Molecular changes in hepatobiliary function and injury after human liver transplantation

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GENERAL
INTRODUCTION
GENERAL INTRODUCTION

1 Orthotopic liver transplantation

Orthotopic liver transplantation (OLT) is the ultimate treatment for patients with end-stage liver disease. Liver transplantation has a good clinical outcome, with 1-year patient survival rates of 80-90%, and 5- and 10-year patient survival rates of 60-70% \(^1\). The transplantation procedure is accompanied by ischemia and subsequent reperfusion (I/R) of the graft which causes cellular and functional damage. Gradually the transplanted liver recovers from the I/R-event and resumes its normal function. After OLT, additional cellular damage may originate from acute rejection. In this thesis, molecular changes in hepatobiliary function as well as cytoprotective pathways are studied during and after liver transplantation in humans.

2 Mechanisms of hepatic injury in liver transplantation

2.1 Ischemia / reperfusion injury

Cold ischemic preservation followed by warm reperfusion is an initial insult to liver grafts during transplantation leading to hepatocellular damage and organ dysfunction. I/R injury is caused by various mechanisms that lead to cell death. Cell death is often divided into two different processes, apoptosis ("programmed" cell death) and necrosis ("accidental" cell death). Both processes will be discussed in more detail in subsequent paragraphs.

Cold ischemic preservation of the liver graft is a condition that results in cell death. Cold ischemia leads to metabolic acidosis due to a shift towards anaerobic metabolism and loss of mitochondrial adenosine triphosphate (ATP) production \(^2-4\). ATP levels are decreased by approximately 80% as early as during the first 12 hours of liver preservation \(^5\). Hydrolysis of ATP delivers energy for various active processes, such as hepatobiliary transport across cell membranes \(^6,7\). Intracellular acidosis and ATP depletion are deleterious processes that can induce the release of lysosomal enzymes and cause proteolysis \(^8,9\). Acidosis, furthermore, can favor the production of harmful reactive oxygen species (ROS: e.g., \(\text{H}_2\text{O}_2\), OH\(^-\), O\(^2-\)) \(^10\). The degree of proteolysis positively correlates with the duration of cold ischemia \(^11\). Severely increased proteolytic activity has been associated with poor postoperative hepatic graft function \(^11-13\).
During cold ischemia, most cells remain vital \(^{(14,15)}\), but they are primed for damage that becomes manifest upon reperfusion of the graft \(^{(16,17)}\). During the re-introduction of oxygenated blood into the graft, metabolism in ischemic hepatic cells changes from an anaerobic into an aerobic process. During this adaptation phase, a relative hyperoxygenated condition exists, which leads to oxidative stress \(^{(18,19)}\). Furthermore, with the recovery of blood flow, inflammatory cells enter the graft and become activated, resulting in additional injury.

In the liver, I/R activates Kupffer cells. These cells are the resident macrophages of the liver, and can produce ROS, proinflammatory cytokines (e.g., TNFα, IL-1, IL-6, PAF, IFN-γ), chemokines, and other mediators that contribute to cell death \(^{(20-22)}\). Next to Kupffer cells, experimental evidence postulated that T-lymphocytes are also activated upon I/R due to the expression of MHC-antigens on sinusoidal endothelial cells \(^{(11,23,24)}\). Together with activated complement factors (e.g., C5a) \(^{(25)}\), Kupffer cells and T-lymphocytes promote the activation and recruitment of polymorphonuclear leukocytes (PMNs) into the liver shortly after recirculation \(^{(28)}\). PMN infiltration leads to the production of even more ROS, and induces the release of additional proteases (e.g., elastase, serine protease, metalloproteinas) causing tissue destruction \(^{(20)}\).

PMN recruitment is assisted by increased sinusoidal endothelial expression of an array of surface adhesion molecules (e.g., family members of selectins and CAMs) \(^{(22)}\). Besides PMNs, red blood cells (RBCs) and platelets in the blood adhere to endothelial cells, leading to damage and sinusoidal congestion \(^{(26,27)}\). Reperfusion after hepatic ischemia also results in enhanced liver tissue concentrations and hepatic venous plasma levels of long-acting vasoconstrictive mediators such as endothelin-1 (ET-1) \(^{(28)}\). Altogether, these actions contribute to the pathophysiological heterogeneous closure of many microvessels, which prolongs ischemia in certain areas of the liver even after reperfusion \(^{(2)}\).

### 2.2 Hepatocellular changes in ischemia / reperfusion

Hepatic cytolysis and cholestasis consistently occurs in patients who have undergone OLT \(^{(29)}\). As discussed in the previous paragraph, different mechanisms of I/R injury, like ROS, tissue pH changes, inflammatory responses and microcirculatory changes contribute to the occurrence of hepatic cell death \(^{(2)}\).

Sinusoidal endothelial cells, can become rounded during cold ischemia, detach and slough into the sinusoidal lumen upon recirculation \(^{(30)}\). The number of endothelial cells that release gets higher as the duration of cold ischemic preservation of the liver prolongs \(^{(31-34)}\). Morphological changes in endothelial cells during
cold preservation result from processes involving the cytoskeleton and extracellular matrix, and appear to be mediated by proteases. Cold preservation results in swelling of hepatocytes, which may be due to a depressed Na\(^+\)/K\(^+\)-ATPase pump and Na\(^+\)/H\(^+\) exchange. Electron microscopy studies demonstrated loss of microvilli at the canalicular hepatocyte membrane during cold ischemia, which was enhanced upon reperfusion of the graft. Reperfusion of the liver also results in the detachment of cholangiocytes from the basement membrane. The amount of bile duct epithelial cells in postoperatively collected human bile was higher when cold ischemic preservation time was longer. In an isolated perfused rat liver model system, \(\gamma\)-glutamyltransferase (\(\gamma\)-GT) levels, a parameter for biliary injury, increased as cold storage time prolonged. PMNs penetrate the biliary ductal basal membrane and probably contribute to bile duct injury.

