Biliary strictures and Liver Transplantation

Clinical and Biomedical Aspects

Kerem Sebib Korkmaz

Colophon

ISBN: 978-90-9027993-0

Layout: Jetske Voorneveld
Cover design: Philip Voorneveld and Jetske Voorneveld

Biliary strictures and Liver Transplantation

Clinical and Biomedical Aspects

Proefschrift

Ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op donderdag 6 februari 2014 klokke 13:45 uur door

Kerem Sebib Korkmaz

Geboren te Den Haag in 1985

Promotiecommissie

Promotor:
Prof. Dr. B. van Hoek

Co-Promotor:
Dr. Ir. H. W. Verspaget

Leden:
Prof. Dr. H. Metselaar
Prof. Dr. U. H. W. Beuers
Prof. Dr. R. J. Porte
Prof. Dr. J. W. de Fijter
Dr. A. G. Baranski
Mw. Dr. M. J. Coenraad

Financial support by Falk B.V. for the studies described in this thesis is gratefully acknowledged. Financial support by Falk B.V., Novo Nordisk B.V., Janssen B.V., Olympus B.V., Roche B.V., Zambon B.V., Tramedico B.V. and the Dutch Society for Hepatology (NVH) for the publication of this thesis is gratefully acknowledged.

Content

Chapter 1

Introduction

Introduction

Liver Transplantation

Orthotopic Liver transplantation (OLT) has evolved into a routine treatment with excellent short and long-term patient survival for various end-stage liver diseases.[1-3] Several improvements, including those in surgical technique and immunosuppression, have contributed to improved survival.[4,5] Possibly, a reduction in infectious complications after OLT may have contributed to this improved patient survival, but this is not well known.[6-9] Improved patient survival may also be attributed to better donor management, organ preservation, as well as intensive care treatment protocols.[10-13] Recently, it has been shown that, next to the antibiotic regimen, donor and recipient genetic polymorphisms in innate immunity contribute to the risk of infection after OLT.[14] In addition, graft loss due to rejection has virtually disappeared since the introduction of tacrolimus.[15,16]

The success of treating patients with end-stage liver disease with liver transplantation has led to an increased demand for donor livers while the number of suitable grafts has remained static over the last decades and this resulted in longer waiting lists.[17]
Many centers have expanded their criteria, in consensus, for potential donor livers. These include extended criteria donor livers and livers from donation after cardiac death (DCD).[18-20] According to the Maastricht criteria a no touch period is warranted in DCD donors to ensure that cardiac arrest had become irreversible as opposed to donors from donation after brain death (DBD), where the circulation in the donor is still intact.[21-23] DCD donors therefore have an additional and inevitable donor warm ischemia time (DWIT), which is defined as the time between circulatory arrest and cold flush in the donor.[24] In addition, a short episode of hypotension between ventilation switch-off and cardiac arrest precedes DWIT. This additional period in DCD of less and no perfusion respectively, and warm ischemia precedes the cold ischemia time (CIT) which starts with cold flush in the donor and persists during transportation. CIT ends at the time of removal of the donor liver from ice at which the recipient warm ischemia time (RWIT)

starts. RWIT ends at reperfusion of the donor liver in the recipient. Unlike DWIT in DCD, the CIT and RWIT are also present in donation after brain death (DBD). Despite donor selection and reduction of ischemia times, livers from DCD donors are more prone to develop severe complications such as primary nonfunction (PNF), delayed graft function and, especially, biliary nonanastomotic strictures (NAS).[25-31]
In 2001, a national protocol in the Netherlands has been introduced for multi-organ donation from DCD donors to fulfill the increasing demand and compensate for the decreasing DBD donation. As to date, approximately 20% to 30% of all OLTs in our country are performed using livers from DCD donors.[32] NAS can occur in up to 35% of grafts using DCD donors and are considered a significant source of morbidity.[33] The exact pathogenesis of the development of NAS remains unclear but several theories exist.[34]

Biliary complications

Biliary complications can occur at any time after OLT. Biliary complications include anastomosis leakage leading to bilomas and stricture formation.[35] Biliary strictures can be divided into two different entities: anastomotic strictures (AS) occur at the surgical notch of the bile duct anastomosis.[36,37] Local inflammation may lead to scarring which in turn leads to narrowing of the anastomosis. NAS are considered as strictures or irregularities occurring at least 1 cm above the anastomosis.[35,38,39] NAS form a heterogeneous group with considerable variation and NAS incidence rates may vary widely, perhaps due to lack of a clear definition. In addition, time of presentation may also vary greatly.[40] NAS may occur early after OLT, i.e. within the first twelve months, or late, even after several years. One study showed that NAS occurring early after OLT (< 1 year) was associated with preservation related factors, such as cold ischemia time (CIT) and WIT as opposed to late presenting NAS, which was more related to the indication for which OLT was performed, especially primary sclerosing cholangitis (PSC).[40] In OLT for PSC, one of the difficulties is that to date there are no diagnostic modalities to distinguish recurrent PSC from NAS related to OLT.
Grafts from DCD are known to develop more NAS than grafts from DBD.[41] NAS – also known as ischemic-type biliary lesions (ITBL) – are associated

with an increased risk of bacterial cholangitis, frequent admissions to the hospital, endoscopic treatment and retransplantation.[42] NAS are most likely the result of a complex mechanism involving ischemic, immunologic and toxic processes which all affect the biliary tree or its vascular system.[43-45] The microvascular supply of the biliary tree, the peri-biliary plexus, stems from the hepatic artery branches and flows into the hepatic sinusoids. A decreased blood flow in the peri-biliary plexus after orthotopic liver transplantation may be involved in the development of NAS.[46,47] This might be the result of microthrombi that develop during the DWIT, CIT and RWIT.[48,49] The most frequently used preservation fluid, University of Wisconsin (UW) solution and Histidine-tryptophan-ketoglutarate (HTK) solution, which was until recently also used in the Netherlands for liver preservation, may be far from perfect.[50] Several attempts are being made to improve preservation and to reduce ischemia-reperfusion injury (IRI) of liver grafts, including machine liver perfusion but also "flush" protocols in which fibrinolytic agents are being used to dissolve microthrombi in the microvascular system of the biliary tree during procurement of the donor liver.[49,51]

Apart from IRI and possible microthrombi, there are several other theories on the development of biliary strictures. One theory encompasses an altered composition of bile after IRI, which occurs after OLT.[44] In a porcine model of DCD liver transplantation, a warm ischemia period of 30 minutes and longer produced bile with a significantly higher bile salt-to-phospholipids ratio after transplantation than livers from donors with 0 or 15 minutes warm ischemia in the donor.[45] Secretion of bile salts might be impaired due to an imbalance between hepatobiliary transporter proteins which secrete bile salts and multidrug-resistance protein 3 (MDR3) which secretes less phospholipids resulting in an increase of the bile salt/phospholipids ratio. Toxic bile salts might lead to injury of the biliary epithelium, especially in case of a reduced 'bicarbonate umbrella'.[52,53] In the past, ABO-incompatibility in liver transplantation was also associated with the development of NAS, indicating that immunological processes are involved in the development of NAS.[54] In ABO-incompatibility this could partially be explained by the fact that the ABH-antigen is consistently present on biliary epithelial cells –in contrast to hepatocytes– which in turn can initiate an immune reaction that causes local

damage to the biliary tissue.[55]
One theory that supports the involvement of the immune system is the loss-of-function mutation of chemokine receptor 5 delta 32 (CCR5Δ32) which has been associated with the development of NAS in recent literature.[43] CCR5Δ32 is a protein, which is located on the surface of macrophages, CD4+, CD8+ but also natural killer cells, and its main function involves attracting immune cells to damaged tissue sites. Impaired functioning of the CCR5Δ32 might lead to less attraction of immune cells to the biliary tract and subsequent healing of injured tissue. However, the contribution of CCR5Δ32 in the development of NAS has been questioned and needs replication in larger cohorts to determine its exact role in the development of NAS.[56]

Activated immune cells also release specific members of the tissue remodeling matrix metalloproteinases (MMP). MMPs have been associated with numerous conditions such as IBD and cancer and there is strong evidence suggesting involvement in IRI as well.[57-59]

Matrix metalloproteinases

Jerome Gross and Charles Lapierre made the first discovery of MMPs in 1962 and the first MMPs discovered in human neutrophilic granulocytes, was in 1968.[60,61] MMPs comprise a large family of proteolytic enzymes that are important in physiological and disease-related extracellular matrix (ECM) remodelling processes.[62] MMPs consist of a signalpeptide or prepeptide, a pro-peptide region, a catalytic domain with a zinc binding region and a hemopexine domain which is connected with a so called hinge region with varying length to the catalytic domain.[63]

The signalpeptide or pre-peptide consists of a sequence of 17-20 hydrophobic amino acids. The hydrophobic portion of the signal peptide is responsible for the secretion of MMPs in the endoplasmic reticulum, from which they can be released into the extracellular space.[64] The propeptide, contains 80 amino acids with an N-terminal hydrophobic rest. Near the C-terminal end of the propeptide is a highly conserved sequence region around cysteine: PRCGVPD.[65,66] The catalytic domain consists of 160 - 170 amino acid

residues and contains binding sites for calcium and zinc ions. The catalytic domain is connected to the hemopexin domain by a so-called hinge region. This connection region is important for the substrate specificity of MMPs which can bind to the substrate itself or establish the binding orientation of the catalytic and hemopexin-domain.[67] The hemopexin-like domain comprises about 200 amino acids that contain four so-called repeats, each with about 48 amino acids. The hemopexin domain seems to be important for substrate specificity of the MMPs and contributes to binding of the substrates which makes it a key player in activating and inhibiting MMPs.[68] MMPs hydrolyze most components of the ECM and play a central role in many biological processes such as normal tissue remodeling, embryogenesis, wound healing and angiogenesis.[69-71] Currently about 26 MMPs have been identified, and most are multidomain zinc endopeptidases. According to their substrate the members of the family are divided in collagenases, stromelysines, gelatinases, membrane-type-(MT)MMPs and others.[72] Stromelysins (MMP-3 and MMP-11 or stromelysin-1 and -2) and matrilysin exhibit the ability to degrade a broad range of substrates, including proteoglycans, fibronectin, laminin, gelatin, collagens-III, -IV, and -V, and elastin.[73] The membrane-type MMPs (MT-MMPs) differ from other MMPs in having a C-terminal transmembrane domain (MT1-, MT2-, MT3- and MT5-MMP) or are anchored to glycosyl phosphatidyl inositol (MT4-MMP, MT6-MMP), which localizes these enzymes to the surface of cells. MT-MMPs have a broad substrate specificity and can degrade interstitial collagens III and I, as well as fibronectin, vitronectin, and cartilage proteoglycans.[74,75] *(Table 1)* In healthy tissue a strict regulation of MMPs is critical in order to maintain proper ECM homeostasis. Among other levels of regulation, MMPs are precisely regulated by their main endogenous protein inhibitors (TIMPs).[76] Disruption of this balance results in serious diseases such as fibrosis, arthritis, and tumour growth. Certain MMPs such as gelatinases (MMP-2, MMP-9) have specific characteristics such as digesting components of connective tissue matrix and type IV collagen.[77] MMPs can no longer be solely thought of as ECM destructionists, but as part of a delicate equilibrium system through which epithelial and immune cells interact with the stroma.

Table 1. List of MMPs and their substrate specificity.[74,75]

Enzyme		Pro-MMP	Substrate
Collagenase			
	Collagenase 1	MMP-1	Type I, II and III collagen
	Collagenase 2	MMP-8	Type I, II and III collagen
	Collagenase 3	MMP-13	Type I collagen
	Collagenase 4	MMP-18	Not found in humans
Gelatinases			
	Gelatinase A	***MMP-2***	***Type IV, V, VII, IX and X collagen, gelatins***
	Gelatinase B	***MMP-9***	***Type IV, V, XI, and XVI collagen, laminin, elastin, decorin***
Stromelysin			
	Stromelysin 1	MMP-3	Basement membrane glycoproteins, fibronectin, E-cadherine, activates plasminogen and MMP-2
	Stromelysin 2	MMP-10	Basement membrane glycoproteins
	Stromelysin 3	MMP-11	Basement membrane glycoproteins
Matrilysin			
	Matrilysin 1	MMP-7	Fibronectin, elastin
	Matrilysin 2	MMP-26	Type IV collagen, fibronectin, fibrinogen
Membrane-type MMPs (MT-MMP)(A)			
	MT1-MMP	MMP-14	Type I, II and III collagen, activates MMP-2 and MMP-13
	MT2-MMP	MMP-15	Fibronectin, laminin, activates MMP-2
	MT3-MMP	MMP-16	Type III collagen, fibronectin
	MT5-MMP	MMP-24	Proteoglycans
	MT4-MMP	MMP-17	Fibrinogen, fibrin
	MT6-MMP	MMP-25	Type IV collagen, fibronectin, fibrin, casein
Others			
	Macrophage elastase	MMP-12	Type IV collagen, elastin, gelatins, fibronectin
	-	MMP-19	Type IV collagen, fibronectin
	Enamelysin	MMP-20	Amelogenin, aggrecan
	-	MMP-21	Type IV, V, VII, IX and X collagen
	CA-MMP	MMP-23	Unknown
	-	MMP-27	Unknown
	Epilysin	MMP-28	Unknown

MMP-2 and MMP-9

The gelatinases A (MMP-2 or 72-kDa type IV collagenase), and B (MMP-9, 92-kDa type IV collagenase) can degrade denatured interstitial collagens (gelatins), type V collagen, and intact type IV collagen, which is an important component of basement membranes.[78] The baseline structure of MMP-2 is homologous to MMP-9 and is constitutively expressed in almost all human tissues but mainly by hepatic stellate cells (HSC), endothelial and epithelial cells. MMP-2 is secreted in its zymogen form (pro-MMP-2) and is tightly regulated by complex signaling through TIMP-2, TIMP-3 and TIMP-4 which all display relevant affinity for the MMP-2 and their adequate secretion is

required for a balanced MMP-2/TIMP ratio and MT1-MMP.[79] The membrane bound activation of pro-MMP-2 ensures that proteolytic activity is localized to specific regions of the cell-surface. MMP-2 cleaves a vast repertoire of substrates, including cytokines, growth factors, and receptors or binding factors but is primarily known for its cleaving properties of gelatin, and types IV, V, VII, IX and X collagen which makes MMP-2 a key player in degrading ECM.[80] Within the catalytic domain of MMP-2 and MMP-9 a threefold sequence consisting of 58 amino acids exists of fibronectin type II that can bind to gelatin and collagen, which makes these MMPs capable of breaking down ECM substrates.[81]

MMP-9 is secreted in monomeric form as zymogen (pro-MMP-9) predominantly from neutrophils and macrophages but the main source in the liver is thought to be the Kupffer cells and activation of pro-MMP-9 is amongst others mediated by the plasminogen activator/plasmin (PA/plasmin) system.[82,83] Its expression level and activity is regulated through TIMP-1 and TIMP-3 but in vivo experiments have shown that MMP-9 activity can also be mediated by trypsin, chemotrypsin, cathepsin B and a variety of cytokines and growth factors including interleukins (IL-1), interferons, epidermal growth factor (EGF), nerve growth factor (NGF), basic fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), platelet derived growth (PDGF), tumor necrosis factor-alpha (TNF-α), transforming growth factor (TGF-β) and the extracellular matrix metalloproteinase inducer (EMMPRIN).[84] The active form of MMP-9 is able to digest decorin, elastin, fibrillin, laminin, gelatin (denatured collagen), and types IV, V, XI and XVI collagen and also activates growth factors like pro-TGF-β and pro-TNF-.[85]

Genetic variants and its functional implications

MMP-2 and MMP-9 have been associated with numerous diseases such as cancer, autoimmune disorders, coronary diseases and diseases, which involve degradation such as Alzheimer's disease.[86] The majority of MMP-2 studies have focused on demonstrating an essential role in promoting cell invasiveness during tumor angiogenesis, arthritis, and atherogenesis, as well as tumor metastasis where levels of MMP-2 expression can be correlated

with tumor grade.[87-89] Studies have shown that natural occurring variants of MMP-2 affect expression and thus might impact progression of pathophysiological processes.[90] Single nucleotide polymorphisms (SNP) in the promoter region of the MMP-2 gene have shown to affect gene regulation. The C -> T transition at –1306 of the MMP-2 promoter gene interrupts a Sp1 binding site.[91] Sp1 is a multifunctional protein that can directly interact with the basal transcription complex or alternatively function as a more general transcription factor and play an important role in directing tissue-specific expression.[92] A variant that abolishes Sp1 binding, such as the MMP-2 –1306 C/T polymorphism, has the potential to affect the level and specificity of gene transcription. One study showed that the reporter gene expression of the T allele in at -1306 was 0.71 lower than the C allele indicating less transcriptional activity.[91] Several other SNPs in the promoter region have been discovered such as the –1575 G/A, –790 C/T and –735 C/T, but these are in complete linkage equilibrium with the –1306 polymorphism.[93,94]
MMP-9 expression is very low in most tissues but increases in response to local secretion of inflammatory cytokines and growth factors, most notably IL-1 and TNF-α, two highly potent inducers of MMP-9 gene activation.[95] MMP-9 has two promoter gene polymorphisms that have proven to be functionally relevant, namely a (CA)n microsatellite polymorphism at position -90 and a SNP polymorphism at position –1562.[96] Other nonsynonomous SNPs have been described but these show no functionally relevant changes in levels or activity.[97] With the –1562C/T polymorphism in the MMP-9 promoter there is a cytosine to thymidine transition at the polymorphic position –1562. Several studies have found that this MMP-9 C->T transition polymorphism exerts an allelic effect with higher transcriptional activity and higher functional MMP-9 serum activity and it has been associated with a higher risk of cardiovascular complications in HIV patients and severe coronary atherosclerosis.[97,98]

Matrix metalloproteinases in liver diseases

MMPs have also been implicated in numerous conditions involving the liver. For example, plasma MMP-2 levels were significantly higher in patients with hepatocellular carcinoma (HCC) and chronic liver diseases as compared to healthy controls and they were more elevated if the Child-Pugh class was

higher.[99] In contrast, MMP-2 is very low in normal liver tissue.[100,101] These findings imply that MMP-2 may be actively involved in both development and progression of various liver diseases or that breakdown and excretion of MMP-2 is impaired in this situation. MMPs are also involved in OLT and studies have shown changed hepatic expression of various members of the MMP/TIMP family during cold preservation injury in both humans and rats.[59] Hepatic ischemic injury leads to swelling of endothelial cells and Kupffer cell activation which in turns leads to secretion of particularly MMP-9 and to a lesser extent MMP-2 via HSC.[102] During OLT MMP-2 plasma levels gradually decrease but MMP-9 plasma levels increase further from the anhepatic phase on until 30 minutes after reperfusion, and are related to the degree of tissue injury and thus seem to be involved in early IRI.[103] Furthermore, after OLT, neutrophil infiltration and matrix degradation was observed, which is accompanied by an increase of MMP-9 in patients with rejection in the first week after OLT, while neither MMP2 level nor MMP9 level are related to peak ALT in the first week after OLT.[103,104] Inhibitors of MMP have been studied in rat models mimicking IRI showing significant improvement in liver function and liver injury.[105,106] In addition, several other studies have studied the relationship between MMP-2 and MMP-9 and the development of acute cellular rejection after OLT suggesting that MMPs might also be involved in immunological processes after OLT.[107, 108]

Outline of the studies described in this thesis

Liver transplantation for end-stage liver disease has become a standard treatment for end-stage liver diseases with excellent long-term results. However, due to scarcity of DBD donor livers many centers have expanded their donor pool by using donor livers with "extended criteria" and DCD livers. **Chapter 2** elaborates on 20 years of OLT for chronic liver disease at the Leiden University Medical Center and describes long-term outcome after OLT using livers from both DBD and DCD. The analysis focuses on differences in patient survival and graft survival in the first and second decade, and after DBD- versus DCD-OLT. Causes of recipient mortality and changes in these parameters were studied. The evaluation not only includes indications for OLT but also parameters of the surgical technique such as intraoperative blood loss.

Influence of other aspects such as ischemia-reperfusion variables for both DBD and DCD donors is described. In addition indications for re-OLT were assessed for both decades.
Biliary complications, especially NAS, frequently occur after OLT and development is often insidious. NAS development after OLT seems predominantly the result of IRI and probably subsequent collagen deposition surrounding the bile ducts. One of the commonly accepted markers of IRI is an increased serum level (peak) of alanine aminotransferase (ALT), which occurs within the first week post OLT. This elevation is more marked for DCD-OLT than for DBD-OLT and the evaluation of the relationship between peak ALT and the development of NAS after OLT is described in **chapter 3**. When there is a clinical suspicion of NAS after OLT, i.e., due to jaundice, fever or itching, invasive procedures such as endoscopic retrograde cholangiography (ERC) or percutaneous transhepatic cholangiography (PTC) or magnetic resonance cholangiography (MRC) are the choices of modality for confirmation. However, these are invasive and expensive and may have side effects. Most centers routinely assess patients in the outpatient clinic with serum liver enzymes and abdominal ultrasound (US). The study on the predictive value of US and serum liver enzymes assessments for detecting the development of NAS post OLT is described in **chapter 4**.

MMP-2 and MMP-9 are the most potent degrading enzymes of type IV collagen; the main component of the extracellular matrix (ECM). Functional single nucleotide polymorphisms (SNPs) of MMP-2 and MMP-9, which affect their activity, may therefore play a role in development of biliary strictures and thereby potentially influence the incidence of NAS. **Chapter 5** reports on the relationship between gene promoter polymorphisms of MMP-2 and MMP-9 in both recipient and donor and the incidence of NAS after OLT. In primary sclerosing cholangitis (PSC) the hallmark of the disease, like in NAS, is stricture formation of the bile ducts. OLT is often indicated in PSC, which is considered a chronic inflammatory disease of the bile ducts. The results of a study on the relationship between gene promoter polymorphisms of MMP-2 and MMP-9 and disease severity in patents with PSC, as defined by patient mortality or OLT, are described in **chapter 6**.
Gene polymorphisms might have the potential to be used as a marker to

evaluate chimerism after OLT. Chimerism in transplantation refers to the coexistence of two different populations of (genetically) distinct cells that originate from both donor and recipient. **Chapter 7** describes the use of MMP-2 and MMP-9 donor/recipient gene promoter profiles in liver biopsies and in blood after OLT as a marker for chimerism. These findings were related to clinical outcomes such as acute cellular rejection.
In **chapter 8** the results of the different studies described in this thesis are summarized and discussed.

References

1. Jain A, Reyes J, Kashyap R, Dodson SF, Demetris AJ, Ruppert K, et al. Long-term sur vival after liver transplantation in 4,000 consecutive patients at a single center. Ann Surg 2000;232(4):490-500.

2. Burroughs AK, Sabin CA, Rolles K, Delvart V, Karam V, Buckels J, et al. 3-month and 12-month mortality after first liver transplant in adults in Europe: predictive models for outcome. Lancet 2006 21;367(9506):225-232.

3. Busuttil RW, Farmer DG, Yersiz H, Hiatt JR, McDiarmid SV, Goldstein LI, et al. Analysis of long-term outcomes of 3200 liver transplantations over two decades: a single-center experience. Ann Surg 2005;241(6):905-916.

4. Jain A, DiMartini A, Kashyap R, Youk A, Rohal S, Fung J. Long-term follow-up after liver transplantation for alcoholic liver disease under tacrolimus. Transplantation 2000 15;70(9):1335-1342.

5. Dawson S, III, Imagawa DK, Johnson C, Cecka M, Terasaki PI, Shackleton CR, et al. UCLA liver transplantation: analysis of immunological factors affecting outcome. Artif Organs 1996;20(10):1063-1072.

6. Ikegami T, Shirabe K, Yoshiya S, Yoshizumi T, Ninomiya M, Uchiyama H, et al. Bacterial sepsis after living donor liver transplantation: the impact of early enteral nutrition. J Am Coll Surg 2012;214(3):288-295.

7. Sun HY, Cacciarelli TV, Singh N. Identifying a targeted population at high risk for infections after liver transplantation in the MELD era. Clin Transplant 2011;25(3):420-425.

8. van Hoek B, de Rooij BJ, Verspaget HW. Risk factors for infection after liver transplantation. Best Pract Res Clin Gastroenterol 2012;26(1):61-72.

9. Patel G, Huprikar S. Infectious complications after orthotopic liver transplantation. Semin Respir Crit Care Med 2012;33(1):111-124.

10. Geissler EK, Schlitt HJ. Immunosuppression for liver transplantation. Gut 2009;58(3):452-463.

11. Schmeding M, Sauer IM, Kiessling A, Pratschke J, Neuhaus R, Neuhaus P, et al. Influence of basiliximab induction therapy on long term outcome after liver transplantation, a prospectively randomised trial. Ann Transplant 2007;12(3):15-21.

12. Goralczyk AD, Hauke N, Bari N, Tsui TY, Lorf T, Obed A. Interleukin 2 receptor antagonists for liver transplant recipients: a systematic review and meta-analysis of controlled studies. Hepatology 2011;54(2):541-554.

13. Schmeding M, Kiessling A, Neuhaus R, Heidenhain C, Bahra M, Neuhaus P, et al. My cophenolate mofetil monotherapy in liver transplantation: 5-year follow-up of a prospective randomized trial. Transplantation 2011 27;92(8):923-929.

14. de Rooij BJ, van Hoek B., ten Hove WR, Roos A, Bouwman LH, Schaapherder AF, et al. Lectin complement pathway gene profile of donor and recipient determine the risk of bacterial infections after orthotopic liver transplantation. Hepatology 2010;52(3):1100-1110.

15. Dumortier J, Guillaud O, Boillot O. Conversion from twice-daily to once-daily tacrolimus in long-term stable liver transplant recipients: A single centre experience on 394 patients. Liver Transpl 2013 8.

16. Florman S. Tacrolimus once-daily formulation in the prophylaxis of transplant rejection in renal or liver allograft recipients: a viewpoint by Sander Florman. Drugs 2007;67(13):1944.

17. Mullhaupt B, Dimitroulis D, Gerlach JT, Clavien PA. Hot topics in liver transplantation: organ allocation--extended criteria donor--living donor liver transplantation. J Hepatol 2008;48 Suppl 1:S58-S67.

18. Dubbeld J, van Hoek B., Ringers J. Use of a liver from donor after cardiac death: is it ap propriate for the sick or the stable? Curr Opin Organ Transplant 2011;16(2):239-242.

19. Pine JK, Aldouri A, Young AL, Davies MH, Attia M, Toogood GJ, et al. Liver transplantation following donation after cardiac death: an analysis using matched pairs. Liver Transpl 2009;15(9):1072-1082.

20. Mittler J, Pascher A, Neuhaus P, Pratschke J. The utility of extended criteria donor organs in severely ill liver transplant recipients. Transplantation 2008 15;86(7):895-896.

21. Daemen JW, Kootstra G, Wijnen RM, Yin M, Heineman E. Nonheart-beating donors: the Maastricht experience. Clin Transpl 1994;303-316.

22. Kootstra G, Daemen JH, Oomen AP. Categories of non-heart-beating donors. Transplant Proc 1995;27(5):2893-2894.

23. Fondevila C, Hessheimer AJ, Flores E, Ruiz A, Mestres N, Calatayud D, et al. Applicability and results of Maastricht type 2 donation after cardiac death liver transplantation. Am J Transplant 2012;12(1):162-170.

24. Kootstra G, Kievit JK, Heineman E. The non heart-beating donor. Br Med Bull 1997;53(4):844-853.

25. Foley DP, Fernandez LA, Leverson G, Anderson M, Mezrich J, Sollinger HW, et al. Biliary complications after liver transplantation from donation after cardiac death donors: an analysis of risk factors and long-term outcomes from a single center. Ann Surg 2011;253(4):817-825.

26. Mathur AK, Heimbach J, Steffick DE, Sonnenday CJ, Goodrich NP, Merion RM. Donation after cardiac death liver transplantation: predictors of outcome. Am J Transplant 2010;10(11):2512-2519.

27. Foley DP, Fernandez LA, Leverson G, Chin LT, Krieger N, Cooper JT, et al. Donation after cardiac death: the University of Wisconsin experience with liver transplantation. Ann Surg 2005;242(5):724-731.

28. Lee KW, Simpkins CE, Montgomery RA, Locke JE, Segev DL, Maley WR. Factors affecting graft survival after liver transplantation from donation after cardiac death donors. Transplantation 2006 27;82(12):1683-1688.

29. Hong JC, Yersiz H, Kositamongkol P, Xia VW, Kaldas FM, Petrowsky H, et al. Liver transplantation using organ donation after cardiac death: a clinical predictive index for graft failure-free survival. Arch Surg 2011;146(9):1017-1023.

30. Mateo R, Cho Y, Singh G, Stapfer M, Donovan J, Kahn J, et al. Risk factors for graft survival after liver transplantation from donation after cardiac death donors: an analysis of OPTN/UNOS data. Am J Transplant 2006;6(4):791-796.

31. Selck FW, Grossman EB, Ratner LE, Renz JF. Utilization, outcomes, and retransplantation of liver allografts from donation after cardiac death: implications for further expansion of

the deceased-donor pool. Ann Surg 2008;248(4):599-607.

32. Buis CI, Hoekstra H, Verdonk RC, Porte RJ. Causes and consequences of ischemic-type biliary lesions after liver transplantation. J Hepatobiliary Pancreat Surg 2006;13(6):517-524.

33. Skaro AI, Jay CL, Baker TB, Wang E, Pasricha S, Lyuksemburg V, et al. The impact of ischemic cholangiopathy in liver transplantation using donors after cardiac death: the untold story. Surgery 2009;146(4):543-552.

34. Cursio R, Gugenheim J. Ischemia-Reperfusion Injury and Ischemic-Type Biliary Lesions following Liver Transplantation. J Transplant 2012;2012:164329.

35. Ryu CH, Lee SK. Biliary strictures after liver transplantation. Gut Liver 2011;5(2):133-142.

36. Albert JG, Filmann N, Elsner J, Moench C, Trojan J, Bojunga J, et al. Long-term follow-up of endoscopic therapy in stenosis of the bilio-biliary anastomosis associated with orthotopic liver transplantation. Liver Transpl 2013 22.

37. Verdonk RC, Buis CI, Porte RJ, van der Jagt EJ, Limburg AJ, van den Berg AP, et al. Anas tomotic biliary strictures after liver transplantation: causes and consequences. Liver Transpl 2006;12(5):726-735.

38. Nishida S, Nakamura N, Kadono J, Komokata T, Sakata R, Madariaga JR, et al. Intrahepatic biliary strictures after liver transplantation. J Hepatobiliary Pancreat Surg 2006;13(6):511-516.

39. Nakamura N, Nishida S, Neff GR, Vaidya A, Levi DM, Kato T, et al. Intrahepatic biliary strictures without hepatic artery thrombosis after liver transplantation: an analysis of 1,113 liver transplantations at a single center. Transplantation 2005 27;79(4):427-432.

40. Buis CI, Hoekstra H, Verdonk RC, Porte RJ. Causes and consequences of ischemic-type biliary lesions after liver transplantation. J Hepatobiliary Pancreat Surg 2006;13(6):517-524.

41. Chan EY, Olson LC, Kisthard JA, Perkins JD, Bakthavatsalam R, Halldorson JB, et al. Ischemic cholangiopathy following liver transplantation from donation after cardiac death donors. Liver Transpl 2008;14(5):604-610.

42. van der Hilst CS, Ijtsma AJ, Bottema JT, van Hoek B., Dubbeld J, Metselaar HJ, et al. The price of donation after cardiac death in liver transplantation: a prospective cost-effective ness study. Transpl Int 2013 11.

43. op den Dries S., Buis CI, Adelmeijer J, van der Jagt EJ, Haagsma EB, Lisman T, et al. The combination of primary sclerosing cholangitis and CCR5-Delta32 in recipients is strongly associated with the development of nonanastomotic biliary strictures after liver transplantation. Liver Int 2011;31(8):1102-1109.

44. Buis CI, Geuken E, Visser DS, Kuipers F, Haagsma EB, Verkade HJ, et al. Altered bile composition after liver transplantation is associated with the development of nonanastomotic biliary strictures. J Hepatol 2009;50(1):69-79.

45. Yska MJ, Buis CI, Monbaliu D, Schuurs TA, Gouw AS, Kahmann ON, et al. The role of bile salt toxicity in the pathogenesis of bile duct injury after non-heart-beating porcine liver transplantation. Transplantation 2008 15;85(11):1625-1631.

46. Heidenhain C, Pratschke J, Puhl G, Neumann U, Pascher A, Veltzke-Schlieker W, et al. Incidence of and risk factors for ischemic-type biliary lesions following orthotopic liver

transplantation. Transpl Int 2010;23(1):14-22.

47. Moench C, Moench K, Lohse AW, Thies J, Otto G. Prevention of ischemic-type biliary lesions by arterial back-table pressure perfusion. Liver Transpl 2003;9(3):285-289.

48. Pascher A, Neuhaus P. Bile duct complications after liver transplantation. Transpl Int 2005;18(6):627-642.

49. Hashimoto K, Eghtesad B, Gunasekaran G, Fujiki M, Uso TD, Quintini C, et al. Use of tissue plasminogen activator in liver transplantation from donation after cardiac death donors. Am J Transplant 2010;10(12):2665-2672.

50. Stewart ZA, Cameron AM, Singer AL, Montgomery RA, Segev DL. Histidine-Tryptophan-Ketoglutarate (HTK) is associated with reduced graft survival in deceased donor livers, especially those donated after cardiac death. Am J Transplant 2009;9(2):286-293.

51. Lang R, He Q, Jin ZK, Han DD, Chen DZ. Urokinase perfusion prevents intrahepatic ischemic-type biliary lesion in donor livers. World J Gastroenterol 2009 28;15(28):3538-3541.

52. Beuers U, Hohenester S, de Buy Wenniger LJ, Kremer AE, Jansen PL, Elferink RP. The biliary HCO(3)(-) umbrella: a unifying hypothesis on pathogenetic and therapeutic aspects of fibrosing cholangiopathies. Hepatology 2010;52(4):1489-1496.

53. Hohenester S, Wenniger LM, Paulusma CC, van Vliet SJ, Jefferson DM, Elferink RP, et al. A biliary HCO3- umbrella constitutes a protective mechanism against bile acid-induced injury in human cholangiocytes. Hepatology 2012;55(1):173-183.

54. Rull R, Garcia Valdecasas JC, Grande L, Fuster J, Lacy AM, Gonzalez FX, et al. Intrahepatic biliary lesions after orthotopic liver transplantation. Transpl Int 2001;14(3):129-134.

55. op den Dries S., Sutton ME, Lisman T, Porte RJ. Protection of bile ducts in liver transplantation: looking beyond ischemia. Transplantation 2011 27;92(4):373-379.

56. Heidenhain C, Puhl G, Moench C, Lautem A, Neuhaus P. Chemokine Receptor-5Delta32 Mutation is No Risk Factor for Ischemic-Type Biliary Lesion in Liver Transplantation. J Transplant 2009;2009:436515.

57. Kubben FJ, Sier CF, Meijer MJ, van den Berg M, van der Reijden JJ, Griffioen G, et al. Clinical impact of MMP and TIMP gene polymorphisms in gastric cancer. Br J Cancer 2006 18;95(6):744-751.

58. Meijer MJ, Mieremet-Ooms MA, van Hogezand RA, Lamers CB, Hommes DW, Verspaget HW. Role of matrix metalloproteinase, tissue inhibitor of metalloproteinase and tumor necrosis factor-alpha single nucleotide gene polymorphisms in inflammatory bowel disease. World J Gastroenterol 2007 7;13(21):2960-2966.

59. Upadhya AG, Harvey RP, Howard TK, Lowell JA, Shenoy S, Strasberg SM. Evidence of a role for matrix metalloproteinases in cold preservation injury of the liver in humans and in the rat. Hepatology 1997;26(4):922-928.

60. Gross J, Lapiere CM. Collagenolytic activity in amphibian tissues: a tissue culture assay. Proc Natl Acad Sci U S A 1962 15;48:1014-1022.

61. Eisen AZ, Jeffrey JJ, Gross J. Human skin collagenase. Isolation and mechanism of attack

on the collagen molecule. Biochim Biophys Acta 1968 25;151(3):637-645.

62. Shapiro SD. Matrix metalloproteinase degradation of extracellular matrix: biological consequences. Curr Opin Cell Biol 1998;10(5):602-608.

63. Hamacher S, Matern S, Roeb E. [Extracellular matrix -- from basic research to clinical significance. An overview with special consideration of matrix metalloproteinases]. Dtsch Med Wochenschr 2004 17;129(38):1976-1980.

64. Bode W. Structural basis of matrix metalloproteinase function. Biochem Soc Symp 2003;(70):1-14.

65. Springman EB, Angleton EL, Birkedal-Hansen H, Van Wart HE. Multiple modes of activation of latent human fibroblast collagenase: evidence for the role of a Cys73 active-site zinc complex in latency and a "cysteine switch" mechanism for activation. Proc Natl Acad Sci U S A 1990;87(1):364-368.

66. Van Wart HE, Birkedal-Hansen H. The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. Proc Natl Acad Sci U S A 1990;87(14):5578-5582.

67. Bode W, Fernandez-Catalan C, Tschesche H, Grams F, Nagase H, Maskos K. Structural properties of matrix metalloproteinases. Cell Mol Life Sci 1999;55(4):639-652.

68. Murphy G, Knauper V. Relating matrix metalloproteinase structure to function: why the "hemopexin" domain? Matrix Biol 1997;15(8-9):511-518.

69. Bauer EA, Stricklin GP, Jeffrey JJ, Eisen AZ. Collagenase production by human skin fibroblasts. Biochem Biophys Res Commun 1975 5;64(1):232-240.

70. Hiden U, Ghaffari-Tabrizi N, Gauster M, Tam-Amersdorfer C, Cetin I, Dieber-Rotheneder M, et al. Membrane-Type Matrix Metalloproteinase 1 (MT1-MMP) Regulates Trophoblast Functions and Is Reduced in Fetal Growth Restriction. Am J Pathol 2013 4.

71. Lafleur MA, Handsley MM, Edwards DR. Metalloproteinases and their inhibitors in angiogenesis. Expert Rev Mol Med 2003;5(23):1-39.

72. Rawlings ND, Barrett AJ, Bateman A. MEROPS: the peptidase database. Nucleic Acids Res 2010;38(Database issue):D227-D233.

73. Overall CM. Molecular determinants of metalloproteinase substrate specificity: matrix metalloproteinase substrate binding domains, modules, and exosites. Mol Biotechnol 2002;22(1):51-86.

74. Nagase H, Visse R, Murphy G. Structure and function of matrix metalloproteinases and TIMPs. Cardiovasc Res 2006 15;69(3):562-573.

75. Iyer RP, Patterson NL, Fields GB, Lindsey ML. The history of matrix metalloproteinases: milestones, myths, and misperceptions. Am J Physiol Heart Circ Physiol 2012 15;303(8):H919-H930.

76. Arthur MJ. Fibrogenesis II. Metalloproteinases and their inhibitors in liver fibrosis. Am J Physiol Gastrointest Liver Physiol 2000;279(2):G245-G249.

77. Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol 2001;17:463-516.

78. Fanjul-Fernandez M, Folgueras AR, Cabrera S, Lopez-Otin C. Matrix metalloproteinases: evolution, gene regulation and functional analysis in mouse models. Biochim Biophys Acta 2010;1803(1):3-19.

79. Woessner JF, Jr. Matrix metalloproteinase inhibition. From the Jurassic to the third millennium. Ann N Y Acad Sci 1999 30;878:388-403.

80. Woessner JF, Jr. The family of matrix metalloproteinases. Ann N Y Acad Sci 1994 6;732:11-21.

81. Westermarck J, Kahari VM. Regulation of matrix metalloproteinase expression in tumor invasion. FASEB J 1999;13(8):781-792.

82. Woessner JF, Jr. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J 1991;5(8):2145-2154.

83. Winwood PJ, Schuppan D, Iredale JP, Kawser CA, Docherty AJ, Arthur MJ. Kupffer cell-derived 95-kd type IV collagenase/gelatinase B: characterization and expression in cultured cells. Hepatology 1995;22(1):304-315.

84. Yu Q, Stamenkovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. Genes Dev 2000 15;14(2):163-176.

85. St-Pierre Y, Van TC, Esteve PO. Emerging features in the regulation of MMP-9 gene expression for the development of novel molecular targets and therapeutic strategies. Curr Drug Targets Inflamm Allergy 2003;2(3):206-215.

86. Hochstrasser T, Ehrlich D, Marksteiner J, Sperner-Unterweger B, Humpel C. Matrix metalloproteinase-2 and epidermal growth factor are decreased in platelets of Alzheimer patients. Curr Alzheimer Res 2012;9(8):982-989.

