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## Non-anastomotic biliary strictures after liver transplantation

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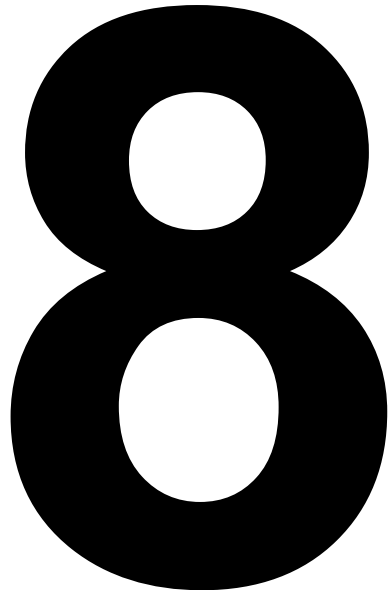
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# **Changes in cholangiocyte bile salt transporter expression and bile duct injury after orthotopic liver transplantation**

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Submitted for publication



## ABSTRACT

*Background* Biliary bile salts have been shown to contribute to bile duct injury after orthotopic liver transplantation (OLT). While cholangiocytes modify bile composition by reabsorption of bile salts (cholehepatic shunt) and contribute to bile flow by active secretion of sodium and water via cystic fibrosis transmembrane conductance regulator (CFTR), there is no formation on the expression of cholangiocyte transporters after OLT. We, therefore, examined the expression of cholangiocyte transporters in liver grafts and correlated this with the development of bile duct injury. *Methods* In 37 adult liver transplant recipients, biopsies were taken from the grafted liver: at the end of cold storage, 3hr after graft reperfusion and at one week after transplantation. Changes in the apical sodium-dependent bile salt transporter (ASBT), the organic solute transporters (OST-alpha and beta), and CFTR were assessed using real time RT-PCR and immunofluorescence staining. Gene expressions were correlated with biliary bile salt secretion as well as with the development of bile duct injury, as assessed by histology. *Results* Compared to normal controls, OST-alpha expression was significantly down-regulated before transplantation and 3hr after graft reperfusion, but levels normalized at one week after OLT. OST-beta expression was more than 8-fold up-regulated at the time of transplantation and levels increased further during the first postoperative week. There were no major changes in the expression of CFTR. Expression of OST-alpha/beta correlated with changes in biliary bile salt secretion. However, OST-alpha/beta and CFTR expression were not correlated with the development of microscopic bile duct injury after OLT. *Conclusions* Liver transplantation is associated with marked differential changes in the expression of the bile salt transporters OST-alpha and OST-beta, while CFTR expression remains stable. Although changes in OST-alpha/beta correlate with biliary bile salt secretion, OST-alpha/beta and CFTR do not correlate with the histological degree of bile duct injury, suggesting that the cholehepatic shunt does not play a major role in the development of bile salt induced bile duct injury after OLT.

## INTRODUCTION

Injury of the biliary system is an important cause of morbidity and mortality after orthotopic liver transplantation (OLT). Bile duct injury can be observed in small (microscopic) bile ducts as well as in the large (macroscopic) bile ducts of the grafted liver, and may lead to bile duct strictures or biliary fibrosis. Injury and stricturing of the larger bile ducts is usually detected by cholangiography and denoted as non-anastomotic strictures (NAS). Insight in the pathogenesis of NAS is still emerging. Various etiologic factors have been identified, including preservation-related injury, immunological injury, and injury of biliary epithelial cells (cholangiocytes) due to bile salt toxicity (1). In both experimental animal studies and clinical studies we and others have previously shown that injury of the small, microscopic, bile ducts, as well as the formation macroscopic bile duct strictures (NAS) is associated with the secretion of relatively toxic bile early after transplantation, characterized by a high bile salt to phospholipid ratio (2-4). Toxic bile salts may aggravate ischemia/reperfusion injury of the biliary epithelium, which may subsequently lead to inflammation and biliary fibrosis or bile duct strictures (5).

