

## LIVER DISEASE

## Adaptive regulation of the ileal apical sodium dependent bile acid transporter (ASBT) in patients with obstructive cholestasis

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**Background/aims:** The apical sodium dependent bile acid transporter ASBT (SLC10A2) contributes substantially to the enterohepatic circulation of bile acids by their reabsorption from the intestine. In the rat, its adaptive regulation was observed in the kidneys, cholangiocytes, and terminal ileum after bile duct ligation. Whether adaptive regulation of the human intestinal ASBT exists during obstructive cholestasis is not known.

**Methods:** Human ASBT mRNA expression along the intestinal tract was analysed by real time polymerase chain reaction in biopsies of 14 control subjects undergoing both gastroscopy and colonoscopy. Their duodenal ASBT mRNA expression was compared with 20 patients with obstructive cholestasis. Additionally, in four patients with obstructive cholestasis, duodenal ASBT mRNA expression was measured after reconstitution of bile flow.

**Results:** Normalised ASBT expression in control subjects was highest (mean arbitrary units (SEM)) in the terminal ileum (1010 (330)). Low ASBT expression was found in colonic segments (8.3 (5), 4.9 (0.9), 4.8 (1.7), and 1.1 (0.2) in the ascending, transverse, descending, and sigmoid colon, respectively). Duodenal ASBT expression in control subjects (171.8 (20.3)) was found to be approximately fourfold higher compared with patients with obstructive cholestasis (37.9 (6.5);  $p < 0.0001$ ). Individual ASBT mRNA expression was inversely correlated with bile acid and bilirubin plasma concentrations. In four cholestatic patients, average ASBT mRNA increased from 76 (18) before to 113 (18) after relief of cholestasis (NS). Immunohistochemical assessment indicated that ASBT protein was expressed on the apical surface of duodenal epithelial cells.

**Conclusion:** Obstructive cholestasis in humans leads to downregulation of ASBT mRNA expression in the distal part of the human duodenum.

Bile acids are amphipathic steroidal compounds derived from the enzymatic catabolism of cholesterol in the liver by cytochrome P450 isoform 7A1 (CYP7A1). In the small intestine, bile acids emulsify dietary fats and lipid soluble vitamins. They participate in the regulation of pancreatic secretion and release of gastrointestinal peptides.<sup>1</sup> Through the coordinated action of several transport proteins expressed in hepatocytes, cholangiocytes, as well as in enterocytes and in proximal tubular cells of the kidney, an efficient enterohepatic circulation of bile acids is maintained.<sup>2</sup> One of these transport proteins, the apical sodium dependent bile acid transporter ASBT (SLC10A2) has been detected in the ileum, caecum, and kidney<sup>3</sup> and mediates the uptake of bile acids from the lumen of the intestine, from renal tubules, and from cholangiocytes. ASBT is a 348 amino acid protein that transports conjugated and unconjugated bile acids with a high efficiency.<sup>3</sup> Subjects with mutations in the ASBT gene suffer from congenital diarrhoea and steatorrhoea due to bile acid malabsorption.<sup>4</sup> Pharmacological inhibition of ASBT leads to interruption of enterohepatic circulation of bile acids with changes in cholesterol and bile acid homeostasis.<sup>5-9</sup> Adaptive induction of asbt mRNA expression, transporter protein, and transport activity was observed in cholic acid fed rats<sup>10</sup> whereas decreased asbt expression was found in the ileum of bile duct ligated rats.<sup>11</sup> In addition, in Wistar rats, a marked reduction in asbt protein expression was observed in microsomal membrane fractions from whole kidney after ligation of the common bile duct (CBDL) with consequent

increased urinary bile acid excretion. These results indicate that asbt is adaptively regulated in different tissues during obstructive cholestasis in the rat.

Lanzini and colleagues<sup>12</sup> studied the effects of cholestasis on intestinal bile acid transport in 14 subjects with chronic cholestasis due to primary biliary cirrhosis before and during ursodeoxycholic acid (UDCA) administration. Prolonged retention of the bile acid analogue <sup>75</sup>Se-homocholeic acid taurine (<sup>75</sup>SeHCAT) in patients with primary biliary cirrhosis was observed compared with healthy controls and patients with Crohn's disease. Retention of <sup>75</sup>SeHCAT decreased with UDCA treatment, inferring that luminal bile acid levels may be involved in the regulation of ASBT gene regulation.

Virtually all bile salt transporter systems are subject to extensive regulation, mainly at the level of gene transcription. These regulatory mechanisms represent adaptive responses to intracellular accumulation of bile salts and other amphipathic molecules. However, little is known about adaptive regulation of ASBT expression in humans. The aim of this study was therefore to investigate expression of ASBT in the duodenum of healthy subjects and compare the results with patients with obstructive cholestasis. Because direct analysis of ASBT gene expression in samples from the

**Abbreviations:** ASBT, apical sodium dependent bile acid transporter; CBDL, common bile duct ligation; UDCA, ursodeoxycholic acid; <sup>75</sup>SeHCAT, <sup>75</sup>Se-homocholeic acid taurine; MRP, multidrug resistance associated protein; ERCP, endoscopic retrograde cholangiopancreatography; PPAR $\alpha$ , peroxisome proliferator activated receptor  $\alpha$

terminal ileum cannot be performed in humans with obstructive cholestasis for obvious ethical reasons, in the first part of this study we analysed ASBT mRNA expression in healthy subjects in different segments of the human intestine. We found that ASBT mRNA was also expressed in the duodenum but to a lesser extent than in the terminal ileum.

