Expression and regulation of transmembrane transporters in healthy intestine and gastrointestinal diseases

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Abbreviations

ABC	ATP binding cassette
AEC	3-amino-9-ethylcarbazole
ANOVA	analysis of variance
ASBT	apical sodium-dependent bile salt transporter
ATP	adenosine-5`-triphosphate
BCRP	breast cancer resistance protein
BPH	benign prostatic hyperplasia
BSEP	bile salt export pump
BXP21	BCRP monoclonal antibody
Caco-2	human colon carcinoma cell line
CAR	constitutive androstane receptor
CDCA	cheodeoxycholic acid
cAMP	cyclic adenosine monophosphate
CBDL	common bile duct ligation
cDNA	complementary DNA
Ct	cycle threshold
CYP450	cytochrome P450
DCA	deoxycholic acid
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ER	endoplasmic reticulum
ERCP	endoscopic retrograde cholangiopancreatography
FCS	(heat inactivated) foetal calf serum
FXR	farnesoid x receptor
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GR	glucocorticoid receptor
GSH	reduced glutathione
HepG2	human hepatoma cell line
HIV	human immunodeficiency virus
HNF	hepatocyte nuclear factor
HUGO	human genome organisation
I-BABP	ileal bile acid binding protein
IBD	inflammatory bowel disease
IFNγ	interferon gamma
IL-1β	interleukin-1β
kDa	kilodalton

LDL	low-density lipoprotein
LPS	lipopolysaccharide
LS180	human colon carcinoma cell line type LS180
mAB	monoclonal antibody
MDCK	Madin-Darby Canine kidney
MDM	monocyte derived macrophage
MDR	multi-drug resistance
mRNA	messenger ribonucleic acid
MRP	multi-drug resistance associated protein
MTX	methotrexate
NBF	nuclear binding fold
NFκB	nuclear factor kappa B
NTCP	Na^+-taurocholate cotransporting protein
OAT	organic anion transporter
OATP	organic anion transporting polypeptide
OCT	organic cation transporter
OST	organic solute transporter
PBC	primary biliary cirrhosis
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
P-gp	P-glycoprotein
PMEA	9- [2-(phosphonomethoxy)ethyl] adenine (=adefovir)
PPARα	peroxisome proliferator activated receptor α
PPRE	peroxisome proliferator response element
PXR	pregnane X receptor
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
RXR	retinoid x receptor
SEM	standard error of the mean
SHP	short heterodimeric protein
SLC	solute carrier
SN-38	active metabolite of irinotecan
SNP	single nucleotide polymorphism
SSZ	sulfasalazine
TNFα	tumor necrosis factor alpha
TURP	transurethral resection of prostate
UDCA	ursodeoxycholic acid

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Summary

Transmembrane transporters mediate energy dependent or independent translocation of drugs, potentially toxic compounds, and of various endogenous substrates such as bile acids and bilirubin across membranes. In this thesis the focus is on two classes of transporters, the ATP-binding cassette (ABC) transporters, which mediate ATP dependent transport and the solute carriers (SLC) which use electrochemical gradients for their transport. The transporters are expressed on membranes of cells of excretory organs (e.g. kidney, liver) and protective barriers (e.g. intestine, blood brain barrier) and influence therefore the absorption, distribution and elimination of compounds. They may reduce the intracellular concentration of drugs.
Transport activity of transmembrane transporters in the intestine depends on the expression level and distribution along the intestine. Transport activity of transporters might be influenced by other factors such as genetic variations, which may present with a dysfunctional phenotype (e.g. single nucleotide polymorphisms; SNPs), or certain disease states, which might adaptively regulate transporter expression on the transcriptional and posttranscriptional level.

The first aim of this thesis was a systematic site-specific analysis of the expression of several ABC transporters and solute carriers along the intestinal tract. Following that, regulation of hepatobiliary transporters in the human intestine during obstructive cholestasis was evaluated.
Studies were performed in close collaboration with the Department of Gastroenterology (University Hospital of Basel).

As described in Chapter 2, transporter mRNA expression was analyzed by real time PCR (Taqman), a method that was previously developed in our laboratory to quantify the expression of transporters using standard curves. Protein expression was assessed by immunohistochemistry, bile acids plasma concentrations were measured by capillary gas chromatography (U. Beuers, Munich).