While hydrophobic bile salts are cytotoxic and may add to bile duct injury, there is substantial evidence that some bile salts have opposing effects and, in fact, are potent inducers of cholangiocyte proliferation and thus stimulate bile duct repair (6-9). Bile salts are actively reabsorbed from the bile by cholangiocytes, a process which is mediated by the apical sodium-dependent bile salt transporter (ASBT) at the ductular membrane of these cells (10, 11). Bile salts are subsequently excreted by cholangiocytes into the peribiliary capillary plexus via the basolateral heteromeric organic solute transporter (OST), consisting of two half transporters OST-alpha and OST-beta (12). Increased uptake of bile salts via ASBT has been linked with an increased expression of the cystic fibrosis transmembrane conductance regulator (CFTR), an anion channel permeable to  $\text{Cl}^-$  and  $\text{HCO}_3^-$ , in the ductular membrane of cholangiocytes (13, 14). Changes in the expression of CFTR may, therefore, be used a marker of bile salt uptake in cholangiocytes.

The roles of ASBT and OST-alpha/beta in bile formation are not yet fully understood, but the emerging concept is that they are involved in cholehepatic shunting of bile salts (9). The cholehepatic shunt may provide an alternative pathway to eliminate biliary bile salt toxicity and prevent further bile duct injury in cholestatic conditions (15). On

the other hand, it has been suggested to play a role in the induction of cholangiocyte proliferation and bile duct repair mechanisms by stimulating the uptake of bile salts by cholangiocytes (6, 7, 16-18). Although changes in the expression of the clinically most relevant hepatocellular bile salt transporters during and after OLT have been well described (2, 3), there is no information on possible changes in the expression of bile salt transporters in cholangiocytes.

We, therefore, aimed to study the expression of the cholangiocyte transporters ASBT, OST-alpha/beta and CFTR during and after OLT and we correlated this with changes in bile composition as well as with the development of microscopic bile duct injury.

## **PATIENTS AND METHODS**

### **Patients**

Thirty-seven adult patients undergoing a liver transplant procedure were included in this study. Surgical technique and perioperative management were as previously described by our group (19, 20). Clinical variables and laboratory data were prospectively collected in a computerized database. Missing data were obtained from the medical records where needed. None of the patients received a statin or ursodeoxycholic acid during the first week after transplantation.

### **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 transplant procedure 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 (21). 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. Bile samples were analyzed for total bile salt concentration as previously described (22). Postoperative secretion of bile salts was defined as concentration multiplied by daily bile concentration per kilogram body weight of the donor.

## 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 graft 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. Tissue and data collection and analyses were performed according to the guidelines of the medical ethical committee of our institution and the Dutch Federation of Scientific Societies.

## Assessment of gene expression

Gene expression was assessed by determining mRNA levels in liver biopsies by using real-time quantitative PCR. Isolation and reverse transcription of RNA was performed as described previously (2). TaqMan gene expression assays (PE Applied Biosystems) for OST-alpha (assay ID Hs00380895\_m1) and OST-beta (assay ID Hs00418306\_m1), ASBT (assay ID Hs01001557\_m1), CFTR (assay ID Hs00357011\_m1) and CK19 (assay ID Hs00761767\_s1) were used to quantify mRNA expression, using the ABI Prism ABI PRISM 7900 HT Sequence detector (Applied Biosystems, Foster City, CA, USA). A probe against ribosomal 18S-RNA (assay ID Hs99999901\_s1) was used as internal control.

## Immunofluorescence staining

Double immunofluorescence labeling for cytokeratin 19 with (Abcam, Cambridge, UK), OST-alpha and OST-beta (both antibodies were kindly provided by Ned Ballatori, University of Rochester School of Medicine, USA) and ASBT (kindly provided by Paul Dawson, Wake Forest University School of Medicine), was performed using cryosections and at appropriate dilutions (12). Secondary antibodies were Fluorescein Isothiocyanate and Tetramethylrhodamine-5-isothiocyanate (Alexa Fluor, Invitrogen, Breda, The Netherlands). Images were taken with a Leica QMRXA fluorescence microscope and Leica Qwin Pro 2003 software.

