Altered bile composition after liver transplantation is associated with the development of Nonanastomotic biliary strictures
Abstract

Nonanastomotic biliary strictures are troublesome complications after liver transplantation. The pathogenesis of NAS is not completely clear, but experimental studies suggest that bile salt toxicity is involved. In 111 adult liver transplant bile samples were collected daily posttransplantation for determination of bile composition. Expression of bile transporters was studied perioperatively. Nonanastomotic biliary strictures were detected in 14 patients (13%) within one year after transplantation. Patient- and donor characteristics and postoperative serum liver enzymes were similar between patients who developed nonanastomotic biliary strictures and those who did not. Secretions of bile salts, phospholipids and cholesterol were significantly lower in patients who developed strictures. In parallel, biliary phospholipids/bile salt ratio was lower in patients developing strictures, suggestive for increased bile cytotoxicity. There were no differences in bile salt pool composition or in hepatobiliary transporter expression.

Conclusion. Although patients who develop nonanastomotic biliary strictures are initially clinically indiscernible from patients who do not develop nonanastomotic biliary strictures, the biliary bile salts and phospholipids secretion, as well as biliary phospholipids/bile salt ratio in the first week after transplantation, was significantly lower in the former group. This supports the concept that bile cytotoxicity is involved in the pathogenesis of nonanastomotic biliary strictures.
Introduction

Biliary complications are a major cause of morbidity and graft failure in patients after liver transplantation (1-3). Nonanastomotic strictures (NAS) of the larger bile ducts are considered to represent the most troublesome biliary complication as they are frequently resistant to therapy (4). The reported incidence of NAS is 5-15% (5-11). The occurrence of NAS can be partly attributed to thrombosis of the hepatic artery. The pathogenesis of NAS that develop in the absence of hepatic artery thrombosis is less clear (1,12). In general, three mechanisms contributing to bile duct injury after liver transplantation have been postulated: preservation or ischemia-related injury (7,13-18), immunological processes (7,19,20) and injury induced by cytotoxicity of biliary bile salts (21-24).

Bile salts have potent detergent properties and may damage cells by affecting the integrity of cellular membranes (22,25). In the biliary tree, the toxic effects of bile salts are usually reduced by the formation of mixed micelles with phospholipids (26,27). Studies in mice and pigs, as well as clinical studies in humans, have indicated that bile formation early after liver transplantation may be disturbed, resulting in more cytotoxic bile with a relatively low phospholipids / bile salt ratio (1,21-24,28,29). We previously showed a strong relationship between this ratio early after liver transplantation and injury of the small bile ducts in the liver (21,24,29). The small bile ducts, however, are lined by distinct cholangiocytes, that have different characteristics compared with cholangiocytes in larger bile ducts, i.e. the location of NAS (30-33). It is unknown whether bile toxicity is also involved in the pathogenesis of transplantation-related injury of the large bile ducts, which may lead to the development of NAS.

In contrast to the cytotoxic properties of bile salts, evidence has accumulated that bile salts may also influence cholangiocyte proliferation and survival, especially in the larger bile ducts (31,34-36). Some bile salts, including taurocholate and tauroliothocholate stimulate cholangiocyte proliferation in vitro and in vivo, and bile salts are considered a survival factor for cholangiocytes in the larger bile ducts (34,35). Cholangiocytes of the large bile ducts are able to take up bile salts from bile via the apical Na+-dependent bile acid transporter (ASBT, gene symbol SLC10A2). After basolateral secretion, bile salts are transported back to hepatocytes and resecreted into bile, thereby contributing to bile flow via the “cholehepatic shunt pathway” (30,37). Bile production and composition, is therefore not exclusively determined by hepatocytes.
It has remained unclear whether bile salts are detrimental or beneficial for cholangiocyte function in large bile ducts after human liver transplantation, and whether or not bile production and composition are involved in the pathogenesis of NAS. In contrast to the small bile ducts, bile salts may not only have toxic effects but could also exert a proliferative restoration or preservation of the biliary epithelial lining of large bile ducts after transplantation. If bile composition is involved in the pathogenesis of NAS, one would expect that the bile composition in the first week after liver transplantation is different in those patients who will develop NAS as compared to patients who will not develop NAS. We tested this hypothesis by prospectively assessing bile production and composition within one week after liver transplantation and the subsequent development of NAS in a large cohort of adult liver transplant recipients.

