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Lectin complement activation pathway and the outcome of orthotopic liver transplantation

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LECTIN COMPLEMENT ACTIVATION
PATHWAY AND THE OUTCOME
OF ORTHOTOPIC LIVER
TRANSPLANTATION



BERT-JAN DE ROOIJ

LECTIN COMPLEMENT ACTIVATION PATHWAY AND
THE OUTCOME OF
ORTHOTOPIC LIVER TRANSPLANTATION

Bert-Jan Felix de Rooij

Lectin Complement Activation Pathway and the Outcome of Orthotopic Liver Transplantation

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LECTIN COMPLEMENT ACTIVATION PATHWAY AND
THE OUTCOME OF
ORTHOTOPIC LIVER TRANSPLANTATION

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 26 september 2019
klokke 10:00 uur

door

Bert-Jan Felix de Rooij
geboren te Leiderdorp in 1976

PROMOTORES

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Prof. dr. ir. H.W. Verspaget

LEDEN PROMOTIECOMMISSIE

Prof. dr. C. van Kooten (voorzitter)

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Dr. M.J. Coenraad

Prof. dr. R.J. Porte (Universiteit Groningen)

Prof. dr. H.J. Metselaar (Erasmus Universiteit Rotterdam)

Er zijn zoveel wegen vanaf de start dus kies je eigen weg en volg je hart
(*Huub van der Lubbe – Je Eigen Weg*)

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VOORWOORD

In dit voorwoord wil ik mijn bijzondere erkenning vastleggen voor iedereen die meegeholpen heeft aan het tot stand komen van dit proefschrift en aan iedereen die me dierbaar is.

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Chapter 1

Introduction and outline of the thesis

INTRODUCTION

Liver transplantation evolved impressively over the past five decades and has gone from an initially futile endeavour to the definitive treatment of most types of liver failure in both children and adults.¹⁻² As liver transplantation matured, indications for liver transplantation have grown to include more causes of acute and chronic liver failure, end-stage cirrhosis, metabolic disorders and selected cancers.³ Consequent to the general experience that developed over the years, the understanding of the prognosis and survival rates after liver transplantation in different populations has improved.⁴ Nowadays one- and five-year patient survival after liver transplantation in the Netherlands is 89% and 75%, respectively.⁵

Improvements in post-transplant survival, for both graft and patients, can be partially attributed to more selective and less toxic immunosuppressive treatment regimens used. The advances in immunosuppressive treatment options decreased the incidence of rejection considerably. Nevertheless, an important remaining adverse effect of this treatment is the increased susceptibility for infections.⁶

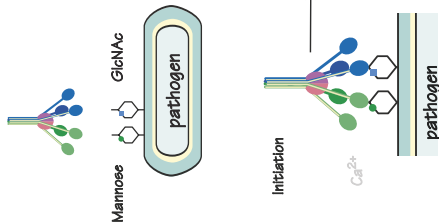
Immunosuppression interferes with the adaptive immune response and consequently the patient becomes critically dependent on the innate immunity, as first line of immunological defence against infections. Even now infection remains a feared complication of liver transplantation and a significant cause of post-transplantation morbidity and mortality. Most infectious complications occur during the first year after transplantation in up to 80% of the patients. Bacterial infections are the most frequent (70%), followed by viral (20%) and fungal infections (8%).⁶⁻⁷

The complement system, first discovered in the 1890s, is an evolutionary conserved and a central subsystem within the innate immunity that consist of more than 30 membrane-bound and soluble proteins. Its origins can be traced back to phyla as ancient as the sea urchin.⁸ It is now clear that the complement system serves as a broadly protective system for the host and contributes to a wide variety of defensive and immune-related functions.

The complement system is a humoral proteolytic cascade-based defence mechanism, consisting of tightly regulated proteolysis of constituent proteins, executed through three separate enzymatic cascades—the alternative, the lectin, and the classical pathway— which are activated in this sequence. All pathways converge on the production of a key complex known as the C3-convertase leading to C3b-mediated opsonisation of the pathogenic microorganism followed by phagocytosis and the formation of a complement membrane-attack-complex (MAC) that directly kills the pathogen (see figure 1, based on⁹ and¹⁰).

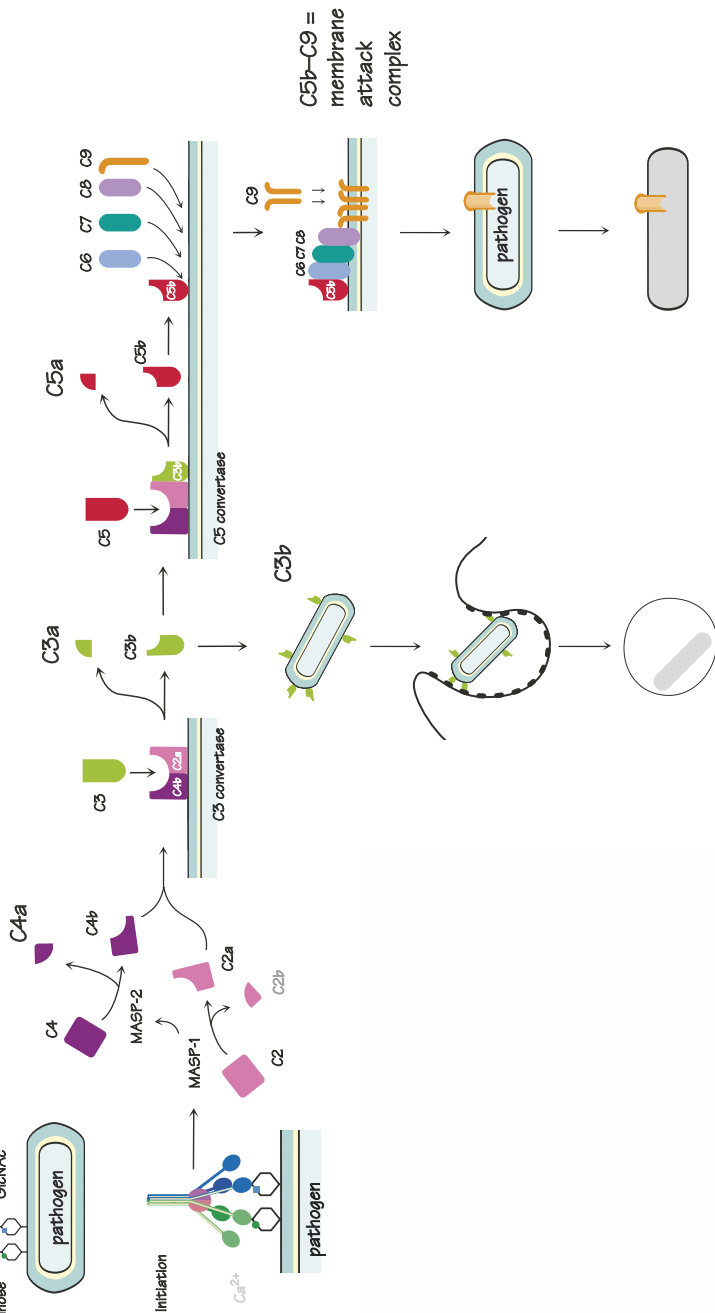
The lectin pathway of complement activation is mediated by mannose-binding lectin (MBL), collectin-10, collectin-11, and the ficolins (ficolin-1, ficolin-2 and ficolin-3) which are soluble pattern recognition molecules. Each of these molecules interact with MBL-associated serine proteases (MASP-1, MASP-2, and MASP-3), originally named after their association with MBL.¹¹ MASP-1 and -2 trigger the activation of the lectin pathway when binding to conserved pathogen-specific structures and altered self-antigens specific structures (see figure 2 based on¹² and¹³). Furthermore, MASPs mediate processes related to coagulation.

MBL or ficolin-2
-MASP's complex



Lectin pathway of complement activation

anaphylatoxins, **C4a**, **C3a** and **C5a**
produce a local inflammatory response



Cytolysis by membrane
attack complex

C3b opsonization enhances phagocytosis

MBL¹⁴, ficolin-2¹⁵ and MASP-2¹⁶ are predominantly produced in the liver and show significant genetic variation in the human population.

Single nucleotide polymorphisms (SNPs) are specific positions in the genome where a variation in the single nucleotide can be found and they represent the most abundant form of genetic variation in a population.

The effect of SNPs depends on the location of the altered amino acid in the protein as well as the nature of the amino acid substitution. If the changes occur within a coding area of the gene or in a regulatory (promotor) region near a gene, this can either lead to the production of an abnormal form of the protein or to an abnormal level of expression of the protein, respectively. Therefore, these SNPs may play a more direct role in disease by ultimately affecting the gene's function. Common SNPs in the genes coding for the three liver-derived members of the lectin complement pathway MBL, ficolin-2 and MASP-2 affect the expression, composition and structure of the proteins with important functional implications which may directly affect disease outcome.

In addition to the complement components described above, the innate immune system also relies on canonical pattern recognition receptors (PRR) to trigger inflammatory responses. These receptors, which recognize invariant 'molecular patterns' on pathogens, include Toll-like receptors (TLRs) and cytosolic PRRs, like nucleotide-binding oligomerization domain-containing protein 2 (NOD2), as shown in figure 3. Evidence has accumulated that indicates an important role for MBL in modulating PRR-related inflammation. For instance, MBL can facilitate enhanced signalling via complement receptors or presentation of bacterial ligands to TLR-2¹⁷ and TLR-4¹⁸ (see figure 3 based on Ip et al.(2008)¹⁷ and Wang (2011)¹⁸). This implies that next to functional polymorphisms in *MBL2*, *FCN2* and *MASP2*, genetic variation in the genes coding for pattern recognition receptors (TLRs and NOD2) and their associated intracellular signalling molecules [MASP-2, C3, MD2 and Myeloid differentiation primary response 88 (MyD88)] also contributes to variations in the immune response against pathogens.

- ◀ **Figure 1.** The complement system is a set of liver-produced plasma proteins that play a role in innate immunity and inflammation. The lectin pathway is activated when Mannose-Binding Lectin (MBL) or ficolin-2, in complex with MASPs, recognize pathogen associated molecular patterns (PAMPs) on microbial surfaces. This activates MASP-1 followed by MASP-2, which results in the formation of a C3 convertase (C4b2a complex). The C3 convertases cleave C3 into the opsonin C3b and the anaphylatoxin C3a. Activation of C3 also leads to the formation of the lectin pathway C5 convertase (C4b2a3b). The C5 convertase cleaves C5 into the anaphylatoxin C5a and C5b, leading to the formation of a terminal membrane attack complex (C5b-9). The view of the pathway is simplified and crosstalk with other molecules and between different pathways has been described. (Adapted from Immunology: A Short Course, Seventh Edition. Richard Coico and Geoffrey Sunshine. © 2015 John Wiley & Sons, Ltd. Published 2015 by John Wiley & Sons, Ltd.)

Liver-produced plasma proteins of the lectin pathway of complement activation

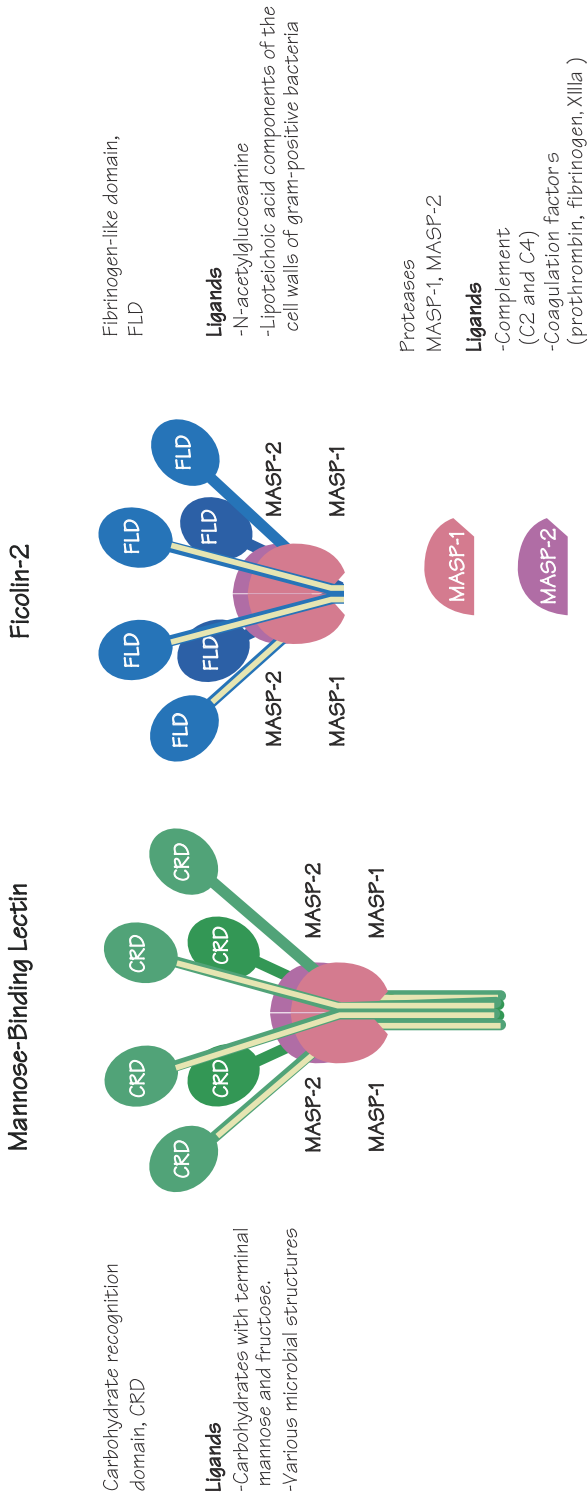
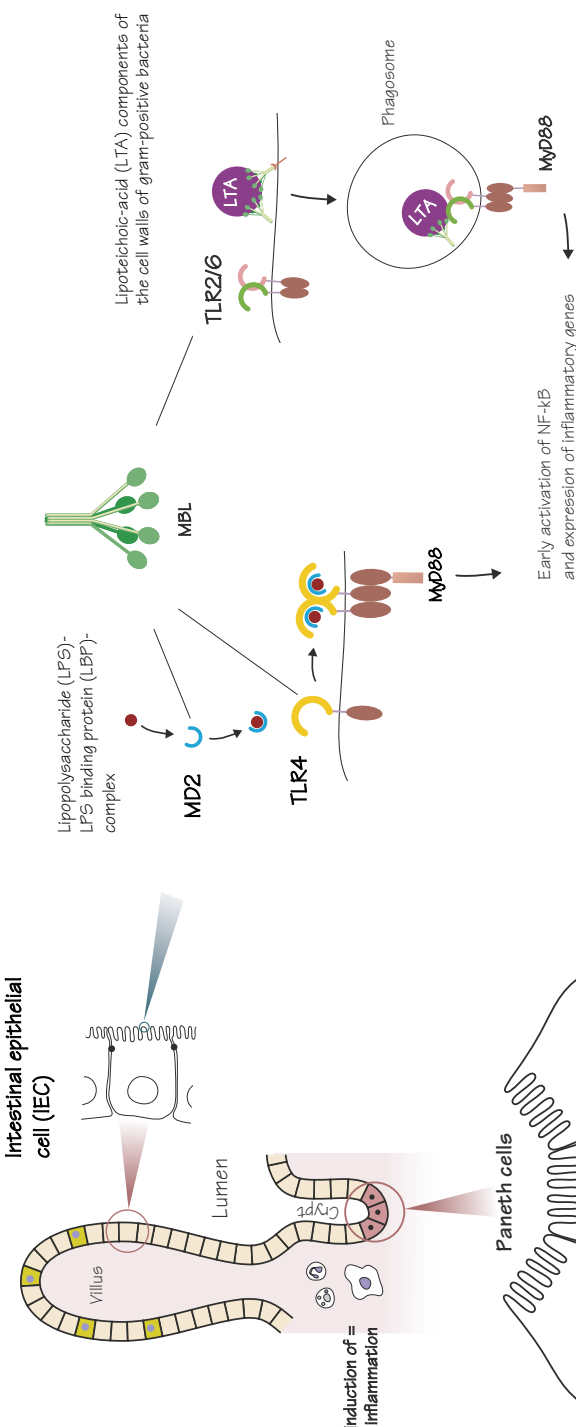


Figure 2. Molecular structure of Mannose-Binding Lectin (MBL) and ficolin-2. The domain organizations between MBL and ficolin-2 are very similar in that both consist of a collagen-like domain and a carbohydrate-binding domain, although their carbohydrate-binding moieties are different. Both MBL and ficolin-2 rely on MBL-associated serine proteases for activation of the complement system. CRD carbohydrate recognition domain; Fibrinogen-like domain, FLD; MASP-1, MASP-2 MBL-associated proteases. Adapted from: Protective molecules and their cognate antibodies: new players in autoimmunity. Zen et al. / Autoimmun Highlights (2010) 1:63–72

MBL affects cytokine expression by modulating TLR-signaling pathways



1. Mannan-binding lectin directly interacts with Toll-like receptor 4 and suppresses lipopolysaccharide-induced inflammatory cytokine secretion from THP-1 cells. Wang et al. / Cellular and Molecular Immunology VOL.8, March 7, 2011
2. Mannose-binding lectin enhances Toll-like receptor and 6 signaling from the phagosome. Ip et al. / JIE 205, January 21, 2008

Figure 3.

OUTLINE AND AIMS OF THE STUDIES DESCRIBED IN THIS THESIS

The combination of a treatment-induced immunocompromised patient and the donor liver, as predominant organ where these complement system associated proteins are synthesized, makes liver transplantation a unique “in vivo” human model to study functional aspects of the components of the lectin pathway of complement activation. This thesis provides a profound insight into the genotype and phenotype profile of mannose-binding lectin (MBL), Ficolin-2 and MASP-2 in relation to liver disease and liver transplantation-related complications.

A summarizing overview of the many transplantation-related, recipient and donor factors involved in the risk for infection after liver transplantation is described first in **chapter two**.

The study described in **chapter three** was performed to elucidate the impact of functional polymorphisms in the genes encoding for MBL, ficolin-2 and MASP-2 in the balance between hepatic (donor) and extrahepatic (recipient) production of these factors.

The role of common functional polymorphisms in genes encoding for MBL, ficolin-2 and MASP-2 and their contribution to the risk of clinically significant bacterial infections after orthotopic liver transplantation (OLT) was investigated in a large group of patients as described in **chapter four**.

An analogous study was performed, as described in **chapter five**, to explore the role of the common functional polymorphisms in the genes encoding for MBL, ficolin-2 and MASP-2 on the risk of cytomegalovirus (CMV) infection which occurs frequently after OLT.

In addition to the role of the genetic variants of the liver-derived proteins of the lectin complement activation pathway, as described in chapter four, **chapter six** describes the independent association between three known genetic variants of intracellular pattern recognition receptor NOD2 and the increased risk of bacterial infections after OLT.

The course of advanced cirrhosis, irrespective of its aetiology, is complicated by cirrhosis-associated immune dysfunction which predisposes to a higher risk of bacterial infection and increased mortality.¹⁹ The liver is the major source of several components of the innate immune system in the body, as described above. While patients with advanced liver disease and ascites are waiting for a liver transplantation they are at risk of developing spontaneous bacterial peritonitis (SBP). The contribution of genetic variation in the genes coding for pattern recognition receptors (lectins, TLRs and NOD2) and their associated intracellular signalling molecules (MASP-2, C3, MD2 and MyD88) of the innate immune system to the susceptibility for SBP in patients with end-stage liver disease and ascites was therefore evaluated, as described in **chapter seven**.

Hepatic ischemia-reperfusion injury (IRI) following OLT results from warm and cold anoxic ischemia between donation and revascularization, followed by reperfusion injury. This enhances susceptibility to infections. In **chapter eight** a study is described to evaluate the relationship of the first-week postoperative peak serum levels of aspartate transaminase (AST), as marker for hepatic ischemia-reperfusion injury, with infection susceptibility after OLT.

Biliary and vascular disorders are common and clinically particularly relevant complications after OLT. Non-anastomotic biliary strictures (NAS) result mainly from ischemia. Impaired blood supply as a result of vascular ischemia may cause injury to the peribiliary glands and vascular plexus.²⁰ The complement and coagulation systems are evolutionary closely related, both structurally and functionally, and are tightly interconnected during the haematological response to injury because both systems are interregulated components of the host defence.²¹ Particularly the lectin complement pathway-associated serine proteases (MASP-1 and MASP-2) represent such a crossroad between coagulation and complement systems.

In **chapter nine** the relationship is described between a mutation in the gene encoding for MASP-2 and two clinical coagulation-associated conditions, i.e., NAS in the donor liver and Budd-Chiari syndrome (BCS) before and after OLT. BCS is a rare disorder caused by obstruction of the hepatic venous outflow tract which can occur in patients with an underlying thrombotic diathesis, both hereditary and acquired, but may also have a variety of other causes, including impaired fibrinolysis.²²

In the summarizing discussion, **chapter ten**, the most relevant findings and results of the studies presented in this thesis are compiled and discussed, and suggestions for further research are proposed.

A thesis summary in Dutch is presented in **chapter eleven**.

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Chapter 2

Risk factors for infection after
liver transplantation

van Hoek B, de Rooij BJ, Verspaget HW

Best Pract Res Clin Gastroenterol. 2012 Feb;26(1):61-72

ABSTRACT

Infection is a common cause of morbidity and mortality after liver transplantation. Risk factors relate to transplantation factors, donor and recipient factors. Transplant factors include ischemia-reperfusion damage, amount of intra-operative blood transfusion, level and type of immunosuppression, rejection, and complications, prolonged intensive care stay with dialysis or ventilation, type of biliary drainage, repeat operations, re-transplantation, antibiotics, antiviral regimen, and environment. Donor risk factors include infection, prolonged intensive care stay, quality of the donor liver (e.g. steatosis), and viral status. For the recipient the most important are MELD score >30, malnutrition, renal failure, acute liver failure, presence of infection or colonization, and immune status for viruses like cytomegalovirus. In recent years it has become clear that genetic polymorphisms in innate immunity, especially the lectin pathway of complement activation and in Toll-like receptors importantly contribute to the infection risk after liver transplantation. Therefore, the risk for infections after liver transplantation is a multifactorial problem and all factors need attention to reduce this risk.

INTRODUCTION

Orthotopic liver transplantation (OLT) has become a routine operation. One- and five-year patient survival is around 90% and 80%, respectively. A major cause of mortality and morbidity after OLT is infection, which occurs in up to 80% of the patients. Bacterial infections are most frequent (70%), followed by viral (20%) and fungal infections (8%).¹⁻³ Clinical symptoms can be blurred or absent due to immunosuppression, often leading to delayed diagnosis. Both donor and recipient factors as well as aspects related to the transplant operation contribute to the risk of infection after OLT. Recently genetic polymorphisms in the innate immune system, from both donor and recipient, have been identified as important risk factors for infection after OLT. The known risk factors for infection after OLT will be discussed.

Transplant factors

The timing of infections after OLT is shown in Table 1,³⁻⁵ divided in early, intermediate (immune suppression related) and late infections. Factors directly related to the OLT procedure contributing to the risk of infection can be either surgical technical issues but can also be preservation-related or graft-related factors (Table 2). The rate of contaminated preservation fluid varies among centers and regions.⁶ Peri-operative antibiotics will usually treat this, but culture-guided therapy sometimes is required.⁷ Particularly the amount of intra-operative blood transfusion is related to the risk of infection immediately after OLT, both from the abdomen and other sources.^{2,8} Initial poor graft function carries an increased infection risk. Partial hepatic necrosis, e.g. due to hepatic artery thrombosis, can lead to abscesses and bile duct injury with bacterial cholangitis. Abdominal fluid collections can become infected after OLT and need meticulous attention with imaging and diagnostic punctures. Both anastomotic and non-anastomotic biliary strictures (NAS) increase the risk of cholangitis. These are frequent complications and NAS more frequently occurs after non-heart-beating (NHB) donation (=DCD: deceased from cardiac death).⁹ Longer ischemia times and genetic factors contribute to the risk of NAS, and thus indirectly contribute to the risk of infection after OLT.¹ Biliary leakage can lead to infected biloma, and this is seen more often with the use of a T-tube and bile duct ischemia, whether or not due to hepatic artery stenosis or thrombosis.¹ The risk of surgical site infection is increased in the case of choledochojejunostomy.¹² In addition, indwelling catheters, invasive interventions, and prolonged dialysis or ventilation increase the risk of bacterial infection³. The antibiotic regimen around OLT has impact on the infection risk. Recent studies recommend oral selective digestive decontamination (SDD) during stay in the ICU.^{13,14} Studies on SDD in OLT show a decrease in gram-negative bacterial infection, with an increased risk for gram-positive infection and resistance and a questionable net effect.¹⁵ Extensive use of antibiotics poses the patient at risk for *Clostridium difficile* or fungal infection. Fungal prophylaxis decreases this latter risk but appears to be only justified in high-risk patients.³ Risk factors for invasive candidiasis apart from heavy immune suppression are prolonged or repeat operations and re-transplantation, high transfusion requirement, previous *Candida* colonization or renal

Table 1. Timing of different infections after liver transplantation^{3,4}

First month
Surgical site, abdomen (infected ascites, abscesses, cholangitis), blood stream, urinary system, respiratory tract, <i>Clostridium difficile</i> colitis, herpes, <i>Candida</i> .
Between one and six months after OLT
Opportunistic infections, often related to over-immunosuppression (e.g. after rejection): a.o. CMV (especially D+/R- serostatus), EBV, HSV 6 and 7, <i>Aspergillus</i> species, <i>Pneumocystis jirovecii</i> , <i>Nocardia</i> , tuberculosis, endemic mycoses, <i>Toxoplasma gondii</i> . Bacterial cholangitis in case of biliary strictures. Hepatitis C virus recurrence.
More than six months after OLT
Community-acquired, especially airway and urine tract in addition to opportunistic infections like varicella-zoster. Bacterial cholangitis in case of biliary strictures. Hepatitis C virus recurrence. More infections in case of graft dysfunction, biliary strictures or recurrent rejection.

failure after OLT, and a choledochojejunostomy. For *Aspergillus* species the risk factors are similar plus fulminant hepatic failure, CMV disease and a prolonged ICU-stay.³

Level and type of immunosuppression

Immunosuppressive treatment of rejection increases the risk of infection, including CMV reactivation. If possible the level of immunosuppression, especially mycophenolate mofetil or azathioprine, is reduced during CMV primo-infection or CMV-recurrence. During a severe infection or if EBV-DNA becomes detectable, reduction of immunosuppression may be needed in order to control the infection, accepting the risk that later on rejection occurs requiring additional immunosuppression. Sirolimus is associated with less cytomegalovirus infections than calcineurin inhibitors but can – if used early – lead to wound dehiscence and infection.^{16,17} While allowing late introduction of calcineurin inhibitors and thus sparing renal function, anti-thymocyte globulin may increase infection risk.¹⁸ When a steroid-free regimen with anti-CD25 is compared to a regimen with prednisolone without anti-CD25, the infection rate in the regimen with anti-CD25 (basiliximab) is lower in two studies,^{19,20} similar in two^{21,22} and increased in one study,²³ with no differences in hepatitis C recurrence.

Recipient factors

A poor condition of the recipient increases the risk for infection: MELD score >30, ICU-stay >48 h prior to transplantation, recipient age, re-transplantation, predicted post-transplant dialysis or probability of reoperation are risk factors for infection (Table 2).²⁴ Malnutrition is

Table 2. Risk factors for infection after liver transplantation.

Transplant factors	Recipient
Ischaemia times, ischaemia-reperfusion damage	Underlying condition of the recipient, i.e. malnutrition
Infected preservation fluid	Co-morbidity, e.g. diabetes, obesity, COPD, renal failure and dialysis
Amount of intra-operative blood transfusion	Colonisation with <i>S. aureus</i> or resistant organisms
Level and type of immunosuppression (e.g. anti-CD25)	Prolonged hospital stay and catheters before OLT
Additional immunosuppression for rejection	Acute liver failure
Indwelling catheters, deep lines	CMV-status and disease (risk for other infections)
Complications like primary non-function, hepatic artery thrombosis, necrosis, biliary strictures	Presence of hepatitis B or C virus or HIV
Prolonged ICU-stay, dialysis, prolonged ventilation	MELD score >30
Type of biliary drainage (Roux-en-Y, T-tube)	Recipient age
Repeat operations and re-transplantation	Previous immunosuppression (autoimmune hepatitis; re-transplantation)
Antibiotic regimen	Previous infection (esp. airway, urine tract)
Viral prophylaxis and monitoring	Immune status for viruses
Environment (other infected patients, building activity, hygienic measures)	Male recipient receiving male donor liver
	Hygienic measures
	Travelling
Genetic polymorphisms in innate immunity	Donor
Lectin pathway of complement activation in donor and donor/recipient mismatch (MBL2, ficolin2, MASP2)	Infection in donor
Toll-like receptors in the recipient	Prolonged ICU-stay
	Quality of the liver graft (e.g. marginal graft)
	Viral status

also a risk factor. Co-morbidity like cystic fibrosis or chronic bronchitis is known to increase the risk for pulmonary infection. Immunosuppression before OLT is a risk factor for infection and mortality, like – especially above the age of 50 years – in autoimmune hepatitis.²⁵ A prolonged (>1 week) pre-transplant hospital stay, long-term intravenous catheters, and ascites before transplantation were found to be associated with infection after OLT.²⁶ Obesity and diabetes appear to contribute to the risk of post-OLT wound infections.¹⁷ Gender plays a role in the susceptibility for many, but not all, infections and may be related to the influence of sex hormones and gender differences in innate immunity.²⁷ As mentioned below, we and others showed that a male recipient of a male liver is at higher risk for bacterial infections than the other combinations of donor and recipient sexes after OLT. If a recipient is colonized with MRSA or ESBL bacteria or if the recipient is infected with *C. difficile*, isolation measures are important to protect the other (transplant) patients. Environmental factors like building activity in the hospital (risk for *Aspergillus fumigatus*) or inadequate handwashing by personnel can also be risk factors for transferring infections to an OLT recipient. Treatment of *Staphylococcus aureus* colonization can decrease morbidity after OLT.^{28,29}

Hepatitis B

In the past hepatitis B virus (HBV) present at OLT almost universally led to recurrence after OLT, often leading to graft failure and recipient death. The long-term administration of anti-HBV immunoglobulins (HBIG) during and after OLT led to a substantial reduction of HBV recurrence, lamivudin further reduced this problem, and the combination allowed OLT to be performed with <5% recurrence.^{30,31} HBV-DNA level at OLT determines the risk of recurrence even with prophylactic treatment³². The addition of adefovir dipivoxil to lamivudin allowed late withdrawal of HBIG in many patients.^{33,34} More recently, entecavir and tenofovir further reduced HBV recurrence after OLT and also allowed treatment of lamivudin-resistant patients.^{35,36} Even entecavir monotherapy was able to prevent HBV recurrence.³⁷ Recipients of a donor liver with anti-HBV core protein positivity can develop HBV after OLT if they are not immune, therefore such recipients also need HBV prophylaxis.³⁸

Hepatitis C

Both in the USA and Europe hepatitis C virus (HCV)-related liver disease has become one of the leading OLT indications. If HCV RNA is present at the time of OLT it persists after OLT. In about 60% of patients this leads to more or less severe chronic active hepatitis. These patients can have an accelerated development of cirrhosis. As in HBV, patients transplanted with HCV can also develop cholestasis and rapid liver failure within weeks after OLT. Co-infection of HBV, HCV genotype 1, rejection therapy, recipient age over 49 years, higher donor age, a steatotic or otherwise marginal donor liver and no HCV therapy were risk factors for severe recurrence in different studies.³⁹ No clear differences in HCV recurrence existed between different induction immunosuppressive therapies.⁴⁰ Ideally HCV is treated before OLT, but often this is not tolerated. Addition of telaprevir or boceprevir improves the results of HCV

treatment in patients before OLT. Studies with combinations of HCV protease and polymerase inhibitors are underway, even without peg-interferon and ribavirin. HCV recurrence after OLT can be treated with peg-interferon and ribavirin with a sustained overall viral response (SVR) rate of 35%, which is lower than in non-transplant HCV patients. The interleukin-28B TT polymorphism is associated with more severe histological HCV recurrence after OLT.⁴¹ While the outcome of OLT for HCV in African American patients and other races is similar,⁴² an African American patient with HCV receiving a liver from a Caucasian donor also has a higher risk of severe HCV recurrence.⁴³ Poly-morphisms in Toll-like receptor 3 may influence the development of rejection after OLT, when HCV is present.⁴⁴ After OLT use of HCV protease inhibitors can lead to extreme elevations of levels of tacrolimus and ciclosporin, and studies on combination therapy with peg-interferon, ribavirin and an HCV protease inhibitor and regimes without interferon after OLT are awaited.⁴⁵

HIV

In the past patients with human immunodeficiency virus (HIV) were excluded from OLT. Nowadays, with the use of highly active anti-retroviral therapy (HAART) HIV replication can be suppressed, and if CD4 lymphocyte counts are normal and no resistance to HAART exists OLT is possible in some patients with end-stage liver disease in HIV. These patients are still more prone to infections after OLT than other recipients and both HAART resistance and drug interactions need a lot of attention. Patients with HIV and HBV can have an excellent outcome after OLT.⁴⁶ In a meta-analysis of liver transplant outcomes in HIV-infected patients those with HBV had a better outcome than those without HBV, while patients without detectable HIV-load at OLT did better than those with detectable HIV-load, while in this study presence of HCV was not a predictor of outcome.⁴⁷ However, most authors agree that after OLT especially in HIV-infected patients HCV recurrence can pose a severe problem, with a worse outcome after OLT in HCV-infected HIV-patients than in HIV-patients without HCV.⁴⁸

Herpes viruses

The donor and recipient immune status for viruses are important risk factors for viral infections. One of the most important viral infections after OLT is cytomegalovirus (CMV) infection: if a donor is IgG anti-CMV positive and the recipient is IgG anti-CMV negative a CMV primo-infection almost universally occurs in the recipient. Since, especially in the acute phase after OLT, this can lead to severe morbidity and even mortality, prophylaxis with valganciclovir is used for this donor positive/recipient negative (D+/R-) CMV-status combination. Most centers use three to six months of valganciclovir prophylaxis in this situation,⁴⁹ and if the primo-infection then becomes manifest it is milder and the recipient is out of the acute phase after OLT. In renal transplantation less CMV infection and disease occurs if six instead of three months of valganciclovir prophylaxis is given. In the first year after OLT frequent monitoring of CMV-DNA by PCR in blood is indicated. In case CMV-DNA becomes positive after prophylaxis in a D+/R- combination (val)ganciclovir is started preemptively. In general,

no prophylaxis with (val)ganciclovir is used in IgG anti-CMV positive recipients,⁵⁰ although some of these patients may require more intensified CMV-DNA monitoring or prophylaxis.

EBV primo-infection or reactivation in the recipient is possible and can lead to post-transplant lymphoproliferative disease (PTLD) in the OLT recipient. This can range from mononucleosis to frank non-Hodgkin lymphoma. Treatment includes cessation of all immunosuppression except prednisolone, and administration of anti-CD20 in case of a B-cell PTLD. It is advisable to monitor EBV-DNA in the first year post-OLT, and in case of a positive and rising EBV-DNA to at least decrease the immunosuppression. Often EBV-DNA becomes undetectable after decreasing the amount of immunosuppression. The donor/recipient status for EBV, the amount of activated natural killer cells and certain underlying autoimmune disorders were found to increase the risk for PTLD.⁵¹

The degree of immunosuppression and D/R status of IgG to other viruses like HSV 1,2, 6,7,8 and VZV determines the risk of developing disease from these viruses. Especially HSV 1 and 2 and VZV can be treated by (val)ganciclovir. Most centers do not use prophylaxis for these viruses, but they use early treatment if required.⁵²⁻⁵⁵

Donor factors

Currently infection of a donor leads to morbidity and mortality in approximately 1% of transplant recipients. Rapid nucleic acid testing for microbial infections in the donor might lead to higher acceptance rates of high-risk donors.⁵⁶ Especially with a longer stay of the donor in the hospital the risk to acquire a nosocomial infection increases. Bacterial infections in the donor are often treated with antibiotics in donor and recipient. Some unknown infections in the donor, like dengue or hepatitis E ⁵⁷, may endanger a transplant recipient. Insufficiently treated or undetected infections in the recipient more often than donor infections lead to sepsis after OLT.⁵⁸ The quality of the graft (e.g. steatosis, donor age) relates to graft function, ICU and hospital stay and infectious complications.

Genetic polymorphisms in the innate immune system

Since adaptive immunity is suppressed by the immunosuppressive medication, the recipient becomes dependent on the innate immune system. This includes pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) on microorganisms. The innate immune system can then kill the pathogen directly in a lymphocyte-independent manner or activate adaptive immunity. In several pathways of innate immunity single nucleotide polymorphisms (SNPs) are known. In some other categories of immunosuppressed patients, e.g. bone marrow recipients, some of these polymorphisms were associated with an increased occurrence or severity of infection. This led to the hypothesis that such SNPs might influence the risk and severity of infection after OLT.

Toll-like receptors

Lipopolysaccharide from gram-negative bacteria is mainly recognized by Toll-like receptor 4 (TLR4). However, in a cohort from the Mayo Clinic no significant associations were found between the TLR4 SNPs D299G and T399I and the risk and outcome of gram-negative infections after OLT.⁵⁹ Toll-like receptor 2 (TLR2) is the major receptor for gram-positive bacterial cell wall components like peptidoglycan and lipoteichoic acid. The common R753Q SNP in the TLR2 gene results in defective intra-cellular signaling and impaired cytokine secretion in response to peptidoglycan, lipopeptides, and other known ligands. The mutation has been suggested to increase to risk of bacterial and viral infections and was found to influence the risk for and outcome of cytomegalovirus and hepatitis C infection after OLT.^{60–62} The homozygous TLR2 Arg753Gln (R753Q) polymorphism impairs recognition of HCV core and NS3 proteins and was shown to be associated with allograft failure and mortality after OLT for chronic HCV.^{60,62} The R753Q polymorphism also paralyzes recognition by TLR2-mediated immune signaling in cells exposed to CMV glycoprotein B.⁶¹ Patients homozygous or heterozygous for the TLR2 R753Q SNP had a higher CMV load and more CMV disease than OLT recipients without this SNP.⁶³ Recently a PCR was developed to detect the N284I and the L412F SNPs in the TLR3 gene that also plays a role in the defense against viruses, and clinical studies in relation to TLR3 polymorphisms are awaited.⁶⁴

Lectin pathway of complement activation

The lectin pathway of complement activation is an evolutionary conserved defense against microorganisms.⁶⁵ It includes mannan-binding lectin (MBL), ficolin-2 (FCN2) and MBL-associated serine protease 2 (MASP2). These proteins are almost exclusively liver-derived and crucial effectors of the innate immune system in the defense against pathogens. Lectins are humoral PRRs that recognize carbohydrate-motifs on microorganisms and elicit innate immunity. These lectins cooperate with phagocytes and other humoral factors, including complement.^{66,67} Polymorphisms in the lectin pathway determine its functional activity and are quite common. Mannan-binding lectin (MBL) and ficolin-2 (FCN2) both can activate the MBL-associated serine protease 2 (MASP2) [68]. FCN2 has similarities in structure and function to MBL and its preferential binding target is N-acetyl-glucosamine, a constituent of bacterial peptidoglycans and a major component of their cell wall.^{69,70} Activated MASP activates the complement cascade and leads to opsonization of microorganisms for phagocytosis or leads to formation of a complement membrane-attack complex (Fig. 1), which leads to 'holes' in the bacterial wall resulting in death of the pathogen.^{71–73} It had been shown that SNPs in the exon1 region of the MBL gene (MBL2) interfere with the oligomerization of the protein and polymorphisms in the promoter regions alter the rate of synthesis of the protein, leading to changes in avidity and protein level of MBL respectively, resulting in MBL deficiency.^{74–76} Ficolin-2 (FCN2) SNPs in the carbohydrate-recognition domain encoding region are associated with decreased (FCN2-B) or increased (FCN2-C) ligand binding of ficolin-2 compared to wild-type ficolin-2 (FCN2-A).⁷⁷

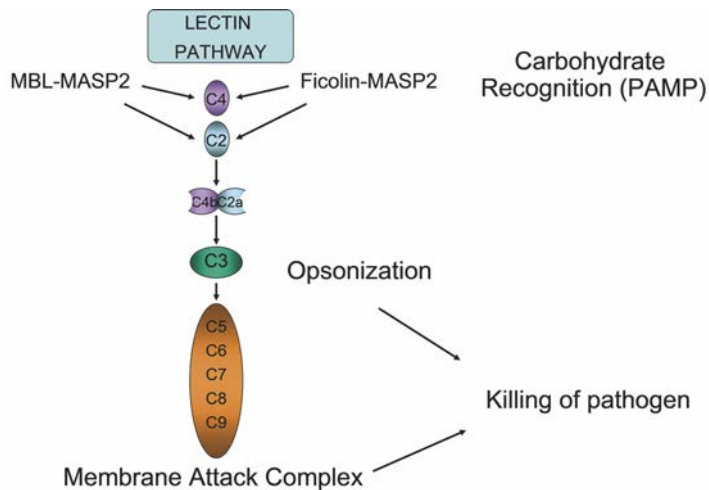


Figure 1. The lectin pathway is activated when either mannose-binding lectin (MBL) or ficolin-2 binds to carbohydrate structures or PAMPs which are present on a large number of pathogens. Upon binding, the associated serine protease MASP2 is responsible for activation of the complement cascade. This will lead to opsonization for phagocytosis and the formation of a membrane-attack complex, both resulting in the killing of the pathogen. Modified from: Dommett et al. Mannose-binding lectin in innate immunity: past, present and future. *Tissue Antigens* (2006) 68: 193–209.

MBL deficiency is associated with more and more severe infections in HIV infection, bone marrow transplantation, pancreatic and renal transplantation, but not in several other conditions, such as pneumococcal infections in randomly included patients, and it may confer protection against tuberculosis.^{78–80} However, MBL deficient patients receiving a pancreas–kidney transplantation had better outcomes because of some protection against ischemia-reperfusion injury and rejection.⁸¹ Two polymorphisms in the MASP2 gene lead to a functional defect in the protease.⁸² One SNP leads to the inability to activate complement,^{83,84} the other SNP is located in the control protein domain2 of MASP2, which is important in stabilizing the structure of the serine protease domain⁸⁵ and is essential for cleavage of complement C4.⁸⁶ Since MBL, ficolin-2 and MASP2 are almost exclusively produced by the liver their impact in OLT is of particular importance.⁸⁷ Our group recently showed that polymorphisms in the lectin pathway of complement activation are important risk factors for bacterial infection post-OLT. Transplantation of an MBL deficient donor liver into an MBL sufficient recipient results in rapid decrease in MBL blood levels, while the functionally important MBL2 SNP in the donor that resulted in low blood levels was also associated with bacterial infections after OLT.⁸⁸ This finding was then confirmed by others.^{89,90} Since the ficolin pathway may compensate for deficiency in the MBL pathway (both activate MASP), high-resolution melting assays were developed to include all known functional SNPs in the lectin pathway of complement activation, including MBL2, FCN2 and MASP2.⁹¹ Recipients receiving a donor liver with mutations in all three components, i.e. MBL2 (XA/O

or O/O), FCN2+6359T, and MASP2+371A, had a cumulative risk of bacterial infection of 75% as compared to only 18% with wild-type donor livers, an observation confirmed in a second cohort. In addition, a genetic (mis)match between donor and recipient conferred a two-fold higher infection risk for each separate gene. The more SNPs leading to a deficient phenotype (with low MBL level and low-binding ficolin levels in the recipient), the higher the risk for bacterial infection (Fig. 2). This was stepwise increase in the risk with the lectin pathway gene profile of the donor and the donor–recipient (mis)match profile independent from other risk factors gender and antibiotic schedule. In addition, patients with the indicated lectin pathway gene polymorphisms and infection had a six-fold higher mortality, of which 80% was infection-related.⁹²

The relationship between cytomegalovirus (CMV) and MBL in solid organ transplantation had been studied mainly in kidney transplantation,^{78,93,94} and only in a small number of patients after OLT.⁹⁵ We investigated the complete MBL–ficolin–MASP gene profile in relation to CMV infection after OLT. It became clear that polymorphisms in the lectin pathway of complement activation also increase the risk of CMV disease. Combined analysis of variant MBL2 (XA/O or O/O) and wild-type FCN2 (FCN2-A) polymorphisms in the donor liver showed an independently associated increased risk of CMV infection for either and both genotypes. This effect was especially strong in IgG anti-CMV donor negative/recipient positive (D-/R+) patients. Moreover, a genetic donor–recipient mismatch for MBL2 and FCN2 increased the CMV risk independently, also combined, and also particularly in CMV D-/R+ patients. For MBL the highest risk for CMV infection was in MBL sufficient recipients of an MBL deficient donor liver. In addition, a FCN2-C (high-affinity) donor liver reduced the chance for CMV infection in a FCN2-A recipient as opposed to the other FCNs genotype combinations.

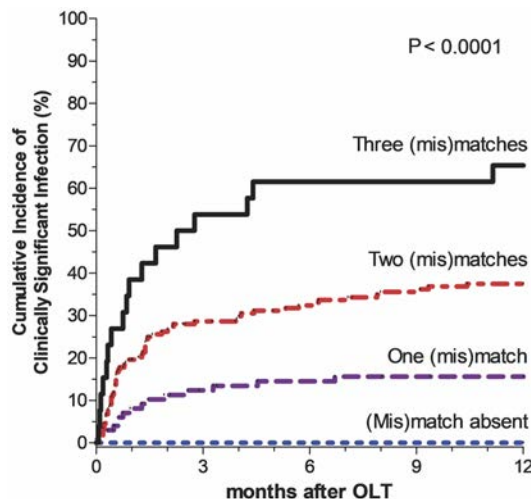


Figure 2. Cumulative incidence of clinically significant infection after orthotopic liver transplantation, according to donor lectin pathway intergenic haplotype. Ref. [92].

Again, there was a stepwise increase in CMV infection risk with the gene profile of the donor and the combined MBL2 and FCN2 donor-recipient mismatch profile, independent from donor-recipient CMV serostatus, also at increasing CMV (re)infection cut-off values of CMV positivity.⁹⁶ These data indicate that a link exists between several components of the lectin pathway of complement activation and the initial immune response to bacteria and CMV after OLT. The association of MBL2 SNPs with CMV and with bacterial infections was similar after OLT. However, with bacterial infections an association with FCN2-B and with MASP2 SNPs was found, while for CMV infection FCN2-C was important and no relation with MASP2 was found.⁹⁶ This illustrates differences in innate immune defense against bacterial and viral (CMV) pathogens after OLT.^{92,96} CMV regulates immune-modulatory genes, that also change innate immunity in favor of CMV survival, e.g. inhibition of NF- κ B leading to decreased cytotoxicity, and inhibition of dendritic cells and their functionality.⁹⁷⁻⁹⁹ This might also explain why CMV infection or reactivation increases the susceptibility to bacterial and pneumocystis infection. Complement activation is potentially detrimental to the host and is kept in place by inhibitors. CMV is able to upregulate the expression of host-encoded (surface) complement inhibitors¹⁰⁰ and counteracts complement activation by incorporation of host cell-derived complement regulatory proteins CD55 and CD59.¹⁰¹ As mentioned, TLR2 Arg753Gln gene polymorphism is associated with CMV replication after OLT⁶³. MBL is able to interact with TLR2 in the phagosome to initiate pro-inflammatory signaling¹⁰², and therefore SNPs in both may affect this signaling and changed immunity against CMV after OLT. A subsequent mechanism by which MBL could be involved in immunity against CMV is that the intracellular interaction of its isoform I-MBL with CMV glycoproteins may disrupt CMV virion assembly or formation, restricting CMV replication.¹⁰³

A possible clinical application of our findings could be to screen for recipient and donor MBL2, FCN2 and MASP2 risk alleles. Subsequently one could intensify the antibiotic strategy or antiviral prophylaxis and more closely monitor high-risk patients. It is unknown if supplementation of recombinant MBL or ficolin will decrease the infection risk post-OLT. It has been shown that recovery of opsonic activity lags behind recovery of MBL serum levels.¹⁰⁴ A study with recombinant MBL in OLT was terminated by the sponsor, as yet for unclear reasons.

It has become clear that certain innate genetic profiles of donor and recipient are important risk factors for infections after OLT.¹⁰⁵ Indeed, as shown, they seem more important than some clinical or environmental factors. Other disturbances in innate immunity may also play a role. This may include changes in – for instance – other Toll-like receptors, Nod-receptors, or RIG-1 like receptors.¹⁰⁶ Recognition of PAMPs by PRRs triggers a cascade of downstream signaling leading to an array of anti-microbial immune responses by cytokines, chemokines and type I interferons, and these responses may also be changed due to polymorphisms in some recipients.¹⁰⁶ Crosstalk between innate and adaptive immunity may also be affected. More investigation into this area is urgently needed. If the genetic make-up of donor and recipient is known this may lead to more personalized prophylaxis around OLT.

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Chapter 3

*Genotype-phenotype correlation of the lectin
pathway of complement activation in patients
before and after orthotopic liver transplantation*

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ABSTRACT

Background and aims

The lectin pathway of complement activation is initiated by pattern recognition receptors such as mannose-binding lectin (MBL) and ficolin-2 which form multimeric complexes with MBL-associated serine protease (MASP)-2. The binding of this complex to pathogen-associated molecular patterns on microbial surfaces, activates MASP-2 which initiates the proteolytic cascade of the complement system by cleaving C4 and C4b-bound C2. MBL, ficolin-2 and MASP-2 are predominately synthesized in the liver and each of their coding genes harbour functional polymorphisms which affect their expression and function. Orthotopic liver transplantation (OLT) is a unique “in vivo” human model to gain insight into the genotype-phenotype profile of MBL, ficolin-2 and MASP-2 in man.