Additionally, we analysed gene expression of multidrug resistance associated protein 3 (MRP3), which is expressed on the basolateral membrane of the enterocyte<sup>13</sup> and which is capable of transporting bile salts,<sup>14</sup> as well as expression of multidrug resistance associated protein 2 (MRP2). Human duodenal MRP2 protein expression was downregulated during cholestasis.<sup>15</sup> Biopsies of the duodenum of patients with obstructive cholestasis were obtained during therapeutic endoscopic retrograde cholangiopancreatography (ERCP).

## PATIENTS AND METHODS

### Patients

Fourteen healthy subjects (seven males, seven females) were enrolled into the first part of the study after giving informed consent. The indication for combined upper and lower gastrointestinal tract endoscopy was a cancer screening programme. Biopsies were obtained from the duodenum, terminal ileum, and from different defined regions of the colon. In the second part of the study, 20 cholestatic patients were enrolled after giving written informed consent. Biopsies were obtained from the duodenum in these patients. Of 10 patients with obstructive tumours, four had carcinomas of the pancreatic head, four had cholangiocarcinomas (Klatskin tumour), and two had metastatic diseases. Ten patients had benign diseases (eight patients with choledocholithiasis and two patients with a benign stenosis of the common bile duct). Obstructive jaundice was defined: (1) on the basis of biochemical parameters (bilirubin,  $\gamma$ -glutamyltransferase, and alkaline phosphatase) and (2) on imaging procedures (ultrasound and ERCP), demonstrating a dilated bile duct system. All control patients had normal values of the above parameters. Patients were only included if they were not taking any medication known to affect ASBT, MRP2, or MRP3 expression. Demographic details are given in table 1.

### Materials

All chemicals were of the highest quality available and were obtained from commercial sources.

### Real time PCR analysis of human ASBT, MRP2, MRP3, PPAR- $\alpha$ , and villin (VIL1) mRNA (TaqMan assay)

Using standard pinch forceps, four intestinal biopsies were taken from each anatomical site (the distal part of the duodenum, terminal ileum, and different segments of the colon) during an interventional ERCP procedure (patients with obstructive cholestasis) or during gastroscopy and colonoscopy (control subjects). Biopsies were immediately submerged in a tube with RNAlater (Ambion) and stored at

-80°C until further processing. For RNA isolation, two biopsies from each donor were homogenised for 30 seconds (Polytron PT 2100; Kinematika AG, Switzerland) and RNA was extracted using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. RNA was quantified with a GeneQuant photometer (Pharmacia, Uppsala, Sweden). After DNase I digestion (Gibco Life Technologies, Basel, Switzerland) 1.5  $\mu$ g of total RNA were reverse transcribed by Superscript (Gibco Life Technologies) according to the manufacturer's protocol, using random hexamers as primers.

TaqMan analysis was carried out on a 7900HT sequence detection system (Applied Biosystems, Rotkreuz, Switzerland). Polymerase chain reaction (PCR) cycling conditions were 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and one minute at 60°C. Each TaqMan reaction contained 10 ng of cDNA in a total volume of 10  $\mu$ l. TaqMan Universal PCR Mastermix from Applied Biosystems was used. Concentrations of primers and probes were 900 nM and 225 nM, respectively. They were designed according to the guidelines of Applied Biosystems with help of the Primer Express 2.0 software (corresponding sequences are listed in table 2). Primers were synthesised by Invitrogen (Basel, Switzerland) and probes by Eurogentec (Seraing, Belgium). All samples were run in triplicate. TaqMan PCR products were confirmed by sequencing (Microsynth GmbH, Balgach, Switzerland). Non-reverse transcribed RNA served as a negative control. No significant amplification was observed in these samples.

For each sample, the number of gene transcripts (ASBT, MRP2, MRP3, peroxisome proliferator activated receptor  $\alpha$  (PPAR $\alpha$ )) and the number of villin transcripts were determined. By calculating the ratio of gene/villin mRNA, gene expression was normalised to enterocyte content. Determination of villin, an enterocyte specific, constitutively expressed protein, can be used to control for the variation in enterocyte content in biopsies.<sup>16, 17</sup> For absolute quantification we used external standard curves. Standards were gene specific cDNA fragments that cover the Taqman primer/probe area and were obtained by PCR amplification. For ASBT, MRP2, and MRP3, we used cDNA from Caco-2 cells as a template whereas for PPAR $\alpha$ , cDNA from duodenal tissue was used. Gene specific PCRs were performed with components from Applied Biosystems (AmpliQ Gold; 10 $\times$  PCR buffer, dNTPs, MgCl<sub>2</sub>). Each reaction contained 25 ng of cDNA and 300 nM of each primer (corresponding sequences are listed in table 3) in a total volume of 25  $\mu$ l. Thermal cycling was conducted using a Mastercycler personal from Eppendorf (Hamburg, Germany). All PCR products were purified by running a 1.5% agarose gel and a subsequent gel extraction (gel extraction kit; Qiagen). Standards were quantified using the PicoGreen reagent (Molecular Probes, Eugene, Oregon, USA) and were checked by sequencing (Microsynth GmbH, Balgach, Switzerland).