87. Niemiec J, Adamczyk A, Malecki K, Ambicka A, Rys J. Tumor grade and matrix metalloproteinase 2 expression in stromal fibroblasts help to stratify the high-risk group of patients with early breast cancer identified on the basis of st gallen recommendations. Clin Breast Cancer 2013;13(2):119-128.

88. Langers AM, Sier CF, Hawinkels LJ, Kubben FJ, van DW, van der Reijden JJ, et al. MMP-2 geno-phenotype is prognostic for colorectal cancer survival, whereas MMP-9 is not. Br J Cancer 2008 3;98(11):1820-1823.

89. Langers AM, Verspaget HW, Hawinkels LJ, Kubben FJ, van DW, van der Reijden JJ, et al. MMP-2 and MMP-9 in normal mucosa are independently associated with outcome of colorectal cancer patients. Br J Cancer 2012 24;106(9):1495-1498.

90. Niu W, Qi Y. Matrix metalloproteinase family gene polymorphisms and risk for coronary artery disease: systematic review and meta-analysis. Heart 2012;98(20):1483-1491.

91. Price SJ, Greaves DR, Watkins H. Identification of novel, functional genetic variants in the human matrix metalloproteinase-2 gene: role of Sp1 in allele-specific transcriptional regulation. J Biol Chem 2001 9;276(10):7549-7558.

92. Zhou Y, Yu C, Miao X, Tan W, Liang G, Xiong P, et al. Substantial reduction in risk of breast cancer associated with genetic polymorphisms in the promoters of the matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-2 genes. Carcinogenesis 2004;25(3):399-404.

93. Harendza S, Lovett DH, Panzer U, Lukacs Z, Kuhnl P, Stahl RA. Linked common polymorphisms in the gelatinase a promoter are associated with diminished transcriptional response to estrogen and genetic fitness. J Biol Chem 2003 6;278(23):20490-20499.

94. Miao X, Yu C, Tan W, Xiong P, Liang G, Lu W, et al. A functional polymorphism in the matrix metalloproteinase-2 gene promoter (-1306C/T) is associated with risk of development but not metastasis of gastric cardia adenocarcinoma. Cancer Res 2003 15;63(14):3987-3990.

95. Nee L, Tuite N, Ryan MP, McMorrow T. TNF-alpha and IL-1 beta-mediated regulation of MMP-9 and TIMP-1 in human glomerular mesangial cells. Nephron Exp Nephrol 2007;107(2):e73-e86.

96. Ye S. Polymorphism in matrix metalloproteinase gene promoters: implication in regulation of gene expression and susceptibility of various diseases. Matrix Biol 2000;19(7):623-629.

97. Zhang B, Ye S, Herrmann SM, Eriksson P, de MM, Evans A, et al. Functional polymorphism in the regulatory region of gelatinase B gene in relation to severity of coronary atherosclerosis. Circulation 1999 13;99(14):1788-1794.

98. Demacq C, Vasconcellos VB, Marcaccini AM, Gerlach RF, Machado AA, Tanus-Santos JE. A genetic polymorphism of matrix metalloproteinase 9 (MMP-9) affects the changes in circulating MMP-9 levels induced by highly active antiretroviral therapy in HIV patients. Pharmacogenomics J 2009;9(4):265-273.

99. Kuyvenhoven JP, van HB, Blom E, van DW, Hanemaaijer R, Verheijen JH, et al. Assessment of the clinical significance of serum matrix metalloproteinases MMP-2 and MMP-9 in patients with various chronic liver diseases and hepatocellular carcinoma. Thromb Haemost 2003;89(4):718-725.

100. Preaux AM, Mallat A, Nhieu JT, D'ortho MP, Hembry RM, Mavier P. Matrix metalloproteinase-2 activation in human hepatic fibrosis regulation by cell-matrix interactions. Hepatology 1999;30(4):944-950.

101. Roomi MW, Monterrey JC, Kalinovsky T, Rath M, Niedzwiecki A. Distinct patterns of matrix metalloproteinase-2 and -9 expression in normal human cell lines. Oncol Rep 2009;21(3):821-826.

102. Upadhya GA, Strasberg SM. Evidence that actin disassembly is a requirement for matrix metalloproteinase secretion by sinusoidal endothelial cells during cold preservation in the rat. Hepatology 1999;30(1):169-176.

103. Kuyvenhoven JP, Molenaar IQ, Verspaget HW, Veldman MG, Palareti G, Legnani C, et al. Plasma MMP-2 and MMP-9 and their inhibitors TIMP-1 and TIMP-2 during human orthotopic liver transplantation. The effect of aprotinin and the relation to ischemia/reperfusion injury. Thromb Haemost 2004;91(3):506-513.

104. Kuyvenhoven JP, Ringers J, Verspaget HW, Lamers CB, van HB. Serum matrix metalloproteinase MMP-2 and MMP-9 in the late phase of ischemia and reperfusion injury in human orthotopic liver transplantation. Transplant Proc 2003;35(8):2967-2969.

105. Defamie V, Laurens M, Patrono D, Devel L, Brault A, Saint-Paul MC, et al. Matrix metalloproteinase inhibition protects rat livers from prolonged cold ischemia-warm Wreperfusion injury. Hepatology 2008;47(1):177-185.

Chapter 2

Improvement of patient survival and its determinants over two decades of liver transplantion for chronic liver disease; a single center experience

Sebib Korkmaz K.[1], Inderson A.[1], Dubbeld J.[2], de Rooij B.-J. F.[1], Maljaars P.W.J.[1], Coenraad M. J.[1], Ringers J.[2], Sarton E.[3], Harinck H. I. J.[4], Baranski A.G.[2] , Voorneveld P.W.[1], Verspaget H.W.[1], van Hoek B.[1]

[1] Department of Gastroenterology and Hepatology
[2] Department of Surgery
[3] Department of Anesthesiology
[4] Department of Intensive Care
Leiden University Medical Center, The Netherlands

Submitted

Abstract

This study evaluates long-term outcomes after orthotopic liver transplantation (OLT) using both livers from donation after brain death (DBD) and cardiac death (DCD). Retrospective analysis was performed on 321 OLTs between September 1992 and March 2011, divided into two decades. Patient survival improved significantly in the second decade with 1, 5- and 10-year survival rates of 91%, 83% and 77%. Graft survival improved similarly. DCD-OLT showed non-significantly lower patient survival and significantly lower graft survival. Most frequent indications for re-OLT were hepatic artery thrombosis (HAT) and biliary non-anastomotic strictures (NAS) in both decades, the latter occurring more often after DCD-OLT. Death due to sepsis declined significantly in the second decade (5-year cumulative incidence (CI) 3.4% vs.15.6%, respectively; $p<0.01$) as well as death due to recurrent hepato-cellular carcinoma (5-year CI 9.7% vs. 1.5%, $p<0.01$). Multivariate analysis revealed that intraoperative blood loss was independently associated with recipient mortality in the first decade (adjusted hazard ratio (aHR):1.07; $p<0.01$) as well as in the second decade in addition to MELD score >30 (aHR: 5.03; $p<0.01$). In conclusion, long-term survival was significantly better in the second decade. DCD-OLT non-significantly impacted patient survival, but graft survival after is lower compared to DBD-OLT due to more NAS.

Introduction

Orthotopic liver transplantation (OLT) has evolved into a routine treatment for end stage liver disease, including acute and chronic liver failure, and for hepatocellular carcinoma (HCC) and metabolic disorders.[1-3] Numerous advances in surgical techniques, organ preservation and post-transplant care have led to a significant increase in OLTs in many centers with improved 5- and 10-year patient and graft survival.[4] Recognition, treatment and prevention of long-term complications such as renal dysfunction due to immuno-suppression, recurrence of primary liver disease and rejection have improved markedly over these years.[5,6] With an increasing number of patients eligible for liver transplantation many centers have accepted livers from extended criteria donors (ECD) in the last decade.[7,8] Apart from these ECD livers, also livers from donation after cardiac death (DCD) are accepted, primarily due to shortage of livers from donation after brain death (DBD). Livers from DCD donors are more susceptible to complications, especially nonanastomotic biliary strictures (NAS).[9] OLT with livers from DCD donors remains controversial in some countries and centers.[10] NAS can occur in up to 25 – 30% after OLT with a liver from a DCD donor,[11,12] which is probably related to an inevitable donor warm ischemia time (DWIT) between cardiac arrest and organ preservation.[13] It remains increasingly important to identify risk factors for graft failure, retransplantation and recipient mortality for both DCD and DBD OLT. The current study analyzes long-term outcome and risk factors for complications and death during 20 years of OLT for chronic liver disease at a single center with livers from both DBD and DCD donors.

Patients and Methods

From September 1992 until March 2011 a total of 321 orthotopic liver transplantations (OLT) for chronic liver disease (CLD) in 275 recipients were performed at the Leiden University Medical Center in the Netherlands. From 2001, in addition to livers from DBD also livers from DCD donors were transplanted, using a strict national protocol that was approved by the Dutch Transplantation Society with informed consent and allocation according to the regular waiting list. All first OLTs performed for CLD performed from September 1992 through 2001 were considered to be in the first decade (n=95), and from January 2002 through March 2011 was considered the second decade (n=180). Follow-up was until December 2011. All data were reviewed retrospectively. Patient, donor and surgical characteristics, as well as graft loss, causes of recipient mortality and retransplantations were obtained from our local medical digital database, medical, anesthetic and surgical patient charts and endoscopy reports.

Medical treatment before and after transplantation

All patients received immunosuppressive agents according to protocol: cyclosporine A or tacrolimus, prednisone during the first half-year. Patients with renal impairment received azathiopurine before 2001, or mycophenolate mofetil from 2001 onwards. Since 2001 basiliximab was given on day 0 and day 4 post OLT. In addition, patients received 24 h of prophylactic antibiotics intravenously (gentamycine, cefuroxim, penicililin G and metronidazol) and 3 weeks of oral selective bowel decontamination (polymyxin/neomycin, norfloxacin and amfotericin B) after OLT. In some cases sirolimus was used after 3 months in which case the calcineurin-inhibitor was lowered or discontinued.

In both decades hepatitis B immunoglobulin (HBIG) was used for OLT in chronic HBV patients and in the second decade nucleoside/nucleotide analogs were added. Patients with HCC received adjuvant systemic doxorubicin before OLT or no treatment in the first decade, whereas in the second decade local tumor ablation while on the waiting list was performed radiofrequency

ablation (RFA), transarterial chemo-embolization (TACE), or percutaneous ethanol injection (PEI). In both decades HCC patients were under radiological surveillance while on the waiting list. This surveillance schedule in the second decade was more intensive with 3-monthly CT scans, compared to 6-monthly ultrasound in the first decade.

Donor surgery and ischemia times

Donor surgery was performed as described previously.[14] In case of DCD donors a donor warm ischemia time (DWIT) was present, defined as the time between circulatory arrest and cold flushing with preservation fluid. Cold ischemia time (CIT) was defined as the time between this preservation flush and the removal of the liver from ice at the time of implantation in the receiving center. The recipient warm ischemia time (RWIT) was defined as the time from removal of the donor liver from ice, until reperfusion.

Recipient surgery and routine follow-up

Lab MELD scores were assessed at the time of OLT to define severity of liver disease. OLT was mostly performed according to standard procedures with 'piggyback'cavo-caval anastomosis, porto-portal and hepatic artery to hepatic artery anastomosis. Only the first 2 years were with venovenous bypass and caval interposition. Blood loss during surgery was accurately measured. In some cases the hepatic artery was anastomosed to the aorta via an iliac conduit. A duct-to-duct biliary anastomosis over an 8-12 Ch stent was performed if possible. The biliary stent was removed endoscopically after 6 weeks with ERCP or earlier as indicated. Further cholangiography was performed as indicated by cholestasis, ultrasound findings or cholangitis. Ultrasound (US) was performed routinely on day 0, 1 and 7, and subsequently at 3, 6, 12 months and yearly after OLT to detect hepatic artery thrombosis (HAT) and bile duct dilatation, which can be an indication for the presence of anastomotic or nonanastomotic strictures (NAS).[15] NAS was defined as any stricture or irregularity occurring >1cm above the bile duct anastomosis requiring intervention. A CT scan was performed in the first week after OLT and in case of abdominal sepsis or suspicion of HAT. A liver biopsy was

performed at 6 months and yearly in case of hepatitis C or recurrent hepatitis B, while in the first decade additional protocol liver biopsies at 1 week, 1 year and yearly were performed in all patients.

Outcome

Primary endpoints were graft survival and patient survival. Graft survival was defined as time from initial transplant to graft loss, patient death, or last follow-up. Patient survival was considered from time of first transplantation to patient death or censored at last known follow-up. Secondary endpoints were causes of recipient mortality and indications for retransplantation.

Statistical analyses

Statistical analysis was performed using SPSS 20. A Student t-test was used for normally distributed continuous variables, Mann-Whitney U test for non-equally distributed variables and Chi-square for categorical variables. Survival analysis was performed using the Kaplan-Meier method, risk factor analysis was performed using univariate and multivariate stepwise forward Cox regression. If a p-value of <0.15 was found in the univariate analysis the parameter was taken into account in the multivariate analysis. A p-value of <0.05 was considered significant.

Ethical statement

This retrospective study was performed according to the guidelines of the local medical ethics board and the Helsinki declaration.

Results

Donor, surgical and recipient variables

A total of 275 first OLTs were performed in nearly 20 years. A total of 46 retransplantations were performed of which 41 patients underwent a first retransplantation, 4 underwent a second retransplantation and one patient underwent a third retransplantation. Livers from DBD donors were used in 278 transplantations and in 43 cases livers from a DCD donor were used. All DCD-OLTs, except one, were performed in the second decade. Median blood loss during surgery in the first decade was 6.8 L whereas median blood loss in the second decade was 3.5 L ($p<0.01$). In line with the decline in blood loss, the amount of used blood products such as Fresh Frozen Plasma (FFP) and erythrocytes and other fluids administered during surgery was lower in the second decade. *(Table 1a)*

Table 1a. Donor and surgical variables at first transplantation.

Donor and surgical variables		Decade of LT			
		Total (n=275)	First decade (n=95)	Second decade (n=180)	*p*-value
Donor variables					
Type of donor n (%)					<0.01
	DBD	234 (85.1)	94 (98.9)	140 (77.8)	
	DCD	41 (14.9)	1 (1.1)	40 (22.2)	
Donor gender n (%)					0.80
	Male	139 (50.5)	47 (49.5)	92 (51.1)	
	Female	136 (49.5)	48 (50.5)	88 (48.9)	
Donor age (median, range)		46 (9 – 78)	41 (9 – 68)	46 (15 – 78)	<0.01
Donor blood type n (%)					0.65
	A	108 (39.9)	39 (41.1)	69 (38.3)	
	B	32 (11.6)	10 (10.5)	22 (12.2)	
	AB	124 (45.1)	44 (46.3)	80 (44.4)	
	O	11 (4.0)	2 (2.1)	9 (5.0)	
Days on ventilation (median, range)		1.5 (0 – 21)	1.3 (0 – 21)	1.5 (0 – 21)	0.38
Surgical variables					
Retransplantation n (%)		41 (14.9)	19 (20.0)	22 (12.2)	
Blood loss (L) (median, IQR)		4.1 (2.4 – 6.9)	6.8 (3.8 – 11.7)	3.5 (2.2 – 5.8)	<0.01
FFP (L) (median, IQR)		2.4 (1.5 – 3.8)	3.6 (2.3 – 6.8)	2.1 (1.5 – 3.0)	<0.01
Packed RBCs (L) (median, IQR)		1.7 (1.0 – 2.8)	2.8 (1.8 – 3.6)	1.5 (0.8 – 2.3)	<0.01

DBD: donation after brain death, DCD: donation after cardiac death, FFP: Fresh Frozen Plasma, RBC: (packed) Red Blood Cells, L: Liters, IQR: Interquartile range

Of the recipients 69.1% were male. Median recipient age at OLT was higher in the second decade compared to the first decade (53 vs. 49 years, respectively; p<0.01). The most frequent indication for OLT was HCC (23.0%) followed by alcoholic liver disease (ALD) (18.2%). HCC was the most common indication for OLT in both decades with a non-significant increase in the second decade (16.8% vs. 26.1%, p=0.10). Other indications for liver transplantation also had not changed significantly in the second decade. Median MELD score was significantly higher in the second decade compared to the first decade (19 versus 14 respectively; Mann Whitney U p<0.01) *(Table 1b)*

Ischemia-reperfusion variables

Ischemia-reperfusion (IR) variables were analyzed for DBD and DCD donors separately. DBD donors had a median CIT of 630 minutes and a median RWIT of 35 minutes for both decades combined. In the first decade DBD donors had a significantly longer median CIT of 720 minutes compared to the second decade which showed a median CIT of 600 minutes (p<0.01). This was also the case for the RWIT (median 36 min vs. 34 min, respectively; p<0.01). Median DWIT in case of livers from DCD donors was 17 minutes. CIT for OLT with a DCD donor was significantly shorter compared to OLT with a DBD donor (DBD: 600 min vs. DCD 486 min; p<0.01). No difference was seen for the RWIT in these groups with medians of 34 minutes for DBD-OLT and 33 minutes for DCD-OLT (p= 0.83), respectively. First week peak AST and peak ALT were significantly higher for DCD donors than for DBD donors in the second decade. A list of ischemia-related variables is shown in table 2.

Table 1b. Baseline recipient variables at first transplantation.

Recipient Variables	Decade of LT			
	Total (n=275)	First decade (n=95)	Second decade (n=180)	*p*-value
Gender n (%)				0.68
Male	190 (69.1)	64 (67.4)	126 (70.0)	
Female	85 (30.9)	31 (32.6)	54 (30.0)	
Recipient age (median, range)	52 (17 – 70)	49 (19 – 68)	53 (17 – 70)	<0.01
MELD score (median, range)	16 (6 – 40)	14 (6 – 40)	19 (6 – 40)	<0.01
Urgency				0.25
T	253 (92.0)	84 (88.4)	169 (93.9)	
H	22 (18.0)	11 (11.6)	11 (6.1)	
Indications for 1st OLT n (%)				
ALD	50 (18.2)	14 (14.7)	36 (20.0)	0.33
HCC	63 (23.0)	16 (16.8)	47 (26.1)	0.10
HCV	33 (12.0)	12 (12.6)	21 (11.7)	0.85
PSC	32 (11.6)	13 (13.7)	19 (10.6)	0.44
HBV	19 (6.9)	10 (10.5)	9 (5.0)	0.13
Metabolic disorders	10 (3.6)	1 (1.1)	9 (5.0)	0.17
PBC	12 (4.4)	6 (6.3)	6 (3.3)	0.35
AIH	10 (3.6)	5 (5.3)	5 (2.8)	0.32
Other	46 (16.7)	18 (18.9)	28 (15.6)	0.50

MELD: Model for End Stage Liver Disease, T: Transplantable, H: High Urgency, ALD: Alcoholic Liver Disease, HCC: Hepatocellular Carcinoma, HCV: Hepatitis C Virus, PSC: Primary Sclerosing Cholangitis, HBV: Hepatitis B Virus, PBC: Primary Biliary Cirrhosis, AIH: Autoimmune Hepatitis

Table 2. Ischemia-reperfusion variables.

Donorvariables					
	First decade‡ Median (IQR)	Second decade‡ Median (IQR)	p-value*	DCD (second decade) Median (IQR)	*p*-value¶*
CIT (min)	720 (540 – 902)	600 (492 – 695)	<0.01	486 (402 – 573)	<0.01
RWIT (min)	36 (33 – 48)	34 (30 – 40)	<0.01	33 (30 – 38)	0.83
1st week peak AST (IU/L)	716 (434–1329)	1004 (324 – 1105)	0.04	2259 (1134 – 3903)	<0.01
1st week peak ALT (IU/L)	396 (225 – 804)	542 (540 – 1938)	0.04	1525 (680– 2818)	<0.01
DWIT (min)				17 (13 – 21)	-

DBD: Donation after brain Death, DCD: Donation after Cardiac Death, CIT: Cold Ischemia Time, RWIT: Recipient Warm Ischemia Time, DWIT: Donor Warm Ischemia Time, AST: Aspartate Aminotransferase, ALT: Alanine Aminotransferase, IQR: Interquartile range, min: minutes

**Mann-Whitney U test; ‡Decade analysis encompasses DBD-OLTs; ¶DCD second decade vs. DBD second decade*

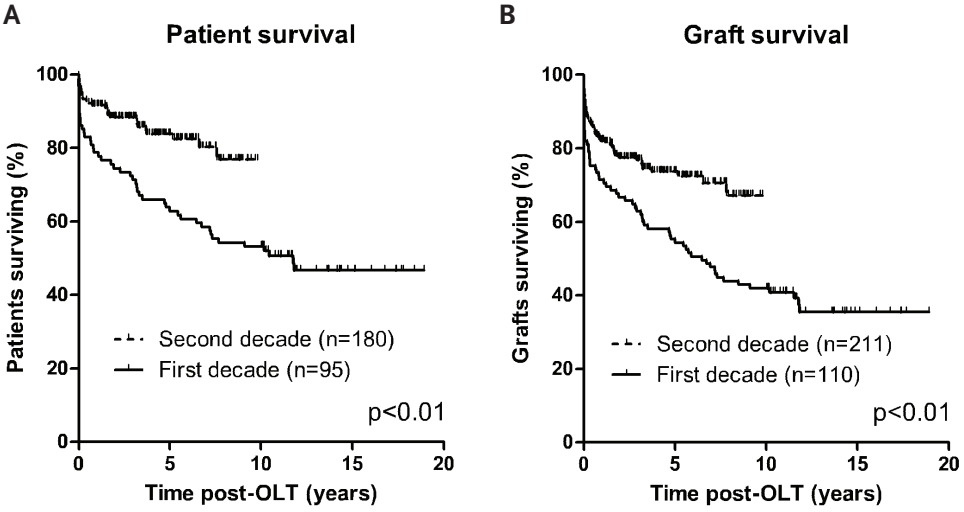
Patient and graft survival

One, 5- and 10-year patient survival after OLT in the first decade was 78%, 63% and 53%, respectively. The second decade showed significant improvement in patient survival rates with 1, 5- and 10-year patient survival rates of 91%, 83% and 77% (log rank p<0.01) *(Figure 1a)*. Graft survival rates also showed a similar improvement in the second decade compared to the first decade (first decade 71%, 54%, 42%, second decade 82%, 74%, 67%, respectively; log rank p<0.01) *(Figure 1b)*. Overall patient survival in the second decade at 1, 5 and 10 years after OLT was not significantly different for OLT with DCD donors compared to DBD donors (DCD: 90.0%, 75.7%, 69.4% vs. DBD: 92.1%, 85.6%, 79.2%, p=0.27) *(supplemental figure 1a)*. One, 5 and 10-year graft survival in case of DCD-OLT in the second decade was worse as compared to DBD-OLT (DBD: 85.6%, 78.1%, 72.6% vs. DCD: 75.0%, 54.7%, 48.6%; p=0.01) *(supplemental figure 1b)*. In the second decade patient survival for patients with a pre-OLT MELD score of >30 had worse survival with 1 and 5 year survival rates of 61.5% and 49.2% compared to survival rates at 1 and 5 year of 95.0% and 88.4% for patients with a MELD score ≤30 (p<0.01, *supplemental figure 2a*). Patients with a MELD score >30 (n=4) in the first decade did not show worse survival compared to patients with a MELD score ≤30. In addition, a MELD score between 20 and 30 did not have an impact on patient survival. (Data not shown)

Retransplantations

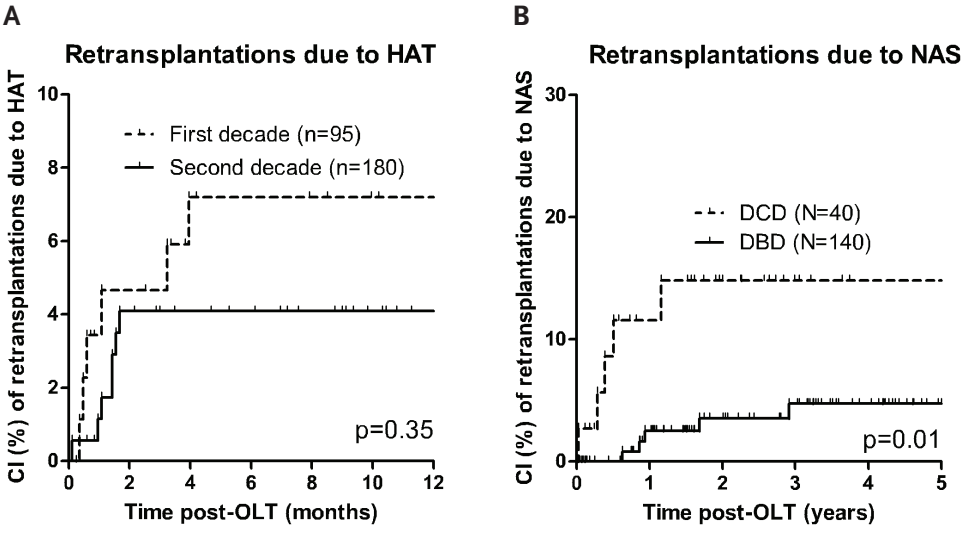
A total of 46 retransplantations were performed, of which 41 first retransplantations. HAT and NAS were the most frequent indications for retransplantation *(Table 3)*. One year cumulative incidence (CI) of retransplantation due to HAT was 7.2% in the first decade compared to 4.1% in the second decade (p=0.35). Five years CI of retransplantation due to NAS in DBD donors was in both decades not significantly different (first decade 6.2% vs. second decade 4.7%, respectively; p=0.75). However, in the second decade 5-year CI of retransplantation due to NAS for OLT with a DCD donor was 14.8% compared to 4.7% for OLT with a DBD donor (p=0.01). *(Figure 2a and Figure 2b)*

Figure 1



Patient (A) and graft (B) survival improved significantly in the second decade compared to the first decade.

Figure 2



A: Retransplantations due to HAT showed a nonsignificant decline in the second decade compared to the first decade (4.1% vs. 7.2%, respectively, p=0.35).
B: Retransplantations due to NAS occurred significantly more often after DCD-OLT than after DBD-OLT (14.8% vs. 4.7%, respectively; p=0.01)

Table 3. Causes of retransplantation after the first transplantation. HAT and NAS are the most frequent causes of retransplantation in both decades.

	First decade				Second decade				
	Years after 1st transplantation				Years after 1st transplantation				
	<1	>1–5	>5	Total (First)	<1	>1–5	>5	Total (Second)	Total
Pts at risk	95	62	44	95	180	146	132	180	275
Indication for 1st retransplantation n (%)									
HAT	6 (6.3)		1 (1.3)	7 (7.4)	7 (3.9)	1 (0.7)		8 (4.4)	15 (5.4)
PNF	2 (2.1)			2 (2.1)	2 (1.1)			2 (1.1)	4 (1.5)
NAS	2 (2.1)	2 (2.4)		4 (4.2)	7 (3.9)	3 (2.1)		10 (5.6)	14 (5.1)
Venous occlusions	1 (1.1)		1 (1.3)	2 (2.1)					2 (1.0)
Recurrence HCV					2 (1.1)			2 (1.1)	2 (1.0)
Rejection	1 (1.1)	1 (1.2)	2 (2.5)	4 (4.2)					4 (1.5)
Total	12 (12.6)	3 (3.6)	4 (5.0)	19 (20)	18 (8.9)	4 (2.7)	0 (0)	22 (12.2)	41 (14.9

HAT: Hepatic Artery Thrombosis, PNF: Primary Nonfunction, NAS: Nonanastomotic Strictures. PNF was defined as non-life sustaining function of the liver requiring retransplantation or leading to death within seven days after liver transplantation.

Table 4. Causes of recipient mortality after OLT in both decades.

	First decade				Second decade				
	Years after 1st transplantation				Years after 1st transplantation				
	<1	>1–5	>5	Total (First)	<1	>1–5	>5	Total (Second)	Total
Pts at risk	95	74	59	95	180	164	154	180	275
Causes of recipient mortality n (%)									
Liver failure	1 (1.1)		1 (1.7)	2 (2.1)	1 (0.6)	1 (0.6)		2 (1.1)	4 (1.5)
Respiratory			1 (1.7)	1 (1.1)		1 (0.6)		1 (0.6)	2 (0.7)
Infection	10 (10.5)	3 (4.1)	4 (6.8)	17 (17.9)	6 (3.3)		1 (0.6)	7 (3.9)	24 (8.7)
Cardiovascular	5 (5.3)		2 (3.4)	7 (7.4)	2 (1.1)	3 (1.8)	2 (1.3)	7 (3.9)	14 (5.1)
Carcinoma (other than liver etiology)		2 (2.7)	2 (3.4)	4 (4.2)	2 (1.1)	2 (1.2)		4 (2.2)	8 (2.9)
Livernecrosis/bleeding	3 (3.2)		1 (1.7)	4 (4.2)	2 (1.1)			2 (1.1)	6 (2.2)
Recurrence primary disease									
HCC	1 (1.1)	6 (8.1)		7 (7.4)	2 (1.2)			2 (1.1)	9 (3.3)
Other	1 (1.1)	4 (5.4)	2 (3.4)	7 (7.4)					7 (2.5)
GVHD					2 (1.1)			2 (1.1)	2 (0.7)
Other					1 (0.6)	1 (0.6)		2 (1.1)	2 (0.7)
Total	21 (22.1)	15 (20.3)	13 (22)	49 (51.6)	18 (10)	8 (4.8)	3 (1.9)	29 (16.1)	78 (28.4)

Infection was the main cause of recipient mortality in both decades. Other causes of recurrence primary disease included cholangiocarcinoma, alcoholic liver disease and recurrence of multiple endocrine neoplasia (MEN) syndrome. HCC: Hepatocellular Carcinoma, GVHD: Graft-versus-Host-Disease

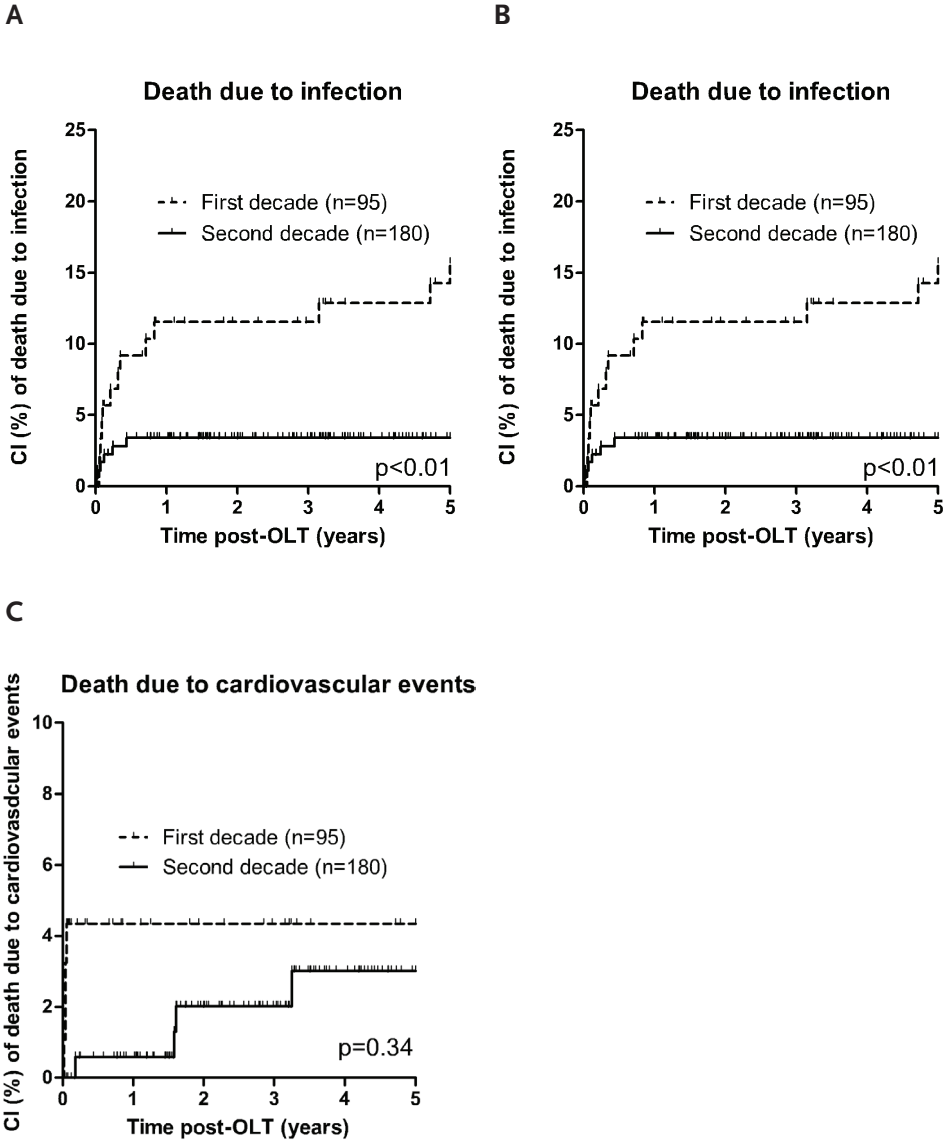
Causes of recipient mortality

Causes of recipient mortality in both decades are given in table 4. Infection was the most prevalent cause of recipient mortality in both decades. Five-year CI of death due to infection post-OLT declined significantly in the second decade compared to the first decade (first decade 15.6% vs. second decade 3.4%; p<0.01) *(Figure 3a)*. Five-year CI of death due to recurrence of HCC declined from 9.7% in the first decade to 1.4% in the second decade (p<0.01). *(Figure 3b)* Death due to cardiovascular events remained stable in the past two decades (5-year CI in first decade 4.3% vs. 3% in the second decade, respectively; p=0.34) *(figure 3c)*. The most frequent cause of mortality in recipients with a pre-OLT MELD >30 in the second decade (n=15) was infection (n=3); In this group one-year CI of death due to infection was 20% compared to 2.0% for patients with a pre-OLT MELD score ≤30 (p<0.01; *supplemental figure 2b*).

Univariate and multivariate analysis

Cox regression analysis of pre-operative and operative risk factors with mortality as outcome was performed for each decade separately. In the first decade blood loss during surgery, recipient age and RWIT were independent risk factors for recipient mortality in the univariate analysis and thus were taken into account in the multivariate analysis. Multivariate analysis showed blood loss during surgery as an independent risk factor for recipient mortality in the first decade (aHR=1.05, p=.0.02). In the second decade blood loss during surgery (aHR= 1.10; p=0.01) as well as MELD score >30 (aHR=5.03; p<0.01) were significant risk factors for recipient mortality. (*Table 5a and 5b)*

Figure 3



A: Five-year cumulative incidence rates of death due to infection post-OLT was 15.6% in the first decade but declined significantly to 3.4% in the second decade. (p=0.01).
B: Recipient mortality caused by recurrent HCC occurred more often in the first decade compared to the second decade (9.7% vs. 1.4%, respectively; p<0.01)
C: Cardiovascular events as cause of recipient mortality remained stable in the second decade. (first decade: 4.3% vs. second decade: 3%, respectively; p=0.34)

Table 5a. Multivariate analysis for risk factors for recipient mortality in the first decade.

Variables			n (%)	Univariate analysis HR (95% CI)	p-value	Multivariate analysis HR (95% CI)	p-value
Year of LT (years)		Continuous		0.95 (0.85 – 1.06)	0.15		
Donor gender							
	Male		47 (49.5)	1.22 (0.70 – 2.15)	0.48		
	Female	Reference	48 (50.5)	1 (reference)			
Donor age (years)		Continuous		0.99 (0.97 – 1.01)	0.26		
Recipient gender							
	Male		64 (67.4)	1.35 (0.73 – 2.52)	0.34		
	Female	Reference	31 (32.6)	1 (reference)			
Recipient age (years)		Continuous		1.04 (1.01 – 1.07)	**0.02**	0.97 (0.94 – 0.99)	0.06
Blood loss during surgery (L)		Continuous		1.06 (1.01 – 1.10)	**0.01**	1.05 (0.99 – 1.08)	**0.02**
CIT (min)		Continuous		1.00 (0.99 – 1.01)	0.48		
RWIT (min)		Continuous		1.02 (1.00 – 1.04)	**0.02**	1.02 (0.99 – 1.05)	0.14
MELD score							
	>30		4 (4.2)	1.14 (0.28 – 4.72)	0.85		
	≤30	Reference	91 (95.8)	1 (reference)			

Blood loss during surgery remained a significant risk factor for recipient mortality (HR: 1.05; p=0.02)
CIT: Cold Ischemic Time, RWIT: Recipient Warm Ischemic Time, MELD: Model for End Stage Liver Disease

Table 5b. Multivariate analysis for risk factors of recipient mortality in the second decade.

Variables			n (%)	Univariate analysis HR (95% CI)	p-value	Multivariate analysis HR (95% CI)	p-value
Year of LT (years)		Continuous		0.92 (0.78 – 1.08)	0.29		
Donor gender							
	Male		140 (77.8)	1.47 (0.70 – 3.07)	0.31		
	Female	Reference	40 (22.2)	1 (reference)			
Donorage (years)		Continuous		1.00 (0.97 – 1.03)	0.99		
Recipient gender							
	Male		126 (70.0)	1.16 (0.51 – 2.61)	0.73		
	Female	Reference	54 (30.0)	1 (reference)			
Recipient age (years)		Continuous		1.01 (0.98 – 1.05)	0.53		
Blood loss during surgery (L)		Continuous		1.10 (1.01 – 1.19)	**0.02**	1.10 (1.02- 1.19)	**0.01**
CIT (min)		Continuous		1.00 (1.00 – 1.00)	0.26		
RWIT (min)		Continuous		1.03 (0.99 – 1.07)	0.18		
MELD score							
	>30		165 (91.7)	5.48 (2.29 – 13.09)	**<0.01**	5.03 (1.96- 12.89)	**<0.01**
	≤30	Reference	15 (8.3)	1 (reference)			

Blood loss during surgery and MELD score >30 were independent risk factors for recipient mortality after OLT. (aHR blood loss = 1.10, p=0.01, aHR MELD score>30= 5.03, p<0.01)
CIT: Cold Ischemic Time, RWIT: Recipient Warm Ischemic Time, MELD; Model for End Stage Liver Disease

Discussion

Many lessons can be learned from this long-term single center study. In the second decade of liver transplantation at our institute 1, 5 and 10-year patient survival rates improved significantly as compared to the first decade (91%, 83% and 77% as compared to 78%, 65% and 53%, respectively). The indications for OLT had not changed in the second decade although a nonsignificant increase in OLT due to HCC was observed, which is in line with indications for OLT across Europe[16,17]. Due to a shortage of donor livers many centers have expanded their criteria for acceptance of potential donor livers.[18,19] Donor age was significantly higher in the second decade as compared to the first decade. Also, the usage of livers from DCD donors was introduced in 2001 in a national protocol with allocation according to the waiting list.[7] Only livers from Maastricht category 3 donors were transplanted, and about 25% of DCD donors were excluded for liver donation. [20,21] Despite the strict DCD protocol, this leads to a nonsignificant difference in recipient mortality for OLT with DCD donors compared to DBD OLT. This is in line with our previously published national 3-year data but in the present study, significantly lower graft survival rates for DCD OLT were observerd compared to DBD.[22,23] Donor variables such as CIT and RWIT were shorter in the second decade for DBD donors, and also peak AST and peak ALT were significantly lower in the second decade for DBD donors. In contrast, despite lower CIT for OLT with livers from DCD donors, peaks of AST and ALT remain significantly higher in DCD OLT compared to DBD OLT indicating more severe ischemia-reperfusion injury (IRI) in DCD OLT. DCD donors are more prone to ischemia damage probably because they are exposed to an additional donor warm ischemic time (DWIT).[24] Possibly, during DWIT microthrombi may develop in the peribiliary plexus and sinusoids, resulting in more IRI as indicated by higher peaks AST and ALT and probably more NAS in DCD OLT than in DBD OLT despite shorter CIT.[25,26] Better preservation techniques are required in order to improve results of DCD OLT.