Methods

We determined MBL, ficolin-2 and MASP2 concentrations and polymorphisms in their coding-genes in three study groups, i.e., healthy controls (n=170), recipients before (n=120) and after OLT (n=111). Subsequently the relationship between genotype and phenotype was assessed.

Results

MBL serum concentrations before and after OLT did not differ, in contrast to ficolin-2 and MASP-2 where serum concentrations before OLT were significantly lower compared to concentrations after OLT. Patients with Child-Pugh B and C cirrhosis did show significantly lower ficolin-2 and MASP-2 concentrations compared to patients with Child-Pugh A cirrhosis; MBL showed a non-significant trend towards lower concentrations. The multilocus MBL genotype A/A and YA/O versus XA/O and O/O, *FCN2* SNP at position -986A>G and *MASP2* SNP at position +371A>C showed strong a genotype-phenotype relationship in each of the three study groups, except for *FCN2* SNP position -986A>G in recipients before OLT. After OLT phenotypes were only associated with the genotype of the donor liver and not with that of the recipient. Patients receiving a liver with an MBL genotype similar to their own had similar MBL levels before and after OLT. MBL genotype mismatches between liver donor and recipient resulted in a significant MBL serum concentration change towards levels compatible with the MBL genotype of the donor liver. In case of ficolin-2 and MASP-2 matching the genotype of patient and donor liver showed a significant change in serum levels towards healthy controls levels.

Conclusions

After OLT the recipient assumes the MBL phenotype of the donor liver and to a lesser extent for ficolin-2 than for MASP-2 the serum levels correlate with the genotype of the liver donor.

INTRODUCTION

The lectin pathway of complement activation is an important component of the innate immune defence. Initiation occurs when germline-encoded humoral pattern recognition molecules: Mannose binding lectin (MBL)¹, ficolins (M-, L-, and H-ficolin; also called ficolin-1, -2, and -3)² and collectin 11 (CL11 or CL-K1)³ in complex with MBL-associated serine proteases (MASPs: MASP-1, MASP-2 and MASP-3)⁴ recognize and bind to evolutionarily conserved structures on pathogens (pathogen-associated molecular patterns). Activation of the proteolytic cascade of the complement system culminates in the destruction and elimination of pathogens via opsonization or direct cell lysis.

MBL and ficolin-2 are structurally and functionally homologous and as well as MASP-2 primarily expressed in the liver. Common single nucleotide polymorphisms (SNPs) in the encoding genes are associated with altered function and expression of these innate pattern recognition molecules⁵⁻⁹ and may impair pathogen recognition, leading to an increased risk of various bacterial¹⁰ and viral infections.

Orthotopic liver transplantation (OLT) is a unique “in vivo” model to study the contribution of MBL-Ficolin-MASP genotypes to protein expression and to evaluate the balance between hepatic (donor) and extrahepatic (recipient) production of these factors.

We assessed the relationship between *MBL2*, *FCN2*, and *MASP2* gene polymorphisms from recipient and donor with MBL, ficolin-2 and MASP-2 serum levels before and after OLT, which in combination is likely to contribute to infection related complications in these patients.

METHODS

Study population

All patients who received OLT at the Leiden University Medical Centre in The Netherlands up to 2005 were taken into consideration for this study. Genomic DNA was extracted routinely from peripheral blood and/or tissue samples, when possible, without given preference to any explicit clinical variables. For this study, 202 patients were identified who underwent OLT between 1992 and 2005, of whom 148 patients whose DNA was available from both donor and recipient. From these patients, 143 were finally included who had at least 30 days of follow-up after liver transplantation, excluding perioperative morbidity and mortality. We were able to retrieve the serum samples of 120 of these patients at either one or two time points before OLT and of 111 patients at time points one, three and/or twelve months after OLT. Data were retrieved from the records of all these patients. The severity of the underlying liver disease was assessed according to the Model of End-stage Liver Disease (MELD) and the Child-Pugh classification. In addition, DNA was extracted from peripheral blood leucocytes of 170 healthy volunteers (37% male, median age 33 years (range 18–72 years), >95% Caucasian) as described before.¹¹ The study was performed on prospectively collected data and plasma samples obtained with informed consent from the patients according to the guidelines of the Medical Ethics Committee, in compliance with the Helsinki Declaration.

Genotyping

Polymorphisms in the *MBL2*, *FCN2*, and *MASP2* genes were determined with the use of high resolution DNA melting assays (HRMA) with the oligonucleotide primers as indicated in supplemental table 1.¹² Briefly, HRMA of PCR products, amplified in the presence of a saturating double-stranded DNA dye (LCGreenPlus, Idaho Technology) and a 3'-blocked probe, identifies both heterozygous and homozygous sequence variants by a change in melting temperature curves, verified by DNA sequenced controls. Genotypic MBL studies have shown that different SNPs in the promoter and exon 1 of the MBL gene (B, C, and D collectively called O, while the wild-type allele for each position is called A) are in strong linkage disequilibrium. The association between MBL multilocus genotype and phenotype is very strong: sufficient functional MBL concentration is associated with YA/YA, YA/XA, XA/XA, and YA/O genotypes, whereas relatively deficient functional MBL concentration is associated with genotypes XA/O and O/O.^{5, 13}

Serologic Analysis

Measurement of MBL, Ficolin-2 and Mannan-binding lectin-associated serine protease-2 concentration by ELISA

MBL concentrations were assessed by sandwich ELISA as described previously.¹⁴ In brief, plates were coated with monoclonal antibody (mAb) 3E7 (anti-MBL mouse IgG1, kindly provided by T. Fujita, Fukushima Medical University School of Medicine, Fukushima, Japan) at a concentration of 5 µg/ml. Serum samples were incubated for 1 h at 37°C, followed by detection with Dig-conjugated mAb 3E7. A calibration line was produced using pooled serum from healthy donors with a known concentration of MBL.

Concentrations of ficolin-2 were assessed using a similar protocol. Plates were coated with mAb GN5 (mouse mAb anti-human Ficolin-2, kindly provided by T. Fujita, Fukushima Medical University School of Medicine, Fukushima, Japan) at a concentration of 5 µg/ml. Samples were incubated for 1 h at 37°C, followed by incubation with Dig-conjugated mAb GN5. Detection of binding of antibodies was as described in the ELISA protocol. A calibration line was produced using pooled human serum from healthy donors with a known concentration of ficolin-2 (kindly provided by Dr D.C. Kilpatrick, Scottish National Blood Transfusion Service Edinburgh, UK), as described previously.¹⁵

Polyclonal antibody directed against a recombinant CCP1-CCP2-SP fragment of human MASP-2 (kindly provided by Dr. P. Gal, Institute of Enzymology, Budapest, Hungary) was produced in rabbits. ELISA plates (high binding microplates from Greiner Bio-One, Frickenhausen, Germany) were coated with 100 µl 2.5 µg/ml antibodies in 0.1 M NaHCO₃, pH 9.6, and incubated overnight at 4°C. Plates were washed three times after each step with 100 µl/well wash buffer [phosphate-buffered saline (PBS), 0.05% Tween-20 from Sigma-Aldrich, St. Louis, MO, USA]. In each case incubation was sustained for 1 h at 37°C. Wells were blocked with 1% bovine serum albumin (BSA) (Fluka & Riedel, Buchs, Switzerland) in PBS. Human serum samples were diluted 1/10 (v/v) in veronal buffer saline containing 0.05% Tween-20,

0.1% gelatin (Reanal Ltd, Budapest, Hungary), 1 M NaCl and 10 mM ethylenediamine tetraacetic acid, pH 7.5. The third layer of our sandwich ELISA was digoxigenin-labelled rabbit IgG anti-human MASP-2 diluted 1:500 (v/v) in detection buffer (PBS, 1% BSA, 0.05% Tween-20). One hundred μ l horseradish peroxidase (HRP)-conjugated sheep anti-digoxigenin antibody (Fab fragments from Boehringer Mannheim, Mannheim, Germany) diluted 1:4000 (v/v) in detection buffer was used as the second antibody. Development was carried out with 2,2'-azino-bis 3-ethylbenzthiazoline-6-sulphonic acid (Sigma-Aldrich) at a concentration of 2.5 mg/ml in 0.1 M citrate/ Na_2HPO_4 buffer, pH 4.2, in the presence of 0.01% H_2O_2 . Absorbance at 415 nm was recorded after 15–30 min incubation at room temperature. The ELISA was standardized using negative controls and dilutions of highly purified recombinant human MASP-2 CCP1-CCP2-SP fragment.¹⁶

Statistical analysis

Data are expressed as means $\pm$ SEM in case of normally distributed data, unless otherwise mentioned. Differences in mean serum levels of MBL, ficolin-2 and MASP-2 between groups were compared using the Mann-Whitney U test or One-way ANOVA followed by Holm-Sidak post-hoc test where appropriate. The mean serum levels before and after OLT matched for each patient were compared using parametric paired Student's *t*-tests. $P < 0.05$ was considered significant.

Data analysis was performed with SPSS Statistical Software Package (version 23.0, SPSS Inc., Chicago, IL) and Prism 7 for Windows (GraphPad Software, San Diego, CA, USA) statistical software products.

RESULTS

The genotype-phenotype correlation of MBL, ficolin-2 and MASP-2 were studied in healthy controls ($n=170$), liver transplant recipients before ($n=120$) and after OLT ($n=111$).

MBL2-MBL genotype-phenotype association

The mean MBL levels in blood for the three groups (healthy controls, transplant recipients before and after OLT) were: 557 ng/ml (median 260; range 17–2620), 921 ng/ml (median 930; range 26–2329) and 856 ng/ml (median 729; range 29–2398) respectively (figure 1A). There was no significant change in the MBL level of the patients after OLT compared to pre-OLT on the population level. We observed a non-significant trend to a lower MBL level in relation to a higher Child-Pugh classification in patients before OLT (figure 1B).

The mean concentration of MBL in relation to the multilocus MBL genotype A/A and YA/O versus XA/O and O/O was consistently and statistically significantly lower in the latter genotype group in each of the three study groups (figure 1C, 1D and 1E). As expected the multilocus genotype of the recipient showed no significant relation with MBL levels after transplantation (supplemental figure 1A), due to the donor liver origin of the MBL production.

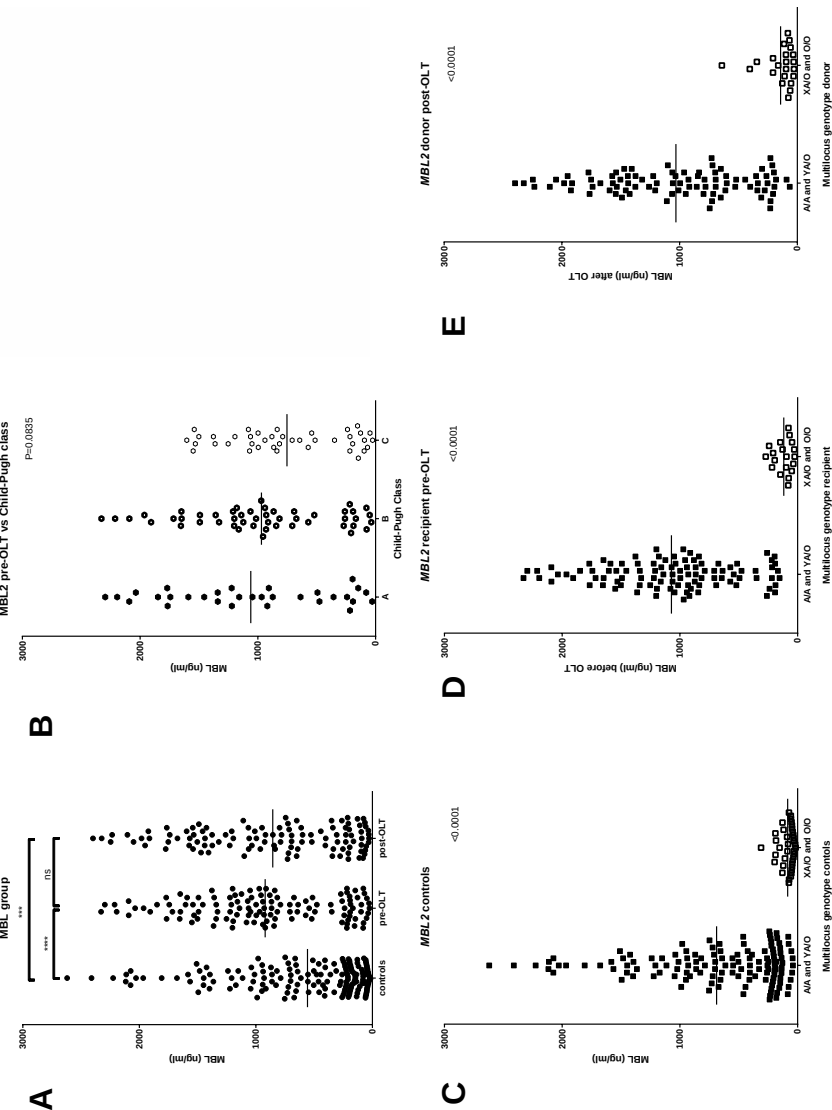


Figure 1. MBL concentrations and multilocus MBL geno-phenotype correlation MBL concentration in A. healthy controls, recipients before and after OLT B. recipients before OLT categorized as Child- Pugh class A, B or C. multilocus MBL genotype-phenotype correlation C. in healthy controls D. recipient before OLT stratified according the multilocus MBL genotype of the recipient E. recipient after OLT stratified according the multilocus MBL genotype of the donor liver Plots show the mean. $P < 0.05$ was considered significant.

FCN2-ficolin-2 genotype-phenotype association

The mean concentration of circulating ficolin-2 for the three study groups (healthy controls, transplant recipients before and after OLT) were: 3598 ng/ml (median 3684; range 562-6810), 1306 ng/ml (median 1126; range 6-4815) and 3549 ng/ml (median 3513; range 283-6570) respectively (figure 2A). There was a significantly higher level of ficolin-2 in recipients after OLT as compared to their pre-OLT level ($P<0.0001$). Furthermore, Child-Pugh B and C cirrhotic patients before OLT showed significantly lower ficolin-2 levels compared to patients with Child-Pugh A cirrhosis (figure 2B).

Also a strong *FCN2*-ficolin-2 genotype-phenotype association was observed with the SNP at position -986A>G in healthy controls, transplant recipient before OLT and after OLT, the latter with the genotype of the donor liver with lower ficolin-2 serum levels in AG and GG as compared to AA genotype (figure 2C, 2D and 2E). The genotype of the recipient showed no significant relation with ficolin-2 levels after OLT (supplemental figure 1B). The other *FCN2* SNPs showed no correlation with the ficolin-2 levels in either study group (supplemental figure 2A-E).

MASP2-MASP-2 genotype-phenotype association

The mean MASP-2 concentration for the three study groups (healthy controls, recipient before and after OLT) were: 1165 ng/ml (median 1077; range 80-2939), 788 ng/ml (median 663; range 102-2358) and 1093 ng/ml (median 1030; range 236-2408) (figure 3A) respectively. There was one (repeatedly) extreme outlier of 10839 ng/ml in the recipient group before OLT which was left out in further analysis. Similar to ficolin-2 the MASP-2 level in the patients after OLT were significantly higher as compared to their pre-OLT levels ($P<0.0001$). Again Child-Pugh B and C cirrhotic patients before OLT showed significantly lower levels compared to patients with Child-Pugh A cirrhosis (figure 3B).

The strongest *MASP2*-MASP-2 genotype-phenotype association was observed at SNP +371A>C in healthy controls, recipients before and after OLT, the latter with the liver donor genotype (figure 3C, 3D and 3E), with higher levels in AC and CC than in AA genotype. The genotype of the recipient and MASP-2 levels after transplantation showed no correlation (supplemental figure 1C).

There also was a significant genotype-phenotype relation with SNP +120A>G in each of the three study groups, but there were only a few heterozygotes (AG) in each group (supplemental figure 2F) and only one single homozygote in total. The G allele was associated with a lower expression.

Serum concentration in orthotopic liver transplant recipients stratified according to the lectin genotype of the donor liver

Patients receiving a liver with a multilocus MBL genotype identical to their own showed highly similar MBL levels before and after OLT. In contrast, MBL genotype mismatches between liver donor and recipient resulted in a significant serum concentration change that was compatible to the MBL genotype of the donor liver (figure 4A).

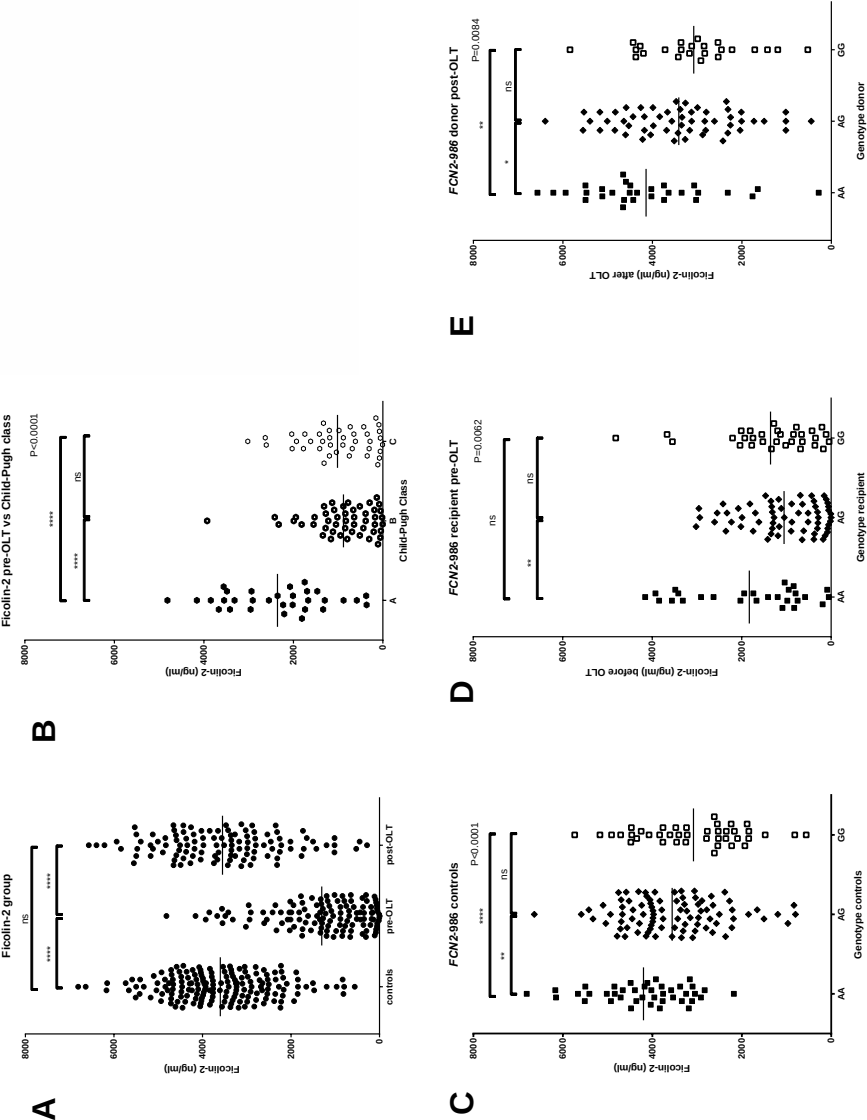


Figure 2. Ficolin-2 concentrations and *FCN2*-986 genotype-phenotype correlation Ficolin-2 concentrations in A. healthy controls, recipients before and after OLT B. recipients before OLT categorized as Child-Pugh class A, B or C *FCN2*-986 genotype-phenotype correlation in C. healthy controls D. recipient before OLT according to the genotype of the recipient E. recipient after OLT according to the genotype of the donor Plots show the mean. $P < 0.05$ was considered significant.

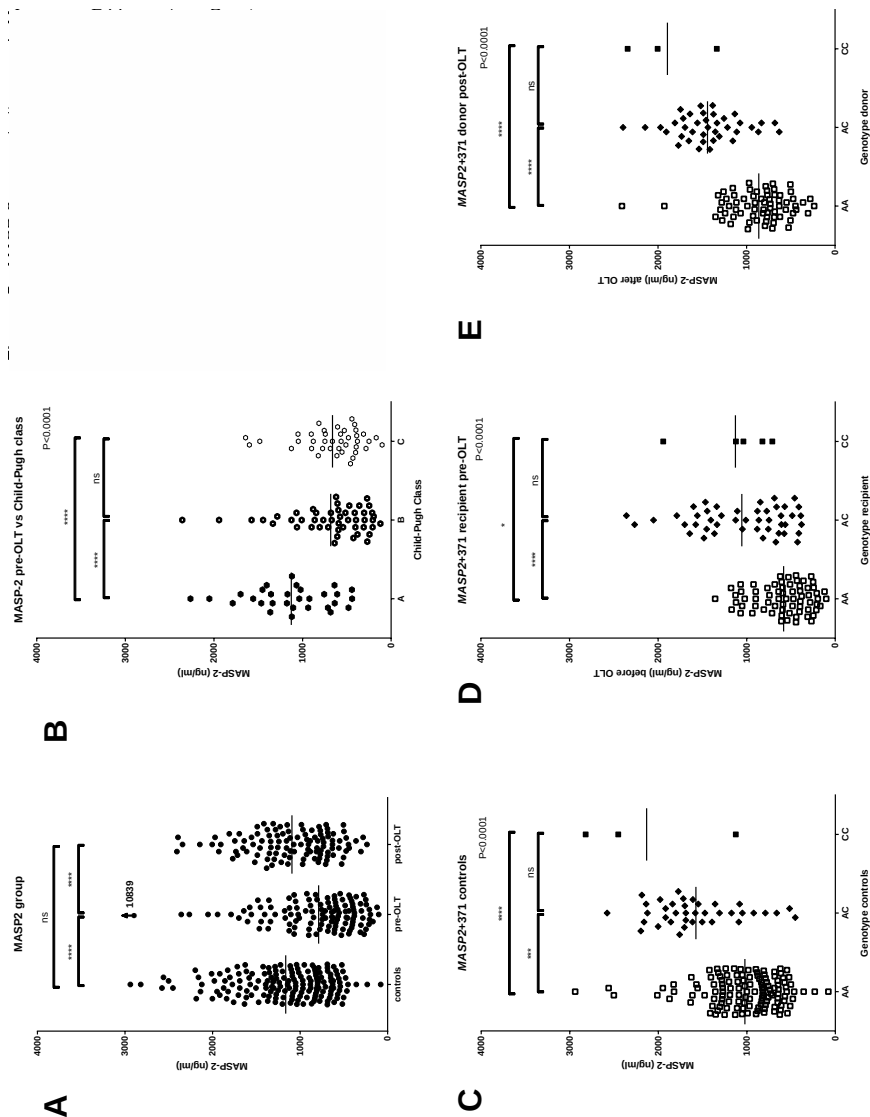


Figure 3. MASP-2 concentrations and MASP2+371 genotype-phenotype correlation MASP-2 concentrations in A. healthy controls, recipients before and after OLT B. recipients before OLT categorized as Child-Pugh class A, B or C MASP2+371 genotype-phenotype correlation in C. healthy controls D. recipient before OLT according to the genotype of the recipient E. recipient after OLT according to the genotype of the donor Plots show the mean. $P < 0.05$ was considered significant.

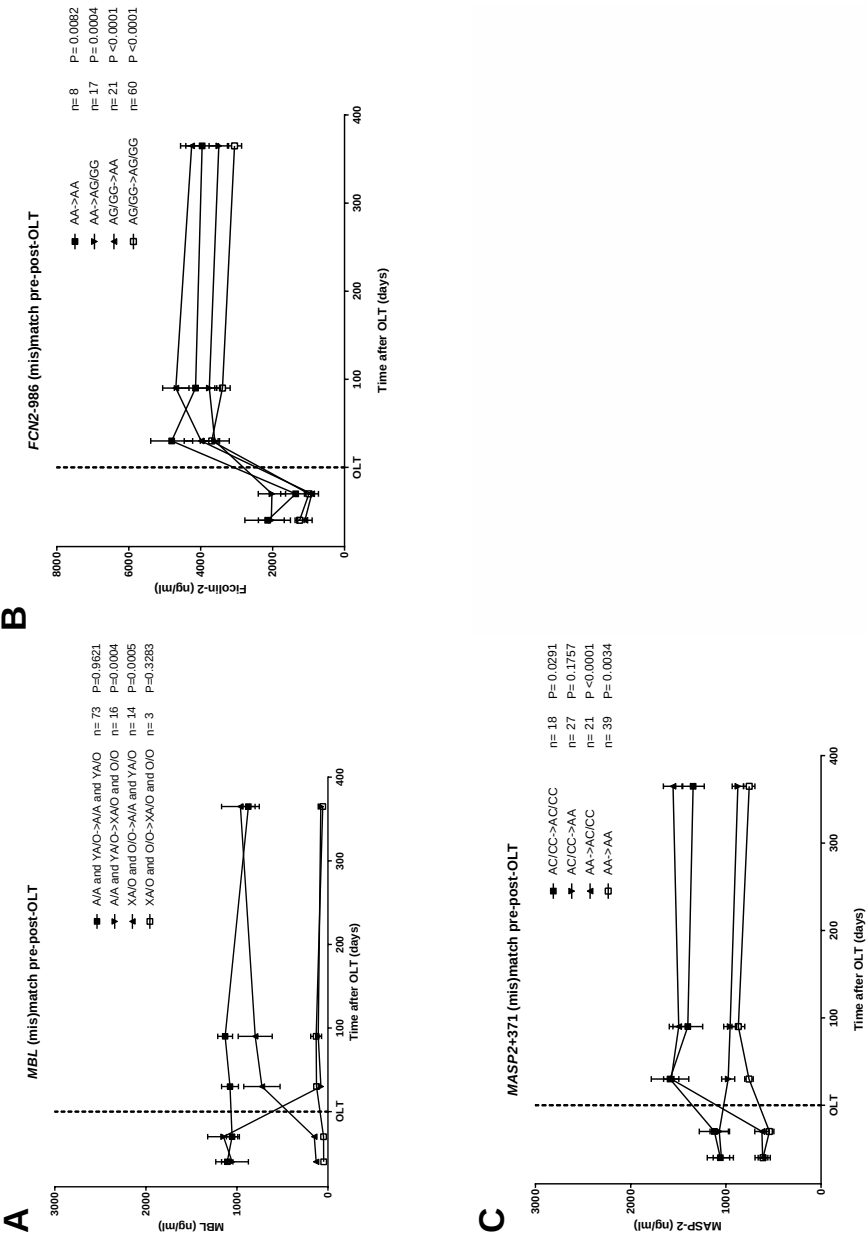


Figure 4. Serum concentrations in liver transplant recipients stratified according to the polymorphism genotype of the liver pre-transplantation and post-transplantation. Paired t-test was used compare serum concentrations before and after transplantation. Error bars show the standard error of the mean. A. BL wildtype [A/A or YAO/O]; MBL-variant [XAO/O or O/O]. B. FCN2 wildtype [AG/GG]. C. MASP2 wildtype [AC/CC]; MASP2-variant [AA].

FCN2 polymorphism -986A>G showed a consistent and significant genotype-phenotype serum association in all three study groups. Heterozygous (AG) and homozygous variant (GG) genotypes within each of these three groups showed no significant difference in serum levels and were therefore clustered in a combined variant genotype (AG/GG) for stratification. Stratification on basis of the genotype of patient and donor liver showed a significant recovering change in ficolin-2 serum level towards healthy control levels, both in patients receiving a liver with a *FCN2* genotype similar to their own as well as in case of genotype mismatch (figure 4B).

A similar approach for *MASP2*+371A>C was followed. There was no significant difference in MASP-2 serum level in the patients before and after OLT amongst *MASP2*+371A>C heterozygotes (AC) and variants (CC) so they were clustered in a combined variant genotype (AC/CC). There was a distinct *MASP2*-MASP-2 genotype-phenotype association before and after OLT, however all patients showed the expected significant recovery of MASP-2 levels after OLT irrespective of the (mis)match of the *MASP2* genotype, except for the AC/CC recipients receiving an AA donor liver whose MASP-2 serum levels did not change (figure 4C).

DISCUSSION

Previous studies suggest that MBL¹⁰, ficolin-2¹⁷ and MASP-2¹⁸ are mainly produced by the liver.¹⁹ Their genes harbour functional polymorphic sites that regulate both expression as well as function. We therefore assumed that orthotopic liver transplantation (OLT) would provide a unique “in vivo” model to study the MBL-Ficolin-MASP genotype-phenotype association and the balance between hepatic (donor) and extrahepatic (recipient) production of these factors.

In the first place, the present study confirms and extends our previous observation that the *MBL2* genotype of the donor liver determines the serum MBL concentration after transplantation.¹⁰ Patients receiving a liver with an MBL genotype equivalent to their own showed similar MBL serum concentrations before and after OLT. However, MBL genotype mismatches between liver donor and recipient resulted in an impressive post-OLT serum concentration change in the recipients that was compatible with the MBL genotype of the donor liver.

Although the present study clearly indicates that MBL is mainly produced by the liver, one might expect MBL levels in patients with liver disease to decrease with advancing hepatic failure as estimated by the Child-Pugh classification. In our study though, we observed that MBL levels in recipients before OLT are almost similar, even slightly higher, as compared to MBL levels in recipients after OLT. This observation suggests that there is either considerable hepatic reserve -and thus MBL quantities are maintained even during relative hepatic decompensation-, or that there is relevant extra-hepatic contribution to serum MBL anyway.

There are two studies that support the assumption of a substantial hepatic reserve in patients with advanced liver cirrhosis, which -in conformity with our study- also demonstrate

increased MBL levels in these patients as compared to healthy individuals.²⁰⁻²² However, the exact reason for the increased MBL production is unclear. It may reflect the on-going systemic inflammation seen in patients as cirrhosis progresses, a process that in coexistence with cirrhosis-induced immunodeficiency is referred to as cirrhosis-associated immune dysfunction syndrome. A systemic inflammation is most likely caused by the recurring release of endogenous intracellular damage-associated molecular patterns from necrotic or apoptotic liver cells and pathogen-associated molecular patterns released from the leaky gut, resulting in activated circulating immune cells and increased serum levels of pro-inflammatory cytokines.²³

Several studies suggest extra-hepatic sources of MBL production. Mullighan et al. performed a retrospective study concerning patients undergoing allogeneic haemopoietic stem cell transplantation and found associations between the *MBL2* genotype of the donor as well as the recipient and the risk of infection post-transplant.²⁴ However, a study by Kilpatrick et al. concerning two cases of allogeneic haematopoietic stem cell transplantation from MBL 'sufficient' donors to MBL 'deficient' recipients did not find an increase in recipient MBL levels post-transplant.²⁵ In addition, Chaudhry et al. showed that MBL deficiency in 98 recipients was not corrected by hematopoietic cell transplantation using a donor with normal MBL production.²⁶ Data from experimental studies performed *in vitro*²⁷ and in rodents²⁸⁻³⁰ demonstrate expression of MBL in the small intestine, kidney, as well as in lymphoid and myeloid cells. However, our data demonstrate that recipients of liver transplants from a donor with an MBL-variant genotype show a strongly diminished concentration of serum MBL (average 88 ng/ml [range 77-100] for A/A recipients of O/O livers) indicating that extrahepatic production of serum MBL takes place only to a very limited extent (figure 5).

Although evidence is lacking, there might also be a possibility that increased MBL levels are the result of a lower turnover rate in patients with liver cirrhosis, as is the case with immunoglobulins.³¹

Functional MBL circulates as higher order multimers (tetramers, pentamers and hexamers) of the structural homotrimeric unit of MBL peptides (subunits) that self-associate into a collagen-like triple helix. Three missense mutations in the gene encoding MBL disrupt the collagen-like helical structure of the MBL peptide and interfere with oligomerization of MBL peptides or assembly of MBL peptide triplets into multimers.³²⁻³³ The ELISA assay we used for detection has a high affinity for the high molecular weight multimeric circulating MBL complexes, whereas the lower molecular weight variant are less well recognized.¹⁴ So the interindividual differences in MBL levels found can be largely explained by polymorphisms of the *MBL2* gene. As a consequence the variation of MBL levels within individuals are relatively small.

The mean level of MBL in the control group is remarkably low as compared to the levels we found in recipients before and after OLT, but also compared to levels in healthy controls described by Berger et al., who used the same antibody and protocol.³⁴ The main difference between the control study group and the two patient study groups is the use of heparinized plasma in case of the control group instead of serum. Because of this dissimilarity we

selected ten patients, from which six had a sufficient-coding genotype and four a deficient-coding genotype, and measured the MBL concentration of each patient in both serum and heparinized plasma samples which were drawn at the same time point and found no significant difference in concentration between serum and plasma (Supplemental figure 3). We cannot fully exclude degradation due to multiple freeze-thaw cycles, but according to a study performed by Minchinton et al. MBL levels in plasma and serum did not significantly change after taken through three freeze-thaw cycles on subsequent days.³⁵ Furthermore, the same heparinized samples of the control group were used to measure ficolin-2 and MASP-2 serum concentrations, in which no significant difference between controls and recipients after OLT was found (aside from the difference we observed in recipients before OLT, most likely caused by cirrhosis).

The median concentration of ficolin-2 has generally been reported to be between 3000 and 4000 ng/ml³⁶ and most normal values fall within the range 1000 to 6000 ng/ml.³⁷ The ficolin-2 levels we found in both healthy controls and recipients after OLT were in good agreement with these findings. The ficolin-2 levels before OLT were significantly lower compared to levels in healthy controls (and recipients after OLT), apparently caused by the underlying liver disease, which corresponds with findings in two other studies.^{22, 38}

The *FCN2*-ficolin-2 genotype-phenotype correlation was previously described by Hummelshoj et al⁷ in 157 unrelated adult Caucasians. In our 170 healthy controls and recipients before OLT we did find a similar association with ficolin-2 levels and the SNP at position -986A>G (also at -602G>A, and -557A>G in the promoter and +6424G>T in exon 8, but not at position -4A>G). Ficolin-2 levels after OLT only showed a genotype-phenotype

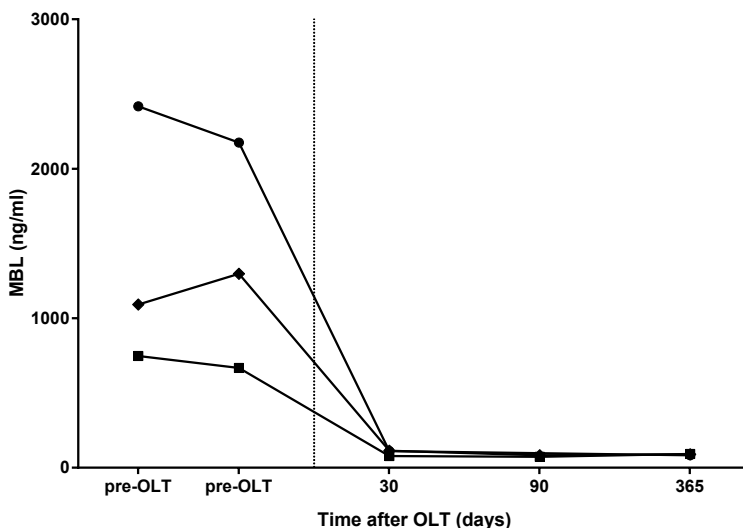


Figure 5. Serum MBL concentration before and after OLT in A/A recipients of O/O donor livers. Each line represents one individual.

relation with the genotype of the donor liver, and not with that of the recipient, similarly to data obtained for MBL. Put together with the reduced levels in patient with a more severe degree of cirrhosis this observation indicates the substantial hepatic involvement in the production of ficolin-2.

Stratification for the *FCN2* genotype of the recipient and liver donor did not reveal the distinct mismatch-related change in serum level we observed with MBL. Apparently, the consequence of cirrhosis of the liver is of greater influence than the effect of its *FCN2* -986A>G genotype on ficolin-2 levels.

As for the *MASP2*-*MASP2* genotype-phenotype association, we could reproduce the gene-dose dependent relation between the SNP at position +371A>C and corresponding *MASP2* levels in healthy controls, recipients before OLT and the donor liver after OLT, but not with the recipients genotype after OLT. Despite the distinct *MASP2*-*MASP2* genotype-phenotype association before and after OLT, stratification by pairing recipient and donor *MASP2* genotype did not reveal the diametrical change in serum concentration compatible with the genotype of the liver in case of a genotype mismatch, as was noticed with MBL. The influence of a healthy donor liver on synthesis of *MASP2* was larger than the genotypic effect of the SNP.

After OLT the genotype of the donor liver correlates with the ficolin-2 and *MASP2* levels in the recipient in a similar fashion as in the healthy controls, which suggests the liver as the main source of *MASP2*. Additional indirect evidence for liver origin of these proteins is that serum levels of both ficolin-2 serum and *MASP2* before transplantation did show a significant decrease in Child-Pugh B and C cirrhotic patients compared to controls. We did not find a genotypic association with the significant decrease of serum levels in Child-Pugh B and C cirrhotic patients (not shown), again indicating a disease related effect on the impaired liver production. The influence of the functional polymorphisms regulating expression of ficolin-2 and *MASP2* thus seems to be surpassed by the impaired liver function in cirrhosis.

As mentioned earlier, MBL serum levels measured with the specific assay used fairly well correlate with MBL complex activity,³⁹ due to the functional implications of the mutations on oligomer formation in conjunction with the specific technical properties of the assay used. However, this does not apply for ficolin-2 levels, since the actual ficolin-2 serum concentration within each genotype does not influence the ficolin-2 binding profile. The binding affinity of ficolin-2 towards N-acetyl glucosamine, a pathogen-associated molecular pattern specifically recognized by ficolin-2, is influenced by polymorphisms in exon 8 of the *FCN2* gene.⁷ In addition, ficolin-2 insufficiency cannot be reliably established by a single serum protein measurement because of changes in status from 'normal' to 'insufficient' or vice versa during periods of active disease.⁴⁰ In addition, quantitative serological data do not fully reflect the activity of the lectin pathway, especially when all the different recognition molecules that can activate the lectin pathway are taken into account. Standardized functional complement activation assays are available only for a part of the molecules involved. Therefore, identification of functional genetic variants of the lectin pathway of complement activation which affect an individual's phenotype is still more feasible to use

for exploring associations with clinical associations than for exactly measuring functional activity of the lectin pathway components.

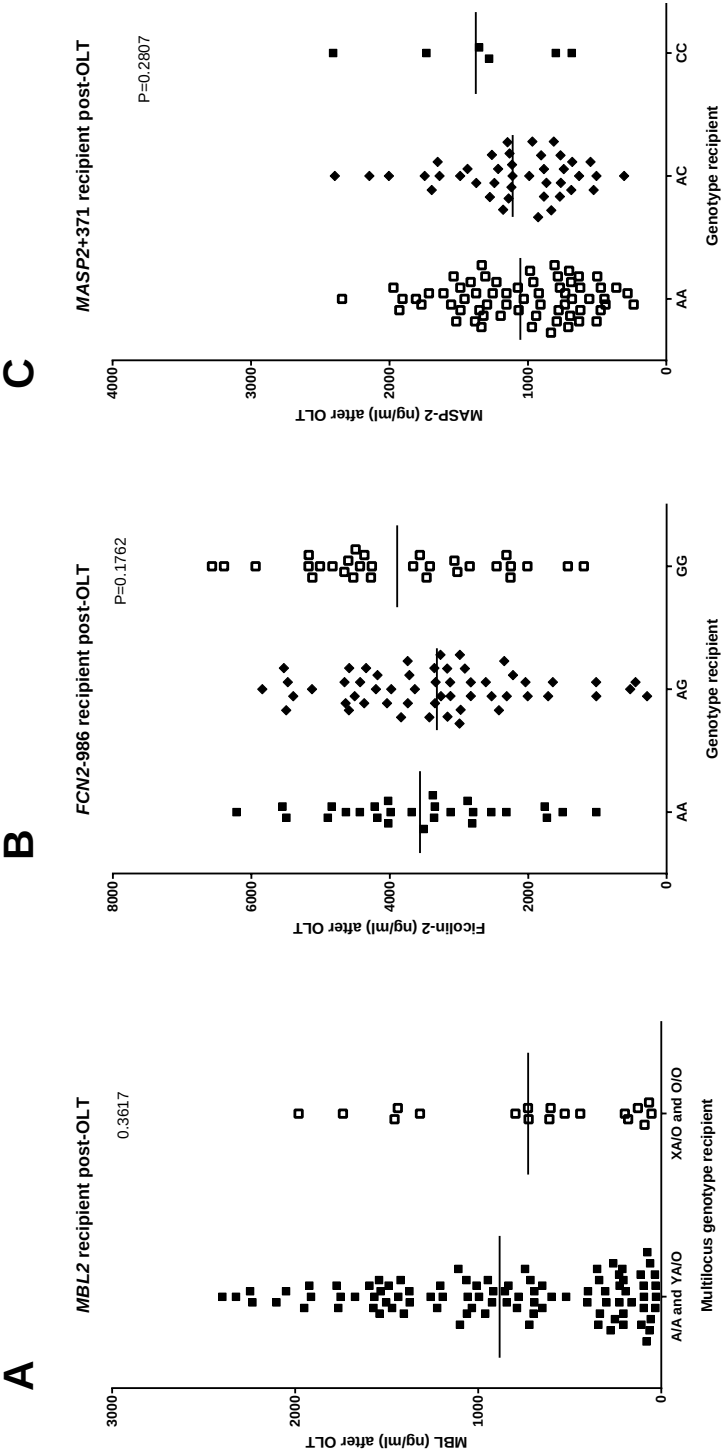
In conclusion, we confirm previous observations that the *MBL2* genotype of the donor liver determines the serum MBL concentration after transplantation. For both ficolin-2 and MASP-2, the lower pre-OLT levels in the recipient, due to impaired production related to liver failure, recover after OLT in a donor-genotype-dependent manner.

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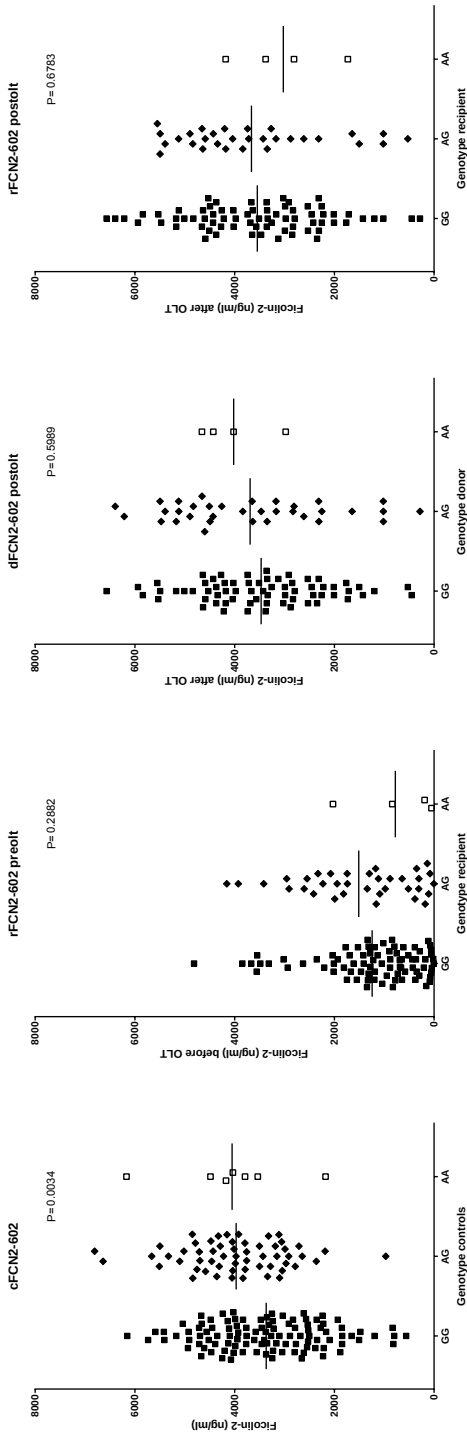
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SUPPORTING DATA



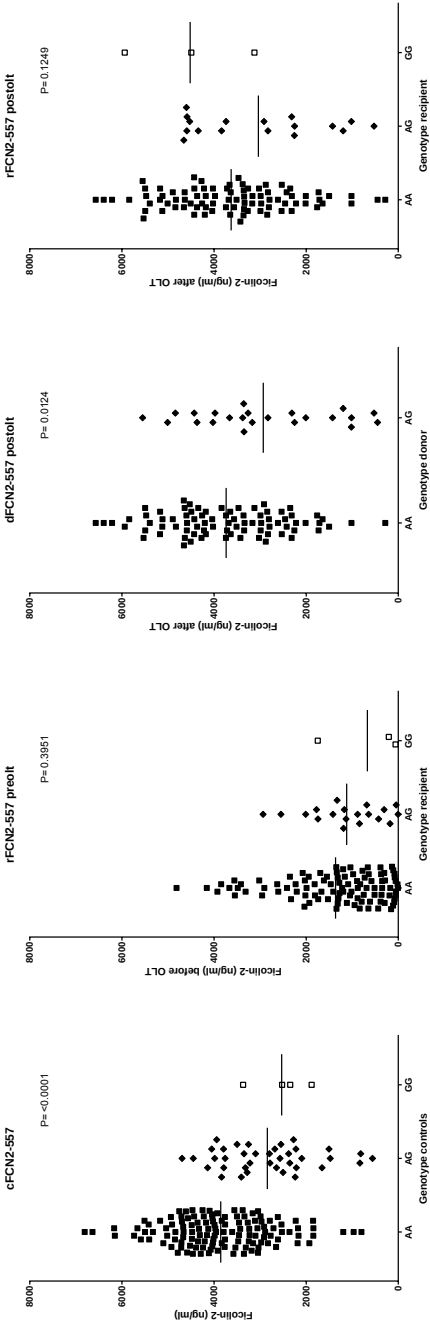
Supplemental figure 1. Genotype-phenotype association between the genotype of the recipient and serum concentration after OLT. (A) MBL-MBL, (B) FCN2-ficolin-2 and (C) MASP2-MASP-2. Lines indicate mean. $P < 0.05$ was considered significant.

A

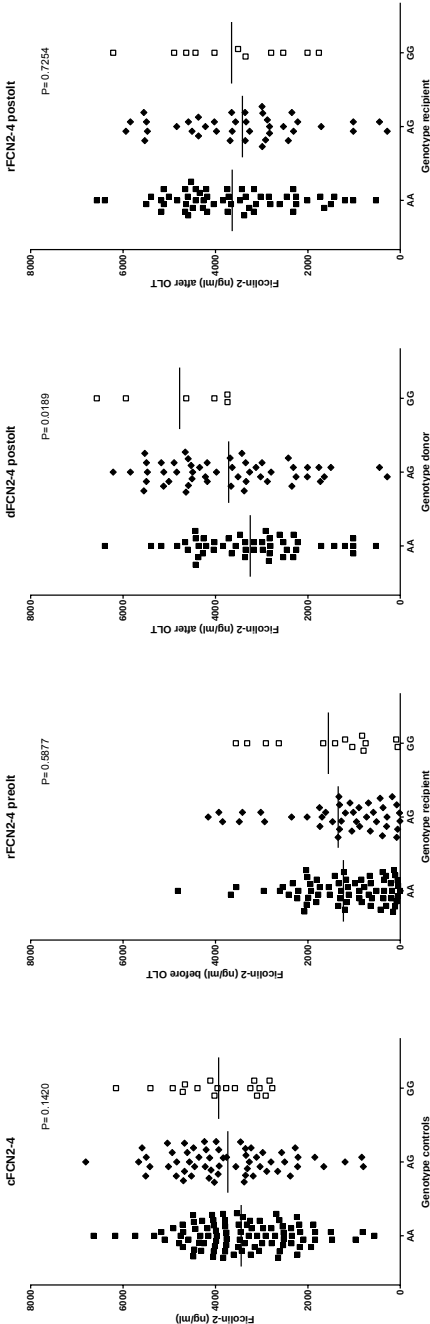


Supplemental figure 2. Scatter dot plots of ficolin-2 and MASP-2 serum levels in healthy controls, recipients before and after OLT, stratified by *FCN2*-602 rs3124953 genotype (A), *FCN2*-557 rs3811140 genotype (B), *FCN2*-4 rs17514136 genotype (C), *FCN2*+6359 rs17549193 genotype (D), *FCN2*+6424 rs7851696 genotype (E), and *MASP2*+120 rs72550870 genotype (F). Serum levels in recipients after OLT were stratified for both genotype of the donor liver and recipient. Plots show the mean. Mann-Whitney U test or One-way ANOVA followed by Holm-Sidak post-hoc test was used for comparing means between groups. $P < 0.05$ was considered significant.