In patients, it was found that cold storage less than 10 hours was associated with a biliary stricture rate of 7%. Cold storage times in excess of 10 to 12 hours were associated with a biliary stricture rate of about 30%. When cold preservation times were extended beyond 13 hours, non-anastomotic biliary strictures were observed in as high as 52% of the recipients.

Isolated rat cholangiocytes have been shown to be more sensitive to reperfusion damage than hepatocytes. This is possibly due to the fact that cholangiocytes produce about 5 times more ROS during reoxygenation, and contain about 7 times lower cellular stores of reduced glutathione (anti-oxidant) than hepatocytes. Evidence exists that the biliary epithelium requires a much longer time to recover from I/R injury than hepatocytes and sinusoidal endothelial cells.

While biochemical serum markers of hepatocellular injury, such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT), usually rapidly decrease after transplantation, serum \(\gamma\)-GT and alkaline phosphatase (ALP) levels generally continue to rise, reaching peak values in the second or third postoperative week.

Although ischemia and subsequent reperfusion cause direct injury to the biliary epithelium, it does not explain the continued rise of \(\gamma\)-GT and ALP levels until several weeks after OLT. Experimental studies in pigs have suggested that hydrophobic bile salts may play a role in the occurrence of bile duct injury during transplantation. These observations were based on the exogenous administration of bile salts to donor animals or to the preservation solution. Data on the role of endogenous bile salts in the pathogenesis of bile duct injury after OLT, however, are missing.
2.3 Acute cellular rejection

The immune rejection is mediated by T-lymphocytes via direct and indirect alloresponses \(^{(44-47)}\). The alloresponse can be divided into two components: (i) allorecognition and (ii) immune effector mechanisms \(^{(47)}\). During allorecognition, major histocompatibility complex (MHC) antigens are recognized by T cells. T cells recognize foreign MHC antigens either directly or indirectly. Direct allorecognition occurs when host T cells recognize intact donor MHC antigens presented on the surface of donor antigen presenting cells (APCs, e.g. macrophages, dendritic cells). Indirect allorecognition occurs when host T cells recognize donor-derived MHC peptides presented after processing by host APCs \(^{(48)}\). Initiation of T cell responses to the transplant occurs via the recognition of antigens presented on donor passenger leukocytes that infiltrate the recipient’s lymphoid organs \(^{(43)}\).

Following alloantigen recognition, CD4\(^+\) T cells (T-helper cells) circulate to the graft and cross the endothelium under the influence of chemotactic cytokines (e.g., chemokines) and interactions between cell surface molecules (e.g., selectins, integrins, endothelial adhesion molecules) \(^{(49)}\). Infiltrated T cells encounter their specific MHC antigen and subsequently release a mixture of proinflammatory cytokines (e.g., IFN\(\gamma\), TNF\(\beta\), IL-2, IL-4, IL-5), that will attract effector cells like monocytes/macrophages and CD8\(^+\) T cells (CTLs) into the graft. Cytokines like IFN\(\gamma\) are also able to induce MHC expression on endothelial and epithelial cells of the graft \(^{(50,51)}\). Furthermore CD4\(^+\) T cells interact with B lymphocytes that subsequently secrete antibodies \(^{(47,52)}\). Altogether, the foreign antigens that are recognized by the recipient’s immune system, lead to a potent immunological reaction and cause graft injury by inducing apoptosis and necrosis of donor cells \(^{(53,54,55)}\).

Although cytotoxic cell infiltrates are frequently found in liver grafts, the presence of CTLs in the liver does not always lead to clinically overt rejection of the transplant. In 59% of the patients a so-called condition of subclinical liver graft rejection exists during the first two postoperative weeks. In this condition, CTL infiltration of the graft does not result in deterioration of hepatic function. In subclinically rejected human kidney transplants it has been suggested that hyperexpression of the protease inhibitor-9 (PI-9) in the graft may have protected against pro-apoptotic effects of granzyme B enzymes that are released by CTLs \(^{(56)}\). The expression of PI-9 in human liver transplants however, has not been studied before.
2.4 Apoptotic and necrotic cell death

Cell death is often divided into two different processes, necrotic and apoptotic cell death. Necrotic cell death results from metabolic disruption with energy depletion (loss of ATP). The uncontrolled degradation in necrosis with cellular swelling and loss of membrane integrity ensues an inflammatory response. Apoptosis, on the other hand, is an ATP-dependent process that is a tightly controlled (programmed) mechanism. Apoptosis is characterized by profound morphological changes of the cell and, specifically, the nucleus. Typical features of apoptosis are the sequential occurrence of cell shrinkage, loss of cell-cell contact, membrane blebbing and chromatin condensations. The nuclear DNA of apoptotic cells is often fragmented into oligonucleosomal-sized units ("laddering"). Eventually, the apoptotic cell breaks into small membrane-surrounded fragments (apoptotic bodies) which are cleared by surrounding cells.

In apoptosis, different intracellular pathways are involved. In Figure 1, a schematic overview is given of receptor-mediated, mitochondria-mediated and granzyme B/perforin-mediated pathways.