In line with improved patient survival, graft survival has also improved in the second decade. With improved immunosuppressive medication such as tacrolimus, therapy-resistant and chronic rejections have virtually disappeared,

illustrated by the fact that no retransplantations for chronic rejection had to be carried out in the second decade.
However, the retransplantation rate has remained stable in the second decade as compared to the first with HAT and NAS being the most frequent indications for retransplantation in both decades. With the introduction of DCD donors retransplantations due to NAS were more frequent than for DBD donors (5-year CI DCD 13.4% vs. DBD 4.3%; p=0.01), and this was responsible for the significantly worse graft survival and nonsignificantly lower patient survival compared to OLT with a DBD donor. As recently shown, the higher morbidity rate leads to higher cost per life year for OLT with a DCD donor compared to a DBD donor.[27]

Death due to infection post-OLT patient was the most prevalent cause of death in both decades. However, the cumulative incidence of death due to infection declined from 15.6% in the first decade to 3.4% in the next decade. This suggests that, although some risk factors for post-OLT infection are not amenable to intervention, such as polymorphisms in innate immunity, other interventions have likely resulted in reducing post-OLT infections.[28,29] For instance, improved tailoring of immunosuppression therapy has led to avoidance of overdosing of immunosuppressive therapy, which probably also resulted in a reduction of mortality due to sepsis.[30-32]
Additionally, the reduction of blood loss during surgery in the second decade may have contributed to the reduced infection rate since it has been shown that intra-operative blood loss during OLT is associated with post-OLT mortality due to septicemia, probably partially related to a less immuno-suppressed state and less reoperation.[33-36]. Moreover, blood losss during surgery reflects surgical performance and is known to impact patient survival.[33,37-39] The current study showed that in both decades blood loss during surgery to be an independent risk factor for recipient mortality.
The second most prevalent cause of patient mortality after OLT was recurrence of hepatocellular carcinoma (HCC). A significant decrease in mortality due to recurrence of HCC was noticed in the second decade despite less stringent transplantation criteria in that decade (including down staging to within Milan criteria) at our institute.[40] The reduced mortality can most likely be attributed to the introduction of adjuvant treatment by local tumor

ablation before OLT with radiofrequency ablation, transarterial chemo-embolization and percutaneous ethanol injection combined with radiological surveillance.[41,42] Non-hepatic malignancies as cause of recipient mortality showed a non-significant decrease in the second decade compared to the first decade which might be due to less immunosuppression. Remarkably, patient mortality due to cardiovascular events remained stable in the second decade as compared to the first decade. More awareness and better management of risk factors like obesity, smoking, hypertension, diabetes and hyperlipidemia are needed before and after OLT to diminish long-term cardiovascular morbidity and mortality.[43]

A MELD score >30 was an independent predictor for worse patient survival compared to patients with a MELD score <30 in the group of patients that were transplanted in the second decade. This is in line with some previous reports indicating worse post-transplant survival with pre-OLT MELD scores of >30, although other studies do not report an impact of MELD score on post-transplantation mortality.[44,45] The fact that MELD score was not an independent risk factor for survival in the first decade is probably due to small numbers. This is probably due to the fact that recipients with very high MELD scores were usually denied OLT in the first decade. In the first two weeks after OLT, infection was the most frequent cause of recipient mortality in recipients with MELD>30 and the increased mortality during this period was responsible for the difference in patient survival compared to recipients with lower MELD scores. This increased susceptibility for infections is in line with a previous study that reported intra-abdominal bacterial infections in 42% of patients with a pre-OLT MELD score of >40.[46]

In conclusion, a marked improvement in not only short-term but also long-term patient survival after OLT has been achieved over the last 20 years in our center. The main reasons for better survival were less death due to post LT infection, less recurrence of HCC and better surgical and anesthetic techniques as reflected by less blood loss during surgery. Based on the risk factors for mortality, further improvements in treatment protocols by reducing blood loss during surgery and better selection and treatment of recipients especially in the case of high MELD scores may result in further improvements

in patient survival rates. Other aspects, such as further reduction in immunosuppression in tolerant recipients, improved prevention and treatment of sepsis and especially the prevention of NAS in OLT with DCD donors along with early indentification and treatment of cardiovascular risk factors may also contribute to an even better long-term patient survival.

References

1. Adam R. [Fifteen years of liver transplantation in Europe]. Bull Acad Natl Med 2007 Nov;191(8):1607-1613.

2. Burroughs A, McNamara D. Liver disease in Europe. Aliment Pharmacol Ther 2003 Nov; 18 Suppl 3:54-59.

3. Burroughs AK, Sabin CA, Rolles K, Delvart V, Karam V, Buckels J, et al. 3-month and 12-month mortality after first liver transplant in adults in Europe: predictive models for outcome. Lancet 2006 Jan 21;367(9506):225-232.

4. Aberg F, Isoniemi H, Hockerstedt K. Long-term results of liver transplantation. Scand J Surg 2011;100(1):14-21.

5. Kotlyar DS, Campbell MS, Reddy KR. Recurrence of diseases following orthotopic liver transplantation. Am J Gastroenterol 2006 Jun;101(6):1370-1378.

6. Geissler EK, Schlitt HJ. Immunosuppression for liver transplantation. Gut 2009 Mar;58(3):452-463.

7. Dubbeld J, van Hoek B., Ringers J. Use of a liver from donor after cardiac death: is it appropriate for the sick or the stable? Curr Opin Organ Transplant 2011 Apr;16(2):239-242.

8. Hong JC, Yersiz H, Kositamongkol P, Xia VW, Kaldas FM, Petrowsky H, et al. Liver transplantation using organ donation after cardiac death: a clinical predictive index for graft failure-free survival. Arch Surg 2011 Sep;146(9):1017-1023.

9. Foley DP, Fernandez LA, Leverson G, Anderson M, Mezrich J, Sollinger HW, et al. Biliary complications after liver transplantation from donation after cardiac death donors: an analysis of risk factors and long-term outcomes from a single center. Ann Surg 2011 Apr;253(4):817-825.

10. Reich DJ, Hong JC. Current status of donation after cardiac death liver transplantation. Curr Opin Organ Transplant 2010 Jun;15(3):316-321.

11. Buis CI, Hoekstra H, Verdonk RC, Porte RJ. Causes and consequences of ischemic-type biliary lesions after liver transplantation. J Hepatobiliary Pancreat Surg 2006;13(6):517-524.

12. Ryu CH, Lee SK. Biliary strictures after liver transplantation. Gut Liver 2011 Jun;5(2):133-142.

13. Chan EY, Olson LC, Kisthard JA, Perkins JD, Bakthavatsalam R, Halldorson JB, et al. Ischemic cholangiopathy following liver transplantation from donation after cardiac death donors. Liver Transpl 2008 May;14(5):604-610.

14. Baranski A.G. Surgical Technique of the Abdominal Organ Procurement: Step by Step. 2009 ed. Springer, 2008.

15. Sebib Korkmaz K., ten Hove W.R., Verspaget H.W., Dubbeld J., Wolterbeek R., van Erkel A., et al. Sequential liver chemistry profiling and abdominal ultrasound assessments to predict biliary strictures after liver transplantation. The Open Transpl Journal 2012 Feb 22;5:1-5.

16. Adam R, Karam V, Delvart V, O'Grady J, Mirza D, Klempnauer J, et al. Evolution of indications and results of liver transplantation in Europe. A report from the European Liver Transplant Registry (ELTR). J Hepatol 2012 Sep;57(3):675-688.

17. Mazzaferro V, Chun YS, Poon RT, Schwartz ME, Yao FY, Marsh JW, et al. Liver transplantation for hepatocellular carcinoma. Ann Surg Oncol 2008 Apr;15(4):1001-1007.

18. Foster R, Zimmerman M, Trotter JF. Expanding donor options: marginal, living, and split donors. Clin Liver Dis 2007 May;11(2):417-429.

19. Durand F, Renz JF, Alkofer B, Burra P, Clavien PA, Porte RJ, et al. Report of the Paris consensus meeting on expanded criteria donors in liver transplantation. Liver Transpl 2008 Dec;14(12):1694-1707.

20. Muiesan P, Girlanda R, Jassem W, Melendez HV, O'Grady J, Bowles M, et al. Single-center experience with liver transplantation from controlled non-heartbeating donors: a viable source of grafts. Ann Surg 2005 Nov;242(5):732-738.

21. Muiesan P. Can controlled non-heart-beating donors provide a solution to the organ shortage? Transplantation 2003 May 27;75(10):1627-1628.

22. Dubbeld J, Hoekstra H, Farid W, Ringers J, Porte RJ, Metselaar HJ, et al. Similar liver transplantation survival with selected cardiac death donors and brain death donors. Br J Surg 2010 May;97(5):744-753.

23. Meurisse N, Vanden Bussche S, Jochmans I, Francois J, Desschans B, Laleman W, et al. Outcomes of liver transplantations using donations after circulatory death: a single-center experience. Transplant Proc 2012 Nov;44(9):2868-2873.

24. Pine JK, Aldouri A, Young AL, Davies MH, Attia M, Toogood GJ, et al. Liver transplantation following donation after cardiac death: an analysis using matched pairs. Liver Transpl 2009 Sep;15(9):1072-1082.

25. Lang R, He Q, Jin ZK, Han DD, Chen DZ. Urokinase perfusion prevents intrahepatic ischemic-type biliary lesion in donor livers. World J Gastroenterol 2009 Jul 28;15(28):3538-3541.

26. Hashimoto K, Eghtesad B, Gunasekaran G, Fujiki M, Uso TD, Quintini C, et al. Use of tissue plasminogen activator in liver transplantation from donation after cardiac death donors. Am J Transplant 2010 Dec;10(12):2665-2672.

27. van der Hilst CS, Ijtsma AJ, Bottema JT, van Hoek B., Dubbeld J, Metselaar HJ, et al. The price of donation after cardiac death in liver transplantation: a prospective cost-effectiveness study. Transpl Int 2013 Feb 11.

28. de Rooij BJ, van Hoek B., ten Hove WR, Roos A, Bouwman LH, Schaapherder AF, et al. Lectin complement pathway gene profile of donor and recipient determine the risk of bacterial infections after orthotopic liver transplantation. Hepatology 2010 Sep;52(3):1100-1110.

29. de Rooij BJ, van der Beek MT, van Hoek B., Vossen AC, Rogier Ten HW, Roos A, et al. Mannose-binding lectin and ficolin-2 gene polymorphisms predispose to cytomegalovirus (re)infection after orthotopic liver transplantation. J Hepatol 2011 Oct;55(4):800-807.

30. Schmeding M, Kiessling A, Neuhaus R, Heidenhain C, Bahra M, Neuhaus P, et al. Mycophenolate mofetil monotherapy in liver transplantation: 5-year follow-up of a prospective randomized trial. Transplantation 2011 Oct 27;92(8):923-929.

31. Schmeding M, Sauer IM, Kiessling A, Pratschke J, Neuhaus R, Neuhaus P, et al. Influence of basiliximab induction therapy on long term outcome after liver transplantation,

    a prospectively randomised trial. Ann Transplant 2007;12(3):15-21.

32. Patel G, Huprikar S. Infectious complications after orthotopic liver transplantation. Semin Respir Crit Care Med 2012 Feb;33(1):111-124.

33. Kappa SF, Gorden DL, Davidson MA, Wright JK, Guillamondegui OD. Intraoperative blood loss predicts hemorrhage-related reoperation after orthotopic liver transplantation. Am Surg 2010 Sep;76(9):969-973.

34. Kaplan J, Sarnaik S, Gitlin J, Lusher J. Diminished helper/suppressor lymphocyte ratios and natural killer activity in recipients of repeated blood transfusions. Blood 1984 Jul;64(1):308-310.

35. Kaido T, Mori A, Ogura Y, Ogawa K, Hata K, Yoshizawa A, et al. Pre- and perioperative factors affecting infection after living donor liver transplantation. Nutrition 2012 Nov;28(11-12):1104-1108.

36. Hendriks HG, van der Meer J, de Wolf JT, Peeters PM, Porte RJ, de JK, et al. Intraoperative blood transfusion requirement is the main determinant of early surgical re-intervention after orthotopic liver transplantation. Transpl Int 2005 Jan;17(11):673-679.

37. Wu WC, Smith TS, Henderson WG, Eaton CB, Poses RM, Uttley G, et al. Operative blood loss, blood transfusion, and 30-day mortality in older patients after major noncardiac surgery. Ann Surg 2010 Jul;252(1):11-17.

38. Ramos E, Dalmau A, Sabate A, Lama C, Llado L, Figueras J, et al. Intraoperative red blood cell transfusion in liver transplantation: influence on patient outcome, prediction of requirements, and measures to reduce them. Liver Transpl 2003 Dec;9(12):1320-1327.

39. de Boer MT, Christensen MC, Asmussen M, van der Hilst CS, Hendriks HG, Slooff MJ, et al. The impact of intraoperative transfusion of platelets and red blood cells on survival after liver transplantation. Anesth Analg 2008 Jan;106(1):32-44, table.

40. Cescon M, Cucchetti A, Ravaioli M, Pinna AD. Hepatocellular carcinoma locoregional therapies for patients in the waiting list. Impact on transplantability and recurrence rate. J. Hepatol 2013 Mar;58(3):609-618.

41. Forner A, Llovet JM, Bruix J. Hepatocellular carcinoma. Lancet 2012 Mar 31;379(9822):1245-1255.

42. Sourianarayanane A, El-Gazzaz G, Sanabria JR, Menon KV, Quintini C, Hashimoto K, et al. Loco-regional therapy in patients with Milan Criteria-compliant hepatocellular carcinoma and short waitlist time to transplant: an outcome analysis. HPB (Oxford) 2012 May;14(5):325-332.

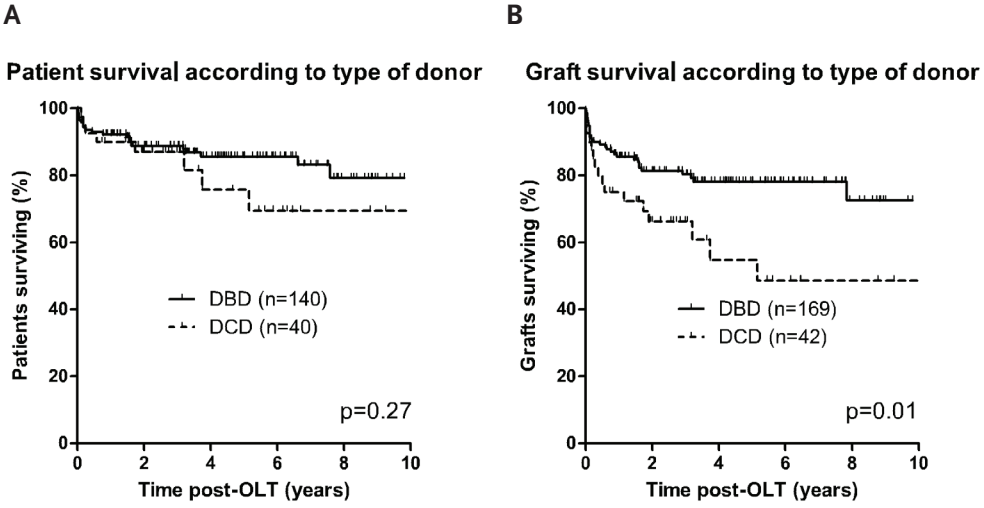
43. Raval Z, Harinstein ME, Skaro AI, Erdogan A, DeWolf AM, Shah SJ, et al. Cardiovascular risk assessment of the liver transplant candidate. J Am Coll Cardiol 2011 Jul 12;58(3):223-231.

44. Oberkofler CE, Dutkowski P, Stocker R, Schuepbach RA, Stover JF, Clavien PA, et al. Model of end stage liver disease (MELD) score greater than 23 predicts length of stay in the ICU but not mortality in liver transplant recipients. Crit Care 2010;14(3):R117.

45. Goldberg D, French B, Thomasson A, Reddy KR, Halpern SD. Waitlist survival of patients with primary sclerosing cholangitis in the model for end-stage liver disease era. Liver Transpl 2011 Nov;17(11):1355-1363.

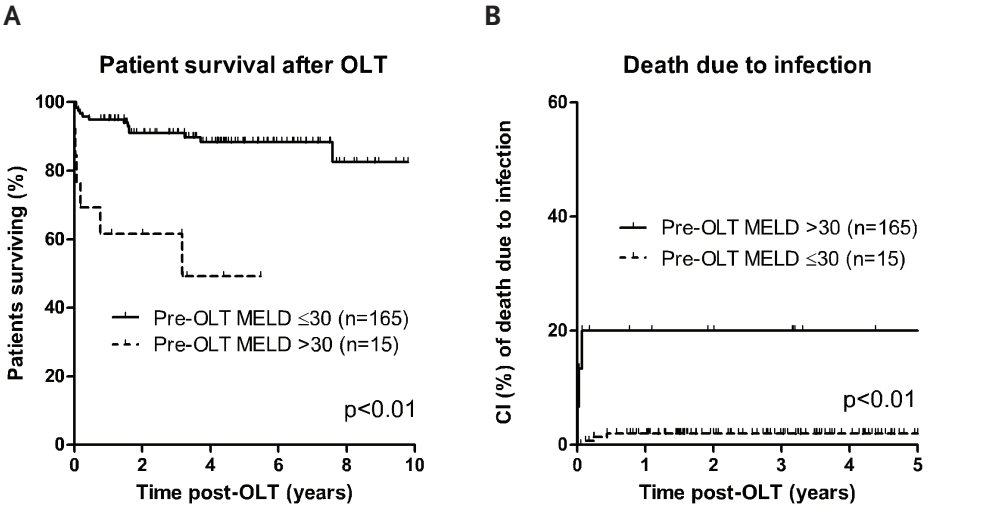
46. Alexopoulos S, Matsuoka L, Cho Y, Thomas E, Sheikh M, Stapfer M, et al. Outcomes after liver transplantation in patients achieving a model for end-stage liver disease score of 40 or higher. Transplantation 2013 Feb 15;95(3):507-512.

Supplemental Figure 1



A: Patient survival was nonsignificantly lower after DCD-OLT compared to DBD-OLT
B: Graft survival was significantly lower after DCD-OLT compared to DBD-OLT

Supplemental Figure 2



A: MELD score >30 impacted patient significantly after OLT
B: Patients with a pre-OLT MELD score >30 died more often due to infection compared to patients with a pre-OLT MELD score ≤30

Chapter 3

Peak alanine aminotransferase as predictor of nonanastomotic biliary stricture formation after cardiac death liver donation

Sebib Korkmaz K.[1], de Rooij B.-J.F.[1], Sutton M.E.[3*], Dubbeld J.[2], Coenraad M.J.[1], Inderson A.[1], Voorneveld P.W.[1], Verspaget H.W.[1], Porte R.J.[3], van Hoek B.[1]*

[1] Leiden University Medical Center, Dept of Gastroenterology and Hepatology, Leiden, The Netherlands
[2] Leiden University Medical Center, Dept of Surgery, Leiden, The Netherlands
[3] University of Groningen, University Medical Center Groningen, Dept of Hepatobiliary surgery and Liver Transplantation, Groningen, The Netherlands
[*] Both authors contributed equally

Submitted

Abstract

Scarcity of donor livers for orthotopic liver transplantation (OLT) has led to the use of livers from donation after cardiac death (DCD). DCD livers are known to have more ischemia-reperfusion injury (IRI) reflected by high peak alanine aminotransferase (ALT) within the first week post-OLT and carry a high risk of biliary nonanastomotic strictures (NAS). It is unknown whether an association exists between peak ALT and the risk to develop NAS in DCD and brain death donated OLT (DBD). We retrospectively reviewed two cohorts of OLT performed with livers from DBD (n=401) or DCD (n=97) from two liver transplantation centers between 2000 and 2012. Optimal cut-off value of peak ALT for development of NAS post DCD-OLT was established at ≥1300 IU/L. In the combined cohorts, four-year cumulative incidence of NAS after DCD-OLT was 51.4.1% in patients with high ALT peak post-OLT, compared to 11.6% in patients with low ALT peak. ($p<0.001$). The independent ohorts showed similar results. No such relation was observed post DBD-OLT. Multivariate analysis revealed peak ALT ≥1300 IU/L to be independently associated with development of NAS post DCD-OLT (adjusted hazard ratio (aHR) = 4.96 confidence interval (CI) (1.73 – 14.24)) adjusted for cold ischemic time (CIT), primary sclerosing cholangitis (PSC) and donor age. Peak ALT is a good clinical marker for the risk to develop NAS post DCD-OLT and can be used to distinguish high-risk from low-risk patients.

Introduction

Orthotopic liver transplantation (OLT) has evolved into routine treatment for advanced liver disease with excellent short and long-term survival.[1,2] An increasing number of patients eligible for liver transplantation and a decreasing number of donors after brain death (DBD) have led to the expansion of criteria for acceptance of potential liver grafts in the last decade.[3,4] OLT with livers from donation after cardiac death (DCD) has become common. However, OLT with a liver from a DCD donor carries a high risk for development of nonanastomotic biliary strictures (NAS).[5] NAS can occur in up to 25 – 45 % after OLT with a DCD donor and is considered a major cause of morbidity and reduced graft survival.[6-8] Early recognition of an increased risk to develop NAS may be valuable to provide timely intervention. Several ischemic parameters have been evaluated such as cold ischemic time (CIT), recipient warm ischemic time (RWIT) and donor warm ischemia time (DWIT) as potential predictors of NAS in DCD and DBD donors, but clinical use of these parameters for predicting NAS has remained controversial.[9,10] Livers retrieved from DCD have an inevitable DWIT between cardiac arrest and organ preservation, which may lead to a higher peak alanine aminotransferase (ALT) and asparate aminotransferase (AST) in the first week after OLT.[11] It is likely that the higher incidence of NAS after OLT with DCD livers is largely the results of the additional ischemia-reperfusion injury (IRI) due to the DWIT.[12-14] We therefore hypothesized that a relation may exist between first week post-OLT peak aminotransferases and the risk for NAS development, especially after DCD OLT. This hypothesis was examined in two independent centers for OLT with DBD and DCD livers.

Methods and Materials

A total of 498 first consecutive OLTs for chronic liver diseases were included from two liver transplantation centers with a minimum follow-up of 7 days. From the Leiden University Medical Center (LUMC; cohort A) a total of 176 OLTs could be included in the time-period of October 2001 until March 2011 next to 322 OLTs from the University Medical Center Groningen (UMCG: cohort B) performed in the time-period of July 2000 until June 2012. Patient follow-up for both groups was until July 2012. This included OLTs using livers from DBD as well as– from 2001 on within a strict protocol – DCD donors.

Donor surgery

In case of DCD donors a donor warm ischemia time (DWIT) was measured, defined as the time between circulatory arrest and cold flush with preservation fluid in the donor. Cold ischemia time (CIT) was defined as the time between cold flush with preservation fluid in the donor and removal of the liver from ice during the transplantation procedure. The recipient warm ischemia time (RWIT) was defined as the time of removal of the donor liver from ice until reperfusion of the donor liver in the recipient.

Recipient surgery and routine follow-up

In both centers OLT with standard technique of 'piggy-back'cavo-caval anastomosis, porto-portal and hepatic artery to hepatic artery anastomosis was perfomed in most recipients. In some cases the hepatic artery was anastomosed to the aorta via an iliac conduit. A duct-to-duct biliary anastomosis -over a 8-12 Ch stent in the LUMC, no stent in UMCG- was performed if possible.
The biliary stent was removed endoscopically with endoscopic retrograde cholangiography (ERC) at 6 weeks or earlier as indicated. In the first year blood liver biochemistry was performed daily in the first two weeks, weekly in the following two weeks, monthly thereafter in the first year, and then every three months. In both cohorts, ultrasound (US) was performed routinely on day 0, 1 and 7, and subsequently at 3, 6, 12 months and yearly after OLT. ERC or magnetic resonance cholangiography (MRC) and other imaging studies were

performed when indicated. A liver biopsy was performed per protocol at 6 months in the LUMC, and further as indicated in both centers. In both cohorts pre-OLT baseline parameters, including laboratory model for end-stage liver disease (MELD) scores were evaluated (n=449). Due to missing variables 49 MELD scores could not be computed (cohort A n=8, cohort B n=41).

IRI and NAS

The degree of hepatocellular injury was evaluated by postoperative serum levels of alanine aminotransferase (ALT). Serum ALT was determined during the 7 consecutive days after OLT and measured by routine biochemical methods. The highest level of peak ALT was evaluated individually and reflected IRI. NAS was considered as any treated stricture or irregularity of the intra- or extrahepatic bile ducts occurring at least 1 cm above the anastomosis post-OLT. Analysis of NAS development was performed in both the combined cohort as well the individual cohorts. Nonanastomotic biliary strictures that did not require intervention and anastomotic strictures were not included in the definition of NAS for the current study. In addition, cases of hepatic artery thrombosis (HAT) occurring after OLT were also excluded.

Statistical analyses

Statistical analysis was performed using SPSS 20.0. A Student t-test was used for normally distributed continuous variables, Mann-Whitney U test was used for variables that were not normally distributed, and Chi-square test was done for categorical variables. The optimal cutoff-value for peak ALT is defined as the point with the most significant split for association with NAS or no NAS as determined by log-rank test. Using the calculated cut-off value, a peak ALT below this value was considered as mild IRI whereas a peak ALT above this value was considered as severe IRI. Cumulative incidence curves were established using one minus survival incidence rates according to the Kaplan-Meier method and risk factor analysis was performed using univariate and multivariate stepwise forward Cox regression analysis. If a p-value of <0.20 was found in the univariate analysis the parameter was taken into account in the multivariate analysis. A p-value of <0.05 was considered statistically significant.

Results

Cohort A consisted of 176 OLTs, with 138 DBD donor livers and 38 DCD donor livers. Cohort B consisted of 322 OLTs with 263 DBD donor livers and 59 DCD donor livers. Median follow-up in cohort A from OLT until development of NAS was 5.0 months (range 1 – 84) and median follow-up in cohort B from OLT until development of NAS was 4.9 months (range 0.3 – 57).

Donor and surgical variables

Median donor age for DCD donors was significantly lower compared to DBD donors in cohort A (45 vs. 51, respectively; p=0.002) as well in cohort B (42 vs. 50, respectively; p=0.003). Cold ischemic time (CIT) was significantly shorter for DCD donors than DBD donors in cohort A (DBD 598 min vs. DCD 498 min; p<0.01) and a similar trend was seen in cohort B (DBD 468 min vs. DCD 451 min; p=0.110). Recipient warm ischemia time (RWIT) was not significantly different for both types of donors in both cohorts. Median donor warm ischemia time (DWIT) in case of DCD-OLT was 17 minutes in both cohorts. Median peak ALT was significantly higher after OLT using DCD donors than after DBD-OLT in cohort A (DBD: 543 IU/L vs. DCD: 1172 IU/L; p<0.001) and the same outcome was seen in cohort B (DBD: 814 IU/L vs. DCD: 1783 IU/L; p<0.001). Similar results were observed for median peak AST after DCD-OLT compared to DBD-OLT in cohort A (DBD: 1004 IU/L vs. 2048 IU/L, p<0.001) as well cohort B (DBD: 1066 IU/L vs. DCD 2618 IU/L, p<0.001). Due to the strong correlation between peak AST and peak ALT further analysis was performed for peak ALT only (Pearson's coefficient=0.845, p<0.001). In general both cohorts were comparable but some incidental differences were observed between cohort A and B. For instance, CIT was significantly longer for DBD-OLT in cohort A compared to cohort B (p<0.001). In cohort B, peak ALT was significantly higher after DBD-OLT compared to cohort A (p<0.001). Though a trend to a higher rate of NAS development was seen in cohort A compared to cohort B this did not reach statistical difference (χ^2=2.35, p=0.184). *(Table 1)*

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

Characteristic	Cohort A (n=176)		*p*	Cohort B (n=322)		*p*
	DBD (n=138)	DCD (n=38)		DBD (n=263)	DCD (n=59)	
Donor age (median, range)	51 (16–78)	45 (15–70)	**0.002**	50 (14–86)	42 (14–65)	**0.003**
Donor gender % (n)			0.103			0.249
Male	47.8 (66)	63.2 (24)		50.2 (132)	59.3 (35)	
Female	52.2 (72)	36.8 (14)		49.8 (131)	40.7 (24)	
Recipient age (median, range)	53 (21–70)	54 (18–69)	0.978	52 (17–68)	54 (19–68)	0.168
Gender % (n)			0.109			0.650
Male	66.7 (92)	80.5 (33)		57.8 (152)	61.0 (36)	
Female	33.3 (46)	19.5 (8)		42.2 (111)	39.0 (23)	
MELD (median, range)	18 (6–40)	19 (6–35)	0.711	15 (6–40)	12 (7–40)	0.079
Diagnosis preOLT % (n)			0.908			0.760
ALD	20 (28)	19.5 (7)		15.2 (40)	13.6 (8)	
HCC	25 (35)	29.3 (10)		2.7 (7)	1.7 (1)	
PSC	10 (12)	12.2 (5)		20.9 (55)	20.3 (12)	
PBC	2.9 (4)	5.3 (2)		5.7 (15)	8.5 (95)	
HBV	5.7 (8)	2.4 (1)		5.3 (14)	1.7 (1)	
AIH	2.9 (4)	2.6 (1)		8.0 (21)	5.1 (3)	
HCV	12.1 (17)	12.2 (5)		8.3 (21)	10.2 (6)	
Metabolic	4.3 (6)	7.3 (3)		8.7 (23)	6.8 (4)	
Other	17.1 (24)	9.8 (4)		25.5 (67)	32.2 (19)	
NAS % (n)	12.3 (17)	42.1 (16)	**<0.001**	14.8 (39)	27.1 (16)	**0.034**
CIT (median, range)	598 (268–1090)	498 (296–728)	**<0.001**	468 (150–854)	451 (318–580)	0.110
RWIT (median, range)	33 (16–71)	33 (20–53)	0.873	45 (27–93)	44 (29–79)	0.666
DWIT (median, range)	-	17 (11–31)		-	17 (7–78)	
Peak AST (median, range)	1004 (46–10454)	2048 (200–11808)	**<0.001**	1066 (64–14750)	2618 (221–19590)	**<0.001**
Peak ALT (median, range)	543 (7–4483)	1172 (89–4664)	**<0.001**	814 (69–8242)	1783 (219–11105)	**<0.001**

DBD = Donation after brain death, DCD = Donation after cardiac death, MELD = Model for End-Stage Liver Disease, OLT = Orthotopic Liver Transplantation, ALD = Alcoholic Liver Disease, HCC = Hepatocellular Carcinoma, PSC = Primary Sclersoing Cholangitis, PBC = Primary Biliary Cirrhosis, HBV = Hepatitis B Virus, AIH = Auto-Immune Hepatitis, HCV = Hepatitis C Virus, NAS = Nonanastmotic strictures, CIT = Cold ischemic time, RWIT = Recipient warm ischemic time, DWIT = Donor warm ischemia time, AST = Aspartate aminotransferase, ALT = Alanine aminotransferase

IRI and NAS

In the combined cohort NAS developed in 33.0% after DCD-OLT and in 14.0% after DBD-OLT (χ^2=19.43, p<0.001). Optimal cut-off value of serum ALT was calculated using log-rank statistics in the combined cohort (A and B) for DCD-OLT (n=97) and was established at ≥1300 IU/L *(supplemental figure 1)*. Using the calculated cut-off value, a low peak ALT of <1300 IU/L was considered as mild IRI whereas a high peak ALT ≥1300 IU/L was considered as severe IRI. After DCD-OLT, severe IRI preceded NAS development in 49.1% cases compared to 10.0% in the mild IRI group (χ^2=16.27, p<0.001). *(Table 2)* Four-year cumulative incidence (CLI) of NAS development was 51.4% in case of severe IRI compared to 11.6% when mild IRI occurred after DCD-OLT (log-rank p<0.001; *Figure 1a*). No association could be found between peak ALT ≥1300 IU/L and NAS after DBD-OLT, neither in the individual cohorts nor for the combined group. In addition, no other optimal cut-off value for NAS development after DBD-OLT could be identified. Based on the cut-off value determined for DCD-OLT four-year cumulative incidence of NAS after DBD-OLT in the combined group was 9.4% when peak ALT was ≥1300 IU/L compared to 6.0% when peak ALT was <1300 IU/L (log-rank p=0.999; *Figure 1b*). The defined cut-off value of peak ALT ≥1300 IU/L was also evaluated after DCD-OLT in the participating cohorts individually. NAS developed significantly more often following severe IRI after DCD-OLT than after mild IRI in cohort A (73.7% vs. 10.5%; χ^2=15.55, p<0.001) and similar results were seen in cohort B (36.8% vs. 9.5%; χ^2=5.11, p=0.032). Four-year CLI of development of NAS after DCD-OLT in cohort A was 74% when severe IRI preceded compared to 12.5% for mild IRI (log-rank p<0.001; *Figure 2a*). Cohort B revealed similar incidence rates for development of NAS after DCD-OLT of 39.1% after severe IRI compared to 11.1% in case of mild IRI (log-rank p=0.056; *Figure 2b*).

Univariate and Multivariate analysis

Cox regression analysis for risk of NAS development was performed for DCD and DBD donors separately. For DCD-OLT, donor age, CIT and peak ALT ≥1300 were significantly associated with NAS in univariate analysis at the

p<0.20 value and were thus included in the multivariate analysis. PSC as indication for OLT, as well as the center at which OLT was performed were not associated with development of NAS in the univariate analysis for DCD-OLT. Multivariate analysis showed peak ALT ≥1300 to be the single independently associated factor for the development of NAS after DCD-OLT, adjusted for CIT and donor age. (adjusted hazard ratio (aHR) = 4.96, confidence interval (CI) = 1.73 – 14.24), p=0.003) *(Table 3)*. Multivariate analysis revealed PSC as indication for OLT to be the only independently associated parameter for the development of NAS after DBD-OLT (aHR = 2.18, CI = 1.22 – 3.88, p = 0.008). *(Table 4)*

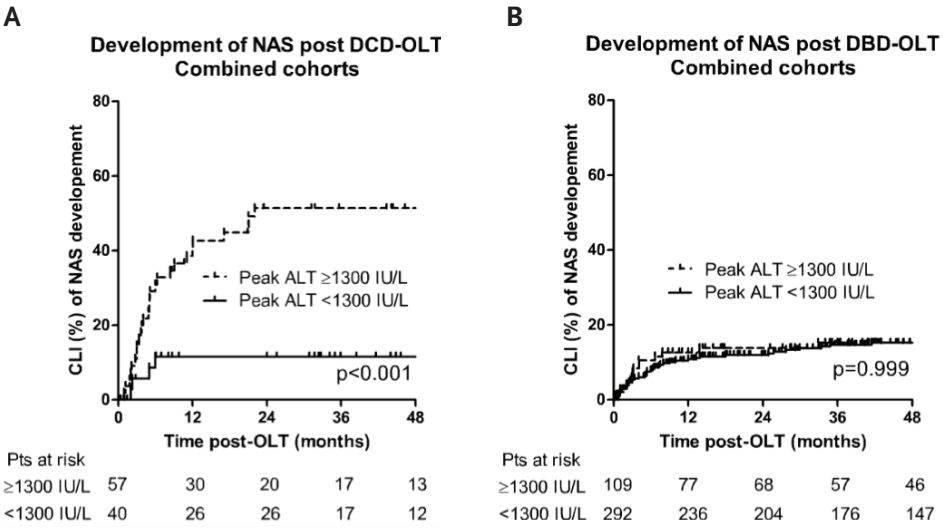
Table 2. Two-by-two table representing development of NAS respective of degree of ischema-reperfusion injury (IRI).

	Combined cohorts (A and B)			
Graft type	DBD		DCD	
	No NAS % (n)	NAS % (n)	No NAS % (n)	NAS % (n)
Degree IRI				
Mild	85.6 (250)	14.4 (42)	90.0 (36)	10.0 (4)
Severe	87.2 (95)	12.8 (14)	50.9 (26)	49.1 (28)
	χ^2=0.16,p=0.749		**χ^2=16.27,p=<0.001**	

	Cohort A				Cohort B			
Graft type	DBD		DCD		DBD		DCD	
	No NAS % (n)	NAS % (n)	No NAS % (n)	NAS % (n)	No NAS % (n)	NAS % (n)	No NAS % (n)	NAS % (n)
Degree IRI								
Mild	87.3 (96)	12.7 (14)	89.5 (17)	10.5 (2)	84.6 (154)	15.4 (28)	90.5 (19)	9.5 (2)
Severe	89.3 (25)	10.7 (3)	26.3 (5)	73.7 (14)	86.4 (70)	13.6 (11)	63.2 (24)	36.8 (14)
	χ^2=0.08,p=1.000		**χ^2 =15.55,p<0.001**		χ^2=0.14,p=0.851		χ^2=5.11,p=0.032	

DBD = Donation after Brain Death, DCD = Donation after Cardiac Death, NAS = Nonanastomotic strictures, IRI = Ischemia-reperfusion Injury

Figure 1



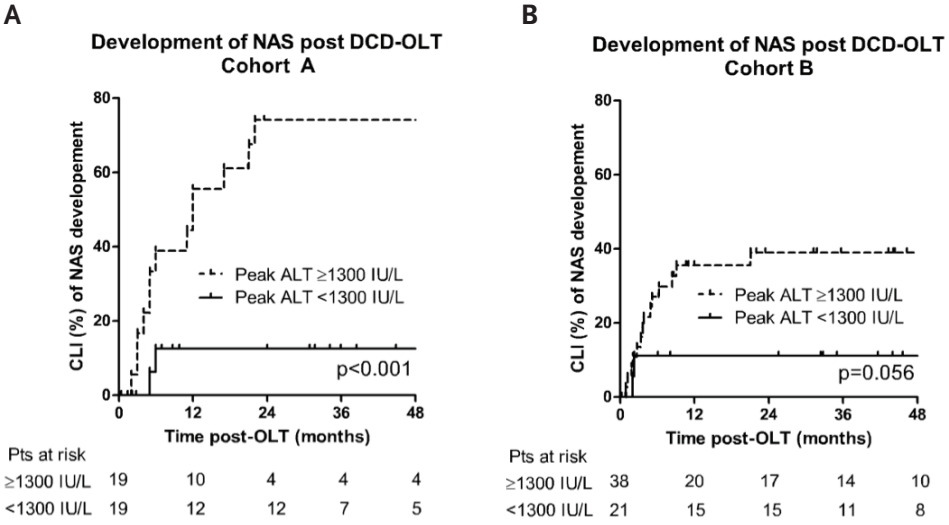
Cumulative incidence (CLI) of non-anastomotic strictures (NAS) development after orthotopic liver transplantation (OLT) with donor livers from donation after cardiac death (DCD) (A) reached 51.4% at 48 months when peak alanine aminotransferase (ALT) ≥1300 IU/L (n=57) compared to 11.6% when peak ALT <1300 IU/L (n=40) (p<0.001). (B) CLI rates of NAS after DBD-OLT did not differ between recipients with mild or severe ischemia-reperfusion injury (IRI). CLI rates were calculated using one minus survival incidence rates with the Kaplan-Meier test and compared using log-rank test.

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

			Univariate analysis		Multivariate analysis	
Variables DCD -OLT		% (n)	HR (95% CI)	p-value	HR (95% CI)	p-value
Donor age	Continuous		1.03 (1.00 – 1.06)	0.044	1.02 (1.00 – 1.05)	0.101
Donor gender	Male	60.8 (59)	1.48 (0.71 – 3.07)	0.295		
	Female (reference)	39.2 (38)	1 (reference)			
Recipient age at OLT	Continuous		0.79 (0.97 – 1.04)	0.791		
Recipient gender	Male	69.1 (67)	0.78 (0.37 – 1.61)	0.496		
	Female (reference)	30.9 (30)	1 (reference)			
MELD score	Continuous		1.02 (0.99 – 1.06)	0.240		
Peak ALT	Severe ≥ (1300 IU/L)	58.8 (57)	5.51 (1.93 – 15.73)	**0.001**	4.96 (1.73 – 14.24)	**0.003**
	Mild (<1300 IU/L)	41.2 (40)	1 (reference)			
CIT	Continuous		1.00 (0.99 – 1.01)	0.165	1.00 (1.00 – 1.01)	0.259
RWIT	Continuous		0.98 (0.95 – 1.02)	0.317		
DWIT	Continuous		1.01 (0.98 – 1.04)	0.581		
PSC as indication	PSC	17.5 (17)	1.29 (0.50 – 3.34)	0.604		
	Other indications	82.5 (80)	1 (reference)			
Study Center	Cohort B	60.8 (59)	0.66 (0.33 – 1.31)	0.232		
	Cohort A	39.2 (38)	1 (reference)			

HR = Hazard ratio, CI = Confidence intervals, OLT=Orthotopic liver transplantation, MELD = Model for End-stage Liver Disease, ALT = Alanine aminotransferase, DWIT= Donor warm ischemic time, RWIT = Recipient warm ischemic time, CIT = Cold ischemic time, PSC = Primary Sclerosing Cholangitis

Figure 2



Cumulative incidence (CLI) rates of non-anastomotic strictures (NAS) development respective of degree of ischemia-reperfusion injury (IRI) in cohort A (A) (n=38) and cohort B (B) (n=59). CLI rates were calculated using one minus survival incidence rates with the Kaplan-Meier test and compared using log-rank test.

