Clavien** and **highly learned mr. Dutkowski**, thank you both for your hospitality and introducing me into the world of hypothermic oxygenated machine perfusion. I had a lovely time! **Very learned mrs. Schlegel,** dear Andrea, without your efforts, my short time in Zürich would probably have been less efficient. Thanks for all the late night dinners, I still quietly laugh when I think about 'there's a donor, I need to transplant' as a replacement for 'there's a fracture, I need to fix it'.

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En dan nu: BUBBELS!!!

CURRICULUM VITAE

Cornelia Johanna (given name Renée) Verhoeven was born on April 21st 1987 in Tilburg, The Netherlands. After graduating from secondary school in 2005, she studied Dutch law at Tilburg University. In 2007 she also started to study medicine at the Erasmus University Medical Center, Rotterdam, The Netherlands. During her study in 2009, she qualified for participating in a Research Master Programme of The Netherlands Institute for Health Sciences, for which she attended the Summer Programme at Harvard School of Public Health in Boston, United States of America in 2011.

After completing her Research Master in 2012, she was allowed to extend her research into the current PhD project on biomarkers in liver transplantation at the department of Surgery (promotor: Prof.dr. J.N.M. IJzermans) and the department of Gastroenterology and Hepatology (promotor: Prof.dr. H.J. Metselaar). In 2014 she performed research at the laboratory of Hepatobiliary and Transplantation surgery at the UniversitätsSpital Zürich, Switzerland (supervisors: Prof.dr. P.A. Clavien and Prof.dr. P. Dutkowski). As member of the committee of (bio)medical sciences of the Royal Dutch Academy of Sciences (KNAW), she was responsible for accreditation of Research Master programmes in the Netherlands from 2011 through 2015. Currently, she holds a position as member of the basic science committee of the International Liver Transplantation Society.

In August 2016, while finishing her PhD thesis, she obtained her medical doctors' degree at the Eramus University Medical Center. She will continue her scientific and clinical career as a post-doctorate researcher in Head and Neck oncology and as resident in Ear-, Nose-, and Throat Surgery at the University Medical Center in Groningen, The Netherlands, under supervision of Prof.dr. B.F.A.M. van der Laan.