**Patients and Methods**

**Patients**

Between August 2000 and December 2004 a total of 222 liver transplants were performed at the University Medical Center Groningen. After excluding children (<18 years; n=70) and non heart-beating donor liver transplants (n=5), 147 patients were potential candidates for the study. Thirty six cases were excluded, because of graft loss within 90 days (n=22), initial poor graft function (defined as in (38,39); n=12), or hepatic artery thrombosis (confirmed by either Doppler ultrasound or angiography; n=2). This resulted in a study population of 111 liver transplant procedures. Surgical technique and perioperative management were as previously described by our group (5,40,41). Clinical variables and laboratory data were prospectively collected in a computerized database. Tissue and data collection was performed according to the guidelines of the medical ethical committee of our institution and the Dutch Federation of Scientific Societies.

**Diagnosis of NAS**

NAS was defined as any stricture, dilatation, or irregularity of the intra- or extrahepatic bile ducts of the liver graft, occurring within the first year after transplantation (Figure 1). The diagnosis NAS was based on at least one adequate imaging study of the biliary tree, after exclusion of hepatic artery thrombosis by either Doppler ultrasound or conventional angiography. Imaging studies of the arterial vasculature were repeated over time if no other explanation for the...
NAS was found and to confirm patency of the hepatic artery (5). Severity of NAS was graded according to a semi-quantitative scale, as described previously (5). Isolated strictures at the bile duct anastomosis were not included in this analysis. The time of first presentation of NAS was recorded for all patients.

Figure 1. Postoperative cholangiography in liver transplant recipients. (A) Example of normal cholangiogram, with smooth lining and equal filling of the biliary tree. (B) Example of non anastomotic biliary strictures (NAS), characterized by diffuse strictures and irregularities of both the extra- and intrahepatic bile ducts on both sides of the liver with intrahepatic dilatations.

Collection of Liver Biopsies
Specimens of liver tissue were obtained during routine diagnostic biopsies of the liver grafts. According to our protocol, three consecutive needle biopsies were collected: at the end of cold preservation, approximately 3 hours after reperfusion, and 1 week after transplantation. An aliquot of the biopsy specimen was immediately snap-frozen for isolation of total RNA, the remaining material was used for routine histological analysis. Pieces of normal liver tissue from hepatic resections for colorectal metastasis were collected after obtaining informed consent and served as controls (n=9). All liver biopsies were snap-frozen and stored at -80°C until further processing.
Collection and Analysis of Bile Samples

Before transplantation, the gallbladder was removed and the bile ducts were flushed with preservation fluid on the backtable during preparation for implantation. During the transplantation an open tip silicon catheter was inserted in the recipient common bile duct and placed retrograde through the anastomosis. Via this open biliary tube, bile flow was entirely diverted outside the patient into a collection bag that was placed below the horizontal bed level (42). Interruption of the enterohepatic circulation in the patient was prevented by re-administration of bile via a percutaneous feeding jejunostomy catheter. Samples of bile were collected daily in the first postoperative week between 8:00 and 9:00 am. Bile samples were frozen and stored at -80°C until further processing. None of the patients received a statin or ursodeoxycholic acid during the first week after transplantation. Bile samples were analyzed for total bile salts, phospholipids, and cholesterol contents. Total bile salt concentrations were measured with fluorescent method using 3α-hydroxysteroid dehydrogenase (43). Phospholipid and cholesterol concentrations in bile were assayed spectrophotometrically, using commercially available enzymatic methods (Wako Chemicals GmbH, Neuss, Germany; and Roche Diagnostics GmbH, Mannheim, Germany; respectively). Postoperative secretion of bile components was defined as concentration multiplied by daily bile production per kilogram body weight of the donor. Bile salt composition of bile samples was determined by capillary gas chromatography in a 50µL bile sample on a Hewlett-Packard gas chromatograph (HP 5880A) equipped with a 50 m x 0.32 mm CP-Sil-19 fused silica column (Chrompack B.V., Middelburg, The Netherlands) (44). Subsequently the hydrophobicity of the bile salt pool was determined by the Heuman index (45).