In receptor-mediated apoptosis, death factors and death receptors are involved that are located on the cell membrane. Death receptors in the liver include Fas (CD95) and tumor necrosis factor-receptor (TNF-R)\(^{(57)}\). Death receptors are

![Diagram](image)
transmembrane proteins that contain an extracellular ligand-binding N-terminal, a membrane spanning domain and an intracellular C-terminal that contains the death domain essential for signalling apoptosis. Binding of Fas by its ligand (CD95L, FasL) or by anti-Fas antibodies results in receptor oligomerization, leading to recruitment of the adapter protein, Fas-associated protein with death domain (FADD) to the death domain of Fas. FADD mediates recruitment of caspases (cysteine aspartyl-specific proteases, such as caspase-8) that activates a death signalling cascade. Active caspase-8 is involved in the cleavage and activation of effector caspase-3. Caspase-3 is regarded as one of the central executioner molecules and is responsible for cleaving various proteins thereby disabling important cellular, structural and repair processes (Figure 1).

Inflammatory cells, cholangiocytes and Kupffer cells are the main sources of TNFα. Upon activation of TNF-R by TNFα, recruitment takes place of the adapter protein TNF-R-associated protein with death domain (TRADD). TRADD recruits signalling proteins like FADD and TNF-associated factor-2 (TRAF-2). Binding of receptor-interacting protein (RIP) to TRAF-2 initiates the activation of survival pathways like nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) (Figure 1).

Since in hepatocytes only a small amount of caspase-8 is formed, a mitochondrial amplification loop is essential to induce apoptotic cell death in hepatocytes. The inner membrane of the mitochondria contains protein complexes of the respiratory chain and the ATP synthase. During apoptotic stimuli (i.e. hypoxia), the permeability of the mitochondrial membrane is disrupted which results in the release of pro-apoptotic factors (i.e. cytochrome c) from the intermembrane space into the cytosol. Opening of mitochondrial membranes may be initiated by several pro-apoptotic Bcl-2 family members. The Bcl-2 family consist of both pro- (Bax, Bak and Bid) and anti- (Bcl2, Bcl-XL and A1/Bfl-1) apoptotic members that can interact and regulate mitochondria-controlled apoptosis (Figure 1).

Cells contain several pathways to protect themselves against deleterious stimuli. Among such protective molecules, the transcription factor NF-κB has been demonstrated to play an important role in the prevention of cell death provoked by inflammatory cytokines such as TNFα, IL-1β and endotoxins (lipopolysaccharide, LPS). The NF-κB inducing kinase (NIK) is a common mediator in the NF-κB signalling cascade. Activation of NIK often occurs after its binding to TRAF-2. As a consequence, an IkB kinase complex (IKK) is activated which is involved in the phosphorylation and thereby inactivation of NF-κB inhibitors. Upon phosphorylation, IkBs are degraded allowing release of NF-κB which exposes a nuclear localization signal sequence and permits translocation of NF-κB to the nucleus.
In the nucleus, NF-κB binds to κB binding sites in promoters on target genes resulting in transcription of anti-apoptotic genes like inducible nitric oxide synthase (iNOS) and inhibitor of apoptosis protein (IAP) family members (Figure 1) (58).

Besides NF-κB, other signalling pathways exist like the p38 mitogen-activated protein kinase (p38 MAPK) cascade. MAPK activity is regulated through a MAPK kinase (MAPKK). Activation of TNF-R results in activation of p38 MAPKs through TRAF sequestering (Figure 1) (58).

In granzyme B / perforin-mediated apoptosis, granzyme B is delivered into hepatic target cells via transmembrane pores formed by perforin. In the target cell, granzyme B can initiate apoptosis through different pathways: (i) by direct or caspase 8-mediated cleavage of caspase 3, or (ii) by activation of the pro-apoptotic Bcl-2 family member Bid and subsequent induction of mitochondrial collapse leading to release of cytochrome c (Figure 1).

In the subsequent paragraphs, two endogenous cytoprotective molecules (heme oxygenase-1 (HO-1) and protease inhibitor-9 (PI-9) are discussed that may inhibit apoptotic cell death.

3 Protective mechanisms against hepatobiliary injury

3.1 The heme oxygenase-1 system

Oxidative stress is an important mechanism of cellular injury during I/R. It is increasingly recognized that cells respond to oxidative stress by the activation of various cytoprotective genes and pathways. Heme oxygenase-1 (HO-1) has been proposed as a graft survival gene (60,61). The HO-1 gene is encoded on chromosome 22q12 (62). Up-regulation of HO-1 is considered to be one of the most critical cellular protection mechanisms against oxidative stress (63,64).

Heme oxygenases are ubiquitous enzymes that catalyzes the oxidative degradation of heme. The heme protein itself, represents a potentially harmful iron chelate, which promotes lipid peroxidation and free radical formation (65). Heme proteins are a major constituent of hemoglobin in red blood cells (RBCs). RBCs appear to be very susceptible for ROS (19). In I/R injury, lysated RBCs release free heme molecules which generate free radicals formation that leads to an exacerbation of the oxidative stress process, aggravating cell damage (20).