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

Variables DBD-OLT		% (n)	Univariate analysis HR (95% CI)	p-value	Multivariate analysis HR (95% CI)	p-value
Donor age	Continuous		1.01 (0.99 – 1.03)	0.251		
Donor gender	Male	49.4 (198)	0.99 (0.59–1.67)	0.991		
	Female (reference)	50.6 (203)	1 (reference)			
Recipient age at OLT	Continuous		0.98 (0.96–1.00)	0.080	0.98 (0.96 – 1.01)	0.140
Recipient gender	Male	60.8 (244)	1.68 (0.95 – 3.03)	0.074	1.66 (0.92 – 3.00)	0.091
	Female (reference)	39.2 (157)	1 (reference)		1 (reference)	
MELD score	Continuous		1.00 (0.97–1.03)	0.979		
Peak ALT	Severe ≥ (1300 IU/L)	72.8 (292)	1.00 (0.55 – 1.83)	1.000		
	Mild (<1300 IU/L)	27.2 (109)	1 (reference)			
CIT	Continuous		1.00 (1.00–1.00)	0.836		
RWIT	Continuous		1.01 (0.98 – 1.03)	0.553		
PSC as indication	PSC	16.7 (67)	2.52 (1.44 – 4.41)	**0.001**	2.18 (1.22 – 3.88)	**0.008**
	Other indications	83.3 (334	1 (reference)		1 (reference)	
Study Center	Cohort B	65.6 (263)	1.17 (0.66 – 2.07)	0.586		
	Cohort A	34.4 (138)	1 (reference)			

HR = Hazard ratio, CI = Confidence intervals, OLT=Orthotopic liver transplantation, MELD=Model for End-stage Liver Disease, ALT = Alanine aminotransferase, DWIT= Donor warm ischemic time, RWIT = Recipient warm ischemic time, CIT = Cold ischemic time, PSC = Primary Sclerosing Cholangitis

Discussion

The current data shows that ischemia-reperfusion injury (IRI), defined as peak serum ALT ≥1300 IU/L post OLT is strongly associated with the development of NAS after DCD-OLT with a 4-year cumulative incidence rate of NAS of 51.4%. The incidence of NAS in DCD-OLT in case of peak ALT <1300 IU/L was not different from the incidence of NAS in DBD-OLT. In the multivariate analysis peak ALT ≥1300 IU/L was independently associated with the development of NAS after DCD-OLT. This association was observed after adjustment not only for ischemia times, PSC as indication for OLT, recipient gender and recipient age, but also the center at which OLT was performed, which were no independent risk factors. After DBD-OLT, peak ALT was not associated with the development of NAS, whereas PSC was a risk factor in these patients.

NAS is a common problem after OLT leading to considerable morbidity and mortality. It has been hypothesized that IRI may play an important role in the development of NAS.(15) DCD grafts are known to be more prone to IRI due to an additional donor warm ischemic time (DWIT) and to have more biliary complications after OLT. (8) Several markers have been associated with the development of NAS after OLT such as cold ischemic time (CIT), recipient warm ischemic time (RWIT) but also chemokine receptors such as chemokine receptor 5 Δ32 (CCR5Δ32) and matrix metalloproteinase-2. (MMP-2)[16,17] In The Netherlands currently about 20% of OLT is with DCD donation since in 2001 a strict national protocol was implemented (18). An incentive was the increasing demand of donor livers and decreasing DBD donation in our country. In this national protocol only Maastricht category 3 donors below 55 years of age, with a DWIT below 30 minutes, a body mass index <28, and a mean arterial pressure <50 mm Hg for maximum 15 minutes were accepted, which excludes about 25% of DCD donors. In addition, CIT was kept as short as possible which explains the shorter CIT in DCD versus DBD OLT. These livers were allocated according to the regular waiting list. Using these criteria we reported in a previous study similar one- and 3-year patient survival for DCD (85% and 80%, respectively) and DBD (86.3% and 80.8%, respectively) OLT, and graft survival rates that did not differ

significantly (74% and 68% versus 80.4% and 74.5%, respectively).[3] The role of prolonged ischemia times is most extensively described for DBD-OLT but definitions of CIT and RWIT vary between centers and there are mixed results on using ischemic times as potential predictors for NAS after DBD-OLT and relatively few data exist on potential predictors for development of NAS after DCD-OLT.[19-21]

Ischemia times, especially CIT, are kept shorter for DCD compared to DBD in order to reduce IRI and hopefully compensate for DWIT-induced injury. However, ischemia times are not indicative for reperfusion damage and there is evidence that most IRI-induced hepatic injury develops due to an excess of reactive oxygen species after restoration of blood flow.[22-24] Serum AST and ALT peaks within the first 7 days after DBD-OLT and DCD-OLT are considered markers of IRI.[25,26] Despite a strict protocol in reducing CIT and RWIT for DCD donors, OLT with DCD donors still have higher peak AST and ALT and more NAS post-operatively than DBD-OLT, indicating more IRI in DCD-OLT. Microthrombi in the peri-biliary plexus and sinusoids may develop during the DWIT, and may be partially responsible for development of NAS and high ALT respectively in DCD-OLT. [27,28] Current preservation solutions and techniques may be insufficient to flush out all microthrombi. Several attempts are being made to improve preservation and reduce IRI of liver grafts using machine liver perfusion, but also fibrinolytic agents are used to dissolve microthrombi in the donor liver.[15,29] Other factors than microthombi may also be responsible for both the increased incidence of NAS and higher ALT in DCD-OLT as compared to DBD-OLT. Current data indicate that reducing IRI in DCD-OLT to the extent that peak ALT is below 1300 UI/L will probably diminish the incidence of NAS to around 10%, but will not completely eliminate NAS. Likewise since after DBD-OLT peak ALT ≥1300 IU/L was not a predictive factor for the development of NAS, other factors than IRI probably play a role in the development of NAS in liver grafts from DBD donors. This is consistent with the idea that NAS is most likely the result of a complex mechanism involving ischemic, immunologic and toxic processes which all affect the biliary tree or vascular supply.[27,30,31]

The current study has certain limitations. We used only ALT and not AST as marker for IRI occurring in the liver. AST is derived from mitochondria in liver cells but is also produced in heart, skeletal muscles and brain cells. After surgery, AST can also be elevated due to damage of the abdominal muscles during surgery, and this makes it less specific as a parameter of IRI after OLT. Furthermore, CCR5Δ32 determination and MMP-2 polymorphisms are not included although described by us in the past. These might prove useful in combination with ALT as risk factors for NAS, but are less readily available. The exact role of CCR5Δ32 needs to be determined in larger cohorts, but studies have shown that this receptor may be involved in the late occurrence of NAS. This may indicate that several entities of NAS may exist and further studies should be performed in this field.[32,33]

In conclusion, our data show that serum peak ALT ≥1300 IU/L is strongly and independently associated with the development of clinically relevant NAS after DCD-OLT and can be used in classifying patients as high-risk or low-risk for developing NAS. The current data indicate that the higher risk of NAS after DCD as compared to DBD is likely the result of more severe IRI due to DWIT in DCD-OLT when compared to DBD-OLT. Our observations also imply that in DBD-OLT and in DCD-OLT with a low peak ALT other mechanisms than in DCD-OLT with high peak ALT (i.e. ≥1300 IU/L) may play an important role in development of NAS.

References

1. Adam R, McMaster P, O'Grady JG, Castaing D, Klempnauer JL, Jamieson N, et al. Evolution of liver transplantation in Europe: report of the European Liver Transplant Registry. Liver Transpl 2003;9(12):1231-1243.

2. Jain A, Reyes J, Kashyap R, Dodson SF, Demetris AJ, Ruppert K, et al. Long-term survival after liver transplantation in 4,000 consecutive patients at a single center. Ann Surg 2000;232(4):490-500.

3. Dubbeld J, Hoekstra H, Farid W, Ringers J, Porte RJ, Metselaar HJ, et al. Similar liver transplantation survival with selected cardiac death donors and brain death donors. Br J Surg 2010;97(5):744-753.

4. Merion RM, Pelletier SJ, Goodrich N, Englesbe MJ, Delmonico FL. Donation after cardiac death as a strategy to increase deceased donor liver availability. Ann Surg 2006;244(4): 555-562.

5. Buis CI, Hoekstra H, Verdonk RC, Porte RJ. Causes and consequences of ischemic-type biliary lesions after liver transplantation. J Hepatobiliary Pancreat Surg 2006;13(6):517-524.

6. Foley DP, Fernandez LA, Leverson G, Chin LT, Krieger N, Cooper JT, et al. Donation after cardiac death: the University of Wisconsin experience with liver transplantation. Ann Surg 2005;242(5):724-731.

7. Foley DP, Fernandez LA, Leverson G, Anderson M, Mezrich J, Sollinger HW, et al. Biliary complications after liver transplantation from donation after cardiac death donors: an analysis of risk factors and long-term outcomes from a single center. Ann Surg 2011;253(4):817-825.

8. Meurisse N, Vanden Bussche S, Jochmans I, Francois J, Desschans B, Laleman W, et al. Outcomes of liver transplantations using donations after circulatory death: a single-center experience. Transplant Proc 2012;44(9):2868-2873.

9. Pirenne J, Van GF, Coosemans W, Aerts R, Gunson B, Koshiba T, et al. Type of donor aortic preservation solution and not cold ischemia time is a major determinant of biliary strictures after liver transplantation. Liver Transpl 2001;7(6):540-545.

10. Heidenhain C, Pratschke J, Puhl G, Neumann U, Pascher A, Veltzke-Schlieker W, et al. Incidence of and risk factors for ischemic-type biliary lesions following orthotopic liver transplantation. Transpl Int 2010;23(1):14-22.

11. Serracino-Inglott F, Habib NA, Mathie RT. Hepatic ischemia-reperfusion injury. Am J Surg 2001;181(2):160-166.

12. Guichelaar MM, Benson JT, Malinchoc M, Krom RA, Wiesner RH, Charlton MR. Risk factors for and clinical course of non-anastomotic biliary strictures after liver transplantation. Am J Transplant 2003;3(7):885-890.

13. Pascher A, Neuhaus P. Bile duct complications after liver transplantation. Transpl Int 2005;18(6):627-642.

14. Cameron AM, Busuttil RW. Ischemic cholangiopathy after liver transplantation. Hepatobiliary Pancreat Dis Int 2005;4(4):495-501.

15. Lang R, He Q, Jin ZK, Han DD, Chen DZ. Urokinase perfusion prevents intrahepatic ischemic-type biliary lesion in donor livers. World J Gastroenterol 2009 28;15(28):3538-3541.

16. Iacob S, Cicinnati VR, Dechene A, Lindemann M, Heinemann FM, Rebmann V, et al. Genetic, immunological and clinical risk factors for biliary strictures following liver transplantation. Liver Int 2012;32(8):1253-1261.

17. Ten Hove WR, Korkmaz KS, op den DS, de Rooij BJ, van HB, Porte RJ, et al. Matrix metalloproteinase 2 genotype is associated with nonanastomotic biliary strictures after orthotopic liver transplantation. Liver Int 2011;31(8):1110-1117.

18. Dubbeld J, van Hoek B., Ringers J. Use of a liver from donor after cardiac death: is it appropriate for the sick or the stable? Curr Opin Organ Transplant 2011;16(2):239-242.

19. Segev DL, Kucirka LM, Nguyen GC, Cameron AM, Locke JE, Simpkins CE, et al. Effect modification in liver allografts with prolonged cold ischemic time. Am J Transplant 2008;8(3):658-666.

20. Nakamura N, Nishida S, Neff GR, Vaidya A, Levi DM, Kato T, et al. Intrahepatic biliary strictures without hepatic artery thrombosis after liver transplantation: an analysis of 1,113 liver transplantations at a single center. Transplantation 2005 27;79(4):427-432.

21. Padbury RT, Attard A, Mirza DF, Olliff S, Gunson BK, Mayer AD, et al. Extended preservation of the liver with UW solution--is it justifiable? Transplantation 1994 27;57(10):1490-1493.

22. Schlegel A, Rougemont OD, Graf R, Clavien PA, Dutkowski P. Protective mechanisms of end-ischemic cold machine perfusion in DCD liver grafts. J Hepatol 2012 11.

23. van Golen RF, van Gulik TM, Heger M. Mechanistic overview of reactive species-induced degradation of the endothelial glycocalyx during hepatic ischemia/reperfusion injury. Free Radic Biol Med 2012 15;52(8):1382-1402.

24. Schlegel A, Rougemont O, Graf R, Clavien PA, Dutkowski P. Protective mechanisms of end-ischemic cold machine perfusion in DCD liver grafts. J Hepatol 2013;58(2):278-286.

25. Shaked A, Nunes FA, Olthoff KM, Lucey MR. Assessment of liver function: pre- and peritransplant evaluation. Clin Chem 1997;43(8 Pt 2):1539-1545.

26. Glanemann M, Langrehr JM, Stange BJ, Neumann U, Settmacher U, Steinmuller T, et al. Clinical implications of hepatic preservation injury after adult liver transplantation. Am J Transplant 2003;3(8):1003-1009.

27. op den DS, Sutton ME, Lisman T, Porte RJ. Protection of bile ducts in liver transplantation: looking beyond ischemia. Transplantation 2011 27;92(4):373-379.

28. Chan EY, Olson LC, Kisthard JA, Perkins JD, Bakthavatsalam R, Halldorson JB, et al. Ischemic cholangiopathy following liver transplantation from donation after cardiac death donors. Liver Transpl 2008;14(5):604-610.

29. Hashimoto K, Eghtesad B, Gunasekaran G, Fujiki M, Uso TD, Quintini C, et al. Use of tissue plasminogen activator in liver transplantation from donation after cardiac death donors. Am J Transplant 2010;10(12):2665-2672.

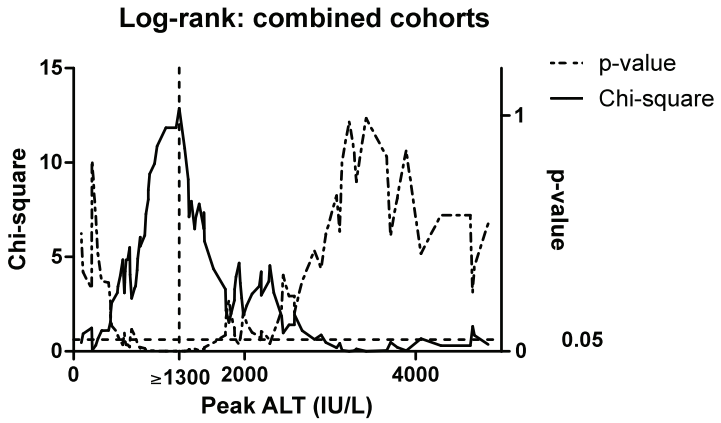
30. Buis CI, Geuken E, Visser DS, Kuipers F, Haagsma EB, Verkade HJ, et al. Altered bile composition after liver transplantation is associated with the development of nonanastomotic biliary strictures. J Hepatol 2009;50(1):69-79.

31. Yska MJ, Buis CI, Monbaliu D, Schuurs TA, Gouw AS, Kahmann ON, et al. The role of bile salt toxicity in the pathogenesis of bile duct injury after non-heart-beating porcine liver transplantation. Transplantation 2008 15;85(11):1625-1631.

32. Heidenhain C, Puhl G, Moench C, Lautem A, Neuhaus P. Chemokine Receptor-5Delta32 Mutation is No Risk Factor for Ischemic-Type Biliary Lesion in Liver Transplantation. J Transplant 2009;2009:436515.

33. op den Dries S, Buis CI, Adelmeijer J, van der Jagt EJ, Haagsma EB, Lisman T, et al. The combination of primary sclerosing cholangitis and CCR5-Delta32 in recipients is strongly associated with the development of nonanastomotic biliary strictures after liver transplantation. Liver Int 2011;31(8):1102-1109.

Supplemental Figure 1



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

Chapter 4

Sequential liver chemistry profiling and abdominal ultrasound assessments to predict biliary strictures after liver transplantation

Kerem Sebib Korkmaz[1], W. Rogier ten Hove[1], Hein W. Verspaget[1], Jeroen Dubbeld[2], Ron Wolterbeek[3], Arian van Erkel[4], Bert-Jan F. de Rooij1, Minneke C. Coenraad[1], Jan Ringers[2], Bart van Hoek[1]

Departments of Gastroenterology and Hepatology[1], Transplantation Surgery[2], Medical Statistics[3] and Interventional Radiology[4], Leiden University Medical Center, Leiden, The Netherlands

The Open Transplantation Journal 2012;22(5):1-5

Abstract

Background: After orthotopic liver transplantation (OLT) early detection of biliary strictures is important. Our aim was to evaluate the predictive value of routine serum liver chemistry profiling and abdominal ultrasound as non-invasive diagnostic tools in detecting biliary strictures after OLT.

Methods: We performed a retrospective study in which 141 primary OLTs, performed between 1992 and 2007 with more than 1 year follow-up, were included. Routinely assessed serum levels of alkaline phosphatase, alanine-aminotransferase, aspartate-aminotransferase, gamma-glutamyl transpeptidase and bilirubin at 3, 6, 9 and 12 months, and abdominal ultrasounds performed at 3, 6 and 12 months after OLT were evaluated. All biliary strictures requiring intervention occurring after 3 months were included. Time-dependent Cox regression analysis was performed to identify predictive factors for the development of biliary strictures.

Results: Eighteen grafts developed non-anastomotic strictures (12.8%) and 18 grafts (12.8%) developed anastomotic strictures requiring intervention. An elevated gamma-glutamyl transpeptidase (HR 1.24 per 100 IU/L; $p = 0.05$) and dilated bile ducts on ultrasound (HR 3.45; $p < 0.01$) were found to have an independent predictive value for the development of biliary strictures requiring intervention. Bilirubin and the other studied liver enzymes were not independently predictive.

Conclusion: Dilated bile ducts on ultrasound and elevated gamma-glutamyltranspeptidase after OLT are independent predictive factors for the development of biliary strictures requiring intervention. Routine assessment by serum gGT and US at 3-month intervals during the first year post-OLT is useful to screen for biliary strictures post-OLT.

Introduction

Biliary complications are common after orthotopic liver transplantation (OLT), with a reported prevalence of 6% to 35%.[1-4] Biliary strictures occurring at the surgical anastomosis are classified as anastomotic strictures (AS), whereas strictures in the donor biliary tree are referred to as non-anastomotic strictures (NAS). Stricture formation is often insidious and usually only then detected when it leads to clinical symptoms as cholestasis, with serum liver enzyme abnormalities, intrahepatic bile duct dilatation and/or infection.[5] The definite diagnosis is made by endoscopic retrograde cholangiopancreatography (ERCP), percutaneous transhepatic cholangiography (PTC) or by magnetic resonance cholangiopancreatography (MRCP).[6,7] Although ERCP and PTC are considered the gold standard, they are invasive procedures and associated with considerable morbidity. ERCP and PTC are often immediately performed when clinical symptoms such as jaundice or cholangitis are present.8 Several uncontrolled series evaluated the efficacy of ERCP in predicting biliary complications.[8,9] Most of these were evaluated in settings where patients present themselves with symptoms such as cholangitis due to a biliary stricture. It is therefore important to detect early signs of stricture formation before such symptoms develop. Transplantation centers differ in how they routinely assess these problems. Liver biochemistry and abdominal ultrasound (US) may be useful for early detection of rejection, recurrence of primary disease (e.g., primary sclerosing cholangitis (PSC) or hepatitis) and bile duct pathology, such as stricture formation, since abnormalities may be present long before patients develop symptoms such as fever or abdominal pain. Only few studies addressed the predictive value of routinely assessed serum liver chemistry profiles and abdominal ultrasonography (US) after OLT as predictors for the occurrence of biliary strictures in a post-transplant population.10 Although some studies did evaluate the prognostic value of liver chemistry and US, this was not in a time-dependent way and it remained unclear whether clinicians should routinely assess these diagnostic modalities in a post-transplant population for the early detection of biliary strictures, and at what time interval.[10,11] The risk of developing biliary strictures varies over time, probably in association with the liver chemistry profile and US findings. The aim of the present study was to evaluate the

predictive value of routinely assessed serum liver chemistry and abdominal ultrasound as non-invasive predictors for the development of biliary strictures requiring intervention after OLT.

Patients and Methods

Patients

We examined 141 consecutive first OLTs with at least one year of follow-up and complete data on serum liver chemistry and enzymes and upper abdominal ultrasonography (US) performed between September 1992 and April 2007 at the Leiden University Medical Centre. Re-transplantations (n=31) were excluded. Clinical data were obtained from the medical digital records, the hepatological and surgical patient charts, and endoscopy reports. Followup was up to August 2008 with a median of 5.2 years (range 1.0 -15.6). OLT was performed according to standard procedures with cavo-caval, porto-portal, and hepatic artery to hepatic artery anastomosis. A duct-to-duct biliary anastomosis over a 8-12 Ch stent was performed, if possible. The biliary stent was removed after 6 weeks or removed earlier as indicated. In some cases the hepatic artery was anastomosed to the aorta via an iliac conduit. All patients received immunosuppressive agents according to protocol: cyclosporin A or tacrolimus, prednisone during the first half-year and patients with renal impairment received azathioprine before 2001 or mycophenolate mofetil from 2001 on. From 2001 on, basiliximab was given post OLT. In some cases sirolimus was used after month 3 in which case the calcineurin-inhibitor was discontinued. All patients received ursodeoxycholic acid in the first 3 months after transplantation.

Biochemical variables

Serum liver enzyme levels of alkaline phosphatase (ALP), alanine-aminotransferase (ALAT), aspartate-aminotransferase (ASAT) and gamma-glutamyltranspeptidase (GGT) were determined daily during the first two weeks and weekly for two months, after that at 3, 6, 9, and 12 months post-operatively. The same was done for bilirubin. Only the latter 4 time points were included in the study because in the first three months after liver transplantation liver enzymes are very susceptible to change due to procedure-related causes such as ischemia-reperfusion damage, early vascular complications such as hepatic artery thrombosis, rejection and infections. Therefore, the first three months

of liver chemistry assessments, ultrasounds and biliary strictures after transplantation were excluded. The upper limit of normal serum level was for ALP 120 IU/L, for ASAT, ALAT and GGT the upper limits of normal were 40 IU/L, 40 IU/L and 51 IU/L, respectively. The upper limit for bilirubin (total) was 17 μmol/L.

Imaging variables

US was performed routinely on day 0, 1 and 7, and subsequently at 3, 6 and 12 months after OLT. The USs performed at 3, 6 and 12 months were included in this study. These US were performed by different experienced radiologists. Due to lack of clear definition in the literature of dilated intrahepatic bile ducts a clear definition has been used by our radiologists with expertise in liver transplantation. In our institute a common bile duct of >7 mm and an intrahepatic bile duct of >2mm on ultrasound were considered dilated and prompted either direct intervention by ERCP or PTC or additional MRCP which in turn might prompt ERCP or PTC. Other reported ultrasound findings of the biliary tree, such as sludge or a thickened biliary wall, were not taken into account in our analysis Routine abdominal CT scan was performed after 3 to 7 days post-OLT and routine liver biopsies were performed at 6 months after transplantation. Additional liver biopsies were taken on indication. Virology monitoring, including CMV-DNA, was performed frequently in the first year.

Clinical variables

Presentation of a biliary stricture (anastomotic and non-anastomotic) was with clinical symptoms such as cholangitis, pruritus or jaundice and/or abnormal liver chemistry. Diagnostic tests to confirm the diagnosis were performed on indication but not included in the present analyses. Only strictures occurring more than three months after OLT that required intervention by ERCP, PTC or surgery were included in this study. From 2001 on, routine ERCP with stent-removal was performed at week 6 post-OLT in case of a duct-to-duct anastomosis. Accompanying the stent removal, a cholangiogram was also performed and possible strictures occurring at this time-point

were excluded due to exclusion of liver enzymes and ultrasound findings in the first three months for the above-mentioned reasons. All strictures were treated endoscopically with ERCP and dilation and/or stenting, and percutaneously with percutaneous transhepatic cholangiodrainage (PTCD) or with surgical intervention.

Statistical analyses

We have used a time-dependent Cox regression model to evaluate the diagnostic value of liver enzymes and routine US assessments in predicting biliary strictures. The Cox proportional-hazards regression model for time-to-event data (e.g. the development of biliary strictures) takes into account the variable changes of variables over time, e.g. changes of liver enzymes and bile duct dilatation on US. Time dependent predictors (covariates) for stricture development in this study were liver chemistry variables ALP, GGT, ASAT, ALAT and bilirubin, obtained with an interval of three months at 3, 6, 9 and 12 months post OLT and US performed 3, 6 and 12 months post OLT. Recipient characteristics, like etiology of liver disease and procedure-related variables were baseline characteristics. Variables at a $p<0.20$ level in the univariate analysis were included in the multivariate analysis. Coefficients were considered significant when $p<0.05$ in the multivariate analysis. The reported hazard ratios and p-values are per 100 international unit elevation for the liver enzymes. Bilirubin levels are reported per 10 μmol/L elevation.

Ethical committee

All data were obtained as part of patient care according to a strict protocol after OLT. There was permission from the local ethics committee to use these data.

Results

Patients and biliary strictures

Baseline characteristics of recipients like etiology of liver disease and procedure-related variables are presented in *Table 1*. Non-anastomotic biliary strictures requiring intervention developed in 18 of the 141 grafts (12.8%). Median time from OLT to NAS was 8.5 months (range 3-29). Median follow-up after the diagnosis of NAS was 5.5 years (range 0.0 – 11.6). Anastomotic strictures developed in 18 out of 141 grafts (12.8%). Median time from OLT to AS was 5.5 months (range 3-72). Median follow-up after the diagnosis of an anastomotic stricture was 2.8 years (range 0.6-15.3). Forty-one percent of the patients who developed a biliary stricture, both anastomotic and non-anastomotic, had biliary ducts dilatation on US whereas only 15% of the patients who had no biliary stricture requiring intervention had biliary ducts dilatation on US ($p = 0.001$, $\chi^2 = 11.65$). Patients who developed a biliary stricture requiring intervention had a mean lag time of 5.3 months (standard error of the mean (SEM) = 0.75) from OLT to aberrant US whereas for patients who did not develop biliary strictures requiring intervention this was 6.2 months (SEM = 0.85). A total of twenty-one ERCPs and twelve PTCs for the management of strictures were performed in the included cases. In three cases a surgical intervention to resolve the stricture was necessary. There was no difference between the duct-to-duct type anastomosis and the Roux-en-Y anastomosis in the occurrence of biliary strictures ($p = 0.88$). Other possible risk factors as primary liver disease, such as PSC, PBC and HCV, or donation after cardiac death are also listed in *Table 2*.

Liver chemistry and abdominal ultrasound

Elevation of serum liver enzymes ALP, GGT, ASAT and ALAT above the upper limit of normal occurred in respectively 57.4%, 71.6%, 53.9%, and 61.0% of the patients at 3, 6, 9 or 12 months after OLT. Bilirubin was elevated in 41.6% of the cases. There was a significant relationship between the level of GGT and the development of biliary strictures requiring intervention, both in the univariate and in the multivariate analysis (hazard ratios 1.35 and 1.25,

$p < 0.001$ and $p = 0.04$, respectively), as presented in *Tables 2* and *3*. ALP above the upper limit of normal was also found to be a significant indicator for the development of biliary strictures in the univariate analysis ($p < 0.001$), but not in the multivariate analysis ($p = 0.15$). Elevated ALAT and ASAT were not associated with biliary strictures in the univariate analysis ($p = 0.61$. and $p = 0.62$ respectively). Elevation of bilirubin was not significant in both univariate and multivariate analysis for the prediction of the development of biliary strictures ($p = 0.08$ and $p = 0.32$ respectively *(Table 3)* Regarding the US assessments a significant relationship was found between dilated bile ducts on abdominal ultrasound and the successive development of a biliary stricture requiring intervention in both the univariate (hazard ratio = 4.48, $p < 0.001$) and multivariate analysis (hazard ratio = 3.45, $p < 0.01$). *(Table 3)*

Table 1. Baseline characteristics of 141 orthotopic liver transplants.

	N (141)
Recipient data	
- Male/ Female	91/50
- Median age (years) (range)	50 (16 – 70)
Etiology of liver disease	
Hepatitis B/C cirrhosis	10/22
Biliary cirrhosis (PSC/PBC)	30 (22/8)
Alcoholic cirrhosis	25
Hepatocellular carcinoma	19
Other	35
Donor and OLT procedure data	
DBD / DCD donor	135/6
Choledochocholedochostomy (duct-to-duct) / Roux-en-Y hepaticojejunostomy	132/9
Donor warm ischemic time (DCD donors) (minutes) (range)	17 (11 – 23)
Cold ischemic time (minutes) (range)	605 (268 – 1095)
Recipient warm ischemic time (minutes) (range)	35 (16 - 90)

Table 2. Univariate analysis of potential predictors for the development of biliary strictures (BS). Time-dependent analysis was used to calculate the predictive value of routinely performed liver chemistry profile assessments and dilated bile ducts on abdominal ultrasound (US) for detecting BS requiring intervention after OLT (n=141). The hazard ratios for liver enzymes are shown per 100 IU/L increase. The hazard ratio for bilirubin is shown per 10 µmol/L increase.

Clinical Variables	Hazard Ratio (95% CI)	P-Value
Dilated bile ducts on US	4.48 (1.97 –10.12)	< 0.001
GGT	1.35 (1.22–1.49)	< 0.001
ALP	1.55 (1.22–1.89)	< 0.001
ALAT	1.13 (0.74–1.82)	0.61
ASAT	1.19 (0.61– 2.45)	0.62
Bilirubin	1.07 (0.99 –1.16)	0.08
Gender	0.64 (0.33–1.40)	0.23
Donation after cardiac death	1.61 (0.38–6.80)	0.51
Underlying liver disease		
- PSC	0.04 (0.00 –18.72)	0.31
- HCV	0.94 (0.38– 2.29)	0.89
- PBC	1.13 (0.27 – 4.73)	0.87
Type of surgical anastomosis (duct-to-duct/Roux-en-Y)	0.90 (0.26 – 4.5)	0.88
Cold IschemicTime (CIT)	0.99 (0.99 –1.00)	0.28
Age (at OLT)	0.97 (0.95 – 1.00)	0.14

Table 3. Multivariate time-dependent Cox regression analysis for liver enzymes and dilated bile ducts on abdominal ultrasound (US) for detecting presence of BS requiring intervention after OLT (n=141). Gamma-glutamyltranspeptidase (GGT) and US remain significant predictors for the development of BS. The Hazard ratio for GGT shows the risk of having a stricture with each 100 IU/l increase. The hazard ratio for bilirubin was calculated for each 10 µmol/L increase.

Clinical Variables	Hazard Ratio (95% CI)	P- value
Dilated bile ducts on US	3.45 (1.46 – 8.17)	< 0.01
GGT	1.24 (1.00 – 1.54)	0.05
ALP	1.34 (0.89 – 2.01)	0.15
Bilirubin	0.92 (0.79 – 1.08)	0.32
Age	0.97 (0.94– 1.00)	0.62

Discussion

Biliary strictures frequently complicate orthotopic liver transplantation and lead to significant morbidity, graft loss and mortality. Early diagnosis and prompt intervention is therefore of great clinical importance. Cholangiography remains the most sensitive and specific assessment in diagnosing biliary strictures but is invasive. The most commonly used and least invasive diagnostic modalities after OLT are serum liver chemistry profile determinations and abdominal ultrasound.[12,13,14,15] The prognostic values of abdominal ultrasound and liver enzymes in detecting biliary strictures have been evaluated before. Hussaini et al.[11], for example, showed that US was a valuable tool to diagnose biliary strictures with a sensitivity and specificity of 77% and 67%, respectively. Que et al.[18] found US to detect biliary strictures with a sensitivity and specificity of 90% and 91% respectively, but reported GGT and ALP to be of poor diagnostic value even at 10-folds the upper limit of normal. However, many transplantation centers still differ in the way they routinely assess liver biochemistry and US to detect biliary strictures and therefore different protocols for follow-up of transplanted patients are used. From previous studies it remained unclear whether routine assessment of these modalities can predict biliary strictures. In an outpatient clinic, patients often present themselves without symptoms., even if biliary strictures are present, but they have abnormal liver enzymes and US. Previous studies evaluated liver chemistry using sensitivity and specificity of US and liver enzymes in relation to the presence of biliary strictures. Although this is a common way to evaluate the diagnostic value of clinical tools it has several limitations: sensitivity and specificity only apply if the assessment of the liver biochemistry profile and biliary strictures occur simultaneously. Routine assessments of liver biochemistry and abdominal ultrasound in an outpatient clinic often precede the detection of a biliary stricture. In most previous studies sensitivity and specificity were based on dichotomized variables, e.g. liver enzymes were elevated or not. However, liver enzymes are continuous variables and change over time and probably concomitantly become elevated during stricture formation. This means that the risk of developing a biliary stricture for each patient varies along with the changes in liver enzymes or ultrasound findings. It is therefore more appropriate to use a time-dependent

regression model for exploring predictive relationships by using quantities such as liver enzymes that vary over time.[19] The current findings are in accordance with non-transplantation studies in which GGT corresponds with the presence of biliary strictures, while mixed data are reported on the predictive value of liver chemistry for the presence of biliary complications after OLT.[8,10,12,18] We found an independent association between the increased serum level of GGT assessed at fixed routine time-points and the risk of detecting a biliary stricture requiring intervention. Time-dependent analysis calculates the hazard ratio for developing biliary strictures per 1 IU/L of elevated GGT, which in our study was 1.0022. Thus, an elevation of 100 IU/L (i.e., 151 U/L) would result in $1.00217^{100} = 1.24$ or a 24% increased risk. In formula terms, the hazard ratio for any elevation of GGT can be calculated ($1.0022^{(\text{elevation above upper limit in IU/L})}$) for the development of a biliary stricture. Abdominal ultrasound is a non-invasive, readily available and economic diagnostic tool. However, several studies observed that ultrasound is not very sensitive in detecting biliary strictures in a post-transplant population, whereas few studies reported the opposite.[3,8,11,13] We found bile duct dilatation on abdominal ultrasound to be a powerful predictor of subsequent development of biliary strictures requiring therapy, exemplified by the high hazard ratio of 3.45 in the multivariate analysis. In clinical practice the calculated hazard ratios of both bile duct dilatation on US and the elevation of GGT are multiplied. In our example GGT levels 100 IU/L above the reference range together with dilated bile ducts on US result in a hazard ratio of 4.3 (3.45 x 1.24), which indicates that the risk of developing a biliary stricture requiring intervention is 4.3 more likely compared to the standard risk. Other modalities such as MRCP are also non-invasive tools which are widely used in diagnosing biliary strictures. However, MRCP is usually considerably more expensive than US and often has a waiting list, which makes it less applicable as a routine diagnostic tool. MRCP can be considered if other diagnostic tools provide no conclusive information and if there is less urgency in performing an ERCP. We realize that our study has some limitations. We decided to use a follow-up of one year after the 3-month time point since most strictures develop within the first year after OLT. Liver enzymes within the first three months were not included because early after transplantation many variables influence liver enzyme levels, such as ischemia-reperfusion damage,

rejection and infection. A shorter follow-up would have weakened the statistical analysis. One should be careful using ultrasound findings which are not defined forehand. Unfortunately, there is no clear definition of dilated bile ducts in the literature and we therefore used a definition, which is being used by our radiologist with expertise in bile duct dilatation and liver transplantation. Further prospective studies are needed to define a cut-off value for bile duct dilatation. Our timedependent regression analysis showed that detection of dilated bile ducts on US or elevated GGT are independent predictive factors for the development of biliary strictures requiring intervention in the first year after OLT. To our knowledge there are no other studies that evaluate the usefulness of routinely assessing liver chemistry and performing ultrasound after OLT. Routine assessment by serum GGT and US at 3-month intervals during the first year post-OLT is useful to screen for biliary strictures post-OLT. Elevated GGT or dilated bile ducts on US in the first year post-OLT should prompt cholangiography and may allow timely intervention before complications like cholangitis develop.

References

1 Verdonk RC, Buis CI, Porte RJ, van der Jagt EJ et al. Anastomotic biliary strictures after liver transplantation: causes and consequences. Liver Transpl 2006; 12(5):726-735.

2 Nishida S, Nakamura N, Kadono J et al. Intrahepatic biliary strictures after liver transplantation. J Hepatobiliary Pancreat Surg 2006; 13(6):511-516.

3 Barriga J, Thompson R, Shokouh-Amiri H et al. Biliary strictures after liver transplantation. Predictive factors for response to endoscopic management and longterm outcome. Am J Med Sci 2008; 335(6):439-443.

4 Pascher A, Neuhaus P. Bile duct complications after liver transplantation. Transpl Int 2005; 18(6):627-642.

5 Colonna JO, Shaked A, Gomes AS et al. Biliary strictures complicating liver transplantation. Incidence, pathogenesis, management, and outcome. Ann Surg 1992; 216(3):344-350.

6 Morelli J, Mulcahy HE, Willner IR et al. Long-term outcomes for patients with postliver transplant anastomotic biliary strictures treated by endoscopic stent placement. Gastrointest Endosc 2003; 58(3):374-379.

7 Lee SH, Ryu JK, Woo SM et al. Optimal interventional treatment and long-term outcomes for biliary stricture after liver transplantation. Clin Transplant 2008; 22(4):484-493.

8 Shastri YM, Hoepffner NM, Akoglu B et al. Liver biochemistry profile, significance and endoscopic management of biliary tract complications post orthotopic liver transplantation. World J Gastroenterol 2007; 13(20):2819-2825.

9 Shah SR, Dooley J, Agarwal R et al. Routine endoscopic retrograde cholangiography in the detection of early biliary complications after liver transplantation. Liver Transpl 2002; 8(5):491-494.

10 Zoepf T, Maldonado-Lopez EJ, Hilgard P et al. Diagnosis of biliary strictures after liver transplantation: which is the best tool? World J Gastroenterol 2005; 11(19):2945-2948.

11 Hussaini SH, Sheridan MB, Davies M. The predictive value of transabdominal ultrasonography in the diagnosis of biliary tract complications after orthotopic liver transplantation. Gut 1999; 45(6):900-903.

12 Ben-Ari Z, Weiss-Schmilovitz H, Sulkes J et al. Serum cholestasis markers as predictors of early outcome after liver transplantation. Clin Transplant 2004; 18(2):130-136.

13 Zemel G, Zajko AB, Skolnick ML et al. The role of sonography and transhepatic cholangiography in the diagnosis of biliary complications after liver transplantation. Am J Roentgenol 1988; 151(5):943-946.

14 Kok T, Van der Sluis A, Klein JP et al. Ultrasound and cholangiography for the diagnosis of biliary complications after orthotopic liver transplantation: a comparative study.J Clin Ultrasound 1996; 24(3):103-115.

15 Uzochukwu LN, Bluth EI, Smetherman DH et al. Early postoperative hepatic sonography as a predictor of vascular and biliary complications in adult orthotopic liver transplant patients.Am J Roentgenol 2005; 185(6):1558-1570.

16 Li S, Stratta RJ, Langnas AN, Wood RP et al. Diffuse biliary tract injury after orthotopic liver transplantation.Am J Surg 1992; 164(5):536-540.

17 Rull R, Garcia Valdecasas JC, Grande L et al. Intrahepatic biliary lesions after orthotopic liver transplantation. Transpl Int 2001; 14(3):129-134.

18 Que Y, Kaneko J, Sugawara Y et al. Role of protocol ultrasonography for detecting biliary stricture in adult living donor liver transplantation recipients. Biosci Trends 2007; 1(1):62-65.

19 Kalbfleisch JD, Prentice RL. The Statistical Analysis of Failure Time Data. New York Wiley; 1980

Chapter 5

Matrix metalloproteinase 2 genotype is associated with nonanastomotic biliary strictures after orthotopic liver transplantation

W. Rogier Ten Hove[1], Kerem Sebib Korkmaz[1], Sanna op den Dries[2], Bert-Jan F. de Rooij[1], Bart van Hoek[1], Robert J. Porte[2], Johan J. van der Reijden[1], Minneke J. Coenraad[1], Jeroen Dubbeld[3], Daniel W. Hommes1 and Hein W. Verspaget[1]

[1] Department of Gastroenterology and Hepatology, Leiden University Medical Center, Leiden, the Netherlands
[2] Department of Hepatobiliary Surgery and Liver Transplantation, University Medical Center Groningen, Groningen, the Netherlands
[3] Department of Surgery, Leiden University Medical Center, Leiden, the Netherlands

Liver International 2011 Sep;31(8):1110-7

Abstract

Background: Nonanastomotic biliary strictures (NAS) are a serious complication after orthotopic liver transplantation (OLT). Matrix metalloproteinases (MMPs) are involved in connective tissue remodelling in chronic liver disease and complications after OLT.

Aim: To evaluate the relationship between MMP-2 and MMP-9 gene polymorphisms and NAS.