## Assessment of bile duct injury

The degree of bile duct injury was assessed histologically and expressed as the bile duct injury severity score (BDISS) (2). The BDISS is a semi-quantitative grading of small bile duct injury, based on the following three components: bile duct damage (graded as 0=absent, 1=mild, 2=moderate, 3=severe; modified from the Banff criteria for acute rejection (23)); ductular proliferation (graded 0–3, using a similar scale as stated above), and cholestasis (grade 0–3, using a similar scale as stated above). This resulted in a minimal BDISS of zero and a maximum score of nine points. Based on the median BDISS (five points), patients were divided into two groups: mild (BDISS<5) and severe small bile duct injury (BDISS>5). All histology examinations were performed by one experienced pathologist who was unaware of the other study data.

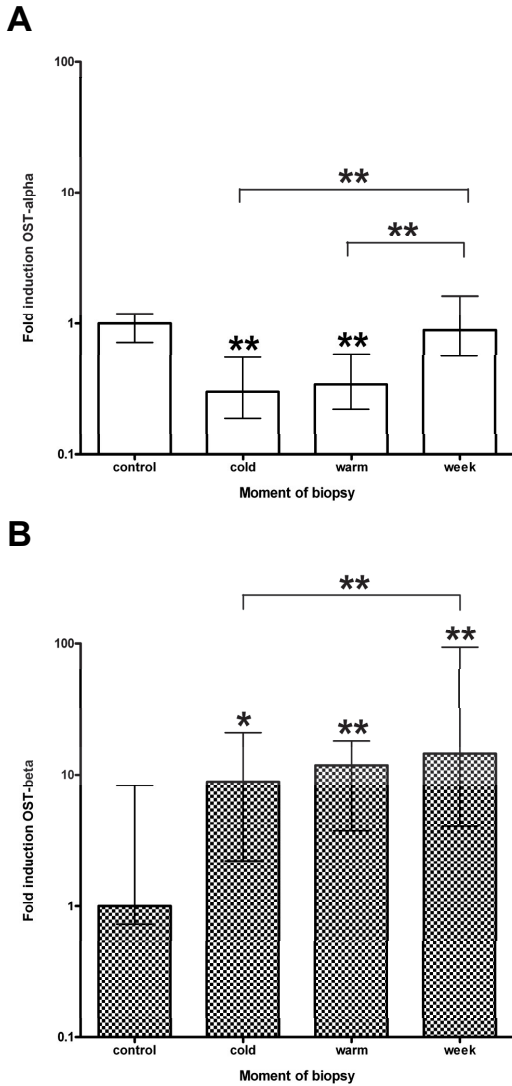
## Statistical methods

Data was analyzed using SPSS software version 16.0 for Windows. Categorical variables were presented as numbers with percentages and compared using Pearson's chi-square test. Continuous variables were presented as medians with interquartile range (IQR) and compared using the Mann-Whitney *U* test. For correlation analyses the Pearson test was used. All *P*-values were considered as statistically significant at a level of less than 0.05.

## RESULTS

### Changes in OST-alpha, OST-beta and ASBT mRNA expression in OLT

To determine the effect of cold preservation and ischemia/reperfusion injury on gene expression of the cholangiocyte bile salt transporters, we measured mRNA expression for OST-alpha, OST-beta and ASBT in the three sequential liver graft biopsies. Compared to normal control samples, OST-alpha mRNA expression was significantly down-regulated before transplantation and after graft reperfusion, but expression levels restored to normal values at one week after OLT (Figure 1A). OST-beta mRNA expression was more than 8-fold up-regulated at the end of cold preservation and after graft reperfusion, and expression levels increased further during the first week after OLT (Figure 1B). We were unable to detect ASBT mRNA transcript levels in any of the liver biopsies.



**Figure 1**

Relative transcript levels of OST-alpha and OST-beta in liver transplant biopsies. Levels of mRNA coding for OST-alpha (A) and OST-beta (B) were determined by RT-PCR in three sequential liver biopsies: at the end of cold preservation (cold) , 3hr after graft reperfusion (warm) and at one week after liver transplantation. The numbers of transcripts were normalized to control levels and compared using the Mann-Whitney U test. Data shown are medians and IQR. \* P < 0.05; \*\* P < 0.01.