HO-1 catalyzes the rate-limiting step in heme metabolism that leads to the formation of equimolar amounts of free divalent iron (Fe\(^{2+}\)), biliverdin and carbon
monoxide (CO) (Figure 2). This oxidation reaction involves a sequence of transformations that consumes three molecules of O$_2$ and seven electrons, provided by NADPH-cytochrome P-450 reductase $^{(66)}$:

(i) Fe$^{2+}$, a potent reductor, is bound by iron regulatory proteins that stimulate synthesis of ferritin, thereby preventing Fe$^{2+}$-dependent oxidative stress $^{(66-68)}$.

(ii) Biliverdin is converted into bilirubin by the enzyme biliverdin reductase. Bilirubin may protect transplanted organs by inhibition of complement, lymphocyte proliferation, IL-2 production and antibody-dependent and independent cell-mediated cytotoxicity $^{(69)}$. Bilirubin may also contribute to graft protection through inhibition of leukocyte adhesion to endothelial cells by suppression of P-selectin expression and chemotaxis $^{(69)}$. Bilirubin and biliverdin both have the ability to scavenge ROS $^{(70-72)}$.

(iii) CO has been shown to serve as an endogenous regulator for maintaining microvascular blood flow of the liver $^{(73,74)}$, to inhibit platelet aggregation $^{(69)}$, and to inhibit apoptosis $^{(75)}$. Inhibition of apoptosis is mediated via activation of the p38 MAPK cascade (Figure 1) $^{(75)}$.

Three isoforms of heme oxygenase so far have been identified: the inducible HO-1, also known as heat shock protein-32 (HSP-32), the consecutively expressed HO-2, and the not fully defined HO-3 $^{(20)}$.

Animal studies have suggested that exogenous induction of HO-1 before transplantation may confer cytoprotective and immune regulatory functions $^{(19,61,76)}$, and could become a novel and powerful strategy to protect (marginal) liver grafts from I/R injury $^{(60,64)}$ and early acute cellular rejection $^{(77)}$. Mouse hearts that were transplanted into immunosuppressed rats upregulate the expression of HO-1 immediately after transplantation and survive indefinitely, whereas HO-1$^{-/-}$ mouse hearts transplanted under the same immunosuppressive regimen were rejected within 3-7 days $^{(78)}$. The mechanism of protection of HO-1 against immune-mediate injury leading to graft rejection however, remains unclear.

There is increasing evidence that HO-1 is not exclusively cytoprotective $^{(79)}$. Excessive (about 15-fold) overexpression of HO-1 in a hamster fibroblast (HA-1) cell-line causes oxidative injury due to the accumulation of free divalent iron $^{(80)}$. Furthermore, it has been shown in rats that highly increased (about 8- to 9-fold) HO-1 activity contributes to endotoxin-induced shock, due to the increased production of the potent vasorelaxant CO $^{(81)}$. In the literature, it is suggested that HO-1 may mediate cytoprotection within a narrow window of over-expression $^{(82)}$. There is, however, no information on the role of HO-1 in I/R injury in human liver transplantation.
3.2 The protease inhibitor-9 pathway

Acute rejection of the transplanted liver is characterized by infiltration of CD8+ T- lymphocytes (CTLs) into the graft which can lead to (i) portal inflammation, (ii) bile duct inflammation/damage and (iii) subendothelial inflammation of portal veins or terminal hepatic venules \( ^{(83)} \). CTLs induce target cell death by at least two effector pathways: the granule exocytosis pathway in which the granzyme B/perforin molecules collaborate to induce target cell apoptosis and the Fas/FasL pathway in which the cross linking of Fas by the FasL displayed by CTLs result in target-cell demise (Figure 1) \( ^{(84)} \).

Granzyme B is delivered into hepatic target cells via transmembrane pores formed by perforin. In the target cell, granzyme B can initiate apoptosis through different pathways: (i) by direct or caspase 8-mediated cleavage of caspase 3, or (ii) by activation of the pro-apoptotic Bcl-2 family member Bid and subsequent induction of mitochondrial collapse leading to release of cytochrome c (Figure 1) \( ^{(84)} \).

The cytosolic serine proteinase inhibitor-9 (PI-9) has been shown to effectively inhibit granzyme B activity \( ^{(85,86)} \). PI-9 has been detected in several cell types including lymphocytes, dendritic cells, and endothelial cells \( ^{(85,87,88)} \). Recently it was suggested in human kidney transplantation that hyperexpression of PI-9 could resist a fatal attack of CTLs and provide an explanation for the silence of histologically apparent cytotoxic infiltrates in subclinical rejection of kidney allografts \( ^{(56)} \). It is unknown whether similar processes occur in liver transplantation.

4 Recovery of hepatobiliary function after liver transplantation

4.1 Bile formation

One important function of the liver is the formation of bile. It is the route for the excretion of a range of compounds such as cholesterol, bilirubin and xenobiotics \( ^{(89)} \). In addition, the production of bile is necessary to support the dietary lipid absorption and absorption of fat soluble vitamins (A, D, E and K) in the intestine \( ^{(90)} \). Secretion of bile (choleresis) is a sensitive and reliable indicator for assessing the viability of the transplanted liver. In adults, approximately 600 to 1200 mL of bile is produced daily \( ^{(91)} \). Immediately after OLT however, bile production is drastically declined due to I/R damage, but it rapidly recovers during the first postoperative days in viable grafts. In initial poor-functioning (IPF) grafts or in primary non-functioning (PNF) liver transplants, recovery of bile production is delayed or absent, respectively \( ^{(3)} \).
After transplantation, liver parenchymal injury steadily diminishes and bile flow gradually increases. The recovery of the graft however, invariably is accompanied by the occurrence of transient biliary damage during the first two to three post-operative weeks. Recovery of bile flow depends mainly on the secretion of bile salts. Bile salts are potent detergents. Under normal circumstances the cytotoxic effects of bile salts are antagonized by phospholipids. When the recovery of phospholipid secretion is not as rapid as that of bile salt secretion, bile could become more cytotoxic with a relative excess of bile salts. We hypothesized that this could lead to bile duct injury and explain the occurrence of transient post-operative bile duct injury.