RNA Extraction and Reverse Transcription Polymerase Chain Reaction

Isolation and reverse transcription of RNA was performed as described previously (21). Messenger RNA levels of following hepatobiliary transporters were analyzed: the most prominent bile salt uptake system (NTCP, Na+-dependent taurocholate cotransporting polypeptide: gene symbol SLC10A1) and secretion system (BSEP, bile salt export pump: gene symbol ABCB11), the phospholipid translocator (MDR3, multidrug resistance protein 3: gene symbol ABCB4) and the main canalicular organic anion transporter and driving force of the bile salt independent bile flow (MRP2, multidrug resistant associated protein-2: gene symbol ABCC2). Additionally, cholesterol 7α-hydroxylase (gene symbol CYP7A1) was analyzed by real-time polymerase chain reaction.
(PCR), using the ABI PRISM 7900 HT Sequence detector (Applied Biosystems, Foster City, CA, USA). Nucleotide sequences of Primers (Invitrogen, Paisly, Scotland) and Probes (Eurogentec, Herstal, Belgium) were designed using Primes Express software (Applied Biosystems, Foster City, CA, USA). Probes were 5’ labeled by a 6-carboxy-fluoresceine (FAM) reporter and 3’ labeled with a 6-carboxy-tetra-methyl-rhodamin (TAMRA) quencher and are listed in Table 1. Messenger RNA copy numbers of genes were normalized to those of 18S rRNA. Real time PCR data were analyzed using the comparative cycle threshold (CT) method (46).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Alternative Name</th>
<th>Primers and Probes</th>
<th>PCR Product (bp)</th>
</tr>
</thead>
</table>
| SLC10A1 | NTCP            | sense 5’-TGA TAT CAC TGG TCC TGG TTC TCA -3’  
antisense 5’-GCA TGT ATT GTG GCC GTT TG -3’  
probe 5’ FAM-TCC TTG CAC CAT AGG GAT CGT CCT CA -TAMRA 3’ | 74               |
| ABCB11  | BSEP            | sense 5’-ACA TGC TTG CGA GGA CCT TTA -3’  
antisense 5’-GGA GGT TCG TGC ACC AGG TA -3’  
probe 5’ FAM-CCA TCC GGC AAC GCT CCA AGT CT -TAMRA 3’ | 105              |
| ABCB4  | MDR3            | sense 5’-CTA TGG AAT TAC TTT TAG TAT CTC ACA AGC ATT -3’  
antisense 5’-AGC GCA TAT GTC TCA CAA T -3’  
probe 5’ FAM-TTT TCC TTA TGC CGG TTG TTT -TAMRA 3’ | 100              |
| ABCC2  | MRP-2           | sense 5’-TGC AGC CTC CAT AAC CAT GAG -3’  
antisense 5’-CTT CTG CCT CTC GCC TAT TCA -3’  
probe 5’ FAM-CAG CTT TCG TCG AAC ACT TAG CCG CA -TAMRA 3’ | 139              |
| CYP7A1 |                 | sense 5’-GAG AAG GCA AAC GGG TGA AC -3’  
antisense 5’-GGT ATG ACA AGG TAT TGG TGA TGA -3’  
probe 5’ FAM-TGG ATT TCC ATA CCT GGG CTG TGC TCT-TAMRA 3’ | 181              |
| 18S    |                 | sense 5’-CGG CTA CCA CAT CCA AGG A -3’  
antisense 5’-CCA ATT ACA GGG CCT CGA AA -3’  
probe 5’ FAM-CGC GCA AAT TAC CCA CTC CCG A -TAMRA 3’ | 109              |

Table 1. Sequences of Primers and Probes Used for Real-Time PCR Analysis
Statistical Analysis
Collection of laboratory values from the central laboratory database was conducted as described previously (46). Continuous variables were presented as medians with interquartile range (IQR) or means with standard error of the mean (SEM) when appropriate. Categorical variables were presented as numbers with percentages and compared using Pearson’s chi-square test. Comparison of continuous variables was performed using the Mann-Whitney U test. Area under the curve (AUC) was analyzed by the trapezium method. The level of significance was set at 0.05. Statistical analysis was performed using SPSS 14.0 (SPSS, Chicago, IL, USA).