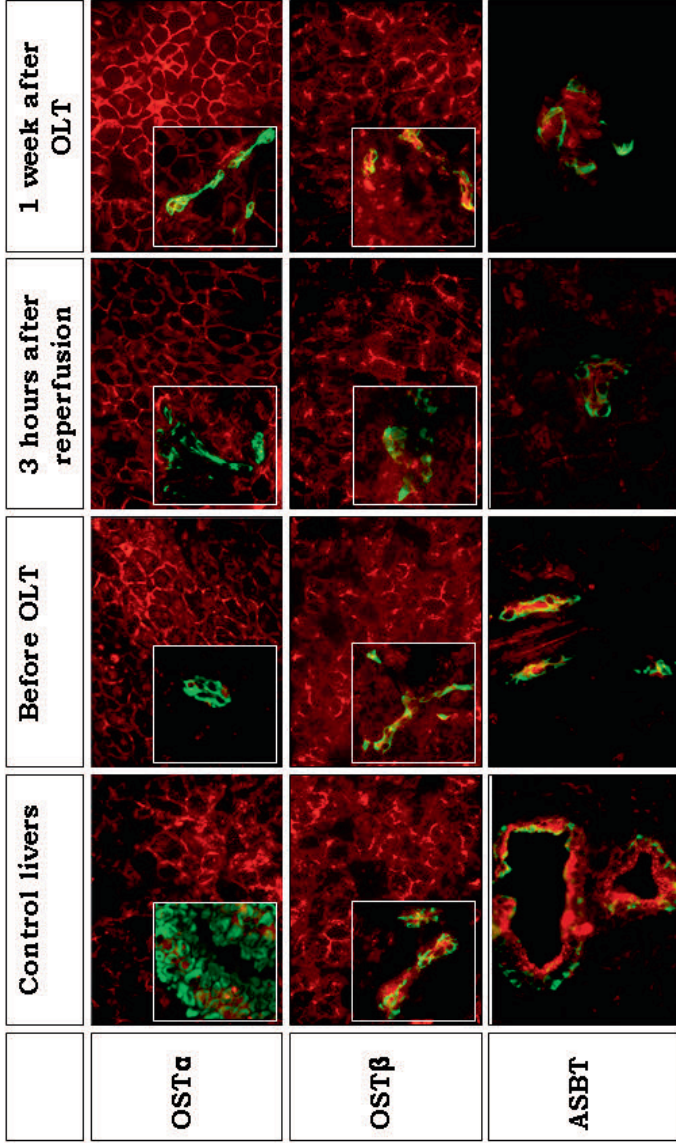


## **Protein expression and cellular localisation of OST-alpha, OST-beta and ASBT**

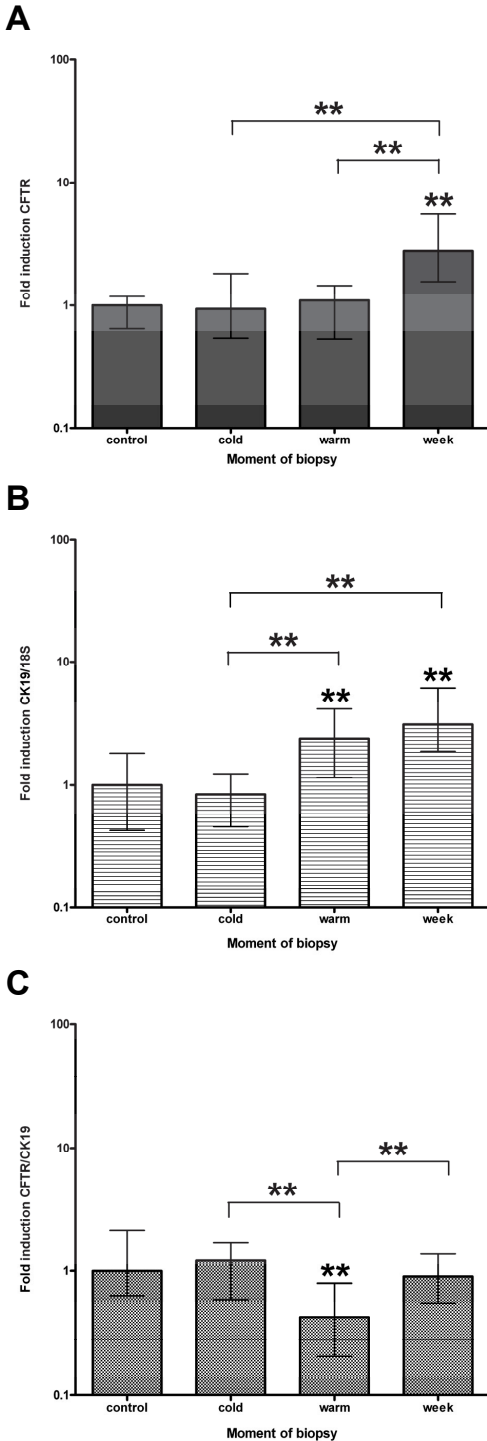
To determine whether the observed changes in mRNA expression were paralleled by changes in protein expression and to determine the cellular localization of OST-alpha, OST-beta and ASBT in liver graft biopsies, we performed immunofluorescence staining for these bile salt transporters. Co-staining for CK19 was used as a marker for cholangiocytes. Overall, the immunofluorescence staining pattern was similar to that of mRNA expression for OST-alpha and OST-beta (Figure 2). OST-alpha and OST-beta protein expression could be detected in the basolateral membranes of both cholangiocytes and hepatocytes. Immunostaining of OST-alpha was reduced at the time of transplantation, but recovered during the first postoperative week. OST-beta immunostaining was enhanced in all three sequential biopsies. In contrast to the undetectable ASBT mRNA transcripts, ASBT was detected by immunostaining in the apical membranes of cholangiocytes.