Standard curves were generated by plotting the obtained Ct values (cycle number when a sample reaches the threshold)

**Table 1** Patient characteristics of the two study groups

| Characteristic            | Icteric patients (n = 20) | Controls (n = 14) | p Value |
|---------------------------|---------------------------|-------------------|---------|
| Age (y)*                  | 67.4 (2.8)                | 59.8 (2.7)        | NS      |
| BMI (kg/m <sup>2</sup> )* | 24.1 (0.8)                | 28.4 (1.6)        | 0.015   |
| Sex (M/F)                 | 10/10                     | 7/7               | NS      |
| Diagnosis                 | 10 T/10 BS                | 3 G/3 O/8 U       |         |
| Bilirubin ( $\mu$ mol/l)  | 235 (42–640)              | 11 (5–17)         | <0.001  |
| Bile acids ( $\mu$ mol/l) | 122.6 (20.5–401)          | 1.7 (0.5–3.2)     | <0.001  |

Values are mean (SEM).

BMI, body mass index; T, tumour; BS, biliary stone; G, mild gastritis; E, mild oesophagitis; U, macroscopically unaffected mucosa.

**Table 2** Primers and probes for TaqMan analysis

| Gene           | Primer         | Sequence                             |
|----------------|----------------|--------------------------------------|
| Villin (VIL1)  | Forward primer | 5'-CATGAGCCATGCGCTGAAC-3'            |
|                | Probe          | 5'-TCATCAAAGCCAAAGCAGTACCCACCAAG-3'  |
|                | Reverse primer | 5'-TCATTCTGCACCTCCACCTGT-3'          |
| ASBT (SLC10A2) | Forward primer | 5'-ACGCAGCTATGTTCCACCATC-3'          |
|                | Probe          | 5'-TTCAGCTCTCCTTCACTCCTGAGGAGCTC-3'  |
|                | Reverse primer | 5'-GCGGGAAGGTGAATACGACA-3'           |
| MRP2 (ABCC2)   | Forward primer | 5'-ACTGTGGCTTGTCTGTCCA-3'            |
|                | Probe          | 5'-CTCAATATCACACAAACCCTGAACTGGCTG-3' |
|                | Reverse primer | 5'-CAACAGCCACAATGTTGGTCTCTA-3'       |
| MRP3 (ABCC3)   | Forward primer | 5'-GGTGGATGCCAACAGAGAA-3'            |
|                | Probe          | 5'-CCAACCGGTGGCTGAGCATCG-3'          |
|                | Reverse primer | 5'-GCAGTCCCACGAACTCC-3'              |
| PPAR $\alpha$  | Forward primer | 5'-CATTACGGAGTCCACGGCT-3'            |
|                | Probe          | 5'-AGGCTGCAAGGGCTTCTTCGGC-3'         |
|                | Reverse primer | 5'-ACACCAGCTTGAGTCGAATCG-3'          |

ASBT, apical sodium dependent bile acid transporter; MRP, multidrug resistance associated protein; PPAR $\alpha$ , peroxisome proliferator activated receptor  $\alpha$ .

as a function of the log(10) of the corresponding dilution factor and using linear regression. Curves were linear with correlation coefficients of 0.996, 0.998, 0.999, 0.997, and 0.998 for ASBT, villin, MRP2, MRP3, and PPAR $\alpha$ , respectively. The corresponding PCR efficiencies (calculated as  $10^{(1/-\text{slope})}-1$ ) were 93%, 95.5%, 89.1%, 95%, and 97.1%, respectively. Each standard curve also included no template controls.

The sensitivity of all TaqMan assays was found to be adequate for quantifying all transcripts in the analysed intestinal tissues. Only in samples from the sigmoid colon did expression of ASBT mRNA appear to be so low that accurate quantification of transcript numbers was not possible for all biopsies. The precision of the ASBT determination was assessed by measuring 10 aliquots of duodenal cDNA (each in triplicates). The intra-assay coefficient of variation (CV) was 4.69%. Interassay CV was not determined as for every gene all samples could be placed onto one 384 well plate. Accuracy of the method was determined by measuring a standard with a known amount of template (n=10 in triplicates). Deviation of the mean value from the true value was 14.85%.

### Immunohistochemical assessment

For immunohistochemical assessment of ASBT expression in human intestinal tissue, a polyclonal rabbit antihuman ASBT (generous gift from Dr PA Dawson, Wake Forest University Baptist Medical Center, Winston-Salem, North Carolina, USA) was used. This antibody was raised against the carboxyl terminal 39 amino acids of human ASBT that was expressed as a glutathione S-transferase-ASBT fusion protein. The human ASBT antibody has been previously used to measure ASBT protein expression in human ileal biopsies.<sup>18</sup>

Human intestinal tissue was mounted in OCT compound (Sakura Finetek, Zooterwoude, the Netherlands), frozen in liquid nitrogen, and stored at  $-70^{\circ}\text{C}$ . Sections (5  $\mu\text{m}$ ) of human intestine were air dried overnight and a periodate-lysine-paraformaldehyde solution (3%) was used for post fixation. Then sections were washed with washing solution (TBS/NaCl, Tween 0.05%) and incubated with goat serum (Vectastain, Elite kit; Vector Laboratories, Burlingame, California, USA) for 30 minutes at room temperature as blocking solution. Tissue sections were incubated with a 1:400 dilution of the polyclonal rabbit antihuman ASBT overnight at  $4^{\circ}\text{C}$ . Samples were washed three times with washing solution and incubated with the goat antirabbit IgG secondary antibody for 30 minutes at room temperature. After three washes with the washing solution, a perhydryal solution ( $\text{H}_2\text{O}_2$  (0.3%), sodium azide (0.1%) in phosphate buffered saline) was used to destroy endogenous peroxidase activity. Staining was performed with the avidin/biotinylated enzyme complex (ABC method) according to the manufacturer's instructions (Vectastain, Elite kit; Vector Laboratories). For detection, 3-amino-9-ethylcarbazole, which forms a red end product, was used (Biogenex, San Ramon, California, USA). Sections, which served as negative controls, were incubated only with the goat antirabbit IgG secondary antibody. Biopsies from normal terminal ileum were used as a positive control.