Bile formation depends on the complementary interactions between two different polarized cell types: hepatic parenchymal cells (hepatocytes), which account for about 80% of the liver mass, and bile duct epithelial cells (cholangiocytes), which form about 2-5% of the liver weight. Bile flow is mainly driven by the secretion of bile salts against a steep (up to about 1000-fold) concentration gradient, and their counterions (Na⁺, K⁺) into the bile by hepatocytes. This is regarded the bile salt dependent bile flow (BSDF), and accounts for about 60% of bile flow. The remainder 40% fraction of human bile flow is bile salt independent (BSIF), and is mediated by secretion processes in both hepatocytes and cholangiocytes. The BSIF is driven by the canalicular secretion of glutathione disulfide and inorganic electrolytes and ductular secretion of inorganic electrolytes (e.g., Cl⁻ and HCO₃⁻). The secreted compounds that generate bile flow induce the movement of water across the apical membranes of both hepatocytes and cho-
Figure 3. Schematic overview of transporter systems in hepatocytes, cholangiocytes and enterocytes. (A) At the basolateral, i.e., sinusoidal, plasma membrane of hepatocytes, two bile salt (BS) uptake transporters are located. The main BS uptake transporter is the Na\(^+\)-dependent taurocholate cotransporting polypeptide NTCP. The Na\(^+\)-independent transporters organic anion transporting polypeptides OATP-A and OATP-C are, to a lesser extent than NTCP, involved in the sinusoidal hepatic uptake of BS. The OATPs mainly regulate the transport of a variety of organic anions (oa). BS transport by NTCP is driven by a coupled Na\(^+\)/K\(^+\)-ATPase pump. Canalicular, i.e. apical, secretion of BS is mediated by ABCB11 (or bile salt export pump, BSEP). ABCG2 (or multidrug resistance-associated protein type 2, MRP2) is to a much lesser extent than ABCB11 involved in biliary BS secretion. The ABCG2 transporter mainly regulates the biliary secretion of organic anions (oa). The multidrug resistance-associated protein type 3 (MRP3) may be involved in the basolateral output of BS. Biliary secretion of phospholipids (Pl) is mediated by ABCB4 (or multidrug resistance protein type 3, MDR3). The cystic fibrosis transmembrane regulator CFTR, mediates biliary Cl\(^-\) output in cholangiocytes. The apical Na\(^+\)-dependent bile salt transporter (ASBT) facilitates the uptake of BS in both cholangiocytes and enterocytes. BS efflux from the basolateral membrane of cholangiocytes may involve OATP-A, MRP3 and a truncated isof orm of ASBT (t-ASBT). Via the periductular capillary plexus, BS return to the liver for re-uptake in hepatocytes, completing the cholehepatic shunt of BS (i). From the basolateral membrane of enterocytes BS may be excreted by MRP3 and t-ASBT to be delivered back to the liver via the blood circulation, which is referred to as the enterohepatic loop of BS (ii). (B) Cholesterol is acquired through intestinal absorption and through synthesis, which mainly occurs in the liver. At the apical membrane of enterocytes, cholesterol (Ch) and plant sterols (PS, taken in via the diet) are taken up by the Niemann-Pick C1-like protein 1 (NPC1L1). PS cannot be used by the body and are excreted into the gut lumen by ABCGS/G8. In enterocytes, absorbed Ch is predominantly packaged into chylomicrons or may be delivered to high density lipoproteins (HDL) by ABCA1 for transport to the liver. The liver takes up Ch from chylomicron remnants, intermediate density lipoproteins (IDL) and low density lipoproteins (LDL) by receptor-mediated endocytosis by both the LDL-receptor (LDLR) and the LDL-receptor related protein (LRP). The scavenger receptor-B1 (SR-BI) is responsible for the uptake of Ch from HDL. In the liver, Ch is either stored, or distributed to peripheral tissues after incorporation into very low density lipoproteins (VLDL), or may be secreted into bile as free Ch by ABCGS/G8, or secreted into the bile after conversion into BS by ABCB11. In peripheral tissues ABCA1 delivers Ch to HDL for transport back to the liver, which is referred to as reverse cholesterol transport (RCT).
languocytes and their tight junctions until restoration of iso-osmolality \(^{(95-97)}\). Moreover, anions like Cl\(^{-}\) and HCO\(_3\)^{-}, alkalinisate the bile composition \(^{(95)}\).

### 4.2 Bile salts

Primary bile salts are synthesized in the liver from cholesterol by the progressive modification of the ring structures with hydroxyl (-OH) groups and the oxidation and shortening of the side chain \(^{(98)}\). Conversion of cholesterol into bile salts is a process requiring a series of enzymatic steps \(^{(96)}\). In humans, approximately 90% of the bile salt synthesis route runs via the microsomal cholesterol 7 alpha-hydroxylase (CYP7A1) enzyme pathway, which is regulated by the bile salt pool size (the amount of bile salts in the body) and by the level of dietary cholesterol \(^{(98)}\). The alternative bile salt synthesis route is managed by the mitochondrial sterol 27-hydroxylase (S27H) enzyme \(^{(99)}\).