Results
Development of NAS
NAS was diagnosed in 14 of the 111 liver transplant recipients (13%) at a median time interval of 2.4 months (IQR 1.3 - 4.0 months) after transplantation. Signs of NAS were mild/moderate in 12 patients and severe in 2 patients. There were no significant differences in donor and recipient characteristics or surgical variables in patients who developed NAS compared to patients who did not develop NAS (Table 2).

Serum Markers of Hepatocellular Injury and Cholestasis
Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the first week after transplantation, as markers of ischemia reperfusion injury, were similar in patients who did or did not develop NAS (Figure 2). Similarly, gamma glutamyltransferase (γGT) and alkaline phosphatase (ALP), markers of cholestasis, were not different between the two groups in the first postoperative week (Figure 2).
Biliary Secretion of Bile Salts, Phospholipids and Cholesterol

Bile production increased 7-fold during the first week after transplantation in both groups (Figure 3). Biliary bile salt secretion increased after transplantation in both groups. Bile flow increased in linear fashion with the higher bile salt secretion rate in both groups. The bile salt independent bile flow (Y-intercept) and the bile salt dependent bile flow (slope) were similar in both groups (NAS group: flow = 0.028 x BS-secretion + 1.12, r²=0.47; Controls: flow = 0.024 x BS-secretion + 1.67, r²= 0.56). However, in patients who did not develop NAS, the increase in bile salt secretion was over 1.5 fold higher compared to patients who did develop NAS (99 ± 23 µmol/day/kg versus 166 ± 27 µmol/day/kg at day 8) (Figure 3). In parallel with the relatively reduced bile salt secretion, secretion of phospholipids and cholesterol was also significantly lower in patients developing NAS (Figure 3). In patients who developed NAS, the secretion of
biliary phospholipids during the first week after transplantation was even more compromised than the secretion of bile salts. This resulted in a significantly lower biliary phospholipid / bile salt ratio in the patients developing NAS, compared to patients who did not develop NAS (Figure 4).

Figure 3. Comparison of median (IQR) daily bile production (panel A), bile salt (BS; panel B) secretion, phospholipid (PL; panel C) secretion, and cholesterol (CH; panel D) secretion during the first 8 days after liver transplantation in patients who later developed non anastomotic biliary strictures (NAS, closed squares) and patients who did not develop NAS (open triangles). Overall BS, PL and CH secretion, as determined by the area under the curve (AUC), was significantly lower in the patients who developed NAS.
Figure 4. Comparison of the mean biliary phospholipid / bile salt (PL/BS) ratio in the first 8 days after liver transplantation in patients who developed non anastomotic biliary strictures (NAS, closed squares) and patients who did not develop NAS (open triangles). The PL/BS ratio was significantly lower in patients who developed NAS.

Figure 5. Composition of bile salts in bile at day 3 after transplantation in patients who later developed non anastomotic biliary strictures (NAS, dark bars) and patients who did not develop NAS (open bars). There were no significant differences in the absolute amounts of the various bile salts between the two groups of patients. Abbreviations: DC: deoxycholate, C: cholate, CDC: chenodeoxycholate, UDC: ursodeoxycholate.
Table 2. Comparison of Donor, Recipient, Surgical and Postoperative Variables of Liver Grafts With or Without Non Anastomotic Strictures (NAS).