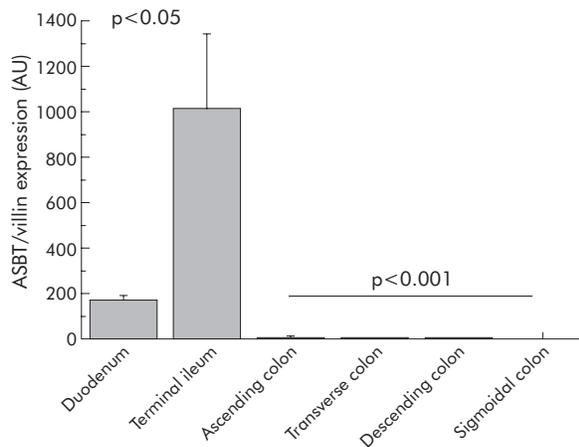
### Bilirubin and bile acid plasma concentrations

Blood samples from subjects were obtained shortly before the endoscopic procedure. Bilirubin plasma concentrations were measured by a modified Malloy-Evelyn method (BIL-T; Roche Diagnostics, Mannheim, Germany). Fasting plasma levels of bile acids were determined as described previously.<sup>19</sup>

**Table 3** Primers for cDNA standards

| Gene           | Primer         | Sequence                         |
|----------------|----------------|----------------------------------|
| Villin (VIL1)  | Forward primer | 5'-AGAAAGCCAATGAGCAGGAGAA-3'     |
|                | Reverse primer | 5'-ATGGATGTGGCATCGAACTTC-3'      |
| ASBT (SLC10A2) | Forward primer | 5'-CATCTCTGGTTGCTCTCGTTGTTTC-3'  |
|                | Reverse primer | 5'-TGATGTCTACTTTTCGTCAGGTTGAA-3' |
| MRP2 (ABCC2)   | Forward primer | 5'-CCAATCTACTCTCACTTCAGCGAGA-3'  |
|                | Reverse primer | 5'-AGATCCAGCTCAGGTGCGTACC-3'     |
| MRP3 (ABCC3)   | Forward primer | 5'-TCTATGCAGCCACATCACGG-3'       |
|                | Reverse primer | 5'-GTCACCTGCAAGGAGTAGGACAC-3'    |
| PPAR $\alpha$  | Forward primer | 5'-AGGAAGCTGTCCTGGCTCAG-3'       |
|                | Reverse primer | 5'-CGTCCAAAACGAATCGCG-3'         |

ASBT, apical sodium dependent bile acid transporter; MRP, multidrug resistance associated protein; PPAR $\alpha$ , peroxisome proliferator activated receptor  $\alpha$ .

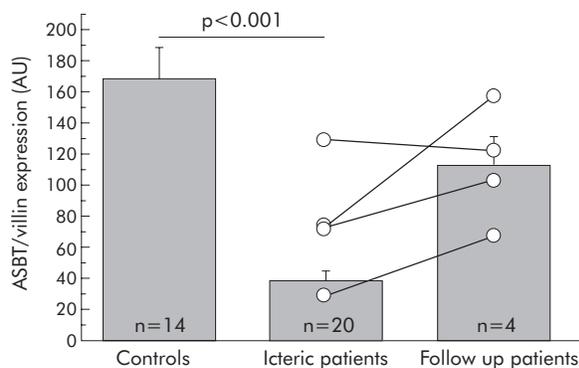


**Figure 1** mRNA expression of apical sodium dependent bile acid transporter (ASBT) in different gut segments. Data represent the means (SEM) of biopsies from 14 healthy subjects. AU, arbitrary units.

In brief, bile acids were extracted with Bond-Elut C18 cartridges (Analytichem International, San Diego, California, USA), solvolysis was performed to cleave sulphate groups, and enzymatic hydrolysis was performed to deconjugate bile acid amidates. Deconjugated bile acids were isolated by extraction on Lipidex 1000 (Packard Instruments, Groningen, the Netherlands) and were then methylated and trimethylsilylated for gas chromatography. Capillary gas chromatography was performed using a Carlo Erba Fractovap 4160 gas chromatograph (Carlo Erba Instruments, Hofheim, Germany). Bile acid derivatives were separated on a fused silica capillary CP Sil 19 CB column coated with chemically bonded OV-1701 (25 m × 0.33 mm; Chrompack, Middelburg, the Netherlands). Hydrogen was the carrier gas ( $P = 0.6 \text{ kg/cm}^2$ ). A temperature programme from 140°C to 270°C with 8°/min was started after on-column injection. Eluted bile acid derivatives were detected by a flame ionisation detector. Fasting plasma samples were stored at -20°C until analysed.