For basolateral uptake and canalicular secretion of bile salts in hepatocytes, different transporter proteins are involved. The hepatic uptake of bile salts is mainly mediated by the Na\(^{+}\)-dependent taurocholate cotransporting polypeptide (NTCP, gene symbol SLC10A1). Hepatobiliary secretion of bile salts is predominantly carried out by the bile salt export pump (BSEP, gene symbol ABCB11). Both NTCP and BSEP transporters will be discussed later (Figure 3A).

Before bile salts are excreted into the bile, they are conjugated with either taurine (predominantly in rodents) or glycine (predominantly in humans) \(^{(100)}\). The primary bile salts are cholate (3\(\alpha\)OH, 7\(\alpha\)OH, 12\(\alpha\)OH-trihydroxy bile salt) and chenodeoxycholate (\(\alpha\)OH, 7\(\alpha\)OH-dihydroxy bile salt). In the intestine, the primary bile salts are converted by bacterial 7\(\alpha\)-dehydroxylation into the secondary bile salts deoxycholate (3\(\alpha\)OH, 12\(\alpha\)OH-dihydroxy bile salt) and lithocholate (3\(\alpha\)OH-monohydroxy bile salt), respectively. Lithocholate is changed by intestinal bacteria into the tertiary bile salt ursodeoxycholate (3\(\alpha\)OH, 7\(\beta\)OH-dihydroxy bile salt) \(^{(90)}\).

Bile salts are detergent molecules. Their solubilizing properties depend on the extent of hydrophobicity. Hydrophobic bile salts are more detergent than hydrophilic bile salts. Based on a hydrophobicity index, the rank order of decreasing hydrophobicity capacity is: lithocholate, deoxycholate, chenodeoxycholate, cholate and ursodeoxycholate \(^{(101)}\). In man, the bile salt pool has a more hydrophobic composition. To prevent bile salt cytotoxicity, the detergent properties of these molecules are antagonized by phospholipids. Phospholipids together with bile salts form polymolecular aggregates, known as mixed micelles. Since bile is predominantly an aqueous environment, it consists of about 80% of water,
micelles also play an important role in the transportation of lipid constituents (e.g. cholesterol) in bile.

4.3 Phospholipids
Phospholipids are the major components of cellular membranes and are prominent components of plasma lipoproteins. Phosphatidylcholine and phosphatidylethanolamine are the two most abundant phospholipids present in eukaryotic cell membranes. In bile, more than 95% of the phospholipids are phosphatidylcholine. Phosphatidylcholine is made by two alternate pathways, by the CDP-choline pathway and by the methylation of phosphatidylethanolamine. Phospholipids are translocated to the bile by the ABCB4 carrier protein (Figure 3A), which will be discussed in subsequent paragraphs. For phospholipid secretion, it is generally accepted that no bile-salt independent output exists. Also biliary phospholipid and cholesterol excretion have been demonstrated to be coupled. Phospholipids are a carrier and a solvent of cholesterol in hepatic bile, and may drive cholesterol output. In the bile, phosphatidylcholine together with bile salts form polymolecular aggregates. An absence of phosphatidylcholine in bile leads to the formation of bile with a cytotoxic composition due to the unantagonized detergent actions of bile salts, which forms the source of peculiar cholestatic diseases. A disproportionate recovery of bile salt to phospholipid secretion after liver transplantation could perhaps lead to bile duct injury.

4.4 Cholesterol
Cholesterol serves as a precursor for bile salts and steroid hormones, as was described in the preceding paragraph. Furthermore, cholesterol is an essential structural constituent of cellular and intracellular membranes. Cholesterol metabolism is a complex process and is incompletely understood. Briefly, cholesterol is acquired through dietary intestinal absorption and through synthesis from acetyl-CoA, which mainly occurs in the liver. Absorbed cholesterol is packaged into chylomicrons for transport to the liver via the lymph stream (Figure 3B). In the liver, cholesterol is either (i) stored, or (ii) distributed to peripheral tissues via the blood stream after incorporation into very low density lipoproteins (VLDL), or (iii) secreted into bile either as free cholesterol or, after conversion, as bile salts (Figure 3B). In peripheral tissues (e.g., macrophages), the ABCA1 transporter delivers cholesterol to high density lipoproteins (HDL) for the transport back to the liver, which is referred to as reverse cholesterol transport (RCT) (Figure 3B). Of the cholesterol present in bile, approximately 60-80% is reab-
sorbed in the intestine, and transported back to the liver, completing the circula-
tion of cholesterol in the body (113).

For basolateral uptake and apical output of cholesterol in hepatocytes, different
transport mechanisms are involved. The hepatic uptake of cholesterol from chy-
lomicrons and low density lipoproteins (LDL), is performed by receptor-mediated
decoytosis by both the LDL-receptor (LDLR) and the LDL-receptor related pro-
tein (LRP) (Figure 3B) (114). Selective uptake of cholesterol from HDL particles, is
carried out by the scavenger receptor-BI (SR-BI) (Figure 3B) (114). For apical out-
put of cholesterol in bile, the ABCG5/ABCG8 half transporters are probably invol-
ved, as will be discussed below.

Disturbances in cholesterol homeostasis can result in increased plasma and bili-
ary cholesterol concentrations. Increases in plasma cholesterol can lead to the
accumulation of cholesterol in macrophages located in the artery wall, causing
atherosclerosis (114). Excessive cholesterol secretion in bile can result in the preci-
pitation of cholesterol crystals, which can result in the formation of gallstones
(115). Little is known about the recovery of hepatobiliary cholesterol secretion after
liver transplantation.