<table>
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<tr>
<th></th>
<th>NAS (n = 14)</th>
<th>Control OLT (n = 97)</th>
<th>P-value</th>
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<td><strong>Donor variables</strong></td>
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<tr>
<td>Age (years)</td>
<td>47 (39 - 57)</td>
<td>48 (37 - 58)</td>
<td>0.98</td>
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<tr>
<td>Gender (male/female)</td>
<td>6 / 8 (43% / 57%)</td>
<td>44 / 53 (45% / 55%)</td>
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<tr>
<td>Gender match (donor/recipient)</td>
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<tr>
<td>M/M</td>
<td>2 (14%)</td>
<td>28 (29%)</td>
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<td>F/F</td>
<td>5 (35%)</td>
<td>24 (25%)</td>
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<tr>
<td>M/F</td>
<td>4 (29%)</td>
<td>16 (17%)</td>
<td></td>
</tr>
<tr>
<td>F/M</td>
<td>3 (21%)</td>
<td>29 (30%)</td>
<td></td>
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<tr>
<td>Body weight donor</td>
<td>72.5 (65 - 86.3)</td>
<td>70 (65 - 80)</td>
<td>0.54</td>
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<td><strong>Laboratory variables</strong></td>
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<td></td>
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<td>Hemoglobin (mmol/L)</td>
<td>7.1 (5.8 - 8.4)</td>
<td>7.0 (6.2 - 7.8)</td>
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<td>Total bilirubin (umol/L)</td>
<td>8.4 (5.5 - 10.5)</td>
<td>10.1 (6.0 - 16.0)</td>
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<td>Alanine Aminotransferase (U/L)</td>
<td>20 (13 - 25)</td>
<td>23 (15 - 45)</td>
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<td>Gamma Glutamyl transferase (U/L)</td>
<td>25 (20 - 63)</td>
<td>22 (14 - 38)</td>
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<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>55 (50 - 86)</td>
<td>53 (39 - 66)</td>
<td>0.31</td>
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<tr>
<td>Cause of death</td>
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<td></td>
<td>0.89</td>
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<td>Cerebral Vascular Accident</td>
<td>11 (79%)</td>
<td>72 (74%)</td>
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<tr>
<td>Trauma</td>
<td>2 (14%)</td>
<td>19 (20%)</td>
<td></td>
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<tr>
<td>Miscellaneous</td>
<td>1 (7%)</td>
<td>6 (6%)</td>
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<td><strong>Recipient variables</strong></td>
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<tr>
<td>Age (years)</td>
<td>54 (44 - 58)</td>
<td>50 (40 - 55)</td>
<td>0.31</td>
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<tr>
<td></td>
<td>Male (n=5)</td>
<td>Female (n=9)</td>
<td>Total (n=14)</td>
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<td>--------------------------------</td>
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<td>--------------</td>
</tr>
<tr>
<td><strong>Gender (Male/Female)</strong></td>
<td>5 / 9</td>
<td>57 / 40</td>
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<td><strong>Disease</strong></td>
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<tr>
<td>Primary Sclerosing Cholangitis</td>
<td>4 (30%)</td>
<td>20 (21%)</td>
<td>24 (16%)</td>
</tr>
<tr>
<td>Primary and Secondary Biliary</td>
<td>2 (14%)</td>
<td>9 (6%)</td>
<td>11 (7%)</td>
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<td>Cirrhosis</td>
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<tr>
<td>Viral hepatitis</td>
<td>0</td>
<td>20 (21%)</td>
<td>20 (14%)</td>
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<td>Autoimmune hepatitis</td>
<td>2 (14%)</td>
<td>10 (10%)</td>
<td>12 (8%)</td>
</tr>
<tr>
<td>Alcoholic cirrhosis</td>
<td>2 (14%)</td>
<td>10 (10%)</td>
<td>12 (8%)</td>
</tr>
<tr>
<td>Viral hepatitis</td>
<td>2 (14%)</td>
<td>5 (5%)</td>
<td>7 (5%)</td>
</tr>
<tr>
<td>Other</td>
<td>2 (14%)</td>
<td>23 (16%)</td>
<td>25 (16%)</td>
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<td><strong>Child Pugh Classification (A/B/C)</strong></td>
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<td>19 / 38 / 37</td>
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<td><strong>Re-transplantation</strong></td>
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<td>15 (16%)</td>
<td>18 (12%)</td>
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<td><strong>Surgical variables</strong></td>
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<td>Preservation Solution</td>
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<tr>
<td>High viscosity (UW)</td>
<td>14 (0%)</td>
<td>88 (91%)</td>
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<tr>
<td>Low viscosity (HTK)</td>
<td>0 (100%)</td>
<td>9 (9%)</td>
<td></td>
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<tr>
<td>Cold ischemia time (minutes)</td>
<td>500 (406 - 595)</td>
<td>489 (409 - 587)</td>
<td></td>
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<tr>
<td>Warm ischemia time (minutes)</td>
<td>48 (42 - 54)</td>
<td>45 (40 - 51)</td>
<td></td>
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<tr>
<td>Revascularization time (minutes)</td>
<td>78 (64 - 98)</td>
<td>93 (80 - 109)</td>
<td></td>
</tr>
<tr>
<td>Bile duct reconstruction (duct to duct / Roux-Y)</td>
<td>11 / 3</td>
<td>76 / 21</td>
<td></td>
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<tr>
<td><strong>Postoperative variables</strong></td>
<td></td>
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<tr>
<td>ICU-length of stay (days)</td>
<td>2.5 (1.0 - 10.8)</td>
<td>2 (1.0 - 6.3)</td>
<td></td>
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<tr>
<td>Acute rejection</td>
<td>5 (36%)</td>
<td>35 (36%)</td>
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</table>
Figure 6. Relative gene expression of the bile transporters ABCB11 (panel A), SLC10A1 (panel B), ABCB4 (panel C) and ABCC2 (panel D) in human liver grafts. A comparison was made between patients who developed non-anastomotic biliary strictures (NAS, dark bars) and patients who did not develop NAS (open bars). Genes of interest were standardized for 18S rRNA. In livers that later developed NAS, a significant decrease in ABCB11 mRNA expression was found immediately after transplantation, compared to pretransplant values. This decrease was not observed in livers that did not develop NAS. In both groups mRNA expression of the bile salt transporters ABCB11 and SLC10A1 increased significantly during the first week after transplantation. However, there were no significant differences between the two groups. Before: before reperfusion. After: 3 hours after reperfusion. One week: one week after liver transplantation. *) p<0.05, when compared to values before transplantation. **) p<0.05, when compared to values after reperfusion.
Figure 7. Relative CYP7A1 gene expression one week after transplantation in livers of patients who developed non anastomotic biliary strictures (NAS) and patients who did not develop NAS. CYP7A1 catalyzes the conversion of cholesterol into 7α-hydroxycholesterol and is considered to be the rate-controlling step in bile salt synthesis.