### Statistics

All values are expressed as means (SEM). The impact of different parameters (bilirubin and bile acid concentration, body mass index, age, and sex) on the variability of ASBT expression was investigated by multilinear regression analysis. ASBT expression in icteric patients was compared with



**Figure 2** Comparison of apical sodium dependent bile acid transporter (ASBT) mRNA expression in patients with obstructive cholestasis (n = 20) compared with control subjects (n = 14) and follow up patients (n = 4). Data are means (SEM). In the scatterplot, lines connect individual ASBT mRNA expression pre- and post reconstitution of bile flow in four follow up patients. AU, arbitrary units.

that of healthy controls by analysis of variance (ANOVA). Regional ASBT mRNA expression was compared with expression in the duodenum by repeated measurement ANOVA with linear contrasts. Correlation of serum bilirubin and bile acid concentrations was performed using Spearman's rank correlation coefficient ( $\rho$ ). Differences in demographic characteristics between icteric patients and controls were analysed by ANOVA of the  $\chi^2$  test, as appropriate. All comparisons were performed as two sided comparisons using the SPSS for Windows software (version 12.0). Level of significance was  $p < 0.05$ .

## RESULTS

### Expression pattern of ASBT mRNA in the human intestine

Human ASBT mRNA expression was studied in 14 control subjects (seven women and seven men) who were undergoing a combined gastroscopy and colonoscopy. Results were normalised by calculation of the ASBT/villin ratio. Normalised ASBT expression (mean (SEM) arbitrary units) was 171.8 (20.3) in the duodenum, 1010 (330) in the terminal ileum, 8.3 (5) in the ascending colon, 4.9 (0.9) in the transverse colon, 4.8 (1.7) in the descending colon, and 1.1 (0.2) in the sigmoid colon, respectively (fig 1).

### Duodenal expression of ASBT mRNA and PPAR $\alpha$ mRNA

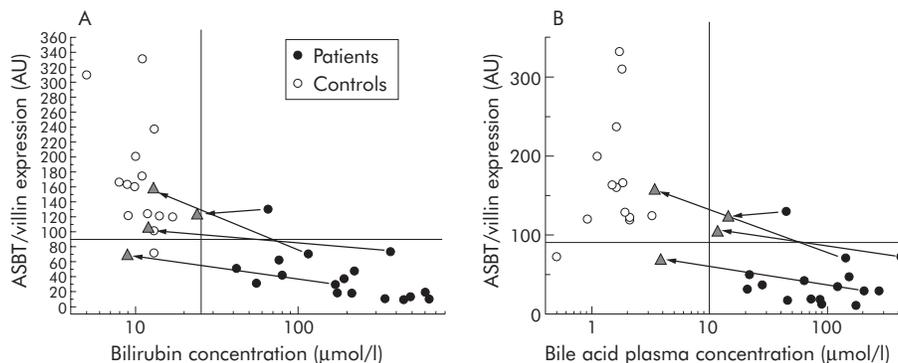
Adaptive regulation of ASBT expression in obstructive cholestasis was investigated by quantification of ASBT mRNA levels in duodenal biopsies of 20 patients with obstructive cholestasis and compared with levels obtained in 14 control subjects. As shown in fig 2, ASBT mRNA expression (ASBT/villin ratio (mean (SEM))) was about fourfold lower in patients with obstructive cholestasis (37.9 (6.5)) compared with control subjects (171.8 (20.3);  $p < 0.001$ ). Patients with obstructive cholestasis due to a tumour showed a trend for lower ASBT mRNA expression compared with patients with a benign aetiology of obstructive cholestasis (31.2 (7.3) for tumour induced and 44.6 (10.8) for benign obstruction); however, the difference was not statistically significant. Plasma bilirubin levels were 301.6 (64.9) and 112.6 (20.0) mmol/l ( $p = 0.02$ ) and bile acid levels were 168.4 (45.3) and 76.8 (22.8)  $\mu\text{mol/l}$  (NS) for patients with and without tumours, respectively. PPAR $\alpha$  mRNA expression was not significantly different between cholestatic patients and controls.

### Correlation of duodenal ASBT mRNA expression with bilirubin and bile acid plasma concentration

Bilirubin and bile acid plasma concentrations were inversely correlated with ASBT mRNA expression ( $\rho = -0.863$ ,  $p < 0.001$  and  $\rho = -0.722$ ,  $p < 0.001$ , respectively). The correlation with ASBT mRNA expression was similar for bilirubin and bile acid concentrations. Using ASBT mRNA expression, an almost perfect separation was obtained between icteric patients and healthy controls (fig 3A, B).

### Effect of reconstitution of bile flow on ASBT mRNA expression

In five of the 20 icteric patients, follow up gastroscopy was performed between 10 and 34 weeks after reconstitution of bile flow (follow up endoscopy was only performed when medically indicated). Three of the five patients received stents into the common bile duct and two patients had a Whipple operation, one due to a tumour in the head of the pancreas and one due to a cholangiocarcinoma. The patient who had the Whipple operation due to a tumour in the head of the pancreas could not be included in the follow up analysis because duodenal biopsies could not be obtained after



**Figure 3** Individual correlation of bilirubin (A) and bile acid (B) plasma concentrations with apical sodium dependent bile acid transporter (ASBT) mRNA expression in patients with obstructive cholestasis and control subjects. Data are means (SEM). Arrows connect individual values pre- and post-reconstitution of bile flow in four follow up patients. AU, arbitrary units.