5 The hepatobiliary transporters

5.1 ATP-binding cassette transporters

The ATP-binding-cassette (ABC) transporters are a family of proteins which
mediate transport of a wide variety of substrates across different cellular mem-
branes (114). Active ABC transporters consists of a single polypeptide with two ATP-
binding domains and 12 or more membrane spanning helices (full-transporter).
In the ATP-binding domain the highly conserved Walker A and Walker B motifs
are present which are involved in ATP binding and hydrolysis. The energy deri-
ved from the hydrolysis of ATP is used for the transport of various compounds
(116). Some ABC-transporters contain two polypeptides each with one ATP-binding
site and 6 membrane spanning helices (half-transporters). These half-transpor-
ters need to dimerize with other half-transporters to be able to transport substra-
tes (114). To date, 48 ABC transporters have been identified in the human geno-
me. Based on their structure and homology, they are classified in 7 groups
(ABCA-ABCG). The ABC transporters that are studied in this thesis will be discus-
sed in the next paragraphs.
5.2 The ABCB4 and ABCB11 transporters

The ABCB4 (or multidrug resistance protein type 3, MDR3) and ABCB11 (or bile salt export pump, BSEP) transport proteins are both located at the canalicular phospholipid double membranes of hepatocytes (Figure 3A). Either of them are full-transporters (117). The genes encoding the ABCB4 and ABCB11 proteins are located on chromosome 7q21 and 2q24, respectively (116).

The ABCB4 protein acts as a phospholipid flippase. It translocates phosphatidylcholine, the predominant biliary phospholipid, from the inner to the outer leaflet of the hepatocyte membrane that faces the bile (118). Biliary phospholipids protect the canalicular membrane against the detergent action of bile salts. Mutations of the gene encoding the ABCB4 protein underlie the disease progressive familial intrahepatic cholestasis type 3 (PFIC3). In the majority of this disorder, the ABCB4 protein is not expressed (89,119). This leads to a lack of phospholipids in the bile, while biliary bile salt secretion unrelentingly proceeds. In these subjects, the bile composition is detrimental. Hepatocytes and cholangiocytes are damaged because the bile salt monomers extract phospholipids from the outer leaflets of the canalicular cell membranes.

In patients with the cholestatic syndrome PFIC3, serum gamma-glutamyltransferase (γ-GT) activity is usually markedly elevated. The liver histology shows extensive bile duct proliferation, portal and periportal inflammation and fibrosis (89,92). In association with the nature of the ABCB4 gene mutation, patients with PFIC3 present at different ages, ranging from infancy to adolescence. Finally they develop liver cirrhosis, and become candidate for liver transplantation.

Besides PFIC3, other cholestatic syndromes exist in patients with ABCB4 gene mutations such as intrahepatic cholestasis of pregnancy (ICP) and peculiar forms of cholesterol gallstone disease (107,119,120). Women with ICP have a normal phenotype, but develop intrahepatic cholestasis during the third trimester of pregnancy. It is suggested that hormones in this trimester cause the heterozygous mutations in the ABCB4 gene to disturb the intracellular trafficking of the synthesized ABCB4 protein. The protein in this way does not reach the canalicular hepatocyte membrane and is therefore unable to perform its function and hence results in manifest disease (89,119,120).

Rosmorduc et al. (121) reported mutations in different domains of the ABCB4 gene in adults, that were associated with cholesterol cholelithiasis. They hypothesize that when residual ABCB4 activity and subsequent biliary phospholipid secretion into the bile decrease below a critical threshold, ABCB4 gene mutations may lead to cholesterol gallstone formation.
The ABCB11 (BSEP) protein mediates the secretion of bile salts across the canalicular hepatocyte membrane into the bile. Also the ABCC2 protein (or multidrug resistance-associated protein type 2, MRP2), to a lesser extent than ABCB11, is involved in the output of bile salts. The ABCC2 transporter mainly regulates the hepatic output of other organic anions such as bilirubin and estrogens (Figure 3A).

Mutations in the ABCB11 gene are associated with progressive familial intrahepatic cholestasis type 2 (PFIC2). This cholestatic disease is characterized by low biliary bile salt concentrations, elevated serum bile salt concentrations and normal γ-GT levels\(^{122}\). Histologically, the liver shows inflammatory activity with lobular and portal fibrosis. In early stages bile duct loss occurs. Hepatocellular carcinoma can develop in later stages. PFIC2 progresses to persistent and progressive cholestasis requiring liver transplantation during the first decade of life\(^{89,119}\).

Between 95-99% of the excreted bile salts are actively reabsorbed in the distal ileum of the intestine by the apical Na\(^+\)-dependent bile salt transporter ASBT (gene symbol SLC10A2) to be delivered back to the liver via the portal blood\(^{90}\). This is referred to as the enterohepatic loop of bile salts (Figure 3A). Basolateral (sinusoidal) re-uptake of bile salts in hepatocytes is mainly mediated by members of the solute carrier (SLC) superfamily. These transporters are not directly ATP-dependent for their function\(^{6}\). Sinusoidal uptake of bile salts is mainly mediated by the Na\(^+\)-dependent taurocholate cotransporting polypeptide (NTCP, gene symbol SLC10A1). A coupled Na\(^+\)/K\(^+\)-exchange provides the energy required for bile salts to traverse across the basolateral hepatocyte phospholipid bilayer (Figure 3A). To a lesser extent, the organic anion transporting polypeptides: OATP-A and OATP-C (solute carrier (SLC) family 21 gene symbols: SLC21A3 and SLC21A6, respectively) in humans are involved in the sinusoidal uptake of bile salts. The OATP proteins also regulate the hepatic uptake of endogenous substrates such as estrogens, thyroid hormones and bilirubin (Figure 3A)\(^{89,122}\).