B

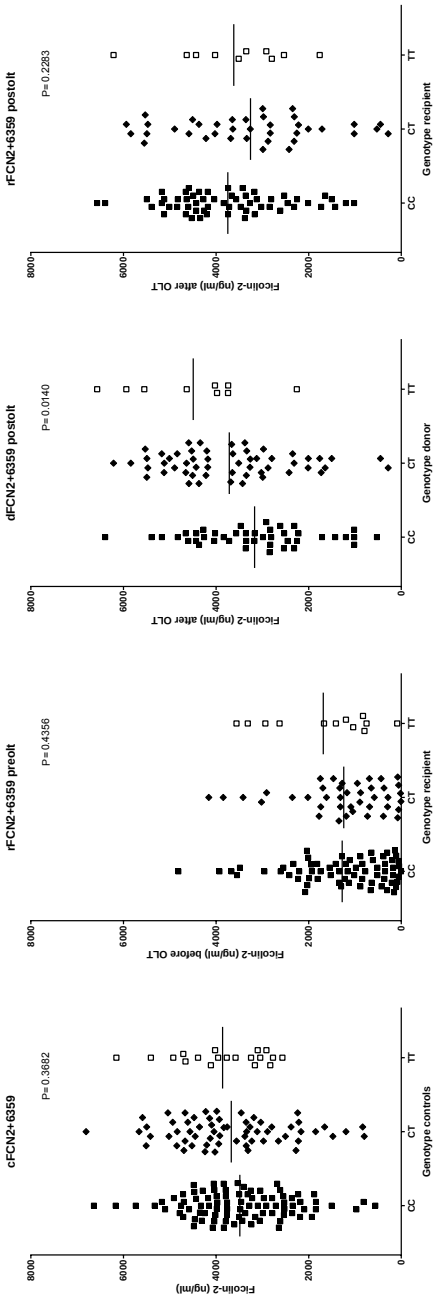


C

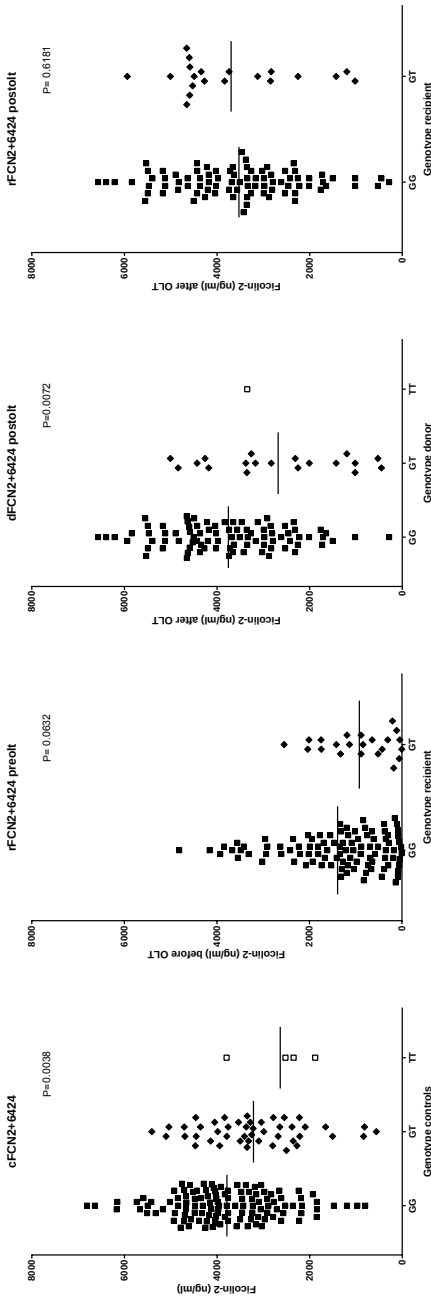


Supplemental figure 2. (continued)

D

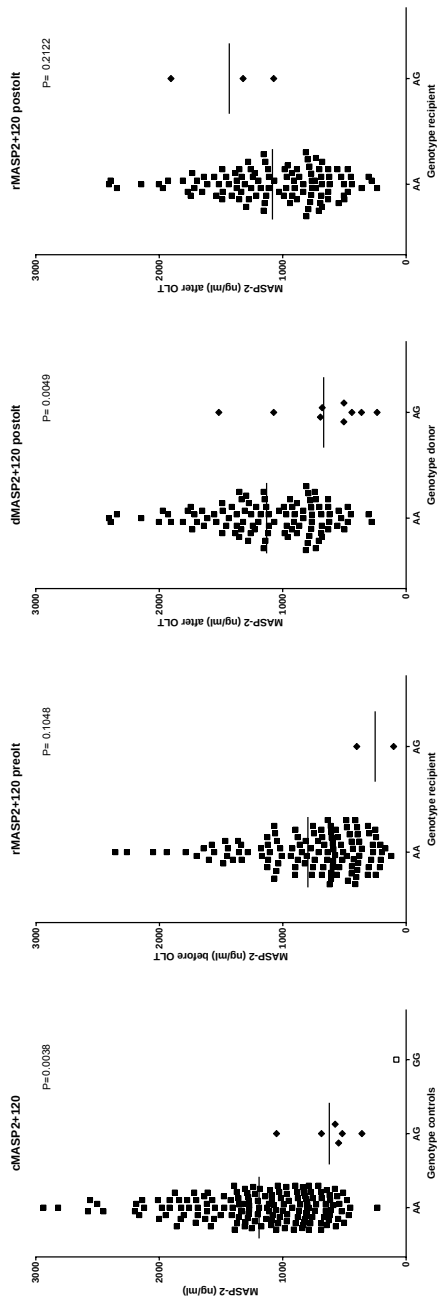


E

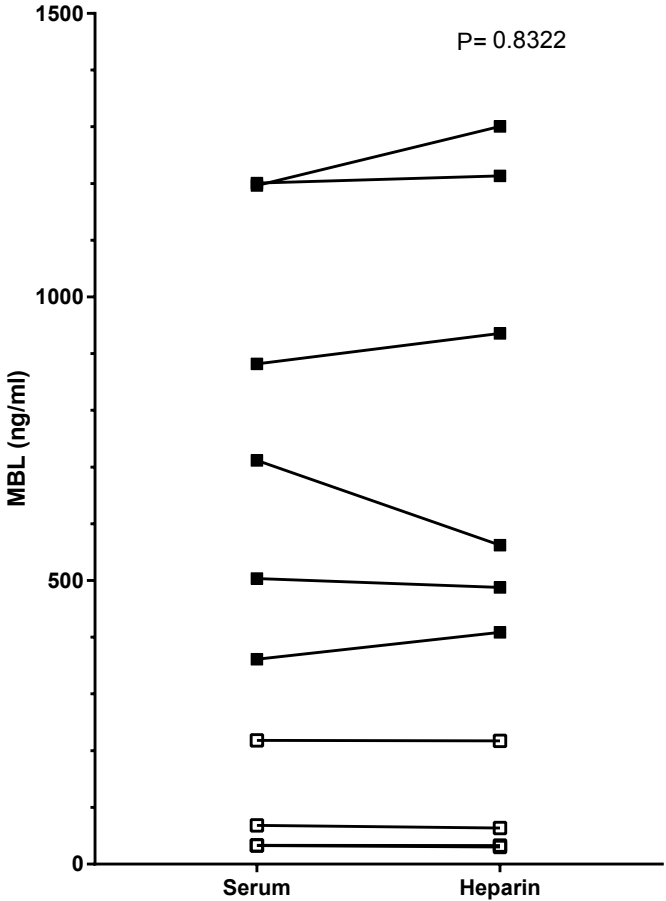


Supplemental figure 2. (continued)

F



Supplemental figure 2. (continued)



Supplemental figure 3. MBL concentration in 10 serum and heparinized plasma pairs. Each line represents one individual. closed squares represent the sufficient-coding genotype; open squares represent the deficient-coding genotype. Paired t-test.

Supplemental Table 1. Oligonucleotide Primers for Polymerase Chain Reaction - High Resolution DNA Melting Assay

Gene	SNP	dbSNP ID	forward primer	reverse primer	sequence	
					3'-blocked probe (C6 spacer)	
<i>MBL2</i> promoter exon1*	H/L -550	rs11003125	CCAGGGCCAAACGTAGTAAGA	CCAGCCGAGAATTAACCTGGA	CAGGCAAGCCTGTCTTAAACACCAAGG	
	Y/X* -221	rs7096206	TTCCTAAGCTAACAGGCATAA	GCACTATGATGAGCAGTGGG	CCACGAAAGCATGTTTATAGTCTT	
	D (codon 52) +223	rs5030737				
<i>FCN2</i> promoter	B (codon 54) +230	rs1800450	CTGCAGTGATTGCCTGTAGC	GCCCAACACGTACCTGGTTC	GGCAAAGATGGGCGTGATGGCACCAAGGGA	
	C (codon 57) +239	rs1800451				
exon 8	-986	rs3124952	TGTTTCTCGCGTGCGCTTTG	CACTGCACCTCCCACTACCA	CATCCCGATGGCAGCAGGTGGCAGG	
	-602	rs3124953	AGGCTCCCACTCTTCTCTC	ATGCGTGTGTCTCACTGTT	CTGTTCAITGTGCCCTGTGCTCTACAT	
	-557	rs3811140	CATGTGCCCTGTGCTCTA	AGGCTGCCAGGGTGA AAA	GGAAA CAGTGGACACACACGCAATTTCC	
	-4	rs17514136	TAAGAGGCGAGGCACCTTTTG	GCCATGCCCAAGGAAAGAGAG	GCAAAGACCAGAAAGAGATGGAGCTGG	
	+6359	rs17549193	GATGTTACTGCTGTAAACGATGC	GGTGAGAGAGGACTGGTGTGTT	GAGATTCCCTGACGTTCCACAACAAC	
<i>MASP2</i> exon 1 exon 9	+6424	rs7851696	CCAGGACAATGATCTTAACACC	CACATGGCAGTTTTTTGTACCAC	GGAAATTGTGCTGTGATGTTTCAGGG	
	+120		TGCATAGAAGGCCTCGAACC	GGCAAGGACACTTTCTACTCGC	CTCGTTGGAGTAGCCGGAGCGGAAGG	
	+371	rs12711521	AGCTTTGTAGTGGTCACTCC	CCCTCCCATGCTTCTTCTT	CCACTGGGTAGATCATCAGGAGGGCCA	

*Vossen, R.H., et al., High-throughput genotyping of mannose-binding lectin variants using high-resolution DNA-melting analysis. Hum Mutat, 2010. 31(4); p. E1286-93.

Chapter 4

Lectin complement pathway gene profile of
donor and recipient determine the risk of bacterial
infections after orthotopic
liver transplantation

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Bouwman LH, Schaapherder AF, Porte RJ, Daha MR,
van der Reijden JJ, Coenraad MJ, Ringers J,
Baranski AG, Hepkema BG, Hommes DW, Verspaget HW

ABSTRACT

Background

Infectious complications after orthotopic liver transplantation (OLT) are a major clinical problem. The lectin pathway of complement activation is liver-derived and a crucial effector of the innate immune defense against pathogens. Polymorphisms in lectin pathway genes determine their functional activity.

Methods

We assessed the relationship between these polymorphic genes and clinically significant bacterial infections, i.e., sepsis, pneumonia, and intra-abdominal infection, and mortality within the first year after OLT, in relation to major risk factors in two cohorts from different transplant centers. Single-nucleotide poly-morphisms in the mannose-binding lectin gene (MBL2), the ficolin-2 gene (FCN2), and the MBL-associated serine protease gene (MASP2) of recipients and donors were determined.

Results

Recipients receiving a donor liver in the principal cohort with polymorphisms in all three components i.e., MBL2 (XA/O; O/O), FCN2+6359T, and MASP2+371A, had a cumulative risk of an infection of 75% as compared to 18% with wild-type donor livers ($P = 0.002$), an observation confirmed in the second cohort ($P = 0.04$). In addition, a genetic (mis)match between donor and recipient conferred a two-fold higher infection risk for each separate gene. Multivariate Cox analysis revealed a stepwise increase in infection risk with the lectin pathway gene profile of the donor (hazard ratio = 4.52; $P = 8.1 \times 10^{-26}$) and the donor-recipient (mis)match genotype (hazard ratio = 6.41; $P = 1.9 \times 10^{-27}$), independent from the other risk factors sex and antibiotic prophylaxis (hazard ratio >1.7 and $P < 0.02$). Moreover, patients with a lectin pathway gene polymorphism and infection had a six-fold higher mortality ($P = 0.9 \times 10^{-28}$), of which 80% was infection-related.

Conclusion

Donor and recipient gene polymorphisms in the lectin complement pathway are major determinants of the risk of clinically significant bacterial infection and mortality after OLT.

INTRODUCTION

The occurrence of infectious complications is a major clinical problem after orthotopic liver transplantation (OLT).¹ Immunosuppressive agents that prevent graft rejection interfere with the adaptive immune response and thereby increase the susceptibility to infections. These drugs do not affect, however, the innate immune system that is crucial for the first line of immunological defense.

Lectins, humoral pattern recognition molecules of the innate immune system, recognize pathogen-associated carbohydrate motifs on microorganisms and elicit activation of multiple processes of innate immunity. In order to execute the elimination of microorganisms, these lectins, such as mannose-binding lectin (MBL) and ficolins, cooperate with phagocytes and other humoral factors, including complement. Upon pathogen binding, both lectins activate the complement system via MBL-associated serine proteases (MASPs), leading to C3b-mediated opsonization of the microorganism followed by phagocytosis and the formation of a complement membrane attack complex that directly kills the pathogen.^{2,3}

Common single-nucleotide polymorphisms (SNPs) in the genes coding for these three members of the lectin complement pathway result in liver-derived protein variants with important functional implications. Structural variants in the exon 1 region of the MBL gene (MBL2) interfere with the oligomerization of the protein and polymorphisms in the promoter regions alter the rate of synthesis of the protein, leading to changes in level, avidity, and pattern recognition of the lectin.^{4,5} These polymorphisms are known to be associated with increased susceptibility to infections in conditions accompanied by an immature or compromised adaptive immune system.⁶⁻⁹ In a proof-of-concept study, we previously showed that gene polymorphisms of MBL from the donor liver are associated with the risk of a clinically significant infection after OLT, an observation that recently has been confirmed independently.^{10,11} Ficolin-2 has similarities in structure and function to MBL and its preferential binding target is N-acetyl-glucosamine,¹² a constituent of bacterial peptidoglycans and a major component of their cell wall.¹³ Polymorphisms in the promoter region of the ficolin-2 (FCN2) gene are associated with differences in ficolin-2 serum levels. Structural amino acid substituting polymorphisms within the carbohydrate recognition domain encoding region of the FNC2 gene are associated with altered ligand binding of ficolin-2.¹⁴ MASP-2 is the serine protease associated with MBL and ficolin-2 that is essential for activation of the complement cascade.¹⁵ Two polymorphisms in the MASP2 gene that change the amino acid sequence are known to lead to a functional defect in the protease that prevents its interaction with the lectins.¹⁶ One SNP leads to the inability to activate complement,^{17,18} and the other SNP is located in the complement control protein domain 2 of MASP2, which is important in stabilizing the structure of the serine protease domain¹⁹ and is essential for effective cleavage of complement C4.²⁰

The risk of infection after transplantation of a solid organ is the combined effect of all of the factors that contribute to a patient's susceptibility to infection, i.e., the net state of immunodeficiency,²¹ in which not only the immunosuppressive therapy but also the genetic

predisposition of recipient and donor organ are likely to play a role. Given the fact that MBL, as well as ficolin-2 and MASP-2, are almost exclusively synthesized in the liver^{10,22} and that the studied gene polymorphisms are quite common in the Caucasian population, there is a realistic chance that a patient in need of liver transplantation will receive a liver from a donor with one or more genetic alterations in the components of the lectin complement pathway.

We evaluated our unchallenged hypothesis that an intergenic interaction between MBL2, FCN2, and MASP2 genes, representing the liver-specific lectin complement cascade, from the donor and recipient contributes to the susceptibility for bacterial infections and associated mortality in OLT recipients.

PATIENTS AND METHODS

Patient Inclusion

All patients who received OLT at the Leiden University Medical Center in The Netherlands were taken into consideration for the principal study. Genomic DNA was extracted routinely from peripheral blood and/or tissue samples, when possible, without given preference to any explicit clinical variables. For this study, 202 patients were identified who underwent OLT between 1992 and 2005, of whom we were able to unselectively retrieve 148 patients whose DNA was available from both donor and recipient. From these patients, 143 were finally included who had at least 7 days of follow-up after liver transplantation, excluding perioperative complication morbidity and mortality. The confirmation study consisted of patients who received OLT at the University Medical Center Groningen between 2000 and 2005. From the 212 available patients, 178 unselected patients could be retrieved for whom we had DNA from both recipient and donor, and 167 had at least 7 days of follow-up after transplantation.

The study was performed with informed consent from the patients according to the guidelines of the Medical Ethics Committee of the Leiden University Medical Center and according to the guidelines of the Medical Ethics Committee of the University Medical Center Groningen and in compliance with the Helsinki Declaration.

Patient Follow-Up

All patients in the principal study 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. Patients in the confirmation study received standard immunosuppressive therapy consisting of basiliximab combined with a calcineurin inhibitor with or without corticosteroids and/or mycophenolate mofetil. With respect to the immunosuppressive therapy, azathioprine was used until 2001, and thereafter mycophenolate mofetil was given in case of impaired renal function. From 2001, basiliximab was also used on days 0 and 4. In addition, all patients received 24 hours of prophylactic antibiotics intravenously: gentamycin, cefuroxim, penicillin G, and metronidazol

in the principal study; amoxicillin-clavulanate and ciprofloxacin in the confirmation study. The patients in the principal study also received 3 weeks of selective digestive tract decontamination (polymyxin/neomycin, norfloxacin, and amfotericin B) after OLT. After surgery, all patients were intensively monitored according to standardized protocols for any infection, rejection, or poor function of the new liver. After hospital discharge, frequent regular visits and standard procedures in case of suspicion of an infection after OLT, including additional visits upon febrile temperature, are operational in both transplant centers.

Clinical Variables

The general patient database, original patient reports, transplantation databases, and microbiology records were evaluated to identify episodes of clinical and laboratory-confirmed bacterial infections within the first year after transplantation, without knowledge of the genotypes. The identified infections were considered clinically significant bacterial infections (CSI) when they complied with the Centers for Disease Control and Prevention criteria²³ for diagnosing infection. All infections found could be categorized into sepsis, including symptomatic urinary tract infection (urosepsis); pneumonia; and intra-abdominal infections, i.e., cholangitis and peritonitis.

Demographic and clinicopathological characteristics of the recipient at the time of OLT (age, sex, indication for liver transplantation, cytomegalovirus serostatus, Child-Pugh classification, and laboratory Model for End-Stage Liver Disease [MELD] score), donor information (age, sex, cytomegalovirus serostatus, and donor type), and posttransplant follow-up data (immunosuppressive regimen, acute cellular rejection according to the Banff scheme²⁴) were also collected from the transplantation databases.

Genotyping

We genotyped a total of 13 SNPs in the MBL2, FCN2, and MASP2 genes, with known functional implications on protein level or function, which are common in the Caucasian population,^{4,5,14,16} with the use of high-resolution DNA melting assays with the oligonucleotide primers as indicated in Supporting Table 1.²⁵⁻²⁷ In brief, high-resolution melting analysis of polymerase chain reaction products amplified in the presence of a saturating double-stranded DNA dye (LCGreenPlus, Idaho Technology, Inc., Salt Lake City, UT) and a 30-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.

MBL-Deficiency Polymorphisms

Genotypic MBL studies have shown that each of the three exon 1 variants (B, C, and D, which are collectively called O, whereas the wild-type is called A) is in strong linkage disequilibrium with a different promoter haplotype. The association between MBL genotype

and phenotype is very strong: sufficient MBL levels are associated with YA/YA, YA/XA, XA/XA, and YA/O genotypes, and insufficient/deficient MBL levels are associated with O/O and XA/O genotypes.^{28,29}

Statistical Analysis

Associations between baseline characteristics of the liver transplant recipients, donors, and posttransplant follow-up data and CSI were analyzed by using the log-rank and two-tailed Student t tests.

The probability of clinically significant infection within the first year after transplantation according to MBL2, FCN2, and MASP2 gene variants was determined with cumulative incidence curves using Kaplan-Meier analysis, and the differences between groups were assessed by log-rank test and Cox regression analysis. Patients were censored at the date of the last follow-up, death, or liver retransplantation.

The multivariate Cox proportional hazards regression analysis was used to evaluate the independence of the MBL2, FCN2, and MASP2 SNPs or the quantity of gene polymorphisms. The forced entry method, including all variables, as well as the backward elimination regression method (Wald statistic) was applied. Results were considered statistically significant when P values were <0.05. Bonferroni correction for multiple comparison tests was not performed because SNPs were selected on the basis of a deducible hypothesis. All analyses were performed with the SPSS statistical software package (version 16.02; SPSS, Inc., Chicago, IL).

RESULTS

Principal Study

The principal study consisted of 143 patients who received OLT (Table 1) of which 59 (41%) encountered a CSI within the first year after transplantation. The MBL2, FCN2, and MASP2 genotype distribution of recipients and donors were analyzed in relation to the cumulative incidence of CSI in the first year after OLT (Supporting Table 2).

Donor

Patients receiving a liver from an MBL-deficient donor (XA/O or O/O) had an increased cumulative incidence of CSI compared to those receiving a wild-type liver (Table 2). In addition, patients receiving a donor liver with at least one copy of the minor T-allele of FCN2 SNP rs17549193 (+6359C→T) also had an increased cumulative CSI incidence. Interestingly, the absence of the minor C-allele (homozygosity for the major A-allele) of MASP2 SNP rs12711521 (+371A→C) in the donor liver was also accompanied with an increased incidence of CSI.

The joint genetic effect of risk-conferring variants of MBL2, FCN2, and/or MASP2 present within the donor liver were clustered as the lectin pathway gene profile (Table 2). This profile, including the number of risk-conferring gene variants, showed an ever-increasing

Table 1. Characteristics of Orthotopic Liver Transplant Recipients and Donors

Variable	Principal Study			Confirmation Study		
	CSI (n = 59)	No CSI (n = 84)	P Value†	CSI (n = 36)	No CSI (n = 131)	P Value†
Age (median, range)*						
Recipient	48 (16-69)	48 (17-67)	0.95	48.5 (20-65)	52 (18-70)	0.20
Donor	45 (15-65)	42 (9-71)	0.68	44.5 (16-72)	49 (14-70)	0.48
Donor type, no. (%)						
Donation after brain death	52 (88)	76 (90)		33 (92)	124 (95)	
Donation after cardiac death	7 (12)	8 (10)	0.83	3 (8)	7 (5)	0.48
Sex of donor and recipient, no. (%)						
Female/female	5 (9)	16 (19)		7 (19)	37 (28)	
Female/male	15 (25)	28 (33)	0.36	9 (25)	34 (26)	0.53
Male/female	10 (17)	15 (18)	0.20	6 (17)	21 (16)	0.48
Male/male	29 (49)	25 (30)	0.02	14 (39)	39 (30)	0.21
Underlying disease, no. (%)						
Viral	16 (27)	24 (29)		5 (14)	16 (12)	
HBV/HCV	7/9	12/12		4/1	6/10	
Alcoholic	10 (17)	17 (20)	0.94	4 (11)	15 (12)	0.74
Cholestatic	13 (22)	20 (24)	0.93	13 (36)	34 (26)	0.84
Other disease‡	20 (34)	23 (27)	0.44	14 (39)	66 (50)	0.44
Type of immunosuppression, no. (%)						
Prednisone/CNI (+ azathioprine)	30 (51)	37 (44)		6 (17)	28 (21)	
Prednisone/CNI/basiliximab (+ MMF)	29 (49)	47 (56)	0.51	30 (83)	103 (79)	0.54
Acute cellular rejection, no. (%)						
No	51 (86)	68 (81)		19 (53)	65 (50)	
Yes	8 (14)	16 (19)	0.46	17 (47)	66 (50)	0.62
Child-Pugh classification, no. (%)						
A	11 (19)	24 (29)		5 (14)	27 (21)	

Table 1. (continued)

Variable	Principal Study			Confirmation Study		
	CSI (n = 59)	No CSI (n = 84)	P Value†	CSI (n = 36)	No CSI (n = 131)	P Value†
B	25 (42)	33 (39)	0.21	18 (50)	64 (49)	0.46
C	23 (39)	27 (32)	0.13	13 (36)	40 (30)	0.35
Lab MELDs (median, range)*	17 (6-40)	14.5 (6-40)	0.12	14 (6-40)	14 (6-40)	0.47
CMV serostatus donor/recipient, no. (%)						
D-/R-	9 (16)	11 (13)		9 (25)	23 (18)	
D-/R+	23 (39)	32 (38)	0.80	16 (44)	48 (37)	0.76
D+/R-	7 (12)	9 (12)	0.90	6 (17)	22 (17)	0.58
D+/R+	20 (34)	32 (38)	0.96	5 (14)	38 (29)	0.06
Clinically significant infection, no. (%)						
LCBI	27 (46)			24 (67)		
PNEU	6 (10)			3 (8)		
IAB	26 (44)			9 (25)		
Microbiologic findings, no. (%)						
Gram positive	33 (56)			19 (53)		
Gram negative	15 (25)			10 (28)		
Mixed infections	7 (12)			1 (3)		
Culture negative	4 (7)			6 (17)		
Median time from OLT to diagnosis						
Days (range)	29 (1-339)			23.5 (2-317)		

CMV, cytomegalovirus; CNI, calcineurin inhibitor; CSI, clinically significant bacterial infection; HBV, hepatitis B virus; HCV, hepatitis C virus; IAB, intra-abdominal infection; LCBI, laboratory-confirmed bloodstream infection; MELD, Model for End-Stage Liver Disease; MMF, mycophenolate mofetil; OLT, orthotopic liver transplantation; PNEU, pneumonia.

* P values are for two-tailed Student t test analysis.

† P values were calculated with the use of the log-rank test.

‡ Other diseases included predominantly autoimmune hepatitis, cryptogenic cirrhosis, and metabolic disorders.

§ Calculated laboratory MELD.

Table 2. Univariate Analysis of the Association of Liver Donor Polymorphisms with the Cumulative Incidence of Clinically Significant Bacterial Infection in the Principal Study (N = 143)

Gene	dbSNP ID	Donor Variant Genotype	Cumulative Incidence of Clinically Significant Infection				
			Variant Absent (Reference) % (n)	One Variant % (n)	Two Variants % (n)	Three Variants % (n)	LR (χ^2) P Value [‡]
MBL2*		XA/O and O/O	37 (41/112)	58 (18/31)			5.5 0.02
FCN2	rs17549193	CT and TT	31 (18/59)	49 (41/84)			4.6 0.03
MASP2	rs12711521	AA	31 (17/54)	47 (42/89)			4.0 0.05
Lectin pathway gene profile [†]							
		MBL2 & MASP2+371AA	29 (12/42)	42 (34/82)	68 (13/19)		12.2 0.002
		FCN2+6359T & MASP2+371AA	18 (4/22)	39 (27/69)	54 (28/52)		8.5 0.01
		MBL2 & FCN2+6359T	28 (13/47)	43 (33/77)	68 (13/19)		10.0 0.007
		MBL2+FCN2+MASP2	18 (3/17)	33 (20/60)	50 (27/54)	75 (9/12)	14.7 0.002
		LR (χ^2) / P value [§]	Reference	1.7 / 0.20	5.3 / 0.02	9.4 / 0.002	

* Five single-nucleotide polymorphisms in MBL2 were genotyped: MBL2 –550 (H/L), rs11003125; MBL2 –221 (X/Y), rs7096206; MBL2 codon 52 (D), rs5030737; MBL2 codon 54 (B), rs1800450; and MBL2 codon 57 (C), rs1800451. Structural variants D, B, and C are collectively called O; A is considered the wild type; Secretor haplotypes were constructed: A/A and YA/O versus XA/O and O/O.

[†] The presence of multiple intergenic variants within the individual were clustered as the lectin pathway gene profile.

[‡] P Values were calculated with the use of the log-rank test.

[§] P Values calculated with the use of the log-rank test compared to variant absent.

cumulative risk for developing CSI with increasing numbers of variants: 18% CSI with no genetic variant, 33% in those with one, 50% in those with two, and 75% in those with three variants ($P < 0.002$; Table 2 and Fig. 1).

Recipient

The genotype distribution in OLT recipients (Supporting Table 2) showed no significant association with the occurrence of CSI either for the independent SNPs or for the number of risk-conferring variants (not shown). However, some remarkable inter-actions between the genotype of the donor and the recipient with the occurrence of CSI were found. Recipients with an MBL-sufficient or wild-type MBL genotype (A/A and YA/O) receiving an MBL-insufficient (XA/O and O/O) donor liver developed significantly more CSI than the other patients (61% [17/28] versus 37% [42/115], respectively, $P < 0.006$). For the SNPs in FCN2 and MASP2 genes, we found that absence of the minor allele in FCN2 SNP rs17549193 and the absence of homozygosity for the major allele in MASP2 SNP rs12711521 in both recipient and donor showed a clear trend toward less CSI (FCN2: 28%[11/39] versus 46% [48/104] and MASP2: 22%[5/23] versus 45% [54/120], both $P < 0.06$).

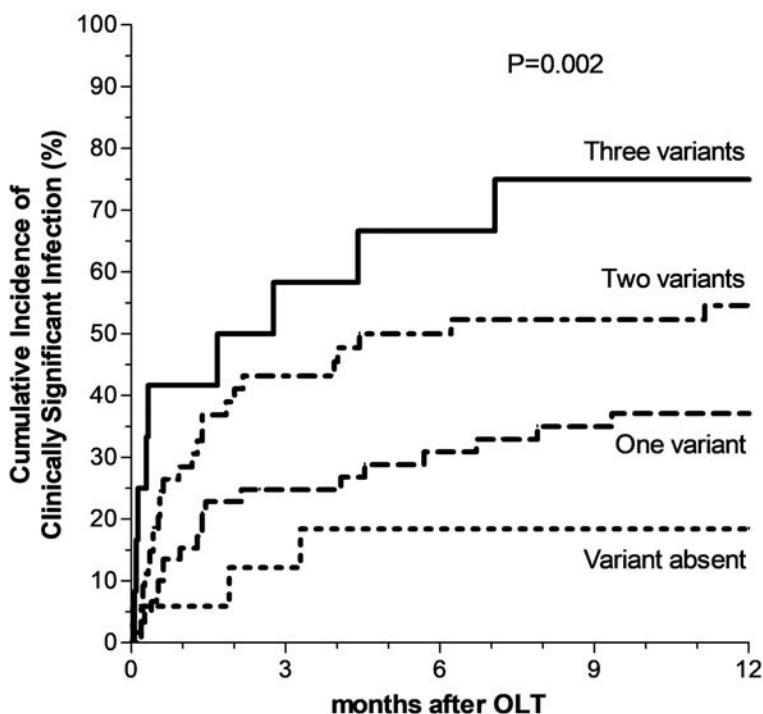


Figure 1. Cumulative incidence of CSI after OLT, according to donor lectin pathway gene profile in the principal study. The endpoint was the time to the diagnosis of CSI in 143 recipients, with censoring of the data for these recipients at the date of last follow-up (death or second transplantation). P value was calculated with the use of the log-rank test.

Confirmation Study

The demographic characteristics of the patients in the confirmation study were comparable to those of the patients in the principal study (Table 1), even though there was a difference in selective digestive tract decontamination, type of intravenous antibiotic prophylaxis, and immunosuppressive therapy. The frequencies for the various SNPs of the recipients were similar compared to the principal group (Supporting Table 2). The cumulative incidence of CSI within the first year was significantly lower (22% [36/167] versus 41% [59/143], $P < 0.007$) and the percentage of transplanted donor livers with an MBL-deficient genotype was significantly lower in this confirmation group compared to the principal study (13% [22/167] versus 22% [31/143], $P = 0.05$; Supporting Table 2). Nevertheless, the lectin pathway gene profile of the donor liver in this confirmation group showed a similar significant association with the cumulative incidence of CSI (56% [5/9] with three variants, 26% [15/57] with two variants, 15% [12/81] with one variant, and 20% [4/20] when genetic variants were absent, log-rank = 8.2; $P = 0.04$). Furthermore, the effect of the donor-recipient genotypic match was also confirmed. MBL mismatch, i.e., a sufficient recipient and an insufficient donor liver, conferred a significantly increased risk for developing clinically significant infection compared to the other MBL combinations (40% [8/20] versus 19% [28/147], $P = 0.03$), whereas again a lower risk of CSI was associated with absence of the minor T-allele in FCN2 SNP rs17549193 (10% [5/50] versus 27% [31/117], $P < 0.03$) and the absence of homozygosity for the major A-allele in MASP2 SNP rs12711521 (9% [2/23] versus 24% [34/144], $P = 0.11$) in both recipient and donor.

Combined Cohorts

In the univariate regression models, a significant association was found for the separate donor gene polymorphisms with CSI of the combined data from both cohorts, in particular for MBL2 (XA/O and O/O) and FCN2 (rs17549193), and less so for MASP2 (rs12711521) (Table 3). In addition, the lectin pathway gene profile of the donor liver showed a significant stepwise association with CSI. In the presence of three variants, 67% CSI was found; 38% CSI was found in the case of two variants, 23% CSI in the case of one variant, and 19% CSI was found when genetic variants in the lectin pathway were absent ($P < 0.001$). The only other factors associated with the infection risk were found to be male sex of the donor and recipient, the antibiotic prophylactic regimen used, and acute cellular rejection.

Multivariate analysis Cox regression analyses and the backward elimination procedure, taking all demographic and clinicopathological characteristics into account, indicated that the three individual lectin pathway genes of the donor were independently associated with the infection risk, next to sex of the donor-recipient combination and the prophylactic antibiotic regimen, whereas acute cellular rejection lost its significance (Table 3).

A final adjusting multivariate model was derived using the lectin pathway gene profile and the backward elimination procedure, which indicated that recipients had an even higher CSI risk if they received a donor liver with two or three genetic variants up to an adjusted

Table 3. Univariate and Multivariate Analysis for the Association of Risk Factors of Clinically Significant Bacterial Infection in all OLT Patients (N = 310)

Variable		Univariate Models		
		LR (χ^2)	% CSI (n)	P Value
Donor <i>MBL2</i>	XA/O and O/O	12.3	49 (26/53)	<0.001
	A/A and YA/O	Ref	27 (69/257)	
Donor <i>FCN2</i> [rs17549193]	CT and TT	7.0	59 (63/106)	0.008
	CC	Ref	23 (32/141)	
Donor <i>MASP2</i> [rs12711521]	AA	2.6	34 (68/202)	0.11
	AC and CC	Ref	25 (27/108)	
Donor lectin pathway gene profile		23.7		<0.001
	3 variants	12.3	67 (14/21)	<0.001
	2 variants	4.3	38 (42/111)	0.04
	1 variant	0.2	23 (32/141)	0.65
	no variants	Ref	19 (7/37)	
Sex		10.0		0.02
	Male donor and male recipient	9.0	40 (43/107)	0.003
	Male donor and female recipient	3.2	31 (16/52)	0.07
	Female donor and male recipient	1.2	28 (24/86)	0.268
	Female donor and recipient	Ref	18 (12/65)	
Type of immunosuppression	P / CNI / B	1.8	28 (59/209)	0.19
	P / CNI	Ref	36 (36/101)	
Antimicrobial prophylaxis	GCPM + SDD	16.9	41 (59/143)	<0.001
	ACC, No SDD	Ref	22 (36/167)	
Acute cellular rejection	Yes	4.9	23 (25/107)	0.03
	No	Ref	35 (70/203)	
Lab MELD score		1.0	31 (95/310)	0.57
Donor age		0.99	31 (95/310)	0.25
Recipient age		0.99	31 (95/310)	0.45
Donor type	DCD	1.0	40 (10/25)	0.33
	DBD	Ref	30 (85/285)	
CMV serostatus donor/recipient		1.6		0.67
	D+/R+	0.8	26 (25/95)	0.37
	D+/R-	0.1	30 (13/44)	0.72
	D-/R+	0.0	33 (39/119)	0.96
	D-/R-	Ref	35 (18/52)	
Underlying disease		1.10		0.78
	Other disease	1.0	28 (34/123)	0.32
	Cholestatic	0.1	33 (26/80)	0.79
	Alcohol	0.1	30 (14/46)	0.77
	Viral	Ref	34 (21/61)	

ACC, amoxicillin-clavulanate and ciprofloxacin; B, basiliximab; CI, confidence interval; CMV cytomegalovirus; CNI, calcineurin inhibitor; CSI, clinically significant infection; D, donor; DBD, donation after brain death; DCD, donation after cardiac

Multivariate Model		Multivariate Model (Backward Wald)		Multivariate Model		Final Multivariate Model (Backward Wald)	
HR (95% CI)	P Value	HR (95% CI)	P Value	HR (95% CI)	P Value	HR (95% CI)	P Value
2.22 (1.37-3.62)	0.001	2.15 (1.35-3.42)	0.001				
1.00 (Reference)		1.00 (Reference)					
1.72 (1.10-2.69)	0.02	1.72 (1.11-2.64)	0.01				
1.00 (Reference)		1.00 (Reference)					
1.45 (0.91-2.33)	0.12	1.56 (1.00-2.45)	0.05				
1.00 (Reference)		1.00 (Reference)					
					<0.001		<0.001
				4.57 (1.78-11.74)	0.002	4.52 (1.81-11.31)	0.001
				2.40 (1.05-5.49)	0.04	2.35 (1.05-5.25)	0.04
				1.26 (0.54-2.95)	0.59	1.24 (0.54-2.82)	0.61
				1.00 (Reference)		1.00 (Reference)	
	0.02		0.02		0.03		0.03
2.54 (1.30-4.96)	0.006	2.39 (1.25-4.55)	0.008	2.43 (1.24-4.75)	0.01	2.28 (1.20-4.35)	0.01
1.55 (0.70-3.46)	0.28	1.44 (0.68-3.09)	0.34	1.53 (0.67-3.50)	0.31	1.42 (0.65-3.10)	0.37
1.39 (0.67-2.88)	0.37	1.38 (0.69-2.80)	0.37	1.34 (0.64-2.81)	0.43	1.32 (0.65-2.68)	0.44
1.00 (Reference)		1.00 (Reference)		1.00 (Reference)		1.00 (Reference)	
0.80 (0.48-1.33)	0.38			0.82 (0.49-1.36)	0.44		
1.00 (Reference)				1.00 (Reference)			
1.92 (1.18-3.14)	0.009	2.14 (1.41-3.26)	<0.001	2.00 (1.22-3.28)	0.006	2.21 (1.45-3.35)	0.002
1.00 (Reference)		1.00 (Reference)		1.00 (Reference)		1.00 (Reference)	
0.76 (0.45-1.29)	0.31			0.76 (0.45-1.28)	0.31		
1.00 (Reference)				1.00 (Reference)			
1.00 (0.97-1.03)	0.99			1.00 (0.98-1.03)	0.96		
1.00 (0.99-1.02)	0.95			1.00 (0.99-1.02)	0.92		
0.99 (0.97-1.01)	0.30			0.99 (0.97-1.01)	0.31		
0.89 (0.43-1.86)	0.76			0.86 (0.41-1.79)	0.68		
1.00 (Reference)				1.00 (Reference)			
	0.60				0.69		
0.71 (0.37-1.37)	0.30			0.77 (0.40-1.49)	0.43		
0.95 (0.44-2.09)	0.90			0.94 (0.43-2.07)	0.88		
1.01 (0.55-1.84)	0.98			1.06 (0.58-1.94)	0.86		
1.00 (Reference)				1.00 (Reference)			
	0.99				0.99		
0.93 (0.51-1.70)	0.80			0.96 (0.53-1.76)	0.91		
0.99 (0.53-1.87)	0.99			1.03 (0.55-1.92)	0.93		
0.93 (0.45-1.90)	0.84			0.95 (0.46-1.93)	0.88		
1.00 (Reference)				1.00 (Reference)			

death; GCPM, gentamycin, cefuroxim, penicillin G, and metronidazol; HR, hazard ratio; LR, log-rank; OLT, orthotopic liver transplantation; P, prednisone; R, recipient; SDD, selective digestive tract decontamination.

hazard ratio (HR) of 4.52 (confidence interval [CI] = 1.81-11.31), again independent from sex and antibiotic prophylaxis, which were also found to have significant and independent HRs of more than 2.21.

The combined genotypes of the donor and the recipient showed even stronger association with CSI than the donor genes alone. Although CSI risk is related to the donor MBL genotype, the risk is even higher when the recipient genotype is taken into account. Thus, receiving an MBL-insufficient liver when having previously had an MBL-sufficient liver almost doubles the risk of CSI as compared to the other donor/recipient MBL combinations (52% [25/ 48] versus 27% [70/262], respectively; $P < 0.0001$). Similarly increased infection risks were found for the FCN2 and MASP2 donor-recipient combinations, as described in Table 4 and Supporting Table 3. The different genotypic donor-recipient combinations also gave rise to (mis)match genotypes associated with increasing infection risk scores from 0% in those without a variant to 65% in those with three variants within the lectin pathway gene profile (Table 4 and Fig. 2). Because the multivariate model revealed that the individual (mis) matches were independently associated with the infection risk, all donor-recipient (mis)-match variant genotypes were included in the final multivariate model, which showed an even higher infection risk profile for two or three variants as compared to one or no variant, with adjusted HRs of 2.74 (CI = 1.56-4.82) and 6.41 (CI = 3.19-12.89), respectively, than that for the donor gene profile alone.

Mortality

The all-cause mortality rate in the first year after OLT for recipients who received a donor liver with one or more variants in the lectin complement path-way was significantly higher in patients who encountered a CSI (28% [25/88] versus 4% [8/185] in those without a CSI; Fig. 3). In the absence of a genetic variant in the lectin pathway of the donor liver ($n = 37$), none of the recipients died in the first year of follow-up, despite a CSI rate of 19%. These differences in CSI-associated mortality persisted after adjustment for the D-MELD score,³⁰ the product of donor age and preoperative laboratory MELD score (unadjusted HR = 7.34; 95% CI = 3.31-16.29), whereas the HR adjusted for D-MELD > 1600 was 7.35 (95% CI = 3.31-16.32). A similar association with mortality was found in the patients with a (mis)match in the MBL2, FCN2, and MASP2 genes between donor and recipient. Presence of one or more (mis)matches was accompanied by a 26% (25 of 95 patients) mortality rate, 80% (20 of 25 patients) of which was infection-related, in case of a clinically significant infection when compared to only a 4% (8 of 209 patients) mortality rate in patients without an infection (unadjusted HR = 7.71 [95% CI = 3.47-17.10]; adjusted for D-MELD > 1600, HR = 7.81 [95% CI = 3.52-17.33], both $P < 0.001$). None of the remaining six patients without a genetic (mis)match had died during follow-up.

Table 4. Univariate and Multivariate Analysis for the Association of Risk Factors of Clinically Significant Bacterial Infection in all OLT Patients Stratified According Donor- Recipient Genotypes (N = 310)

Variable		Univariate Model			Multivariate Models			Final Multivariate Models		
		LR (χ^2)	% CSI (n)	P Value	HR (95% CI)	P Value	HR (95% CI)	P Value	HR (95% CI)	P Value
Donor-recipient genotypic group	MBL2*	15.0	52 (25/48)	<0.001	2.58 (1.62-4.10)	<0.001				
	Other combinations	Ref	27 (70/262)		1.00 (Reference)					
	FCN2†	8.8	36 (79/221)	0.003	2.33 (1.36-4.00)	0.002				
	CC donor and recipient	Ref	18 (16/89)		1.00 (Reference)					
	MASP2‡	5.4	33 (88/264)	0.02	2.65 (1.22-5.73)	0.01				
(Mis)match profile‡	AA donor and/or recipient	Ref	15 (7/46)		1.00 (Reference)					
	AC and CC donor and recipient	33.3		<0.001						
	3 variants	6.1	65 (17/26)	0.01			6.41 (3.19-12.89)			<0.001
	2 variants	2.8	36 (63/177)	0.09			2.74 (1.56-4.82)			<0.001
	1 variant	1.0	15 (15/101)	0.31			1.00 (Reference)			
Sex	No variants	Ref	0 (0/6)				1.00 (Reference)			
	Male donor and male recipient	7.6	40 (43/107)	0.006	1.77 (1.18-2.67)	0.006	1.75 (1.16-2.63)			0.007
	Female donor and or recipient	Ref	26 (52/203)		1.00 (Reference)		1.00 (Reference)			
Antimicrobial prophylaxis	GCPM + SDD	16.9	41 (59/143)	<0.001	2.17 (1.43-3.29)	<0.001	2.15 (1.42-3.26)			<0.001
	ACC, No SDD	Ref	22 (36/167)		1.00 (Reference)		1.00 (Reference)			

ACC, amoxicillin-clavulanate and ciprofloxacin; CI, confidence interval; CSI, clinically significant infection; GCPM, gentamycin, cefuroxime, penicillin G, and metronidazole; HR, hazard ratio; LR, log-rank; OLT, orthotopic liver transplantation; SDD, selective digestive tract decontamination.

* The presence of a deficient donor MBL2 secretor haplotypes in a sufficient recipient haplotype conferred the highest risk compared to other donor-recipient combinations.

† The presence of the reduced infection genotype in FCN2 and MASP2 in both the donor and recipient showed a clear trend to less clinically significant infections as opposed to the other genotypes.

‡ The presence of donor-recipient genotypic groups associated with clinically significant infections within the individual were clustered as lectin pathway gene profile.

§ Backward elimination regression method was used, adjusting for recipient age (continuous), sex combinations (4 categories), type of immunosuppression, antimicrobial prophylaxis, acute cellular rejection (yes/no), Lab MELD score (continuous), donor age (continuous), donor type, cytomegalovirus serostatus (4 categories), and underlying disease (4 categories).

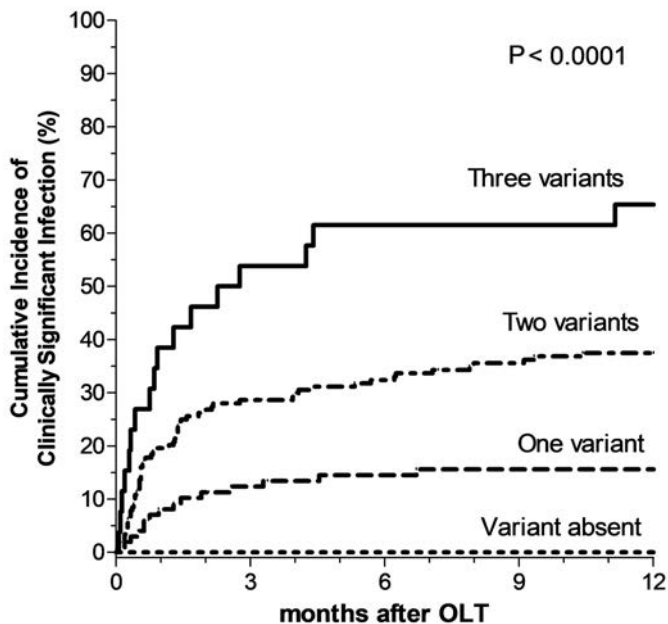


Figure 2. Cumulative incidence of CSI after OLT, according to donor-recipient genotype. The endpoint was the time to diagnosis of CSI in 310 recipients from both cohorts, with censoring of the data for these recipients at the date of last follow-up (death or second transplantation). *P* value was calculated with the use of the log-rank test.

DISCUSSION

The presence of common functional gene polymorphisms in MBL2, FCN2, and MASP2, which affect the composition, structure, and function of the respective proteins, was found to confer an increased risk of CSI after liver transplantation. Thus, the multifactorial antimicrobial lectin complement activation pathway is of eminent importance to the risk of bacterial infections such as sepsis, peritonitis, and pneumonia, after OLT.

Earlier studies already indicated that MBL deficiency of the donor liver is accompanied by an increased risk of infections after liver transplantation.^{10,11} We now showed that the minor T-allele of FCN2 SNP rs17549193 (+6359C→T) and homozygosity for the major A-allele, or the absence of the minor allele, of MASP2 SNP rs12711521 (+371A→C), which are the other main components of the lectin complement activation pathway, also have a significant impact on this infection risk. Diverse combined SNPs in the MBL2 gene, in conjunction with SNPs in the FCN2 and MASP2 genes of the donor liver, constitute a genetic profile of the lectin complement activation pathway which carry a gene dose-dependent risk for bacterial infection in the first year after OLT, as demonstrated and confirmed in the two separate cohorts.

The recipient lectin complement pathway gene pro-file seemed not to convey a major clinical risk itself. However, MBL-sufficient recipients receiving an MBL-insufficient donor

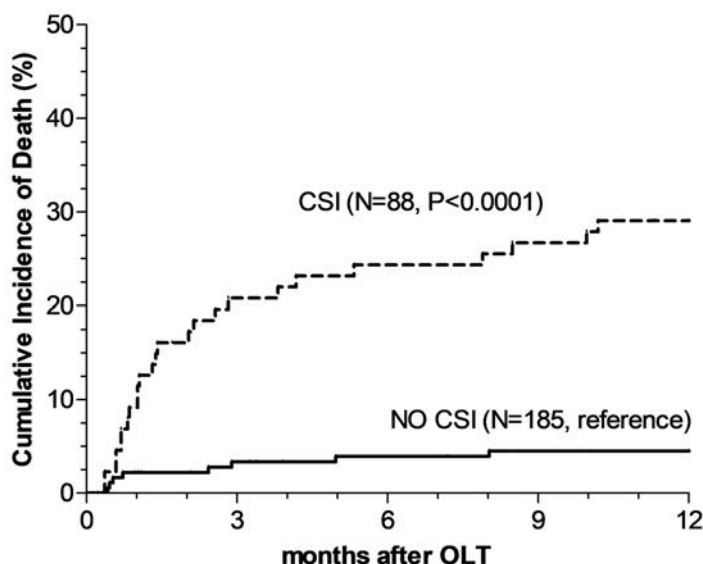


Figure 3. Cumulative incidence of death within the first year after OLT in recipients with a genetic variant in the donor lectin pathway genes, according to the occurrence of CSI. The endpoint was the time to death in 273 recipients from both cohorts, with censoring of the data for these patients at the date of last follow-up, P value was calculated with the use of the log-rank test. In the absence of a genetic variant in the donor lectin pathway genes none of the recipients ($n = 37$) died in the first year of follow-up. P value was calculated with the use of the log-rank test.

liver were found to be at high risk for these infections. In addition, combined donor and recipient FCN2 and MASP2 genotype analyses showed that when there is no match in the allele associated with reduced infection, the relative risk of CSI is also highly increased.

The essential components of the lectin pathway of complement activation that we studied are mainly produced in the liver.^{10,22} After liver transplantation, the adaptive immunity of the recipient is reduced by immunosuppression and the recipient will, to a major extent, be dependent on the lectin complement activation pathway of the donor liver. The functional SNPs in these polymorphic genes may thus lead to reduced complement activation and opsonization, which results in increased susceptibility to infections in patients with an immature or compromised adaptive immune system. Our study is the first to show that the interplay between the genotype of three members of the lectin complement pathway in both donor and recipient has a major impact on the risk of developing infections and on related death in immunocompromised OLT recipients.