## **Changes in CFTR mRNA expression during and after OLT**

At the time of transplantation CFTR mRNA expression was similar to that in normal control biopsies, but levels were 2-fold higher at one week after OLT (Figure 3A). To assess whether the increased levels of CFTR mRNA were a result of bile duct proliferation or rather caused by an increased expression for CFTR mRNA per cell, CFTR mRNA expression was correlated with CK19 mRNA expression levels. Expression of CK19 appeared to be significantly up-regulated at one week after OLT, compared to levels in pretransplant biopsies (Figure 3B). When levels of CFTR mRNA at one week after OLT were corrected for changes in CK19 mRNA levels, no major changes were observed, suggesting that the observed increase in CFTR mRNA was caused by ductular proliferation rather than increased expression per cholangiocyte (Figure 3C).



**Figure 2** Immunofluorescence staining with CK19 co-labeling of OST-alpha, OST-beta and ASBT. Pictures shown are representative for 3 individual liver slide preparations. OST-alpha and OST-beta were equally present in the basolateral membranes of cholangiocytes and hepatocytes. OST-alpha protein expression seems to be reduced during cold and warm preservation and recovered after one week. OST-beta is induced on protein level as well, during cold and warm preservation, and one week after OLT. ASBT was only revealed in cholangiocytes, though did not reveal clear differences in expression.



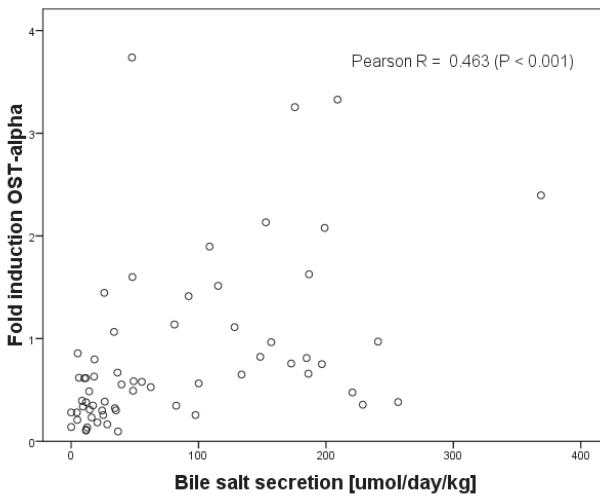
**Figure 3**

Relative transcript levels of CFTR, CK19 and CFTR normalized for CK19. Levels mRNA coding for CFTR and CK19 were quantified by RT-PCR in three sequential liver biopsies: at the end of cold preservation (cold), 3hr after graft reperfusion (warm) and at one week after liver transplantation. The numbers of transcripts were normalized to control levels and compared using the Mann-Whitney U test. Data shown are medians and IQR. \*\* P < 0.01. (A) Uncorrected CFTR mRNA expression levels in the three sequential biopsies. (B) mRNA expression of the cholangiocyte marker CK19 in the three sequential liver biopsies. (C) CFTR mRNA expression corrected for CK19 mRNA expression.