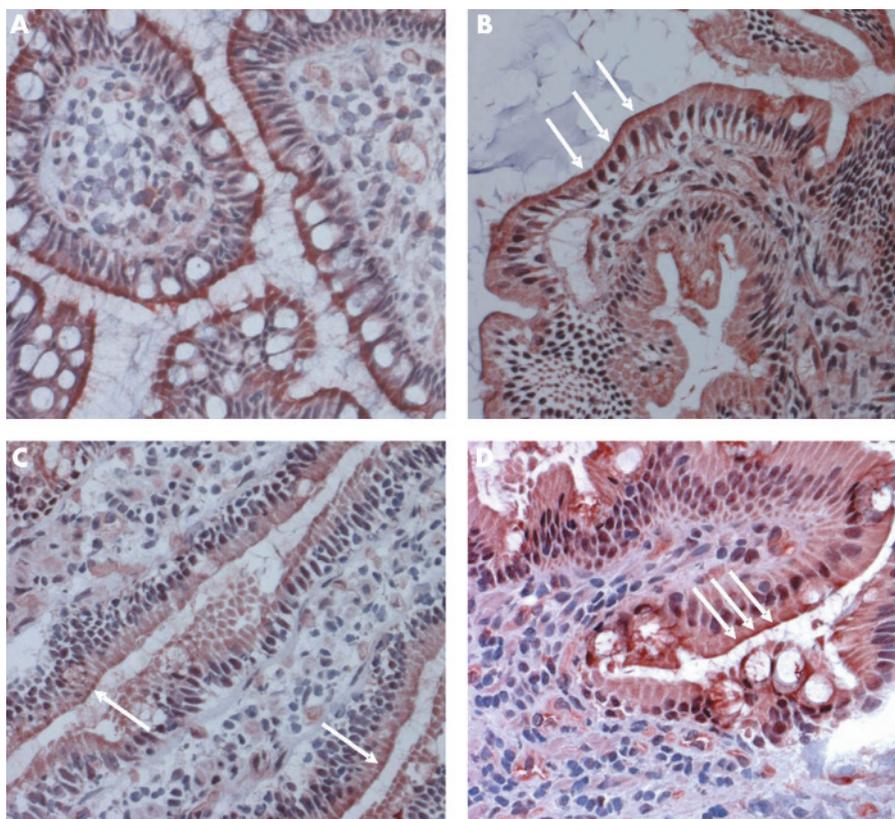
complete duodenopancreatectomy. In the patient with cholangiocarcinoma, only a partial duodenopancreatectomy was performed so duodenal biopsies were obtained in the follow up endoscopy.

At follow up endoscopy, bilirubin and bile acid plasma concentrations had normalised in all patients and three of the four patients showed increased expression of ASBT mRNA compared with the baseline value obtained. These three follow up patients with bilirubin (bile acid) plasma concentrations of 373 (431), 170 (204), and 115 (170)  $\mu\text{mol/l}$  before endoscopic intervention showed an increase in ASBT expression from 73 to 103, 29 to 68, and 71 to 157 after reconstitution of bile flow, respectively. One patient, who

had a bilirubin (bile acid) level of 65 (32.2)  $\mu\text{mol/l}$  before intervention and 24 (14.5)  $\mu\text{mol/l}$  after reconstitution of bile flow showed an ASBT expression of 130 before intervention, which decreased slightly to 122 after reconstitution of bile flow ( $n = 4$ ). The difference in ASBT expression before (76 (18)) and after (113 (18)) reconstitution of bile flow for these four follow up patients was not significant (paired  $t$  test;  $p = 0.15$ ).

#### Immunohistochemistry of ASBT

Immunohistochemistry analysis in healthy subjects revealed clear staining of ASBT in ileal and duodenal mucosa (fig 4A and B, respectively). Staining of duodenal mucosa was clearly



**Figure 4** (A–D) Immunohistochemical localisation of apical sodium dependent bile acid transporter (ASBT) protein on the apical membrane of the ileum and duodenum of humans using a polyclonal rabbit antihuman ASBT antibody. Clear staining of ileal (A) and duodenal (B) epithelial cells of control subjects. Staining of duodenal epithelial cells in a patient with obstructive cholestasis was almost abolished before (C) and restored after (D) reconstitution of bile flow (all pictures at 200-fold magnification).

less intense than that from ileal tissue. In the duodenal mucosa of icteric patients, staining for ASBT was almost completely abolished (fig 4C). After reconstitution of bile flow, a more intense staining was found in the same tissue (fig 4D).

### Duodenal expression of MRP2 mRNA and MRP3 mRNA

Additionally, we investigated whether there was also adaptive regulation of MRP2 and MRP3 mRNA expression in the distal part of the duodenum.

Patients with obstructive cholestasis showed significantly lower MRP2 mRNA expression (0.21 (0.02)) compared with control subjects (0.32 (0.03),  $p = 0.004$ ). After reconstitution of bile flow in four patients with obstructive cholestasis, MRP2 expression increased to 0.28 (0.02) (NS). No difference between patients with obstructive cholestasis and control subjects was observed with regard to MRP3 expression (data not shown).

## DISCUSSION

Bile acids undergo extensive enterohepatic and cholehepatic circulation through the coordinated action of several transport proteins in hepatocytes, cholangiocytes, and enterocytes.<sup>20</sup> The uptake of bile acids at the apical membrane of enterocytes by the apical sodium dependent bile acid transporter ASBT (SLC10A2) reflects an important mechanism for enterohepatic circulation of bile acids. Human ASBT is an efficient transport system for conjugated and unconjugated bile acids.<sup>3</sup> In the present study we were able to show that human ASBT mRNA and protein are expressed in the duodenum of the human intestine but to a lesser extent than in the terminal ileum. In addition, significant downregulation of duodenal ASBT mRNA expression was demonstrated in patients with complete or near complete obstruction of bile duct compared with control subjects. Finally, restoring bile flow to the duodenum increased ASBT mRNA expression in a subpopulation of patients with bile duct obstruction.