Apart from the lectin pathway polymorphisms, univariate and multivariate analyses showed that the male-male donor-recipient sex combination and the type of antimicrobial prophylaxis were consistently found to be accompanied by an increased infection risk. The regimen with selective digestive tract decontamination showed significantly more infections after OLT, as reported before in several other studies.³¹⁻³⁴ In the multivariate

analyses, the lectin pathway gene profile was found to convey the risk of infection independent from the prophylactic antibiotic regimen. Also noteworthy was the association of the male-male donor-recipient sex combination as an independent risk factor for CSI after liver transplantation. Sex differences in terms of infection and sepsis have been observed in several clinical and epidemiological studies with a predominance of risk in male patients, leading to lower proinflammatory innate immune responses and a worse prognosis with sepsis.^{35,36} These findings indicate that male patients receiving a male donor liver should be monitored more intensively and perhaps receive more preemptive antibiotic treatment because of the increased infection risk.

The present study further revealed an important contribution of the MBL2 gene donor-recipient mis-match in the occurrence of CSI. The impact of the MBL2 gene on the increased infection risk was particularly seen in MBL-sufficient recipients whose liver was replaced by an MBL-insufficient donor liver. This raises the question as to whether MBL supplementation might be beneficial. However, the MBL-insufficient recipient does not seem to profit from MBL supplementation, i.e., transplantation of an MBL-sufficient liver. This is in line with the observation that MBL protein substitution in other conditions seems to be ineffective; for example, neutropenic MBL-deficient children who were treated with MBL substitution still encountered neutropenic fever and sepsis.³⁷ Substitution only appeared to be beneficial in some case reports and preclinical studies in knockout mice.^{38,39}

Finally, the high mortality risk in the first year after OLT in patients with one or more gene polymorphisms in the lectin complement pathway who encountered an infectious event as opposed to those without infection illustrates the major clinical impact of these polymorphisms, in particular because of the high percentage of infection-related deaths. A similar association with survival was recently reported in a small group of patients with only the MBL2 exon 1 gene mutations of the donor liver.⁴⁰

Our findings account for up to 84% (80/95) but not for all infections observed in the patients who underwent OLT. This might arise for several reasons. For example, other low-allele-frequency SNPs in the lectin pathway genes might also have an impact, but these can only be examined in a considerably larger study population. Furthermore, the lectin pathway is not the only innate immune response to bacterial infections in immunocompromised patients. Toll-like receptors (TLRs) and Nod-like receptors, for instance, also play a role in the immune response to bacterial infections,⁴¹ but their function and gene polymorphisms have not been studied extensively in OLT. Interestingly, MBL is able to interact with TLR2 in the phagosome to initiate proinflammatory signaling,⁴² which thereby might also play a role in infection after OLT.

Gene association studies have several potential limitations which should be taken into consideration when interpreting the results. One is that selection bias may arise from the fact that not all patients were included (patients were excluded because DNA was absent or because of perioperative morbidity or mortality within the first 7 days after transplantation). However, frequencies for the studied SNPs in recipients were comparable in both cohorts. Another limitation is that the study may suffer from bias due to population stratification.

In our study, however, a similar association was observed in a second independent cohort, despite differences in treatment regimes and donor genotype frequencies. An additional theoretical limitation is the possibility that the evaluated polymorphisms may not be directly associated with CSI, but instead may be associated with other factors that influence that clinical endpoint. However, the multivariate analyses identify each of the separate SNPs, the number of risk-conferring SNPs, sex, and antimicrobial prophylaxis as independent risk factors for infection.

In conclusion, the genetic profile of the lectin complement activation pathway has a major impact on bacterial infection after liver transplantation. These observations also confirm the importance of the liver as primary source of the lectin complement pathway constituents: MBL, FCN2, and MASP2. Further studies on these genetic risk factors in liver transplantation could contribute to novel infection prevention strategies and improvement of postoperative outcome. This should be evaluated in prospective intervention studies. Such an approach based on lectin complement pathway genes might in time lead to more personalized treatment protocols and improved survival after OLT.

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SUPPORTING TABLES

Supporting Table 1. Oligonucleotide Primers for Polymerase Chain Reaction - High Resolution DNA Melting Assay

Gene	SNP	dbSNP ID	sequence		
			forward primer	reverse primer	3'-blocked probe (C6 spacer)
MBL2 promoter exon1*	H/L	-550	CCAGGCGCAACGTAGTAAGA	CCAGCCGAGAATTAACTGGA	CAGGCAAGCCTGTCTTAAAAACACCAAGG
	Y/X*	-221	TTCCTAAGCTAACAGGCATAA	GCACTATGATGAGCAGTGGG	CCACGAAAGCAATGTTTATAGTCTT
	D (codon 52)	+223			
FCN2	B (codon 54)	+230	CTGCAGTGATTGCCTGTAGC	GCCCAACACGTACCTGTGTC	GGCAAAGATGGCGCTGATGGCACCAAGGGA
	C (codon 57)	+239			
promoter		-986	TGTTTCTGCGTGGCTTTTG	CACTGCACCTCCCACTACCA	CATCCGATGGCAGCAGGTGGCAGG
		-602	AGGCTCCCACTCTTCTCTC	ATGCGTGTGTGTCCTGTT	CTGTTTCATGTGCCCTGTGCTCTACAT
		-557	CATGTGCCCTGTGCTCTA	AGGCTGCCAGGGGTGAAAA	GGAAACAGTGGACACACACGCAATTTTCC
		-4	TAAGAGGGCAGGCACCTTTTG	GCCATGCCCAGGAAGAGAG	GCAAAGACCAGAAAGAGATGGAGCTGG
exon 8	B	+6359	GATGTTACTGCCCTGTAAACGATGC	GGTGGAGAAAGGACTGGTTGTT	GAGATTCCCTGACGTTCCACAACAAC
MASP2 exon 1 exon 9	C	+6424	CCAGGACAATGATCTTAACACC	CACATGGCAGTTTTTTGTACAC	GGAAATTGTGCTGTGATGTTTCAGGG
		+120	TGCATAGAGGCCTCGAACC	GGCAAGGACACTTTCTACTCGC	CTCGTTGGAGTAGCCGGAGCGGAAGG
		+371	AGCTTTGTAGGTGTCCTCC	CCCTCCCATGCTTCTTCTT	CCACTGGGTAGATCATCAGGAGGGCCA

* Reference 27

Supporting Table 2. Frequencies of Lectin Pathway Gene Polymorphisms in Orthotopic Liver Transplant Recipients and Donors

				Principal Study				Confirmation Study			
				Recipient		Donor		Recipient		Donor	
dbSNP ID	SNP	Genotype	CSI (n=59)	No CSI (n=84)	CSI (n=59)	No CSI (n=84)		CSI (n=36)	No CSI (n=131)	CSI (n=36)	No CSI (n=131)
MBL2											
rs11003125	-550	C→G	21	31	23	39		13	50	12	49
		LL	CC								
		HL	CG	44	33	40		19	61	18	57
		HH	GG	9	3	5		4	20	6	25
rs7096206											
	-221	G→C	34	51	30	45		20	84	25	82
		YY	GG								
		YX	CG	28	27	34		13	39	10	39
		XX	CC	5	2	5		3	8	1	10
exon1											
		AA	38	58	31	52		26	68	18	81
		AB	10	14	15	19		4	28	13	28
	A/O	AC	0	2	0	3		0	6	0	2
		AD	7	5	8	7		4	22	3	16
	O/O	BB	1	0	2	0		0	2	1	1
		BC	0	0	1	0		0	0	0	0
		BD	1	2	2	0		2	2	0	2
		CC	0	0	0	0		0	0	0	1
		DC	0	0	0	0		0	2	0	0
		DD	2	3	0	3		0	1	1	0
secretor haplotype* ^a											
		A/A and YA/O	50	72	41	71		31	110	28	117
		XA/O and O/O	9	12	18	13		5	21	8	14
FCN2											
rs3124952	-986	A→G	11	22	17	21					
		AA									
		AG	31	41	33	46					

Supporting Table 2. (continued)

Principal Study										Confirmation Study						
					Recipient		Donor		Recipient		Donor					
					CSI (n=59)	No CSI (n=84)	CSI (n=59)	No CSI (n=84)	CSI (n=36)	No CSI (n=131)	CSI (n=36)	No CSI (n=131)				
dbSNP ID	SNP	Genotype														
rs3124953	-602	G→A	GG	17	21		9	17								
				42	52		42	50								
				16	29		14	30								
rs3811140	-557	A→G	AA	1	3		3	4								
				50	69		46	67								
				5	15		13	17								
rs17514136	-4	A→G	GG	4	0		0	0								
				30	49		22	43								
				23	24		35	35								
rs17549193 ^a	+6359	C→T	T236M	6	11		2	6								
				32	49		18	41								
				22	25		37	36								
rs7851696	+6424	G→T	GG	5	10		4	7								
				49	68		49	69								
				10	16		9	15								
MASP2	N/A		TT	0	0		1	0								
rs12711521 ^a	+371	G→T	D371Y	57	83		54	80								
				2	1		5	4								
				0	0		0	0								
				0	6		1	3								
			AC	22	29		16	34								

Supporting Table 2. (continued)

dbSNP ID	SNP	Principal Study				Confirmation Study			
		Recipient		Donor		Recipient		Donor	
		Genotype	CSI (n=59)	No CSI (n=84)	CSI (n=59)	No CSI (n=84)	CSI (n=36)	No CSI (n=131)	CSI (n=36)
AA		37	49	42	47	24	86	26	87

CSI denotes clinically significant infection.

*Five single nucleotide polymorphisms in *MBL2* were genotyped: MBL2 -550 (H/L), rs11003125; MBL2 -221 (X/Y), rs7096206; MBL2 codon 52 (D), rs5030737; MBL2 codon 54 (B), rs1800450 and MBL2 codon 57 (C), rs1800451. Structural variants D, B and C are collectively called O; where A is considered wild type; Secretor haplotypes were constructed: A/A and YA/O versus XA/O and O/O.

^a SNP distribution (%) of the recipients in both cohorts were comparable: MBL secretor haplotype [A/A and YA/O] / [XA/O and O/O]; principal 85/15, confirmation 84/16; FCN2 rs17549193 CC/CT/TT: principal 57/33/10, confirmation 57/37/6; MASP2 rs12711521 CC/AC/AA: principal 4/36/60, confirmation 2/32/66.

Supporting Table 3. Univariate and Multivariate Analysis for the Association of Risk Factors of Clinically Significant Bacterial Infection in all OLT Patients (N=310)

Variable		Univariate Models			Multivariate Model†	
		LR (χ^2)	% CSI (n)	P Value	HR (95% CI)	P Value
Donor-Recipient Genotypic group	<i>MBL2</i> *	12.3		<0.001		0.001
	XA/O and O/O donor and A/A and YA/O recipient	14.9	52 (25/48)	<0.001	2.67 (1.61-4.43)	<0.001
	XA/O and O/O donor and recipient	0.08	20 (1/5)	0.78	0.54 (0.070-4.09)	0.55
	A/A and YA/O donor and XA/O and O/O recipient	0.4	31 (13/42)	0.55	0.97 (0.51-1.83)	0.91
	A/A and YA/O donor and recipient	Ref	26 (56/215)		1.00 [Reference]	
	<i>FCN2</i> **	10.8		0.01		0.009
	CT or TT donor and CC recipient	11.0	41 (36/87)	0.01	2.84 (1.55-5.20)	0.001
	CT or TT donor and recipient	4.9	33 (27/82)	0.03	1.91 (1.01-3.62)	0.02
	CC donor and CT or TT recipient	3.3	31 (16/52)	0.07	1.86 (0.92-3.88)	0.09
	CC donor and recipient	Ref	18 (16/89)		1.00 [Reference]	
	<i>MASP2</i> **	7.3		0.06		0.15
	AA donor and AC and CC recipient	7.7	40 (27/68)	0.006	2.71 (1.14-6.48)	0.03
	AA donor and recipient	3.9	31 (41/134)	0.05	2.41 (1.06-5.45)	0.04
	AC and CC donor and AA recipient	3.7	32 (20/62)	0.05	2.45 (1.00-5.97)	0.05
	AC and CC donor and recipient	Ref	15 (7/46)		1.00 [Reference]	
	(Mis)match profile***	33.3		<0.001		
	3 variants	6.1	65 (17/26)	0.01		
	2 variants	2.8	36 (63/177)	0.09		
	1 variant	1.0	15 (15/101)	0.31		
	no variants	Ref	0 (0/6)			
Gender		10.0		0.02		0.04
	Male donor and male recipient	9.0	40 (43/107)	0.003	2.54 (1.29-4.99)	0.007
	Male donor and female recipient	3.2	31 (16/52)	0.07	1.71 (0.76-3.84)	0.20

Multivariate Model (Backward Wald)		Multivariate Model†		Final Multivariate Model (Backward Wald)	
HR (95% CI)	P Value	HR (95% CI)	P Value	HR (95% CI)	P Value
	0.002				
2.51 (1.55-4.07)	<0.001				
0.54 (0.073-3.99)	0.54				
1.01 (0.55-1.87)	0.97				
1.00 [Reference]					
	0.008				
2.82 (1.56-5.10)	0.001				
1.95 (1.04-3.66)	0.04				
1.85 (0.91-3.75)	0.09				
1.00 [Reference]					
	0.07				
3.09 (1.33-7.17)	0.009				
2.52 (1.12-5.65)	0.03				
2.41 (1.01-5.74)	0.05				
1.00 [Reference]					
			3.0 E-06		1.8E-06
		6.27 (3.07-12.80)	4.8E-07	6.26 (3.11-12.61)	2.9E-07
		2.74 (1.54-4.86)	5.9E-04	2.75 (1.57-4.84)	4.4E-04
		1.00 [Reference]		1.00 [Reference]	
	0.04		0.03		0.04
2.40 (1.26-4.59)	0.008	2.43 (1.24-4.74)	0.009	2.31 (1.22-4.40)	0.01
1.62 (0.76-3.48)	0.21	1.66 (0.74-3.70)	0.22	1.55 (0.73-3.30)	0.25

Supporting Table 3. (continued)

Variable		Univariate Models			Multivariate Model†	
		LR (χ^2)	% CSI (n)	P Value	HR (95% CI)	P Value
Type of immunosuppression	Female donor and male recipient	1.2	28 (24/86)	0.268	1.53 (0.74-3.19)	0.26
	Female donor and female recipient	Ref	18 (12/65)		1.00 [Reference]	
	P / CNI / B	1.8	28 (59/209)	0.19	0.75 (0.44-1.27)	0.29
	P / CNI	Ref	36 (36/101)		1.00 [Reference]	
Antimicrobial prophylaxis	GCPM + SDD	16.9	41 (59/143)	<0.001	1.84 (1.11-3.05)	0.02
Acute cellular rejection	ACC, No SDD	Ref	22 (36/167)		1.00 [Reference]	
	Yes	4.9	23 (25/107)	0.03	0.74 (0.43-1.28)	0.28
	No	Ref	35 (70/203)		1.00 [Reference]	
Lab MELD score		1.0	31 (95/310)	0.57	1.00 (0.98-1.03)	0.81
Donor age		0.99	31 (95/310)	0.25	1.00 (0.99-1.02)	0.91
Recipient age		0.99	31 (95/310)	0.45	0.99 (0.97-1.01)	0.48
Donor type	DCD	1.0	40 (10/25)	0.33	1.00 (0.48-2.11)	1.0
CMV serostatus donor/recipient	DBD	Ref	30 (85/285)		1.00 [Reference]	
		1.6		0.67		0.55
Underlying disease	D+/R+	0.8	26 (25/95)	0.37	0.64 (0.33-1.25)	0.19
	D+/R-	0.1	30 (13/44)	0.72	0.88 (0.40-1.94)	0.75
	D-/R+	0.0	33 (39/119)	0.96	0.88 (0.47-1.64)	0.69
	D-/R-	Ref	35 (18/52)		1.00 [Reference]	
		1.10		0.78		0.97
	Other disease	1.0	28 (34/123)	0.32	0.87 (0.47-1.62)	0.66
	Cholestatic	0.1	33 (26/80)	0.79	0.89 (0.47-1.69)	0.72
	Alcohol	0.1	30 (14/46)	0.77	0.99 (0.48-2.05)	0.97
	Viral	Ref	34 (21/61)		1.00 [Reference]	

† Adjusted for recipient age (continuous), gender combinations (4 categories), type of immunosuppression, antimicrobial prophylaxis, acute cellular rejection (yes/no), Lab MELD score (continuous), donor age (continuous), donor type, CMV (4 categories), underlying disease (4 categories).

Multivariate Model (Backward Wald)		Multivariate Model†		Final Multivariate Model (Backward Wald)	
HR (95% CI)	P Value	HR (95% CI)	P Value	HR (95% CI)	P Value
1.56 (0.77-3.16)	0.22	1.41 (0.68-2.91)	0.36	1.42 (0.71-2.85)	0.32
1.00 [Reference]		1.00 [Reference]		1.00 [Reference]	
		0.73 (0.44-1.20)	0.22		
		1.00 [Reference]			
2.12 (1.39-3.22)	<0.001	1.83 (1.12-3.00)	0.02	2.12 (1.40-3.22)	4.1E-04
1.00 [Reference]		1.00 [Reference]		1.00 [Reference]	
		0.74 (0.44-1.26)	0.27		
		1.00 [Reference]			
		1.00 (0.98-1.03)	0.75		
		1.00 (0.98-1.01)	0.91		
		0.99 (0.98-1.01)	0.43		
		1.02 (0.50-2.11)	0.95		
		1.00 [Reference]	0.64		
		0.71 (0.37-1.37)	0.31		
		0.99 (0.45-2.15)	0.97		
		0.96 (0.52-1.77)	0.90		
		1.00 [Reference]	0.99		
		0.94 (0.51-1.73)	0.84		
		0.94 (0.50-1.78)	0.85		
		1.04 (0.51-2.14)	0.91		
		1.00 [Reference]			

Chapter 5

Mannose-binding lectin and ficolin-2 gene
polymorphisms predispose to
cytomegalovirus (re)infection after
orthotopic liver transplantation

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ABSTRACT

Background

The lectin pathway of complement activation is a crucial effector cascade of the innate immune response to pathogens. Cytomegalovirus (CMV) infection occurs frequently in immunocompromised patients after orthotopic liver trans-plantation (OLT). Single-nucleotide polymorphisms (SNPs) in the lectin pathway genes determine their liver-derived protein level and functional activity. We examined the association between these SNPs and the risk for CMV infection in OLT.

Methods

OLT patients (n = 295) were genotyped for recipient and donor SNPs in mannose-binding lectin (*MBL2*), Ficolin-2 (*FCN2*) and MBL-associated serine protease (*MASP2*) genes.

Results

Combined analysis of independently associated variant *MBL2* [HR 1.65, p <0.02] and wild-type *FCN2* [1.85; p <0.02] SNPs in the donor liver showed an increased risk of CMV infection for either and both risk genotypes [HR 2.02 and HR 3.26, respectively, p = 0.004], especially in CMV Donor-/Recipient+ (D-/R+) patients [HR 4.7 and HR 10.0, respectively, p = 0.01]. A genetic donor-recipient mismatch for *MBL2* and *FCN2* increased the CMV risk independently, also combined [HR 5.35; p <0.001], particularly in CMV D-/R+ patients [HR 16.6; p = 0.009]. Multivariate Cox analysis showed a consistent stepwise increase in CMV infection risk with the gene profile of the donor [up to HR 2.77; p <0.005] and the combined *MBL2* and *FCN2* donor-recipient mismatch profile [up to HR 4.57; p <0.001], independent from donor-recipient CMV serostatus, also at higher CMV (re)infection cut-off values.

Conclusions

MBL2 and *FCN2* risk alleles of donor liver and recipient constitute independent risk factors for CMV infection after OLT. Patients with these risk genes probably need intensified CMV monitoring and anti-viral therapy.

INTRODUCTION

Cytomegalovirus (CMV) is an important cause of opportunistic infection in immunocompromised individuals and can have severe manifestations.¹ The administration of immunosuppressive drugs after orthotopic liver transplantation (OLT) has a broad range of inhibitory effects on the adaptive immune system, which prevent graft rejection but increase the susceptibility to opportunistic infection, including CMV infection. After OLT, the patient thus becomes critically dependent on the innate immunity and the complement system is a pivotal component of this innate defense against pathogens. The structurally similar pattern-recognition receptors mannose-binding lectin (MBL) and Ficolin-2 bind carbohydrate ligands on pathogens and initiate the lectin pathway of complement activation via MBL-associated serine proteases (MASPs), predominantly through MASP-2. Common single nucleotide polymorphisms (SNPs) in the genes coding for these three members of the lectin complement pathway result in diverse protein variants and altered protein levels with potentially important functional implications, as recently shown by us for bacterial infections after OLT.²

Structural variations in the exon 1 region of the MBL gene (*MBL2*) interfere with the oligomerization of the protein and polymorphisms in the promoter regions alter the rate of synthesis of the protein, leading to changes in avidity and protein level of the lectin, respectively.^{3,4} Ficolin-2 (*FCN2*) gene polymorphisms within the carbohydrate-recognition domain encoding region of the *FCN2* gene are associated with a decreased (*FCN2-B*) or increased (*FCN2-C*) ligand binding of Ficolin-2 compared to wild-type Ficolin-2 (*FCN2-A*).⁵ The relation between MBL and CMV in solid organ transplantation has been previously studied, mainly in kidney transplantation,^{6–8} and only in a small number of patients after liver transplantation.⁹ Data concerning the relation between development of CMV infection and other components of the lectin pathway of complement, i.e., the Ficolins and the MASPs, have never been reported before. The combination of an immunocompromised patient and a new donor liver, as the major site where these proteins are synthesized, makes OLT a unique in vivo patient model to study the MBL–Ficolin–MASP gene profile in relation to CMV infection.

MATERIALS AND METHODS

Study population

Data were retrieved from the records of all patients undergoing orthotopic liver transplantation (OLT) at the Leiden University Medical Center (LUMC, from 1992 to 2006) and the University Medical Center Groningen (from 2000 to 2006) in The Netherlands. Follow-up data were collected until the end of December 2006. The study was performed on prospectively collected data and samples obtained with informed consent from the patients according to the guidelines of both Medical Ethics Committees, in compliance with the Helsinki Declaration.

For the present study, 426 patients were identified who underwent OLT from whom we were able to unselectively include 310 patients of whom DNA was available from both donor and recipient, and who had at least 7 days of follow-up after transplantation, thus excluding patients with perioperative morbidity and mortality. From 248 cases, blood CMV pp65 antigen follow-up within the first year after OLT was available. CMV-DNA quantification data were available in 47 cases because of transition from pp65 in the LUMC from June 2002 onwards. Thus, a final cohort of 295 patients undergoing an OLT was used for the present study.

Covariates and definitions

Data on covariates of interest were identified from the general patient database and from transplantation databases and included: age of recipient and donor at the time of OLT, gender, primary liver disease, donor and recipient CMV IgG anti-body status, acute cellular rejection, and date of death or last follow-up. Allograft rejection was diagnosed by liver allograft biopsy using the Banff classification scheme¹⁰ and these patients received additional immunosuppression (corticosteroids and other), which is known to increase the risk of CMV reactivation. Donor (D) and recipient (R) CMV serology were coded as a 4-level covariate (D-/R-, D-/R+, D+/R-, D+/R+).

Assessment of CMV infection and disease

CMV pp65 lower matrix protein had been detected routinely in leukocytes from ethylenediaminetetraacetic acid (EDTA) blood samples. Laboratory evidence for CMV infection was defined as a result of one or more cells positive for CMV pp65 antigen per 50,000 leukocytes. CMV-DNA loads had been determined prospectively in EDTA plasma by a validated quantitative real-time polymerase chain reaction (PCR) using the I-Cycler IQ DNA detection system (Bio-Rad, Veenendaal, The Netherlands), as previously described¹¹ with a detection limit of 0.90 log₁₀.

Both CMV screenings were performed twice a week during the first 21 days post-transplantation. Thereafter, the test was performed at each outpatient visit, at least every other week until postoperative day 90. Subsequently, screening was performed at least once every month until postoperative day 365. Since all patients had an undetectable CMV load at the time of transplantation, any subsequent detection of pp65 or CMV-DNA was considered CMV (re)infection in our study. Time to first positive CMV test result was calculated from the date of trans-plantation until the date of the first positive test. Those with persistently negative CMV assays were censored at day 365 and patients who died within 365 days after OLT were censored on the day of death.

Primary CMV infection was defined as the detection of CMV infection in an individual previously found to be CMV seronegative. Recurrent infection was defined as new detection of CMV infection resulting from either reactivation of latent virus (endogenous) or reinfection (exogenous) in a patient who has previously had a documented infection¹². CMV disease was

defined as the presence of clinical signs and symptoms compatible with the diagnosis, i.e., presence of fever (temperature, $>38^{\circ}\text{C}$), neutropenia ($<1.5 \times 10^9/\text{L}$), or thrombocytopenia ($<100,000/\text{mm}^3$), accompanied by the isolation of CMV from blood.¹² Tissue invasive CMV disease was defined when tissue samples were found to be positively stained for CMV.

Immunosuppression and antibiotic prophylaxis

Patients received routine immunosuppressive therapy consisting of corticosteroids, a calcineurin inhibitor (cyclosporine or tacrolimus) and either azathioprine or mycophenolate mofetil and/or basiliximab. With respect to the immunosuppressive therapy, azathioprine was used until 2001, and thereafter mycophenolate mofetil was given in case of impaired renal function. From 2001 on, basiliximab was used on days 0 and 4. In addition, patients received 24 h of pro-phylactic antibiotics intravenously (gentamycin, cefuroxim, penicillin G, and metronidazol) and 3 weeks of selective digestive tract decontamination (polymyxin/neomycin, norfloxacin, and amphotericin B) after OLT (131 patients) or amoxicillin–clavulanate and ciprofloxacin without selective digestive tract decontamination (164 patients).

CMV prophylaxis

All patients transplanted before 2002 received anti-CMV prophylaxis irrespective of their CMV status: D+/R- received 3 weeks of intravenous ganciclovir (induction), followed by oral acyclovir up to 3 months after OLT (maintenance). Patients with other serostatus combinations received oral acyclovir for 3 months. From 2002 onwards, CMV prophylaxis was restricted to patients with a D+/R- serostatus combination: these patients received prophylaxis with valganciclovir for 3 months, while the other D/R combinations did not receive prophylaxis. In both periods, preemptive therapy was used, meaning that a primary infection was treated upon first positivity of CMVpp65 test or PCR, while reactivations were treated if a relevant increase in CMVpp65 or CMV-DNA occurred after first positivity. The use of acyclovir, ganciclovir, and valganciclovir as prophylaxis was included as one variable to consider the combined effects of these medications in the analyses.

Genotyping and MBL-deficiency single-nucleotide polymorphisms

Genomic DNA was from peripheral blood and/or tissue samples. SNPs in the *MBL2*, *FCN2*, and *MASP2* genes were determined with the use of high-resolution DNA melting assays (HRMA) with the oligonucleotide primers as indicated in Supplementary Table 1.^{2,13,14} Briefly, HRMA of PCR products, amplified in the presence of a saturating double-stranded DNA dye (LCGreenPlus, Idaho Technology) and a 30-blocked probe, identifies both heterozygous and homozygous sequence variants by a change in melting temperature curves, verified by DNA sequenced controls.

Genotypic MBL studies have shown that different SNPs in the promoter and exon 1 of the MBL gene (B, C, and D collectively called O, while the wild-type allele for each position is called A) are in strong linkage disequilibrium. The association between MBL genotype

Table 1. Characteristics of orthotopic liver transplant recipients and donors (n = 295).

Variable	n (%)
Age (median, range)	
Recipient	51 (16-70)
Donor	46 (13-72)
Gender	
Male recipient	181 (61.4)
Male donor	146 (49.5)
Primary liver disease	
Viral	57 (19.3)
Alcoholic	44 (14.9)
Cholestatic	78 (26.4)
Other disease§	116 (39.3)
Immunosuppressive regimen	
Prednisone/CNI (+ azathioprine)	94 (31.9)
Prednisone/CNI/basiliximab (+ MMF)	201 (68.1)
Antiviral prophylaxis	
None	126 (42.7)
Acyclovir	127 (43.1)
Ganciclovir + Acyclovir	8 (2.7)
Valganciclovir	34 (11.5)
Acute cellular rejection	103 (34.9)
Child-Turcotte-Pugh class	
A	65 (22.0)
B	134 (45.4)
C	96 (32.5)
Lab MELD score (median, SD)	14 ± 9
CMV serostatus	
D+/R+	90 (30.5)
D+/R-	42 (14.2)
D-/R+	114 (38.6)
D-/R-	49 (16.6)

CNI, calcineurin inhibitors; MMF, mycophenolate mofetil; MELD, Model for End-Stage Liver Disease; CMV, cytomegalovirus; D, donor; R, recipient.

§Other diseases included predominately autoimmune hepatitis, cryptogenic cirrhosis, and metabolic disorders

and phenotype is very strong: sufficient functional MBL concentration is associated with YA/YA, YA/XA, XA/XA, and YA/O genotypes, whereas relatively deficient functional MBL concentration is associated with XA/O and O/O genotypes.^{15,16}

Statistical analysis

In the univariate analysis, pre-transplantation and post-transplantation covariates were analyzed for their association with CMV infection. Cox proportional-hazards survival

analysis was used for both discrete categorical variables and continuous variables. For survival analysis, cases were censored at the date of the last follow-up, death, or liver re-transplantation. The multivariate Cox proportional hazards regression analysis was used to evaluate the independence of covariates with a p value <0.15 in the univariate analyses. Results were considered statistically significant when p values were <0.05 . Bonferroni correction for multiple comparison tests was not performed because SNPs were selected on the basis of a deducible hypothesis, i.e., the reported associations with bacterial infections² and preliminary studies on CMV in OLT⁹ consequence of the SNP on the function of the respective proteins and the liver as their production organ. All analyses were performed with SPSS Statistical Software Package (version 16.02, SPSS Inc., Chicago, IL).

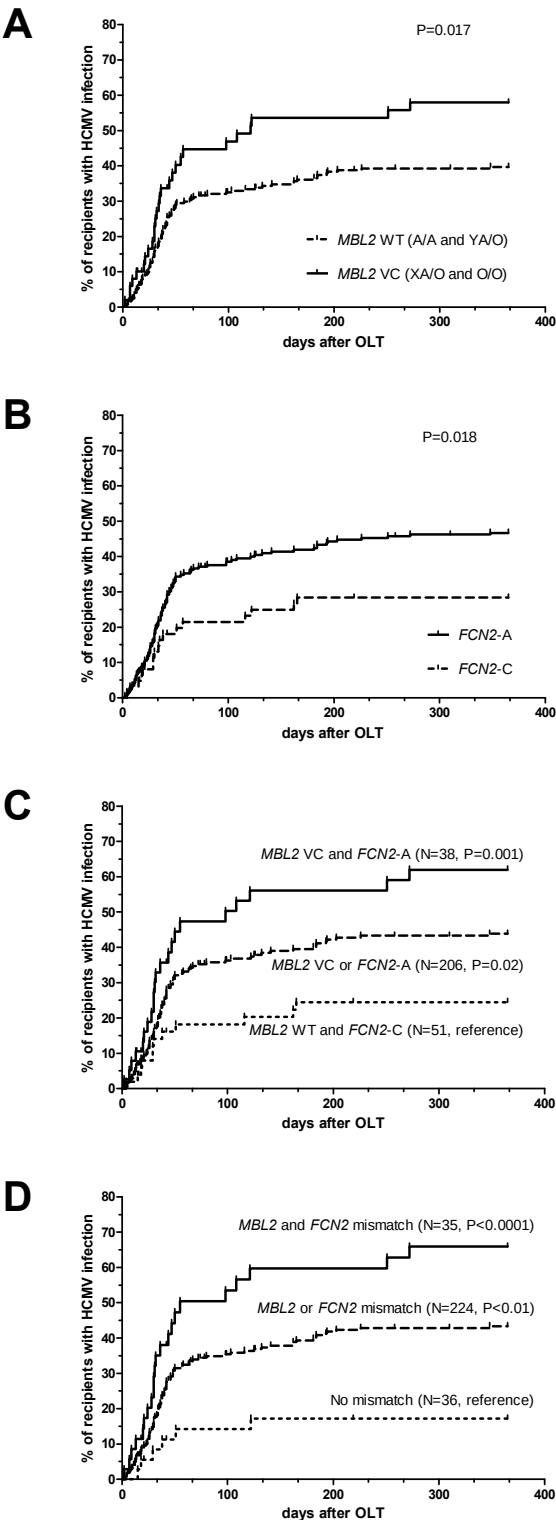
RESULTS

We evaluated 295 orthotopic liver transplantation recipient–donor pairs. Pre- and post-transplantation clinicopathological features of the patients are shown in Table 1. In 120 out of the 295 patients (35%), there was laboratory evidence of CMV infection within the first year after OLT, of which 90 (75%) were recurrent infections and 30 (25%) were primary infections. The median time to infection was 36 days (range 1–348 days) after transplantation. Out of these 120 patients, 37 (31%) [13% (37/295) of all patients] had CMV disease. All 295 recipients and their donor livers were tested for nine functional polymorphisms in the *MBL2*, *FCN2*, and *MASP2* genes (Supplementary Table 2).

MBL2 and FCN2 polymorphisms of the donor are associated with CMV infection

The *MBL2*, *FCN2*, and *MASP2* genotype distributions of the donor livers were analyzed in relation to the cumulative incidence of CMV infection within the first year post-OLT. In a univariate Cox proportional hazards model, patients receiving a liver from an MBL deficient donor (XA/O or O/O) were found to have a significantly increased risk of CMV infection compared to those receiving a wild-type liver [54% (27/50) vs. 38% (93/245) HR 1.65; CI, 1.07–2.54 $p = 0.02$] (Fig. 1A). In addition, there was a significant association with increased CMV infection in patients receiving a donor liver with an absence of the minor T-allele of *FCN2* SNP rs7851696 (*FCN2*-A) compared to a donor liver with at least one copy of the minor T-allele (*FCN2*-C) [44% (103/232) vs. 27% (17/63) [HR 1.85; CI, 1.11–3.13 $p < 0.02$] (Fig. 1B). In particular, the recurrent CMV infection in seropositive recipients was increased with an MBL deficient compared to a wild type donor liver [75% (24/37) vs. 40% (66/167), respectively, $p = 0.005$], and it was again found to be increased when receiving a *FCN2*-A donor liver compared to *FCN2*-C donor liver [49% (78/159) vs. 29% (12/42), $p = 0.02$].

The *FCN2*-B (rs17549193) and *MASP2* donor gene polymorphisms showed no significant association with CMV infection. Moreover, none of the gene polymorphisms related with CMV infection in the donor showed a direct association in the recipients (Supplementary Table 2). However, the genotype of the recipient in relation to the donor genotype was found to be a major determinant regarding the occurrence of CMV infection after OLT.



Recipients with a sufficient (wild type) MBL genotype (A/A and YA/O) receiving a donor liver with a deficient MBL genotype (XA/O and O/O) developed significantly more frequently a CMV infection than the other patients [58% (26/45) vs. 38% (94/250), respectively, $p = 0.006$]. Conversely, CMV infection was less frequent in low-binding *FCN2*-A recipients who received a high-binding *FCN2*-C donor liver compared to the other genotype combinations [22% (10/46) vs. 44% (110/249) $p = 0.009$].

Combined analysis of MBL2 and FCN2-C SNP in the risk of CMV infection

The combination of MBL deficiency genotypes (XA/O or O/O) and presence of *FCN2*-A in the donor liver revealed a significant relation with CMV infection (Table 2). To extend the findings of the univariate model, the combined impact of donor *MBL2* and *FCN2*-A genotype on the cumulative probability of CMV infection was assessed as shown in Fig. 1C.

In order to determine the risk of true CMV reactivation, we additionally analyzed the rates of CMV infection in the subgroup of seropositive recipients who received a liver from a seronegative donor, i.e., the D/R+ subgroup. In this particular group, cumulative CMV infection occurred significantly more often in recipients who received a liver from an MBL deficient (XA/O or O/O) and *FCN2*-A (54%, HR 10.0, $p = 0.004$) donor or in those with either one of these genotypes in the donor liver (35%, HR 4.7, $p = 0.04$) compared to those with the *MBL2* wild type and the presence of *FCN2*-C (9%, reference) (Supplementary Fig. 1).

Furthermore, in these D-/R+ patients, an *MBL2* mismatch, i.e., a sufficient recipient with a deficient donor liver, conferred a significantly increased risk for developing CMV reactivation compared to the other *MBL2* combinations [59% (10/17) vs. 27% (26/97) respectively, $p = 0.003$]. The *FCN2* mismatch, i.e., a *FCN2*-A recipient and a *FCN2*-C donor liver showed a trend toward less frequent CMV reactivation when compared to the other combinations [17% (4/23) vs. 35% (32/91) respectively, $p = 0.09$].

The combined *MBL2* and *FCN2* mismatch profile showed a notably higher risk for developing CMV infection when compared to the combined donor SNPs alone (5.35 vs. 3.26, see Tables 2 and 3, respectively). Notably, the rates of reactivation in the CMV D-/R+ subgroup increased with the number of mismatches compared to no mismatches (mismatch in both *MBL2* and *FCN2* 58%, HR 16.6, $p = 0.009$; mismatch in *MBL2* or *FCN2* 33%, HR 7.3, $p = 0.05$).

CMV disease in all OLT patients showed no significant relation with the number of variants present in the donor liver [10% (5/51) with no genetic variant; 13% (27/206) in those with one, and 13% (5/38) in those with two variants] or the combined mis-match profile [8%

- ◀ **Figure 1.** Kaplan-Meier estimation of human cytomegalovirus (HCMV) infection in liver transplant recipients with functional lectin pathway polymorphisms. In the donor liver: **A** *MBL2* variant carriers (VC) compared to *MBL2* wild types (WT). **B** minor T-allele of *FCN2*-C SNP compared to absence of the minor T-allele (*FCN2*-A). **C** combination of donor *MBL2* (XA/O or O/O) and *FCN2* (*FCN2*-A or *FCN2*-C). In donor-recipient: **D** combined *MBL2* and *FCN2* donor-recipient mismatch profile. p values were calculated with the use of the log-rank test.

Table 2. Univariate and multivariate analysis of the association of risk factors of CMV infection after OLT according to combined donor MBL2 and FCN2 genotypes (n = 295).

Variable	CMV infection % (n)	Univariable Model		Adjusted Multivariable Model	
		HR (95% CI)	p value	HR (95% CI)	p value
Donor genotypes*					
MBL2 XA/O and O/O and FCN2-A	58 (22/38)	3.26 (1.61-6.60)	0.001	2.77 (1.35-5.67)	0.005
MBL2 XA/O and O/O or FCN2-A	42 (86/206)	2.02 (1.10-3.70)	0.02	2.03 (1.10-3.75)	0.02
MBL2 A/A and YA/O and FCN2-C	24 (12/51)	1 [Reference]		1 [Reference]	
CMV serostatus					
D+/R+	60 (54/90)	14.49 (4.53-46.40)	<0.001	16.64 (4.90-56.50)	<0.001
D+/R-	64 (27/42)	13.88 (4.21-45.82)	<0.001	13.01 (4.02-42.10)	<0.001
D-/R+	32 (36/114)	6.31 (1.94-20.48)	0.002	6.48 (1.98-21.23)	0.002
D-/R-	6 (3/49)	1 [Reference]		1 [Reference]	
Age recipient					
Continuous	41 (120/295)	1.00 (0.98-1.01)	0.59		
Age donor					
Continuous	41 (120/295)	1.00 (0.99-1.01)	0.88		
Gender recipient					
Male	45 (81/181)	1.48 (1.01-2.18)	0.04	1.39 (0.93-2.09)	0.11
Female	34 (39/114)	1 [Reference]		1 [Reference]	
Gender donor					
Male	40 (59/146)	1.06 (0.74-1.51)	0.77		
Female	41 (61/149)	1 [Reference]			
Immunosuppressive regimen					
P/CNI/BAS (+ MMF)	42 (85/201)	1.21 (0.82-1.80)	0.34		
P/CNI (+ AZA)	37 (35/94)	1 [Reference]			
Acute rejection					
Yes	36 (37/103)	0.75 (0.51-1.11)	0.15	0.94 (0.63-1.40)	0.75
No	43 (83/192)	1 [Reference]		1 [Reference]	
Antiviral treatment					
Yes	41 (69/169)	0.90 (0.63-1.30)	0.59	0.62 (0.41-0.93)	0.02
None	40 (51/126)	1 [Reference]		1 [Reference]	
Primary liver disease					
Viral	49 (28/57)	1.38 (0.86-2.21)	0.19	1.06 (0.64-1.75)	0.82
Alcoholic	55 (24/44)	1.63 (0.99-2.68)	0.06	1.11 (0.67-1.85)	0.69
Cholestatic	31 (24/78)	0.78 (0.47-1.28)	0.32	0.73 (0.44-1.21)	0.23
other	38 (44/116)	1 [Reference]		1 [Reference]	

OLT, orthotopic liver transplantation; CMV, cytomegalovirus; D, donor; R, recipient; HR, hazard ratio; CI confidence interval; P, prednisone; CNI, calcineurin inhibitors; BAS, basiliximab; MMF, mycophenolate mofetil; AZA, azathioprine; *Four single nucleotide polymorphisms in MBL2 were genotyped: rs11003125; MBL2 -221 (X/Y), rs7096206; MBL2 codon 52 (D), rs5030737; MBL2 codon 54 (B), rs1800450 and MBL2 codon 57 (C), rs1800451. Structural variants D, B and C are collectively called O; where A is considered wild type; Secretor haplotypes were constructed: A/A and YA/O versus XA/O and O/O.

Table 3. Univariate and multivariate analysis of the association of risk factors of CMV infections in all OLT patients stratified according to donor–recipient genotypes (n = 295).

Variable	CMV infection % (n)				Univariate Model			Multivariate Model†			Final Multivariate Model†
	HR (95% CI)	p value	HR (95% CI)	p value	HR (95% CI)	p value	HR (95% CI)	p value	HR (95% CI)	p value	Model†
Donor-Recipient Genotypic mismatch	MBL2* XA/O and O/O donor and A/A and YA/O recipient	58 (26/45)	1.84 (1.19-2.84)	0.006	1.73 (1.10-2.71)	0.02					
	Other combinations	38 (94/250)	1 [Reference]			1 [Reference]					
	FCN2* Other combinations	44 (110/249)	1.33 (1.07-1.65)	0.009	1.29 (1.04-1.61)	0.02					
	FCN2-C donor and FCN2-A recipient	22 (10/46)	1 [Reference]			1 [Reference]					
Combined MBL2 and FCN2 mismatch profile‡	MBL2 and FCN2 mismatch	63 (22/35)	5.35 (2.17-13.20)	<0.001					4.57 (1.83-11.40)	<0.001	
	MBL2 or FCN2 mismatch	41 (92/224)	2.97 (1.30-6.78)	<0.01					3.09 (1.34-7.11)	<0.01	
	No mismatch	17 (6/36)	1 [Reference]						1 [Reference]		

OLT, orthotopic liver transplantation; CMV, cytomegalovirus; HR, hazard ratio, CI, confidence interval.

*The presence of a deficient donor *MBL2* secretor haplotypes in a sufficient recipient haplotype conferred the highest risk compared to other donor–recipient combinations.

‡The presence of a *FCN2*-A (low binding) recipient receiving a *FCN2*-C (high binding) donor liver showed a clear trend to less CMV infections as opposed to the other genotypes.

§The presence of donor–recipient mismatch associated with CMV within the individual was clustered as mismatch profile groups.

†Adjusted for CMV serostatus (four categories), gender recipient, acute rejection (yes/no), antiviral prophylaxis (yes/no), underlying disease (four categories), as in Table 2.

(3/36) with no mismatch; 13% (29/224) in those with one, and 14% (5/35) in those with two mismatches]. Occurrence of CMV disease in the D-/R+ subgroup showed a trend to increase with the number of variants present in the donor liver but this did not reach statistical significance [9% (2/23) with no genetic variant; 12% (9/78) in those with one and 23% (3/13) in those with two variants]. The mismatch profile showed an increase of symptomatic CMV disease in case of an *MBL2* mismatch [29% (5/17) vs. 9% (9/97) respectively, $p = 0.02$] but no relation with *FCN2* mismatches (data not shown). The combined mismatch profile also showed a trend to increase with the number of mismatches but this did not reach statistical significance [6% (1/18) with no mismatch; 12% (10/84) in those with one and 25% (3/12) in those with two mismatches].

Covariates

Analysis of other factors associated with development of CMV infection, using the univariate Cox proportional hazards model, is shown in Table 2. In this model, the four strata based on the CMV serostatus of donor and recipient showed a strong association with infection. Patients who were CMV D+/R- had the highest cumulative incidence of CMV infection (64%), followed by CMV D+/R+ (60%), CMV D-/R+ (32%), and CMV D-/R- (6%). CMV disease was most frequently observed in the CMV D+/R+ patients with a cumulative incidence of 17% (15/90) and 17% (7/42, one with suspected CMV hepatitis) in CMV D+/R-, followed by 12% (14/114, one with CMV gastritis) in CMV D-/R+, and 2% (1/49) in CMV D-/R- patients. Male gender of the recipient was significantly associated with more frequent CMV infection. Furthermore, a (non-significant) trend was observed concerning the type of primary liver disease in relation to the risk of HCMV infection.

Multivariate analysis

In the multivariate Cox proportional hazards model, adjusting for risk factors associated with CMV infection ($p < 0.15$), both donor *MBL2* (XA/O or O/O) genotypes [HR 1.56 (CI 1.01–2.42), $p < 0.05$] and *FCN2*-A [HR 1.69 (CI 1.01–2.86), $p < 0.05$] showed an independent relation with CMV infection. In the multivariate Cox proportional hazards model, adjusting for risk factors associated with CMV infection, the gene-dose related combinations of genetic variations in *MBL2* and *FCN2* in the donor liver [either gene HR 2.03, $p < 0.02$; both genes HR 2.77, $p < 0.005$], donor–recipient CMV serostatus pairs [HR 6.47, $p < 0.002$], and antiviral treatment [HR 0.62, $p < 0.02$] remained the only significant independent predictors of a CMV infection after OLT (Table 2). All other clinicopathological factors lost their predictive impact on the incidence of CMV. Despite the fact that antiviral prophylaxis was not associated with CMV infection in the univariate analysis ($p = 0.59$), it was included in the multivariate analysis as a possible confounder. Since the multivariate model showed that the individual mismatches in *MBL2* and *FCN2* were independently associated with an increased CMV infection risk (Table 3), both donor–recipient mismatch genotypes were included in the final multivariate model. An even higher CMV infection risk profile, compared to the donor gene profile alone (Table 2), was found for one or two mismatches as compared to no mismatch, with adjusted

Table 4. Univariate and multivariate analysis for the association of risk factors by extent of CMV positivity according to MBL2 and FCN2 genotype combinations in all OLT patients (n = 295).

Variable	CMV infection % (n)	Pp65 ≥3 or CMV-DNA log ≥2*				Pp65 ≥10 or CMV-DNA log ≥3*			
		Univariate Model		Multivariate Model†		CMV infection % (n)		Univariate Model	
		HR (95% CI)	p value	HR (95% CI)	p value			HR (95% CI)	p value
Donor genotypes	MBL2 XA/O and O/O and FCN2-A	3.05 (1.39-6.66)	0.005	2.55 (1.15-5.65)	0.02	34 (13/38)	2.60 (1.11-6.10)	1.99 (0.83-4.73)	0.12
	MBL2 XA/O and O/O or FCN2-A	1.76 (0.90-3.42)	0.10	1.80 (0.91-3.53)	0.09	24 (49/206)	1.54 (0.76-3.15)	1.48 (0.72-3.03)	0.29
	MBL2 A/A and YA/O and FCN2-C	1 [Reference]		1 [Reference]		18 (9/51)	1 [Reference]	1 [Reference]	
	Combined MBL2 and FCN2 mismatch	6.26 (2.10-18.62)	<0.001	5.18 (1.72-15.62)	0.003	37 (13/35)	6.43 (1.83-22.58)	4.64 (1.30-16.54)	0.02
MBL2 and FCN2 mismatch profile	MBL2 or FCN2 mismatch	3.31 (1.21-9.07)	0.02	3.43 (1.24-9.49)	0.02	25 (55/224)	3.58 (1.12-11.44)	3.27 (1.01-10.54)	0.05
	No mismatch	1 [Reference]		1 [Reference]		8 (3/36)	1 [Reference]	1 [Reference]	

OLT, orthotopic liver transplantation; CMV, cytomegalovirus; HR, hazard ratio; CI, confidence interval.

*Pp65 or CMV-DNA log increase in pre-OLT CMV positive recipients and any positive pp65 or CMV-DNA in pre-OLT CMV negative recipients.

†Adjusted for CMV serostatus (four categories), gender recipient, acute rejection (yes/no), antiviral prophylaxis (yes/no), underlying disease (four categories), as in Table 2.

hazard ratios of 3.09 [CI 1.34–7.11] and 4.57 [CI 1.83–11.40], respectively. To substantiate these observations, we performed similar analyses at increasing cut-off values of pp65 and CMV-DNA for CMV positivity. The univariate and multivariate analysis results, broken down by extent of CMV positivity, showed a consistent association between *MBL2* and *FCN2* genotype combinations, particularly the donor–recipient mismatches, and CMV infection (Table 4).