Bile Salt Pool Analysis

In a subset of 22 patients (9 NAS and 13 controls) bile salt pool composition at postoperative day 1, 2, 3 and 7 was analyzed using gas chromatography. This analysis did not reveal any significant differences between the two groups. Amounts of the various bile salts at postoperative day 3, when the difference in phospholipids / bile salt ratio between the two groups was most pronounced, are shown in Figure 5. In addition, no differences in biliary hydrophobicity, as reflected by the Heuman index, were found at any time point between the two groups.

Hepatic Expression of Bile Transporters and CYP7A1

Perioperative changes in the hepatic expression of hepatobiliary transporters are presented in Figure 6. Compared to preoperative values, mRNA levels of the bile salt transporter ABCB11 were significantly decreased at 3 hrs after reperfusion in livers that developed NAS, whereas this change was not observed in livers that did not develop NAS. In both groups, mRNA levels of the bile salt transporters ABCB11 and SLC10A1 increased significantly during the first week after transplantation. In contrast, no significant changes were observed in the hepatic expression of ABCB4, the phospholipid translocator, and ABCC2. There were no significant differences in transporter expression between the two groups at any time point.
In parallel with the low bile salt secretion, expression of CYP7A1 (the rate-controlling enzyme in de novo bile salt synthesis) at one week after transplantation was substantially lower in patients who developed NAS, compared to those who did not (Figure 7).