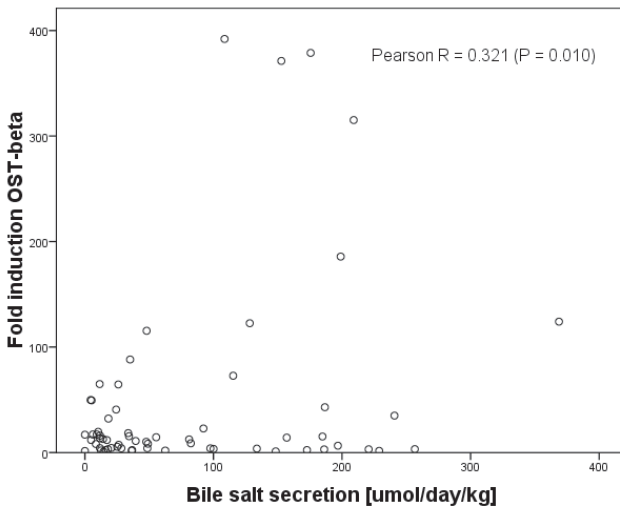
## Correlation between OST-alpha/beta and CFTR expression and bile salt secretion

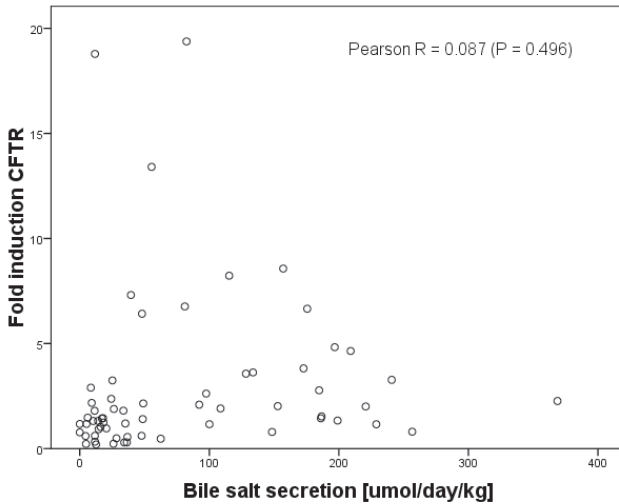
To determine whether OST-alpha, OST-beta or CFTR gene expression was related to the biliary bile salt secretion, we correlated OST-alpha, OST-beta and CFTR mRNA levels with biliary bile salt secretion at one week after OLT. OST-alpha/beta mRNA levels correlated significantly with the biliary bile salt secretion one week after OLT. There was no correlation between CFTR mRNA levels and the biliary bile salt secretion one week after OLT (Figure 4).

A



B



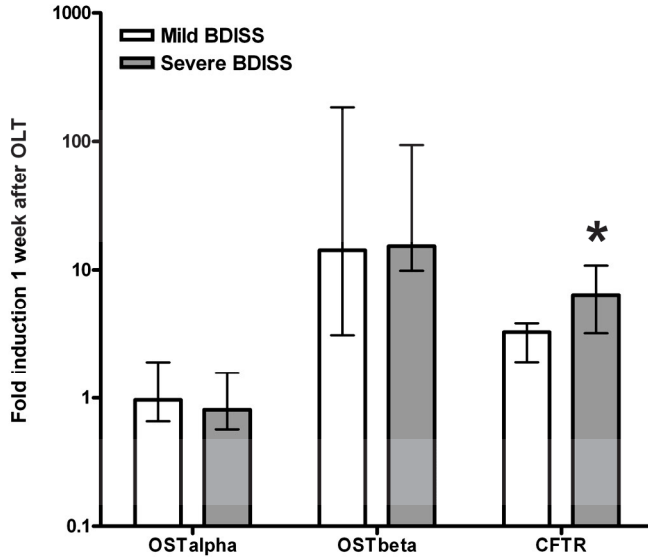
**C****Figure 4**

Correlation between OST-alpha, OST-beta and CFTR, and the biliary bile salt secretion one week after OLT. OST-alpha/beta mRNA levels correlated significantly with the biliary bile salt secretion one week after OLT. There was no correlation between CFTR mRNA levels and the biliary bile salt secretion one week after OLT.