Downregulation of human duodenal ASBT mRNA may be compared with the adaptive response of bile acid transport proteins during obstructive cholestasis in the terminal ileum, the kidney, as well as in the cholangiocytes of the rat.<sup>11–21</sup> In addition to downregulation of asbt in the terminal ileum, the intestinal absorption rate of taurocholate in bile duct ligated rats was lower and the absorption rate was inversely correlated to serum bile acid concentrations compared with sham operated rats.<sup>11</sup> Patients with mutations in the ASBT gene can suffer from congenital diarrhoea and steatorrhoea, which is explained by an interrupted enterohepatic bile acid circulation.<sup>4</sup> Further clinical data indicate that in patients with primary sclerosing cholangitis, biliary enrichment of UDCA decreases with increasing cholestasis.<sup>22</sup> Moreover, in patients with bile duct obstruction and external biliary drainage, intestinal absorption of UDCA was decreased, before relief of cholestasis.<sup>23</sup> These data suggest that in humans, intestinal bile acid absorption is reduced during obstructive cholestasis.

What factors influence ASBT gene expression in the enterocyte are largely unknown. Animal studies suggest that luminal bile acids may be one such regulatory factor. Reduction of intestinal bile acid concentration during fasting<sup>24</sup> or after biliary diversion<sup>25</sup> or bile duct ligation<sup>26</sup> is accompanied by a decrease in ileal bile acid transport. Stravitz *et al* demonstrated that increasing intestinal bile acid concentrations by cholic acid feeding leads to an increase in the sodium dependent transport rate.<sup>10</sup> Furthermore, adaptive induction of asbt mRNA levels and transporter protein as well as increased asbt transport activity by cholic acid fed rats was documented.<sup>10</sup>

In contrast with these observations, other authors have demonstrated in rats that the ileal taurocholate absorption rate correlated inversely to serum bile acid concentrations after bile duct ligation and after biliary diversion compared with sham operated animals.<sup>11</sup> The authors concluded that cholestasis leads to decreased, and biliary diversion to increased, active ileal absorption of taurocholate, in which the systemic bile acid load seems to be the decisive factor. In the kidney, bile acid excretion in urine increased progressively in both cholestatic animal models but also in clinical cholestatic disorders.<sup>21–27</sup> In Wistar rats, a marked reduction in asbt protein expression in microsomal membrane fractions from whole kidney after CBDL was observed, resulting in reduced levels of asbt expression on the luminal membrane of the proximal tubule of the kidney, a change that is associated with a diminished capacity to reabsorb bile acids from the glomerular filtrate.<sup>21</sup> In the same study, upregulation of Mrp2 protein expression on the apical membrane of the rat renal proximal tubule was shown. MRP2 is associated with an increased ability to excrete divalent organic anions such as bile salt sulphates and glucuronides (bilirubin) that accumulate during cholestasis and would therefore facilitate extrahepatic pathways for bile acid and bilirubin excretion during cholestasis. This adaptive response of asbt and Mrp2 in the kidney of the rat does not seem to be regulated by luminal bile acids.

Here we showed that ASBT mRNA expression correlated inversely with bilirubin as well as with bile acid plasma concentrations, which were both used as markers for obstructive cholestasis.

Bile acids are synthesised from cholesterol in the liver; their production is a major mechanism of cholesterol elimination and important for the maintenance of cholesterol homeostasis.<sup>28</sup> Downregulation of ASBT expression in obstructive cholestasis might therefore be of clinical interest. In a previous publication, it was shown that inhibition of ileal bile acid transport with SC-435, a competitive inhibitor of ASBT, lowered plasma cholesterol levels: (1) by inactivating the hepatic farnesoid x receptor and (2) by stimulating cholesterol 7 $\alpha$ -hydroxylase (CYP7A1).<sup>29</sup> This cytochrome is the rate limiting enzyme of chenodeoxycholic acid synthesis. It is located in the endoplasmic reticulum of the hepatocyte. In patients with biliary obstruction, a rise in serum 7 $\alpha$ -hydroxycholesterol was observed after biliary drainage.<sup>30</sup> The 7 $\alpha$ -hydroxylation rate was significantly lower for patients with obstructive cholestasis compared with healthy subjects.<sup>31</sup> These data suggest that in the case of a substantial bile acid malabsorption, the activity of 7 $\alpha$ -hydroxylase in the liver and synthesis of bile acids is increased.

Other authors suggest that inhibition of ASBT with SC-435 reduces low density lipoprotein cholesterol and ApoB by enhanced plasma clearance of low density lipoprotein ApoB.<sup>6,9</sup> Treatment of Caco-2 cell monolayers with 25-hydroxycholesterol significantly inhibited Na<sup>+</sup> dependent <sup>3</sup>H-taurocholate uptake. Inhibition in hASBT activity was associated with reduction in both the level of hASBT mRNA and its promoter activity.<sup>32</sup> asbt protein expression did not change in rats but rose by 31% in rabbits when they were fed with cholesterol (cholest-5-en-3 $\beta$ -ol).<sup>33</sup> Administration of SC-435 to apo E<sup>-/-</sup> mice lowered serum total cholesterol by 35% and reduced aortic root lesion area by 65%. The authors concluded that specific inhibition of asbt could be a novel therapeutic approach for treatment of hypercholesterolaemia, resulting in a decreased risk for atherosclerosis.<sup>7</sup> Taken together, all of these data suggest that expression of ASBT in the enterocyte seems to be an important component in cholesterol homeostasis and lipid metabolism in humans.