**Discussion**

In a prospective clinical study, we evaluated the potential role of bile composition and especially the relative contribution of bile salts and phospholipids in the development strictures of the large bile ducts, or NAS, after otherwise successful liver transplantation. Interestingly, the overall biliary secretion of bile salts, phospholipids and cholesterol during the first week after transplantation was significantly lower in patients who later developed NAS, compared to patients who did not develop NAS. The secretion of phospholipids was relatively more affected than bile salt secretion, resulting in a lower biliary phospholipids / bile salt ratio in patients who developed NAS. These findings indicate that the development of strictures of the large bile ducts is preceded by abnormal bile composition early after transplantation, several weeks before clinical symptoms of bile duct injury appear. This study supports the hypothesis that early changes in bile composition contribute to the relatively late stricturing of the large bile ducts, leading to the radiological diagnosis of NAS after transplantation.

In the current study the incidence of NAS up to one year after transplantation was 13%. This rate is similar to data reported in most previous studies (6,7,10,46) but higher than reported in some others (15,18,47). Variations in the reported incidence of NAS among different studies can be explained by differences in study design (retrospective versus prospective) and differences in the diagnostic criteria used.

Bile salts possess potent detergent properties and as such, are potentially cytotoxic (48,49). In case of relative excess of bile salts, either due to increased bile salt secretion or reduced secretion of phospholipids, micellar bile salts may cause cholangiocyte injury, pericholangitis and periductal fibrosis (50,51). In previous studies we have shown that toxic bile composition early after transplantation, characterized by a low biliary phospholipid / bile salt ratio, is associated with histological signs of injury of the small bile ducts in the liver (21,24,29). The role of bile salt toxicity in the pathogenesis of injury of the small intrahepatic bile ducts was also demonstrated in an experimental study using a liver transplant model in mice (24). Livers transplanted from Abcb4/-+ mice, which have only 50% expression of
the phospholipids translocator Abcb4, into wild-type recipients developed signs of severe injury of the small intrahepatic bile ducts within two weeks after transplantation (24). In the current study we focused on the development of NAS, which is a disease of the large bile ducts (5,52). Our results suggest for the first time that bile salt toxicity is also involved in the development of large bile duct injury, leading to the clinical and radiological diagnosis of NAS. Despite the observed low phospholipid / bile salt ratio in patients developing NAS, reflecting bile toxicity, the overall biliary secretion of bile salts in these patients was lower than in patients who did not develop NAS. This observation was not expected and introduces the intriguing possibility that, apart from relative bile salt toxicity, relative bile salt deprivation could (also) contribute to cholangiocyte injury and the development of NAS. There is substantial evidence that bile salts are potent inducers of cholangiocyte proliferation and thus bile duct repair (31,34-36). Uptake of bile salts by cholangiocytes is mediated by the transporter ASBT (SLC10A2) at the ductular membrane of these cells (30,37). In contrast to cholangiocytes of the small bile ducts, cholangiocytes in larger bile ducts do express ASBT and, therefore, these cells can re-absorb bile salts from bile (30,37). This important difference between cholangiocytes from small and large bile ducts may explain why a previous clinical study focusing on posttransplant injury of the small bile ducts did not reveal a relationship between small bile duct injury and reduced bile salt secretion. Collectively, these observations raise the possibility that the pathogenesis of biliary injury after liver transplantation is different for small and large bile ducts. In this respect it would have been interesting to study the expression of ASBT (SLC10A2) in the large bile ducts in the current study. However, it is difficult to take serial biopsies of the large bile ducts in patients and we were unable to detect ASBT (SLC10A2) mRNA expression in liver biopsies, which mainly contain small bile ducts (data not shown).

Some bile salts have a more pronounced effect on cholangiocyte proliferation than others. Taurocholate, for example, may enhance proliferation, while ursodeoxycholate may reduce the proliferative effects of other bile salts (36,53). In the current study we found no differences in the bile salt pool composition in patients who developed NAS, compared to those who did not. Therefore, we have no evidence to suggest that differences in composition of the bile salt pool are involved in the altered overall bile salt secretion or in the pathogenesis of NAS after liver transplantation.