The results presented in this thesis include systematic site specific analysis of quantitative expression of the human multidrug resistance transporters, such MDR1 (ABCB1), breast cancer resistance transporter (BCRP; ABCG2) and multidrug resistance associated transporters (MRP1-5; ABCC1-5) as well as the apical sodium dependent bile acid transporter ASBT (SLC10A2) along the the intestinal tract. These data are shown in Chapter 3. As the mRNA expression levels of the investigated transporters change along the intestinal tract, these findings might be of interest to develop target strategies for orally administered drugs.

Also, this systematic site specific analysis of MDR transporters serves as a preparation for a prospective clinical study in patients with Inflammatory Bowel Disease (IBD), which will investigate MDR transporter gene expression in intestinal biopsies (intestinal epithelial cells, intestinal macrophages) in newly diagnosed IBD patients compared to treatment refractory IBD patients, patients in remission and disease free controls. This study design will help to evaluate, if MDR transporters vary due to interindividual differences, inflammatory processes and/or pharmacological treatment and might serve as an explanation for patients with IBD not responding to drug treatment.

Intestinal macrophages play a central role in the orchestration of innate immune response reactions in the gut. As anti-inflammatory as well as immunosuppresive drugs such as glucocorticoids, methotrexate, cyclosporine, 6-mercaptopurine and sulfasalazine, which all of them are used in the treatment of IBD patients, are substrates of MDR transporters, a method for isolation of CD14+ peripheral blood cells (monocytes), their ex vivo cultivation and differentiation into macrophages was established. In Chapter 4, first results demonstrate the influence of the differentiation process of monocytes into monocyte derived macrophages (MDM) and the stimulation of MDM with bacterial products (LPS) on MDR transporter expression. At present, the effect of budesonide, methotrexate, 6-mercaptopurine and sulfasalazine on the transporter expression in ex vivo cultivated human MDM is evaluated.

Adaptive regulation of hepatobiliary transport systems during obstructive cholestasis with a disrupted enterohepatic circulation has been demonstrated in the intestine only for rodents before. The results presented in Chapter 3 showed, that ASBT, which contributes substantially to the enterohepatic circulation of bile acids by their reabsorption from the intestine, is adaptivelly regulated in the human duodenum during obstructive cholestasis. Our findings are of clinical importance as we have shown for the first time that ASBT is expressed in the human duodenum. These results may indicate species specific differences to rodents, and that changes in the ASBT gene expression can be measured in the duodenum during obstructive cholestasis. Adaptive regulation of ASBT in the intestine has clinical implications for the bile acid homeostasis and also for the lipid metabolism.
BCRP mediates energy dependent efflux of drugs and potentially toxic compounds, and of various endogenous substrates such as bile acids. Here, expression of human BCRP mRNA was shown to be highest in the duodenum with a continuos decrease along the intestinal tract

down to the rectum. BCRP mRNA and protein expression in the duodenum was found to be decreased during obstructive cholestasis when compared to control subjects and BCRP expression increased after reconstitution of bile flow. In consequence, reduced intestinal BCRP expression during obstructive cholestasis might influence the accumulation of bile acids, food-derived carcinogens and the pharmacokinetics of various drugs that are transported by BCRP.

In an isolated project with the background of observed therapy resistance to antibiotics in the treatment of patients with chronic prostatitis, MDR expression in prostatic tissue in regard to inflammation was evaluated in 50 patients that underwent transurethral resection of prostate. In this study group, neither inflammation nor localization of inflammation in prostate tissue (acinar versus interstitial) influenced MDR transporter expression.

Aims of the thesis

Reabsorption of bile salts from the intestinal lumen is a critical step for bile salt homeostasis in the body. Several transporters are involved in this process and mediate energy dependent or independent efflux of drugs, potentially toxic compounds, and of various endogenous substrates such as bile acids and bilirubin. The aim of the present thesis was to examine the regulation of hepatobiliary transport systems in the human gut during obstructive cholestasis with a disrupted enterohepatic circulation. A dysregulation of these transport systems in the gut might be of clinical relevance for drug treatment regimens during obstructive cholestasis.
The following questions have been addressed:

- systematic site-specific analysis of several ABC transporters and solute carriers expression along the intestinal tract
- regulation of hepatobiliary transporters in the human intestine on the transcriptional and posttranscriptional level during obstructive cholestasis