DISCUSSION

The present study revealed the association of polymorphisms in liver-derived members of the lectin pathway of complement activation with the occurrence of CMV infection after liver transplantation. Notably, liver transplant patients with single-nucleotide *MBL2* polymorphisms (XA/O or O/O) in the donor liver had significantly increased rates of CMV infection compared to recipients of *MBL2* wild type donor livers. Furthermore, the major allele of the *FCN2* SNP rs7851696 (*FCN2*-A) of the donor liver increased the chance of CMV infection compared to the minor allele (*FCN2*-C). The joint genetic effect of these *MBL2* and *FCN2* genotypes in the donor liver and donor–recipient mismatches were even stronger. The involvement of mannose-binding lectin in the immune response against CMV after solid organ transplantation was suggested earlier.^{6–9} For example, the study from Cervera et al.⁶ reported that low-MBL genotypes of the pre-transplant CMV positive recipient were associated with a higher proportion of CMV disease in renal transplantation. Despite the small number of patients (13%) who developed CMV disease in our OLT group, we also found a trend between genotypically variant donor livers or donor–recipient mismatches and the development of CMV disease in pre-transplant CMV positive recipients.

Our results indicate there is a pathogenetic link between several components of the lectin pathway of complement activation and the initial immune response against CMV after OLT, as we recently also reported for bacterial infections.² Both lectins i.e., MBL and Ficolin-2 are known to exert their effects via binding of specific carbohydrates presented on the surface of pathogens, leading to the activation of the complement cascade and enhanced phagocytosis in the host. CMV glycoproteins consist of proteins which are covalently linked to carbohydrates, like mannose and N-acetylglucosamine. The single nucleotide polymorphisms in the *MBL2* gene are associated with changes in avidity and production level of this lectin.³ The *FCN2*-C genotype was previously reported to be associated with stronger N-acetyl-glucosamine binding capacity compared to the wild type (*FCN2*-A).⁵ Therefore, gene polymorphisms of both lectins of the donor liver may very well contribute to their functional activity in the innate immune response against CMV after OLT.

The lectin complement pathway genes of the OLT recipients were not found to be intrinsically related to the risk of CMV infection, but a major indirect contribution related to the genetic constitution of the donor liver was observed. Particularly, MBL sufficient recipients receiving an MBL deficient donor liver were found to be at high risk for CMV infections, especially for CMV reactivation in the D/R+ subgroup. In addition, an *FCN2*-C donor

liver reduced the chance for CMV infection in an *FCN2*-A recipient as opposed to the other *FCN2* genotype combinations. These findings are to some extent similar to the observations we made previously with bacterial infections,² i.e., an association with the variant *MBL2* genotype and the donor recipient mismatch. In contrast, however, with bacterial infections, an association with *FCN2*-B and with *MASP2* was found which was not observed with the CMV infection. These observations illustrate intrinsic differences in the involvement of the lectin complement pathway in bacterial versus viral (CMV) infections after OLT.

Viruses have developed different strategies to evade complement-mediated destruction. Modulation of host immune responses is a common strategy for promoting virus persistence and avoiding clearance. CMV is known to encode numerous immunomodulatory genes and is capable of altering several innate immune responses in favor of its own survival, for instance cytotoxicity, inhibition of NF- κ B, etc..^{17–19} Complement activation is potentially harmful to the host by inducing tissue damage and is therefore carefully kept in place by regulators of complement activation. Inhibition of complement activation is important for CMV replication. So far no virus-encoded complement inhibitors have been identified for CMV, but several studies show that CMV upregulates the expression of host-encoded (surface) complement inhibitors²⁰ and counteracts complement activation by incorporation of host cell-derived complement regulatory proteins CD55 and CD59.²¹ Previous research also showed that the Toll-like receptor 2 (TLR2) Arg753Gln gene polymorphism seems to be associated with CMV replication after liver transplantation.²² Interestingly, MBL is able to interact with TLR 2 in the phagosome to initiate proinflammatory signaling²³ and, although speculative, this might also play a role in CMV infection after OLT.

In addition, there may be a subsequent mechanism by which MBL could be involved in CMV replication. MBL naturally occurs in two forms, S-MBL and I-MBL, which are synthesized mainly in the liver and translated from a single form of mRNA. S-MBL activates complement via the lectin pathway, whereas I-MBL acts as a putative intracellular cargo lectin for glycoprotein transport between the endoplasmic reticulum and Golgi apparatus.²⁴ The intracellular interaction of I-MBL with HCMV glycoproteins may thereby disrupt CMV virion assembly or formation, restricting CMV replication and transmission.

Liver transplant recipients who were initially seronegative for CMV, but received a graft from a CMV positive donor, are at greatest risk for CMV infection, similar to the observations with other organ transplants.²⁵ These D+/R- OLT patients always receive CMV prophylaxis and constitute only a small percentage [14% (42/ 295)] of our patients. Probably therefore, no association with the lectin and pathway genes was found in this group. Of the CMV seropositive recipients, only some will develop CMV reactivation and the optimal strategy for prevention of CMV infection in this subgroup has not yet been established. In our study, in CMV seropositive recipients, who received a liver from a CMV negative donor, reactivation of CMV occurred significantly more often when the donor liver was MBL-deficient. The presence of the minor allele of the *FCN2*-C SNP of the donor liver limited CMV infection compared to the presence of the major A-allele.

The impact of the lectin pathway genes in the CMV (re)infection process was reinforced by our observation that their contribution remained impressive even at higher cut-off values in the CMV seropositive recipients. In the CMV seronegative recipients, any CMV detection is already indicative for infection and necessitates treatment. Although the associations we found are observational findings, it is tempting to speculate that in CMV negative donors, due to the primary exposure of the liver to CMV from the recipient, the mentioned CMV-induced immune evasion responses have not yet been modulated by the virus, and therefore functional MBL and *FCN2*-C from the donor might still exert their initial protective effect.

At present, genetic risk factors for CMV infections are insufficiently understood, but a possible clinical application of our findings would be to screen for presence of *MBL2*–*FCN2* risk alleles in the donors for the CMV serostatus D-/R+ patients and subsequently intensify viral load or antigenemia monitoring in high risk patients.

There are some theoretical limitations to our study. Firstly, it is a retrospective study; nevertheless, frequencies of the studied SNPs associated with MBL deficiency and of the *FCN2*-C SNP in both donors and recipients were comparable to the frequency reported by others in healthy Caucasian populations.^{26–29} Furthermore, the two independent cohorts showed a consistent association and were comparable with regard to the genetic pro-file and the occurrence of CMV infection/reactivation (Supplementary Fig. 2) but separately did not have enough power, i.e. numbers, to be conclusive. Secondly, despite a relatively large combined patient cohort, only a small number of patients (13%) developed CMV disease or tissue invasive disease (two patients). These numbers were too small to perform robust statistical analyses on CMV disease in the different subgroups. Therefore, we limit our interpretation to the observation that donor and recipient *MBL2* and *FCN2* polymorphisms contribute to the CMV infection and/or reactivation in OLT recipients.

In conclusion, the present study strongly suggests an association between the occurrence of CMV infection and donor/recipient *MBL2* and *FCN2* gene polymorphisms in liver transplant patients, independent from other risk factors. These observations may have potential clinical implications, as recently discussed regarding this component of the innate immune system and infections after liver transplantation in general.³⁰ Therefore, further studies on these genetic risk factors in liver transplantation, including longer follow-up, more patient cohorts, and patients with invasive CMV disease, could contribute to novel CMV infection prevention strategies in these patients.

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SUPPLEMENTARY DATA

Supplemental Table 1. Oligonucleotide Primers for Polymerase Chain Reaction - High Resolution DNA Melting Assay

Gene	SNP	dbSNP ID	sequence		
			forward primer	reverse primer	3'-blocked probe (C6 spacer)
MBL2	promoter	-550	CCAGGGCCCAACGTAGTAAGA	CCAGCCCAGAAATTAAGTGA	CAGCAAGCCTGTCTAAACACCAAGG
		-221	TTCCCTAAGCTAACAGGCATAA	GCACTATGATGAGCAGTGGG	CCACGGAAAGCATGTTTATAGTCTT
	exon 1*				
FCN2		D (codon 52)			
		+223			
		rs5030737			
FCN2	exon 8	B (codon 54)			
		+230	CTGCAGTGATTGCCTGTAGC	GCCCAACACGTACCTGGTTC	GGCAAAAGATGGGCGTGATGCACCAAGGGA
		C (codon 57)			
MASP2		+239			
		rs1800451			
MASP2	exon 8	B	GATGTTACTGCCTGTAACGATGC	GGTGGAAGAAGGACTGGTTGTT	GAGATTCCCTGACGTTCCACACAAC
		+6359			
		C			
MASP2		+6424	CCAGGACAATGATCTTAACACC	CACATGGCAGTTTTTTGTACCAC	GGAAATTGTGCTGTGATGTTTCAGGG
		rs7851696			
MASP2	exon 1	+105	TGCATAGAAGGCCTCGAACC	GGCAAGGACACTTTTCTACTCGC	CTCGTTGGAGTAGCCGGAGCGGAAGG
		rs72550870			
	exon 9	+371	AGCTTTGTAGTGGTCACTCC	CCCTCCCATGCTTCTTTCTT	CCACTGGGTAGATCATCAGGAGGGCCA
		rs12711521			

*Ref. 14

Supplemental Table 2. Frequencies of Lectin Pathway Gene Polymorphisms in Orthotopic Liver Transplant Recipients and Donors

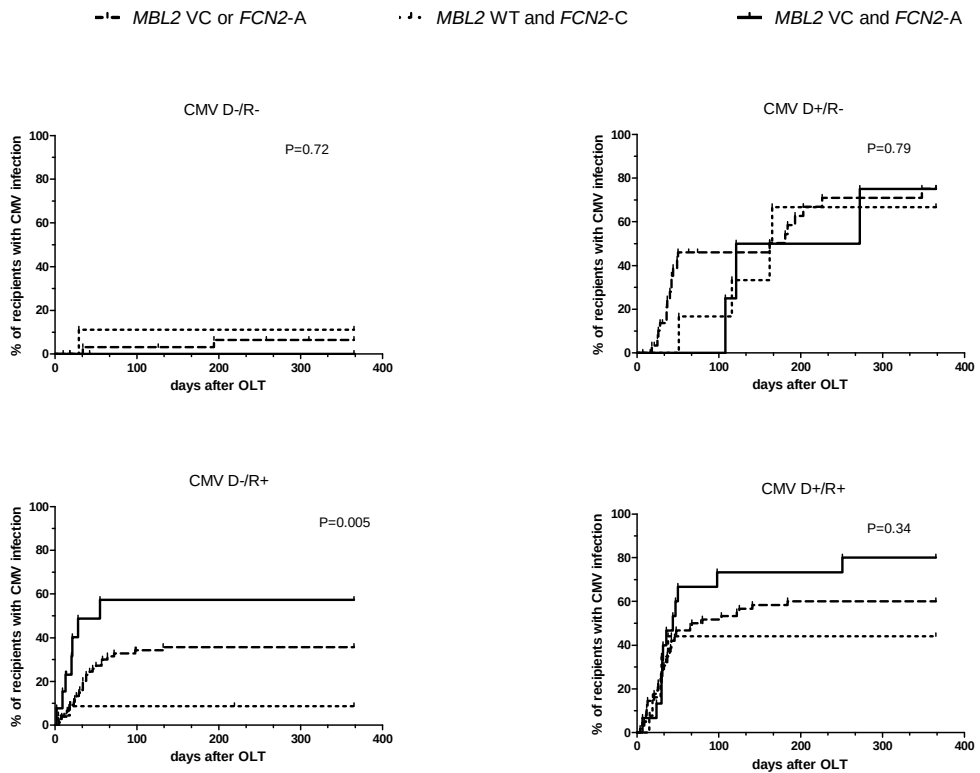
dbSNP ID	SNP	Genotype	Donor		Recipient	
			HCMV %(n=120)	No HCMV %(n=175)	HCMV %(n=120)	No HCMV %(n=175)
<i>MBL2</i> secretor haplotype ^{a, b}		A/A and YA/O	38 (93)*	62 (152)	43 (107)	57 (144)
		XA/O and O/O	54 (27)*	46 (23)	30 (13)	70 (31)
<i>FCN2</i> rs17549193	+6359 (B)	C → T				
		T236M	40 (55)	60 (84)		
		CT	41 (52)	59 (75)		
		TT	45 (13)	55 (16)		
		GG	44 (103)*	56 (129)	41 (96)	59 (136)
rs7851696 ^b	+6424 (C)	G → T				
		A258S	28 (17)*	72 (43)	38 (23)	62 (37)
		TT	0 (0)*	100 (3)	33 (1)	67 (2)
<i>MASP2</i> rs72550870	+105	A → G				
		D105G	41 (114)	59 (164)		
		AG	35 (6)	65 (11)		
rs12711521	+371	GG	0 (0)	0 (0)		
		G → T				
		D371Y	39 (74)	61 (117)		
		AA	44 (41)	56 (53)		
		AC				
		CC	50 (5)	50 (5)		

HCMV denotes Human cytomegalovirus.

^aFive single nucleotide polymorphisms in *MBL2* were genotyped: MBL2 -550 (H/L), rs11003125; MBL2 -221 (X/Y), rs7096206; MBL2 codon 52 (D), rs5030737; MBL2 codon 54 (B), rs1800450 and MBL2 codon 57 (C), rs1800451. Structural variants D, B and C are collectively called O; where A is considered wild type; Secretor haplotypes were constructed: A/A and YA/O versus XA/O and O/O.

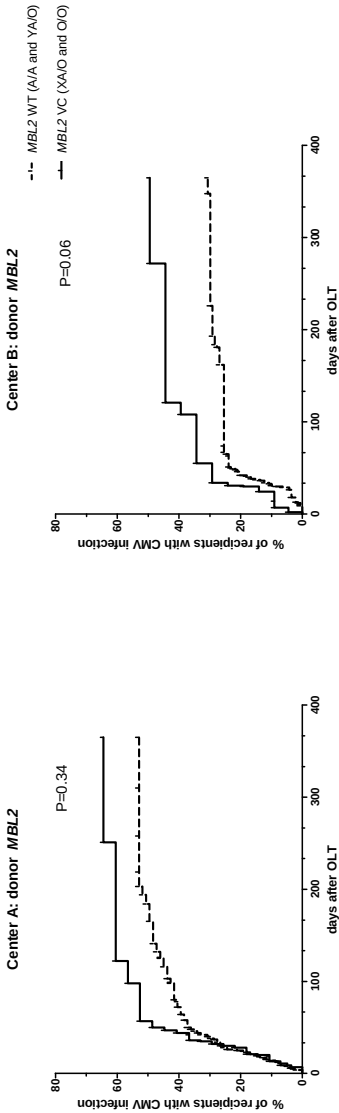
^b SNP distribution (%) of the donors and recipients were comparable to the frequency reported by others in healthy Caucasian populations.

*p Values <0.05, calculated with the use of the univariate Cox proportional-hazards survival analysis

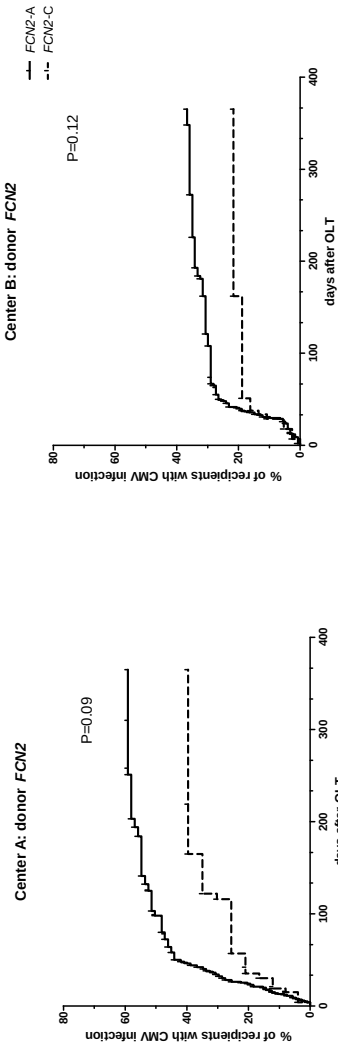


Supplemental Figure 1. Kaplan-Meier estimation of cytomegalovirus (CMV) infection in liver transplant recipients with functional lectin pathway polymorphisms in the donor liver according to the four strata based on the CMV serostatus of donor (D) and recipient (R). *MBL2* wild types (WT): A/A or YA/O; *MBL2* variant carriers (VC): XA/O or O/O; *FCN2*-A: absence of the minor T-allele; *FCN2*-C: presence of minor T-allele. P values were calculated with the use of the log-rank test.

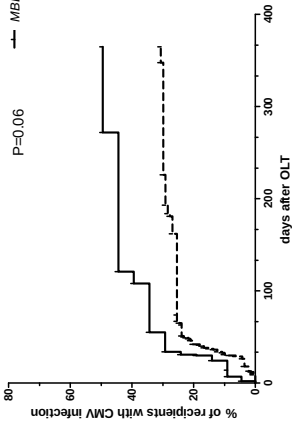
A



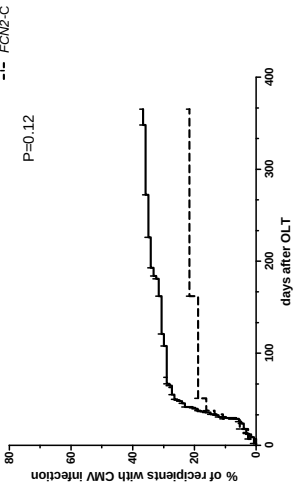
B



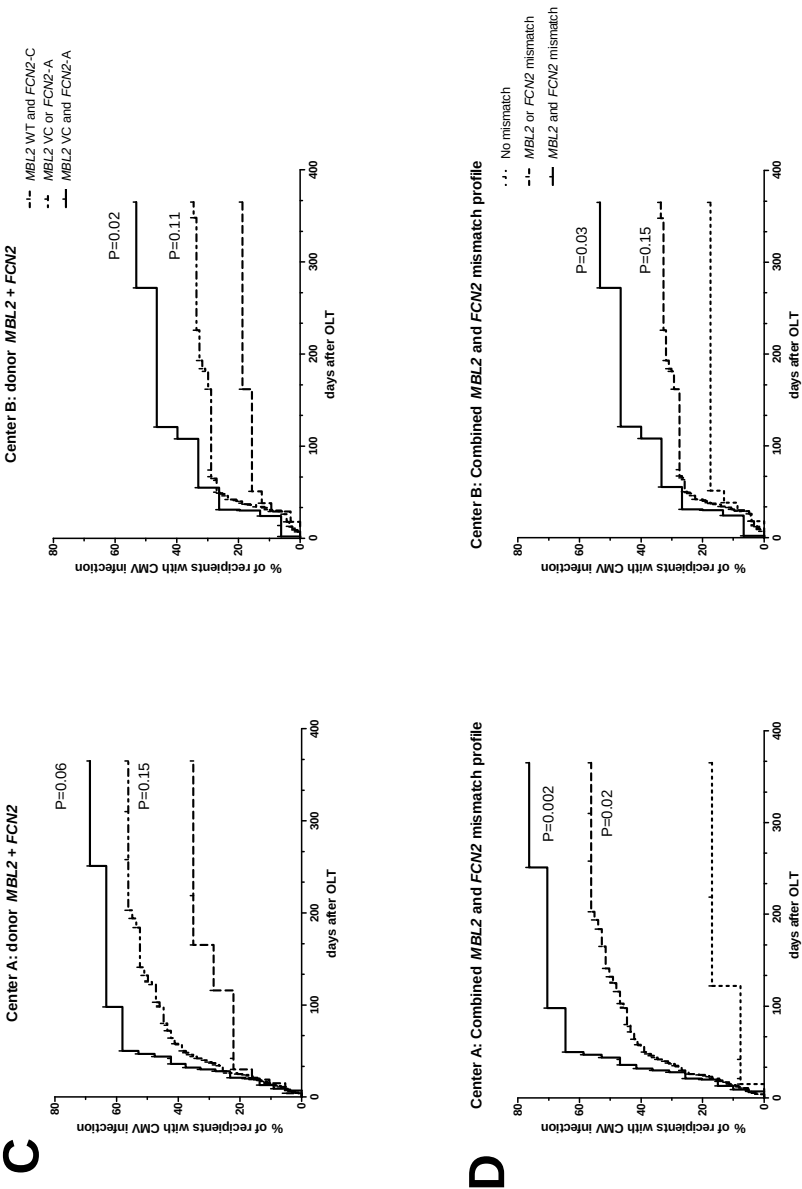
Center B: donor *MBL2*



Center B: donor *FCN2*



Supplemental Figure 2. Kaplan-Meier estimation of cytomegalovirus (CMV) infection in liver transplant recipients with functional lectin pathway polymorphisms in the donor liver for both independent centers, P values were calculated with the use of the log-rank test. A) *MBL2* variant carriers (VC) compared to *MBL2* wild types (WT). B) minor T-allele of *FCN2*-C SNP compared to absence of the minor T-allele (*FCN2*-A). C) combination of donor *MBL2* (X/A/O or O/O) and *FCN2* (*FCN2*-A or *FCN2*-C). D) combined *MBL2* and *FCN2* mismatch profile.



Supplemental Figure 2. (continued)

Chapter 6

Recipient's Genetic R702W *NOD2* Variant Is Associated with an Increased Risk of Bacterial Infections after Orthotopic Liver Transplantation

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ABSTRACT

Background

Orthotopic liver transplantation (OLT) is accompanied by a significant postoperative infection risk. Immunosuppression to prevent rejection increases the susceptibility to infections, mainly by impairing the adaptive immune system. Genetic polymorphisms in the lectin complement pathway of the donor have recently been identified as important risk determinants of clinically significant bacterial infection (CSI) after OLT. Another genetic factor involved in innate immunity is NOD2, which was reported to be associated with increased risk of spontaneous bacterial peritonitis in cirrhotic patients.

Methods

We assessed association of three genetic *NOD2* variants (R702W, G908R and 3020insC) with increased risk of CSI after OLT. 288 OLT recipient-donor pairs from two tertiary referral centers were genotyped for the three *NOD2* variants. The probability of CSI in relation to *NOD2* gene variants was determined with cumulative incidence curves and log-rank analysis.

Results

The R702W *NOD2* variant in the recipient was associated with CSI after OLT. Eight out of 15 (53.3%) individuals with a mutated genotype compared to 80/273 (29.3%) with wild type genotype developed CSI ($p=0.027$, univariate cox regression), illustrated by a higher frequency of CSI after OLT over time ($p=0.0003$, log rank analysis). Multivariate analysis (including the donor lectin complement pathway profile) showed independence of this R702W *NOD2* association from other risk factors (HR 2.0; $p=0.04$). The other *NOD2* variants, G908R and 3020insC, in the recipient were not associated with CSI. There was no association with CSI after OLT for any of the *NOD2* variants in the donor.

Conclusion

The mutated *NOD2* R702W genotype in the recipient is independently associated with an increased risk of bacterial infections after liver transplantation, indicating a predisposing role for this genetic factor impairing the recipient's innate immune system.

INTRODUCTION

Results of liver transplantations have steadily improved over the last decades with 5-years patient survival at 70%-80% in recent years. This success is attributable to improved operative techniques, earlier detection and treatment of post-operative complications and new immunosuppressive drugs.¹ However, infections still represent an important clinical problem after orthotopic liver transplantation (OLT) with significant patient morbidity and mortality.² There are several contributing factors for this high infection risk, for example the immunosuppressive agents that prevent rejection increase the susceptibility to infections, mainly by interference with the acquired immune system. In addition, previous studies showed that genetic polymorphisms in the lectin complement pathway of the donor also contributes significantly to the risk of infection after liver transplantation.^{3,4} With the current study we assessed whether genetic polymorphisms in NOD2 (nucleotide-binding oligomerization domain containing 2), also pivotal in the innate immunity, are associated with infectious complications and mortality after OLT.

NOD2 is an intracellular receptor, recognizing bacterial peptidoglycan, present in macrophages, dendritic cells and certain intestinal epithelial cells, including Paneth cells, that play an important role in innate immunity.⁵⁻⁷ Three genetic *NOD2* variants are known to be strongly associated with Crohn's disease.^{8,9} The same *NOD2* variants are also involved in several infectious conditions: an increase in sepsis related mortality in the ICU has been described¹⁰ and recently the occurrence of spontaneous bacterial peritonitis in liver cirrhosis patients was reported to be increased among *NOD2*-carriers and associated with reduced survival.¹¹ *NOD2* was also found to be associated with increased risk of graft versus host disease and transplant related mortality in haematopoietic stem cell transplantation,¹² and in kidney transplantation mutated *NOD2* haplotypes are associated with higher rates of death with functioning graft.¹³

Based on the above we hypothesized that genetic variants in *NOD2* could be associated with increased risk of bacterial infections after liver transplantation, potentially leading to impaired graft- and patient survival.

METHODS

The study was approved by the Medical Ethics Committee from the University Medical Center Groningen, the Netherlands and the Medical Ethics Committee from the Leiden University Medical Center, the Netherlands. From all patients written informed consent was obtained, all in compliance with the Helsinki Declaration

Study population

The study population consisted of 307 OLT recipient-donor pairs. From both participating centers only those patients whose DNA was available from both donor and recipient, and who had at least 7 days of follow-up after liver transplantation were included. 140 patients received OLT at the Leiden University Medical Center (LUMC) in the Netherlands between

1992 and 2005, and 167 patients received OLT at the University Medical Center Groningen (UMCG) in the Netherlands between 2000 and 2005. The cohort has recently been described in a previous study.⁴

The LUMC 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. The UMCG patients received standard immunosuppressive therapy consisting of basiliximab combined with a calcineurin inhibitor with or without corticosteroids and/or mycophenolate mofetil. Until 2001 azathioprine was used instead of mycophenolate mofetil in case of impaired renal function, and basiliximab on day 0 and 4 was introduced in 2001. All patients received 24 hours of prophylactic antibiotics intravenously: gentamycin, cefuroxim, penicillin G, and metronidazol in the LUMC, amoxicillin-clavulanate and ciprofloxacin in the UMCG. In addition, the LUMC patients received 3 weeks of selective digestive tract decontamination (polymyxin/neomycin, norfloxacin, and amfotericin B) after OLT. After surgery, all patients were intensively monitored according to standardized protocols for any infection, rejection, or poor function of the new liver. After hospital discharge, frequent regular visits and standard procedures in case of suspicion of an infection after OLT, including additional visits upon febrile temperature, are operational in both transplant centers.

Clinical Variables

The general patient database, original patient reports, transplantation databases, and microbiology records were evaluated to identify episodes of clinical and laboratory-confirmed bacterial infections within the first year after transplantation. The identified infections were considered clinically significant bacterial infections (CSI) when they complied with the Centers for Disease Control and Prevention criteria for diagnosing infection.¹⁴ All infections found were categorized into sepsis (including symptomatic urinary tract infection - urosepsis), pneumonia; and intra-abdominal infections, i.e., cholangitis and peritonitis.

Demographic and clinicopathological characteristics of the recipient at the time of OLT (age, sex, indication for liver transplantation, Child-Pugh classification, and laboratory Model for End-Stage Liver Disease [MELD] score), donor information (age, sex), and posttransplant follow-up data (immunosuppressive regimen, acute cellular rejection according to the Banff scheme¹⁵) were also collected from the transplantation databases. Clinical characteristics of donors and recipients are shown in Table 1.

Genotyping of the *NOD2* variants

Genomic DNA was extracted routinely from peripheral blood and/or tissue samples.

All donors and recipients were genotyped for the *NOD2* SNPS rs2066844 (R702W), rs2066845 (G908R), and rs2066847 (frame-shift mutation 1007fs, or 3020insC) by means of Applied Biosystems Taqman (ABI 7900 HT)[®] technology according to the manufacturer's recommendations at the Laboratory of Genetics at the UMCG in January 2012.

Table 1. Characteristics of all Eligible Orthotopic Liver Transplant Recipients and Donors in the Study (N=307).

Variable	
Age (median, IQR)	
Recipient	51 (41-56)
Donor	46 (34-55)
Gender donor/recipient, N (%)	
Male/male	105 (34.2)
Male/female	52 (16.9)
Female/male	85 (27.7)
Female/female	65 (21.2)
Primary liver disease, N (%)	
Viral	59 (19.2)
Alcoholic	46 (15.0)
Cholestatic	79 (25.7)
Other disease [§]	123 (40.1)
Child-Pugh class, N (%)	
A	67 (21.8)
B	138 (45.0)
C	102 (33.2)
Lab MELD score (median, IQR)	15 (11-21)
Donor-type, N (%)	
Donation after cardiac death	24 (7.8)
Donation after brain death	283 (92.2)
Maintenance immunosuppression, N (%)	
Prednisone / CNI / basiliximab (+ MMF)	206 (67.1)
Prednisone / CNI (+ azathioprine)	101 (32.9)
Acute cellular rejection, N (%)	
Yes	107 (34.9)
No	200 (65.1)
Bacterial infection, N (%)	94 (30.6)
LCBI	52 (55.3)
PNEU	10 (10.6)
IAB	32 (34.0)

IQR denotes interquartile range; MELD, Model for End-Stage Liver Disease; CNI, calcineurin inhibitors; MMF, mycophenolate mofetil; LCBI, laboratory-confirmed bloodstream infection; PNEU, pneumonia; IAB, intra-abdominal infection. § Other diseases included predominately autoimmune hepatitis, cryptogenic cirrhosis and metabolic disorders.

For rs2066844 and rs2066845 pre-made assays were provided by applied biosystems (AB ID:C_11711717468_20 and AB ID C_11717466_20 respectively). The genotyping assay for rs2066847 was custom developed by Applied Biosystems (forward primer GTCCAATAACTGCATCACCTACCT; reverse primer CAGACTTCCAGGATGGTGTTCATTC; VIC-labeled MGB probe CAGGCCCTTGAAAG; FAM-labeled MGB probe CAGGCCCTTGAAAG).

The results of the genotyping assay were analyzed using the SDS 2.3 software compatible with the Applied Biosystems Taqman®. Results obtained from SDS were exported to excel and SPSS for further statistical analyses.

Statistical analysis

Associations between baseline characteristics of the liver transplant recipients, donors, and posttransplant follow-up data and CSI were performed with the use of univariate Cox regression models. The probability of clinically significant infection within the first year after transplantation in relation to *NOD2* gene variants was determined with cumulative incidence curves using Kaplan-Meier analysis, and the differences between groups were assessed by log-rank test. Patients were censored at the date of the last follow-up, death, or liver retransplantation. The multivariate Cox proportional hazards regression analysis was used to evaluate the independence of the potential association of R702W, G908R and 3020insC with infection. Factors associated with infection with a P value of less than 0.15 in the univariate analysis were entered in the multivariate model and non-significant factors were removed by means of a backward-selection procedure. In this multivariate analysis it was also assessed whether associations were independent from previously described associated genetic lectin pathway variants in the donor.⁴ Results were considered statistically significant when P values were <0.05. Bonferroni correction for the number of SNPs tested was not performed because SNPs were selected on the basis of a deducible hypothesis. All analyses were performed with the SPSS statistical software package (version 16.02; SPSS, Inc., Chicago, IL).

RESULTS

Prevalence of NOD2 variants in recipients and donors

Overall more than 95% of all individuals were successfully genotyped for the three *NOD2* variants R702W (rs2066844), G908R (rs2066845) and 3020insC (2066847). In individuals with all variants successfully genotyped any *NOD2* variant was present in 39/287 (13.6%) of the recipients of an OLT and in 54/289 (18.7%) of the liver donors ($p=0.15$).

The minor allele frequency (MAF) in R702W was higher in donors compared to recipients (5.1% and 2.8%, $p=0.050$). In G908R MAF was 2.9% in recipients and 2.0% in donors ($p=0.35$) and in 3020insC MAF was 1.2% in recipients and 2.2% in donors ($p=0.18$). Table S1 illustrates genotype distribution for all three SNPs in recipients and donors stratified for occurrence of CSI.

NOD2 variants in recipients and donors in relation to infectious complications

R702W in the recipients, successfully determined in 288 cases, was significantly associated with the development of infectious complications after liver transplantation. In recipients with one or two risk alleles (genotype CT and genotype TT) for R702W the CSI frequency was 53.3% (8 out of 15) as opposed to 29.3% (80 out of 273) in recipients with the wildtype

(CC) genotype (HR 2.1, $p = 0.03$, univariate cox regression). The development of a CSI over time was also significantly higher in individuals with one or to mutated alleles in R702W ($p < 0.001$, log rank analysis, Figure 1.). One recipient carrying the TT genotype developed infection early after transplantation. When grouping the two risk genotypes (CT and TT) together, the association was borderline nonsignificant ($p=0.070$, univariate cox regression; $p = 0.063$, log rank analysis; $p=0.096$ in multivariate analysis). There was no significant relation between site-specific infections (i.e., laboratory-confirmed bloodstream infection, pneumonia or intra-abdominal infection) and mutated alleles in R702W (data not shown).

The association of recipient *NOD2* R702W showed the same trend direction in both independent sub cohorts. In cohort 1 the only individual with TT genotype and two out of six (33.3%) individuals with CT genotype compared to 33 out of 151 (21.9%) individuals with CC genotype developed infection after transplantation ($p < 0.001$, log rank analysis). In cohort 2 the similar, although not statistically significant, trend was observed, i.e., five out of eight (62.5%) individuals with CT genotype developed infection after OLT compared to 47 out of 122 (38.5%) individuals with the CC genotype ($p = 0.40$, log rank analysis).

In the other two *NOD2* variants, G908R and 3020insC, recipient genotypes were equally distributed between patients that did or did not develop a CSI ($p = 0.13$ and $p = 0.96$ respectively). Regarding the donor genotypes there was no significant association with the number of CSI after transplantation for any of the three *NOD2* variants (R702W $p = 0.26$, G908R $p = 0.16$, 3020insC $p = 0.65$; Table S1).

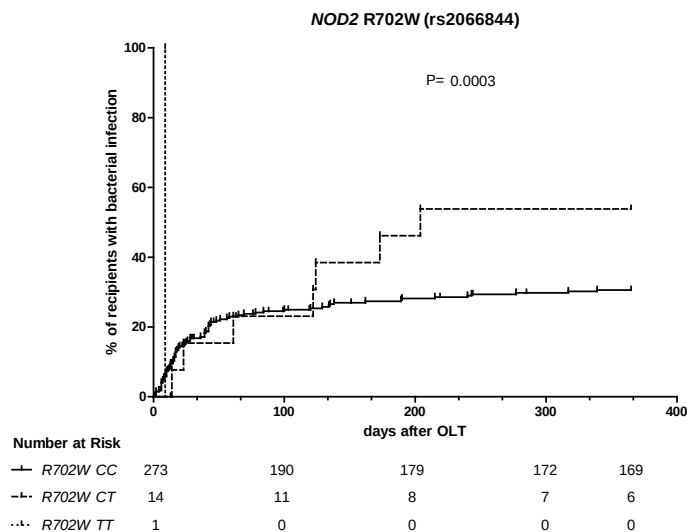


Figure 1. Development of clinically significant infection over time in relation to the recipient *NOD2* R702W genotype. Occurrence of clinically significant infection in days after OLT in relation to the recipient *NOD2* R702W genotype. CC = wildtype, TT homozygote for mutation, CT = heterozygote. The numbers below the Kaplan – Meier curves represent the number of individuals 'at risk' per genotype at particular time points after transplantation. doi: 10.1371/journal.pone.0072617.g001

We also assessed whether there was a difference in occurrence of CSI in individuals carrying one or more of either *NOD2* variants in the recipient or donor. Twelve out of 39 (30.8%) individuals with one or more *NOD2* variant in the recipient developed CSI compared to 76 out of 248 (30.6%) individuals without recipient *NOD2* variant ($p = 0.97$). Similarly, regarding the donor *NOD2* status no difference was observed in the occurrence of CSI: 14 out of 54 (25.9%) individuals with one or more *NOD2* variant in the donor developed CSI compared to 70 out of 235 (29.8%) individuals without donor *NOD2* variant ($p = 0.48$)

Multivariate analysis

Based on the observed *NOD2* association a multivariate analysis was performed to assess whether the R702W risk allele was a risk factor for the development of CSI independent from recently described associations in the lectin pathway donor profile and other potential risk factors. This analysis showed that the association of the minor allele of *NOD2* R702W in the recipient is indeed independent from the lectin pathway profile of the donor and the only other significant contributing factor, namely the gender of the recipient donor pair (HR 2.0, $p = 0.04$; Table 2)

NOD2 variants in recipient and donor in relation to patient survival

None of the *NOD2* variants in donor or recipient was found to be associated with patient survival the first year after OLT. In total 27 patients deceased within one year after transplantation: one recipient carried the R702W variant, two recipients carried the G908R variant and two recipients carried the 3020insC variant. R702W, G908R and 3020insC was present in five, zero and one donor(s) respectively.

DISCUSSION

In the current study it was found that the existence of the *NOD2* variant R702W in the recipient is associated with an increased risk of bacterial infections after liver transplantation. This association was found to be independent of clinical variables, like gender, that are associated with increased bacterial infections. Furthermore, this *NOD2* R702W association was shown to be independent of the previously described infection risk mutations in the lectin complement pathway of the donor liver.⁴

Liver transplant recipients with one or two risk alleles for R702W (genotype CT and genotype TT) in the recipient developed more often a clinically significant infection, i.e., sepsis, pneumonia or abdominal infection, than individuals with a wild type genotype. It is interesting to note that one individual with homozygote TT risk for *NOD2* R702W variant in the recipient developed infection early after transplantation. The recipients with heterozygote CT risk genotype developed infection more than three months after transplantation. We observed no association with *NOD2* variants in recipient or donor with patient survival in the first year after liver transplantation.

Table 2. Univariate and Multivariate Analysis to Infections risk after orthotopic liver transplantation (N=288)

Variable	Bacterial infection % (N)	Univariate			Multivariate		
		HR	CI (95%)	c	HR	CI (95%)	P Value
Age							
Recipient	30.6 (88/288)	0.99	0.98-1.0	0.37			
Donor	30.6 (88/288)	0.99	0.98-1.0	0.11			
Gender recipient				0.02			0.02
Male/male	40.8 (40/98)	2.6	1.4-5.0	0.004	2.6	1.4-5.0	0.004
Male/female	30.0 (15/50)	1.8	0.84-3.8	0.13	1.5	0.68-3.2	0.32
Female/male	27.6 (21/76)	1.6	0.79-3.3	0.19	1.5	0.71-3.0	0.30
Female/female	18.8 (12/64)	ref			ref		
Primary liver disease				0.49			
Viral	37.5 (21/56)	1.5	0.86-2.6				
Alcoholic	26.3 (10/38)	1.1	0.52-2.1				
Cholestatic	33.3 (25/75)	1.3	0.77-2.2				
Other disease [§]	26.9 (32/119)	ref					
Child-Pugh class				0.14			
C	36.8 (35/95)	1.8	0.99-3.4	0.05			
B	30.0 (39/116)	1.4	0.76-2.6	0.29			
A	22.2 (14/63)	ref					
Lab MELD score	31 (88/288)	1.0	0.97-1.0	0.72			
Donor-type							
Donation after cardiac death	37.5 (9/24)	1.3	0.65-2.6	0.47			
Donation after brain death	29.9 (79/264)	ref					
Immunosuppression							
Prednisone/CNI/basiliximab (+ MMF)	28.1 (55/196)	0.74	0.48-1.1	0.18			
Prednisone/CNI (+ azathioprine)	35.9 (33/92)	ref					

Table 2. (continued)

Variable	Bacterial infection % (N)	Univariate			Multivariate		
		HR	CI (95%)	CI (95%)	HR	CI (95%)	CI (95%)
Acute cellular rejection							
Yes	24.0 (24/100)	0.60	0.38-0.95	0.03			
No	34.0 (64/188)	ref					
CMV seropositivity							
D+/R+	26.4 (23/87)	0.79	0.42-1.5	0.47			
D+/R-	30.8 (12/39)	0.95	0.45-2.0	0.89			
D-/R+	32.5 (37/114)	1.1	0.59-1.9	0.85			
D-/R-	33.3 (16/48)	ref					
Lectin complement pathway donor profile [‡]							
3 variants	63.2 (12/19)	1.8	1.4-2.3	<0.001	1.8	1.4-2.3	<0.001
2 variants	39.8 (41/103)	4.1	1.6-10.4	0.003	4.1	1.6-10.5	0.003
1 variant	21.7 (28/129)	2.5	1.1-5.5	0.03	2.3	1.0-5.1	0.047
0 variants	18.9 (7/37)	1.2	0.5-2.6	0.73	1.1	0.47-2.5	0.859
Recipient R702W		ref			ref		
TT	100 (1/1)	2.1	1.1-4.2	0.03	2.0	1.0-4.0	0.040
CT	50.0 (7/14)	18.1	2.4-136.6	0.005			
CC	29.3 (80/273)	1.7	0.80-3.8	0.16			

HR denotes hazard ratio; CI, confidence interval; MELD, Model for End-Stage Liver Disease; CNI, calcineurin inhibitors; MMF, mycophenolate mofetil
‡The presence of donor-recipient genotypic groups associated with bacterial infections within the individual were clustered as lectin pathway donor profile; MBL2: XA/O and O/O donor and A/A and YA/O recipient; FCN2: CT or TT donor and or recipient; MASP2: AA donor and or recipient

The risk of infections after transplantation of a solid organ is the resultant of the net state of an impaired immune defense. Multiple factors contribute to the increased risk of infections, including the outcome of transplantation with or without technical complications, graft dysfunction and the intensity of immunosuppression.^{16,17} Genetic predisposition of recipient and/or donor organ influencing the innate immune system is likely to play a central role in further increasing the risk of infection. For example, we recently reported that genetic variants in the lectin complement pathway of the donor increases the risk of CSI after transplantation. Components of the lectin pathway of complement activation, including mannan binding lectin 2 (MBL2), ficolin 2 (FCN2) and Mannan-binding lectin serine protease 2 (MASP2), are primarily produced in the liver. Therefore, functional SNPs in these polymorphic genes present in the donor liver would lead to reduced complement activation and opsonisation resulting in an increased infection risk. We now show that the *NOD2* R702W variant in the recipient is another innate risk factor for bacterial infections after OLT. *NOD2* is a critical component of the intestinal immunological barrier. In the initial period after transplantation this intestinal barrier is already compromised due to the postoperative state of the patient^{18,19}. Furthermore, bacterial translocation in cirrhosis is increased due to structural changes of the intestinal mucosa, including dilatation of intercellular space and vascular congestion as well as mucosal oxidative damage,²⁰ and probably these structural changes also contribute to a decreased barrier function during the recovery phase of the gut post transplantation.

As an intracellular receptor *NOD2* (nucleotide-binding oligomerization domain containing 2) recognizes bacterial peptidoglycans. This receptor is present in macrophages, dendritic cells and certain intestinal epithelial cells, including Paneth cells, and is known to play an important role in the innate immunity. The three *NOD2* variants R702W, G908R, 3020insC are all located in the coding region of *NOD2* and lead to an altered amino acid sequence, either due to non-synonymous SNPs that results in amino acid exchanges or through an insertion that results in a frame-shift mutation. Mutations in *NOD2* hence lead to a decreased activation of the NK- κ B pathway upon stimulation by bacterial peptidoglycans.^{8,9} *NOD2* is also involved in modulation of Toll-like receptor (TLR) signaling,²¹ and regarding this latter the recipient TLR2 R753Q variant has also been associated with presentation with septic shock and infection recurrence with gram-positive bacterial infections after OLT.²² The observation that this *NOD2* R702W variant is associated with CSI after OLT independently from the lectin-binding complement activation system also indicates that there might be an interplay between these antibacterial pathways which merits further functional studies.

Our findings complement the earlier identification of *NOD2* being a risk factor in multiple infectious conditions and transplantation related complications. *NOD2* has, for instance, been reported to be associated with increased sepsis-related mortality in patients admitted to an Intensive Care Unit.¹⁰ Another recent study reported an increased risk of spontaneous bacterial peritonitis (SBP) in cirrhotic patients carrying *NOD2* variants accompanied by a reduced patient survival time after SBP.¹¹ The same group also reported an increased susceptibility towards SBP in patients carrying TLR2 polymorphisms.²³ In transplant literature

NOD2 has been linked to increased risk of graft versus host disease and transplant-related mortality in hematopoietic stem cell transplantation.^{12,24} However, other reports could not confirm these results.²⁵ In kidney transplantation mutated *NOD2* haplotypes are associated with higher rates of death with functioning graft, but not with rejection and graft failure.¹³ Fishbein et al. found that Crohn's disease-associated *NOD2* variants were associated with decreased graft and patient survival after small bowel transplantation.²⁶ Other studies on small bowel transplantation were inconsistent,²⁷ although one study confirmed the R702W variant, the same variant that was related to CSI in the current study, was associated with early rejection and decreased patient survival.²⁸

There is an increasing interest in the use of biomarkers as predictors for outcome in solid organ transplantation. Various studies tried to estimate or predict the occurrence of rejection and chronic allograft damage by correlating genomic, transcriptomic and proteomic information from donor and recipient with clinical phenotypes. Genomic analysis, for instance, revealed that mutations in the innate immune system protein Toll-like receptors in donor and/or recipient were associated with reduced risk and severity of allograft rejection in liver, lung and kidney.²⁹ With increasing knowledge about factors involved in the risk of developing infections after liver transplantation such biomarkers might also be a useful tool to identify patients at risk for infections after transplantation. Especially considering the relatively high prevalence of bacterial infections after liver transplantation and the considerable impact on patient morbidity and mortality such predictors could have a considerable clinical impact. The current study adds to that knowledge by reporting the association of the *NOD2* R702W variant in the recipient with increased risk of bacterial infections after liver transplantation. However, the frequencies of the three *NOD2* variants we found are low but comparable to the frequencies reported in the literature³⁰ and implicates difficult assessment of association of the variants with clinical outcomes.

In conclusion, the mutated *NOD2* R702W genotype in the recipient is associated with an increased risk of bacterial infections after liver transplantation. This association is independent of clinical variables and mutations in the donor liver of the lectin complement pathway. This indicates a possible predisposing role for this recipient *NOD2* genetic risk factor impairing the innate immune system after orthotopic liver transplantation.

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SUPPLEMENTARY DATA

Table S1. Frequencies of NOD2 Polymorphisms in Orthotopic Liver Transplant Recipients and Donors

dbSNP ID	SNP	Genotype	Recipient				Donor			
			CSI	No CSI	HR	P value	CSI	No CSI	HR	P value
NOD2 R702W*	R702W	CàT			2.128	0.0270			1.401	0.264
		CC	80	193			75	191		
		CT	7	7			11	17		
		TT	1	0			0	1		
G908R**	R908R	GiàC			0.338	0.130			0.046	0.162
		GG	84	191			87	197		
		CG	2	15			0	12		
		CC	0	0			0	0		
3020insC***	c.3020insC	neg	89	201	0.962	0.957	84	199	0.763	0.645
		pos	2	5			3	10		

HR denotes hazard ratio.

* Recipient: undetermined 19/307 cases (6.2%); donor: undetermined 12/307 cases (3.2%); overall 29/614= 4.7%.

** Recipient: undetermined 15/307 cases (4.9%); donor: undetermined 11/307 cases (3.6%); overall 26/614= 4.2%.

*** Recipient: undetermined 10/307 cases (3.3%); donor: undetermined 11/307 cases (3.6%); overall 21/614= 3.4%.

Chapter 7

*Genetic variation in innate immunity pattern
recognition receptors and their signaling
molecules is not associated with spontaneous
bacterial peritonitis in liver cirrhosis*

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ABSTRACT

Background & Aims

Spontaneous bacterial peritonitis (SBP) is a frequent and life-threatening complication in patients with advanced liver cirrhosis. In these patients the complement system, as part of innate immunity, is assumed to be impaired, thereby facilitating the development of SBP.

In this study we investigated whether genetic variation in the genes coding for pattern recognition receptors (lectins, TLRs and NOD2) and their associated intracellular signaling molecules (Masp-2, C3, MD2 and MyD88) of the innate immune system contribute to susceptibility for SBP in patients with end-stage liver disease.

Methods:

Patients with end-stage liver disease (n = 280) were genotyped for SNPs in *MBL2*, *FCN2*, *MAS2P*, *C3*, *TLR2*, *TLR4*, *MD2*, *MYD88* and *NOD2*. These and clinical parameters were related to a history with or without SBP.

Results:

Nineteen percent of the patients had a history of SBP. Child-Pugh class and aetiology of liver disease showed a significant relation with the development of SBP. Univariate analysis revealed significantly more frequent SBP in the patients homozygous (GG) for *TLR4* D299G compared to homozygous wildtype (AA). However, in multivariate analysis only Child-Pugh class was a predictor for developing SBP (Child-Pugh A vs B: OR 4.9. P=0.013; A vs C: OR 6.8 P=0.003).

Conclusions:

None of the studied polymorphisms in pattern recognition receptors and their signalling molecules of the innate immune system was found to be associated with the development of SBP. The Child-Pugh classification was confirmed to be a strong independent risk factor for developing SBP.

INTRODUCTION

Spontaneous bacterial peritonitis (SBP) is one of the most frequent and life-threatening complications of patients with advanced liver cirrhosis. Even today the development of SBP is associated with a one-year mortality rate up to 20%.¹

The proposed hypothesis for the development of SBP in cirrhosis is alteration in compartments that are involved in promoting pathological bacterial translocation. These compartments include the gut microbiome, intestinal barrier function and the immune system. There are multiple influencing factors that have an impact on these compartments and genetic susceptibility of the host is assumed to be one of the key players.²

The lectin complement pathway plays an important role in initiating complement activation, opsonisation and elimination of pathogens. Single nucleotide polymorphisms (SNPs) in the genes coding for the liver-derived components of the lectin complement pathway result in diverse protein variants and altered protein levels. These can potentially have important functional implications, especially in immunocompromised persons, as previously shown by us for bacterial³ and cytomegalovirus (CMV) infections⁴ after orthotopic liver transplantation (OLT). The impact of these variants on SBP development, however, has not been elucidated yet.