Methods: MMP-2 (– 1306 C/T) and MMP-9 (– 1562 C/T) gene promoter polymorphisms were analysed in 314 recipient–donor combinations. Serum levels of these MMPs were determined in subgroups of patients as well. NAS were identified with various radiological imaging studies performed within 4 years after OLT and defined as any stricture, dilation or irregularity of the intra- or extrahepatic bile ducts of the liver graft followed by an intervention, after exclusion of hepatic artery thrombosis and anastomotic strictures.

Results: The average incidence of NAS was 15%. The major clinical risk factor for the development of NAS was PSC in the recipient. The presence of the MMP-2 CT genotype in donor and/or recipient was associated with a significantly higher incidence of NAS, up to 29% when both donor and recipient had the MMP-2 CT genotype ($P = 0.003$). In the multivariate analyses, pre-OLT PSC (hazard ratio 2.1, $P = 0.02$) and MMP-2 CT genotype (hazard ratio 3.5, $P = 0.003$) were found to be independent risk factors for the development of NAS after OLT. No obvious association was found between NAS and the MMP-9 genotype and serum levels of the MMPs.

Conclusion: MMP-2 CT genotype of donor and recipient is an independent risk factor, in addition to PSC, for the development of NAS after OLT.

Introduction

Biliary complications are a common feature after orthotopic liver transplantation (OLT), with a reported incidence of up to 35%. Leaks and strictures are the most common complications, often requiring endoscopic, radiological or surgical intervention[1–9]. Anastomotic strictures result from surgical or local ischaemic causes. Main categories of risk factors for nonanastomotic biliary strictures (NAS) include ischaemia related injury, immunologically induced injury and cytotoxic injury by bile salts. A higher incidence of NAS is reported in patients transplanted for primary sclerosing cholangitis (PSC) and patients who suffered from a postoperative CMV infection[10–17]. Donation after cardiac death (DCD) procedures are also reported to have an increased risk of NAS compared with donation after brain death procedures[18,19]. NAS are often referred to as ischaemic-type biliary lesions, based on the resemblance with biliary abnormalities observed after hepatic artery thrombosis. The reported incidence of NAS varies in different publications from 1 to 19%[4,5,20–25]. If untreated, NAS may lead to cholestasis, severe graft dysfunction, septic complications, secondary cirrhosis and graft loss[7,23,26].

Matrix metalloproteinases (MMPs) comprise a large family of proteolytic enzymes that are important in physiological and disease-related extracellular matrix remodelling processes[27–30]. MMP-2 and MMP-9 are capable of digesting components of the connective tissue matrix and type IV collagen within basement membranes. These MMPs are considered to play an important role in cancer development, tissue remodelling, fibrosis and inflammation, including cirrhosis and liver trans-plantation[31–35]. We showed previously, for example, that serum MMP-2 levels increased, whereas MMP-9 levels decreased in relation to the severity of the cirrhosis[31]. These serum MMP levels were subsequently found to change irrespective of their gene polymorphisms in late phase injury or rejection (I/R) after liver transplantation[36]. The aim of the present study was to assess whether a relationship exists between MMP-2 and MMP-9 gene promoter polymorphisms in the donor and recipient DNA with the development of NAS after OLT.

Patients and Methods

Patients

All adult patients who received a liver transplant at the Leiden University Medical Center (LUMC) and University Medical Center Groningen (UMCG) in the Netherlands were eligible for inclusion. For this study, 202 patients were identified from the transplantation databases who underwent OLT at the LUMC between 1992 and 2005, of whom we were able to include 147 patients whose DNA was available from both donor and recipient, and who had at least 7 days of follow-up after liver transplantation. Also, patients who received OLT be-tween 2000 and 2005 at the UMCG were eligible for the study because data were available. Of the 224 available patients, 167 unselected patients could be included of whom we had DNA from both recipient and donor, and who had at least 7 days of follow-up after trans-plantation. Genomic DNA was extracted routinely from peripheral blood and/or tissue samples without given preference to any explicit clinical variables. All patients received standard immunosuppressive therapy consisting of corticosteroids, a calcineurin inhibitor (i.e., cyclosporine or tacrolimus) with or without mycophenolate mofetil or azathioprine and/or basiliximab. Azathioprine was used until 2001, and thereafter mycophenolate mofetil was given in case of impaired renal function. Demographical and clinicopathological characteristics of the recipient at the time of OLT (age, gender, indication for liver transplantation, laboratory MELD score), donor information (age, gender and donor type), transplantation procedure variables (warm and cold ischaemia time) and post-transplant follow-up data of up to 4 years were collected from the transplantation databases. This study was performed with informed consent from the patients according to the guidelines of the Medical Ethics Committee of both participating centres and in compliance with the Helsinki Declaration.

Nonanastomotic strictures

In this study, only biliary strictures followed that by an intervention were included. If a biliary stricture was suspected from clinical findings, liver

function tests or abdominal ultrasound, further imaging of the biliary tract was performed. In both centres, a biliary drain was placed routinely after OLT and cholangiography was performed if clinically indicated and in the LUMC, cholangiography was also performed routinely 6 weeks after OLT.
All imaging studies of the biliary tree, performed within 4 years after OLT, were included [direct cholangiography via the biliary drain, percutaneous transhepatic cholangiodrainage (PTCD), ERCP as well as MRCP]. For the purpose of this study, NAS were defined as follows: any stricture, dilation or irregularity of the intrahepatic or extrahepatic bile ducts of the liver graft, either with or without biliary sludge formation, at least 1 cm above the biliary anastomosis and treated endoscopically with ERCP and dilation and/or stenting, percutaneously with PTCD or by surgical intervention. Hepatic artery thrombosis by either Doppler ultrasound or conventional angiography as well as isolated strictures/stenoses at the bile duct anastomosis and related dilations were, by definition, excluded from this analysis.

Genotyping

Genomic DNA was extracted by routine methods from peripheral blood leucocytes and/or tissue samples. In addition, DNA samples from the blood or tissue of the liver donor were obtained from the Eurotransplant Reference Laboratory or freshly isolated MMP-2: high-resolution DNA melting analysis.

MMP2 –1306 C/T (rs243865) genotyping, as most relevant SNP, was performed with the use of high-resolution DNA melting assay[37]. Sequences of the polymerase chain reaction (PCR) primers were 5'-CCAGTGCCTCTT-GCTGTTTT-3' (forward) and 5'- GACTTCTGAGCTGAGACCTGA-3' (reverse). The unlabelled probe was designed according to the wild-type (C) genotype and had the following sequence: 5'- CCACCCAGCACTCCA CCTCTTTAGCTC-3'. The probe had a 3'-amino-C7 modification to prevent DNA polymerase extension during PCR. In brief, high-resolution melting analysis of PCR products amplified in the presence of a saturating double-stranded DNA dye (LCGreenPlus, Idaho Technology, Salt Lake City, Utah, USA) and a 3'-blocked probe, identified both heterozygous and homozygous sequence variants. Heterozygotes were identified by a change in melting curve shape, and

different homozygotes are distinguished by a change in melting temperature. In each experiment, sequence-verified control donors for each genotype were used. MMP-9: PCR-RFLP genotyping.

The MMP-9 SNP C/T at position – 1562 (rs3918242) was determined with PCR analysis followed by restric-tion enzyme fragment length polymorphisms (RFLP) analysis, the principles of which are described elsewhere[36], and confirmed by direct sequence analysis of four patients. Briefly, the region flanking the SNP was ampli-fied with outer primers 5'- ATGGCTCATGCCCG-TAATC-3' and 5'-TCACCTTCTTCAAAGCCCTATT-3' followed by RFLP analysis with SphI to produce 352, 35212071145 or 2071145 bp fragments in case of CC, CT and TT genotype respectively. Genotypes CC, CT and TT are easily identified from the migration pattern on agarose gels[36,38–40].

Determination of serological MMP levels

From two subgroups of patients included in our study, we also assessed the serological levels of MMP-2 and MMP-9 before and after transplantation. This pretransplantation group consisted of 47 patients (30 males) with chronic liver disease of various aetiologies, including 27 patients who eventually underwent an OLT. Their median age was 46 years (range 16–68). Fourteen patients had chronic viral hepatitis, 14 patients had cholestatic liver disease, 10 patients had alcohol-related liver disease and the remaining nine patients had miscellaneous liver diseases. From the group of 27 OLT patients, serum samples 1 month after transplantation were evaluated. All serum samples had been stored at – 80 °C until use. MMP-2 and MMP-9 concentrations were determined using highly specific enzyme-linked immunosorbent assays, which measures the pro-enzyme, active- and inhibitor complexed forms, as described previously[31,36].

Statistical analysis

Data were analysed using SPSS 17.0 software (SPSS Inc.; Chicago, IL, USA). Characteristics of the liver transplant recipients, donors and post-transplant follow-up data with the risk of developing NAS were analysed using the

log-rank and two-tailed Student's t-tests. Differences in the serological levels of MMP were analysed using ANOVA. Genotype frequencies were analysed by generating two-by-three contingency tables and statistical analysis was performed using the χ^2-test or the Fisher's exact test, where appropriate. Comparison of time with NAS was made using Kaplan–Meier statistics with a log-rank test. Univariate and multivariate analyses were performed using Cox's proportional hazards method. Variables associated with an increased risk of NAS at the $P < 0.15$ level in the univariate logistic regression analysis were included in the backward stepwise multivariate logistic regression model. P-values ≤ 0.05 were considered statistically significant.

Results

The study population consisted of 314 OLT donor/ recipient combinations of which 48 (15%)developed NAS within the first 4 years after transplantation.

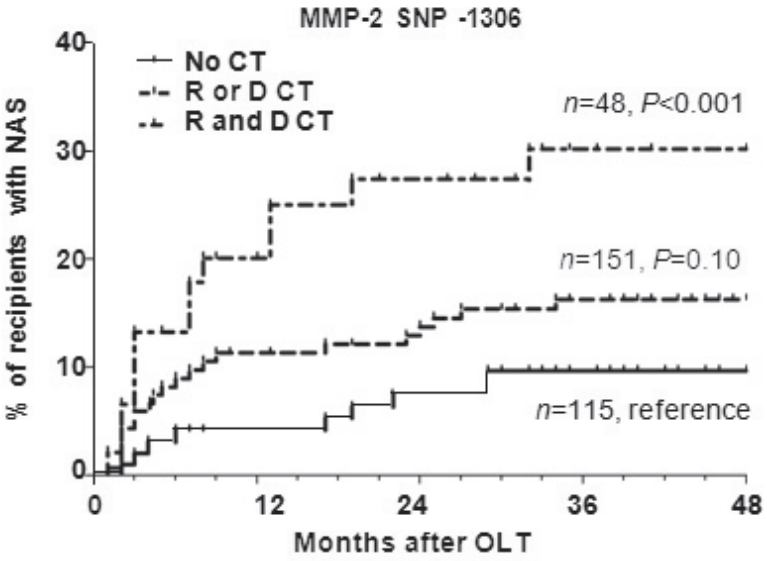
MMP-2 genotype and NAS

The frequencies of MMP-2 and MMP-9 gene promoter polymorphisms in recipients and in donors vs. the occurrence of NAS are given in *Table 1*. Evaluation whether the MMP genotype is reflected in the serum level indicated that in patients with liver disease no such relation exists. Specifically, MMP-2 levels in the pre-OLT serum of recipients with a CC (n = 32) genotype was 5123 ± 553 ng/ml, whereas for those with a CT or TT genotype (n = 15), these levels were 5347 ± 886 (NS). For MMP-9, these levels were 129 ± 16 (n = 36) vs. 156 ± 28 (n = 11) respectively (NS).

The presence of MMP-2 CT genotype in the recipient as well as in the donor was significantly associated with the development of NAS. Furthermore, the cumulative presence of MMP-2 CT genotype in both recipient and donor vs. the occurrence of NAS is shown in *Tables 2* and *3*. In the group of patients that developed NAS, the absence of a CT genotype was more frequent (21%) than in the patients that did not develop NAS (39%) and for CT in donor and recipient, exactly the opposite was observed (29% vs. 13%, *Table 2*). If CT genotype was present in neither recipient nor donor, the risk of developing NAS was 9% (10/115). When MMP-2 CT genotype was present in either donor or recipient, NAS developed in 16% (24/151) of cases. The occurrence of NAS increased to 29% if MMP-2 CT genotype was present in both recipient and donor (14/48; P <0.003, *Table 3*). *Figure 1* shows the cumulative incidence of NAS within 48 months after OLT related to the presence of MMP-2 CT genotype in recipient and donor. We also evaluated whether this association between genotype MMP-2 and NAS was reflected in the serum levels. One month after OLT, the MMP-2 level in patients with NAS was showed a trend to be lower [i.e., 1892 ± 431 ng/ml (n = 5) vs. 2869 ± 287 (n = 22), P = 0.06], compared with the patients without NAS. Interestingly, a similar trend was observed in relation to the MMP-2 genotype, i.e. lower in relation to the

presence of CT [2969 ± 452 vs. 2540 ± 349 vs. 2396 ± 448 for no CT in donor or recipient (n = 10), CT in donor or recipient (n = 15) and CT in donor and recipient (n = 2), respectively, NS].

Figure 1



Cumulative incidence of NAS within 48 months after OLT related to the presence of MMP-2 CT genotype in recipient (R) and donor (D). MMP, matrix metalloproteinase; NAS, nonanastomotic biliary strictures; OLT, orthotopic liver transplantation.

Further assessment of the impact of the MMP-2 genotypes and NAS-related morbidity by including re-OLTs showed a similar stepwise increase in relation to the MMP-2 genotype from 14% (16/115) to 20% (30/151) and 38% (18/48) respectively (χ^2 11.66, P = 0.003). By including death in the follow-up, this increased to 26% (30/115), 29% (44/151) and 44% (21/48) respectively (25.18, P = 0.08). In a similar manner, the MMP-9 genotype distribution of recipient and donor vs. the occurrence of NAS was evaluated. However, no significant correlation was found between MMP-9 genotype and the development of NAS *(Table 1)* or with the serum levels of MMP-9 (data not shown).

Table 1. Frequencies of matrix metalloproteinase polymorphisms in orthotopic liver transplant recipients and donors (n = 314)

Genotype				Recipient		Donor	
dbSNP ID	SNP			NAS % (n)	No NAS % (n)	NAS % (n)	No NAS % (n)
MMP-2 rs243865	– 1306	C → T	CC	46 (22)	61 (162)	44 (21)	53
			CT	52 (25)	33 (87)	56 (27)	41
			TT	2 (1)	6 (17)	0 (0)	6
				P < 0.03, χ^2 7.2		P = 0.05, χ^2 5.9	
MMP-9 rs3918242	– 1562	C → T	CC	67 (32)	71 (185)	75 (36)	78
			CT	33 (16)	27 (71)	23 (11)	21
			TT	0 (0)	2 (5)	2 (1)	1
				P = 0.46, χ^2 1.6 (n = 309)		P = 0.66, χ^2 0.9 (n = 312)	

Multivariate analysis of MMP-2 genotype and covariates

The development of NAS was significantly higher when PSC was the indication for OLT [15/48 cases (31%) vs. 33/ 257 cases (13%)], as expected. No significant association was found between the occurrence of NAS and other transplant characteristics, such as gender and age (both of recipient and donor), laboratory MELD score, length of warm or cold ischaemia time or DCD procedures. However, it should be noted that only 25 DCD procedures were included in this cohort. Also, in relation to immunosuppressive therapy, there was no association found with the development of NAS, i.e. patients on corticosteroids with a calcineurin inhibitor with or without basiliximab had a similar risk of developing NAS [13% (27/209) vs. 20% (21/105), respectively, NS].

Early (≤ 12 months) and late (12–48 months) onset of NAS was also looked at for all studied risk factors. NAS was diagnosed in 33 cases (10.5%) within the first year after OLT and in 15 cases (4.8%) from 12 to 48 months after OLT.

Table 2. Comparison of donor, recipient and procedure variables between patients with and without nonanastomotic biliary strictures after orthotopic liver transplant (n =314)

	Total	NAS (n = 48)	%	No NAS (n = 266)	%	P-value
Donor variables						
Age (in years, median, range)	44 (16–72)	43	(16–67)	44	(9–72)	0.80
Gender						
Female	153	29	60	124	47	0.08
Male	161	19	40	142	53	
Recipient variables						
Age (in years, median, range)	48 (16–70)	45	(16–61)	48	(17–70)	0.08
Gender						
Female	122	16	33	106	40	0.39
Male	192	32	67	160	60	
Primary liver disease						**0.04**
Post viral cirrhosis	59	4	8	55	21	
Alcoholic cirrhosis	46	5	10	41	15	
PSC	57	15	31	42	16	
Other cholestatic disease*	28	6	13	22	8	
Other disease†	124	18	38	106	40	
Laboratory MELD score (median, range)		15 (6–40)		15 (6–40)		0.98
OLT procedure variables						0.50
DCD	25	5	10	20	8	
DBD	289	43	90	246	92	
WIT in minutes (mean± SD)	44 ± 13 n = 296	42 ± 10 n = 46		44 ± 13 n = 250		0.37
CIT in minutes (mean± SD)	573 ± 188 n = 299	595 ± 183 n = 46		561 ± 189 n = 253		0.38
MMP-2 [rs243865] CT						**0.003**
No CT present	115	10	21	105	39	
CT in recipient or donor	151	24	50	127	48	
CT in recipient and donor	48	14	29	34	13	

Age, MELD scores, WIT and CIT differences were evaluated by Student's t-test; frequency distribution data were analysed by χ^2 or Fisher's exact tests, where appropriate. CIT, cold ischaemia time; time between the start of cold perfusion of graft in the donor and the end of cold preservation of the liver graft; DBD, donation after brain death; DCD, donation after cardiac death; MELD, model for end-stage liver disease; MMP, matrix metalloproteinase; NAS, nonanastomotic biliary lesions; OLT, orthotopic liver transplantation; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; SD, standard deviation; SSC, secondary sclerosing cholangitis; WIT, warm ischaemia time; time between the end of cold ischaemic preservation of the liver graft and portal vein reperfusion in the recipient.

**Other cholestatic disease comprise PBC and SSC.*

†Other diseases include predominantly autoimmune hepatitis, cryptogenic cirrhosis and metabolic disorders.

Table 3. Univariate and multivariate analysis for the association of risk factors of nonanastomotic biliary lesions in orthotopic liver transplant patients

				Univariate analysis		Multivariate analysis (n = 299)	
Risk factor		NAS	%	HR (95% CI)	P-value	Adjusted HR (95% CI)	P-value
MMP-2 [rs243865] CT					0.01		0.008
Donor and recipient	Vs. none	14/48	29	3.48 (1.55 –7.84)	0.003	3.48 (1.54 – 7.87)	0.003
Donor or recipient	Vs. none	24/151	16	1.84 (0.88 –3.84)	0.11	1.64 (0.78 – 3.46)	0.20
None	Reference	10/115	9	1 (reference)		1 (reference)	
Age recipient	Continuous	48/314		0.98 (0.96 –1.01)	0.13		
Age donor	Continuous	48/314		1.00 (0.98 –1.02)	0.72		
Gender recipient	Female	16/122	13	0.74 (0.41 – 1.35)	0.32		
	Male	32/192	17	1 (reference)			
Gender donor	Female	29/153	19	1.53 (0.86 – 2.73)	0.15		
	Male	19/161	12	1 (reference)			
Primary liver disease	PSC	15/57	26	2.0 (1.11 – 3.76)	0.02	2.14 (1.13 –4.06)	0.02
	Other	33/257	13	1 (reference)			
Laboratory MELD score	Continuous	48/314		1.00 (0.97 –1.04)	0.84		
Procedure	DCD	5/25	20	1.50 (0.60 –3.80)	0.39		
	DBD	43/289	15	1 (reference)			
CIT	Continuous	46/299		1.000 (1.000 –1.002)	0.15	1.001 (1.000 –1.003)	0.08
WIT	Continuous	46/296		0.99 (0.96 –1.01)	0.38		

CI, confidence interval; CIT, cold ischaemia time; time between the start of cold perfusion of graft in the donor and the end of cold preservation of the liver graft; DBD, donation after brain death; DCD, donation after cardiac death; HR, hazard ratio; MELD, model for end-stage liver disease; MMP, matrix metalloproteinase; NAS, nonanastomotic biliary strictures; OLT, orthotopic liver transplantation; PSC, primary sclerosing cholangitis; WIT, warm ischaemia time; time between the end of cold ischaemic preservation of the liver graft and portal vein reperfusion in the recipient. Univariate and backward multivariate analyses were performed using Cox's proportional hazards method.

Cold ischaemia time (CIT) was significantly longer for the group with early development of NAS (631 ± 179 vs. 520 ± 174 min; P = 0.05). Interestingly, the effect of increased CIT and the incidence of NAS was particularly present in the first 12 months after OLT (hazard ratio 1.002; P = 0.03). Late occurrence of NAS was observed relatively more frequently when patients were transplanted for PSC. With PSC as the indication for liver transplantation, the occurrence of NAS within the first 12 months after OLT was 27% (9/33) as opposed to 40% (6/15) from the late onset NAS. A pre-OLT diagnosis of PSC was found to be accompanied particularly with an increased risk of late onset NAS (hazard ratio 3.1; P = 0.03). Multivariate Cox regression analyses and the backward elimination procedure, taking all patient and transplant characteristics into account with an increased risk of NAS (at the level of P ≤ 0.15), indicated that the presence of MMP-2 CT genotype in donor and recipient was an independent risk factor for the development of NAS with a higher hazard ratio than PSC as primary liver disease (3.5 vs. 2.1, respectively; *Table 3*).

Discussion

In the present study, we report a strong association between the presence of MMP-2 CT genotype in donor and/or recipient and the development of NAS after OLT. In fact, MMP-2 genotype was a greater risk factor for NAS after OLT than PSC. The presence of the MMP-2 CT genotype in donor and/or recipient was found to increase the NAS incidence stepwise from 9% when absent, increasing to 16% when present in either recipient or donor, further increasing to 29% when present in both donor and recipient. In contrast, no association was found between MMP-9 genotype and the development of NAS.

Nonanastomotic strictures are considered to be the most troublesome biliary complication after OLT, associated with high retransplant rates in up to 20% of patients[5,7,25,41]. Interestingly, further assessment of the impact of the MMP-2 genotypes and NAS-related morbidity in our patients, by including re-OLTs, also revealed a stepwise increase in relation to the MMP-2 genotype from 14 to 38% and by including death in the follow-up, this increased even up from 26 to 44%. Apparently, the MMP-2 CT genotype also contributes to the morbidity accompanying NAS in the OLT patients.

Various risk factors for NAS have been identified, suggesting a multifactorial origin[9,14]. The main categories include ischaemia-related injury, immune-mediated injury such as ABO compatibility, pre-existing disease (especially PSC) and toxic injury by bile salts[42,43]. In addition to clinicopathological factors, we were also interested in the impact of the gelatinases MMP-2 and MMP-9 in the development of NAS.

Matrix metalloproteinases comprise a large family of proteolytic enzymes involved in physiological and dis-ease-related connective tissue remodelling processes and the gelatinases MMP-2 and MMP-9 are considered to play an important role in inflammation, degradation and remodelling processes in the liver[29,31,44]. MMP activity is regulated by various factors and controlled by activation of latent pro-enzymes and by interaction with endogenous inhibitors such as tissue inhibitors of metalloproteinases (TIMPs). Recently, several single nucleotide polymorphisms (SNP) in the gene promoter regions of

MMPs have been found with an impact on the transcription rate[30,32]. The C/T transition at position –1306 in the promoter of MMP-2, which abolishes the Sp 1 binding site, and leads to decreased mRNA transcription and protein expression, is generally accepted to be the most relevant SNP for MMP-2. Other SNPs of MMP-2 have been reported as well, e.g. –1575 G/A, –790 C/T and –735 C/T, but these are in almost complete linkage (dis)equilibrium with –1306 C/T and thus provide no additional information[30,32,33]. In several studies, an association was demonstrated between MMP-2 polymorphisms and the development of cancer. It has even been suggested that MMP-2 represents a potential target for tumour therapeutics (32, 33).

In the MMP-9 gene, an SNP at position –1562 is because of a C to T substitution in the promoter region. In vitro studies have shown that this transition results in loss of binding of a nuclear repression protein and increased transcriptional activity in macrophages, associated with the severity of coronary atherosclerosis.
Although other SNPs in the MMP-9 gene have been described, they were mainly nonsynonomous located in the exon part of the gene and found not to affect the activity or level of the enzyme[34,35]. In contrast, in cardiovascular disease, for example, the –1562 T allele was found to be associated with increased MMP-9 plasma levels[35].

In the liver, the hepatic stellate cell seems to be the main cellular source of MMP-2 and when activated these cells are involved in the synthesis of matrix proteins and in the regulation of matrix degradation leading to liver fibrosis. Following liver injury, the stellate cells become activated and can express a wide range of MMPs and TIMPs, but in particular MMP-2[44–46]. Increased mRNA expression of MMP-2 was reported in liver biopsies of patients with cirrhosis[44]. We found previously serum levels of MMP-2 to be increased in patients with chronic liver disease and strongly correlated with serum markers indicative of a poor liver function[31]. After OLT, a gradual decrease of MMP-2 levels were found they remained higher, however, than found in healthy controls and increased with recurrent liver dis-ease. MMP-9 is released predominantly from neutrophils and macrophages, but the principal source in the liver is thought to be the Kupffer cell, the resident macrophage

References

1. Park JS, Kim MH, Lee SK, et al. Efficacy of endoscopic and percutaneous treatments for biliary complications after cadaveric and living donor liver transplantation. Gastro-intest Endosc 2003; 57: 78–85.

2. Qian YB, Liu CL, Lo CM, Fan ST. Risk factors for biliary complications after liver transplantation. Arch Surg 2004; 139: 1101–5.

3. Rerknimitr R, Sherman S, Fogel EL, et al. Biliary tract complications after orthotopic liver transplantation with choledochocholedochostomy anastomosis: endoscopic findings and results of therapy. Gastrointest Endosc 2002; 55: 224–31.

4. Sawyer RG, Punch JD. Incidence and management of biliary complications after 291 liver transplants following the introduction of transcystic stenting. Transplantation 1998; 66: 1201–7.

5. Thethy S, Thomson BN, Pleass H, et al. Management of biliary tract complications after orthotopic liver transplan-tation. Clin Transplant 2004; 18: 647–53.

6. Turrion VS, Alvira LG, Jimenez M, et al. Management of the biliary complications associated with liver transplanta-tion: 13 years of experience. Transplant Proc 1999; 31: 2392–3.

7. Guichelaar MM, Benson JT, Malinchoc M, et al. Risk factors for and clinical course of non-anastomotic biliary strictures after liver transplantation. Am J Transplant 2003; 3: 885–90.

8. Rizk RS, McVicar JP, Emond MJ, et al. Endoscopic manage-ment of biliary strictures in liver transplant recipients: effect on patient and graft survival. Gastrointest Endosc 1998; 47: 128–35.

9. Buis CI, Hoekstra H, Verdonk RC, Porte RJ. Causes and consequences of ischemic-type biliary lesions after liver transplantation. J Hepatobiliary Pancreat Surg 2006; 13: 517–24.

10. Sheng R, Zajko AB, Campbell WL, Abu-Elmagd K. Biliary strictures in hepatic transplants: prevalence and types in patients with primary sclerosing cholangitis vs those with other liver diseases. Am J Roentgenol 1993; 161: 297–300.

11. Distante V, Farouk M, Kurzawinski TR, et al. Duct-to-duct biliary reconstruction following liver transplantation for primary sclerosing cholangitis. Transpl Int 1996; 9: 126–30.

12. Feith MP, Klompmaker IJ, Maring JK, et al. Biliary recon-struction during liver transplantation in patients with primary sclerosing cholangitis. Transplant Proc 1997; 29: 560–1.

13. Schmitz V, Neumann UP, Puhl G, et al. Surgical complica-tions and long-term outcome of different biliary recon-structions in liver transplantation for primary sclerosing cholangitis-choledochoduodenostomy versus choledocho-jejunostomy. Am J Transplant 2006; 6: 379–85.

14. Colonna JO, Shaked A, Gomes AS, et al. Biliary strictures complicating liver transplantation. Incidence, pathogen-esis, management, and outcome. Ann Surg 1992; 216: 344–50.

15. Halme L, Hockerstedt K, Lautenschlager I. Cytomegalo-virus infection and development of biliary complications after liver transplantation. Transplantation 2003; 75: 1853–8.

16. Kowdley KV, Fawaz KA, Kaplan MM. Extrahepatic biliary stricture associated with cytomegalovirus in a liver trans-plant recipient. Transpl Int 1996; 9: 161–3.

17. Torras J, Llado L, Figueras J, et al. Biliary tract complica-tions after liver transplantation: type, management, and outcome. Transplant Proc 1999; 31: 2406.

18. Maheshwari A, Maley W, Li Z, Thuluvath PJ. Biliary complications and outcomes of liver transplantation from donors after cardiac death. Liver Transpl 2007; 13: 1645–53.

19. Dubbeld J, Hoekstra H, Farid W, et al. Similar liver transplantation survival with selected cardiac death donors and brain death donors. Br J Surg 2010; 97: 744–53.

20. Campbell WL, Sheng R, Zajko AB, Abu-Elmagd K, Deme-tris AJ. Intrahepatic biliary strictures after liver transplanta-tion. Radiology 1994; 191: 735–40.

21. Feller RB, Waugh RC, Selby WS, et al. Biliary strictures after liver transplantation: clinical picture, correlates and out-comes. J Gastroenterol Hepatol 1996; 11: 21–5.

22. Ward EM, Kiely MJ, Maus TP, Wiesner RH, Krom RA. Hilar biliary strictures after liver transplantation: cholan-giography and percutaneous treatment. Radiology 1990; 177: 259–63.

23. Sanchez-Urdazpal L, Gores GJ, Ward EM, et al. Diagnostic features and clinical outcome of ischemic-type biliary complications after liver transplantation. Hepatology 1993; 17: 605–9.

24. Pascher A, Neuhaus P. Bile duct complications after liver transplantation. Transpl Int 2005; 18: 627–42.

25. Buis CI, Verdonk RC, van der Jagt EJ, et al. Non-anasto-motic biliary strictures after adult liver transplantation part one: radiological features and risk factors for early versus late presentation. Liver Transpl 2007; 13: 708–1.

26. Rull R, Garcia Valdecasas JC, Grande L, et al. Intrahepatic biliary lesions after orthotopic liver transplantation. Transpl Int 2001; 14: 129–34.

27. Shapiro SD. Matrix metalloproteinase degradation of ex-tracellular matrix: biological consequences. Curr Opin Cell Biol 1998; 10: 602–8.

28. Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol 2001; 17: 463–516.

29. Verspaget HW, Kuyvenhoven JP, Sier CF, van Hoek B. Matrix metalloproteinases in chronic liver disease and liver transplantation. In Lendeckel U, Hooper NM, eds Proteases in Biology and Disease 5: Proteases in Gastrointestinal Tissues. Dordrecht, the

Netherlands: Springer, 2006; 209–34.

30. Harendza S, Lovett DH, Panzer U, et al. Linked common polymorphisms in the gelatinase a promoter are associated with diminished transcriptional response to estrogen and genetic fitness. J Biol Chem 2003; 278: 20490–9.

31. Kuyvenhoven JP, van Hoek B, Blom E, et al. Assessment of the clinical significance of serum matrix metalloproteinases MMP-2 and MMP-9 in patients with various chronic liver diseases and hepatocellular carcinoma. Thromb Haemost 2003; 89: 718–25.

32. Price SJ, Greaves DR, Watkins H. Identification of novel, functional genetic variants in the human matrix metallo-proteinase-2 gene: role of Sp1 in allele-specific transcrip-tional regulation. J Biol Chem 2001; 276: 7549–58.

33. Miao X, Yu C, Tan W, et al. A functional polymorphism in the matrix metalloproteinase-2 gene promoter (-1306C/T) is associated with risk of development but not metastasis of gastric cardia adenocarcinoma. Cancer Res 2003; 63: 3987–90.

34. Wu J, Zhang L, Luo H, et al. Association of matrix metalloproteinases-9 gene polymorphisms with genetic susceptibility to esophageal squamous cell carcinoma. DNA Cell Biol 2008; 27: 553–7.

35. Zhang B, Ye S, Herrmann SM, et al. Functional polymorph-ism in the regulatory region of gelatinase B gene in relation to severity of coronary atherosclerosis. Circulation 1999; 99: 1788–94.

36. Ten Hove WR, de Rooij BJF, van Hoek B, et al. MMP-2 and MMP-9 serum levels change but their gene promotor polymorphisms are not associated with late phase I/R injury or rejection after ort hotopic liver transplantation. Open Transplant Journal 2008; 2: 66–72.

37. Zhou LM, Wang L, Palais R, Pryor R, Wittwer CT. High-resolution DNA melting analysis for simultaneous muta-tion scanning and genotyping in solution. Clin Chem 2005; 51: 1770–7.

38. Kubben FJ, Sier CF, Meijer MJ, et al. Clinical impact of MMP and TIMP gene polymorphisms in gastric cancer. Br J Cancer 2006; 95: 744–51.

39. Ye S, Dhillon S, Ke X, Collins AR, Day IN. An efficient procedure for genotyping single nucleotide polymorph-isms. Nucleic Acids Res 2001; 29: E88–8.

40. Meijer MJ, Mieremet-Ooms MA, van Hogezand RA, et al. Role of matrix metalloproteinase, tissue inhibitor of metal-loproteinase and tumor necrosis factor-alpha single nu-cleotide gene polymorphisms in inflammatory bowel disease. World J Gastroenterol 2007; 13: 2960–6.

41. Sanchez-Urdazpal L, Gores GJ, Ward EM, et al. Ischemic-type biliary complications after orthotopic liver transplan-tation. Hepatology 1992; 16: 49–53.

42. Buis CI, Geuken E, Visser DS, et al. Altered bile composi-tion after liver transplantation is associated with the devel-opment of nonanastomotic biliary strictures. J Hepatol 2009; 50: 69–79.

43. Geuken E, Visser D, Kuipers F, et al. Rapid increase of bile salt secretion is associated with bile duct injury after human liver transplantation. J Hepatol 2004; 41: 1017–25.

44. Milani S, Herbst H, Schuppan D, et al. Differential expres-sion of matrixmetalloproteinase-1 and -2 genes in normal and fibrotic human liver. Am J Pathol 1994; 144: 528–37.

45. Kuyvenhoven JP, Verspaget HW, Gao Q, et al. Assessment of serum matrix metalloproteinases MMP-2 and MMP-9 after human liver transplantation: increased serum MMP-9 level in acute rejection. Transplantation 2004; 77: 1646–52.

46. Benyon RC, Arthur MJ. Extracellular matrix degradation and the role of hepatic stellate cells. Semin Liver Dis 2001; 21: 373–84.

47. Sankary HN, McChesney L, Hart M, Foster P, Williams J. Identification of donor and recipient risk factors associated with nonanastomotic biliary strictures in human hepatic allografts. Transplant Proc 1993; 25: 1964–7.

48. Brandsaeter B, Schrumpf E, Bentdal O, et al. Recurrent primary sclerosing cholangitis after liver transplantation: a magnetic resonance cholangiography study with analyses of predictive factors. Liver Transpl 2005; 11: 1361–9.

Chapter 6

MMP-2 is a disease modifying gene in Primary Sclerosing Cholangitis

Kerem Sebib Korkmaz[1], Bert-Jan de Rooij[1], Bart van Hoek[1], Marcel Janse[2], Minneke J. Coenraad[1], Johan J. van der Reijden[1], Rinse K. Weersma[2], Robert J. Porte[3], Philip W. Voorneveld[1], Andrzej G. Baranski[4], Hein W. Verspaget[1]

[1] Department of Gastroenterology and Hepatology, Leiden University Medical Center, Leiden, The Netherlands
[2] Department of Gastroenterology and Hepatology, University Medical Center Groningen, Groningen, The Netherlands,
[3] Department of Hepatobiliary Surgery and Liver Transplantation, University Medical Center Groningen, Groningen, The Netherlands
[4] Department of Surgery, Leiden University Medical Center, Leiden, The Netherlands

Liver International 2013 Jun 4. doi: 10.1111/liv.12237. [Epub ahead of print]

Abstract

Background: Primary sclerosing cholangitis (PSC) is a chronic inflammatory disease of the bile ducts, frequently necessitating orthotopic liver transplantation (OLT), often accompanied by inflammatory bowel disease (IBD). Matrix metalloproteinases (MMPs) are associated with fibrotic diseases due to involvement in tissue remodeling.

Aim: Evaluate the contribution of MMP-2 and -9 promoter polymorphisms to disease severity in PSC, as assessed by death or need for OLT.

Methods: MMP-2 (–1306 C/T) and -9 (–1562 C/T) promoter polymorphisms were analyzed in 132 PSC patients. Follow-up was from onset PSC until death, OLT or end of follow-up.

Results: Twenty-year cumulative incidence (CI) of death or OLT for PSC patients with MMP-2 CT genotype was 86.5% compared to 52.8% for CC genotype (p=0.030) and reached 100% at 11.3 years for TT genotype. In patients with IBD, CIs were similar: 20-years CI of death or OLT for MMP-2 CT genotype was 86.0% compared to 49.0% for CC genotype and 100% at 11.3 years for TT genotype. Patients without IBD showed a similar trend in 20 year CI for MMP-2 CT (77.8%) compared to CC (57.8%) and CI for TT genotype reached 100% at 9.3 years. Multivariate analysis showed, along with age at diagnosis, a stepwise increase in hazard ratio for MMP-2 T-allele polymorphism for death or OLT. MMP-9 genotype was not associated with disease severity in PSC.

Conclusion: MMP-2 C to T –1306 promoter polymorphism in PSC is an independent risk factor for disease severity as reflected by need for OLT or disease progression leading to mortality.

Introduction

In primary sclerosing cholangitis (PSC) the chronic inflammation of the bile ducts leads to fibrosis and eventually often to liver cirrhosis. As to date, the etiology of PSC remains unknown and no specific treatment can delay or arrest the progressive course of this disease. Therefore, orthotopic liver transplantation (OLT) remains the only curative option for endstage disease. Furthermore, PSC is often accompanied by inflammatory bowel disease (IBD), particularly ulcerative colitis (UC), affecting almost 70% of the PSC patients.[1,2] Mean survival after onset of PSC until OLT, death by cholangitis, cholangiocarcinoma (CCA) or liver failure is reported to be 15 to 18 years.[1,3] The pathogenesis of PSC most likely involves genetic, environmental, immune and remodeling factors each contributing to inflammation, cell dysfunction and, in later stages, development of fibrosis. Genome-wide association studies (GWAS) on PSC have implicated the role of several genes such as FUT2, GPR35 and TCF4 as potential key players for the intracellular processing of bacterial pathogens in the intestinal mucosa.[4,5]. We have previously identified three UC susceptibility loci to be associated with PSC involving both the innate immune system (REL, CARD9) as well as the adaptive system (IL-2 pathway).[6] Moreover, mutations of the takeda G-protein-coupled bile acid receptor 5 (TGR5) gene located at 2q35 (TGR5) has been proposed as a plausible disease gene in PSC due to the expression in monocytes and macrophages, particularly since TGR5 has been reported to inhibit inflammatory cytokines from activated macrophages, including Kupffer cells.[7-10] The identified genes are involved in disease development but are not necessarily involved in disease modification. The number of studies that describe demarcation markers for the clinical course of this potentially life-threatening disease is limited.[11-13] Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes which are involved in the degradation, processing and remodeling of the extracellular matrix (ECM).[14,15] Dysregulation of MMPs is a feature of numerous conditions such as cancer and various inflammatory and fibrotic processes.[16,17] Interestingly, previous studies have shown an impact of MMP-3 genotype on disease progression in PSC although from genome-wide association studies (GWAS) this gene was not identified to be associated with the development of the

disease.[4,18] MMP-2 and -9 are known to play a key role in the degradation of type IV collagen and are also involved in the cleavage of galectin-3 which in turn is involved in chemoattraction and migration of cells, and in apoptosis.[19,20] Collagen type IV is a major component of the basal membrane, which can also be found around bile ducts, and is considered to be essential for the development of intrahepatic bile ducts.[21] In this study, the gene promoter polymorphisms of MMP-2 and MMP-9 were studied in relation to the clinical course of PSC patients using OLT or death as markers of severity of disease.

Patients and Methods

For this study a total of 132 adult patients with PSC of whom DNA was available could be included from the Leiden University Medical Center (LUMC, n=69) and the University Medical Center Groningen (UMCG, n=63). Both centers are tertiary referral centers and offer liver transplantation as treatment for end-stage PSC. Genomic DNA was extracted routinely from peripheral blood and/or tissue samples without given preference to any explicit clinical variable. All patients were routinely checked at the outpatient clinic and PSC patients were screened regularly for the need for OLT. All patients were prospectively included and followed up in the centers until July 2012 and the combined endpoint death or first OLT was considered as primary end-point for this study. Clinical data including death or OLT were obtained from medical records and hepatological and surgical patient charts. This study was performed according to the guidelines of the Medical Ethics Committee of both participating centers and in compliance with the Helsinki Declaration.