Beside the focus on regulation of hepatobiliary transport systems in the human intestine an important research topic of our lab is the investigation of molecular mechanisms for insufficient or failed therapy for patients with inflammatory bowel disease (IBD).
As macrophages play an important role in host defense, particularly in the inflammatory process of acute and chronic disease, a project was started to investigate the influence of IBD drugs and cytokines on the regulation of MDR transporter gene expression in peripheral blood monocytes and macrophages. The following goals have been set:

- Establishment of a method for isolation of CD14+ peripheral blood cells (monocytes), *ex vivo* cultivation and differentiation into macrophages
- Influence of differentiation into macrophages and stimulation of bacterial products (LPS) on MDR transporter expression

In an isolated project the expression of multidrug resistance transporters was studied in prostatic tissue of patients diagnosed with benign prostatic hyperplasia (BPH) undergoing transurethral resection of the prostate. The following question was addressed:

- Influence of inflammation on MDR transporter expression in human prostatic tissue

1. Introduction

1.1 Physiology of bile salt transporters

Bile formation is an osmotic secretory process that is driven by the active concentration of bile salts (major organic solutes in bile) and other biliary constituents in the bile canaliculi. Bile salts are concentrated up to 1000 fold in bile using an active transport by hepatocytes against a concentration gradient when secreted into bile ductuli. As it passes along the bile ductules and ducts bile is modified by secretory and absorptive processes and is further concentrated up to 10-fold in the gallbladder before reaching the intestine. In the small intestine bile salts emulsify dietary fats and lipid-soluble vitamins. They regulate pancreatic secretion and the release of gastrointestinal peptides (Koop et al., 1996). In addition, bile is an important route of elimination for environmental toxins, carcinogens, drugs and their metabolites (xenobiotics) and serves also as the major route of excretion for endogenous compounds and metabolic products such as cholesterol, bilirubin and hormones (Trauner and Boyer, 2003).

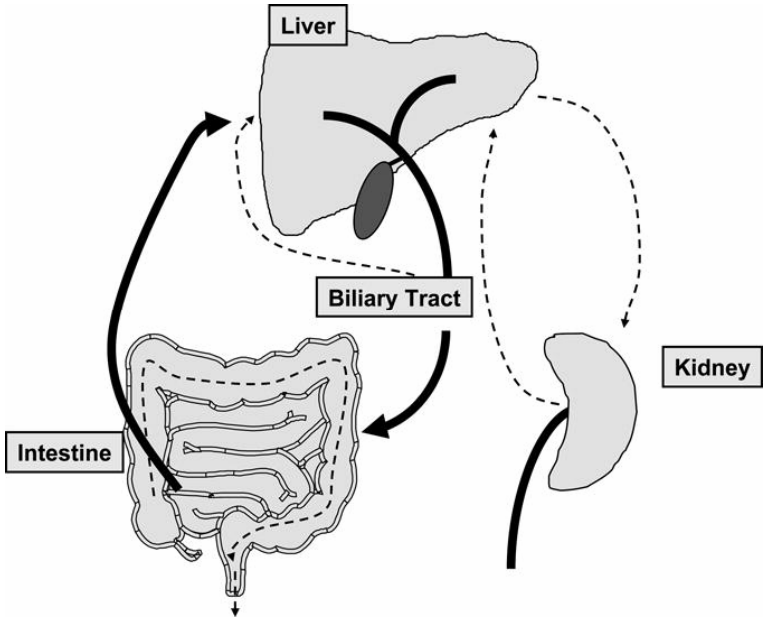

Figure: Trauner M et al., J Clin Gastroenterol 2005;39: S111-24

Figure represents normal bile secretion. Many biliary compounds (eg bile salts, bilirubin, cholesterol) secreted into bile undergo an extensive enterohepatic circulation, ie, are reabsorbed in the (small) intestine, taken up again by the liver and secreted into bile. Some compounds repeat this cycle for several

times before becoming eliminated by the feces. Additional “cycles” include a cholehepatic cycle between bile duct epithelial cells (cholangiocytes) and hepatocytes, and a nephrohepatic cycle between kidney cells (proximal renal tubules) and liver hepatocytes, the latter preventing the loss of glomerularely filtered bile salts into urine.

Bile salts undergo extensive enterohepatic circulation. After reabsorbtion in the proximal small intestine by sodium independent absorption and in the distal ileum by active sodium-dependent absorption, bile salts return to the liver via the portal circulation. This efficient enterohepatic circulation ensures that from the total bile salt pool of adult humans (3-4 g), which circulates 6-10 times per 24 hours through the enterohepatic pathway only 0.5 g bile salts are lost per 24 hours through fecal excretion. The loss is compensated by de novo synthesis from cholesterol (Meier and Stieger, 2002).