The third component of complement, C3, is a central player of the complement system and the liver is responsible for 90% of systemic complement production.⁵ Low C3 levels in cirrhotic ascites were reported to predispose to develop SBP.⁶ Increased prevalence of the C3fast (F) allele has been associated with some immune-mediated disorders.⁷

Besides initiating complement activation, evidence has accumulated that indicates to an important role for MBL in modulating inflammation. MBL can facilitate enhanced signaling via complement receptors or presentation of bacterial ligands to Toll-like receptor (TLR)-2⁸ and Toll-like receptor 4 (TLR4).⁹ This implies that even if SNPs in *MBL2*, *FCN2* and *MASP2* are absent, the presence of SNPs influencing expression or function of TLRs and their associated intracellular signaling molecules might potentially influence the susceptibility to SBP.

We therefore evaluated whether genetic variation in the genes coding for these pattern recognition receptors (lectins, TLRs and NOD2) and their associated intracellular signaling molecules (Masp-2, C3, MD2 and MyD88) contributes to variations in susceptibility to SBP in patients with end-stage liver disease.

METHODS

Study population

The study was performed on prospectively collected data and samples obtained with informed consent from the patients according to the guidelines of both Medical Ethics Committees, in compliance with the Helsinki Declaration. Data were retrieved from the records of all patients on the waiting list who received orthotopic liver transplantation (OLT) at the Leiden University Medical Center from 1992 to 2006 and the University Medical

Center Groningen from 2000 to 2006 in The Netherlands. Follow-up data was collected until the moment of transplantation.

For the present study, 426 patients with end-stage liver cirrhosis were identified who underwent OLT of whom we were able to unselectively include a final cohort of 280 patients from whom DNA was available. The severity of the underlying liver disease was assessed according to the Model of End-stage Liver Disease (MELD) and the Child-Pugh classification.

Assessment of spontaneous bacterial peritonitis

The diagnosis of SBP was made if the ascitic fluid polymorphonuclear cell count was greater than 250 /mm³ (0.25×10^9 /l), following the criteria of the International Ascites Club.¹⁰

Genotyping

Genomic DNA was extracted from peripheral blood and/or tissue samples prior to liver transplantation. SNPs in the *MBL2*, *FCN2* and *MASP2* gene were determined using a previously described high resolution DNA melting assays (HRMA).³ Genotypic MBL studies have shown that different SNPs in the promoter and exon 1 of the MBL gene (B, C and D collectively called O, whereas the wild-type allele for each position is called A) are in strong linkage disequilibrium. The association between MBL genotype and phenotype is very strong: sufficient functional MBL concentration is associated with YA/YA, YA/XA, XA/XA and YA/O genotypes, whereas relatively deficient functional MBL concentration is associated with XA/O and O/O genotypes.¹¹⁻¹²

SNPs in the *TLR4* (rs4986790 and rs4986791), *TLR2* (rs5743708), *MyD88* (rs6853) and *MD2* (rs11466004) gene were determined using high resolution DNA melting assays (HRMA) with the oligonucleotide primers as indicated in Supplemental Table 1.

Complement C3 'slow' and 'fast' allotypes (rs 2230199) were genotyped with an tetra-primer amplification refractory mutation system PCR (T-ARMS-PCR) described by Benedetta Peruzzi et al.¹³

NOD2 SNPs rs2066844 (R702W), rs2066845 (G908R), and rs2066847 (frame-shift mutation 1007fs, or 3020insC) were genotyped as described earlier.¹⁴

Statistical analysis

Statistical analysis was performed with SPSS Statistical Software Package (version 23.0, SPSS Inc., Chicago, IL) and Prism 6 for Windows (GraphPad Software, San Diego, CA, USA) statistical software products. The contributions of polymorphisms and clinical characteristics (e.g. age, gender, primary liver disease, Child-Pugh class) to the development of SBP were analysed using univariate analysis (Pearson's χ^2 test or the Fisher's exact test, where appropriate).

Multivariate analysis was performed using binary logistic regression to evaluate the independence of covariates with a p value <0.20 in the univariate analyses. P values <0.05 were considered statistically significant. [All polymorphisms adhered to the Hardy-Weinberg expectations ($P > 0.05$)].

Table 1. Characteristics of Patients Prior To Orthotopic Liver Transplant (n=280).

Variable	n (%)	SBP n=53 (%)	No SBP n=227 (%)	P-value
Age (median, range)	51 (16-70)	51 (20-65)	51 (16-70)	0.77
Gender, male	173 (61.8)	33 (62.3)	141 (61.7)	0.20
Primary liver disease				0.031
Cholestatic	74 (26.4)	8 (15.1)	66 (29.1)	
Viral	56 (20.0)	9 (17.0)	47 (20.7)	
Alcoholic	43 (15.4)	14 (26.4)	29 (12.8)	
Other disease [§]	107 (38.2)	22 (41.5)	83 (37.4)	
Child-Pugh class				<0.003
A	62 (22.1)	3 (5.7)	59 (26.0)	
B	124 (44.3)	25 (47.2)	99 (43.6)	
C	94 (33.6)	25 (47.2)	69 (30.4)	
Lab MELD score (median, SD)	14±8	15 (8.6)	14 (8.3)	0.10

[§] Other diseases included predominately autoimmune hepatitis, cryptogenic cirrhosis and metabolic disorders.
MELD Model for End-Stage Liver Disease.

RESULTS

In the present study 280 cirrhotic patients before OLT could be included in the analysis and 53 (19%) of these had encountered SBP. Table 1 summarizes the baseline characteristics of these patients.

The Child-Pugh classification showed a significant association with a history of SBP (Child-Pugh A: 4.8%; B: 20.3%; C: 26.3%). The underlying primary liver disease also showed a significant relation with SBP, with significantly more SBP in the alcoholic liver disease subgroup as compared to patients with cholestatic liver diseases (Table 1).

The association of SNPs in the candidate genes *MBL2*, *FCN2*, *MASP2*, *C3*, *TLR2*, *TLR4*, *MD2*, *MyD88* and *NOD2* with SBP was explored. The genotype distribution of all genes was comparable with the expected allele frequencies in the normal Caucasian population. The frequencies and distribution of the genotypes of innate immunity receptor gene polymorphisms and their signal molecules and the odds ratios for the association with SBP are shown in Table 2 and 3 respectively.

SBP was significantly more frequent in the two patients homozygous (GG) for *TLR4* D299G compared to homozygous wildtype (AA), see Table 2. Allele frequency (A vs G) showed no significant relation ($p=0.91$) with SBP. There was no statistically significant association between any of the other analysed SNPs and the risk of SBP.

Subsequently multivariate analysis was performed including covariates from the univariate analyses with a p value <0.20 . Child-Pugh classification and primary liver disease were included together with the subsequent individual SNPs: *TLR4* (rs4986790), *NOD2* (rs2066847), *MASP2* (rs72550870) and *MD2* (rs11466004) (Table 4). Non-significant covariates

Table 2. Frequencies of Innate Immunity Receptor Gene Polymorphisms in patients prior to OLT

Receptors	SNP	Genotype	SBP %(n)	No SBP %(n)	P-value
MBL2					
Secretor haplotype ^a	A/A and YA/O	19 (44)	81 (189)	1.00	
		XA/O and O/O	19 (9)	81 (38)	
FCN2					
rs17549193	+6359 (B)	CC	19 (31)	81 (128)	0.52
		CT	20 (20)	80 (80)	
		TT	10 (2)	90 (19)	
rs7851696	+6424 (C)	GG	18 (39)	82 (179)	0.70
		GT	22 (13)	78 (45)	
		TT	25 (1)	75 (3)	
TLR2					
rs5743708	+753	GG	19(47)	81 (200)	0.68
		AG	24 (5)	76 (16)	
		AA	0 (0)	100 (2)	
TLR4					
rs4986790	+299	AA	19 (46)	81 (192)	0.01*
		AG	13 (4)	87 (26)	
		GG	100 (2)	0 (0)	
rs4986791	+399	CC	20 (46)	80 (189)	0.72
		CT	14 (5)	86 (30)	
		TT	25 (1)	75 (3)	
NOD2					
rs2066844	+702	CC	19 (47)	81 (198)	0.61
		CG	29 (4)	71 (10)	
		GG	0 (0)	100 (1)	
rs2066845	+908	GG	20 (49)	80 (198)	0.92
		GC	19 (3)	81 (13)	
		CC	0	0	
rs2066847	3020insC	WT	19 (49)	81 (212)	0.11
		HET	43 (3)	57 (4)	

^aFive single nucleotide polymorphisms in *MBL2* were genotyped: *MBL2* -550 (H/L), rs11003125; *MBL2* -221 (X/Y), rs7096206; *MBL2* codon 52 (D), rs5030737; *MBL2* codon 54 (B), rs1800450 and *MBL2* codon 57 (C), rs1800451. Structural variants D, B and C are collectively called O; where A is considered wild type; Secretor haplotypes were constructed: A/A and YA/O versus XA/O and O/O.

rs17549193 n=280 SBP:53 No SBP:227
rs7851696 n=280 SBP:53 No SBP:227
rs5743708 n=270 SBP:52 No SBP:218
rs4986790 n=270 SBP:52 No SBP:218;
rs4986791 n=274 SBP:52 No SBP:222
rs2066844 n=260 SBP:51 No SBP:209
rs2066845 n=263 SBP:52 No SBP:211
rs2066847 n=268 SBP:52 No SBP:216

Table 3. Frequencies of Innate Immunity Signal Molecule Gene Polymorphisms in patients prior to OLT

SNP		Genotype	SBP %(n)	No SBP %(n)	P-value
Signal Molecules					
MASP2					
rs72550870	+105	AA	18(48)	82 (218)	0.10
		AG	36 (5)	65 (9)	
		GG	0 (0)	0 (0)	
rs12711521	+371	AA	20 (37)	80 (142)	0.54
		AC	16 (15)	84 (76)	
		CC	10 (1)	90 (9)	
C3					
rs2230199		SS	18 (32)	82 (141)	0.84
		FS	21 (18)	79 (66)	
		FF	22 (2)	78 (7)	
MD2					
rs11466004	+157	CC	20 (51)	80 (199)	0.19
		CT	7 (1)	93 (14)	
		TT	0	0	
MYD88					
rs6853		AA	20 (43)	80 (167)	0.63
		AG	15 (9)	85 (50)	
		GG	14 (1)	86 (6)	

complement component 3 (C3) allotypes, called C3F (fast) and C3S (slow)

rs72550870 n=280 SBP:53 No SBP:227

rs12711521 n=280 SBP:53 No SBP:227

rs2230199 n=266 SBP:52 No SBP:214

rs11466004 n=265 SBP:52 No SBP:213

rs6853 n=276 SBP:53 No SBP:223

were excluded stepwise, resulting in Child-Pugh classification as the only independent risk factor for developing SBP (Child-Pugh A vs B: OR 4.9. P=0.013; A vs C: OR 6.8 P=0.003).

DISCUSSION

In this report the possible association between frequencies and distribution of the genotypes of innate immunity receptor gene polymorphisms and their signal molecules and their odds ratios for the association with spontaneous bacterial peritonitis (SBP) was explored in patients with liver cirrhosis on the waiting list for liver transplantation.

In the current study these polymorphisms appeared to have no role in the genetic susceptibility for SBP in these patients, and only a higher Child-Pugh class was associated with a history of SBP.

An association between MBL and SBP was demonstrated by Yuen et al. who showed that patients with chronic hepatitis B virus-related cirrhosis with SBP have a marked increase

Table 4. Multivariate binary logistic regression of parameters associated with SBP development in Cirrhotic patients prior to OLT

Variable	OR (B)	P-value	OR (B)	P-value	OR (B)	P-value	OR (B)	P-value
Primary liver disease								
Viral								
reference								
Alcoholic	2.142	0.127						
Cholestatic	0.728	0.563						
Other disease [§]	1.596	0.307						
Child-Pugh class								
reference								
A								
B	5.006	0.014	4.908	0.012	5.334	0.008	5.026	0.011
C	6.359	0.011	6.509	0.003	6.836	0.003	6.919	0.003
MD2 rs72550870			0.508	0.247				
MD2 rs11466004					0.280	0.229		
NOD2 rs2066847							3.036	0.168
TLR4 rs4986790							0.947	0.912

in *MBL2* codon 54 point mutation (B-variant) rate and a decrease in serum MBL level in comparison to cirrhosis patients without SBP.¹⁵ Further analyses in our cohort of patients did not reveal a specific relation of SBP with the innate immunity polymorphisms tested in the subgroup with viral hepatitis-related cirrhosis (not shown). The difference with Yuen's study might be explained by the relatively low numbers of HBV patients in our cohort (26 out of the 56 patients with virus-related cirrhosis). Another explanation might be the difference in frequency of the haplotypes between ethnic groups with, e.g., LYPB being the common variant haplotype in Caucasians (12%) and even more so in Asians (22%), but very rare in Africans¹⁶⁻¹⁷. Based on these data this *MBL2* mutation at least has no causal relation with SBP in Caucasian patients.

So far no studies had examined the association of polymorphisms in *FCN2* or *MASP2* with SBP. In the present study we did not find such an association.

Further downstream in the complement pathway cascade is C3. It has been shown that low C3 levels in cirrhotic ascites predispose to spontaneous bacterial peritonitis.⁶ An increased prevalence of the C3F allele has been associated with some immune-mediated disorders. However, we did not find an association for the two common allelic variants of C3 with development of SBP.

We previously showed that recipient's genetic R702W *NOD2* variant is associated with an increased risk of bacterial infections after orthotopic liver transplantation.¹⁴ To substantiate their role in SBP in cirrhosis prior to OLT we determined the prevalence of three *NOD2* variants (rs2066844: R702W; rs2066845: G908R and rs2066847: 3020insC) but did not find such an association.

Appenrodt and colleagues reported an increased risk of SBP and death in 150 patients with cirrhosis and ascites who carried any risk allele of the three *NOD2* variants.¹⁸ These findings were partially confirmed in a prospective study of 175 patients by Bruns and colleagues.¹⁹ In particular, the minor allele frequency of *NOD2* variant 3020insC was significantly increased in the 29 patients with SBP. A subgroup analysis showed an association between culture-positive SBP (n=10) and SNPs 3020insC and G908R. The median survival did not differ significantly in patients carrying *NOD2* risk alleles. A subgroup analyses in patients without hepatocellular carcinoma (HCC) at study entry, however, did show a modest association with death. In our cohort of 280 patients we did find an increased frequency of *NOD2* variant 3020insC in patients developing SBP (43% vs 19%) but this difference did not reach statistical significance.

With regard to *TLR2*, Appenrodt and colleagues used the same patient cohort consisting of 150 patients with cirrhosis and ascites to genotype for *TLR2* gene variants -16934 (rs4696480), R753Q (rs5743708), P631H (rs5743704) and the *TLR2* GT microsatellite polymorphism in intron 2.²⁰ SBP was significantly more frequent in patients with the *TLR2* -16934 TT genotype and in carriers with two long tandem GT repeat alleles (>20). Bruns and colleagues could not confirm the above findings in their study of 120 patients, but did find an association with *TLR2* R753Q and SBP.²¹

The *TLR2* R753Q polymorphism has also been found to be involved in a variety of infectious diseases and infection recurrence in patients with Gram-positive bacterial infections after liver transplantation.²² However, we did not find a relation of *TLR2* R753Q with SBP in our cohort.

Toll-like receptor 4 (TLR4) and its co-receptor MD-2 recognize bacterial lipopolysaccharide (LPS) and signal the innate immune response. Two known *TLR4* polymorphisms, replacing Asp-299 with Gly (rs4986790) and Thr-399 with Ile (rs4986791), cause LPS hyporesponsiveness and have been proposed to be associated with a variety of infectious and non-infectious diseases.

TLR4 genetic polymorphisms, mainly D299G, have been associated with increased predisposition to infection in several populations. Guarner and colleagues evaluated 111 cirrhotic patients with ascites in retrospect and observed a trend towards a higher incidence of bacterial infections and a significantly higher number of infections per patient in patients with the *TLR4* D299G polymorphism, compared to wild-type patients.²³

We did find an association between *TLR4* D299G and SBP. Namely, the two patients homozygous (GG) for *TLR4* D299G both developed SBP. Nevertheless, the correlation was not gene-dose dependent and the allele frequency A vs G did not differ significantly ($P=0.43$). Since only two out of 280 (0.7%) patients are homozygous carriers the clinical relevance seems unclear and should be looked into in a larger group of patients.

MD-2 is required for TLR4-mediated responses and enables TLR2-mediated responses to lipopolysaccharide and enhances TLR2-mediated responses to Gram-positive and Gram-negative bacteria and their cell wall components. Heterozygosity at the polymorphic site *MD2* rs11466004 has been reported to be associated with lower levels of IL-10 secretion.²⁴

MyD88 is a key downstream adapter for most TLRs and interleukin-1 receptors (IL-1Rs). Absence of MyD88 in MyD88-deficient mice resulted in a dramatic reduction of host resistance to several infectious agents.²⁵

Although quite rare, children with autosomal recessive MyD88 deficiency suffer from life-threatening, often recurrent pyogenic bacterial infections.²⁶

Heterozygosity at the polymorphic site *MyD88* rs6853 leads to defective production of TNF- α , IL-12 and NO, but is associated with resistance to human pulmonary tuberculosis.²⁷ Until now no studies had examined the association of polymorphisms in *MD2* or *MyD88* with SBP. In the current study we did not find such an association.

We did find a relation between SBP and the severity of liver disease according to the Child-Pugh classification. This finding is in accordance with several other studies.²⁸ Especially liver insufficiency seems to be the main factor since chronic pre-hepatic portal hypertension without liver insufficiency does not lead to increased SBP incidence.²⁹

We are aware of the fact that the present study contains a probable selection bias since the data used in this study were retrieved from the records of patients on the waiting list for OLT with patients who died while waiting for OLT not being included. However, the incidence of SBP in those patients did not differ from those included (not shown). Despite this and

the retrospective nature of this study the frequencies of the studied SNPs were comparable to the frequencies reported by others and did not deviate from Hardy-Weinberg equilibrium. Furthermore, the incidence of SBP in our cohort is comparable with that reported by others.¹⁸

SBP has a heterogeneous group of causative pathogens. A monogenic affected pattern recognition receptor only results in a impaired response if its specific pathogen-associated molecular patterns is encountered. Consequently, finding a causal relation or association between a SNP and SBP is highly dependent on the type of the bacterial pathogen causing SBP. Even though several patients had multiple polymorphisms this did not result in more SBP. Apparently there is sufficient redundancy in sensing pathogens by the innate immune system.

In conclusion, we have studied the association of SBP and polymorphisms in a selection of genes of the innate immunity coding for several pattern recognition receptors (lectins, TLRs and NOD2) and their associated intracellular signaling molecules (Masp-2, C3, MD2 and MyD88). Our observations exclude a major role for the investigated polymorphisms as a risk factor for developing SBP. Therefore, development of preventive or therapeutic strategies for SBP directed at defects in these components of innate immunity does not seem warranted.

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SUPPLEMENTARY DATA

Supplemental Table 1. Oligonucleotide Primers for Polymerase Chain Reaction - High Resolution DNA Melting Assay

Gene	dbSNP ID	SNP	sequence		
			forward primer	reverse primer	3'-blocked probe (Amino Linker C6)
MBL2	rs11003125	H/L	-550 CCAGGGCCAACTAGTAAGA	CCAGCCAGAATTAACTGGA	CAGGCAAGCCTGTCTTAAACACCAAGG
	rs7096206	Y/X	-221 TTCCCTAAGCTAACAGGCATAA	GCACTATGATGACAGTGGG	CCACGGAAAGCATGTTTATAGTCTT
	rs5030737	D (codon 52)	+223 CTGCAGTGATTGCCTGTAGC	GCCCAACACGTACCTGGTTC	GGCAAAGATGGGCGGTGATGCGACCAAGGGA
	rs1800450	B (codon 54)	+230		
	rs1800451	C (codon 57)	+239		
FCN2	rs17549193	B	+6359 GATGTTACTGCCTGTACGATGC	GGTGGAGAAGGACTGGTTGTT	GAGATTCCCTGA ~~C~~ GGTTCACAAAC
	rs7851696	C	+6424 CCAGGACAATGATCTTAACACC	CACATGGCAGTTTTTGTACCAC	GGAAATTGT ~~G~~ CTGTGATGTTTCAGGG
TLR2	rs5743708		+753 CTGGAGCCCAATTGAGAAAAA	CGCAGCTCTCAGATTTTACCC	TTCTGCAAGCTG ~~C~~ GGAAGATAATGAAC
TLR4	rs4986790		+299 CCTGTGCAATTTGACCATTG	CACACTCACCAGGGAAAATG	TACTACTCGATG ~~G~~ TATTATTGACTTA
	rs4986791		+399 TGGCTTGAGTTTCAAGGTTG	TGTTCTAAGCCCCAAGAAGTTTGA	TTTTGGGACAA ~~C~~ CAGCCTAAAGTAT
MASP2	rs72550870		+105 TGCATAGAAGGCCCTCGAACC	GGCAAGGACACTTTTCTACTCGC	CTCGTTGGAGTAG ~~C~~ CGGAGCGGAAGG
	rs12711521		+371 AGCTTTGTAGGTGCTCACTCC	CCCTCCCATGCTTCTTCTT	CCACTGGGTAGAT ~~C~~ ATCAGGAGGGCCA
MD2	rs11466004		+157 TGCTCTTTTGCTTGGAGTTTG	CCTGGGTACCCTTAAACACAGG	CATCTACACCAA ~~C~~ CTAATTCAATTA
MYD88	rs6853	A>G	UTR-3 GCACCTGTCCCCCTTTAATA	CCTGGGTACCCTTAAACACAGG	TTAAAGCCATCTC ~~A~~ AGAGGCATCTTCT

Chapter 8

*Postoperative peak serum aspartate
aminotransferase is an independent predictor of
increased infection risk after liver transplantation*

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ABSTRACT

Hepatic ischemia-reperfusion injury (IRI) is inevitable in liver transplantation and enhances susceptibility to infections. We evaluated postoperative serum levels of aspartate transaminase (AST), as an IRI marker, in 261 patients after orthotopic liver transplantation (OLT) and determined the contribution to bacterial and CMV infection susceptibility. Log-rank statistics was used for optimal cut-off point analysis. The contribution to the risk of infection was assessed with multivariate Cox regression analysis.

The optimally discriminating peak AST level related to bacterial infection was 1516 IU/L (log-rank 15.36, $P < 0.001$) with an adjusted hazard ratio for bacterial infection risk in the $AST \geq 1500$ group of 2.08 ($P = 0.009$). For CMV infections the optimal peak AST resulted in four cut-off points, each related to a different donor–recipient CMV serostatus group, with the strongest association in recipients of organs from CMV seropositive donors. Patients with a peak $AST \geq 1600$ had an increased risk of CMV infection [51% versus 36% in patients with a peak $AST < 1600$, log-rank test $P = 0.03$]. Adjusted hazard ratio for CMV infection in the $AST \geq 1600$ group was 1.70 ($P = 0.03$).

In conclusion, elevated peak AST is independently associated with an increased infection risk in OLT recipients. Thus, AST evaluation might be useful for infection risk estimation in OLT patients.

INTRODUCTION

Hepatic ischemia/reperfusion injury (IRI) is inevitable in liver transplantation and despite the improvements in surgical techniques, postoperative care, and immunosuppression, leads to serious clinical problems in approximately 10%-30% of liver transplants.¹

IRI is considered one of the main factors responsible for initial poor graft function (IPGF) after liver transplantation.² Even moderate reperfusion damage, which does not severely affect the graft, can impair long-term hepatic recovery affecting the patient's recovery after transplantation and might even enhance patient susceptibility to infections.³ IRI has a complex pathophysiology with a number of contributing factors. Postoperative serum levels of aspartate transaminase (AST) are in general used as a marker of graft injury.

Shortage of adequate organs has led to the use of extended criteria donor (ECD) livers (increased age or donation after cardiac death (DCD)) to accommodate the transplant demand. However, ECD livers, by definition, are much more prone to IRI, IPGF and primary graft non-function (PGNF) after transplantation.⁴

In patients with cirrhosis the degree of liver dysfunction is related to abnormalities of the immune response, which increase the susceptibility to infection.⁵

Most infections occur during the immediate period after liver transplantation and the period of highly intense immunosuppression. Infectious complications remain an important and inevitable cause of morbidity and mortality among liver recipients. In this context it is therefore essential to be able to predict infection risk in order to execute surveillance for specific risk factors.

In the current study we evaluated whether hepatic IRI contributes to the susceptibility for bacterial and CMV infections after orthotopic liver transplantation.

MATERIALS AND METHODS

Patients, study protocol and transplant variables

We retrospectively studied 261 consecutive patients, who received orthotopic liver transplantation (OLT) at the Leiden University Medical Center and the University Medical Center Groningen (from 2000 to 2006) in The Netherlands. The study was performed on prospectively collected data and samples obtained with informed consent from the patients according to the guidelines of both Medical Ethics Committees, in compliance with the Helsinki Declaration.

Clinical variables

Demographic and clinical characteristics of the recipient at the time of OLT, donor information, and post-transplant follow-up data were collected from the transplantation databases. The degree of hepatocellular injury was evaluated by postoperative serum levels of aspartate aminotransferase (AST). The AST was determined at 7 consecutive days after OLT and measured by routine methods. The upper limit of normal serum level for AST was 40 IU/L.

Bacterial infections

The general patient database, original patient reports, transplantation databases and microbiology records were (re)evaluated to identify episodes of clinical and laboratory confirmed bacterial infections within the first year after transplantation, according to Centers for Disease Control and Prevention criteria for diagnosing infection.⁶ To exclude the potential effect on AST serum levels during infection due to hepatic involvement, patients with an identified bacterial infection within the first 7 days after OLT were excluded from the analysis.

CMV infection

CMV pp65 lower matrix protein had been detected routinely in leukocytes from EDTA blood samples. Laboratory evidence for CMV infection was defined as a result of one or more cells positive for CMV pp65 antigen per 50,000 leukocytes. CMV-DNA loads had been determined prospectively in EDTA plasma, as previously described⁷ with a detection limit of 0.90 log₁₀.

Both CMV screenings were performed twice a week during the first 21 days post-transplantation. Thereafter, the test was performed at each outpatient visit, at least every other week until postoperative day 90. Subsequently, screening was performed at least once every month until postoperative day 365. Since all patients had an undetectable CMV load at the time of transplantation, any subsequent detection of pp65 or CMV-DNA was considered a CMV infection in our study, regardless of their CMV serostatus.⁸ From 206 cases blood CMV pp65 antigen follow-up within the first year after OLT was available. CMV-DNA quantification data were available in 47 cases because of transition from pp65 in the LUMC from June 2002 onwards. Patients with a first positive CMV test result within 7 days of transplantation were excluded from the analysis.

Immunosuppression, antibiotic and antiviral prophylaxis

Patients received cyclosporine or tacrolimus-based immunosuppressive therapy, corticosteroids and either azathioprine or mycophenolate mofetil and/or basiliximab. With respect to the immunosuppressive therapy, azathioprine was used until 2001, and thereafter mycophenolate mofetil was given in case of impaired renal function. From 2001 on, basiliximab was used on days 0 and 4. In addition, patients received 24 h of systemic prophylactic antibiotics (gentamycin, cefuroxim, penicillin G, and metronidazol) and 3 weeks of selective digestive tract decontamination (polymyxin/neomycin, norfloxacin, and amphotericin B) after OLT (89 patients) or amoxicillin–clavulanate and ciprofloxacin without selective digestive tract decontamination (163 patients). Anti-CMV prophylaxis before 2002 consisted of 3 weeks of intravenous ganciclovir (induction), followed by oral acyclovir up to 3 months after OLT (maintenance) for cytomegalovirus donor-positive/recipient-negative (D+/R-) patients. Patients with other serostatus combinations received oral acyclovir for 3 months. From 2002 onwards, CMV prophylaxis was restricted to patients with a D+/R-serostatus combination: these patients received prophylaxis with valganciclovir for 3 months, while the other D/R combinations did not receive prophylaxis. In both periods,

preemptive therapy was used, meaning that a primary infection was treated upon first positivity of CMVpp65 test or PCR, while reactivations were treated if a relevant increase in CMVpp65 or CMV-DNA occurred after first positivity. The use of acyclovir, ganciclovir, and valganciclovir as prophylaxis was included as one variable to consider the combined effects of these medications in the analyses.

Genotyping

DNA from donors and recipients was isolated routinely from blood or tissue samples. Single-nucleotide polymorphisms (SNPs) in the lectin pathway genes: mannose-binding lectin (*MBL2*), Ficolin-2 (*FCN2*), and MBL-associated serine protease (*MASP2*) were determined with the use of high-resolution melt curve analysis as previously described.^{9, 10}

Statistical Analysis

All statistics were calculated using SPSS version 16.0.2 (SPSS Inc., Chicago, IL). Log-rank statistics was used for optimal cut-off point analysis. The primary endpoint, infection, was described with the use of Kaplan–Meier estimates and was compared between groups with the use of the log-rank test. Univariate and multivariate survival analyses were performed using the Cox's proportional hazards method. All variables associated with infection with a P value of less than 0.15 in the univariate analysis were included in the multivariate model. In all survival analyses, patients without an infection were censored at day 365 and patients who died within 365 days after OLT were censored on the day of death. Two-sided hypotheses and tests were used for all statistical inferences. A P value of less than 0.05 was considered to indicate statistical significance.

RESULTS

A total of 261 patients who underwent orthotopic liver transplantation (OLT) and had aspartate aminotransferase (AST) measurements at 7 consecutive days after OLT were evaluated in this study of which the pre- and post-transplantation clinicopathological features are shown in Table 1.

AST and the risk of bacterial infection

From the 261 cases 9 patients were excluded because the bacterial infection was within 7 days after OLT. In 61 out of the 252 patients (24%) there was bacterial infection within 8-365 days after OLT. The median time to bacterial infection was 39 days (IQR 16-92 days) after transplantation.

The relation of peak AST as a continuous parameter preceding a bacterial infection was significant in the univariate analysis, with an HR of 1.000148 per 1 IU/L (95% CI 1.000050-1.000246, $P=0.003$). Thus, an elevation of 1000 IU/L (i.e., 1040 IU/L) would result in $1.000148^{1000} = 1.16$ or a 16% increased risk. Determination of the optimally discriminating peak AST level in relation to bacterial infection in the univariate analysis revealed an

Table 1. Characteristics of Orthotopic Liver Transplant Recipients and Donors (N=261)

Variable	
Age (median, IQR)	
Recipient	51 (41-56)
Donor	46 (34-56)
Gender recipient, N (%)	
Male	161 (61.7)
Female	100 (38.3)
Gender donor, N (%)	
Male	132 (50.6)
Female	129 (49.4)
Primary liver disease, N (%)	
Viral	44 (16.9)
Alcoholic	35 (13.4)
Cholestatic	68 (26.1)
Other disease ^s	114 (43.7)
Child-Pugh class, N (%)	
A	57 (21.8)
B	118 (45.2)
C	86 (33.0)
Lab MELD score (median, IQR)	14 (11-21)
Donor-type, N (%)	
Donation after cardiac death	25 (9.6)
Donation after brain death	236 (90.4)
Cold ischemia time* (median, IQR)	510 (419-613)
Warm ischemia time** (median, IQR)	44 (36-50)
Maintenance immunosuppression, N (%)	
Prednisone / CNI / basiliximab (+ MMF)	207 (79.3)
Prednisone / CNI (+ azathioprine)	54 (20.7)
Acute cellular rejection, N (%)	
Yes	90 (34.5)
No	171 (65.5)
Antimicrobial prophylaxis***, N (%)	
GCPM + SDD	89 (35)
ACC, No SDD	163 (65)
CMV serostatus****, N (%)	
D+/R+	69 (28.2)
D+/R-	38 (15.5)
D-/R+	95 (38.8)
D-/R-	43 (17.6)
Antiviral treatment****, N (%)	
None	118 (48.2)
Acyclovir	89 (36.3)
Ganciclovir+ Acyclovir	5 (2.0)
Valganciclovir	33 (13.5)

Table 1. (continued)

Variable	
Bacterial infection days 8-365, N (%)	61 (24)
CMV infection days 8-365, N (%)	95 (39)
Both bacterial and CMV infection days 8-365, N (%)	20 (9)

IQR denotes interquartile range; MELD, Model for End-Stage Liver Disease; CNI, calcineurin inhibitors; MMF, mycophenolate mofetil; GCPM, Gentamycin, Cefuroxim, Penicillin G and Metronidazol; SDD, Selective Digestive tract Decontamination and ACC, Amoxicillin-Clavulanate and Ciprofloxacin;
CMV, Human cytomegalovirus; D, donor; R, recipient.

[§] Other diseases included predominately autoimmune hepatitis, cryptogenic cirrhosis and metabolic disorders. * N=254; ** N=255; *** N=252; **** N=245;

optimum at 1516 IU/L (log-rank 15.36, $P < 0.0001$, Figure 1A). Consequently, patients with a peak AST ≥ 1500 had a significantly higher rate of bacterial infection after 7 days post-OLT than those with a peak AST < 1500 [43% (21/49) versus 20% (40/203) log-rank test $P < 0.0001$].

In the multivariate analysis the adjusted hazard ratio for bacterial infection in the AST ≥ 1500 group as compared to the AST < 1500 , was 2.079 (95% CI 1.199 to 3.603; $P = 0.009$). The only other factor at study inclusion, other than the peak AST ≥ 1500 , which remained independently associated with an increased risk of bacterial infection after OLT in the multivariate analysis was the lectin complement pathway mismatch profile (Table 2 and supplemental Table 1).

AST and the risk of CMV infection

From the 253 cases with available systemic CMV detection follow-up 8 patients were excluded because of a first positive CMV test result within 7 days. In 95 out of the 245 patients (39%) there was initial laboratory evidence of CMV infection between days 8 and 365 after OLT. The median time to CMV infection was 37 days (IQR 28-58 days) after transplantation.

The relation of peak AST as a continuous parameter in the univariate analysis with CMV infection was significant with an HR of 1.000095 per 1 IU/L (95% CI 1.000006-1.000184, $P = 0.04$).

For the association with CMV infection incidence, the optimal cut-off point value for the peak AST resulted in the identification of four cut-off points (Figure 1B). Each of these cut-off points correlated with a different donor–recipient CMV serostatus pairs. The strongest association of CMV infection with IRI typically occurred in recipients of organs from CMV seropositive donors. Patients with a peak AST ≥ 1600 were found to have a significantly increased risk of CMV infection compared to those with a peak AST < 1600 [51% (25/49) versus 36% (70/196) log-rank test $P = 0.03$]. Recipients of organs from CMV seronegative donors showed no association with a peak AST ≥ 1600 (28.6% (6/27) versus 18.9% (21/111) log-rank test $P = 0.638$). Patients at high risk for CMV infection (CMV serostatus: D+/R-) showed an increased infection risk after 7 days with a peak AST ≥ 1295 compared to those with a peak AST < 1295 (100% (10/10) versus 57.1% (16/28) log-rank test $P = 0.004$].

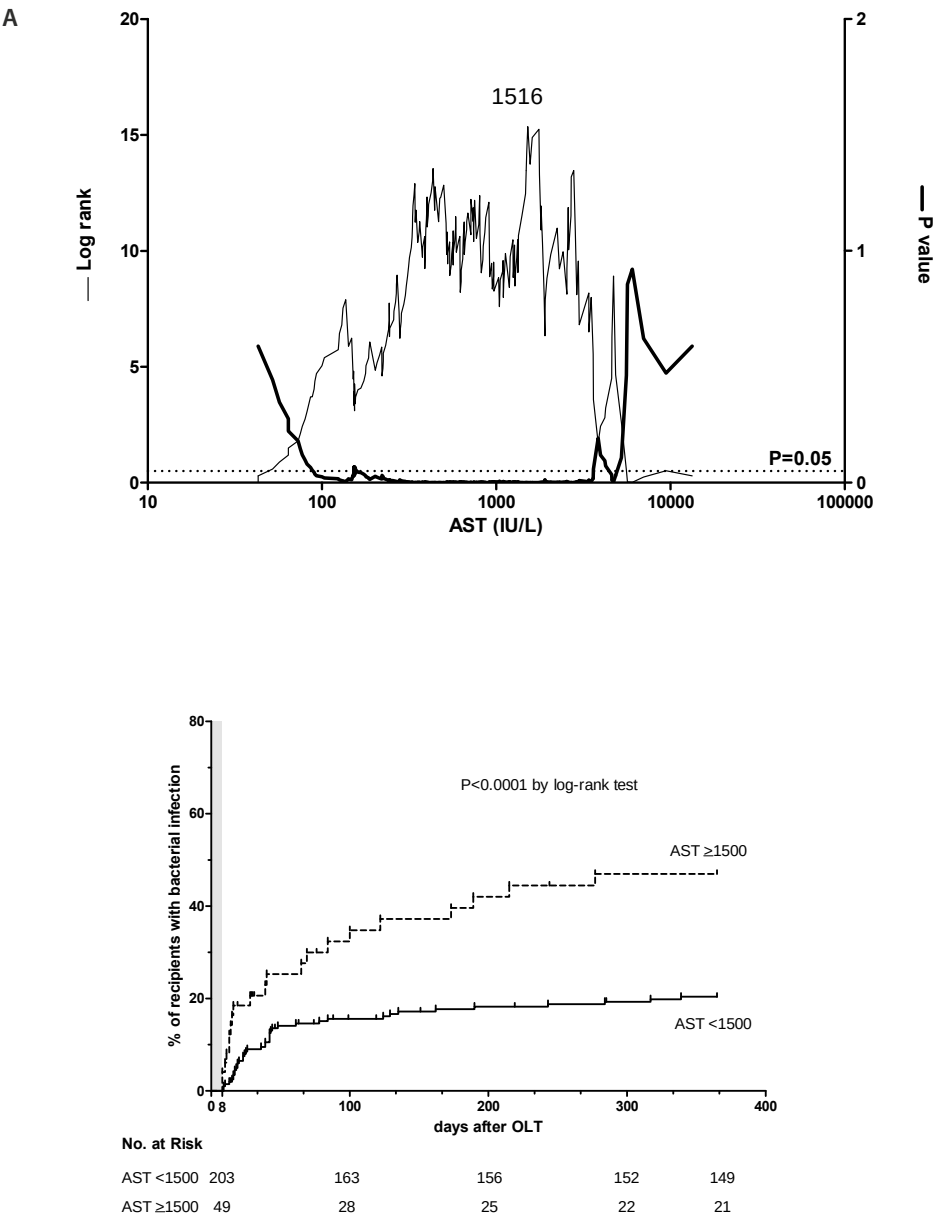


Figure 1. Optimal cut-off analysis and corresponding Kaplan-Meier estimation of bacterial infection (A) and cytomegalovirus (CMV) infection (B) for peak AST in liver transplant recipients. (A) Determination of the optimally discriminating peak AST level within the first 7 consecutive days after OLT in relation to bacterial infection (8-365 days after OLT) in the univariate analysis revealed an optimum at 1516 IU/L. (B) For the association with CMV infection incidence (8-365 days after OLT) the optimal cut-off point value for the peak AST within the first 7 consecutive days after OLT resulted in the identification of four cut-off points. Each of these cut-off points correlated with a different donor–recipient CMV serostatus pairs. Both bacterial and CMV infection (8-365 days after OLT) were described with the use of Kaplan–Meier estimates and was compared between groups with the use of the log-rank (LR) test.

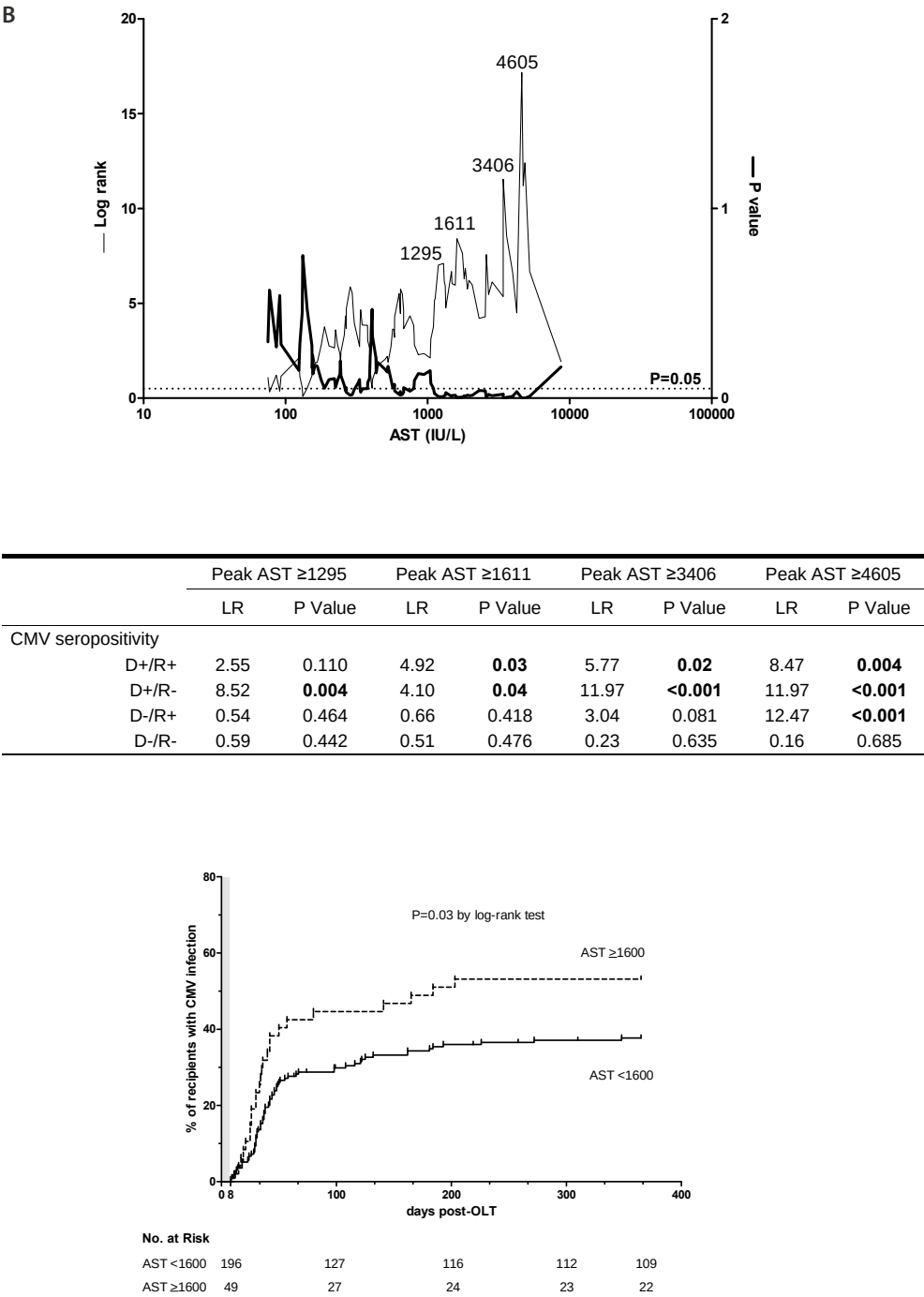


Figure 1. (continued)

Table 2. Univariate and Multivariate Cox Proportional Hazard Models in Patients Assessing Associations With Bacterial Infection

	Bacterial infection % (N)	Univariate			Multivariate†		
		HR	CI (95%)	P Value	HR	CI (95%)	P Value
Lectin pathway mismatch profile‡							
2 or 3 variants	34 (54/161)	5.138	2.337-11.297	<0.001	4.711	2.139-10.375	<0.001
No or 1 variant	8 (7/91)	ref			ref		
Peak AST							
≥1500	43 (21/49)	2.754	1.622-4.676	<0.001	2.074	1.199-3.603	0.009
<1500	20 (40/203)	ref			ref		

In the multivariate analysis the adjusted hazard ratio for CMV infection in the $AST \geq 1600$ group as compared to the $AST < 1600$ was 1.699 (95% CI 1.047 to 2.754; $P=0.03$). Transplant-related factors other than the week one peak $AST \geq 1600$ that were independently associated with an increased risk of CMV infection were *donation after cardiac death (DCD)* and seropositivity for CMV (in the recipient, the donor, or both) (Table 3, and supplemental Table 2).

Increasing cut-off values of pp65 (>3) and CMV-DNA ($\log > 2$) for CMV positivity showed a consistent association (41% (20/49) versus 22% (44/196), log-rank test $P=0.003$). Even higher cut-off values of pp65 (>10) and CMV-DNA ($\log > 3$) for CMV positivity showed an association (31% (15/49) versus 15% (30/196), log-rank test $P=0.004$). Multivariate analysis results broken down by respective extent of CMV positivity showed consistent positive associations between peak $AST \geq 1600$ and CMV infection risk. (HR 2.133; 95% CI 1.190-3.823, $P=0.001$ and HR 1.696; 95% CI 1.035-2.779, $P=0.04$, respectively).

DISCUSSION

The results of this study show that the degree of IRI as indicated by the level of serum aspartate transaminase (AST) in the first week after OLT has a strong association with the risk of infections after OLT. The peak AST was found to be an independent risk factor for bacterial as well as CMV infection after liver transplantation.

Hepatic IRI has a complex pathophysiology with a number of contributing factors which potentially increase the susceptibility to infection. The ischemic insult has been reported to lead to sublethal cell injury, formation of proinflammatory mediators (like TNF- α) and the recruitment and activation of macrophages, neutrophils, and lymphocytes that further enhance the injury. Microcirculatory disturbances lead to underperfused areas in the liver. The end result is cellular death via a combination of apoptosis and necrosis¹¹ which combined with immunosuppression may provide a focus for bacterial infections

In some cases IRI even leads to IPGF. IPGF is a serious clinical complication after OLT, with an important impact on the course of the disease and long-term benefit.¹²

The degree of liver dysfunction is related to several abnormalities of the immune response, all of which increase the susceptibility to infection.⁵ In addition, IRI is probably also involved in development of non-anastomotic biliary strictures (NAS), which can lead to cholangitis, sepsis and death or retransplantation.

Two previously published papers concluded that there was no relation between IRI and bacterial infection incidence after OLT. The first study by Bilbao et al. showed that infection occurred in 39% of recipients with severe IRI ($AST > 5000$) compared to 19% in mild and moderate ($AST < 5000$) injury, which almost reached statistical significance ($p=0.06$).¹³ Data published by Glanemann et al. showed that bacterial infection occurred in 43.3% of recipients with minor injury ($AST < 1000$), 53% in moderate ($AST 1000-5000$) and 58.3% severe injury ($AST > 5000$) which, in contrary to the authors conclusions, did reach overall statistical significance if we recalculated it (Chi-square 6.70, $p=0.035$), but indeed showed no significant

Table 3. Univariate and Multivariate Cox Proportional Hazard Models in Patients Assessing Associations With CMV Infection

	CMV infection % (N)	Univariate			Multivariate†		
		HR	CI (95%)	P Value	HR	CI (95%)	P Value
Donor-type							
Donation after cardiac death	52 (12/23)	1.876	1.023-3.439	0.04	3.068	1.512-6.222	0.002
Donation after brain death	37 (83/222)	ref			ref		
CMV seropositivity				<0.001			<0.001
D+/R+	61 (42/69)	19.027	4.601-78.685		19.963	4.766-83.621	
D+/R-	68 (26/38)	18.677	4.427-78.792		20.455	4.790-87.356	
D-/R+	26 (25/95)	6.555	1.552-27.675		7.983	1.872-34.038	
D-/R-	5 (2/43)	ref			ref		
Peak AST							
≥1600	51 (36/70)	1.663	1.099-2.518	0.02	1.699	1.028-2.808	0.04
<1600	34 (59/175)	ref			ref		

difference between the three subgroups.¹⁴ The main difference between our study and these two above mentioned studies, which demonstrated at least a trend towards significance, is that they used commonly but arbitrary AST cut-off values. In addition, both studies date from a period in which ECD graft and DCD grafts were not used for transplantation.

Reactivation of latent CMV is of significant concern in immunocompromised transplant patients. In our study we found that a peak AST ≥ 1600 (corresponding with mild to severe IRI) increased the risk of CMV infection in recipients of organs from CMV seropositive donors. Although this is an observational study there is experimental evidence which support our findings.

Endothelial cells (ECs) are a site of CMV latency and persistence.¹⁵ IRI is critically involved in endothelial damage and the degree of endothelial cell (ECs) detachment has been shown to correlate with the duration of cold ischemia in numerous models and clinical studies.¹⁶ Detachment of infected transplant-derived ECs by IRI might thus be responsible for systemic virus dissemination via blood. Furthermore, animal studies have shown that in for example renal transplantation IRI activates the enhancer domain of the human cytomegalovirus major immediate early promoter.¹⁷ IRI might similarly be a positive factor for activating CMV replication in livers from CMV seropositive donors in the human setting.

In CMV seropositive recipients obtaining a liver from a seronegative donor the increased CMV infection risk was correlated with considerable higher peak AST levels. Previous studies show that IRI induce an intense systemic inflammatory response which precipitate inflammatory damage in organs not involved in the initial ischemic insult.¹⁸ Although speculative, these remote effects of IRI might explain the different peak AST threshold for increased CMV infection risk in CMV serostatus D-/R+ patients. The higher peak AST indicative of much more severe IRI, might be required to execute remote CMV reactivation in the recipient.

In concordance with previous studies, AST levels were significantly higher in *donation after cardiac death* (DCD) grafts compared to DBD grafts.^{19,20} Nevertheless, DCD grafts were independently associated with an increased risk of CMV infection, despite a comparable CMV serostatus distribution (data not shown). Further analyses showed that only recipients of seropositive DCD grafts were at increased risk, with 9 out of 10 resulting in CMV infection, especially in seronegative recipients (D+/R-) (3 out of 3). An explanation might be the prolonged ischemic time in DCD grafts. As mentioned earlier, IRI can activate cytomegalovirus replication. The extent of endothelial cell detachment in DCD grafts is probably also increased, making it a potential source for CMV dissemination.