PSC diagnosis and OLT

Patients with clinical suspicion of PSC were (re)evaluated with either endoscopic retrograde cholangioscopy (ERCP) or magnetic resonance cholangiography (MRCP). In case of clear characteristics on the cholangiogram PSC was classified as large duct PSC. A liver biopsy was performed on patients with

clinical symptoms but without clear characteristics of PSC on the cholangiogram, in which case a small duct PSC was the diagnostic feature. OLT was performed according to standard procedures. Post OLT standard immunosuppressive therapy was given consisting of corticosteroids, a calcineurin inhibitor with or without mycophenolate mofetil or azathiopurine. From 2001 on, basiliximab was given during and day 4 post OLT.

MMP-2: high-resolution DNA melting analysis

MMP2 –1306 C/T (rs243865) as most relevant SNP was assessed using high-resolution DNA melting assay, as described previously.[22] In brief, sequences of the primers used in the polymerase chain reaction (PCR) were 5'-CCAGT-GCCTCTTGCTGTTTT-3' (forward) and 5'- GACTTCTGAGCTGAGACCTGA-3' (reverse). The wild-type (C) genotype sequence: 5'-CCACCCAGCACTCCAC-CTCTTTAGCTC-3' was assessed using an unlabelled probe designed accordingly, with a 3'-amino-C7 modification to prevent DNA polymerase extension during PCR. High-resolution melting analysis of PCR products amplified in the presence of a saturating double stranded DNA dye (LCGreenPlus, Idaho Technology, Salt Lake City, Utah, USA) and a 3'-blocked probe to identify both heterozygous and homozygous sequence variants distinguished by differences in the melting curve shape. In each experiment, sequence-verified control donors for each genotype were used. Observed frequencies in our PSC patients were CC=70, CT=53 and TT=9, which is in Hardy-Weinberg equilibrium.

MMP-9: PCR-RFLP genotyping

The SNP C/T at position –1562 (rs3918242) of the MMP-9 gene promoter was determined by PCR-RFLP, also previously described.(19) The SNP flanking region was amplified using primers 5'-ATGGCTCATG-CCCGTAATC-3' and 5'-TCACCTTCTTCAAAGCCCTATT-3' followed by restriction analysis with Sph I to produce 352+207+145 bp fragments that identify CC, CT and TT genotypes by the migration patterns on agarose gels.(22-24) Observed frequencies in our PSC patients were CC=102, CT=29 and TT=1, which is in Hardy-Weinberg equilibrium.

Statistical analysis

Data were analyzed using SPSS 20.0 software (SPSS Inc.; Chicago, IL, USA). Log-rank statistics were used for follow-up data. Analysis of Variance (ANOVA) test was used for normal distributions. Non-parametric Mann-Whitney U test was used to analyze medians in case of non-normal distributions. Univariate and multivariate analysis were performed using Cox's proportional hazards method. Variables associated with an increased risk at the $p<0.20$ level in the univariate analysis were taken into account in the multivariate analysis. A p-level of ≤ 0.05 was considered statistically significant for the multivariate analyses.

Results

Clinical features

A total of 132 patients were included with a median age of 38 years at onset of PSC and a median follow-up of 12.5 years. Sixty PSC patients (45.5%) were transplanted with a median age at OLT of 49 years. Eleven patients (8.3%) died before receiving transplantation of which 7 developed CCA (5.3%). Median follow-up from PSC diagnosis to OLT was 8.2 years (range 0.4 – 21 years). Of all PSC patients 80.3% had concomitant IBD, with a predominance of UC (64.4%). Large duct PSC was diagnosed using ERCP in 72 cases (54.5%) and in 36 patients (27.3%) the diagnosis was by MRCP. Small ducts PSC was diagnosed in 24 (18.2%) patients based on liver biopsy when no clear characteristics of PSC were present on the cholangiogram. A list of baseline characteristics of the patients is given in *table 1*.

MMP genotype association

MMP-2 CT and TT genotypes within the PSC patients were significantly associated with a higher incidence of OLT or death during follow-up. The cumulative incidence of patients experiencing OLT or death in 20 years after diagnosis was 86.5% for the CT genotype compared to 52.8% for the CC genotype (CT vs. CC log-rank p=0.030). Patients with a TT genotype had cumulative incidence of death or OLT of 100% in 11.3 years after diagnosis, which was higher compared to patients with CT or CC genotype (overall comparison, logrank p=0.027, *figure 1a*). In addition, the MMP-2 promoter polymorphisms showed comparable results when PSC patients had IBD as concomitant disease, indicated by the cumulative incidence of death or OLT within 20 years of 86.0% for the CT genotype group compared to 49.0% for PSC patients with IBD and a CC genotype (CT vs. CC log-rank p=0.038). Accordingly, PSC patients with a TT genotype also showed comparable results when IBD was concomitant, with a cumulative incidence of death or OLT of 100% in 11.3 years compared to (overall comparison log-rank p=0.021).
PSC patients with the MMP-2 CT (n=9) or TT (n=1) genotype but without IBD as concomitant disease had a similar trend in the cumulative incidence

of OLT or death of 77.8% for CT and 100% for TT compared to 57.8% for the CC genotype (n=16), although due to small numbers this difference did not reach statistical significance (p=0.437, *figure 1c*). In patients undergoing OLT the Model for End-stage Liver Disease (MELD) scores immediately preceding OLT were not significantly different in the CT or TT genotype group compared to the CC group (median MELD score CC group 11 (n=25); CT group 12 (n= 31); TT group 10 (n=4), ANOVA p=0.732). Furthermore, only 18% (11/60) had a MELD score >17 indicating more severe liver disease in these patients (CC=4; CT=6; TT=1, ANOVA p=0.625).

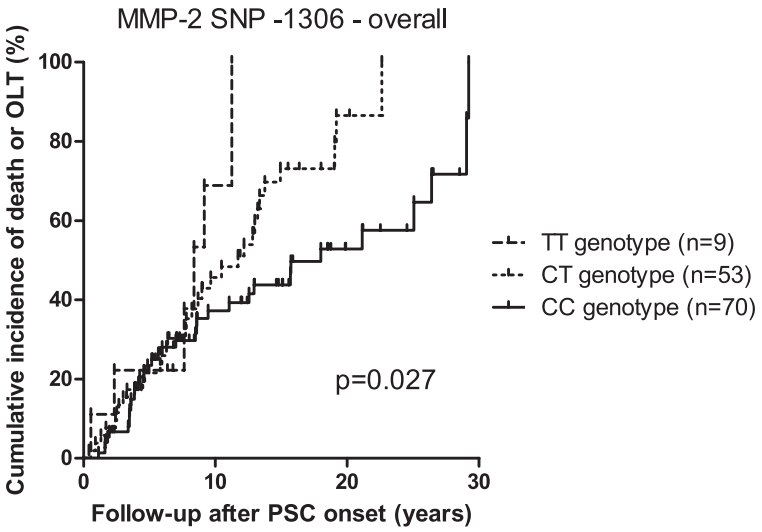
The MMP-9 gene promoter polymorphisms were evaluated similarly but no association was found of these polymorphisms and the risk of OLT or death as indicators of disease severity. No association was found for MMP-2 or MMP-9 genotypes and the development of CCA in PSC patients.

Table 1. Baseline characteristics

Characteristic		Total n=132
Age at onset of PSC (median, range)		38 (18 – 75)
Endpoint	Death % (n)	8.3 (11)
	OLT % (n)	45.5 (60)
Age at OLT (median, range)		49 (21 – 68)
Follow-up after PSC (median,range)		12.5 (0.6 – 30)
Gender % (n)	Male	66.7 (88)
	Female	33.3 (44)
IBD % (n)	UC	64.4 (85)
	CD	10.6 (14)
	Indeterminate	5.3 (7)
	No IBD	19.7 (26)
MELD score before OLT (median, range)		11 (6 – 34)
Type of PSC (%)	Small duct	18.2 (24)
	Large duct	81.8 (108)

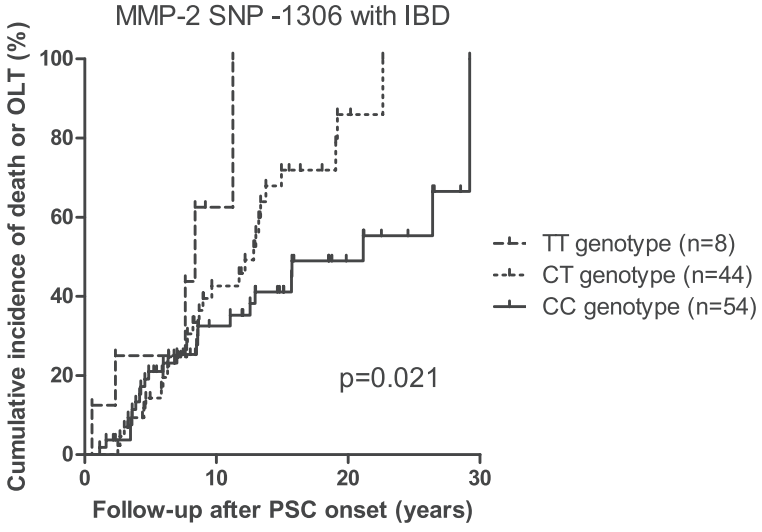
PSC, primary sclerosing cholangtitis; OLT, orthotopic liver transplantation; MELD, Model for End-Stage Liver Disease; IBD, Inflammatory Bowel Disease; UC, Ulcerative Coltis; CD, Crohn's Disease.

Figure 1a



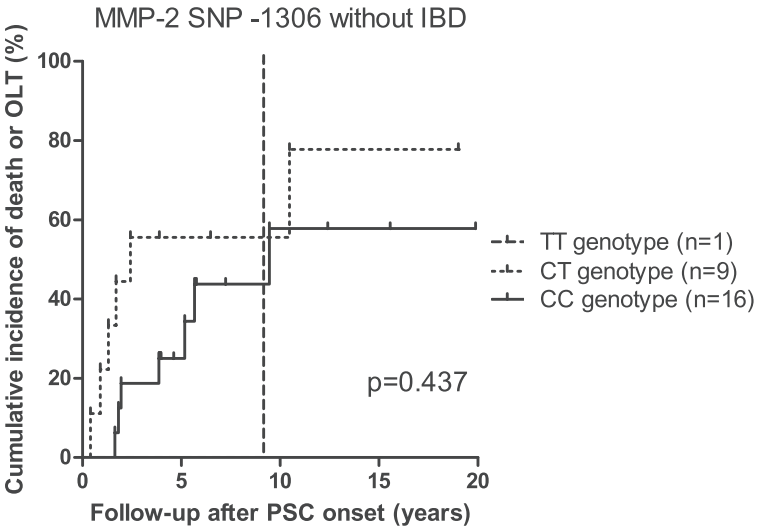
The cumulative incidence of death or OLT after onset of PSC. Cumulative incidence during 20-years of follow-up after diagnosis of PSC was significantly higher in PSC patients with MMP-2 CT genotype (86.5%) compared to the CC-genotype (52.8%; p=0.030). Patients with a TT genotype had a cumulative incidence of 100% after 11.3 years (overall comparison; log-rank p=0.027)

Figure 1b



The cumulative incidence of death or OLT in relation to IBD and MMP-2 genotype. Cumulative incidence of death or OLT during 20-years of follow-up PSC patients with the MMP-2 CT/TT genotype and IBD as concomitant disease was 86.0% compared to 49.0% for PSC patients with IBD and wild-type MMP-2 promoter (CC) (CT vs. CC, log-rank p=0.038). Patients wit a TT genotype reached a cumulative incidence of 100% after 11.3 years (overall comparison, log-rank p=0.021)

Figure 1c



PSC patients with the MMP-2 CT/TT genotype but no IBD as concomitant disease had a cumulative incidence of death or OLT at twenty years of 77.8% compared to 57.8% for the CC genotype (overall comparison; log-rank p=0.437)

Table 2. Univariate and multivariate analysis of risk factors for death or OLT in PSC patients. Multivariate analysis showed that age as well the presence of MMP-2 CT or TT genotype in PSC patients were significant risk factors for death or OLT.

			Univariate analysis		Multivariate analysis	
Variables		Total	HR (95%CI)	p-value	HR (95%CI)	p-value
Gender	Male	88/132	1.05 (0.63 – 1.76)	0.852		
	Female	44/132	1(reference)			
Age at PSC onset	Continuous		1.03 (1.01 – 1.05)	0.001	1.03 (1.01 – 1.05)	0.004
MMP-2 (rs243865)	Overall			0.031		0.019
	TT	9/132	2.65 (1.08 – 6.51)	0.031	2.87 (1.16 – 7.09)	0.023
	CT	53/132	1.74 (1.05 – 2.88)	0.034	1.80 (1.09 – 2.99)	0.022
	CC	70/132	1 (reference)		1 (reference)	
MMP-9 (rs3918242)	Overall			0.560		
	TT	1/132	2.13 (0.29 – 15.56)	0.456		
	CT	29/132	1.27 (0.72 – 2.23)	0.408		
	CC	102/132	1 (reference)			
Type of PSC	Small duct	24/132	0.92 (0.50– 1.69)	0.790		
	Large duct	108/132	1 (reference)			
IBD	IBD	106/132	0.54 (0.30– 0.97)	0.039	0.61 (0.33 – 1.12)	0.112
	No IBD	26/132	1 (reference)			

Univariate and multivariate analysis

In the univariate Cox regression analysis, the age at diagnosis of PSC, IBD and MMP-2 CT/TT genotype were found to be associated at the $p<0.20$ level and were thus taken into account in the multivariate analysis. No significant association was found for gender, subtype of PSC (e.g., large or small duct) or MMP-9 genotypes and the risk of OLT or death in the univariate analysis. Multivariate Cox regression analysis revealed age at onset of PSC as well as MMP-2 CT and TT genotype promoter polymorphisms to be significant and independent risk factors for OLT or death for PSC patients (age at onset hazard ratio (adjusted HR) 1.03, $p=0.004$, MMP-2 CT adjusted HR 1.80, $p=0.022$, MMP-2 TT adjusted HR=2.87, $p=0.023$). *(Table 2)*

Discussion

In this study we found the MMP-2 that the T-allele of the –1306 C/T promoter polymorphisms is an important, gene-dose dependent and clinically independent diseasemodifying gene in PSC, also when the disease was accompanied by inflammatory bowel disease. This disease modifying effect was not observed with MMP-9 and therefore underlines the contribution of MMP-2 in progressive liver diseases accompanied with fibrotic tissue formation. PSC is still a disease of unknown origin but several hypotheses exist on the pathogenesis of PSC. One theory derives from the strong association of PSC with IBD and suggests that damage to the biliary tract might result from aberrant lymphocyte trafficking from the intestinal mucosa to the liver.[25] Other studies suggest that cholangiocyte dysfunction involving mutations of the TGR5 gene, with impaired protection from toxic bile salts by the bicarbonate "umbrella" contributes to a decreased inhibition of inflammatory cytokines released from Kupffer cells.[7,9,26] Although many of the above mentioned loci have been implicated in the pathogenesis of PSC, description of genes that contribute to disease progression is limited. This might be due to the fact that GWAS studies only focus on disease development rather than on disease outcome. Furthermore, investigating genetic associations with disease progression requires patients with long-term follow-up in contrast to disease association studies. Interestingly, genetic variants of MMP-3 have previously been associated with disease development and severity, although the gene did not emerge from the GWAS studies.[4,5,27] Jurdan et al. showed that genetic variants of MMP-3 were associated with increased disease progression and this progression was even more emphasized when PSC patients had concurrent UC.[28] Satsangi et al. showed the MMP-3 5A allele to be more frequently present in PSC patients compared to UC patients and healthy controls suggesting the potential involvement of MMP-3 in PSC development.[29] However, so far MMP-2 and MMP-9 were not investigated as potential disease modifiers in PSC. Matrix metalloproteinases are a large group of proteolytic enzymes involved in remodeling the extracellular matrix (ECM) in physiological as well as pathological conditions. MMP-2 and MMP-9 are thought to play a key role in the degradation of all kinds of ECM components, such as type IV collagen, a main component of

ECM. The C/T transition at position 1306 in the promoter of MMP-2, which abolishes the Sp 1 binding site and leads to decreased mRNA transcription and protein expression, is generally accepted as yet to be the most relevant SNP for MMP-2 and in almost complete linkage disequilibrium with several other single nucleotide polymorphisms in the MMP-2 promoter region.[30] In the current study we have shown a stepwise increase in hazard ratio for death or OLT in the multivariate analysis for the C/T transition for MMP-2 independent of age at which PSC was diagnosed. Moreover, small or large duct PSC did not have an impact on survival or need for OLT in our population. In a previous study we have shown that MMP-2 CT genotype was clearly associated with the development of nonanastomotic biliary strictures (NAS) within 4 years after OLT.[22] We also found that for patients who developed NAS after OLT, serum levels of MMP-2 tended to be lower for the CT/TT-genotype group compared to the CC-genotype group. The C/T transition likely leads to decreased activity of MMP-2, which may lead to decreased cleavage of collagen. In case of PSC, where the inflammation stimulus is continuously present, the pivotal role of a decreased locoregional MMP-2 activity may be even more emphasized with subsequent collagen formation and deposition. Approximately 70% - 90% of PSC patients have IBD.[31] Interestingly, treatment of IBD with medication or even colectomy does not stop or alter the natural course of PSC in these patients.[2,32] This is in line with our multivariate analysis where IBD was found to have no independent association and thus no impact on the progression of PSC. However, PSC patients without concomitant IBD also showed similar cumulative incidences of 77.8% for the CT genotype compared to 57.8% for PSC patients with CC genotype and no IBD. Only one patient without IBD had a TT genotype and had an event at 9.2 years. Although this did not reach statistical significance, most likely due to small numbers, it clearly shows that the MMP-2 CT/TT genotype is generally and consistently involved in disease progression in PSC. Remarkably,in contrast to the association found with MMP-2 there was no association between the MMP- 9 promoter polymorphisms and PSC disease severity. This finding is in line with our previous observation where we found no association between the MMP-9 promoter polymorphisms and the development of NAS after OLT.[22] These observations might imply that MMP-9 polymorphisms are less likely to contribute to fibrotic processes in the liver.

The current study has some potential limitations. For instance, we evaluated MMP-9 –1562 C/T as most relevant SNP. Although several SNPs of MMP-9 are known, only two of them seem to be functional, a –1562 C/T substitution (rs 3918242) and a microsatellite SNP at –90(CA)n (rs 3222264).[33,34] One study previously showed that the MMP-9 –1562C/T polymorphism increased MMP-9 expression in vitro.[35] However, in our previous study MMP-9 –1562C/T was not associated with the development of NAS and the present study also showed no association with disease progression in patients with PSC. Secondly, no MMP was associated with PSC in any of the performed GWAS studies so far. However, our current study is hypothesis driven based on our previous report on MMP-2 C/T polymorphisms and NAS development. The role of MMP-2 in NAS was previously observed, indicating the importance of MMP-2 in the turn-over of collagen surrounding bile ducts. Furthermore, in the present study we found a clear gene dose-dependent relationship in the C/T polymorphism with disease progression in PSC patients. We realize that the current study has a relative small sample size (n=132) and larger studies are indicated to confirm the results of the present study. These studies might preferably also include longitudinal assessment of circulating levels and tissue expression of MMP-2 in PSC patients in relation to genotype, disease progression and fibrosis formation. It is important, however, to realize that the genotype of the PSC patient does not change over time, whereas we know from our previous studies that the MMP-2 levels change with disease progression, i.e., fibrosis and cirrhosis, irrespective of the phenotype and genotype, and that these levels change after OLT but these levels depend on donor and recipient mismatch of the MMP-2 genotype.[22,36,37] Furthermore, we did not determine serum IgG4 levels to distinguish for possible IgG4-related sclerosing cholangitis (IgG4-SC). However, involvement of IBD is highly unusual in IgG4- SC, while IBD was present in 80.4% of the cases in our population. In addition, no patients had involvement of other organs such as the pancreas, which is common in IgG4-mediated disease.

In summary, the MMP-2 –1306 C/T gene promoter polymorphism in PSC patients is an independent risk factor for a more severe phenotype resulting in more frequent and earlier OLT or patient's mortality. Our current study is

in line with previous studies on MMPs and PSC severity and indicates the important role of MMPs in regulating liver matrix homeostasis in PSC. These findings contribute to our understanding of the pathophysiologic processes involved in PSC and may provide an additional diagnostic tool to identify high-risk patients for timely referral to a hospital with transplantation facilities.

References

1. Ponsioen CY. Recent insights in primary sclerosing cholangitis. J Dig Dis 2012 Jul;13(7):337-341.

2. Papatheodoridis GV, Hamilton M, Mistry PK, Davidson B, Rolles K, Burroughs AK. Ulcerative colitis has an aggressive course after orthotopic liver transplantation for primary sclerosing cholangitis. Gut 1998 Nov;43(5):639-644.

3. Chapman R, Cullen S. Etiopathogenesis of primary sclerosing cholangitis. World J Gastroenterol 2008 Jun 7;14(21):3350-3359.

4. Folseraas T, Melum E, Rausch P, Juran BD, Ellinghaus E, Shiryaev A, et al. Extended analysis of a genome-wide association study in primary sclerosing cholangitis detects multiple novel risk loci. J Hepatol 2012 Aug;57(2):366-375.

5. Ellinghaus D, Folseraas T, Holm K, Ellinghaus E, Melum E, Balschun T, et al. Genome-wide association analysis in sclerosing cholangitis and ulcerative colitis identifies risk loci at GPR35 and TCF4. Hepatology 2012 Jul 23.

6. Janse M, Lamberts LE, Franke L, Raychaudhuri S, Ellinghaus E, Muri BK, et al. Three ulcerative colitis susceptibility loci are associated with primary sclerosing cholangitis and indicate a role for IL2, REL, and CARD9. Hepatology 2011 Jun;53(6):1977-1985.

7. Kawamata Y, Fujii R, Hosoya M, Harada M, Yoshida H, Miwa M, et al. A G proteincoupled receptor responsive to bile acids. J Biol Chem 2003 Mar 14;278(11):9435- 9440.

8. Keitel V, Reinehr R, Gatsios P, Rupprecht C, Gorg B, Selbach O, et al. The G-protein coupled bile salt receptor TGR5 is expressed in liver sinusoidal endothelial cells. Hepatology 2007 Mar;45(3):695-704.

9. Keitel V, Donner M, Winandy S, Kubitz R, Haussinger D. Expression and function of the bile acid receptor TGR5 in Kupffer cells. Biochem Biophys Res Commun 2008 Jul 18;372(1):78-84.

10. Keitel V, Haussinger D. Perspective: TGR5 (Gpbar-1) in liver physiology and disease. Clin Res Hepatol Gastroenterol 2012 Apr 18.

11. Invernizzi P. Primary sclerosing cholangitis is changing clinical spectrum and old biomarkers disclose an innovative role: the case of alkaline phosphatase. Dig Liver Dis 2011 Apr;43(4):268-269.

12. Dobric S, Popovic D, Nikolic M, Andrejevic S, Spuran M, Bonaci-Nikolic B. Antineutrophil cytoplasmic antibodies (ANCA) specific for one or several antigens: useful markers for subtypes of ulcerative colitis and associated primary sclerosing cholangitis. Clin Chem Lab Med 2012 Mar;50(3):503-509.

13. Reinhard L, Rupp C, Riedel HD, Ruppert T, Giese T, Flechtenmacher C, et al. S100A9 is a biliary protein marker of disease activity in primary sclerosing cholangitis. PLoS One 2012;7(1):e29821.

14. Shapiro SD. Matrix metalloproteinase degradation of extracellular matrix: biological consequences. Curr Opin Cell Biol 1998 Oct;10(5):602-608.

15. Vargova V, Pytliak M, Mechirova V. Matrix metalloproteinases. EXS 2012;103:1-33.

16. Zhou Y, Yu C, Miao X, Tan W, Liang G, Xiong P, et al. Substantial reduction in risk of breast cancer associated with genetic polymorphisms in the promoters of the matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-2 genes. Carcinogenesis 2004 Mar;25(3):399-404.

17. Pytliak M, Vargova V, Mechirova V. Matrix metalloproteinases and their role in oncogenesis: a review. Onkologie 2012;35(1-2):49-53.

18. Ellinghaus D, Folseraas T, Holm K, Ellinghaus E, Melum E, Balschun T, et al. Genome-wide association analysis in sclerosing cholangitis and ulcerative colitis identifies risk loci at GPR35 and TCF4. Hepatology 2012 Jul 23.

19. Nangia-Makker P, Raz T, Tait L, Hogan V, Fridman R, Raz A. Galectin-3 cleavage: a novel surrogate marker for matrix metalloproteinase activity in growing breast cancers. Cancer Res 2007 Dec 15;67(24):11760-11768.

20. Nangia-Makker P, Wang Y, Raz T, Tait L, Balan V, Hogan V, et al. Cleavage of galectin-3 by matrix metalloproteases induces angiogenesis in breast cancer. Int J Cancer 2010 Dec 1;127(11):2530-2541.

21. Yasoshima M, Sato Y, Furubo S, Kizawa K, Sanzen T, Ozaki S, et al. Matrix proteins of basement membrane of intrahepatic bile ducts are degraded in congenital hepatic fibrosis and Caroli's disease. J Pathol 2009 Feb;217(3):442-451.

22. Ten Hove WR, Sebib Korkmaz K, op den Dries S., de Rooij BJ, van Hoek B., Porte RJ, et al. Matrix metalloproteinase 2 genotype is associated with nonanastomotic biliary strictures after orthotopic liver transplantation. Liver Int 2011 Sep;31(8):1110-1117.

23. Kubben FJ, Sier CF, van Duijn W., Griffioen G, Hanemaaijer R, van de Velde CJ, et al. Matrix metalloproteinase-2 is a consistent prognostic factor in gastric cancer. Br J Cancer 2006 Apr 10;94(7):1035-1040.

24. Meijer MJ, Mieremet-Ooms MA, van Hogezand RA, Lamers CB, Hommes DW, Verspaget HW. Role of matrix metalloproteinase, tissue inhibitor of metalloproteinase and tumor necrosis factor-alpha single nucleotide gene polymorphisms in inflammatory bowel disease. World J Gastroenterol 2007 Jun 7;13(21):2960-2966.

25. Grant AJ, Lalor PF, Salmi M, Jalkanen S, Adams DH. Homing of mucosal lymphocytes to the liver in the pathogenesis of hepatic complications of inflammatory bowel disease. Lancet 2002 Jan 12;359(9301):150-157.

26. Beuers U, Hohenester S, de Buy Wenniger LJ, Kremer AE, Jansen PL, Elferink RP. The biliary HCO(3)(-) umbrella: a unifying hypothesis on pathogenetic and therapeutic aspects of fibrosing cholangiopathies. Hepatology 2010 Oct;52(4):1489-1496.

27. Janse M, Lamberts LE, Franke L, Raychaudhuri S, Ellinghaus E, Muri BK, et al. Three ulcerative colitis susceptibility loci are associated with primary sclerosing cholangitis and indicate a role for IL2, REL, and CARD9. Hepatology 2011 Jun;53(6):1977-1985.

28. Juran BD, Atkinson EJ, Schlicht EM, Larson JJ, Ellinghaus D, Franke A, et al. Genetic poly morphisms of matrix metalloproteinase 3 in primary sclerosing cholangitis. Liver Int 2011 Jul;31(6):785-791.

29. Satsangi J, Chapman RW, Haldar N, Donaldson P, Mitchell S, Simmons J, et al. A functional polymorphism of the stromelysin gene (MMP-3) influences susceptibility to primary sclerosing cholangitis. Gastroenterology 2001 Jul;121(1):124-130.

30. Price SJ, Greaves DR, Watkins H. Identification of novel, functional genetic variants in the human matrix metalloproteinase-2 gene: role of Sp1 in allele-specific transcriptional regulation. J Biol Chem 2001 Mar 9;276(10):7549-7558.

31. Boonstra K, van Erpecum KJ, van Nieuwkerk KM, Drenth JP, Poen AC, Witteman BJ, et al. Primary sclerosing cholangitis is associated with a distinct phenotype of inflammatory bowel disease. Inflamm Bowel Dis 2012 Mar 8.

32. Aoki CA, Bowlus CL, Gershwin ME. The immunobiology of primary sclerosing cholangitis. Autoimmun Rev 2005 Mar;4(3):137-143.

33. Van den Steen PE, Dubois B, Nelissen I, Rudd PM, Dwek RA, Op den akker G. Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9). Crit Rev Biochem Mol Biol 2002 Dec;37(6):375-536.

34. Shimajiri S, Arima N, Tanimoto A, Murata Y, Hamada T, Wang KY, et al. Shortened microsatellite d(CA)21 sequence down-regulates promoter activity of matrix metalloproteinase 9 gene. FEBS Lett 1999 Jul 16;455(1-2):70-74.

35. Zhang B, Ye S, Herrmann SM, Eriksson P, de MM, Evans A, et al. Functional polymorphism in the regulatory region of gelatinase B gene in relation to severity of coronary atherosclerosis. Circulation 1999 Apr 13;99(14):1788-1794.

36. Kuyvenhoven JP, van HB, Blom E, van DW, Hanemaaijer R, Verheijen JH, et al. Assessment of the clinical significance of serum matrix metalloproteinases MMP-2 and MMP-9 in patients with various chronic liver diseases and hepatocellular carcinoma. Thromb Haemost 2003 Apr;89(4):718-725.

37. ten Hove W.R., de Rooij B.-J.F., van Hoek B., Kuyvenhoven J.Ph., Meijer M.J.W., van den Berg M., et al. MMP-2 and MMP-9 serum levels change but their gene promoter polymorphisms are not associated with late phase I/R Injury or rejection after orthotopic liver transplantation. The Open Transplantation Journal 2008 Oct 20;2:66-72.

Chapter 7

Acute cellular rejection is associated with matrix metalloproteinase-2 genotype chimerism after orthotopic liver transplantation

Kerem Sebib Korkmaz[1], W. Rogier ten Hove[1], Bert-Jan F. de Rooij[1], Bart van Hoek[1], Johan J. van der Reijden[1], Minneke J. Coenraad[1], Jeroen Dubbeld[2], Hein W. Verspaget[1]

[1] Department of Gastroenterology and Hepatology, Leiden University Medical Center, Leiden, The Netherlands

[2] Department of Surgery, Leiden University Medical Center, Leiden, The Netherlands

Transplantation Proceedings 2013 Mar;45(2):558-63

Abstract

Purpose: Chimerism in transplantation medicine refers to the coexistence of cells of donor and recipient origin. Their existence in relation to possible pathological mechanisms remains largely unknown. We used donor/recipient mismatches for matrix metalloproteinases (MMP) gene polymorphisms in liver biopsies and in blood as a marker for chimerism after orthotopic liver transplantation (OLT). The second aim of this study was to evaluate these polymorphisms in relation to clinical outcome such as ischemia/reperfusion injury (IRI) and acute cellular rejection (ACR).

Methods: MMP-2 and MMP-9 promoter polymorphism donor/recipient mismatches were determined in 147 OLT patients. The relationship between these MMP polymorphism mismatches in donor and recipient DNA with the development of IRI and ACR after OLT was evaluated. Liver biopsy specimens and peripheral blood samples were subsequently evaluated for the presence of chimerism, also in relation to these complications.

Results: MMP polymorphism donor/recipient mismatches were found in 53.7% (MMP-2) and 35.5% (MMP-9) of the OLT patients but no relation was observed with IRI or ACR. Chimerism in liver biopsy specimens was found to be present in 28.8% (MMP-2) and 16.2% (MMP-9) of the cases. Liver chimerism in MMP-2 was found to be significantly associated with ACR after OLT (χ^2 6.4, p=0.01). Multivariate analysis revealed MMP-2 chimerism to be an independent risk factor for ACR after OLT adjusted for MELD score (HR=3.83, p=0.03). In addition, evidence of donor chimerism was found in peripheral blood samples of the recipients in some cases.

Conclusion: Chimerism after liver transplantation can be found in liver biopsy specimens and in peripheral blood. MMP donor/recipient polymorphism mismatches are good markers for assessing chimerism after OLT. In the multivariate analysis liver chimerism in MMP-2 was found to be significantly associated with ACR after OLT.

Introduction

Chimerism after solid organ transplantation is a fascinating phenomenon. In the earliest days of organ transplantation, back in the 1960s, Medawar hypothesized that chimerism could lead to graft tolerance.[1] The existence of chimerism after transplantation have been studied by many, but the clinical significance remains unraveled.[2-5] Previously, we have addressed different aspects of chimerism within the liver, both after orthotopic liver transplantation (OLT) and after allogeneic bone marrow transplantation (BMT).[6,7] In these earlier studies we used in situ hybridization techniques targeting Y-chromosomes. A major drawback of this technique is that only a small fraction of sex mismatched donor/recipient combinations are relevant to be studied, i.e., male recipients receiving a female liver and female recipients receiving a male BMT. In the present study we have used a different technique to investigate chimerism after OLT, which allowed us to include a larger number of liver transplant recipients. Gene polymorphisms of matrix metalloproteinases (MMP) 2 and 9 were analyzed. We previously reported on MMP polymorphisms in relation to different complications of liver transplantation and found preliminary indications that chimerism did occur.[8,9] Mismatches between donor and recipients were selected to study the presence of chimerism in liver biopsy specimens and in peripheral blood after liver transplantation. The aim of the present study was to further explore the existence of chimerism in MMP-2 and MMP-9 promoter polymorphism donor/recipient mismatches in both liver biopsy specimens and in peripheral blood after OLT. The association between these MMP mismatches and the clinical occurrence of ischemia/reperfusion injury (IRI) and acute cellular rejection (ACR) after OLT was also assessed.

Patients and Methods

Patients

All patients who received a liver transplant at Leiden University Medical Center (LUMC) between 1992 and 2005 were eligible for inclusion. Of these 202 patients, donor and recipient DNA was available of 147 patients with at least 7 days of follow-up after OLT. Demographical and clinicopathological characteristics of the study population were obtained from the transplantation database. The study was performed according to the guidelines of the Medical Ethics Committee of the Leiden University Medical Center and in compliance with the Helsinki Declaration.

Genotyping

Genomic DNA was extracted by routine methods from peripheral blood leukocytes and/or tissue samples. In addition, DNA samples from the blood of the liver donors were obtained from the Eurotransplant Reference Laboratory or freshy isolated from donor blood or spleen tissue. Also, DNA was isolated from liver biopsy tissue of the allograft in the recipients, obtained several months (median 17, range 5 to 48) after OLT.

MMP-2: high resolution DNA melting analysis (HRMA)

MMP-2 –1306 C/T promoter SNP was determined using a high resolution DNA melting assay with the 5'-CCACCCAGCACTCCACCTCTTTAGCTCF-3' wild-type (C) gene probe, and the primers 5'-CCAGTGCCTCTTGCTGTTTT-3' (forward) and 5'-GACTTCTGAGCTGAGACCTGA-3' (reverse).[9,10] This –1306 C/T MMP-2 gene promoter polymorphism was also determined by tetra-primer amplification refractory mutation systempolymerase chain reaction (PCR) analysis, the principles of which are described elsewhere9, and confirmed by direct sequence analysis of 4 patients. Briefly, the region flanking the SNP was amplified with outer primers 5'-ACCAGACAAGCCTGAACTTGTCTGA-3' and 5'-TGTGACAACCGTCTCTGAGGAATG-3' together with inner allelic specific primers 5'-ATATTCCCCACCCAGCACGCT-3' and

5'-GCTGAGACCTGAAGAGCTAAAGAGTTG-3'. Genotypes CC, CT and TT (542+379; 542+379+211; 542+211 bp, respectively) are easily identified from the migration pattern on agarose gels.
This common functional polymorphism abolishes an Sp1 binding site within the promoter region of MMP-2. In brief, high-resolution melting analysis of PCR products amplified in the presence of a saturating double-stranded DNA dye (LCGreenPlus, Idaho Technology) and the 3'-blocked probe, identifies both heterozygous and homozygous sequence variants. Heterozygotes and homozygotes are distinguished by differences in the melting curve shape, due to differences in melting temperature. In each experiment, sequence-verified control donors for each genotype were used. MMP-2 genotype distribution was as follows: in recipients CC 67.3% (n=99), CT 27.9% (n=41), TT 4.8% (n=7) and in donors CC 45.6% (n=67), CT 49.7% (n=73), TT 4.8% (n=7).

MMP-9: PCR-Restriction Fragment Length Polymorphism (RFLP) genotyping.

The SNP C/T at position –1562 of the MMP-9 gene promoter was determined by PCR-RFLP. The SNP flanking region was amplified using primers 5'-ATGGCTCATG-CCCGTAATC-3' and 5'-TCACCTTCTTCAAAGCCCTATT-3' followed by restriction analysis with Sph I to produce 352, 352+207+145 or 207+145 bp fragments in case of CC, CT and TT genotype, respectively, which are easily identified from the migration patterns on agarose gels.[8,11-13]
MMP-9 genotype distribution was as follows: in recipients CC 75.9% (n=107), CT 23.4% (n=33), TT 0.7% (n=1) and in donors CC 73.0% (n=103), CT 27.0% (n=38) and none of the donors had TT genotype.

Assessment of MMP gene mismatch and chimerism in liver biopsy specimens and peripheral blood samples

Mismatch in the MMP-2 or -9 genes is defined as a non-identical genotype in OLT recipient and donor. Chimerism is defined as the presence of an MMP genotype signal in the DNA of the liver biopsy after OLT that originates from the recipient and in the blood DNA of the recipient after OLT when an MMP genotype signal appears from the donor.

Ischemia and reperfusion injury (IRI) and acute cellular rejection (ACR)

The degree of hepatocellular IRI was evaluated by measurement of aspartate aminotransferase (AST) during the first week after OLT. Patients were classified into 2 groups depending on whether the serum AST peak was lower than 1,500 IU/L (no or mild IRI) or higher than 1,500 IU/L (more severe IRI), respectively.
Liver biopsies were taken according to our protocol at approximately 1 week, 3 months, 6 months and one year after OLT, or when there was a suspicion of ACR, and then each year. ACR was graded according to the Banff scheme. The rejection had to be clinically relevant for this study, i.e. histologically confirmed and treated with additional immunosuppression.

Statistical Analysis and Ethical committee

Genotype frequencies were analyzed by generating two- by-two contingency tables. Statistical analysis was performed using the Chi-square test or Fischer's Exact test, where appropriate, using SPSS software (SPSS Inc; Chicago, IL, USA). Univariate and multivariate analysis was performed using Cox regression analysis. Variables entering the univariate analysis that were significant at the $p<0.20$ level were taken into account in the multivariate analysis. Variables in the multivariate analysis were considered to be significant at P- values of ≤ 0.05.
This retrospective study was approved by the institutional medical ethics review board and is in accordance with the Helsinki Declaration.

Results

Patients

Our study population consisted of 147 OLT donor/recipient combinations; median age at OLT was 48 years (range 16 – 69 years) with a predominance of male recipients(65.3%). A list of baseline characteristics is given in *Table 1*. MMP polymorphism mismatches between recipient and donor were found in 53.7% for MMP-2 (79/147) and in 35.5% for MMP-9 (50/141).
No statistically significant relation was found between the absence or presence of a mismatch at –1306 C/T MMP-2 or –1562 C/T MMP-9 in relation to the development of IRI or ACR after OLT, as illustrated in *Table 2*.

Table 1. Baseline characteristics

Characteristic	Total n=147
Recipient age at OLT (median, range)	48 (16 – 69)
Donor age	43 (9 – 71)
Recipient gender % (n)	
Female	34.7 (51)
Male	65.3 (96)
Donor gender % (n)	
Female	44.9 (66)
Male	55.1 (81)
MELD score before OLT (median, range)	15 (4 – 50)
CIT (median, range)	649 (224 – 1200)
WIT(median, range)	36 (16 – 127)

OLT: Liver transplantation, MELD: Model for end stage liver disease;
CIT: Cold ischemic time; WIT: Warm ischemic time

Chimerism in liver biopsy specimens

Of the 79 MMP-2 mismatches, liver biopsy specimens of 59 cases could be adequately studied for the presence of chimerism, which was found in 28.8% (17/59, *Figure 1*). For the MMP-9 mismatches, 50 in total, liver tissue of 37 patients could reliably be scored for chimerism, which was found in 16.2% (6/37). The presence of chimerism in liver tissue was investigated in relation to IRI and ACR. A statistically significant association was found between chimerism for MMP-2 (but not MMP-9) and the occurrence of ACR, i.e., 41.2% versus 11.9% in the patients without chimerism (χ^2=6.4; P=0.01). No association was found with IRI and MMP-2 or MMP-9 *(Table 3)*.