## Expression of cholangiocyte transporters in relation to the degree of bile duct injury

We next examined whether there were differences in OST-alpha, OST-beta or CFTR gene expression in liver grafts with mild or moderate/severe small bile duct injury, as determined by the BDISS. For this purpose, patients were divided in two groups, based on the BDISS at one week after OLT. Mild small bile duct injury (BDISS <5) was observed in 15 of the 37 (40.5%) patients whereas 9 (24.3%) patients showed severe small bile duct injury (BDISS ≥5). Comparing OST-alpha or OST-beta mRNA levels in these two groups did not reveal significant differences. CFTR mRNA expression was significantly higher in liver grafts with signs of severe small bile duct injury, compared to livers with only mild injury (Figure 5). However, when CFTR mRNA expression levels were normalized for CK19 expression these differences disappeared, suggesting that the observed association between overall CFTR mRNA expression and the BDISS

was caused by an increased number of biliary epithelial cells (ductular proliferation) rather than up-regulation of CFTR gene expression per cell.



**Figure 5**

Relative transcript levels of OST-alpha, OST-beta and CFTR at one week after transplantation in patients with mild and severe bile duct injury, as detected microscopically by the bile duct injury severity score (BDISS). The numbers of transcripts were normalized to control levels and compared using the Mann-Whitney U test. Data shown are medians with the IQR. \*  $P < 0.05$ . There were no differences OST-alpha mRNA and OST-beta MRNA levels between patients with mild (BDISS  $< 5$ ) and severe bile duct injury (BDISS  $\geq 5$ ). Although uncorrected CFTR mRNA levels were significantly higher in patients with signs of severe bile duct injury one week after OLT, this difference disappeared when values were corrected for CK19 expression, suggesting that the observed association between overall CFTR mRNA expression and the BDISS was caused by an increased number of biliary epithelial cells (ductular proliferation) rather than up-regulation of CFTR gene expression per cell.

## DISCUSSION

The aims of this study were to examine changes in the expression of the cholangiocyte transporters ASBT, OST-alpha, OST-beta and CFTR during and after OLT and correlate these with changes in bile composition as well as with the development of bile duct injury. We have observed remarkable changes in the expression of OST-beta and OST-alpha during and early after OLT. While OST-beta mRNA expression was 8-fold up-regulated in donor livers during and after transplantation, compared to normal controls, OST-alpha mRNA expression decreased significantly at 3 hrs after reperfusion, but levels restored to normal by the end of the first postoperative week. CFTR mRNA expression in liver grafts at the time of transplantation was not different from that in controls, but levels increased 2-fold by the end of the first postoperative week. This 2-fold increase, however, disappeared when values were normalized for CK19 expression, suggesting that the observed increase was mainly due to an increase in the number of biliary epithelial cells (ductular proliferation) rather than an increased gene expression per cell. Despite the changes in the expression of OST-alpha and OST-beta correlated with the bile salt secretion, expression for these transporters did not correlate with the histological detection of bile duct injury, suggesting that changes in the cholehepatic shunt during OLT do not play a major role in the pathogenesis of biliary epithelial cell injury in liver transplants.

Interestingly, we observed a strong differential expression of the two subunits of the heteromeric bile salt transporter OST-alpha/beta in liver transplants. While OST-beta mRNA expression was up to 8-fold increased, OST-alpha expression was reduced by half at 3hr after graft reperfusion. OST-alpha and OST-beta genes are reported to be regulated by bile acids via the farnesoid X receptor (FXR) and liver X receptor-alpha (LXR-alpha) (24-26). Using a model of precision cut human liver slices, Khan et al. have recently also described large variations in the regulation of the OST-alpha and OST-beta genes (27). Especially ligands for FXR, such as chenodeoxycholic acid and lithocholic acid, exerted differential effects on OST-alpha and OST-beta gene expression. Others have found evidence that OST-beta regulation is indeed more sensitive to bile salts than OST-alpha (28, 29). Induction of OST-beta is thought to be anti-cholestatic, by acting as an adaptive bile salt overflow system, thereby increasing the hepatocholangiocyte bile salt flux and reducing the accumulation of intracellular bile salts (28, 30). In parallel with the observed regulatory effects of bile salts on OST-