We recognize limitations to the current study. Firstly, because of the retrospective nature of data collection, there might be a chance of bias. Secondly, although we excluded infections that occurred in the first 7 days post-OLT, we cannot totally exclude that for instance reactivation of CMV in the graft resulted in higher AST serum levels due to hepatic involvement before its systemic detection. However, several studies showed there were no significant differences found between patients with active CMV infection and patients without active CMV infection with regard to AST serum values.^{21,22} Furthermore, if CMV

infection would lead to a high AST peak before systemic detection it would still predict an imminent infection and could be useful for prediction of those patients at risk for CMV.

Hepatic IRI is inevitable in liver transplantation but attenuation is of crucial importance since its major influence on early hepatic function and should therefore be kept to a minimum.

Optimizing graft quality by preconditioning, organ preservation, and postconditioning is currently being investigated. The most promising protective strategy against IRI explored during the last few years seems to be preconditioning, which appears to increase the resistance of liver cells to ischemia and reperfusion events. Preconditioning or pharmacological interventions that mimic these effects most likely have the greatest potential to improve clinical outcome in liver transplantation.

In addition to an impaired long-term hepatic recovery and the risk of primary graft non-function (PGNF), early hepatic dysfunction (IPGF) IRI also increases the risk of both bacterial and CMV infection post-OLT.

According to our data AST has value as a non-invasive marker in identifying patients who are at increased risk of infections after OLT. The findings generated from this study could help to design an effective approach to preventive interference for both bacterial and CMV infection in persons who have undergone OLT. Recipients of organs from CMV seropositive donors or a DCD graft are especially at increased risk for CMV infection after hepatic IRI.

In summary, we have shown that an elevated peak AST is independently associated with increased infection risk in liver transplant recipients. We suggest that AST, an inexpensive, convenient clinical test, could be useful in the laboratory evaluation of infectious risk estimation of OLT patients.

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SUPPLEMENTAL DATA

Supplemental Table 1. Univariate and Multivariate Cox Proportional Hazard Models in Patients Assessing Associations With Bacterial Infection

Variable	Bacterial infection % (N)	Univariate			Multivariate		
		HR	CI (95%)	P Value	HR	CI (95%)	P Value
Age							
Recipient	24 (61/252)	0.990	0.970-1.010	0.320			
Donor	24 (61/252)	0.987	0.971-1.004	0.124	0.990	0.973-1.007	0.230
Gender recipient							
Male	27 (41/153)	1.349	0.790-2.303	0.272			
Female	20 (20/99)	ref					
Gender donor							
Male	30 (39/129)	1.860	1.103-3.137	0.020	1.478	0.870-2.510	0.150
Female	18 (22/123)	ref		0.453	ref		
Primary liver disease							
Viral	17 (7/41)	0.672	0.292-1.542				
Alcoholic	20 (7/35)	0.856	0.373-1.966				
Cholestatic	31 (20/64)	1.300	0.729-2.318				
Other disease ^s	24 (27/112)	ref		0.832			
Child-Pugh class							
A	22 (12/55)	ref					
B	26 (30/116)	1.217	0.623-2.377				
C	23 (19/81)	1.089	0.529-2.244				
Lab MELD score	24 (61/252)	0.995	0.965-1.025	0.723			
Donor-type							
Donation after cardiac death	35 (8/23)	1.537	0.731-3.232	0.257			
Donation after brain death	23 (53/229)	ref					
Cold ischemia time	25 (61/245)	1.001	0.999-1.002	0.506			
Warm ischemia time	24 (60/247)	1.014	0.992-1.035	0.215			

Supplemental Table 1. continued

Variable	Bacterial infection % (N)	Univariate			Multivariate		
		HR	CI (95%)	P Value	HR	CI (95%)	P Value
Maintenance immunosuppression							
Prednisone / CNI / basiliximab (+ MMF)	26 (51/199)	1.439	0.731-2.835	0.293			
Prednisone / CNI (+ azathioprine)	19 (10/53)	ref					
Antimicrobial prophylaxis							
GCPM + SDD	31 (28/89)	1.750	1.057-2.897	0.030	1.411	0.839-2.374	0.194
ACC, No SDD	20 (33/163)	ref			ref		
Acute cellular rejection							
Yes	21 (19/89)	0.772	0.449-1.328	0.350			
No	26 (42/163)	ref					
Lectin pathway mismatch profile [‡]							
2 or 3 variants	34 (54/161)	5.138	2.337-11.297	0.000047	4.711	2.139-10.375	0.00012
No or 1 variant	8 (7/91)	ref			ref		
Peak AST							
≥1500	43 (21/49)	2.754	1.622-4.676	0.00018	2.074	1.199-3.603	0.009
<1500	20 (40/203)	ref			ref		

HR denotes hazard ratio; CI, confidence interval; MELD, Model for End-Stage Liver Disease; CNI, calcineurin inhibitors; MMF, mycophenolate mofetil;

GCPM, Gentamycin, Cefuroxime, Penicillin G and Metronidazole; SDD, Selective Digestive tract Decontamination and ACC, Amoxicillin-Clavulanate and Ciprofloxacin;

AST, aspartate aminotransferase;

[‡] The presence of donor-recipient genotypic groups associated with bacterial infections within the individual were clustered as lectin pathway gene profile; MBL2: XA/O and O/O donor and A/A and YA/O recipient; FCN2: CT or TT donor and or recipient; MASP2: AA donor and or recipient

Supplemental Table 2. Univariate and Multivariate Cox Proportional Hazard Models in Patients Assessing Associations With CMV infection

Variable	CMV infection % (N)	Univariate			Multivariate		
		HR	CI (95%)	P Value	HR	CI (95%)	P Value
Age							
Recipient	39 (95/245)	1.001	0.985-1.018	0.879			
Donor	39 (95/245)	1.001	0.987-1.015	0.882			
Gender recipient							
Male	44 (67/151)	1.746	1.123-2.715	0.013	1.504	0.929-2.434	0.097
Female	30 (28/94)	ref			ref		
Gender donor							
Male	37 (45/121)	0.981	0.656-1.468	0.926			
Female	40 (50/124)	ref					
Primary liver disease				0.030			0.053
Viral	41 (17/41)	1.130	0.638-2.002		0.557	0.297-1.044	
Alcoholic	61 (20/33)	1.984	1.154-3.412		1.262	0.709-2.245	
Cholestatic	30 (20/66)	0.821	0.478-1.411		0.623	0.351-1.104	
Other disease	36 (38/105)	ref			ref		
Child-Pugh class				0.935			
A	39 (22/56)	ref					
B	38 (41/109)	0.942	0.561-1.581				
C	40 (32/80)	1.024	0.595-1.762				
Lab MELD score	39 (95/245)	1.001	0.976-1.026	0.955			
Donor-type							
Donation after cardiac death	52 (12/23)	1.876	1.023-3.439	0.042	3.068	1.512-6.222	0.0019
Donation after brain death	37 (83/222)	ref			ref		
Cold ischemia time	39 (93/241)	1.001	1.000-1.002	0.122	1.001	0.999-1.002	0.481
Warm ischemia time	38 (93/242)	0.984	0.967-1.003	0.092	0.983	0.963-1.002	0.085

Supplemental Table 2. continued

Variable	CMV infection % (N)	Univariate			Multivariate		
		HR	CI (95%)	P Value	HR	CI (95%)	P Value
CMV seropositivity				<0.0001			<0.0001
D+/R+	61 (42/69)	19.027	4.601-78.685		19.963	4.766-83.621	
D+/R-	68 (26/38)	18.677	4.427-78.792		20.455	4.790-87.356	
D-/R+	26 (25/95)	6.555	1.552-27.675		7.983	1.872-34.038	
D-/R-	5 (2/43)	ref			ref		
Maintenance immunosuppression							
Prednisone / CNI / basiliximab (+ MMF)	40 (78/193)	1.391	0.823-2.351	0.218			
Prednisone / CNI (+ azathioprine)	33 (17/52)	ref					
Antiviral treatment							
Yes	39 (50/127)	0.900	0.602-1.347	0.610			
No	38 (45/118)	ref					
Acute cellular rejection							
Yes	32 (28/87)	0.693	0.446-1.077	0.103	1.028	0.637-1.659	0.911
No	42 (67/158)	ref			ref		
Combined MBL2 and FCN2 mismatch profile†							
MBL2* and/or FCN2* mismatch	42 (90/215)	3.101	1.260-7.635	0.014	2.288	0.906-5.778	0.080
No mismatch	17 (5/30)	ref			ref		
Peak AST							
≥1600	51 (36/70)	1.663	1.099-2.518	0.016	1.699	1.028-2.808	0.039
<1600	34 (59/175)	ref			ref		

HR denotes hazard ratio; CI, confidence interval; MELD, Model for End-Stage Liver Disease; CNI, calcineurin inhibitors; MMF, mycophenolate mofetil; AST, aspartate aminotransferase;

†The presence of donor-recipient mismatch associated with CMV within the individual were clustered as mismatch profile groups

MBL2: XA/O and O/O donor and A/A and YA/O recipient and /or FCN2: FCN2-C donor and FCN2-A recipient

*The presence of a deficient donor MBL2 secretor haplotypes in a sufficient recipient haplotype conferred the highest risk compared to other donor-recipient combinations.

‡The presence of a FCN2-A (low binding) recipient receiving a FCN2-C (high binding) donor liver showed a clear trend to less CMV infections as opposed to the other genotypes.

Chapter 9

**Mannan-Binding Lectin-Associated Serine
Protease-2 variant is strongly associated with
the development of non-anastomotic biliary
strictures after liver transplantation and with
Budd-Chiari syndrome**

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ABSTRACT

Non-anastomotic biliary strictures (NAS) after liver transplantation may partially be the consequence of ischemic injury caused by microthrombi formation in the microvascular supply of the biliary tree during ischemia intervals throughout transplantation. In Budd-Chiari syndrome (BCS), thrombosis develops in the hepatic venous outflow tract in patients with an underlying thrombotic diathesis and hypofibrinolysis.

Compelling evidence shows that coagulation and complement systems are co-regulated and highly integrated. Mannose-binding lectin associated protease (MASP)-2 is liver-derived and an essential component of the lectin pathway of complement activation. Natural wild-type pro-urokinase plasminogen activator is also a substrate for MASP-2. Deficiency of MASP-2 after orthotopic liver transplantation (OLT) might therefore contribute to a decreased fibrinolytic potential.

We explored the impact of the *MASP2* D120G mutation on the development of NAS in a cohort of 147 OLTs. The risk of NAS was assessed with the use of multivariate Cox regression analysis. The analysis was replicated in a validation study involving 167 OLTs. The risk of BCS was explored in all patients combined.

In the discovery study the *MASP2* mutation in the donor increased the risk of NAS (adjusted hazard ratio, 6.4; 95% CI, 2.2-19.0; $P=0.008$). In the validation study the *MASP2* mutation also increased the risk of NAS (adjusted hazard ratio, 3.0; 95% CI, 1.0-8.9; $P<0.05$). Three out of the four patients (75%) with BCS as indication for liver transplantation were heterozygous for *MASP2* D120G compared to 4% in patients with other indications for OLT ($P=0.0004$).

This study reveals a causal relationship between the *MASP2* D120G mutation and the development of NAS and BCS after liver transplantation.

INTRODUCTION

Biliary and vascular disorders are common and clinically particularly relevant complications after orthotopic liver transplantation (OLT). The formation of non-anastomotic biliary strictures (NAS) occurs in 5 to 15% of liver transplant recipients.¹ Although the exact pathophysiological mechanism is unknown, NAS is most likely the result of a complex mechanism involving ischemic, immunologic and toxic processes, which all affect the biliary tree or its vascular supply.¹

Recent literature has shown that a decreased blood flow in the microvascular supply of the biliary tree, the peri-biliary plexus, possibly due to microthrombi formation, may be involved in the development of NAS after OLT. Microthrombi may develop during the donor warm en recipient cold and warm ischemia intervals during the pretransplantation preservation procedure.^{2,3}

The coagulation and complement systems are both activated following tissue injury to protect the host from blood loss and against infections.⁴ A number of recent studies show direct interactions between the two systems,^{5,6} among which are links between the coagulation factors and mannan-binding lectin (MBL)-associated proteases (MASPs) of the complement lectin pathway. MASP-1/3 for instance has been shown to mediate coagulation factor-like activities, including thrombin-like activity.⁷ Evidence further shows that natural wild-type pro-urokinase plasminogen activator is a substrate for MASP-2.⁸

MASP-2 is a serine protease associated in a molecular complex with the complement-activating lectins of the lectin pathway of complement (MBL, ficolin-1, ficolin-2, ficolin-3). This enzyme is responsible for complement activation via the lectin pathway by its ability to cleave C4 and C2, leading to formation of the C3 convertase C4b2a. An inherited deficiency of MASP-2, caused by a homozygous mutation (rs72550870) in the *CUB1* domain, is known to prevent the formation of functional MBL–MASP-2 complexes⁹ and is associated with low serum levels of MASP-2.¹⁰ Next to impaired functionality of MASP-2, it also results in an increase of MASP-3 in the MBL–MASP complex, since MASP-3 competes with MASP-2 for binding to MBL.¹¹

Budd–Chiari syndrome (BCS) is a rare disorder caused by obstruction of the hepatic venous outflow tract. The disorder most often occurs in patients with an underlying thrombotic diathesis, both hereditary and acquired, but may also have a variety of other causes,¹² including impaired fibrinolysis.¹³

We hypothesized that the MASP-2 mutation might be associated with conditions of the liver related with increased thrombus formation or decreased fibrinolysis of thrombi, possibly involved in the pathogenesis of NAS, or obstruction of the hepatic outflow tract, as seen in BCS.

In the present study we investigated the putative role of MASP-2 deficiency in the formation of non-anastomotic biliary strictures or Budd–Chiari syndrome.

PATIENTS AND METHODS

For the discovery study, we identified 202 patients who underwent OLT at the Leiden University Medical Center (LUMC) between 1992 and 2006. Out of these 202 liver transplantations we were able to unselectively include 147 patients whose DNA was available from both donor and recipient. To study the correlation between MASP2 genotype and serum concentration, we were able to retrieve serum samples from 139 patients after OLT. For the validation study, we included patients who received OLT between 2000 and 2006 at the University Medical Center Groningen (UMCG). Of the 224 available patients, 167 unselected patients could be included of whom we had DNA from both recipient and donor.

All patients received cyclosporine or tacrolimus-based immunosuppressive therapy, corticosteroids and in case of impaired renal function either azathioprine until 2001 or mycophenolate mofetil thereafter, and from 2001 on, basiliximab was used in addition on days 0 and 4.

Demographical and clinicopathological characteristics of the recipient at the time of OLT, donor information, transplantation procedure variables and post-transplant follow-up data of up to 4 years were collected from the transplantation databases.

The study was conducted in accordance with Dutch legislation and the local ethical committee guidelines of both participating centers, in compliance with the Helsinki declaration.

Non-anastomotic biliary strictures

Non-anastomotic strictures (NAS) were defined as strictures, dilatations or irregularities of the intra- 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.¹⁴ Hepatic artery thrombosis by either Doppler ultrasound or contrast CT or conventional angiography as well as isolated strictures/stenoses at the bile duct anastomosis and related dilations were, by definition, excluded from this analysis. The time of first presentation of NAS was recorded for all patients.

If a biliary stricture was suspected from clinical findings, liver function tests or abdominal ultrasound, further imaging of the biliary tract was performed. In the LUMC, the biliary duct-to-duct anastomosis was made over an endoprosthesis, in the UMCG without if endoprosthesis if possible. Protocol cholangiography was performed routinely 6 weeks after OLT at the time of drain removal. Other cholangiographies were only on indication. All imaging studies of the biliary tree, performed within 4 years after OLT, were included [direct cholangiography via the biliary drain, by percutaneous transhepatic cholangiodrainage (PTCD), by ERCP or as MRCP].

Budd-Chiari syndrome

BCS was defined as hepatic outflow obstruction regardless of the cause or level of obstruction, from the small hepatic veins to the entrance of the inferior vena cava into the right atrium. BCS was confirmed by radiographic imaging (ultrasonography, computed tomography, magnetic resonance imaging, or venography).

Genotyping

MASP2+120 A/G (rs72550870) genotyping was performed with the use of high-resolution DNA melting assay, as part of our studies on the genetics of the lectin pathway of complement activation in OLT.^{15,16} Sequences of the polymerase chain reaction (PCR) primers were 5'-TGCATAGAAGGCCTCGAACC-3' (forward) and 5'-GGCAAGGACACTTCTACTCGC-3' (reverse). The unlabelled probe was designed to match the variant sequence (C) and had the following sequence: 5'-CTCGTTGGAGTAGCCGGAGCGGAAGG-3'. The probe had a 3'-amino-C6 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.

Determination of serological MASP-2 levels

MASP-2 concentrations were assessed by sandwich ELISA as described previously using a rabbit polyclonal antibody directed against a recombinant CCP1-CCP-2-SP fragment of human MASP-2.¹⁷ In brief, plates (Maxisorb; Nunc, Roskilde, Denmark) were coated with rabbit IgG anti-MASP-2 (2.5 µg/ml) in coating buffer (100 mM Na₂CO₃/NaHCO₃ [pH 9.6]) and incubated overnight at 4°C. After each step, plates were washed three times with PBS that contained 0.05% Tween 20. Residual binding sites were blocked by incubation with PBS that contained 1% BSA.

Appropriately diluted serum samples and subsequent detection antibodies were incubated for 1 h at 37°C. Serum samples were diluted 1/40 (v/v) in veronal buffer saline containing 0.05% Tween-20, 0.1% gelatin, 1 M NaCl and 10 mM ethylenediamine tetraacetic acid, pH 7.5. MASP-2-binding to the coating was detected using digoxigenin-labelled rabbit IgG anti-human MASP-2 diluted 1:1000 (v/v) in detection buffer (PBS, 1% BSA, 0.05% Tween-20). Binding of rabbit antibodies was detected using horseradish peroxidase-conjugated sheep anti-dig antibodies (Fab fragments; Roche Diagnostics). Development was carried out with 2,2'-azino-bis 3-ethylbenzthiazoline-6-sulphonic acid (Sigma-Aldrich) at a concentration of 2.5 mg/ml in 0.1 M citrate/Na₂ HPO₄ buffer, pH 4.2, in the presence of 0.01% H₂O₂. Concentrations of MASP-2 were calculated using serial dilutions of a standard serum in which the concentration of MASP-2 was arbitrarily set at 1000 U/ml.

Statistical analysis

All statistics were calculated using SPSS version 16.0.2 (SPSS Inc., Chicago, IL). Comparisons of the risks of NAS were performed with the use of univariate Cox regression models. Multivariate Cox models were used to evaluate the relative hazards associated with the *MASP2* variant. Candidate covariates were selected from the factors associated with NAS in the univariate analysis ($P \leq 0.15$). The cumulative incidence of NAS was calculated using the Kaplan–Meier method and compared using the log rank test. Categorical variables were compared with the Pearson's chi-square test or Fisher's exact test where appropriate.

Serum concentrations were compared among the groups with the Mann–Whitney *U*-test to compare two independent groups. Two-sided hypotheses and tests were used for all statistical inferences. A *P* value of less than 0.05 was considered to indicate statistical significance.

RESULTS

Discovery study

The discovery study included 147 patients who underwent OLT (Table 1). Out of the 147 donors tested, 9 (6.1%) donors were heterozygous for the minor *MASP2*+120 G-allele and 93.9% (138) were homozygous for the wild-type allele. Non-anastomotic strictures (NAS) were observed in 20 of 147 (14%) transplanted livers with a median time of 5.5 months (IQR 3.00–9.75) after transplantation.

Serum levels of MASP-2 after transplantation were significantly lower in patients who received a donor liver heterozygous for the minor G-allele of *MASP2* (Figure 1).

Univariate analysis

To assess the risk of NAS according to the *MASP2* SNP in the donor liver we evaluated the cumulative incidence of NAS. Patients receiving a donor liver heterozygous for the minor G-allele of *MASP2* had a cumulative incidence of NAS of 56%, compared to 11% in the recipients with a wild-type donor liver ($P < 0.001$) (Table 1).

Multivariate analysis

The model revealed that donor *MASP2*+120G (adjusted hazard ratio, 6.40; 95% CI, 2.16–19.00; $P = 0.008$) was a very high-risk factor for NAS (Table 2). The model also included age of the recipient (HR 0.94; 95% CI, 0.91–0.98; $P = 0.004$), primary liver disease (PSC: HR 1.45; 95% CI, 0.43–4.82; $P = 0.55$; other cholestatic disease: HR 4.45; 95% CI, 1.39–14.28; $P = 0.01$) and warm ischemia time (HR 0.94; 95% CI, 0.88–1.01; $P = 0.09$).

Validation study

The characteristics of the patients in the validation study were comparable to those of the patients in the initial study (Table 1). Out of the 167 donors included, 8 (4.8%) donors were heterozygous for the minor *MASP2*+120 G-allele and 95.2% (159) were homozygous for

Table 1. Characteristics of Recipients and Donors in the Discovery and Validation Study

Variable	Discovery Study			Validation Study		
	NAS (N=20)	No NAS (N=127)	P Value†	NAS (N=28)	No NAS (N=139)	P Value†
Recipient Age (mean, SD)	41 (10)	49 (11)	0.016	46 (13)	48 (12)	0.637
Donor Age (mean, SD)	39 (13)	42 (16)	0.312	43 (12)	46 (15)	0.500
Gender of donor and recipient, % (n)			0.880			0.284
Male/male	30 (6)	38 (48)	0.727	36 (10)	31 (43)	0.615
Male/female	25 (5)	17 (22)	0.798	4 (1)	19 (26)	0.165
Female/male	25 (5)	29 (37)	0.673	36 (10)	24 (33)	0.353
Female/female	20 (4)	16 (20)		25 (7)	27 (37)	
Primary liver disease, % (n)			0.149			0.024
Primary sclerosing cholangitis	20 (4)	16 (20)	0.483	39 (11)	16 (22)	0.008
Other cholestatic disease*	20 (4)	7 (9)	0.053	7 (2)	9 (13)	0.977
Other**	60 (12)	77 (98)		54 (15)	75 (104)	
Lab MELD score (mean, SD)	16 (10)	18 (9)	0.525	17 (9)	17 (9)	0.730
Donor-type, % (n)						
Donation after cardiac death	15 (3)	9 (12)	0.372	7 (2)	6 (8)	0.639
Donation after brain death	85 (17)	91 (115)		93 (26)	94 (131)	
Cold ischemia time (mean, SD)	694 (216)	642 (217) ^a	0.208	539 (139)	495 (117) ^b	0.061
Warm ischemia time (mean, SD)	34 (7)	39 (13) ^a	0.136	47 (9)	48 (11) ^b	0.994
Donor MASP2 (rs72550870), % (n)			0.0008			0.009
AA	75 (15)	97 (123)		86 (24)	97 (135)	
AG	25 (5)	3 (4)		14 (4)	3 (4)	

† P values are for univariate Cox regression models
* Other cholestatic disease comprise Primary biliary cirrhosis and Secondary sclerosing cholangitis.
** Other include predominantly post viral cirrhosis, alcoholic cirrhosis, autoimmune hepatitis, cryptogenic cirrhosis and metabolic disorders.
^a n=120 ^b n=138

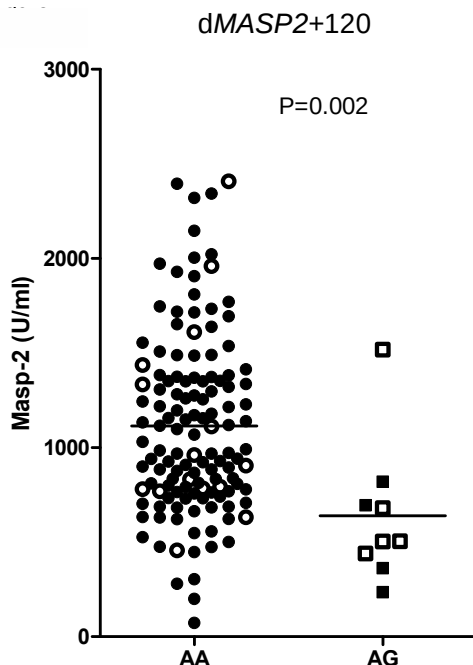


Figure 1. Serological levels of MASP-2 in relation to the donor MASP2 genotype in patients after OLT. Open symbols represent patients who developed NAS

the wild-type allele. NAS was observed in 28 of 167 (16.8%) transplanted livers with a median time of 5.5 months (IQR 2.00-21.50) after transplantation. The association of donor *MASP2* +120AG with the risk of NAS was confirmed in the validation study (adjusted hazard ratio, 3.01; 95% CI, 1.02-8.92; $P < 0.05$) independent from primary liver disease (PSC: HR 3.10; 95% CI, 1.38-6.98; $P = 0.006$; other cholestatic disease: HR 1.09; 95% CI, 0.25-4.76; $P = 0.91$) and cold ischemia time (HR 1.00; 95% CI, 1.00-1.01; $P = 0.03$), which were the other risk factors for NAS in this validation cohort (Tables 1 and 2).

The impact of the donor *MASP2* genotype on the cumulative probability of NAS in the two studies combined is shown in Figure 2. The overall hazard ratio was found to be 4.45 (95% CI, 2.11-9.36; $P < 0.001$), adjusted for recipient age (HR 0.98; 95% CI, 0.96-1.00; $P = 0.08$), primary liver disease (PSC: HR 2.42; 95% CI, 1.26-4.61; $P = 0.008$; other cholestatic disease: HR 2.18; 95% CI, 0.88-5.39; $P = 0.09$), warm ischemia time (HR 1.00; 95% CI, 0.97-1.02; $P = 0.74$) and cold ischemia time (HR 1.00; 95% CI, 1.00-1.00; $P = 0.06$).

MASP2 association with Budd-Chiari syndrome

The risk of Budd-Chiari syndrome (BCS) was explored in patients of discovery and validation study combined. We analyzed 268 out of the 314 recipients based on the exclusion of 46 patients due to retransplantation. Five percent (13 patients) were heterozygous for the minor *MASP2*+120 G-allele and 95% (255) were homozygous for the wild-type allele. Out

Table 2. Multivariate Analyses of the Association of Donor MASP2 Polymorphism with the Risk of NAS in Both Studies

Variable	Discovery Study (N=140)			Validation Study (N=166)		
	Adjusted Hazard Ratio	CI (95%)	P Value†	Adjusted Hazard Ratio	CI (95%)	P Value‡
Donor <i>MASP2</i> (rs72550870) AG versus AA	6.40	2.16-19.00	0.008	3.01	1.02-8.92	0.047

† Hazard ratios have been adjusted for Recipient age, Primary liver disease, and Warm ischemia time

‡ Hazard ratios have been adjusted for Primary liver disease, Cold ischemia time

of the four patients with Budd-Chiari syndrome (BCS) as indication for OLT, 3 patients were heterozygous for the minor G-allele of *MASP2* (Table 3). Of the other indications for OLT, 4% (10) were heterozygous for the minor *MASP2*+120 G-allele compared to 96% homozygous ($P=0.0004$). In addition, after transplantation two out of three patients who developed BCS after OLT received a donor liver heterozygous for the minor G-allele of *MASP2* (Table 3).

DISCUSSION

The current study indicates a strong association between a heterozygous mutation in the *CUB1* domain of the *MASP2* gene and the development of two clinical coagulation-associated conditions in liver transplantation recipients. Firstly, we found that the presence of the *MASP2* mutation in the donor liver was associated with a significantly increased incidence of the development of NAS. Secondly, the frequency of this mutation was significantly higher in patients with BCS as indication for transplantation. Moreover, this frequency was also increased in recipients of donor livers carrying the *MASP2* mutation with post-OLT BCS.

MASP-2 deficiency was originally described in an immunodeficient patient with autoimmune manifestations. The deficiency was due to homozygosity of a missense mutation (rs72550870) in the *CUB1* domain which gives rise to a non-functional protein preventing the formation of functional MBL–MASP-2 complexes.⁹ MASP-2 is best known for its proteolytic ability to cleave complement factor C4 and C2, generating the C3 convertase which cleaves and activates component C3 causing a cascade of subsequent complement cleavage and activation events.^{18;19}

Expression of MASP2 is predominantly localized in the liver which is the only tissue where MASP-2 mRNA has been detected.²⁰⁻²²

Next to impaired functionality of MASP-2, the mutation also results in an increase of MASP-3 in the MBL–MASP complex, since MASP-3 competes with MASP-2 for binding to MBL.¹¹

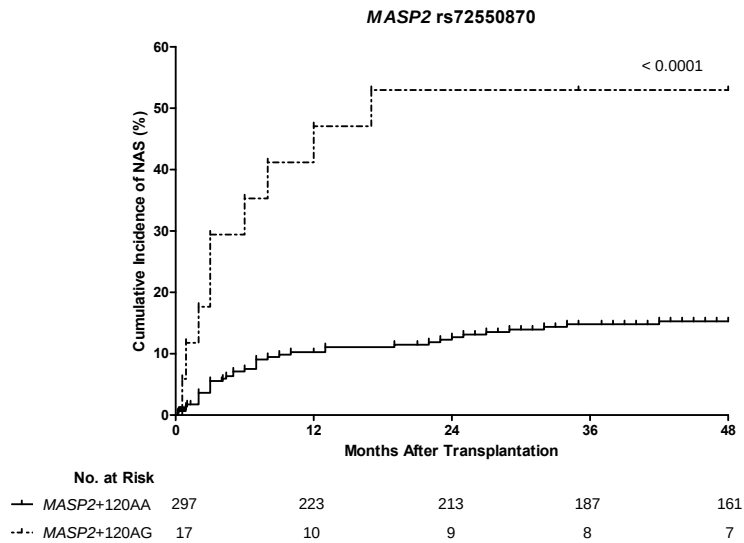


Figure 2. Cumulative incidence of non-anastomotic strictures (NAS) within 48 months after orthotopic liver transplantation, related to MASP2 genotype of the donor liver in patients of both discovery and validation study combined. The end point was the time to the diagnosis of NAS in 314 recipients, with censoring of the data for these recipients at the date of last follow-up (death or second transplantation). P value calculated with the use of the log-rank test.

Table 3. Budd–Chiari Syndrome Association with MASP2 Polymorphism

Variable	Recipient MASP2 (rs72550870)			Donor MASP2 (rs72550870)		
	AG	AA	P Value†	AG	AA	P Value†
Indication for OLT, % (n)						
Budd–Chiari syndrome*	75 (3)	25 (1)	0.0004			
Other disease	4 (10)	96 (254)				
Complication after OLT, % (n)						0.009
Budd–Chiari syndrome**				67 (2)	33 (1)	
Other / none				5 (16)	95 (296)	

† Fisher's exact test

* Budd–Chiari syndrome (BCS)

Recipient MASP2 AG:

1 with Protein C deficiency;

Budd–Chiari syndrome with SLE (anti-cardiolipine)

1 BCS with unknown cause

Recipient MASP2 AA: Low protein C + S during thrombosis, factor V Leiden heterozygosity, oral contraceptives use

** Donor MASP2 AG: 1 patient developed both NAS (1 year) and BCS (7 years) after OLT; 1 BCS with unknown cause

Donor MASP2 AA patient developed both NAS and BCS after OLT

The frequency of the gene mutation in our study was 3.7% in recipients and 5.1% of the donor livers without BCS or NAS, which is comparable with the frequency of 3.6% found in large study among Caucasians.²³

There is compelling evidence that coagulation and complement systems are co-regulated and highly integrated⁴. For example, MASP-1 has thrombin-like activity and is able to cleave fibrinogen and factor XIII.^{7;24;25} In addition, MASP-1 induces fibrin clot formation by converting prothrombin to thrombin which then cleaves fibrinogen. Fibrin clot formation can be initiated by MASP-1 in plasma and results in clots with a prolonged lysis time, which may be related to changes in the clot structure.²⁶ Although MASP-2 was reported to have Xa-like activity and activated prothrombin through cleavage to form thrombin, its activation potential is quite low (about 4–5% the rate observed with factor Xa).²⁷ Remarkably, natural wild-type pro-urokinase acts as a substrate for MASP-2 and is therefore involved in fibrinolysis.⁸

This two-sided effect of MASPs might be advantageous since localized activation of the coagulation system and clot formation serves an important survival function in response to systemic microbial invasion. Generalized intravascular coagulation, as seen in the presence of invasive bloodstream infections, is clearly disadvantageous to the host, with consumption of clotting factors and widespread deposition of fibrin clots throughout the microcirculation. Impaired MASP2 could disturb the equilibrium causing a state of hypercoagulability.

NAS may be the consequence of ischemic injury caused by microthrombi in the microvascular supply of the biliary tree called the peri-biliary vascular plexus or in the sinusoids with indirect damage of the biliary tree.^{2;3} Indeed, a recent case presentation by Farid et al. indicates that portal blood flow may also have an important contribution to the biliary microcirculation and that a compromised portal venous blood supply can predispose to the development of NAS after OLT.²⁸

Portal venous blood supply is also compromised in BCS, a disorder caused by obstruction of the hepatic venous outflow tract. Factors that confer a predisposition to the development of BCS include hypercoagulable states, both hereditary and acquired, and a variety of other causes.^{12;13}

MASP-2 is produced in hepatocytes and released directly from the liver cells into the sinusoids.

MASP-1 and MASP-3 are splice variants of the *MASP1* gene. MASP-1 is primarily expressed in the liver, whereas a more broad tissue expression pattern is observed for MASP-3.²⁹

While preserving donor livers before transplantation, formation of fibrin thrombi may occur in the sinusoids and decreased sinusoidal blood flow can lead to obstruction of the venous vasculature of the liver. Both initiation and promotion of thrombus formation seems to be correlated to mutated MASP-2. Thrombus formation could be augmented due to a decreased local fibrinolytic effect of MASP-2 and increased presence of MASP-3 in the lectin–MASP complex could lead to increased MASP-1/3-catalysed deposition and polymerization of fibrin (Figure 3).

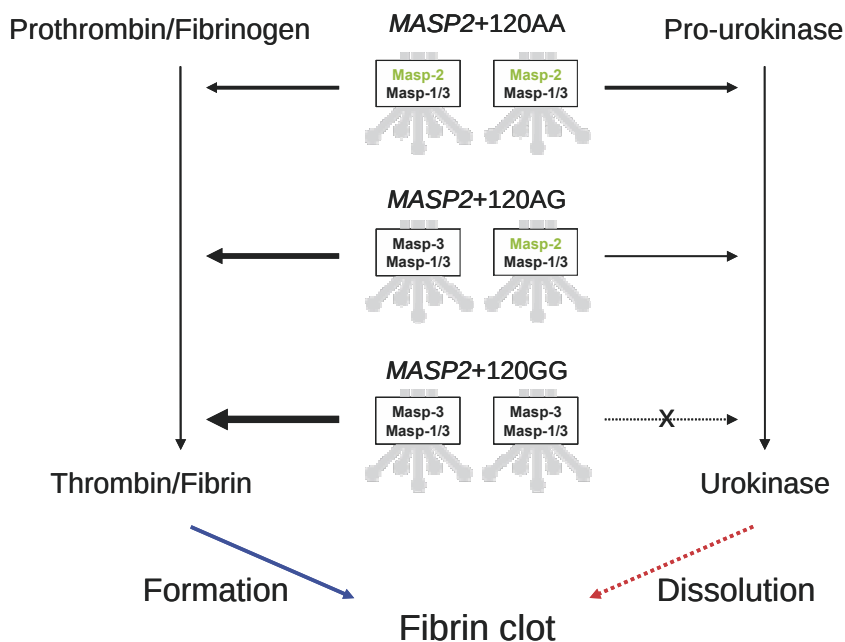


Figure 3. Proposed model on the role of MASP2 and disordered coagulation or fibrinolysis While preserving a donor liver for transplantation, fibrin clots may form in the sinusoids and arterial plexus of the liver. The lectin-MASP complex consists of MASP2 and MASP1 and 3. There is evidence that MASP1 and 3 can mediate coagulation factor like activities, which can lead to clot formation. MASP2 on the other hand can dissolve clots. In case of a MASP2 AG genotype this would result in decreased fibrinolysis. Since there is increased presence of MASP-1 and 3 this could lead to increased MASP1 and 3 catalyzed clot formation.

The existence of a multifactorial etiology for BCS is increasingly recognized.^{30,31} In case of BCS the mutated MASP-2 probably adds up to other coexisting risk factors for BCS.

Based on our findings, there is clinical evidence that BCS and NAS might share a similar pathophysiological mechanism which causes disordered coagulation or fibrinolysis. Although the explanation remains speculative the link between the mutated MASP-2 and NAS might be not far-fetched. As mentioned earlier, evidence has been presented that natural wild-type pro-urokinase is a substrate for MASP-2. Pro-urokinase is the precursor of urokinase, a plasminogen activator which through the proteolytic enzyme plasmin might facilitate fibrinolysis.

In both NAS and BCS, thrombolytic therapy is successfully used to break up or dissolve blood clots. One prospective study reported that perfusion of donor livers with urokinase reduced the incidence of NAS with 75% compared to a historical control group.³²

Similarly, systemic and locally administered thrombolytic agents, like urokinase have been used in the treatment of Budd-Chiari syndrome.³³ Furthermore, in our study two out of the three patients who developed BCS after OLT also developed NAS.

However, possible explanations for the significantly higher incidence of NAS in livers with MASP-2 mutation may also be explained by an immunological imbalance caused by the lack of a functional MASP-2 in these patients. NAS has been associated with various immunological processes, such as pre-existing diseases with a presumed autoimmune component (PSC and autoimmune hepatitis) and its association with functional mutation of the CCR5 gene (CCR5-Δ32).³⁴

Another explanation for the increased NAS incidence in case of this MASP-2 mutation might be that MASP-mediated fibrinogen cleavage leads to the generation of fibrinopeptides A (FPA) and B (FPB) which both have potent chemoattractant effects on neutrophil and fibroblast recruitment.²⁶ Therefore MASP-mediated release of FPA and FPB may play a role in early immune activation after reperfusion.

Furthermore, apoptotic cell clearance might be impaired. During preservation of the liver, ischemic injury results in apoptotic cells which are a rich source of autoantigens that are known targets of autoantibodies in systemic autoimmune diseases. The data available suggest that deficiency of apoptotic cell opsonins such as complement factors delays the *in vivo* uptake of apoptotic material by professional phagocytes.³⁵ This may result in a prolonged exposure of the immune system to apoptotic cells and to a progression of these apoptotic cells into later stages of cell death, thereby increasing the risk of induction of autoimmunity.

In conclusion, this study clearly shows a possibly causal relationship between a mutation in the MASP2 gene and the development of BCS before and after and NAS after orthotopic liver transplantation.

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Chapter 10

Summarizing discussion

SUMMARIZING DISCUSSION

Liver transplantation is the treatment of choice for appropriately selected patients with acute liver failure, end-stage liver disease, some metabolic disorders and hepatocellular carcinoma. Since the first human liver transplantation performed by Thomas Starzl in 1963, progressive improvement of graft and patient survival after liver transplantation has been achieved due to better insight into the immunobiology of allograft rejection, new and more conservative use of immunosuppressive agents and better surgical and medical management. The first liver transplantation in the Netherlands was performed in 1966 in Leiden and since then more than 3000 procedures have been performed. Nowadays one- and five-year patient survival after liver transplantation in the Netherlands is 89% and 75%, respectively.¹

Despite improvements in immunosuppressive therapy, immunosuppression after liver transplantation is accompanied with some adverse effects, including the increased susceptibility for infections.

Nowadays infectious complications are the main cause of morbidity and mortality during the first year after liver transplantation and occur in up to 80% of the patients, despite efforts to prevent this (with use of vaccination, antimicrobial prophylaxis, and pre-emptive therapy) and treatment of infectious complications. Bacterial infections are the most frequent (70%), followed by viral (20%) and fungal infections (8%).²⁻³

The risk for infections after liver transplantation is a multifactorial problem and all factors must be taken into consideration in order to reduce this risk. **Chapter 2** gives an overview of the many, transplantation-related, recipient and donor factors which are involved in the risk for infection after liver transplantation. It also emphasises that there is an increasing body of data to support the role of immune gene-deficiencies in innate immunity in the pathogenesis and predisposition to infectious disease, especially among individuals with immature, defective, or compromised adaptive immune systems, like immune-compromised patients and young children.

The human immune system can be divided into two broad components – the innate immune system and the adaptive immune system – that operate in tandem in order to try and prevent infection. The administration of immunosuppressive drugs has a broad range of inhibitory effects on the adaptive immune system of the recipient, who consequently becomes predominantly dependent on the innate immunity, as first line of immunological defence against infections.

The liver plays a key role in controlling systemic innate immunity through the production of 80–90% of the circulating innate immunity proteins in the body, such as soluble pattern-recognition receptors, complement components and coagulation factors.⁴

Pattern-recognition receptors (PRRs) are germ-line-encoded, invariant receptors that recognize evolutionarily conserved pathogen-associated molecular patterns (PAMPs) among broad groups of microorganisms. PRRs have two main functions: complement activation and microbial cell opsonisation for phagocytosis.

The complement system is a central subsystem within the innate immunity that consist of a large set of plasma and membrane proteins that react with one another as a cascade resulting in opsonization of pathogens, the recruitment of inflammatory cells, and direct killing of pathogens through the formation of a complement membrane attack complex.

There are three separate pathways through which complement can be activated on the surfaces of pathogens, the classical pathway, the lectin pathway, and the alternative pathway. Each is triggered in a distinct manner, but they all converge on the production of a key complex known as the C3-convertase which is essential to cleave C3 and generate large amounts of C3b, the main effector molecule of the complement system.⁵

The lectin pathway of complement activation is initiated when soluble PRRs - mannose-binding lectin (MBL) or ficolins (ficolin-1, ficolin-2 and ficolin-3)- in complex with MBL-associated serine proteases (MASP-1, MASP-2 and MASP-3), recognize foreign patterns of carbohydrate or acetyl groups, respectively.

Common SNPs in the genes coding for the three liver-derived members of the lectin complement pathway, - MBL, ficolin-2 and MASP-2- affect the expression, composition and structure of the proteins with important functional implications that may directly affect disease outcome.

In the general population there is a wide variety in MBL serum concentrations and function⁶ caused by heterozygous polymorphic variation in the gene encoding MBL, which is present in 20% to 40% of the population depending on ethnicity. MBL deficiency is one of the most common human immunodeficiencies and may predispose individuals to a broad range of infectious diseases, especially among affected individuals who largely depend on their innate immune system for antimicrobial immune defences, such as liver transplant recipients.⁷

After liver transplantation, the recipient with a drug-induced immunodeficiency adopts the hepatocyte-derived innate immunity proteins of the donor and for that liver transplantation offers a unique “in vivo” human model to study functional aspects of the components of the lectin pathway of complement activation.

In an attempt to improve the ability to more accurately predict a patient’s risk of infection after transplantation this dissertation/thesis focus is on the effect of genetic variation in pattern recognition receptors and their signalling pathways on susceptibility to infections before and after liver transplantation, with a special emphasis on the lectin pathway of complement activation.

First, we needed to assess whether MBL, ficolin-2 and MASP-2, as key components of the lectin complement pathway, are mainly produced by the liver. Hence, we evaluated the contribution of common single nucleotide polymorphisms (SNP) in the genes coding for three (liver-derived) components involved in the lectin complement pathway: MBL, ficolin-2 and MASP-2, and the balance between hepatic (donor) and extrahepatic (recipient) production of these factors, as described in **chapter 3**. To estimate this genetic contribution to variation in expression we determined MBL, ficolin-2 and MASP-2 concentrations and

potential polymorphisms in their coding-genes - *MBL*, *FCN2* and *MASP2* - in three study groups, i.e. 170 healthy controls, 120 liver transplant recipients before and 111 after OLT.

MBL serum concentrations in recipients before and after OLT did not differ, in contrast to ficolin-2 and MASP-2 where serum concentrations before OLT were significantly lower compared to concentrations after OLT. Furthermore, ficolin-2 and MASP-2 concentrations before OLT were significantly lower in patients with a more severe hepatic dysfunction, in cirrhosis graded as Child-Pugh B and Child-Pugh C classes, compared to patients graded as Child-Pugh class A. Recent published data on MBL, ficolin-2 and MASP-2 serum levels in patients with cirrhosis (of various aetiology) endorse these findings.^{8,9}

In each of the three study groups a strong genotype-phenotype correlation was observed regarding the multilocus *MBL* genotype A/A and YA/O versus XA/O and O/O, *FCN2* SNP at position -986A>G and *MASP2* SNP at position +371A>C, with the exception of *FCN2* SNP position -986A>G in recipients before OLT. After OLT, phenotypes were only associated with the genotype of the donor liver and not with that of the recipient.

As far as MBL genotype-phenotype correlation is concerned we confirmed and extended, in the largest cohort of OLT patients so far, previous observations that the MBL genotype of the donor liver determines the serum MBL concentration after transplantation. We also showed that ficolin-2 and MASP-2 serum levels in recipients after OLT correlate with the genotype of the liver donor and that the lower pre-OLT levels in the recipient recover after OLT. So far, there had been no published data on the dynamics of ficolin-2 or MASP-2 serum levels after OLT.

The conclusions from chapter 3 permitted us to consider the *MBL*, *FCN2* and *MASP2* genotype of the donor liver in recipients after OLT when exploring genetic susceptibility correlations among post-OLT complications.

Chapters 4 and 5 describe our observations as to what extent genetic variation in and interplay between genes coding for MBL, ficolin-2, and MASP-2 from the donor and recipient contributes to the susceptibility for clinically significant bacterial infections (CSI) and cytomegalovirus (CMV) (re) infections in OLT recipients within the first year after OLT. The general patient database, original patient reports, transplantation databases, and microbiology records from two different transplant centres were evaluated to identify episodes of clinical and laboratory-confirmed bacterial infections within the first year after transplantation, without knowledge of the genotypes. Demographic and clinicopathological characteristics of the recipient at the time of OLT (age, sex, indication for liver transplantation, Child-Pugh classification, and laboratory Model for End-Stage Liver Disease [MELD] score), donor information (age, sex), and posttransplant follow-up data (immunosuppressive regimen, episodes of acute cellular rejection) were also collected from the transplantation databases.

The study described in **chapter 4** consisted of a (principal) cohort of 143 patients who received OLT between 1992 and 2006. We determined the genotype frequencies of 13 functionally relevant SNPs in *MBL*, *FCN2* and *MASP2* which are common in the Caucasian

population. The *MBL*, *FCN2*, and *MASP2* genotype distribution of recipients and donors were analysed in relation to the cumulative incidence of clinically significant bacterial infections (CSI), i.e., sepsis, pneumonia or abdominal infection in the first year after OLT. In the principal cohort 41% of the recipients encountered a CSI within the first year after transplantation. Recipients receiving a donor liver with polymorphisms in all three components i.e., *MBL* (XA/O; O/O), *FCN2*+6359T (rs17549193) and *MASP2*+371A (rs12711521), had a cumulative risk of an infection of 75% as compared to 18% with wild-type donor livers. The joint genetic effect of risk-conferring variants of *MBL*, *FCN2*, and/or *MASP2* present within the donor liver were clustered as the "lectin pathway gene profile". This profile, including the number of risk-conferring gene variants, showed an ever-increasing cumulative risk for developing CSI with increasing numbers of variants: 18% CSI with no genetic variant, 33% in those with one, 50% in those with two, and 75% in those with three variants. These results were validated in a confirmation cohort which consisted of 167 OLT recipients with comparable characteristics as the patients from the principal study. Despite a significantly lower cumulative incidence of CSI within the first year and a significant lower percentage of transplanted donor livers with an *MBL*-deficient genotype in this confirmation cohort, the lectin pathway gene profile of the donor liver showed a similar increasing cumulative risk for developing CSI with increasing numbers of variants: 20% when genetic variants were absent, 15% with one variant, 26% with two variants and 56% with three variants. In both the principal and confirmation cohort the genotype distribution of the recipients showed no significant association for the independent SNPs or for the number of risk-conferring variants with the occurrence of CSI. However, in both the principal and confirmation cohort, recipients with an *MBL*-sufficient genotype (A/A and YA/O) receiving an *MBL*-insufficient (XA/O and O/O) donor liver developed significantly more CSI than the other patients (61% versus 37% respectively, $P < 0.006$). We also found that absence of the minor allele in *FCN2* SNP rs17549193 and the absence of homozygosity for the major allele in *MASP2* SNP rs12711521 in both recipient and donor showed a clear trend toward less CSI (*FCN2*: 28% versus 46% and *MASP2*: 22% versus 45% both $p=0.06$), implicating that such a genetic (mis)match between donor and recipient conferred a two-fold higher infection risk for each separate gene. Multivariate Cox analysis of the two cohorts combined revealed a stepwise increase in infection risk with the lectin pathway gene profile of the donor (hazard ratio (HR) 4.52; $p=8.1 \times 10^{-6}$) and the donor-recipient (mis)match genotype (HR 6.41; $p=1.9 \times 10^{-7}$), independent from the other risk factors sex and antibiotic prophylaxis (HR > 1.7 ; $p < 0.02$). In addition, patients with a lectin pathway gene polymorphism and infection had a six-fold higher mortality, of which 80% was infection-related.