Chimerism in peripheral blood

To assess chimerism in peripheral blood, donor/recipient combinations were selected that consisted of a MMP-2 genotype homozygote recipient and either a MMP-2 heterozygote donor or a donor of a different homozygote genotype. 27 patients could be included of whom blood DNA samples were available 3 to 12 years after OLT. Indications of blood chimerism was observed in 18.5% (5/27, *Figure 2*) of these patients, i.e., 6.3% (5/79) of the MMP-2 mismatches and 3.4% (5/147) of the total OLT population, where the donor MMP-2 gene signal was discernable in the recipient's blood DNA, in addition to the recipient's own MMP-2 gene signal. MMP-9 chimerism in blood of the recipients was not observed.

Univariate and Multivariate analysis

Univariate analysis showed that Model for End-stage Liver Disease (MELD) score and MMP-2 chimerism were significant risk factors at the p<0.20 level and were thus taken into account for the multivariate analysis. No significant relationship was found between recipient gender, donor gender, recipient age, donor age, cold ischemic time (CIT) and warm ischemic time (WIT). Multivariate analysis revealed MMP-2 chimerism to be an independent risk factor for ACR after OLT adjusted for MELD score (Hazard ratio (HR) = 3.83, p=0.03) *(Table 4)*.

Table 2. Mismatch at -1306 C/T MMP-2 or -1562 C/T MMP-9 recipient-donor genotype in relation to the development of IRI or ACR after OLT.

Genotype	Mismatch	Complication			
		IRI		ACR	
		no	yes	no	yes
-1306 C/T MMP -2 recipient -donor	no	49 (72.1)	19 (27.9)	59 (86.8)	9 (13.2)
	yes	51 (64.6)	28 (35.4)	64 (81.0)	15 (19.0)
Statistical significance		X^2=0.95; P=0.33		X^2=0.89; P=0.35	
-1562 C/T MMP -9 recipient -donor	no	60 (65.9)	31 (34.1)	77 (84.6)	14 (15.4)
	yes	37 (74.0)	13 (26.0)	43 (86.0)	7 (14.0)
Statistical significance		X^2=0.98; P=0.32		X^2=0.05; P=0.83	

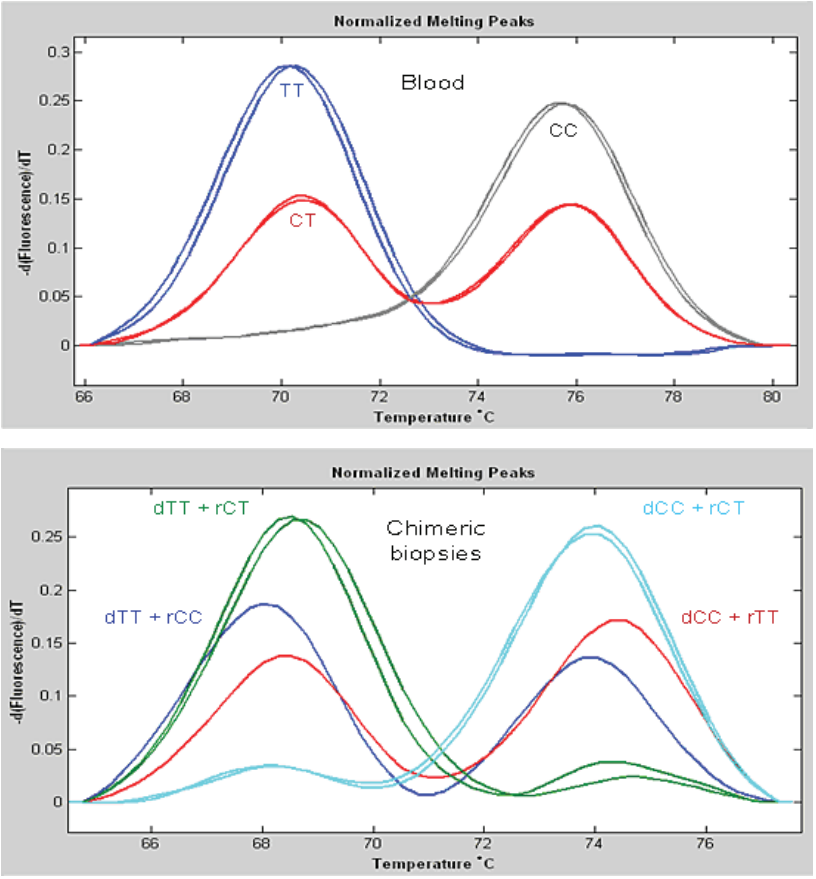
MMP: matrix metalloproteinase, IRI: ischemia-reperfusion injury, ACR: acute cellular injury, OLT: orthotopic liver transplantation

Table 3. MMP chimerism in liver tissue after OLT in relation to IRI and ACR. The presence of MMP-2 chimerism in liver biopsy specimens was significantly associated with ACR after orthotopic liver transplantation.

Genotype	Chimerism	**Complication**			
		IRI		**ACR**	
		no	yes	no	yes
MMP -2 in liver biopsy after OLT	no	31 (73.8)	11 (26.2)	37 (88.1)	5 (11.9)
	yes	11 (64.7)	6 (35.3)	10 (58.8)	7 (41.2)
Statistical significance		X^2=0.49; P=0.48		**X^2=6.4; P=0.01**	
MMP -9 in liver biopsy after OLT	no	22 (71.0)	9 (29.0)	26 (83.9)	5 (16.1)
	yes	5 (83.3)	1 (16.7)	4 (66.7)	2 (33.3)
Statistical significance		X^2=0.39; P=0.48		X^2=0.97; P=0.32	

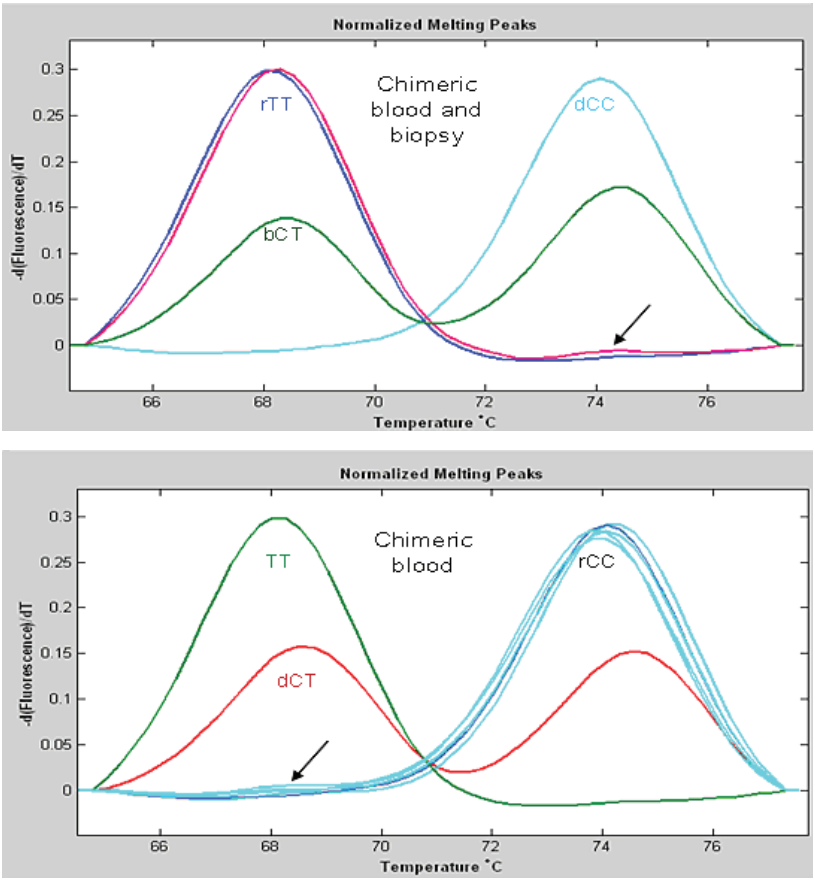
MMP: matrix metalloproteinase, IRI: ischemia-reperfusion injury, ACR: acute cellular injury, OLT: orthotopic liver transplantation

Figure 1. Chimerism in liver biopsy specimens of OLT patients.



Upper: Reference MMP-2 HMRA curves in blood: TT in blue, CC in grey and CT in red
Bottom: Chimeric MMP-2 HMRA curves in biopsies. Donor TT and recipient CC in dark blue; donor TT and recipient CT in green; donor CC and recipient CT in light blue; donor CC and recipient TT in red.

Figure 2: Chimerism in biopsies and blood of OLT patients.



Upper: MMP-2 HMRA curves of a TT recipient and CC donor in dark blue and light blue. Liver biopsy chimerism of the patient as shown by the CT curve in green. Blood chimerism is indicated by the arrow pointing at a minor peak at C in the purple TT curve of the recipient.
Bottom: Blood chimerism in CC recipients with CT donors in light blue, with the arrow pointing at minor chimeric T peaks; donor CT indicated in red, reference TT in green and recipient CC in grey. TT and recipient CT in green; donor CC and recipient CT in light blue; donor CC and recipient TT in red.

Table 4. Multivariate analysis shows that MMP-2 chimerism was an independent risk factor for acute cellular rejection (ACR) after OLT (Hazard ratio (HR) =3.83, p = 0.03).

Variables		Univariate analysis HR (95% CI)	p-value	Multivariate analysis HR (95% CI)	p-value
Recipient gender	Male	0.53 (0.17 – 1.64)	0.27		
	Female (reference)	1 (reference)			
Recipient age at OLT	Continuous	0.98 (0.94–1.03)	0.47		
Donor gender	Male	0.63 (0.19– 2.11)	0.46		
	Female (reference)	1 (reference)			
Donor age	Continuous	1.01 (0.98 – 1.05)	0.52		
MELD	Continuous	1.04 (0.99– 1.10)	0.15	1.03 (0.96 – 1.08)	0.33
MMP -2 chimerism	Yes	4.19 (1.33 – 13.26)	0.02	3.83 (1.18 – 12.40)	0.03
	No (reference)	1 (reference)			
CIT	Continuous	1.00 (0.99–1.00)	0.74		
WIT	Continuous	1.02 (0.97– 1.08)	0.41		

OLT: Orthotopic liver transplantation; MELD: Model for end stage liver disease; MMP: matrix metalloproteinase; CIT: cold ischemic time, WIT; warm ischemic time

Discussion

In the current study we analyzed MMP DNA polymorphisms as a marker for chimerism. Assessment of the MMP-2 and MMP-9 genotypes in DNA of OLT patients showed clear evidence of chimerism, both in liver tissue specimens and in peripheral blood, even years after transplantation.
In a previous study we already described liver chimerism in sex-mismatched donor/recipient mismatches.[6] Combinations selected for that study were male transplant recipients and female donors in whom cells of recipient (male) origin could be readily identified with the use of an Y-chromosome specific in-situ hybridization technique. In addition, patients who received an HLA-mismatched liver transplant were studied and chimerism was evaluated using immunohistochemistry with HLA class I-specific antibodies. To discriminate between cells of recipient and/or donor origin, double staining techniques were used with antibodies against specific cell types and subsets, i.e., endothelial and bile duct epithelial cells, lymphocytes, monocytes and other inflammatory cells. Endothelial cell chimerism was found to be quite common, whereas chimerism for biliary epithelial cells and hepatocytes could be shown only in a minority of cases. The limitation of using Y-chromosomes as a marker of chimerism is that only a small fraction of donor/recipient combinations (i.e., female/male) could be included. HLA staining could not differentiate hepatocytes from inflammatory cells with certainty and another limitation was the occasionally poor quality of liver biopsy samples for immunohistochemical analysis.
MMPs are involved in connective tissue remodeling processes associated with chronic liver disease and complications after OLT.[8,14-16] In earlier studies we investigated, for example, MMP gene polymorphisms and their relation to IRI, ACR and non-anastomotic biliary strictures (NAS) after OLT and found the MMP-2 CT genotype to be an independent risk factor for the development of NAS.[9] Now we specifically studied several single nucleotide polymorphisms (SNP) in the gene promotor regions of MMP-2 and MMP-9, that were determined in DNA samples of peripheral blood leukocytes and/or liver tissue samples, for liver chimerism after OLT. For that purpose MMP gene mismatches were selected for the assessment of chimerism. Using this method we could include a far greater number than with the previously

used methods (79/147 for MMP-2 and 50/141 for MMP-9). RFLP and HRMA for MMP-2 polymorphisms gave identical results and worked well, whereas for MMP-9 only the RFLP SNP analysis was found to be suitable, but this technique was not sensitive enough for blood chimerism. Major advantage of the HRMA technique is that it was suitable for analyzing peripheral blood DNA samples of possible chimeric post-OLT patients. There are, however, also several limitations. The existence of chimerism can only be shown in a qualitative fashion. A quantification of chimerism would be very interesting, both in relation to donor/recipient and procedure-related variables and complications, and in the follow-up of chimerism over time after the transplant procedure. Another limitation of this HRMA technique is that chimerism in liver tissue biopsies cannot be specified for different cell lineages, because tissue samples are processed for DNA extraction.

The MMP gene chimerism observed in liver tissue after OLT was assessed in relation to IRI and ACR, and an association was found between the chimeric MMP-2 genotype and the occurrence of ACR, but not for chimeric MMP-9 due to its low frequency. This was not unexpected, since acute rejection is characterized by a portal mixed inflammatory infiltrate (of recipient origin), in combination with bile duct damage and endothelitis. A functional upregulation in ACR was previously described for MMP-9 and not for MMP-2.[16] The relation of MMP-2 chimeric gene expression and ACR in the post OLT biopsies can simply be explained by the influx of a mixed leukocyte infiltrate of recipient origin. MMP-2 chimerism in the liver biopsies was found to be an independent risk factor for the development of ACR after OLT in the multivariate analysis, even adjusted for MELD score (HR 3.83, p=0.03). Other potential risk factors such as (donor) gender, (donor) age, CIT and WIT were also assessed but no significant association was found.

Indication of chimerism in peripheral blood samples after OLT was found in a minority of patients (18.5% of selected patients, i.e. 6.3% MMP-2 mismatches and 3.4% of the total OLT population) where the donor MMP-2 gene signal was detected in the recipient's blood DNA. As such an interesting observation was that a liver donor DNA signal is discernable in the DNA of circulating blood cells of the recipients. Further analyses need to be done in order to evaluate whether this is an indication of tolerance and where the signal is coming from, i.e., (re)circulating liver (stem)cells or donor

(stem)cells that have migrated to and recirculate from the recipients bone marrow.

In conclusion, this study indicates that chimerism after OLT persists both within the transplanted liver as well as in peripheral blood. There was a relationship between MMP-2 chimerism and ACR, also in the multivariate analysis. The clinical relevance of chimerism in relation to pathological mechanisms such as graft tolerance, ACR or outcome remains unclear and requires further elucidation.

References

1. Medawar PB. Transplantation of tissues and organs: Introduction. Br Med Bull 1965;21:97-99.

2. Bogman MJ, de Waal RM, Koene RA. Persistent expression of donor antigens in endothelium of long-standing skin xenografts and vulnerability to destruction by specific antibodies. Transplant Proc 1987;19:205-207.

3. Sedmak DD, Sharma HM, Czajak CM, et al. Recipient endothelialization of renal allografts. An immunohistochemical study utilizing blood group antigens. Transplantation 1988;46:907-910.

4. Fogt F, Beyser KH, Poremba C, et al. Recipient-derived hepatocytes in liver transplants: A rare event in sex-mismatched transplants. Hepatology 2002;36:173-176.

5. Starzl TE, Demetris AJ, Murase N, et al. Cell migration, chimerism, and graft acceptance. Lancet 1992;339:1579-1582.

6. Ten Hove WR, van Hoek B, Bajema IM, et al.. Extensive chimerism in liver transplants: vascular endothelium, bile duct epithelium, and hepatocytes. Liver Transpl. 2003;9:552-556.

7. Ten Hove WR, Verspaget HW, Barge R, et al.. Liver chimerism after allogeneic blood stem cell transplantation. Transplant Proc. 2007;39:231-236.

8. Ten Hove WR, de Rooij BJF, van Hoek B, et al. Serum levels change but their gene promoter polymorphisms are not associated with late phase I//R injury or rejection after orthotopic liver transplantation. Open Transplant Journal 2008;2:66-72.

9. Ten Hove WR, Sebib Korkmaz K, op den Dries S, et al. Matrix metalloproteinase 2 genotype is associated with nonanastomotic biliary strictures after orthotopic liver transplantation. Liver International 2011; e-pub ahead of print.

10. Zhou LM, Wang L, Palais R, et al. High-resolution DNA melting analysis for simultaneous mutation scanning and genotyping in solution. Clin Chem 2005;51:1770-1777.

11. Kubben FJ, Sier CF, van Duijn W, et al. Matrix metalloproteinase-2 is a consistent prognostic factor in gastric cancer. Br J Cancer 2006;94:1035-1040.

12. Ye S, Dhillon S, Ke X, et al. An efficient procedure for genotyping single nucleotide polymorphisms. Nucleic Acids Res 2001;29:E88.

13. Meijer MJ, Mieremet-Ooms MA, van Hogezand RA, et al. Role of matrix metalloproteinase, tissue inhibitor of metalloproteinase and tumor necrosis factor-alpha single nucleotide gene polymorphisms in inflammatory bowel disease. World J Gastroenterol 2007;13:2960-2966.

14. Shapiro SD. Matrix metalloproteinase degradation of extracellular matrix: biological consequences. Curr Opin Cell Biol 1998;10:602-608.

15. Verspaget HW, Kuyvenhoven JP, Sier CF, et al. Matrix metalloproteinases in chronic liver disease and liver transplantation. Lendeckel U, Hooper NM, editors. Dordrecht, the Netherlands, Springer. Proteases in Biology and Disease 5: Proteases in Gastrointestinal Tissues. 2006:209-234.

16. Kuyvenhoven JP, Verspaget HW, Gao Q, et al. Assessment of serum matrix metalloproteinases MMP-2 and MMP-9 after human liver transplantation: increased serum MMP-9 level in acute rejection. Transplantation 2004;77:1646-1652.

Chapter 8

Summarizing discussion

Introduction

Orthotopic liver transplantation (OLT) is an established therapy with an excellent survival rate. The transplantation program at the Leiden University Medical Center (LUMC) started in 1992 and has led to more than two decades of experience in OLT with current 1, 5 and 10-year survival rates of 91%, 83% and 77% which exceed the average rates in Europe.[1]

Chapter 2 describes the systematic evaluation of potential factors that are involved in the improved patient survival. Indications for which OLT is currently performed are not much different from the first ten years of OLT since the initiation of the liver transplantation (LT) program in our center. Hepatocellular carcinoma (HCC) was found to be the most frequent indication in both the first and the second ten years of OLT at our institute. Blood loss during OLT was much lower in the second decade compared to the first period, which probably reflects improved operative techniques. In addition, causes of recipient mortality were examined and this revealed that in the first decade infection was the most common cause of death post-OLT followed by recurrence of HCC. However, in the second decade death due to infection as well as mortality due to recurrence of HCC declined significantly. This reduced mortality caused by infection is probably indicative of improved early diagnosis of infection and better antibiotic regimens, in addition to better intensive care unit (ICU) treatment. The reduced mortality from HCC recurrence is likely the results of the introduction of adjuvant treatment of HCC before OLT with transarterial chemo-embolization (TACE) and radio frequency ablation (RFA). With OLT currently being the standard treatment for end-stage liver disease for many etiologies, demand has increased disproportionally to the availability of potential donor livers. As a result patients listed on the waiting list have a median waiting period of approximately two years. In an attempt to reduce organ shortage, partially due to a decrease in donation after brain death (DBD), the LT centers in the Netherlands have expanded their donor pool by using donor livers from donation after cardiac death (DCD). DCD livers are known to have more ischemia-reperfusion injury (IRI) due to an additional donor warm ischemia time (DWIT). Since 2001, this national multi-organ donation protocol has been introduced that allows the use of livers from DCD of Maastricht

category 3.[2] DCD livers from donors below 55 years of age, with a DWIT below 30 minutes, a body mass index <28, and a mean arterial pressure <50 mm Hg for maximum 15 minutes were accepted, which excludes about 25% of DCD donors. In addition, in DCD-OLT cold ischemia time (CIT) was kept as short as possible to minimize IRI. In our center this has resulted in significant shorter CITs than for DBDOLT. However, biliary complications such as nonanastomotic strictures (NAS) occur significantly more often after DCD-OLT than after DBD-OLT. NAS often results in cholangitis and retransplantation making it a significant source of morbidity and mortality. In the studied cohort, graft survival was significantly more affected by NAS development after DCD-OLT when compared to DBD-OLT. Patient survival in DCDOLT was not significantly lower than in DBD-OLT but this did not reach statistical significance, which is in line with previous reports.[3]

IRI and NAS development

As to date it is not fully clarified as to how NAS develops after both DCD-OLT and DBD-OLT. It is clear, however, that NAS results from IRI, which most likely involves immunological and toxic factors.[4,5] IRI is reflected by peak alanine aminotransferase (ALT) and aspartate aminotransferase (AST) occurring within the first week post-OLT. Previous reports have used the degree of IRI in relation to clinical outcomes such as infection.[6,7] In these studies, the degree of IRI has been arbitrarily divided in categories that correspond with severity, i.e., mild, moderate and severe. We analyzed the relationship between peak ALT and the development of NAS after DCD-OLT and DBDOLT in patients from two independent liver transplantation centers in the Netherlands, i.e., the University Medical Center in Groningen and the LUMC, as described in **chapter 3**. In this study we determined the optimal cut-off value using log-rank statistics of peak ALT and the development of NAS post DCD-OLT to be at ≥1300 IU/L and categorized grafts in recipients with a peak ALT <1300 IU/L as having mild IRI and recipients with ≥1300 IU/L as having a graft with severe IRI. After DCD-OLT severe IRI showed a clear and independent relationship with the development of NAS. Four-year cumulative incidence of NAS development reached 51.4% in case of severe IRI compared to 11.4% in case of mild IRI. This strong relationship was also evaluated in the two

included cohorts separately, which showed similar results. No independent relationship was found to exist between NAS and ischemia times. Peak-ALT has the advantage of measuring the impact of reperfusion in addition to ischemia. The incidence of NAS after DBD-OLT was approximately 10%. Interestingly, no relationship was observed between peak ALT and NAS development after DBD-OLT in both centers. Moreover, no cut-off value for ALT in relation to development of NAS could be established for DBD-OLT. Remarkably, the incidence of NAS after DCD-OLT in case of mild IRI is similar to the incidence of NAS after DBD-OLT. This finding could imply that other factors are involved in the extra IRI-related NAS induced in DCD-OLT with high peak-ALT than those involved in the development of NAS in DBD-OLT and in DCD-OLT with low peak-ALT.It may also indicate that DWIT exacerbates the similar pathway which results in NAS after both DBD-OLT and DCD-OLT with peak ALT<1300 IU/L. It also implies that a peak-ALT>1300 IU/L can be indicative of a high likelihood for development of NAS in DCD-OLT. Treatment that is able to reduce IRI, and consequently reduces the peak-ALT to below 1300 IU/L, in DCD-OLT may also reduce the NAS incidence of DCD-OLT to that of DBD-OLT.

Clinical markers of biliary stricture formation

The development of biliary strictures, both anastomotic and nonanastomotic, after OLT is often insidious and patients usually present themselves either in the outpatient clinic or in the emergency room with symptoms such as jaundice, fever, itching and/or abdominal pain.[8,9] Most liver transplantation centers offer routine follow-up for patients after OLT in the clinic, including history and physical exam and blood tests for liver enzymes and graft function and -less often- abdominal ultrasound examinations. These routine measurements may harbor early signs of stricture formation. Early detection of NAS is important since prompt intervention can prevent cholangitis and admissions, it may slow progression, and may even prevent or delay retransplantation. However, there is relatively few data on the value of routinely performing blood tests and ultrasound examinations in predicting possible stricture formation.[10-12] The study described in **chapter 4** illustrates that a time-dependent Cox regression model was a valuable tool in establishing a

strong predictive relationship between an elevated gamma-glutamyl transferase (GGT) as well as dilated bile ducts on ultrasound (US) at routine assessments on the one hand and the development of bile duct strictures in the subsequent period of 3 months on the other hand. Other liver enzymes had no independent predictive value next to GGT for the development of biliary strictures. These data suggest that routinely assessing GGT and US are useful screening tools for early detection of biliary stricture formation.This may eventually result in presymptomatic referral for diagnosis and treatment.

Matrix metalloproteinases and biliary complications before and after OLT

IRI is inevitable after OLT in the current setting of organ procurement and preservation procedures. Recent literature has shown that reperfusion likely is responsible for most of the preservation damage that occurs after OLT. This seems for an important part due to the release of reactive oxygen species (ROS) derived from mitochondria.[13,14] Hepatic stellate cell (HSC) activation, for example, may occur due to different stimuli such as ROS, apoptotic bodies and paracrine stimuli, including Kupffer cells.[15,16] HSC are further stimulated by several key cytokines into myofibroblasts which produce type IV collagen.[17] During this process, Kupffer cells and HSC also secrete MMP-2 and MMP-9 which creates a delicate equilibrium between collagen deposition due to inflammation and breakdown of extracellular matrix (ECM).[18] HSC are the main cellular source of MMP-2 in the liver which is capable of degrading type IV collagen and MMP-2 and MMP-9 have been associated with both physiological as well as pathological conditions such as inflammation, cancer and OLT.[19-22]. We and others previously showed that MMP-9 is released in the anhepatic phase and especially early after reperfusion, and that this peak is related to tissue injury.[23,24] In the first week after OLT neither MMP-2 nor MMP-9 levels were related to peak-ALT, while MMP-9 levels increased in case of rejection, produced by neutrophils in the portal triads. We now performed studies on the gene level and found that the -1306 C/T MMP-2 gene promoter polymorphism of both donor and recipient was associated with the development of NAS after OLT. **(Chapter 5)** The risk of developing NAS within 48 months after OLT increased if the C/T polymorphism was present in both donor and recipient and this was an independent risk

factor, even when adjusted for PSC as indication, and for CIT. We also described that the serum levels of MMP-2 were, though non-significantly, lower with the C/T genotype than in the presence of the CC genotype. MMP-2 may thus play a role in late-phase subsequent matrix degradation after OLT. Hypothetically, after IRI a decreased activity of MMP-2 may lead to insufficient degradation of collagen surrounding bile ducts leading to more NAS in the long-term follow-up.

Many chronic inflammatory conditions of the liver lead to fibrosis and cirrhosis and form an indication for OLT. In primary sclerosing cholangitis (PSC) chronic inflammation of the bile ducts leads to scarring and biliary stricture formation which can lead to OLT before cirrhosis ensues.[27] Patients with PSC often develop cholangitis, likely due to reflux of bacterial pathogens from the intestine and 15% to 20% of PSC patients develop cholangiocarcinoma.[28] Remarkably, PSC is accompanied by inflammatory bowel disease (IBD) in approximately 60% to 80% of cases.[29] It is thought that PSC might result from aberrant trafficking of intestinal lymphocytes from the gut to the liver.[30,31] Interestingly, MMPs were not among the discovered genes in genetic studies that tried to discover etiological or pathogenetic factors in PSC. Interestingly MMPs have been previously associated with IBD where serological evaluations revealed that MMP-2 levels increased in patients Crohn's disease (CD) upon effective treatment with Infliximab.[32] In PSC severe scarring of tissue surrounding bile ducts occurs, only later followed by fibrosis and cirrhosis. Therefore different pathways may be involved in etiology and initial pathogenesis of PSC on the one hand and in progression of PSC on the other hand. It is assumable that different stimuli involving liver injury may activate shared pathways in fibrosis, i.e., resulting in NAS after OLT or stricture formation in bile duct diseases such as PSC, and eventually cirrhosis. In **chapter 6** we describe the gene promoter polymorphisms of MMP-2 and MMP-9 in relation to disease severity, as defined by death or OLT, in patients with PSC. PSC patients from the two independent LT centers with the MMP-2 –1306 C/T or T/T genotype showed a clear stepwise increase in hazard of death or OLT compared to patients with the CC (wild type) genotype. This was the case for patients both with and without IBD as concomitant disease, implying that impaired MMP-2 activity is associated with a more severe

phenotype in PSC. Genes such as NOD2, GPR35 and TCF4 have been discovered in genome wide association studies as factors involved in etiology and pathogenesis of PSC, and these are all key players for the intracellular processing of bacterial pathogens in the intestinal mucosa.[33,34] Although MMPs were not among the genes discovered in these studies, several reports revealed MMP-3 to be associated with both disease development as well as disease progression in patients with PSC.[35,36] These observations support our theory that MMPs are involved in PSC and our data add MMP-2 as a new key player in disease progression in patients with PSC. Thus, the studies from **chapter 5 and 6** consistently indicate that MMP-2 is involved in bile duct-related liver injury following IRI or other inflammatory processes like in PSC.

Matrix metalloproteinases and chimerism

Besides complications that may occur within the biliary tract, such as NAS, acute cellular rejection (ACR) is still a complication in about 20% of recipients in OLT. Graft tolerance seems to be variable as indicated by the fact that different levels of immunosuppression are needed in different patients for prevention of rejection.[37-39] Several studies have reported chimerism as a potential tool in diagnosing graft-versus-host disease (GVHD) after liver transplantation.[40-42] Although much debated, it has been hypothesized that chimerism might play a role in the development of graft tolerance and ACR.[43,44] Chimerism of both recipient and donor has been first demonstrated in 1968 with livers that had been transplanted to female recipients from male cadaveric donors and our own study group later confirmed this observation.[45,46] The use of sex-chromosomes in determining the presence of chimerism has limitations since only donor-recipient pairs of the opposite sex can be included. One previous study performed a serial quantitative analysis of donor chimerism (DC) levels in serum samples after LT. The authors observed that high levels of DC was associated with recurrent disease and rejection and suggested that DC could be a marker for graft tolerance.[47] In **chapter 7** we report on the use of similar techniques regarding MMP-2 and MMP-9 gene promoter polymorphisms as markers for the presence of chimerism. This MMP-related chimerism was found in liver biopsy specimens in 28.8% of cases for MMP-2 and 16.8% for

MMP-9. In addition, chimerism was also detectable in peripheral blood after OLT and was observed in 18.5% of the MMP-2 mismatches and in 3.4% of the MMP-9 mismatches. In a multivariate analysis the presence of MMP-2 chimerism was found to be a significant independent risk factor for the development of ACR and even when adjusted for the Model for End-Stage Liver Disease score (MELD). The presence of donor DNA in the circulation of the recipient is consistent with previous studies that have shown that donor cells entering the recipient's circulation and lymph nodes largely induce tolerance in solid graft transplantation.[48-50]

Conclusion and future perspectives

Liver transplantation has evolved from an experimental treatment, with relative high mortality, to the current standard treatment for end-stage liver diseases with excellent survival rates. However, expanding the pool of potential donor livers by using donor livers from DCD is not without consequences. We have shown that livers from DCD donors are far more susceptible for developing NAS after liver transplantation. This is very likely caused by the donor warm ischemia time (DWIT), which is inevitable in the current setting of DCD procurement. A theory states that during DWIT microthrombi might develop in the hepatic sinusoids and peri-biliary microvasculature, which after reperfusion could result in release of ROS from mitochondria. ROS are known to cause severe damage to cells like hepatocytes leading to cell death. In the clinical setting this translates into an increase in ALT in the first week after reperfusion as the result of combined injury resulting from ischemia and reperfusion. We demonstrated that peak ALT above 1300 IU/L is associated with NAS development after DCD-OLT, but not after DBD-OLT. This implies that reducing peak ALT to below 1300 IU/L in DCD-OLT probably will bring back the incidence of NAS after DCD-OLT to that of DBD-OLT. It also indicates that DWIT is responsible for both the higher ALT and the higher incidence of NAS after DCD-OLT. However, reducing IRI will probably not eliminate development of NAS since other factors such as loss of CCR5Δ32 function, toxic bile salts and loss of the bicarbonate "umbrella" have also been reported to contribute significantly.

Activation of Kupffer cells and stellate cells results in collagen deposition and

production of MMPs, especially MMP-9 and MMP-2, which are capable of degrading components of the ECM such as collagen type IV. Our group previously found indications that MMP- 9 is released during early phase IRI and is derived from Kupffer cells and neutrophils. The current results indicate that MMP-2 also plays a prominent role in the long-term progression of two different conditions that probably share common pathways, namely chronic peribiliary inflammation in the context of PSC but also in the development of NAS after OLT. We have shown that the MMP-2 gene promoter polymorphism (–1306 C/T) constitutes a functional SNP indicated by the fact that C/T polymorphisms are characterized by less circulation of MMP-2 in relation to the genotype. We now also demonstrate that these polymorphisms are related to the risk for development of NAS after OLT. Furthermore, in PSC patients we observed a striking increase in disease severity in relation to the MMP-2 C/T and TT genotype as defined by death or OLT. These data suggest a prominent role of MMPs in the degradation of collagen surrounding bile ducts before and after liver transplantation. The studies that are described in this thesis provide further insight into clinical markers for development of NAS post liver transplantation but also evaluate the role of MMPs in stricturing of the bile ducts both before (in PSC) and after (in NAS) liver transplantation.

Currently several protocols using machine perfusion are being developed to reduce IRI during and after procurement of donor livers in order to prevent complications such as graft failure and NAS.[51-55] However, it is unclear when, how and especially where the use of machine perfusion should take place. Several studies reported that back table hypothermic oxygenated machine perfusion could resuscitate severely injured livers.[56,57] There are also promising results in reducing IRI with hypothermic or (sub)normothermic machine perfusion.[58] However, apart from practical limitations, it is unclear whether these interventions will reduce NAS after LT. Some data indicate that use of fibrinolytics during preservation may reduce IRI after OLT.[52,59] Our findings indicate that well-timed stimulation or inhibition of the activity of MMP-2 and -9 might also contribute to the reduction of NAS incidence and may possibly also delay progression in PSC. Further studies in these directions are warranted.

References

1. Adam R, McMaster P, O'Grady JG, Castaing D, Klempnauer JL, Jamieson N, et al. Evolution of liver transplantation in Europe: report of the European Liver Transplant Registry. Liver Transpl 2003;9(12):1231-1243.

2. Dubbeld J, van Hoek B., Ringers J. Use of a liver from donor after cardiac death: is it appropriate for the sick or the stable? Curr Opin Organ Transplant 2011;16(2):239-242.

3. Dubbeld J, Hoekstra H, Farid W, Ringers J, Porte RJ, Metselaar HJ, et al. Similar liver transplantation survival with selected cardiac death donors and brain death donors. Br J Surg 2010;97(5):744-753.

4. Yska MJ, Buis CI, Monbaliu D, Schuurs TA, Gouw AS, Kahmann ON, et al. The role of bile salt toxicity in the pathogenesis of bile duct injury after non-heartbeating porcine liver transplantation. Transplantation 2008 15;85(11):1625-1631.

5. op den Dries S., Sutton ME, Lisman T, Porte RJ. Protection of bile ducts in liver transplantation: looking beyond ischemia. Transplantation 2011 27;92(4):373-379.

6. Glanemann M, Langrehr JM, Stange BJ, Neumann U, Settmacher U, Steinmuller T, et al. Clinical implications of hepatic preservation injury after adult liver transplantation. Am J Transplant 2003;3(8):1003-1009.

7. Bilbao I, Charco R, Hidalgo E, Lazaro JL, Balsells J, Murio E, et al. Risk factors for severe ischemic injury after liver transplantation. Transplant Proc 1997;29(1-2):368-370.

8. Guichelaar MM, Benson JT, Malinchoc M, Krom RA, Wiesner RH, Charlton MR. Risk factors for and clinical course of non-anastomotic biliary strictures after liver transplantation. Am J Transplant 2003;3(7):885-890.

9. Nishida S, Nakamura N, Kadono J, Komokata T, Sakata R, Madariaga JR, et al. Intrahepatic biliary strictures after liver transplantation. J Hepatobiliary Pancreat Surg 2006;13(6):511-516.

10. Zoepf T, Maldonado-Lopez EJ, Hilgard P, Dechene A, Malago M, Broelsch CE, et al. Diagnosis of biliary strictures after liver transplantation: which is the best tool? World J Gastroenterol 2005 21;11(19):2945-2948.

11. Que Y, Kaneko J, Sugawara Y, Tamura S, Makuuchi M. Role of protocol ultrasonography for detecting biliary stricture in adult living donor liver transplantation recipients. Biosci Trends 2007;1(1):62-65.

12. Ben-Ari Z, Weiss-Schmilovitz H, Sulkes J, Brown M, Bar-Nathan N, Shaharabani E, et al. Serum cholestasis markers as predictors of early outcome after liver transplantation. Clin Transplant 2004;18(2):130-136.

13. Schlegel A, Rougemont O, Graf R, Clavien PA, Dutkowski P. Protective mechanisms of end-ischemic cold machine perfusion in DCD liver grafts. J Hepatol 2013;58(2):278-286.

14. Jaeschke H. Reactive oxygen and mechanisms of inflammatory liver injury: Present concepts. J Gastroenterol Hepatol 2011;26 Suppl 1:173-179.

15. Jaeschke H. Mechanisms of Liver Injury. II. Mechanisms of neutrophil-induced liver cell injury during hepatic ischemia-reperfusion and other acute inflammatory conditions. Am J Physiol Gastrointest Liver Physiol 2006;290(6):G1083-G1088.

16. Friedman SL. Mechanisms of disease: Mechanisms of hepatic fibrosis and therapeutic implications. Nat Clin Pract Gastroenterol Hepatol 2004;1(2):98-105.

17. Zeisberg M, Yang C, Martino M, Duncan MB, Rieder F, Tanjore H, et al. Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition. J Biol Chem 2007 10;282(32):23337-23347.

18. Schuppan D, Ruehl M, Somasundaram R, Hahn EG. Matrix as a modulator of hepatic fibrogenesis. Semin Liver Dis 2001;21(3):351-372.

19. Kubben FJ, Sier CF, Meijer MJ, van den Berg M, van der Reijden JJ, Griffioen G, et al. Clinical impact of MMP and TIMP gene polymorphisms in gastric cancer. Br J Cancer 2006 18;95(6):744-751.

20. Woessner JF, Jr. The family of matrix metalloproteinases. Ann N Y Acad Sci 1994 6;732:11-21.

21. Kuyvenhoven JP, van Hoek B., Blom E, van Duijn W., Hanemaaijer R, Verheijen JH, et al. Assessment of the clinical significance of serum matrix metalloproteinases MMP-2 and MMP-9 in patients with various chronic liver diseases and hepatocellular carcinoma. Thromb Haemost 2003;89(4):718-725.

22. Arthur MJ. Fibrogenesis II. Metalloproteinases and their inhibitors in liver fibrosis. Am J Physiol Gastrointest Liver Physiol 2000;279(2):G245-G249.

23. Kuyvenhoven JP, Molenaar IQ, Verspaget HW, Veldman MG, Palareti G, Legnani C, et al. Plasma MMP-2 and MMP-9 and their inhibitors TIMP-1 and TIMP-2 during human orthotopic liver transplantation. The effect of aprotinin and the relation to ischemia/reperfusion injury. Thromb Haemost 2004;91(3):506-513.

24. Upadhya AG, Harvey RP, Howard TK, Lowell JA, Shenoy S, Strasberg SM. Evidence of a role for matrix metalloproteinases in cold preservation injury of the liver in humans and in the rat. Hepatology 1997;26(4):922-928.

25. Benyon RC, Arthur MJ. Extracellular matrix degradation and the role of hepatic stellate cells. Semin Liver Dis 2001;21(3):373-384.

26. Kuyvenhoven JP, Ringers J, Verspaget HW, Lamers CB, van HB. Serum matrix metalloproteinase MMP-2 and MMP-9 in the late phase of ischemia and reperfusion injury in human orthotopic liver transplantation. Transplant Proc 2003;35(8):2967-2969.

27. O'Mahony CA, Vierling JM. Etiopathogenesis of primary sclerosing cholangitis. Semin Liver Dis 2006;26(1):3-21.

28. Goldberg D, French B, Thomasson A, Reddy KR, Halpern SD. Waitlist survival of patients with primary sclerosing cholangitis in the model for end-stage liver disease era. Liver Transpl 2011;17(11):1355-1363.

29. Ponsioen CY. Recent insights in primary sclerosing cholangitis. J Dig Dis2012;13(7):337-341.

30. Grant AJ, Lalor PF, Salmi M, Jalkanen S, Adams DH. Homing of mucosal lymphocytes to the liver in the pathogenesis of hepatic complications of inflammatory bowel disease. Lancet 2002 12;359(9301):150-157.

31. Janse M, Lamberts LE, Franke L, Raychaudhuri S, Ellinghaus E, Muri BK, et al. Three ulcerative colitis susceptibility loci are associated with primary sclerosing cholangitis and indicate a role for IL2, REL, and CARD9. Hepatology 2011;53(6):1977-1985.