alpha/beta expression in rodent and *in vitro* models, we found a correlation between OST-alpha/beta mRNA expression and biliary bile salt secretion at one week after transplantation. Although the large changes in OST-alpha and OST-beta expression in liver transplants observed in the current study suggest that these genes may be differentially regulated by oxidative stress or inflammatory mediators (either in the donor or in the recipient), this concept is not supported by a study performed by Wagner et al., who found no evidence for such regulatory mechanisms of OST-alpha/beta gene expression in a model of common bile duct ligation in mice (31). Immunofluorescence staining for OST-alpha and beta revealed similar changes in protein expression, but also showed that in the human liver these transporters are not solely expressed in cholangiocytes, but can also be detected in hepatocytes. These findings are in line with data obtained from immunofluorescence staining in cell culture experiments performed by Ballatori et al., who showed an equal expression of OST-alpha and OST-beta in human cholangiocytes and hepatocytes (12). Therefore, it cannot be fully deduced from the current study whether the observed changes in OST-alpha/beta mRNA expression are a result of changes in the expression in hepatocytes, in cholangiocytes, or in both cell types. Determination of changes in gene expression within one cell type would require additional sophisticated laboratory techniques, such as laser capture microdissection and determination of mRNA levels in one cell type. Future experiments should include this method to discriminate between changes in gene expression in hepatocytes and cholangiocytes.

Similar to OST-beta, expression of the bile salt transporter ASBT in apical membrane of cholangiocytes has been shown to be regulated in direct proportion to biliary bile salt concentration (16, 17, 32). Unfortunately, we were unable to detect ASBT mRNA transcripts in our liver biopsies. This may be explained by the fact that livers biopsies contain mainly bile ductules (< 15 nm) and small intralobular bile ducts (15–100 nm), which are lined by small cholangiocytes (33). In contrast to large cholangiocytes lining the larger or macroscopic bile ducts, small cholangiocytes do normally not express ASBT. Only during bile duct proliferation expression of ASBT has been observed in small cholangiocytes (16). Interestingly, we were able to detect ASBT protein expression by immunostaining at all three time points, which leaves open the possibility that technical factors may explain the lack of ASBT mRNA detection.



In contrast to ASBT transcripts, CFTR was actually expressed in small bile duct, unlike reports that CFTR is expressed only in cholangiocytes of larger bile ducts (34). CFTR acts in concerted action with ASBT and was significantly induced one week after OLT, primarily due to bile duct proliferation. Indirect, this may indicate that the bile salt reabsorption is increased after OLT. Bile salt absorption triggers CFTR activation and consequently, local salt and water secretion, which may serve to prevent obstruction (14). Nevertheless, there was no significant difference in CFTR expression between livers with a high versus a low DBISS.

In both experimental animal studies and clinical studies we and others have previously shown that injury of the small, microscopic, bile ducts, as well as the formation macroscopic NAS is associated with the secretion of relatively toxic bile early after transplantation, characterized by a high bile salt/phospholipid ratio (2, 3). While changes in bile composition and the subsequent development of biliary injury have been associated with changes in biliary transporter proteins in hepatocytes (2, 3), we could not find any correlation between changes in OST-alpha/beta or CFTR and bile duct injury as measured by the BDISS. These data suggest that the cholehepatic shunt or cholangiocyte mediated changes in bile composition do not play a major role in the development of bile duct injury after OLT. In conclusion, transplantation of the liver is associated with marked differential changes in the expression of the bile salt transporters OST-alpha and OST-beta, while CFTR expression remains rather stable. Although changes in OST-alpha/beta correlated with the biliary bile salt secretion, these biliary bile salt transporters did not correlate with the histological degree of bile duct injury, suggesting that the cholehepatic shunt does not play a major role in the development of bile salt induced bile duct injury after OLT.

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