The results from this study support previous studies that indicate that OLT recipients of *MBL* deficient livers have a higher risk of infections.^{7, 10} Our study extended these findings by showing that recipients with an *MBL*-sufficient genotype receiving an *MBL*-insufficient/deficient liver are at greatest risk of developing infection. Recently published data by Lombardo-Quezada et al. confirm that OLT recipients of *MBL*-deficient livers have a higher

risk of bacterial infections and infection-related mortality in the first year after OLT, regardless of the recipient MBL genotype.¹¹ They did find a slight interplay between donor and recipient MBL genotype regarding the risk of bacterial infection and pneumonia, but only in MBL-deficient donor–MBL-deficient recipient “pairs”. One retrospective study performed by Curvelo et al. studied the predictive value of donor MBL genotyping for bacterial infections and observed no significant increases in the risk of intra-abdominal infections or bacteremia in OLT recipients of MBL-insufficient livers, although that study found significantly more pneumonia in recipients receiving a MBL-deficient liver.¹²

Not much published data is available on the impact of SNPs in *FCN2* and *MASP2* and infections risk in OLT recipients. One study performed by de Mare-Bredemeijer et al. (who also participated in a latter study by Curvelo et al.) evaluated the influence of a broad range of genetic variants in recipient and donor innate immunity receptors on bacterial (and fungal) infections after OLT, including *FCN2* (rs17549193) and *MASP2* (rs12711521).¹³ The MBL genotype was left out because it lacked association in the previously mentioned study. They concluded none of the eventually 50 tested SNPs in innate immunity receptors did influence susceptibility to bacterial infections after OLT. However, the main limitation of that study is the large number of variables (i.e., SNPs) used compared with the sample size of their identification cohort: The low statistical power (roughly 33%) potentially undermines the reliability of that study.

Cytomegalovirus (CMV) reactivation and primo-infection frequently occurs in immunocompromised patients after OLT and can lead to CMV disease. Innate recognition of virus proteins is an important component of the immune response to viral pathogens. Pattern recognizing receptors MBL and ficolin-2 also recognise viral pathogen-associated molecular patterns (PAMPs), including viral glycoproteins. Both possess antiviral activities through complement activation, opsonophagocytosis and direct spatial blocking of virus-receptor interactions and entry. In **chapter 5** we reported our study regarding the contribution of *MBL*, *FCN2*, and *MASP2* gene variants on susceptibility to CMV infections.

We evaluated 295 OLT recipient–donor pairs and genotyped them for recipient and donor SNPs in mannose-binding lectin (*MBL*), ficolin-2 (*FCN2*) and MBL-associated serine protease (*MASP2*) genes. The *MBL*, *FCN2*, and *MASP2* genotype distributions of the donor livers were analyzed in relation to the cumulative incidence of CMV reactivation or primo-infection within the first year post-OLT. In 35% of the patients there was laboratory evidence of CMV viraemia within the first year after OLT, of which 75% were reactivations and 25% were primary infections. Univariate analysis showed a significantly increased risk of CMV viraemia in OLT recipients of an MBL deficient (XA/O or O/O) donor liver compared to receiving a wild-type donor liver (54% versus 38% HR 1.65; $p < 0.02$). In addition, recipients of a wild-type *FCN2*-A “low-binding” donor liver had a significantly increased risk of CMV viraemia compared to recipients of a *FCN2*-C “high-binding” donor liver (44% versus 27% HR 1.85; $p < 0.02$).

There was a significantly increasing cumulative risk of CMV viraemia in recipients who received a donor liver with either or both risk-conferring gene variants: 24% CMV viraemia in

absence of a genetic variant, 42% in those with either one variant (HR 2.02; $p=0.02$) and 58% in those with both variants (HR 3.26; $p=0.001$). This was especially the case in IgG anti-CMV donor negative/recipient positive (D-/R+) patients (HR 4.7 and HR 10.0, respectively; $p=0.01$) in whom factual CMV reactivation occurs. Similar as to what we observed in chapter 4, the presence of a genetic donor–recipient mismatch increased the risk of CMV viraemia significantly for either *MBL* or *FCN2* genotype (HR 2.97; $p<0.01$) and was even higher in case of a mismatch in both (HR 5.35; $p<0.001$), particularly in CMV D-/R+ patients (HR 16.6; $p=0.009$). These observations were retained in a final multivariate Cox analysis which, adjusted for CMV serostatus, gender of the recipient, occurrence of acute rejection, use of antiviral prophylaxis and underlying disease, showed a consistent stepwise increase in CMV viraemia risk with the gene profile of the donor [up to HR 2.77; $p<0.005$] and the combined *MBL* and *FCN2* donor–recipient mismatch profile [up to HR 4.57; $p<0.001$], independent from donor–recipient CMV serostatus, also at higher CMV viraemia cut-off values.

Recently, Lombardo-Quezada et al. also evaluated the role of donor and recipient *MBL* multilocus genotypes in the incidence, severity and outcomes of CMV infection/disease.¹¹ However, they could not detect any significant differences between the incidence of CMV viraemia in OLT recipients of *MBL*-deficient and *MBL*-sufficient donor livers. The foremost dissimilarity between the two studies is the different interpretation method used for *MBL* genotypes stratification into groups with *MBL* serum levels predicted to be sufficient or insufficient.

The combinations of the promoter-221 and exon 1 polymorphisms in the encoding gene are used as the working multilocus genotype. The *MBL* multilocus genotypes YA/YA and YA/XA result in normal serum *MBL* levels, O/O and XA/O genotypes are low/null serum *MBL* levels producers and YA/O and XA/XA genotypes are associated with intermediate/low *MBL2* serum levels. Based on our study we described in chapter 3, we used a strict definition of *MBL* insufficiency and considered only O/O and XA/O genotypes to be *MBL*-insufficient. Lombardo-Quezada et al. however, also considered the XA/XA and YA/O genotype to be *MBL*-insufficient. To genuinely compare between *MBL* genotype–phenotype association studies the method of interpretation and definition of the *MBL* groups should be alike. Another dissimilarity is the assessment and definition of CMV infection. From June 2002 onwards the virological assay used for diagnosing and monitoring CMV viraemia in the LUMC made a transition from the quantitative pp65-antigenemia assay to the quantification of CMV-DNA. Unfortunately, our study comprises this period of transition. Lombardo-Quezada et al. used the quantitative PCR assay to detect presence of CMV DNA and defined CMV infection as CMV DNA >1000 copies/mL. Since all patients in our study had an undetectable CMV load at the time of transplantation, any subsequent detection of pp65 or CMV-DNA was considered CMV reactivation or (in case of D+/R- combination) primo-infection in our study. Perhaps the latter could explain the third dissimilarity, i.e., the different incidence of CMV viraemia between our studies: Lombardo-Quezada et al. found an average of 19%, whereas we found an average of 35% patients with CMV-viraemia.

In the foregoing two chapters we focussed on two soluble PRRs, in **chapter 6** we describe the independent association between genetic variants of an intracellular PRR, namely Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) and the increased risk of clinically significant bacterial infections (CSI) after OLT. NOD2 participates as an intracellular PRR in innate immune responses and is considered a major host defense factor, especially with respect to intracellular infections. NOD2 is active in several types of epithelial cells, including Paneth cells, which are found in the epithelial lining of the intestine. These cells help defend the intestinal wall against bacterial infection. Studies suggest mutations in NOD2 (R702W, G908R and 3020insC) prevents the protein from recognizing bacteria appropriately, allowing these microbes to grow unchecked and invade cells that line the intestine. Mutations in NOD2 are associated with negative outcomes in patients with an acquired (secondary) immunodeficiency disorder (early mortality in sepsis patients, spontaneous bacterial peritonitis and death in patients with liver cirrhosis, and transplant-related mortality and graft versus host disease following allogeneic stem cell transplantation) and is the single most important genetic risk factors for the occurrence of Crohn's disease, a complex disorder that causes inflammation of the digestive system.¹⁴

We evaluated 288 OLT recipient-donor pairs and genotyped both recipient and donor for the probable presence of three genetic *NOD2* variants (R702W, G908R and 3020insC). Univariate analysis revealed that recipients with one or two risk alleles for the *NOD2* R702W variant developed significantly more CSI after OLT compared to recipients with the wildtype genotype, namely 53.3% compared to 29.3% (HR 2.1; $p = 0.03$). The other *NOD2* variants, G908R and 3020insC, in the recipient were not associated with CSI. There was no association with CSI after OLT for any of the *NOD2* variants in the donor. Multivariate analysis indicated that the *NOD2* R702W genotype of the recipient is indeed associated with CSI after OLT (HR 2.0; $P=0.04$), independent from the lectin complement pathway profile of the donor (up to a HR 4.1 in case of three variants; $p = 0.003$) and the only other significant contributing factor, i.e., male recipients of livers from male donors (HR 2.0; $p = 0.04$). This indicates that the *NOD2* R702W variant of the recipient, partially impairing the recipient's innate immune system, plays a possible predisposing role -probably at the level of the gut lining epithelium- in increasing the risk of developing infections.

It may be useful to construct a gene risk profile including genetic risk factors of both recipient and donor to identify those patients at risk for infections after transplantation.

As mentioned before, the liver is the major source of the circulating innate immunity proteins in the body. The course of advanced cirrhosis, irrespective of its etiology, is complicated by cirrhosis-associated immune dysfunction which predisposes to a higher risk of bacterial infection and increased mortality.¹⁵ While patients with advanced liver disease and ascites are waiting for a liver transplantation they are at risk of developing spontaneous bacterial peritonitis (SBP), one of the most frequent and life-threatening complications in patients with advanced liver cirrhosis. Even today the development of SBP is associated with a one-year mortality rate up to 20%.¹⁶ Genetic susceptibility of the host is assumed to be one

of the key players for the development of SBP.¹⁷ We therefore evaluated the contribution of genetic variation in the genes coding for pattern recognition receptors (lectins, TLRs and NOD2) and their associated intracellular signalling molecules (MASP-2, C3, MD2 and MyD88) of the innate immune system to the susceptibility for SBP in patients with end-stage liver disease and ascites as described in **chapter 7**. We included 280 patients with end-stage liver disease of which 19% had encountered SBP and genotyped them for SNPs in *MBL*, *FCN2*, *MAS2P*, *C3*, *TLR2*, *TLR4*, *MD2*, *MYD88* and *NOD2*. There appeared to be no significant association between these polymorphisms and the susceptibility to SBP in these patients, except for the only two patients homozygous for *TLR4* (rs4986790) compared to 238 patients with a homozygous wildtype (100% versus 19%; $P=0.01$). Subsequently multivariate analysis was performed including covariates from the univariate analyses with a p value <0.20 . Child-Pugh classification and primary liver disease (grouped by etiology: cholestatic, viral, alcoholic or other disease) were included together with the following individual SNPs: *TLR4* (rs4986790), *NOD2* (rs2066847), *MASP2* (rs72550870) and *MD2* (rs11466004). Non-significant covariates were excluded stepwise, resulting in Child-Pugh classification as the only independent risk factor for developing SBP. The prevalence of SBP significantly increased according to the Child-Pugh classification [(Child-Pugh A versus B: odds ratio (OR) 4.9, $P=0.013$; A versus C: OR 6.8 $P=0.003$)]. The Child-Pugh class is a good indicator of global liver function in a patient with cirrhosis and a higher class is recognized as independent risk factor for SBP development.¹⁸⁻¹⁹ A possible explanation for the absence of a genetic correlation with SBP in this study could be that a monogenic affected pattern recognition receptor only results in a impaired response if its specific pathogen-associated molecular patterns is encountered. Since SBP has a heterogeneous group of causative pathogens, finding a causal relation or association between a SNP and SBP is highly dependent on the type of the bacterial pathogen causing SBP. Another explanation could be that the remaining response of the adaptive immune system or redundancy in sensing pathogens by the innate immune system or both, is still sufficient to prevent an infection from becoming established. For instance, in our study described in chapter 3 we observed abundant serum levels of MBL in recipients before OLT, despite advanced cirrhosis.

Another important complication of liver transplantation which enhances susceptibility to infections is hepatic ischemia-reperfusion injury (IRI) which results from warm and cold anoxic ischemia between donation and revascularization, followed by reperfusion injury. In **chapter 8** we described the usefulness of first-week postoperative peak serum levels of aspartate transaminase (AST), a marker for hepatic ischemia-reperfusion injury, as an independent predictor of increased infection risk after liver transplantation. We evaluated postoperative serum levels of AST preceding a bacterial infection in 261 patients after OLT and used logrank statistics to determine the optimal discriminating AST cut-off value in relation to bacterial and CMV infection rate. Univariate analysis revealed an optimum at 1516 IU/L for bacterial infection, as a result patients with a peak AST >1500 had a significantly higher rate of bacterial infection after 7 days post-OLT than those with a peak AST <1500 .

(43% versus 20%; $P<0.0001$). Multivariate analysis showed a twofold increase in the risk of bacterial infection in the $AST>1500$ group as compared to the $AST<1500$ group (HR 2.08; $P=0.009$). The presence of two or three variants in the lectin pathway mismatch profile (*MBL*: XA/O and O/O donor and A/A and YA/O recipient; *FCN2*: CT or TT donor and or recipient; *MASP2*: AA donor and or recipient) was the only other factor at study inclusion, which remained independently associated with an increased risk of bacterial infection after OLT (HR 4.70; $P<0.001$). Regarding the risk of CMV infections, the optimal peak AST resulted in four cut-off points, each related to a different donor–recipient IgG anti-CMV serostatus group, with the strongest association in recipients of organs from IgG anti-CMV positive donors. Recipients of IgG anti-CMV positive donor livers, with a peak $AST>1600$ had an increased risk of CMV infection compared to the $AST<1600$ group (51% versus 36%; $P=0.03$). Multivariate analysis indicated a 70% increase in the risk of CMV infection after OLT in the $AST>1600$ group as compared to the $AST<1600$ group (HR 1.70; $P=0.03$). This was independent from donation after cardiac death versus donation after brain death liver transplantation (HR 3.1; $P=0.002$) or receiving an IgG anti-CMV positive donor liver (HR 20; $P<0.001$). These data suggest that peak serum AST in the first week after OLT, an inexpensive, convenient clinical test, could contribute to predicting a patient's risk of infection after transplantation more accurately.

Previously published data by Glanemann et al. on clinical implications of hepatic IRI after OLT suggested there was no significant correlation between IRI and bacterial infections after OLT.²⁰ They showed that bacterial infection occurred in 43.3% of recipients with minor injury ($AST<1000$), 53% in moderate ($AST\ 1000\text{--}5000$) and 58.3% with severe injury ($AST>5000$) which, in contrary to the authors conclusions, did reach overall statistical significance if we recalculated it (Chi-square 6.70, $p=0.035$). There are two main differences between our study and theirs, firstly they used commonly used but arbitrary AST cut-off values. Secondly, their study dates from a period in which the utilization of donation after cardiac death and extended criteria donor livers was not accepted. It is of interest that we did not only see more bacterial infection, but also more CMV viraemia in case of more severe IRI, as reflected by a higher peak AST. A causal relationship between severity of IRI and an increased risk of CMV infection has not been described before. IRI did occur more frequently in recipients of IgG anti-CMV positive donors. Therefore, CMV-induced injury to biliary and vascular structures of the donor liver itself could in theory contribute to the hepatic IRI process. However, immune-mediated injury caused by an active CMV-infection contributing to IRI is less likely, since IRI typically occurs within the first 72 hours after transplantation and the first positive CMV loads were detected after 7 days.

Biliary and vascular disorders are common complications after OLT and an important cause of morbidity and mortality in liver transplant recipients. The formation of non-anastomotic biliary strictures (NAS) occurs in 5% to 15% of liver transplant recipients and results mainly from hepatic ischemia between donor and recipient. Impaired blood supply as a result of vascular ischemia may cause injury to the peribiliary glands and vascular plexus.²¹

Both the complement and the coagulation cascades originate from the same ancestral pathways²² and both serve as a host defense mechanism in an environment with tissue injury and inflammation. Compelling evidence shows extensive cross talk between both systems.²³ The MBL- and FCN-associated serine proteases, MASP-1 and MASP-2 of the lectin pathway of complement activation represent such a crossroad between coagulation and complement systems. Both MASP-1²⁴⁻²⁵ and MASP-2²⁶ are able to produce active thrombin from prothrombin and hence are able to induce and promote clot formation. In contrast, evidence has been presented that natural wild-type pro-urokinase acts as a potent substrate for MASP-2.²⁷ Pro-urokinase is the precursor of urokinase, a serine protease that acts on the endogenous enzymatic breakdown of the fibrin in blood clots. An inherited deficit of MASP-2, caused by a mutation (rs72550870) in the CUB1 domain, renders MASP-2 incapable of binding to MBL and ficolins, abolishing complement activation and reducing MASP-2 serum levels.²⁸ We therefore hypothesized that there might be a causal relation between this mutation in the gene encoding for MASP-2 and two clinical coagulation-associated conditions, i.e., non-anastomotic biliary strictures (NAS) in the donor liver and Budd-Chiari syndrome (BCS). BCS is a rare disorder caused by obstruction of the hepatic venous outflow tract which can occur in patients with an underlying thrombotic diathesis, both hereditary and acquired, but it may also have a variety of other causes, including impaired fibrinolysis.²⁹ Both for NAS and BCS thrombolytic agents, like urokinase have been used in an attempt to break up or dissolve blood clots.

Chapter 9 reports our study on NAS in which we evaluated a group of 147 patients who underwent OLT, eventually entitled as the discovery study, and genotyped them for the presence of the *MASP2*+120 mutation (rs72550870). Among the 147 liver donors tested, 6.1% donors were heterozygous for the minor *MASP2*+120 G-allele and 93.9% were homozygous for the wild-type allele, none were homozygous for this mutation. Non-anastomotic strictures (NAS) were observed in 14% transplanted livers after transplantation. The *MASP2* mutation in the donor significantly increased the risk of NAS (adjusted hazard ratio, 6.4; $P=0.008$). We subsequently performed a validation study including 167 patients after OLT among who 4.8% of the liver donors were heterozygous for the minor *MASP2*+120 G-allele and 95.2% were homozygous for the wild-type allele. NAS was observed in 16.8% transplanted livers after transplantation and was also associated with a *MASP2* mutation in the donor (adjusted hazard ratio 3.0; $P<0.05$). The overall hazard ratio for NAS in the two studies combined was found to be 4.45 ($P<0.001$), adjusted for recipient age (HR 0.98; $P=0.08$), primary liver disease (primary sclerosing cholangitis: HR 2.42; $P=0.008$; other cholestatic disease: HR 2.18; $P=0.09$), warm ischemia time (HR 1.00; $P=0.74$) and cold ischemia time (HR 1.00; $P=0.06$).

The possible association between BCS and *MASP2*+120 mutation was also explored in patients of the discovery and validation study combined. Out of the 268 remaining recipients, after excluding those patients that underwent retransplantation, 5% were heterozygous for the minor *MASP2*+120 G-allele and 95% homozygous for the wild-type allele. Out of the patients with BCS as indication for OLT, 3 out of 4 (75%) were heterozygous for the minor

G-allele of *MASP2* compared to only 4% heterozygous patients with other indications for OLT ($P=0.0004$). In addition, after transplantation two out of three patients who developed BCS after OLT received a donor liver heterozygous for the minor G-allele of *MASP2* and presence of the *MASP2*+120G mutation in both recipient and donor was significantly associated with the development of BCS ($P\leq 0.009$).

The mechanism of action of mutated MASP-2 on disordered coagulation or fibrinolysis we proposed in this chapter is as follows: MASP-1 and MASP-2 each associate as homodimer or heterodimer and one or two of these MASP-dimers form complexes with MBL or ficolins. The *MASP2*+120G mutation not only impairs the functionality of MASP-2 but also results in an increase of MASP-3 in the MBL-MASP complex, since MASP-3 competes with MASP-2 for binding to MBL.³⁰ MASP-3 is the exclusive activator of the alternative pathway pro-factor D³¹ and does not elicit the enzymatic signal upon lectin pathway activation. Since MASP-2 can contribute to dissolve clots by activating urokinase and MASP-1 activates coagulation factors and promotes clot formation, we therefore suggested that a lectin-MASP complex deprived from MASP-2 could disturb the equilibrium causing a state of hypofibrinolysis and hypercoagulability. Although accumulating evidence (mainly shown in purified or plasma based static systems) suggests that MASPs, and in particular MASP-1, are directly involved in the generation of the fibrin clot, the dual role of MASP-2 clearly indicates that further research into complement-coagulation interactions is needed.

CONCLUSIONS AND PERSPECTIVES

Precision medicine takes into account measurements of individual variation. Genomic medicine can be considered a subclass of precision medicine, defined by The National Human Genome Research Institute (NHGRI) as an emerging medical discipline that involves using genomic information about an individual as part of their clinical care (e.g., for diagnostic or therapeutic decision-making) and the health outcomes and policy implications of that clinical use. Genomic medicine is already making an impact in the fields of oncology, pharmacology, rare diseases and infectious disease.

Despite widespread and successful use of organ transplantation as a curative therapy for organ failure, genetic research has yet to make a major impact on transplantation practice in general, aside from genetic testing of HLA in nearly all types of transplants (except liver transplantation, which only requires blood group compatibility) and specific cytochrome P450 (CYP) polymorphisms which can affect drug metabolism.³² This is remarkable because in essence transplantation involves the direct exposure to a foreign donor genome in a recipient, creating unique opportunities to investigate the implications this has on graft function and clinical outcomes.

In the current thesis observations are described as to what extent genetic variation in and interplay between donor and recipient genes coding for innate immunity proteins, with emphasis on the lectin pathway of complement activation, contributes to the risk of common complications concerning orthotopic liver transplantation (OLT), i.e., infections,

ischemia-reperfusion injury and formation of non-anastomotic strictures (NAS), and whether this information may be useful for guiding clinical decision-making in preventive and/or targeted treatment.

Consequent to the conclusions of chapter 3 of this thesis that identification of pathogenic genetic variants of the lectin pathway of complement activation in donor and recipient of liver transplantation has a major impact on their protein level in the recipient, genotyping is the most feasible option for exploring associations with clinical outcome. Chapters 4, 5 and 6 address that genotyping of liver donors and recipients could provide information on the risk of developing a bacterial or CMV infection and of bacterial infection-related mortality after OLT. Recipients receiving a donor liver with one or more pathogenic lectin pathway variants have the highest risk of infection, which is further increased when variants are absent in the recipient. The presence of a *NOD2* variant in the recipient, described in chapter 6, contributes to this infection risk independently. The MASP2 mutation in the donor increased the risk of NAS which consequently can cause cholangitis.

Additionally, non-genetic information, particularly in the absence of any pathogenic variants in both recipient and donor liver, like post-OLT elevations of AST exceeding 1500 U/L or not can be useful to identify patients with an increased risk of developing bacterial infection or CMV viraemia.

In selected “at risk” patients, based on the (non-) genetic parameters, assessing bacterial colonization or prolonging antimicrobial prophylaxis could be considered, as well as intensified clinical follow-up and preemptive antimicrobial therapy. In case of MBL-deficient donor livers our results also suggest the potential use of MBL substitution therapy during a limited time after OLT. Though this approach appears to be safe and substitution twice weekly was safe and resulted in trough levels considered protective in MBL-deficient children with cancer during chemotherapy-induced neutropenia,³³ its feasibility is hindered because recombinant MBL is currently commercially unavailable.

The pathway for the translation and integration of genomics from the laboratory into everyday medicine remains largely undefined. Screening donor and recipient for these pathogenic variants can usually be done using High-Resolution Melting Analysis (HRMA), a highly sensitive, simple and low-cost test, and is feasible within several hours.³⁴ Patients awaiting OLT can be screened for pathogenic variants of interest in advance, while screening of the donor liver genotype could be performed during the liver transplant procedure which generally takes between four and eight hours.

Genetic association studies using a candidate gene approach represent an important tool for studying polygenic transplant-related intermediate phenotypes and outcomes. It is important, however, to remain mindful of their limitations and potential pitfalls in both the design of the study and interpretation of the results. Although several studies have evaluated the same polymorphism for associations with sepsis susceptibility or severity in general, their conclusions have often been conflicting. Methodological inconsistency in the design and execution of many studies may account for some of the contradictory findings.³⁵ Failure to replicate findings caused by differences in allele frequency between

ethnic groups or gene-environment interactions (and its subsequent effect on power) does not negate the validity of the original study, but calls instead for more appropriately powered studies. Studies on liver transplantation usually include relatively small number of patients and therefore could lack adequate statistical power. To improve the quality and reproducibility of studies there is a clear need for wider collaboration among all study groups with appropriately harmonizable data sets to boost statistical power among existing studies, thereby valorizing existing information. This should enable us to further unscramble the genetic structure of transplant related complications and change the way transplanted grafts are (to be) matched and/or recipients are monitored after OLT.

In conclusion, the studies described in this thesis demonstrate that identification of pathogenic genetic variants of the lectin pathway of complement activation in both recipient and donor allograft could contribute to patient-risk stratification and may help to further improve clinical outcomes after OLT by enhancing clinical decision making and treatment of patients in a more precise, personally tailored manner.

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Chapter 11

Nederlandse samenvatting,
List of publications, Curriculum Vitae

NEDERLANDSE SAMENVATTING

Levertransplantatie is de enige levensreddende behandeling voor een selecte groep patiënten met acuut leverfalen, chronisch leverfalen, levertumoren en levensbedreigende metabole aandoeningen. Bij een (orthotope) levertransplantatie wordt de zieke lever uit het lichaam verwijderd en vervangen voor een gezonde donorlever. De eerste levertransplantaties bij de mens vonden plaats in 1963 en werden uitgevoerd door chirurg Dr. Thomas Starzl in het academisch ziekenhuis van de universiteit van Colorado in de Verenigde Staten. De overleving van deze patiënten was maximaal 23 dagen.¹ Sindsdien is overleving na levertransplantatie van zowel de patiënt als van de donorlever aanzienlijk verbeterd, onder andere door verbeteringen en meer selectief gebruik van anti-afstotingsmedicijnen (immunosuppressiva). In 1966 werd in Leiden de eerste levertransplantatie in Nederland verricht en sindsdien zijn in Nederland meer dan 3000 van deze procedures uitgevoerd. Tegenwoordig is de overleving na één jaar en vijf jaar na een levertransplantatie in Nederland respectievelijk 89% en 75%.²

Ondanks het feit dat de resultaten sinds de introductie van levertransplantatie zijn verbeterd, zorgen de geneesmiddelen die gebruikt worden om afstoting te voorkomen en te behandelen voor complicaties omdat ze zijn gericht op het immuunsysteem van de ontvanger. De onderdrukking van het immuunsysteem zorgt voor een verhoogde vatbaarheid voor infecties. Infectieuze complicaties treden in het eerste jaar na transplantatie tot wel bij 80% van de patiënten op en zijn, ondanks de inspanningen op het gebied van preventie en behandeling, momenteel de belangrijkste oorzaak van ziekte (morbiditeit) en sterfte (mortaliteit). Bacteriële infecties komen het meest voor (70%), gevolgd door virale (20%) en schimmelinfecties (8%).³⁻⁴

Het immuunsysteem van de mens kan globaal worden onderverdeeld in twee hoofddelen - het aangeboren en het verworven immuunsysteem - die samenwerken om weerstand te bieden tegen infecties. De anti-afstotingsmedicijnen die worden gebruikt hebben een breed scala van remmende effecten op het verworven immuunsysteem van de ontvanger, waardoor die meer afhankelijk wordt van het aangeboren immuunsysteem.

Het aangeboren immuunsysteem is in staat onderscheid te maken tussen lichaamseigen en lichaamsvreemd met behulp van patroonherkenningsreceptoren (*pattern-recognition receptors*, PRRs). PRRs zijn gecodeerd in het menselijk genoom (DNA) en gericht tegen moleculaire patronen (zogenoemde *pathogen-associated molecular patterns*, PAMPs), die zich op een brede groep van ziekteverwekkende micro-organismen (pathogenen) bevinden en die essentieel zijn voor de overleving van het micro-organisme en door de evolutie heen zijn behouden. Voorbeelden van PRRs zijn lectine receptoren, Nucleotide-binding oligomerization domain-containing protein (NOD)-like receptoren en Toll-like receptoren.

De lever vervult een cruciale rol binnen het aangeboren immuunsysteem aangezien het 80-90% van de circulerende aangeboren immuniteitseiwitten in het lichaam produceert, zoals oplosbare PRRs, complement componenten en stollingsfactoren.⁵

Het complementsysteem is een centraal subsysteem dat deel uitmaakt van het aangeboren immuunsysteem en bestaat uit een groot aantal plasma- en membraaneiwitten,

waarvan C1 t/m C9 de belangrijkste eiwitten zijn. Het complementsysteem kan via drie routes worden geactiveerd, de klassieke route, de lectineroute en de alternatieve route, en elke route heeft zijn eigen “gangmaker”. Activatie zal via een kettingreactie (het achtereenvolgens knippen van eiwitten door andere eiwitten die net voordien zelf geknipt zijn) bij elke van de routes leiden tot de splitsing van het centrale eiwit van het complement systeem, factor C3 met als uiteindelijk resultaat: het herkenbaar maken (opsonisatie) van pathogenen, het aantrekken van ontstekingscellen uit de bloedbaan (chemotaxie) en het direct doden (lysis) van pathogenen door de vorming van een opening in hun celwand (membrane attack complex, MAC).⁶

De lectineroute wordt geactiveerd als de patroonherkenningsreceptoren, mannose-bindend lectine (MBL) en/of ficolines (ficolin-1, ficolin-2 en ficolin-3) met daaraan gekoppeld het enzym MBL-associated serine proteases (MASP-1, MASP-2 en MASP-3), binden aan suikergroepen op het oppervlak van micro-organismen.

De erfelijke informatie van mensen bevindt zich in de kern van elke cel, ligt verpakt in chromosomen en is opgebouwd uit vier bouwstenen aangeduid met de letters A, T, G en C. A staat voor Adenine, T voor Thymine, G voor Guanine en C voor Cytosine. A vormt altijd een paar met T (en omgekeerd) en G vormt een paar met C (en omgekeerd). Lange ketens van deze vier DNA-bouwstenen vormen “het DNA”. Genen zijn stukjes van deze DNA-ketens die één bepaalde eigenschap bepalen. Kleine verschillen in de volgorde van de DNA-bouwstenen tussen mensen liggen, ten dele, aan de basis waarom mensen onderling genetisch, (fysiek en psychisch) van elkaar verschillen. DNA-veranderingen spelen ook een belangrijke rol bij het ontstaan van veel ziekten. Sommige veranderingen verlagen of verhogen juist de kans om bepaalde ziekten te krijgen. Indien een specifieke DNA-verandering bij ten minste 1% van de populatie voorkomt, spreekt men van een polymorfisme. Meer dan 30% van de genen die coderen voor eiwitten zijn polymorf, veel polymorfe genen danken hun hoge frequentie aan selectie door omgevingsfactoren.

De eiwitten MBL, ficoline-2 en MASP-2 worden nagenoeg alleen geproduceerd door de lever en de hoeveelheid en werking van deze eiwitten wordt sterk beïnvloed door polymorfismen in de coderende genen. Na een levertransplantatie krijgt de ontvanger een lever met de genen van de leverdonor en bepalen deze genen voortaan hoeveelheid en eigenschappen van eiwitten die in de lever worden gemaakt. In situaties waarbij het verworven immuunsysteem is verstoord, zoals het geval is bij patiënten na levertransplantatie en behandeling met immunosuppressiva, leiden ongunstige polymorfismen binnen het aangeboren immuunsysteem mogelijk tot een minder efficiënte afweer tegen infecties.

De focus van dit proefschrift ligt op het in kaart brengen van de erfelijke opmaak van het aangeboren immuunsysteem van ontvanger en donor van een lever, met een speciale nadruk op de lectine-route van complementactivatie, en op de vraag of dit gevolgen heeft voor de kans op (infectieuze) complicaties voor en na levertransplantatie.

Hoofdstuk 1 is een algemene introductie tot dit proefschrift, inclusief een kort overzicht met doelstellingen van ieder hoofdstuk.

In **hoofdstuk 2** wordt een overzicht gegeven van de vele, transplantatie-gerelateerde, ontvanger- en donorfactoren die kunnen bijdragen aan een verhoogd risico op infectie na levertransplantatie. In het hoofdstuk wordt ook benadrukt dat uit verschillende onderzoeken is gebleken dat genetische veranderingen binnen het aangeboren immuunsysteem een rol kunnen spelen bij de aanleg en wijze waarop infectieziekten ontstaan, vooral bij personen met een onderontwikkeld (naïef), defect of verzwakt verworven (adaptief) immuunsysteem.

Allereerst wilden we vaststellen dat MBL, ficoline-2 en MASP-2 inderdaad alleen geproduceerd worden door de lever, zoals algemeen wordt aangenomen. In **hoofdstuk 3** werd het onderzoek beschreven naar het effect van functionele polymorfismen in de coderende genen *MBL*, *FCN2* en *MASP2* op de concentratie van respectievelijk, MBL, ficoline-2 en MASP-2 concentratie in het bloed. Aan de hand van drie studiegroepen, bestaande uit: 170 gezonde controles, 120 ontvangers vóór en 111 ontvangers na levertransplantatie, laten we zien dat na transplantatie het genotype van de donorlever de hoogte van de concentratie van zowel MBL, ficoline-2 en MASP-2 bepaalt, onafhankelijk van het genotype van de ontvanger.

Vervolgens hebben we onderzocht of de aanwezigheid van polymorfismen in de genen die coderen voor MBL, ficoline-2 en MASP-2 gevolgen heeft voor de kans op het krijgen van bacteriële infecties na levertransplantatie (**hoofdstuk 4**). Hiertoe hebben we, terugkijkend, bij ontvangers en hun donorlever gekeken in de genen naar aanwezigheid van polymorfismen en geregistreerd of in het eerste jaar na transplantatie een bacteriële infectie was opgetreden. In twee aparte onafhankelijke groepen met respectievelijk 143 en 167 ontvangers na levertransplantatie, konden we aantonen dat ontvangers van een donorlever met een polymorfisme in één van de drie genen *MBL*, *FCN2* en *MASP2* een grotere kans hebben op een bacteriële infectie na transplantatie en dat deze kans toenam als sprake was van meerdere polymorfismen in dezelfde donorlever. Het betrof polymorfismen die zorgden voor of een verminderde productie van goed functionerend MBL (XA/O of O/O), of een minder goed bindend ficoline-2 (*FCN2*-B) of een minder functioneel MASP-2 (*MASP2*+371). De erfelijke opmaak van de ontvanger zelf lijkt ook een rol te spelen. De kans op infectie was namelijk het grootst bij ontvangers van een donorlever met afwijkende genen die zelf voor transplantatie geen afwijkende *MBL*, *FCN2* en *MASP2* genen hadden. De exacte verklaring hiervoor weten we niet, maar mogelijk heeft het te maken met het feit dat ontvangers met van nature aangedane *MBL*, *FCN2* en *MASP2* genen al 'gewend' zijn aan minder effectieve onderdelen van het aangeboren immuunsysteem en ter compensatie andere, ons nog niet bekende, onderdelen van de afweer extra hadden benut. Een ontvanger met van nature gunstige polymorfismen en waarbij dus wellicht een compensatiemechanisme ontbreekt en die een lever krijgt met een verminderde productie van effectief MBL, ficoline-2 en/of MASP-2 heeft daardoor misschien op een moment van sterk onderdrukte afweer ook nog eens een minder optimale aangeboren afweer. Ontvangers van een donorlever met ongunstige polymorfismen die een bacteriële infectie kregen hadden een zesmaal hogere kans om te overlijden.

Naast bacteriële infecties hebben ontvangers na levertransplantatie, zoals eerder genoemd, ook een verhoogde kans op het krijgen van virale infecties, met name op infecties

met het cytomegalovirus (CMV). CMV blijft na een eerste (primo-) infectie levenslang in het lichaam aanwezig en kan opnieuw actief worden zonder ziektesymptomen (meer) te veroorzaken, een zogenoemde latente infectie. Bij patiënten met problemen met het immuunsysteem, zoals na levertransplantatie, kan een primo-infectie met CMV, maar ook re-activatie van een latente infectie, waarbij dus zowel de ontvanger zelf als donorlever de potentiële bron kunnen zijn, leiden tot ernstige infecties van bijna alle inwendige organen.

In **hoofdstuk 5** is de studie beschreven waar we aantonen dat polymorfismen in de genen die coderen voor MBL en ficoline-2 kunnen leiden tot een verhoogde kans op CMV-infecties na levertransplantatie. Met een vergelijkbare aanpak als in hoofdstuk 4 is terugkijkend gekeken naar de *MBL*, *FCN2* en *MASP2* genen van de ontvangers en hun leverdonor in twee verschillende levertransplantatie centra. Van alle patiënten is vervolgens geregistreerd of zij een 'CMV infectie' (primo-infectie of re-activatie) hadden opgelopen na transplantatie. Ontvangers van een levertransplantaat met genen die zorgden voor verminderd functioneel MBL (XA/O of O/O) hadden inderdaad een verhoogde kans op CMV-infecties. Daarnaast hadden ontvangers van een levertransplantaat met het ficoline-2 polymorfisme (*FCN2-C*), dat resulteert in een sterkere binding van ficoline-2 aan micro-organismen, juist minder kans op een CMV infectie na transplantatie ten opzichte van ontvangers van een donorlever met normaal ficoline-2 (*FCN2-A*). De kans op CMV-infecties nam nog verder toe als de ontvanger een levertransplantaat ontving met een ongunstig MBL polymorfisme en een normaal ficoline-2. Dit risico was het grootst bij patiënten die zelf vóór transplantatie gunstige polymorfismen hadden. Bij de patiënten die zelf al ongunstige polymorfismen hadden was dit risico minder groot. Mogelijk speelt een compensatiemechanisme, zoals we eerder beschreven bij hoofdstuk 4 een rol.

Micro-organismen kunnen buiten en binnen de cel een gevaar vormen. Het NOD2-eiwit is een patroonherkenningsreceptor dat zich binnenin verschillende celtypen van het aangeboren immuunsysteem bevindt en in staat is om aanwezigheid van celwandstructuren van bacteriën te herkennen. Binding van NOD2 zorgt voor het opwekken van een algemene immuunrespons om de bacterie te elimineren. Drie verschillende polymorfismen in het *NOD2*-gen leiden ertoe dat het NOD2-eiwit de celwand van bacteriën niet meer goed kan herkennen. In **hoofdstuk 6** beschrijven we de studie naar de relatie van deze *NOD2* polymorfismen met het ontstaan van bacteriële infecties na levertransplantatie. Terugkijkend werd bij 288 ontvanger-donor paren de aanwezigheid van drie verschillende *NOD2* polymorfismen bij ontvanger en donor onderzocht en vervolgens geregistreerd of zij een bacteriële infectie hadden opgelopen in het eerste jaar na transplantatie. Hierbij vonden we dat alleen het *NOD2* polymorfisme R702W van de ontvanger geassocieerd is met meer bacteriële infecties na transplantatie en dat deze associatie onafhankelijk is van eventueel aanwezige polymorfismen in de genen van MBL, ficoline-2 en MASP-2 die staan beschreven in hoofdstuk 4. Het meenemen van dit *NOD2* polymorfisme bij het samenstellen van een genetisch risicoprofiel kan bijdragen aan betere infectierisico inschattingen.

Ernstige schade aan de lever zorgt voor verstoring van alle leverfuncties, zoals productie van belangrijke eiwitten, productie en opslag van energie en brandstoffen, verwerking en afvoer van afvalstoffen. Dit heeft directe en indirecte gevolgen voor het functioneren van het immuunsysteem. Bij levercirrose wordt gezond leverweefsel vervangen door niet-functioneel littekenweefsel, waarbij niet alleen leverfuncties afnemen maar ook de normale structuur van de lever is aangetast en bloed meer moeite heeft om door de lever te stromen. De hoge druk in de bloedvaten van de lever en het geleidelijk niet goed meer functioneren van de lever heeft vaak tot gevolg dat vochtophoping in de buik (ascites) ontstaat. Patiënten met levercirrose zijn meer vatbaar voor infecties en worden als immuundeficiënt beschouwd. Bijkomende deficiënties in het aangeboren immuunsysteem veroorzaakt door polymorfismen zouden een verhoogd risico kunnen geven op het ontstaan van infecties. Een relatief vaak voorkomende en ernstige infectieuze complicatie van cirrose met ascites is het ontstaan van spontane bacteriële peritonitis (SBP), een bacteriële infectie van ascites in de buik zonder aanwezigheid van een andere in de buikholte gelegen ontsteking of maligniteit, waarbij tot 20% van deze groep patiënten binnen een jaar komt te overlijden.

In **hoofdstuk 7** beschrijven we ons onderzoek naar de invloed van polymorfismen van verschillende patroonherkenningsreceptoren en de bijbehorende signaalmoleculen op het ontstaan van SBP. Daartoe hebben we bij 280 patiënten gekeken naar polymorfismen van: lectine receptoren (MBL en ficoline-2), Toll-like receptoren (TLR)2 en TLR4, en NOD2-receptor en de bijhorende signaalmoleculen (MASP-2, C3, MD2 en MyD88) en vervolgens gerelateerd aangevallen van SBP die plaatsvonden voor transplantatie. We laten in onze studie zien dat geen van de onderzochte polymorfismen een belangrijke rol lijkt te spelen bij het ontstaan van SBP. Wel bevestigen we dat het risico op SBP toeneemt met de ernst van levercirrose, ingedeeld in 3 stadia, de zogenaamde Child Pugh classificatie. De afwezigheid van een relatie tussen de onderzochte polymorfismen en SBP zou verklaard kunnen worden door de plek waar patroonherkenningsreceptoren opereren. De Toll-like en NOD-like receptoren bevinden zich op en in immuuncellen die zich, onder andere, in de darmen bevinden en daar bacteriën doden die door de mechanische barrière van de darm heendringen. Als gevolg van levercirrose is de doorlaatbaarheid van de darm voor bacteriën echter verhoogd en kunnen bacteriën zich makkelijker naar de met ascites gevulde buikholte begeven. De bacteriën die SBP veroorzaken zijn meestal darmbacteriën (zoals E.coli en Klebsiella soorten) die deel uit maken van de normale darmflora. De lectine receptoren bevinden zich in lichaamsvloeistoffen, waaronder ascites, en kunnen binden aan bacteriën. Echter blijkt uit onderzoek dat , om nog onduidelijke reden, MBL niet altijd goed kan binden aan E.coli en Klebsiella soorten, twee potentiële SBP verwekkers.⁷ Een andere mogelijkheid is dat de overgebleven respons van het aangeboren en verworven immuunsysteem nog toereikend is om infectie te voorkomen.

Tussen het uitnemen van een donorlever en transplantatie in de ontvanger krijgt de lever geleidelijk een tekort aan zuurstof (ischemie) en voeding. Herstel van de bloedtoevoer naar de lever (reperfusie) heeft echter ook een keerzijde. Reperfusie van het leverweefsel zorgt

voor een steriele ontstekingsreactie en kan extra schade veroorzaken. De combinatie van schadelijke processen heet ischemie/reperfusie-schade (Ischaemia-Reperfusion Injury, IRI) en kan resulteren in minimale schade aan cellen tot aan niet functioneren van de donorlever. Bij beschadiging van levercellen komt onder andere aspartaat-aminotransferase (ASAT of AST) vrij in het bloed. Leverschade door ischemie-reperfusie leidt meestal tot een ASAT-piek in de eerste drie dagen na transplantatie als gevolg van necrose. Meestal daalt ASAT snel in de eerste dagen en herstelt de schade. In de eerste week wordt de hoogte hiervan gebruikt om de mate van ischemie-reperfusie schade na levertransplantatie te bepalen.

In **hoofdstuk 8** beschrijven we de studie waarin we hebben gekeken vanaf welke maximale piekhoogte van ASAT, als indicatie voor de ernst van ischemie-reperfusie schade, in de eerste drie dagen na levertransplantatie, het risico is vergroot op zowel bacteriële als CMV-infecties na de eerste week na transplantatie. Ontvangers met een piek-ASAT-waarde van meer dan 1500 IU/L (verhoging van 38x de bovenste referentiewaarde van 40 IU/L) in de eerste drie dagen na transplantatie hadden twee keer zoveel risico op een bacteriële infectie 7 dagen na transplantatie. Ontvangers met een piek-ASAT van meer dan 1600 IU/L (40x de bovenste referentiewaarde) hadden tweemaal zo vaak een CMV-infectie 7 dagen na transplantatie. Het risico op infectie was onafhankelijk van het genetisch risicoprofiel van de lectine route van complement activatie, zoals beschreven in hoofdstuk 4 en 5. Met name in gevallen waar patiënten geen verhoogd genetisch ontvanger-donor risicoprofiel hebben, zou de hoogte van de ASAT piek bijvoorbeeld gebruikt kunnen worden voor betere inschatting van het risico op infecties.

De laatste jaren wordt steeds duidelijker dat bij verschillende vasculaire aandoeningen, zoals trombose en atherosclerose, zowel activatie van de bloedstolling als ontstekingsactiviteit een belangrijke rol spelen en dat sprake is van wederzijdse interactie tussen deze processen.⁸ Een voorbeeld hiervan zijn de enzymen MASP-1 en MASP-2 die in staat zijn zowel complement als stollingsfactoren te activeren. In **hoofdstuk 9** beschrijven we onze studie naar de associatie tussen een niet-functionerende genetische variant van MASP-2 en twee verschillende ziektebeelden, waarbij obstructie van bloedvaten door bloedstolsels een rol speelt bij het ontstaan. De balans tussen de opbouw en afbraak van stolsels is van groot belang om uitbreiding van stolsels te voorkomen. Zowel MASP-1 en MASP-2 zijn in staat stolling te bevorderen door stollingsfactoren te activeren. MASP-2 is daarentegen ook in staat om urokinase, een enzym dat stolsels afbreekt, te activeren.⁹ We veronderstelden dat halvering van de hoeveelheid aanwezig functioneel MASP-2 resulteert in een verhoogde stollingsneiging door het ontstaan van disbalans tussen de aanmaak en afbraak van stolsels.

Het eerste ziektebeeld waar we naar keken betreft non-anastomotische galwegstricturen (NAS), een ernstige complicatie na levertransplantatie, die wordt gekenmerkt door vernauwingen en verwijdingen van de galwegen in de lever. Hoewel de ontstaanswijze niet geheel duidelijk is, wordt aangenomen dat NAS het gevolg is van meerdere factoren, waaronder zuurstofgebrek (ischemie), schade aangebracht door het immuunsysteem en schadelijke effecten door galzouten. Het ontstaan van stolsels kan bijdragen aan het

ontstaan van ischemie van de galwegen. We hebben terugkijkend bij 147 patiënten na levertransplantatie de aanwezigheid van de *MASP2* D120G variant en het voorkomen van NAS onderzocht. Hierbij vonden we dat ontvangers van een donor lever die heterozygoot (één gen van het genenpaar is aangedaan) was voor de *MASP2* D120G variant wel 6,4 keer zo vaak NAS ontwikkelden na levertransplantatie in vergelijking met afwezigheid van deze variant. We konden deze bevinding bevestigen in een onafhankelijke groep van 167 patiënten die elders een levertransplantatie ondergingen. Naast aanwezigheid van deze *MASP-2* variant bij de donorlever, bleek ook dat primaire scleroserende cholangitis (PSC) - een aandoening waarbij de galwegen binnen en buiten de lever ontstoken zijn - als reden van transplantatie en de duur van 'koude ischemie' - de tijd dat een orgaan gekoeld op ijs ligt voor het wordt getransplanteerd - risicofactoren zijn voor het krijgen van NAS.

Het andere ziektebeeld waar we een associatie vonden is het Budd-Chiari syndroom (BCS), een zeldzaam maar ernstig ziektebeeld waarbij één of meer afvoerende bloedvaten van de lever verstopt zitten. In ongeveer driekwart van de gevallen is de oorzaak trombose, meestal als complicatie van een onderliggende aandoening die gepaard gaat met bloedstollingsstoornissen. In ons cohort bevonden zich vier patiënten die een levertransplantatie hadden ondergaan met het BCS als indicatie. Drie van de vier patiënten bleken heterozygoot te zijn voor het *MASP2* D120G polymorfisme, in de groep patiënten met andere indicaties voor levertransplantatie was 4% heterozygoot, wat overeenkomt met het percentage heterozygoten in een normale populatie. Na levertransplantatie ontstond bij drie andere ontvangers een BCS, twee hiervan hadden een lever gekregen van een donor die heterozygoot was voor het *MASP2* D120G polymorfisme. Bij zowel NAS als BCS lijkt een dus een associatie te bestaan met aanwezigheid van de *MASP2* D120G variant. Het ontstaan van stolsels draagt bij NAS mogelijk bij aan ischemie van de galwegen en bij BCS aan de afvloedbelemmering.

Concluderend tonen de in dit proefschrift beschreven studies aan dat bepaling van genetische polymorfismen van het aangeboren immuunsysteem in zowel ontvanger- als in de donorlever, met name van de componenten van de lectine-route van complement activatie, een bijdrage zouden kunnen leveren aan de identificatie van patiënten die de grootste kans hebben op een infectieuze, galweg- of vasculaire complicatie na levertransplantatie. Identificatie van risicopatiënten kan wellicht helpen de klinische besluitvorming te verbeteren en levertransplantatie patiënten te behandelen op basis van vroege symptomen of zelfs preventief, op een meer precieze, persoonlijk toegesneden manier.

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CURRICULUM VITAE

Bert-Jan Felix de Rooij werd geboren op 8 juli 1976 te Leiderdorp. Na het behalen van zijn HAVO en VWO diploma, begon hij in 1997 met de studie Biomedische Wetenschappen en combineerde deze vanaf 1998 met de studie geneeskunde aan de Universiteit Leiden. Na het behalen van het artsexamen in 2005, begon hij in deeltijd met zijn afstudeerstage voor de studie Biomedische Wetenschappen bij de afdeling Maag-, Darm-, en Leverziekten van het Leids Universitair Medisch Centrum. Het onderzoek naar genetische risicoprofielen bij levertransplantaties onder leiding van prof.dr.ir. H.W. Verspaget en prof.dr. B. van Hoek werd in 2007 voortgezet als promotietraject. In 2011 startte hij zijn opleiding tot maag-darm-leverarts wat hij combineerde met het voortzetten van het onderzoeksproject in het kader van een AGIKO-stipendium van ZonMw. Hij trouwde in 2011 met Nadia van Opstal en is trotse vader van 3 kinderen, Amy, Floris en Laurens.