32. Meijer MJ, Mieremet-Ooms MA, van Duijn W., van der Zon AM, Hanemaaijer R, Verheijen JH, et al. Effect of the anti-tumor necrosis factor-alpha antibody infliximab on the ex vivo mucosal matrix metalloproteinase-proteolytic phenotype in inflammatory bowel disease. Inflamm Bowel Dis 2007;13(2):200-210.

33. Ellinghaus D, Folseraas T, Holm K, Ellinghaus E, Melum E, Balschun T, et al. Genome-wide association analysis in sclerosing cholangitis and ulcerative colitis identifies risk loci at GPR35 and TCF4. Hepatology 2012 23.12

34. Folseraas T, Melum E, Rausch P, Juran BD, Ellinghaus E, Shiryaev A, et al. Extended analysis of a genome-wide association study in primary sclerosing cholangitis detects multiple novel risk loci. J Hepatol 2012;57(2):366-375.

35. Satsangi J, Chapman RW, Haldar N, Donaldson P, Mitchell S, Simmons J, et al. A functional polymorphism of the stromelysin gene (MMP-3) influences susceptibility to primary sclerosing cholangitis. Gastroenterology 2001;121(1):124-130.

36. Juran BD, Atkinson EJ, Schlicht EM, Larson JJ, Ellinghaus D, Franke A, et al. Genetic polymorphisms of matrix metalloproteinase 3 in primary sclerosing cholangitis. Liver Int 2011;31(6):785-791.

37. Starzl TE. Immunosuppressive therapy and tolerance of organ allografts. N Engl J Med 2008 24;358(4):407-411.

38. Starzl TE. Acquired immunologic tolerance: with particular reference to transplantation. Immunol Res 2007;38(1-3):6-41.

39. Starzl TE. Acquired tolerance, allograft "acceptance," and immune suppression. Transplant Proc 2000;32(3):515.

40. Hahn AB, Baliga P. Rapid method for the analysis of peripheral chimerism in suspected graft-versus-host disease after liver transplantation. Liver Transpl 2000;6(2):180-184.

41. Nierhoff D, Horvath HC, Mytilineos J, Golling M, Bud O, Klar E, et al. Microchimerism in bone marrow-derived CD34(+) cells of patients after liver transplantation. Blood 2000 15;96(2):763-767.

42. Pollack MS, Speeg KV, Callander NS, Freytes CO, Espinoza AA, Esterl RM, et al. Severe, late-onset graft-versus-host disease in a liver transplant recipient documented by chimerism analysis. Hum Immunol 2005;66(1):28-31.

43. Starzl TE, Demetris AJ, Trucco M, Murase N, Ricordi C, Ildstad S, et al. Cell migration and chimerism after whole-organ transplantation: the basis of graft acceptance. Hepatology 1993;17(6):1127-1152.

44. Starzl TE. Chimerism and tolerance in transplantation. Proc Natl Acad Sci U S A 2004 5;101 Suppl 2:14607-14614.

45. Kashiwagi N, Porter KA, Penn I, Brettschneider L, Starzl TE. Studies of homograft sex and of gamma globulin phenotypes after orthotopic homotransplantation of the human liver. Surg Forum 1969;20:374-376.

46. Hove WR, van HB, Bajema IM, Ringers J, van Krieken JH, Lagaaij EL. Extensive chimerism in liver transplants: vascular endothelium, bile duct epithelium, and hepatocytes. Liver Transpl 2003;9(6):552-556.

47. Ayala R, Grande S, Albizua E, Crooke A, Meneu JC, Moreno A, et al. Long-term follow-up of donor chimerism and tolerance after human liver transplantation. Liver Transpl 2009;15(6):581-591.

48. Scandling JD, Busque S, Dejbakhsh-Jones S, Benike C, Millan MT, Shizuru JA, et al. Tolerance and chimerism after renal and hematopoietic-cell transplantation. N Engl J Med 2008 24;358(4):362-368.

49. Alexander SI, Smith N, Hu M, Verran D, Shun A, Dorney S, et al. Chimerism and tolerance in a recipient of a deceased-donor liver transplant. N Engl J Med 2008 24;358(4):369-374.

50. Nakao A, Toyokawa H, Kimizuka K, Nalesnik MA, Nozaki I, Bailey RJ, et al. Simultaneous bone marrow and intestine transplantation promotes marrow-derived hematopoietic stem cell engraftment and chimerism. Blood 2006 15;108(4):1413-1420.

51. Moench C, Moench K, Lohse AW, Thies J, Otto G. Prevention of ischemic-type biliary lesions by arterial back-table pressure perfusion. Liver Transpl 2003;9(3):285-289.

52. Lang R, He Q, Jin ZK, Han DD, Chen DZ. Urokinase perfusion prevents intrahepatic ischemic-type biliary lesion in donor livers. World J Gastroenterol 2009 28;15(28):3538-3541.

53. Guarrera JV. Assist devices: machine preservation of extended criteria donors. Liver Transpl 2012;18 Suppl 2:S31-S33.

54. Guarrera JV, Henry SD, Chen SW, Brown T, Nachber E, Arrington B, et al. Hypothermic machine preservation attenuates ischemia/reperfusion markers after liver transplantation: preliminary results. J Surg Res 2011 15;167(2):e365-e373.

55. Henry SD, Nachber E, Tulipan J, Stone J, Bae C, Reznik L, et al. Hypothermic machine preservation reduces molecular markers of ischemia/reperfusion injury in human liver transplantation. Am J Transplant 2012;12(9):2477-2486.

56. Dutkowski P, de RO, Clavien PA. Machine perfusion for 'marginal' liver grafts. Am J Transplant 2008;8(5):917-924.

57. Dutkowski P, Graf R, Clavien PA. Rescue of the cold preserved rat liver by hypothermic oxygenated machine perfusion. Am J Transplant 2006;6(5 Pt 1):903-912.

58. Op den Dries S., Karimian N, Sutton ME, Westerkamp AC, Nijsten MW, Gouw AS, et al. Ex vivo Normothermic Machine Perfusion and Viability Testing of Discarded Human Donor Livers. Am J Transplant 2013 6.

59. Hashimoto K, Eghtesad B, Gunasekaran G, Fujiki M, Uso TD, Quintini C, et al. Use of tissue plasminogen activator in liver transplantation from

Chapter 9

Nederlandse samenvatting

Introductie

Levertransplantatie is een standaard behandeling geworden voor eindstadium leverziekte met een sterk verbeterde korte en lange termijnoverleving. Het Leids Universitair Medisch Centrum (LUMC) voert levertransplantaties uit sinds 1992 en behoort daarmee tot een van de 3 centra in Nederland, naast Groningen en Rotterdam, waar deze behandeling aangeboden wordt. Inmiddels bedraagt de 1, 5 en 10-jaars overleving na levertransplantatie in het LUMC respectievelijk 91%, 83% en 77%, ruim boven het gemiddeld in Europa. Het verbeteren van de chirurgische technieken heeft ertoe bijgedragen dat de overleving op korte termijn sterk verbeterd is. Ook afname van infecties na levertransplantatie, minder acute en chronische afstoting en verbeterde preservatietechnieken van het donororgaan hebben vrijwel zeker significante bijdragen geleverd aan de verbeterde overleving. Ondanks de verbeterde patiënt- overleving na OLT blijven er complicaties. In het bijzonder complicaties aan de galwegen zoals vernauwingen (stricturen) vormen een significant probleem na levertransplantatie waarvoor patiënten frequent worden opgenomen, en in het uiterste geval opnieuw getransplanteerd moeten worden.

De toegenomen beschikbaarheid van levertransplantatie als behandeling, evenals de verbeterde overleving hebben ertoe geleid dat meer patiënten die lijden aan het eindstadium van een leverziekte in aanmerking komen voor levertransplantatie. Het succes van levertransplantatie is slechts mogelijk bij voldoende beschikbaarheid van donororganen. Het aantal hersendode donoren is in Nederland gedaald. Dit heeft geleid tot een toename van de wachttijd voor levertransplantatie welke kan oplopen tot een jaar. De oplossing van het tekort is in grote lijnen tweeledig: enerzijds is het noodzakelijk om meer potentiële donoren te werven, anderzijds is het van groot belang om maximaal gebruik te maken van het huidige aanbod aan donorlevers. Om meer potentiële donoren te werven is goede informatievoorziening voor mensen die donatie overwegen essentieel, en zijn er verschillende campagnes vanuit de overheid en (semi)publieke instanties gestart om dit te realiseren. Ook zou een zogenaamd geen-bezwaar systeem, zoals dit in België en Oostenrijk functioneert, opnieuw overwogen kunnen worden.

Het optimaal benutten van het huidig donoraanbod is complex. Tot 2001 werden in Nederland uitsluitend gebruik gemaakt van donorlevers van hersendode donoren (donation after brain death (DBD)). Deze donoren hebben bij donatie een intacte circulatie waardoor de lever nog voldoende geoxygeneerd wordt. Levers van DBD donoren zijn daarmee van relatief goede kwaliteit. Sinds 2001 wordt er in Nederland ook gebruik gemaakt van organen van hartdode donoren (donation after cardiac death (DCD)) waarbij er kort voor en tijdens donatie geen sprake meer is van een intacte circulatie, zodat de warme donorlever niet meer van zuurstofrijk bloed wordt voorzien. Deze periode lijkt cruciaal in de vorming van stricturen aan de galwegen na levertransplantatie met DCD donoren. Vorming van galwegstricturen na levertransplantatie wordt in grofweg twee typen verdeeld: de anastomotische stricturen (AS), dit zijn stricturen die ter plaatse van de chirurgische naad plaatsvinden, en de nonanastomotische stricturen (NAS). NAS zijn stricturen die zich minimaal 1cm boven de chirurgische naad ontwikkelen in extra- en/of intrahepatische galwegen.

Verbeterde overleving na levertransplantatie

In de afgelopen twee decennia hebben vele factoren bijgedragen aan de verbeterde overleving na levertransplantatie in het LUMC. In **hoofdstuk 2** is er systematisch gezocht naar factoren die hebben bijgedragen aan deze verbetering. De indicaties waarvoor patiënten getransplanteerd worden, zijn in het LUMC in vergelijking met het eerste decennium niet veranderd in het tweede decennium. Het bleek dat hepatocellulair carcinoom de meest voorkomende indicatie was voor levertransplantatie in beide decennia. De chirurgische technieken hebben een sterke verbetering ondergaan in het tweede decennium gekenmerkt door een sterk gedaalde hoeveelheid bloedverlies tijdens de operatie. Vervolgens is er gekeken naar de doodsoorzaken na transplantatie in beide decennia. Hieruit bleek dat in het eerste decennium infectie de meest voorkomende oorzaak was voor overlijden na levertransplantatie, gevolgd door overlijden door terugkeer van de oorspronkelijke ziekte, met name hepatocellulair carcinoom. Beide oorzaken van overlijden kenden een significante daling in het tweede decennium, hoewel infectie nog steeds de belangrijkste oorzaak is van overlijden na transplantatie. Deze

daling van sterfte aan infectie is toe te schrijven aan verbeterde behandelprotocollen direct na levertransplantatie op de intensive care, verbeterde diagnostiek en behandeling met antibiotica. De sterke afname aan sterfte door terugkeer van HCC na OLT was vrijwel zeker het gevolg van de introductie van neo-adjuvante behandeling met transarteriële chemo-embolisatie (TACE) en ablatie van de levertumor middels radiofrequentie (RFA) van patiënten met hepatocellulair carcinoom op de wachtlijst. Vanaf 2001 is er een strikt protocol ingevoerd om ook DCD donorlevers te gebruiken voor levertransplantatie. DCD donoren zijn onderhevig aan strenge selectie eisen, zoals een donorleeftijd van <55 jaar, een circulatiestilstand (donor warme ischemie tijd (DWIT)) van bij voorkeur niet langer dan 30 minuten, een body mass index <28, en een arteriële bloeddruk van <50 mm Hg niet langer dan 15 minuten. Op basis van voorgenoemde selectiecriteria wordt ongeveer 25% van alle DCD donoren geëxcludeerd. Hiernaast wordt getracht preservatietijden zoals de koude ischemie tijd (CIT) die tijdens het transport van het donororgaan optreedt te beperken, met als doel de ischemie-reperfusie schade(IRI) tot een minimum te reduceren. Ondanks deze maatregelen blijkt de IRI na levertransplantatie met DCD donoren significant hoger te zijn dan na levertransplantatie met DBD donoren. Dit uit zich in een hogere piek in de leverenzymen alanine aminotransferase (ALT) en aspartaat aminotransferase (AST) in de eerste week na levertransplantatie. Op de langere termijn blijken levers van DCD donoren ook vaker NAS te ontwikkelen na OLT, en ondergaan deze patiënten vaker retransplantaties door NAS waardoor de transplantaatoverleving significant korter is dan na DBD levertransplantatie. Verdere analyse heeft aangetoond dat de patiëntoverleving niet significant lager is na DCD levertransplantatie ten opzicht van DBD-OLT.

IRI en ontwikkeling van galwegstricturen

Het ontstaan van AS wordt vaak gezien als een chirurgische complicatie door verlittekening op de choledochus-naad, die vaak relatief eenvoudig met endoscopische technieken te behandelen is. NAS vormen na transplantatie een veel groter probleem. NAS ontstaat in ongeveer 20% - 40% na levertransplantatie met DCD donoren. NAS ontstaat waarschijnlijk door een samenspel van ischemische, immunologische en toxische factoren die inspelen op de

bloedvoorziening van de galwegen, ook wel bekend als de peri-biliare plexus, en op de galwegen zelf. Een afgenomen microcirculatie in de peri-biliare plexus kan wellicht een rol spelen bij het ontstaan van NAS. De peri-biliare plexus wordt enkel van bloed voorzien door de arteria hepatica. Men vermoedt dat stase van bloed tijdens de DWIT en CIT in de arteria hepatica leidt tot microtrombusvorming in de peri-biliare plexus. Mogelijk spelen microthrombi ook in de sinusoiden een rol bij het ontstaan van IRI. Na levertransplantatie stijgen de leverenzymen ALT en AST als gevolg van lekkage uit hepatocyten die afsterven tijdens de DWIT, CIT, RWIT en reperfusie. De maximale hoogte (piek) van deze stijging in de eerste week na OLT vormt een goede indicator van IRI van het transplantaat. **Hoofdstuk 3** beschrijft de relatie tussen de piek ALT in de eerste week en het ontstaan van NAS na DCD levertransplantatie in twee transplantatiecentra, namelijk het LUMC en het Universitair Medisch Centrum Groningen (UMCG). In deze twee cohorten gecombineerd is gezocht naar de optimale cut-off waarde die het ontstaan van NAS kan voorspellen na DCD levertransplantatie, en deze werd vastgesteld op ≥1300 IU/L. Hierbij werd de IRI in twee categorieën verdeeld: een piek ALT van <1300 IU/L werd beschouwd als milde IR-schade en een piek ALT ≥1300 IU/L als ernstige IR-schade. In de gecombineerde cohorten bleek dat DCD transplantaten die ernstige IR-schade vertoonden significant vaker NAS ontwikkelden in vergelijking met DCD transplantaten die een milde IR-schade hadden ontwikkeld: De 4-jaars cumulatieve incidentie van NAS ontwikkeling na ernstige IR-schade was 51.4% ten opzichte van 11.6% in het geval van milde IR-schade. Ook in de afzonderlijke cohorten bleek deze relatie duidelijk te bestaan. Echter, er kon geen associatie gevonden worden tussen de piek ALT en het ontstaan van NAS bij DBD transplantaten. Voor dit type donor kon tevens geen cut-off waarde vastgesteld worden voor piek ALT om patiënten met hoge en lage kans op NAS te scheiden. Dit kan erop wijzen dat wellicht andere aspecten, zoals toxische of immunologische schade een rol spelen bij het ontstaan van NAS na DBD transplantatie. In ieder geval blijkt dat zowel IRI als NAS toenemen door de DWIT bij levertransplantatie met hartdode donoren.

Klinische variabelen voor galwegstricturen

De ontwikkeling van NAS is vaak sluipend en patiënten presenteren zich vaak op de eerste hulp of polikliniek pas als er een significante occlusie is van de galwegen, waardoor er klachten ontstaan als koorts, buikpijn, geelzucht, al dan niet gecombineerd met jeuk. De presentatie van patiënten met galwegstricturen, zoals AS of NAS, is gelijk en er zijn geen klinische tekenen die dit onderscheid kunnen maken. De definitieve diagnose wordt gesteld met behulp van endoscopische retrograde cholangio- en pancreaticografie (ERCP) of percutane cholangiografie (PTC); beide onderzoeken zijn echter invasief en gaan gepaard met morbiditeit. MRCP is niet invasief maar wel duur en daarbij is voor interventie is vervolgens alsnog PTC of ERCP nodig. In veel transplantatiecentra wordt een uitgebreid programma aan postoperatieve zorg aangeboden, waarbij patiënten frequent worden gezien op de polikliniek ter controle met als primaire doel de leverfunctie te evalueren, maar ook complicaties zoals afstoting, terugkeer van oorspronkelijke ziekte en galwegstricturen vroeg op te kunnen sporen. Er is slechts een beperkt aantal studies dat de voorspellende waarde van het routinematig screenen van deze patiënten, met behulp van bloedcontroles en echografie bij de opsporing van galwegstricturen geëvalueerd hebben. In **hoofdstuk 4** werd met een tijdsafhankelijke Cox regressie model aangetoond, dat het routinematig bepalen van GGT en het uitvoeren van echo's een sterk voorspellende waarde had voor het optreden van NAS in de daaropvolgende 3 maanden. Andere leverenzymen waren niet onafhankelijk voorspellend voor galwegstricturen. Een verhoogde GGT en gedilateerde galwegen op de echo rechtvaardigen hiermee het gebruik van invasieve methoden, zoals ERCP en PTC om de strictuur aan te tonen en te behandelen.

Matrix metalloproteinasen en galwegstricturen

Matrix metalloproteinasen (MMPs) vormen een groep eiwitklievende enzymen die betrokken zijn bij het hermodelleren van bindweefsel. Inmiddels zijn er meer dan 26 varianten bekend. In gezond weefsel wordt voortdurend weefsel aangemaakt en afgebroken waarbij MMPs een cruciale rol spelen. Onder invloed van verschillende stimuli kan de productie van MMP toe- of afnemen.

Als gevolg van IRI kunnen zuurstofradicalen (reactive oxygen species (ROS)) ontstaan ten gevolge van metabole reacties die bepaalde mononucleaire (Kupffer) cellen kunnen activeren. Deze activeren vervolgens stellate cellen in de lever die vervolgens MMP-2 en MMP-9 produceren. MMP-9 wordt ook in endotheel, de Kupffercellen, neutrofielen en endotheel gevormd. MMP-2 en MMP-9 zijn in staat om type IV collageen af te breken, een van de hoofdcomponenten van bindweefsel en zijn reeds in verband gebracht met verschillende ziektebeelden als tumorvorming, ontstekingsziekten en verbindweefseling (fibrose). *(Figuur 1)* In **hoofdstuk 5** werden single nucleotide polymorfismen (SNP) in de promotorregio van MMP-2 en MMP-9 onderzocht in relatie tot het ontstaan van NAS na levertransplantatie. Hieruit bleek dat patiënten die drager waren van het MMP-2 –1306 CT of TT promoter polymorfisme significant vaker NAS ontwikkelden ten opzichte van patiënten die drager waren van het wild-type (CC) allel. De cumulatieve incidentie nam verder toe indien het CT/TT polymorfisme ook aanwezig was in de donorlever. In een multivariate analyse bleek dat de aanwezigheid van het CT/TT polymorfisme onafhankelijk voorspellend was voor het ontstaan van NAS, gecorrigeerd voor CIT en primaire scleroserende cholangitis (PSC) als indicatie voor levertransplantatie. Een interessante bevinding was het feit, dat bloedspiegels van MMP-2 lager was in patiënten die NAS ontwikkelden ten opzichte van hen zonder NAS en dat deze tevens lager warenindien de patiënten drager waren van het CT/TT polymorfisme. Dit wijst erop dat het polymorfisme waarschijnlijk een functioneel effect heeft op de beschikbaarheid (en mogelijk ook activiteit) van MMP-2.

Naast IRI kan ook een andere oorzaak van ontsteking een bron van MMP activering zijn. Verschillende ontstekingsziekten in de lever kunnen bij aanhoudende ontsteking leiden tot fibrose en ze vormen frequent aanleiding tot levertransplantatie. Een goed voorbeeld is primaire scleroserende cholangitis (PSC), waarbij chronische ontsteking van de galwegen uiteindelijk leiden tot strictuurvorming. PSC gaat bovendien vaak gepaard met inflammatoir darmlijden (IBD). Door verminderde afvloed van gal kunnen patiënten infecties ontwikkelen welke gepaard gaan met ziekenhuisopname, behandeling met antibiotica en endoscopische behandeling van de galwegen. Ongeveer 15% - 20% van de PSC patiënten ontwikkelt kanker van de galwegen. Infecties die elkaar snel opvolgen vormen vaak een reden om de PSC patiënt te transplanteren. Gezien

de voorheen beschreven relatie tussen de ontwikkeling van galwegstricturen na levertransplantatie en genetische polymorfismen van MMP-2, is er gekeken of er een relatie bestond bij andere fibrotische aandoeningen zoals PSC met MMP-2 polymorfismen. In **hoofdstuk 6** werd de ziekte-ernst van PSC patiënten getoetst aan MMP-2 polymorfismen, waarbij ziekte-ernst werd gedefinieerd als overlijden of levertransplantatie. Hieruit kwam naar voren dat PSC patiënten die drager waren van het CT polymorfisme er slechter aan toe waren, en dus vaker overleden of getransplanteerd werden dan patienten die drager waren van het wild-type (CC) allel. Bovendien was de ziekte-ernst nog groter indien PSC patiënten drager waren van het TT allel. De associatie met de aanwezigheid van het T-allel en ziekte-ernst werd bevestigd in subgroepen van PSC patiënten met en zonder IBD. In een multivariate regressie analyse werden de bevindingen bevestigd waarbij een stapsgewijze toename in ziekte-ernst werd gezien naar gelang het aanwezig zijn van T-allel. De resultaten uit deze studie geven aan dat MMP-2 sterk betrokken is bij ziekteprogressie in patiënten met PSC.

Hoofdstuk 5 en **6** onderschrijven het belang van MMP-2 in ziekteprocessen waarbij collageendepositie een cruciale rol speelt zoals bij de ontwikkeling van NAS en PSC.

Chimerisme

Met chimerisme wordt de aanwezigheid van cellen bedoeld met verschillende genetische achtergronden in een individu. Bij transplantatie is deze aanwezigheid vanzelfsprekend, aangezien er een donorlever met een andere genetische achtergrond wordt getransplanteerd. De vormen van chimerisme kunnen nog een tweetal andere vormen aannemen. Studies hebben uitgewezen dat afstoting gerelateerd is aan de aanwezigheid van ontvanger cellen in de donor lever, en de aanwezigheid van cellen afkomstig van de donorlever in de bloedbaan en in perifeer weefsel (met name lymfeklieren) van de ontvanger. Hoewel een laag normale waarde wordt geassocieerd met een vorm van tolerantie van de ontvanger, worden hogere percentages van donor cellen geassocieerd met afstoting, graft-versus-host ziekte en terugkeer van de oorspronkelijke ziekte waarvoor getransplanteerd werd. Chimerisme werd

voor het eerst aangetoond in 1968 na levertransplantatie bij vrouwelijke ontvangers met donor levers afkomstig van mannen. Hierbij werd op basis van de aanwezigheid van het mannelijk geslachtschromosoom aangetoond dat er sprake was van chimerisme. Een nadeel van het gebruik van geslachts-chromosomen bij het aantonen van chimerisme is dat slechts donor-ontvanger combinaties van verschillende sekse gebruikt kunnen worden. In **hoofdstuk 7** werden MMP-2 en MMP-9 promotor polymorfismen bepaald in donor en ontvanger om chimerisme aan te tonen. Deze bepalingen werden verricht op leverbiopten en bloed van de ontvanger. Chimerisme werd aangetroffen in 28.8% van de leverbiopten voor MMP-2 en in 16.8% voor MMP-9. Ook in bloed werd chimerisme aangetroffen, namelijk in 18.5% voor het MMP-2 polymorfisme en 3.4% voor het MMP-9 polymorfisme. Hiernaast werd er een significante associatie gevonden voor de aanwezigheid van chimerisme en het optreden van afstoting. Ook hier werd in een multivariate analyse aangetoond dat deze associatie onafhankelijk was, gecorrigeerd voor de score voor de ernst van de leverziekte waarvoor getransplanteerd is (MELD score).

Conclusie en toekomstperspectief

Levertransplantatie is uitgegroeid van experimentele behandeling tot standaardbehandeling voor eindstadium leverziekte met uitstekende lange termijn overleving. Als gevolg hiervan kunnen meer patiënten in aanmerking komen voor levertransplantatie terwijl het aantal hersendode donoren afneemt. Deze onevenwichtige toename heeft ertoe geleid dat transplantatiecentra beter gebruik maken van het huidig aanbod aan donorlevers: Momenteel worden 25 – 35% van alle transplantaties verricht met hartdode ('DCD') donorlevers. Het gebruik van DCD donorlevers leidt tot minder sterfte op de wachtlijst, maar een nadeel is dat tot wel 35% van alle getransplanteerde DCD levers nonanastomotische galwegstricturen (NAS) ontwikkelt. NAS leidt tot meer opnames en meer retransplantaties welke gepaard gaat met aanzienlijke morbiditeit en zelfs mortaliteit. Het is waarschijnlijk dat NAS ontstaat door een samenspel van IRI (ischemie-reperfusie-schade), immunologische en toxische factoren. IRI ontstaat bij DCD waarschijnlijk doordat tijdens de DWIT extra schade ontstaat, mogelijk door microstolsels gevormd in de kleinste aftakkingen van de peri-biliare plexus. Bij het aansluiten van de donorlever in de ontvanger met reperfusie als gevolg, komen vervolgens ROS vrij die aan het leverweefsel schade kunnen aanrichten waaronder aan hepatocyten. Bij het afsterven van hepatocyten komt ALT vrij, wat zich uit als een piek die te meten is in de eerste week na levertransplantatie. We hebben aangetoond dat een piek ALT van meer dan 1300 IU/L in de eerste week sterk voorspellend is voor het ontstaan van NAS na levertransplantatie met DCD donoren. Deze associatie werd niet gevonden na levertransplantatie met DBD donoren. Het reduceren van de piek ALT tot minder dan 1300 IU/L zal dus de incidentie van NAS na DCD transplantatie waarschijnlijk reduceren tot aan die van levertransplantatie met DBD donoren. ROS kunnen direct en indirect Kupffer cellen en stellate cellen activeren, die op hun beurt respectievelijk MMP-9 en MMP-2 produceren. Deze MMP's zijn in staat om type IV collageen af te breken. In een eerdere studie hebben wij aangetoond dat MMP-9 vrijkomt tijdens de vroege fase van IR-schade, afkomstig van met name Kupffer cellen en neutrofielen. De huidige studies in deze thesis laten zien dat MMP-2 betrokken is bij complicaties die zich op de lange termijn manifesteren zoals ziekteprogressie bij PSC en de ontwikkeling van NAS na levertransplantatie. Het is waarschijnlijk dat

laatstgenoemde ziektebeelden verschillende initiërende reactiepaden hebben maar uiteindelijk kunnen leiden tot eenzelfde strictuurvorming van de galwegen. In de huidige thesis hebben we aangetoond dat het MMP-2 -1306 C/T polymorfisme leidt to een verminderde circulatie van MMP-2, welke duidt op een functionele SNP. Bovendien lopen dragers van het CT/TT polymorfisme een groter risico op het ontwikkelen van NAS en dit effect is groter indien deze polymorfismen ook aanwezig waren in de donorlever. De rol van het MMP-2 C/T polymorfisme komt ook bij PSC patiënten sterk naar voren waarbij dragers van het CT respectievelijk TT allel een stapsgewijze toename lieten zien wat betreft de ernst van het ziektebeeld bij PSC zich uitend als eerder overlijden of vroegtijdiger en in een hoger percentage getransplanteerd worden. Deze data suggereert dat MMP-2 een essentiële rol heeft na IRI en bij de chronische peribiliaire inflammatie en in de afbraak van collageen rondom de galwegen. Dit biedt verder inzicht in mogelijke klinische markers en aangrijpinspunten voor behandeling van ontwikkeling van galwegstricturen, zowel bij primaire sccleroserende cholangitis als na levertransplantatie (NAS).

Inmiddels zijn er meerdere therapieën in ontwikkeling met als voornaamste doel IRI na levertransplantatie te reduceren en daarmee complicaties zoals NAS en het falen van het transplantaat te voorkomen. Een van deze onderzoeken omvat het gebruik van machine perfusie waarbij de donorlever onder fysiologische omstandigheden wordt voorzien van onder andere zuurstof. Recente studies hebben al veelbelovende resultaten getoond met het “verbeteren” van donorlevers. Het is tot op heden echter nog onduidelijk waar en wanneer het gebruik van machineperfusie toegepast zou moeten worden. Ook zijn er nog geen data die ondersteunen dat machineperfusie de ontwikkeling van NAS kan voorkomen. Andere therapieën die in ontwikkeling zijn omvatten het gebruik van stolseloplossende medicijnen die tijdens de uitnameprocedure van DCD donorlevers toegepast kunnen worden om microstolsels op te lossen. Onze data ondersteunen het idee dat MMPs een belangrijke rol spelen in IRI en ontstaan van NAS na levertransplantatie. Dit geeft mogelijk aangrijpingspunten voor het beperken van IRI en NAS na OLT en wellicht ook bij PSC. Andere strategieën gericht op bijvoorbeeld toxische galzouten en immunologische factoren moeten zeker ook onderzocht worden.

Abbreviations

ACR	Acute Cellular Rejection
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
AS	Anastomotic Strictures
CIT	Cold Ischemia Time
CCR5Δ32	Chemokine Receptor 5 delta 32
DCD	Donation after Cardiac Death
DBD	Donation after Brain Death
DWIT	Donor Warm Ischemia Time
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EMMPRIN	Extracellular Matrix Metalloproteinase Inducer
FGF	Fibroblast Growth Factor
HCC	Hepatocellular Carcinoma
HR	Hazard Ratio
HSC	Hepatic Stellate Cells
HTK	Histidine-tryptophan-ketoglutarate (preservation fluid)
IBD	Inflammatory Bowel Disease
IL-1	Interleukin-1
IRI	Ischemia-Reperfusion Injury
ITBL	Ischemic-Type Biliary Lesions
LT	Liver transplantation
MDR3	Multidrug Resistance Protein 3
MMP	Matrix Metalloproteinase
MT-MMP	Membrane-type Matrix Metalloproteinase
NAS	Nonanastomotic Strictures

NGF	Nerve Growth Factor
NK	Natural Killer cells
OLT	Orthotopic Liver Transplantation
PA	Plasmin Activator
PDGF	Platelet Derived Growth Factor
PNF	Primary Nonfunction
PSC	Primary Sclerosing Cholangitis
RFA	Radiofrequency Ablation
ROS	Reactive Oxygen Species
RWIT	Recipient Warm Ischemia Time
Sp-1	Specificity Protein-1
SNP	Single Nucleotide Polymorphism
TACE	Transarterial Chemo Embolization
TGF-β	Transforming Growth Factor β
TIMP	Tissue Inhibitors of Metalloproteinases
TNF-α	Tumor Necrosis factor α
UW	University of Wisconsin (preservation fluid)
VEGF	Vascular Endothelial Growth Factor

List of publications

Kerem Sebib Korkmaz, W. Rogier ten Hove, Hein Verspaget, Jeroen Dubbeld, Ron Wolterbeek, Arian van Erkel, Bert-Jan F. de Rooij, Minneke C. Coenraad, Jan Ringers, Bart van Hoek *Sequential liver chemistry profiling and abdominal ultrasound assessments to predict biliary strictures after liver transplantation.* The Open Transplantation Journal 2012;22(5):1-5

W. Rogier Ten Hove, **Kerem Sebib Korkmaz**, Sanna op den Dries, Bert-Jan F. de Rooij, Bart van Hoek, Robert J. Porte, Johan J. van der Reijden, Minneke J. Coenraad, Jeroen Dubbeld, Daniel W. Hommes and Hein W. Verspaget *Matrix metalloproteinase 2 genotype is associated with nonanastomotic biliary strictures after orthotopic liver transplantation.* Liver International 2011;31(8):1110-7

Kerem Sebib Korkmaz, Bert-Jan de Rooij, Bart van Hoek, Marcel Janse, Minneke J. Coenraad, Johan J. van der Reijden, Rinse K. Weersma, Robert J. Porte, Philip W. Voorneveld, Andrzej G. Baranski, Hein W. Verspaget *Matrix metalloproteinase 2 is a disease modifying gene in primary sclerosing cholangitis.* Liver International 2013 Jun 4. doi: 10.1111/liv.12237. [Epub ahead of print]

Kerem Sebib Korkmaz, W. Rogier ten Hove, Bert-Jan F. de Rooij, Bart van Hoek, Johan J. van der Reijden, Minneke J. Coenraad, Jeroen Dubbeld, Hein W. Verspaget *Acute cellular rejection is associated with matrix metalloproteinase-2 genotype chimerism after orthotopic liver transplantation.* Transplantation Proceedings 2013;45(2):558-63

Voorneveld P.W., Stache V., Jacob R.J., Smolders E., Sitters A.I., Liesker A, **Sebib Korkmaz K.**, Lam S.M., De Miranda N.F., Morreau H., Kodach L.L., Hardwick J.C. *Reduced expression of bone morphogenetic protein receptor IA in pancreatic cancer is associated with a poor prognosis.* British Journal of Cancer. 2013 Oct 1;109(7):1805-12. doi: 10.1038/bjc.2013.486. Epub 2013 Aug 22

Dankwoord

Vanzelfsprekend gaat mijn dank uit naar iedereen die heeft bijgedragen aan de voorbereidingen en de uiteindelijke voltooiing van dit proefschrift. Gaarne zou ik enkele hiervan bij name willen noemen, te beginnen bij mijn promotor en copromoter. Zeer geachte prof. dr. B. van Hoek, beste Bart, het was een groot voorrecht om met je samen te werken in de afgelopen jaren. Je onmetelijke wijsheid, betrokkenheid en geduld hebben ervoor gezorgd dat ik mijn onderzoek op een prettige manier heb kunnen uitvoeren. Je bent een stimulans voor me geweest in moeilijkere tijden en ik heb veel van je geleerd. Ook op persoonlijk vlak blijf je een voorbeeld voor mij en ik had me geen betere promotor kunnen wensen. Ik ben blij dat ik met je heb kunnen samenwerken!
Geachte dr. ir. Verspaget, beste Hein, graag wil ik je danken voor de talloze keren dat je met je precisie blik mij terecht hebt gewezen. Je drang om continu te verbeteren heeft het onderzoek alleen maar beter gemaakt. Ook van jou heb ik erg veel kunnen leren. Bedankt hiervoor.
Bert-Jan, onze samenwerking in de afgelopen jaren heb ik als zeer prettig ervaren en je feedback was altijd van grote waarde voor mij en ik dank je voor alle hulp die je me hebt aangeboden. Dr. ten Hove, beste Rogier, hoewel onze samenwerking van korte duur was, hebben we veel aan elkaar gehad tijdens onze onderzoeksperiode waarvoor dank.
De gezelligheid op de werkvloer heeft niet zelden legendarische proporties aangenomen dankzij mijn directe collegae. Beste Danny, dank voor je hulp en je gezelligheid op de afdeling. Tony, hoewel je later bij de groep kwam hebben we desondanks een gezellige tijd gehad en zal ik onze discussies omtrent statistische analyses niet snel vergeten. Claire, je neemt een deel over van het takenpakket en ook dat zal ongetwijfeld goed komen. Uiteraard gaat mijn dank ook uit naar alle medewerkers van het laboratorium van de Maag-, Darm-, Leverziekten!
Prof. dr. R. J. Porte, beste Robert, graag zou ik u willen bedanken voor het beschikbaar stellen van de Groningse data alsmede uw feedback omtrent de onderzoeken die we samen hebben verricht. Ik hoop dat de samenwerking met het noorden vruchtbaar zal blijven in de toekomst. Michael Sutton, je hebt me goed ontvangen in Groningen waarvoor dank. Onze samenwerking

staat inderdaad voor het (L)UMC(G)!
Jeroen Maljaars, jou zou ik graag willen bedanken voor de ontelbare discussies die we hebben gehad zowel formeel als informeel. Ook jouw scherpe blik op zaken heeft een betere onderzoeker van me gemaakt. Akin, jouw gedrevenheid op het gebied van de hepatologie vormt een inspiratiebron voor velen en ook voor mij waarvoor dank. In het bijzonder wil ik Ron Wolterbeek danken voor de talloze uren die ik bij je heb doorbracht vanwege complexe statistische vraagstukken. Ik heb ongelooflijk veel geleerd van je en dankzij jou is mijn blik op wetenschappelijke artikelen vele malen kritischer geworden.
Mijn paranimfen, Ilse en Philip, zijn uiteraard niet te vergeten. Ilse, jij bracht de nodige female touch in een werkhok (toentertijd) gedomineerd door mannen. Je liet ons ontelbaar vaak vrolijker achter dan dat je ons aantrof. Phil, ik vond het erg leerzaam om met je samen te werken maar vooral ontzettend leuk! Dank voor deze periode en het feit dat ik gebruik heb mogen maken van je connecties in de grafische wereld en je grafische vaardigheden.
Jetske, dank voor je inzet in het grafisch vormgeven van dit proefschrift. Je hebt me erg veel geholpen en het resultaat mag er ook zijn. Het boekje ziet er voortreffelijk uit waarvoor mijn dank groot is.
Uiteraard gebiedt het de promovendus om ook de mensen te danken die voornamelijk in informele sfeer van grote waarde zijn geweest. Allereerst zou ik mijn zeer goede vrienden willen danken die min of meer in hetzelfde schuitje zitten: Mariët, Russ, Wouter, Said en Brian. Onze wegen hebben zich reeds gescheiden maar op een of andere manier vinden we elkaar toch weer. Ook mijn vrienden met wie ik inmiddels onvoorstelbaar veel heb meegemaakt wil ik danken: Ibrahim, Huseyin, Kahraman en Murat, jullie zijn geweldig. Het is een absoluut voorrecht om jullie tot mijn vrienden te rekenen.
Cultureel gezien is het voor mij onmogelijk om aan de familie voorbij te gaan in dit dankwoord. Allereerst zou ik mijn lieve broer Kenan en mijn nagenoeg broer Cuma willen danken voor hun onvoorwaardelijke steun in alle tijden. Kenan, jij bent de beste broer die ik me kan wensen en alleen jij kan me werkelijk laten lachen. Cuma, op jou kan ik altijd rekenen. Bovendien zou ik Ziya, Omit en Meysem willen danken voor hun steun en vertrouwen.
Mama en papa, jullie vormen de basis van mijn leven en dankzij jullie is dit proefschrift mogelijk geworden. Papa, ik heb veel van je geleerd en ik ben ook trots op jou. Je hebt me altijd gesteund en ik ben blij dat ik dit moment

met je kan delen. Mama, een betere moeder is voor mij gewoonweg onmogelijk voor te stellen, je excelleert op alle vlakken. Bedankt en ik hou van jullie. Zonder mijn lieve vrouw Eylem was dit geheel sowieso onmogelijk geweest. Jij bent mijn grote steun en toeverlaat en ik weet dat ik altijd op je kan rekenen. Uiteraard wil ik mijn lieve dochter Aliya ook bedanken. Je bent nieuw op deze wereld en ik zal mijn best doen om je een fantastische toekomst te geven. Voor jullie en dankzij jullie doe ik dit.

Bedankt!

Curriculum Vitae

The author of this thesis was born on July 14, 1985 in The Hague, The Netherlands. After graduating from the Christelijk Gymnasium Sorghvliet he started his medical study in 2004. During the medical course he developed a particular interest for gastroenterology and hepatology but also for scientific research. He started his research internship at the department of Gastroenterology and Hepatology under the supervision of Prof. Dr. B. van Hoek during which he investigated the short- and long-term biliary complications after liver transplantation. After finishing the medical traineeships he decided to start his PhD traineeship (co-supervisor Dr. Ir. H. W. Verspaget), which would elaborate further on the topic of his initially started research project. The results of which are described in the current thesis. In 2013 he started his residency Gastroenterology and Hepatology (supervisor Dr. R. A. Veenendaal) of which the initial phase is currently being done at the Medical Center Haaglanden (supervisors Dr. A. H. Bootsma and mw. Dr. P. H. L. M. Geelhoed-Duyvestijn).