A key question that emerges from this study is: what determines the low bile salt secretion in livers that are developing NAS? Theoretically, reduced biliary bile salt secretion can result
from a) decreased de novo synthesis, b) impaired hepatobiliary transport at the level of the canalicular membrane (ABCB11), and/or c) impaired intestinal bile salt re-absorption and fecal loss of bile salts leading to reduced bile salt pool size. In the classical pathway of de novo bile salt biosynthesis, CYP7A1 catalyzes the conversion of cholesterol into 7α-hydroxycholesterol, which is considered to be the rate-controlling step. In humans, the classical pathway accounts for approximately 80% of total bile salt synthesis (54,55). We observed a lower hepatic expression of CYP7A1 in patients who later developed NAS, compared to those who did not. It is tempting to ascribe the reduced bile salt secretion in patients who developed NAS to the lower expression of CYP7A1. Yet, three issues should be considered in this respect: a) the difference in CYP7A1 expression was striking, but it did not reach statistical significance, in contrast to the difference in bile secretion; b) no information is available on the correlation between CYP7A1 mRNA levels and actual cholate synthesis in the early post-transplant period; and c) it can be anticipated that the amount of bile salts secreted after liver transplantation is increasingly derived from re-absorbed (“conserved”) bile salts from the intestine.

To demonstrate or refute increased intestinal loss of bile salts as an explanation for the differences in biliary bile salt secretion we would have needed the collection of faeces. Although this was not performed, we have other arguments to assume that the observed differences in bile salt secretion are not caused by differences in intestinal bile salt loss. Reduced bile salt pool size due to impaired intestinal reabsorption would be expected to lead to an increased rather than a decreased hepatic CYP7A1 expression. In addition, a previous study from our centre has shown that serum bile salt concentrations increase during the first week after transplantation, which is not compatible with increased intestinal losses (56).

Hepatobiliary secretion of bile salts is an active process which, under normal circumstances, is mainly influenced by the sinusoidal transporter SLC10A1 and the canalicular transporter ABCB11. Theoretically, impaired hepatobiliary transport could have resulted form a reduced expression of these transporter proteins. Compared to pretransplant values, ABCB11 mRNA expression was decreased immediately after transplantation in livers that later developed NAS. Although this decrease was not observed in livers that did not develop NAS, there were no significant differences between the two groups either before or immediately after transplantation. In accordance with previous observations by Geuken et al. (21), we observed an increased mRNA expression of the bile salt transporters SLC10A1 and ABCB11 in both groups after transplantation, while mRNA levels of the phospholipid translocator ABCB4 did...
not change. These findings are compatible with the relatively low biliary phospholipid / bile salt ratio observed early after transplantation. However, there were no significant differences in the expression of the bile transporters between the two groups, suggesting that the observed differences in bile salt secretion cannot be explained by differences in gene transcription. Based on the current study, we cannot exclude that posttranscriptional processes or changes in transporter activity are involved. Unfortunately, we were unable to perform Western blot analyses for quantification of transporter protein levels due to the small amount of liver tissue obtained from needle biopsies.

We also examined whether differences in bile composition between patients who developed NAS and those who did not could be explained by differences in phospholipids secreted per bile salt. Therefore we additionally analyzed the biliary hydrophobicity index and the bile salt independent bile flow. There were no significant differences in the hydrophobicity index or in the bile salt independent bile flow, indicating that these factors cannot explain the observed differences between the two groups (57,58).

Several other factors have been shown to contribute to the development of NAS after liver transplantation, including long cold or warm ischemia times (7,9), inadequate washout and perfusion of the peribiliary capillary plexus (16,17), and immunological injury (19,59). In the current study we found no differences in the duration of cold and warm ischemia in livers with or without NAS. These data support previous suggestions that the pathogenesis of NAS is not only related to a direct ischemic injury of the biliary epithelium (1,12).

In summary, the results of this prospective clinical study strongly support the hypothesis that bile composition is involved in the pathogenesis of NAS after liver transplantation. Patients who developed NAS within one year after liver transplantation were initially clinically indiscernible from patients who did not develop NAS. However, bile composition in this early postoperative period was different in these two groups. Patients who developed NAS were characterized by a reduced biliary secretion of bile salts and phospholipids and a decreased biliary phospholipid / bile salt ratio. We speculate that those early defects in bile formation, possibly genetically based, play a role in the injury of the biliary epithelium of large bile ducts early after transplantation, subsequently leading to the formation of biliary strictures.
Reference List


Bile composition after liver transplantation and NAS