Next to the enterohepatic loop of bile salts, an intrahepatic shunt of bile salts exists. (Un)conjugated bile salts are extracted from the bile by ASBT, which is located at the canalicular membrane of intrahepatic cholangiocytes. The efflux of bile salts into the periductular venous capillary plexus is mediated by a truncated isoform of ASBT (t-ASBT), and/or the multidrug resistance-associated protein type 3 (MRP3, gene symbol ABCC3). Also the organic anion transporting polypeptide OATP-A may be involved in the efflux of bile salts into the intrahepatic circulation\(^{123-129}\). It is suggested that under pathophysiological conditions such as extrahepatic cholestasis, the intrahepatic circulation of bile salts reduces bile

CHAPTER 1

GENERAL INTRODUCTION AND AIM
salt synthesis via a negative feedback mechanism, thereby preventing bile salt cytotoxicity.

We hypothesized that immediately after liver transplantation, the biliary bile salt to phospholipid secretion would be disproportionate with a relative excess of bile salts and could lead to postoperative bile duct injury. These hypothesized differences in recovery of bile salt and phospholipid secretion rates may be explained by a disparity between ABCB4 (MDR3) and ABCB11 (BSEP) activity.

5.3 The ABCG5 and ABCG8 half-transporters

Animal experiments have shown that the two polypeptides Abcg5 and Abcg8 importantly mediate the biliary secretion of cholesterol. ABCG5 and ABCG8 unite to generate a functionally active heterodimeric transport protein. The genes encoding ABCG5 and ABCG8 are highly expressed in cells of the intestine and liver, and are located on chromosome 2p21. The genes are adjacent located in a head-to-head configuration. In enterocytes and hepatocytes, ABCG5 and ABCG8 transport plant sterols (e.g., sitosterol, the major plant sterol) and animal sterols (e.g., cholesterol, the principal sterol in man) into the gut lumen and bile. Thereby they decrease sterol absorption. The intestine is a major barrier to the uptake of plant sterols: less than 5% of dietary plant sterols are absorbed compared to about 40% of the available cholesterol. Mutations in either of the two genes encoding these ABC half-transporters are associated with the disease sitosterolemia. In this disorder, intestinal absorption of dietary plant and animal sterols is strongly increased in addition to a substantially impaired biliary output. Patients with sitosterolemia have markedly enhanced plasma sterol concentrations, that results in the development of xanthomas (depositions of cholesterol) in tendons and skin and premature atherosclerosis.

We examined ABCG5/ABCG8 expression and biliary cholesterol secretion in patients after OLT to evaluate the presumed dimerization and coordinated regulation of both half-transporters, and to assess control strength of ABCG5/ABCG8 in the process of hepatobiliary cholesterol secretion in the human situation.
6 Outline and aim of the thesis

Liver transplantation is associated with I/R and may postoperatively be accompanied by acute rejection of the transplant. Both I/R and rejection represent a continuum of processes that can culminate into graft injury and compromised hepatobiliary function.

The aim of the research described in this thesis was to investigate molecular changes in hepatobiliary function and injury in human OLT. Studies were focused on the recovery of hepatobiliary function (bile formation) in relationship to cellular injury. Moreover, two potentially protective genes and pathways in the liver (HO-1 and PI-9) were studied.

In chapter 2 we examined ABCG5/ABCG8 expression and biliary cholesterol secretion in patients after OLT to evaluate the presumed dimerization and coordinated regulation of both half-transporters, and to assess control strength of ABCG5/ABCG8 in the process of hepatobiliary cholesterol secretion in man.

In chapter 3 we studied the postoperative recovery of bile flow in relation to the occurrence of transient bile duct injury in liver transplant subjects. We hypothesized that immediately after transplantation, the recovery of phospholipid secretion is not as rapid as that of bile salt secretion. The relative excess of bile salts could lead to bile with a more cytotoxic composition and explain the occurrence of biliary injury.

In chapters 4 and 5 we studied the endogenous HO-1 expression in liver transplants. Although HO-1 has been shown to drastically reduce I/R injury in rat liver grafts, HO-1 probably is not exclusively cytoprotective. Each product generated by the action of HO-1 (Fe$^{2+}$, CO and bilirubin) is also able to induce injury. We studied changes in endogenous HO-1 expression levels during liver transplantation and correlated this with immediate postoperative graft injury and function.

In chapter 6 we investigated endogenous expressions of PI-9 and HO-1 during the development of early subclinical rejection. While infiltrating granzyme B-positive T lymphocytes cause hepatic injury during acute cellular rejection, in subclinical rejection the presence of CTLs in the graft does not lead to manifest liver injury. We hypothesized that during early subclinical rejection the activity of CTLs is kept silent by actions of PI-9 and HO-1.

Finally an integrated overview of the results obtained in this research thesis is provided and discussed in chapter 7.


122. Ros J. Expression and regulation of ABC transporter genes during liver regeneration. Department of Gastroenterology, University Medical Centre Groningen, Groningen, The Netherlands, 2002.