Intracellular factors that influence ASBT gene expression remain largely unknown. One potential factor might be

activation of nuclear receptor PPAR $\alpha$ , a ligand activated transcription factor that regulates expression of a number of genes involved in peroxisomal and mitochondrial  $\beta$ -oxidation of fatty acids. PPAR $\alpha$  binds as a heterodimer with the retinoid X receptor to a peroxisome proliferator response element located in the promoter region of target genes. PPAR $\alpha$  was shown to play a critical role in the adaptive response to fasting in mice.<sup>34–36</sup> PPAR $\alpha$  also influences bile acid composition by induction of the sterol 12 $\alpha$ -hydroxylase, which acts at a branch-point in the bile acid synthetic pathway by catalysing the conversion of 7 $\alpha$ -hydroxy-4-cholesten-3-one to 7 $\alpha$ ,12 $\alpha$ -dihydroxy-4-cholesten-3-one. This reaction determines the ratio of cholic acid to chenodeoxycholic acid.<sup>37</sup> In Hepa 1c1c7 cells, which were transiently transfected with an expression plasmid for PPAR $\alpha$  and retinoid X receptor  $\alpha$  and a luciferase reporter construct containing copies of the peroxisome proliferator response element from the rat ACOX gene, inhibition of PPAR $\alpha$  reporter gene expression was shown with increasing concentrations of chenodeoxycholic acid in the presence or absence of Wy-14,643 (= PPAR $\alpha$  ligand).<sup>38</sup> It was concluded that during certain pathophysiological states, where intracellular bile acid concentrations might be elevated, effects on PPAR $\alpha$  dependent target gene regulation are possible.<sup>38</sup> Recently, PPAR $\alpha$  was shown to transactivate the human ASBT gene.<sup>39</sup> Incubation of human hepatoma HepG2 cells with chenodeoxycholic acid resulted in significant induction in PPAR $\alpha$  mRNA levels. In addition, hPPAR $\alpha$  gene expression was upregulated by taurocholic acid in human primary hepatocytes.<sup>40</sup> However, no difference between patients with obstructive cholestasis and control subjects was observed with regard to duodenal PPAR $\alpha$  mRNA expression in our study.

In a previous study, Dietrich *et al* demonstrated that obstructive cholestasis promotes downregulation of intestinal MRP2 protein expression in rats and humans.<sup>15</sup> The reduction was correlated with duration of cholestasis and was reversible after reconstitution of bile flow. In the same study, in patients with obstructive cholestasis, a non-significant decrease in MRP2 mRNA expression was detected compared with control subjects. However, the authors observed that decline of intestinal rat MRP2 mRNA occurred more slowly and gradually than downregulation of its protein expression. Our results showed a significant downregulation of MRP2 mRNA expression compared with control subjects and a non-significant increase after reconstitution of bile flow in four patients. One explanation for the downregulation of MRP2 gene expression in our study compared with Dietrich and colleagues<sup>15</sup> might be higher plasma bilirubin concentrations in our patients with obstructive cholestasis. Another reason might be duration of cholestasis, which was not clearly stated in both studies and therefore may have been different. Other factors such as food derived compounds or substrates might also be involved in the regulation of gene expression. For example, it was shown that grapefruit and orange juice inhibited the transport by MRP2,<sup>41</sup> components of our daily diet, such as the flavonoid epicatechin in tea,<sup>42</sup> chrysin, and its metabolites are substrates of MRP2.<sup>43</sup> Drugs are unlikely to have contributed to the different MRP2 mRNA expression in the present study and the study performed by Dietrich and colleagues<sup>15</sup> because patients with drugs, which are known to affect MRP2 expression, were excluded from both studies. Finally, our data support the results of Dietrich and colleagues<sup>15</sup> which demonstrated an adaptive downregulation of duodenal MRP2 in patients with obstructive cholestasis.

MRP3 is expressed on the basolateral membrane in the proximal small bowel<sup>13</sup> and is capable of transporting bile salts, including taurocholate, glycocholate, taurochenodeoxycholate-3-sulphate, and tauroolithocholate-3-sulphate.<sup>14</sup> This

suggests that MRP3 might be an important transporter in the enterohepatic circulation of bile acids. However, in the present study, we did not observe any difference in human duodenal MRP3 gene expression in patients with obstructive cholestasis compared with control subjects. Similarly, no compensatory upregulation of human duodenal MRP3 expression in cholestatic patients was previously described.<sup>15</sup>

In conclusion, human ASBT mRNA is expressed in the small intestine, predominantly in the terminal ileum but also, to a lesser extent, in the duodenum. Adaptive downregulation of ASBT and MRP2 mRNA in the duodenum can be observed in patients with obstructive cholestasis compared with control subjects. Furthermore, duodenal ASBT mRNA expression levels inversely correlated with bilirubin and bile acid plasma concentrations in patients with obstructive cholestasis as well as in control subjects. This adaptive gene regulation may represent a mechanism preventing the accumulation of hepatotoxic bile acids in cholestasis. After relief of cholestasis, an increase in ASBT mRNA was observed in three of four patients.

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## Adaptive regulation of the ileal apical sodium dependent bile acid transporter (ASBT) in patients with obstructive cholestasis

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