Hepatic uptake, biliary excretion and intestinal reabsorption are mediated by specific transport proteins. More than 80% of conjugated bile salts, which circulate in plasma tightly bound to albumin and lipoproteins (Wolkoff and Cohen, 2003) undergo single-pass extraction by the liver. Hepatic uptake of bile salts on the basolateral membrane in humans is mediated predominantly by the Na^+-taurocholate cotransporting protein (NTCP, SLC10A1) for taurine and glycine conjugated bile salts (Hagenbuch and Meier, 1994; Meier et al., 1997) and the Na^+ independent organic anion-transporting polypeptide (OATP-C) (Kullak-Ublick et al., 2001). The canalicular secretion of conjugated bile salts on the apical membrane of the hepatocyte represents the rate limiting step in the overall bile salt transport from blood into bile. The secretion into bile canaliculi is performed by the bile salt export pump (BSEP), which is a member of the ATP-binding cassette (ABC) transporter gene superfamily. Bsep knockout mice exhibit liver steatosis with mild cholestasis. However, they demonstrate residual bile salt secretion of about 30% compared with wild type mice (Wang et al., 2001b). Mutations in the human BSEP-gene are associated with progressive familial intrahepatic cholestasis (PFIC2) (Strautnieks et al., 1998). These patients secrete less than 1% of biliary salts compared with normal infants (Jansen et al., 1999). The exact contribution of multidrug resistance associated protein 2 (MRP2), which is localized on the canalicular membrane, to canalicular transport of bile salts is currently under investigation (Keppler and Konig, 2000). However, mutations in MRP2 have been reported to underly Dubin-Johnson syndrome that is characterized by a defective excretion of conjugated anions into the bile (chronic conjugated hyperbilirubinemia) (Hashimoto et al., 2002). MRP2 is deficient in rats with an inborn error in the biliary secretion of organic anions, including conjugated bilirubin (Buchler et al., 1996; Paulusma et al., 1996).

BCRP (ABCG2) another ABC transporter is localized on the canalicular membrane of the hepatocyte and physiologic substrates include estrone-3-sulfate, dehydroepiandrosterone sulphate and sulfasalazine (Suzuki et al., 2003). Its role for the hepatobiliary transport has not been completely elucidated, yet.

1.2 Intestinal transporters for reabsorption of bile salts

A critical step for the bile salt homeostasis is the reabsorption of bile salts from the intestine in which different transporters might be involved. In the brush border membrane of jejunal enterocytes in rats the organic anion transporting protein (Oatp3) is expressed (Walters et al., 2000). This sodium independent bile salt transporter transports a large variety of organic anions and bile salts (Kullak-Ublick et al., 2000). However, whether functional expression of bile salt - transporting OATPs also occurs in the brush border membrane of the human intestine remains to be elucidated. Reabsorption of bile salts mainly occurs in the distal ileum where the apical sodium-dependent bile salt transporter (ASBT/SLC10A2) is expressed. Human ASBT transports conjugated and unconjugated bile salts with a higher affinity for CDCA and DCA than for taurocholate (Craddock et al., 1998). Patients with mutations in the ASBT gene can suffer from congenital diarrhea and steatorrhea, concomitant with an interrupted enterohepatic circulation of bile salts (Oelkers et al., 1997). Induction of Asbt mRNA levels, transporter protein and transport activity by cholic acid feeding in rats was observed (Stravitz et al., 1997).

After uptake into the enterocyte, bile salts are shuttled to the basolateral membrane for efflux into the portal circulation. Transcellular transport is probably mediated by the ileal bile acid-binding protein (I-BABP) that is cytoplasmatically attached to ASBT (Gong et al., 1994).

Multidrug resistance associated protein 3 (MRP3) is expressed in increasing levels from the jejunum to the large intestine (Cherrington et al., 2002) and is localized on the basolateral membrane of polarized cells (Konig et al., 1999; Kool et al., 1999). MRP3 transports substrates such as conjugated bile salts and bilirubin glucuronide (Hirohashi et al., 2000; Keppler et al., 2000; Zelcer et al., 2003) and is a potential candidate for bile salt efflux from enterocytes into portal blood. In Caco2-cells bile salts increased the expression of human MRP3 (Inokuchi et al., 2001) and in an in-vitro study with LLC-PK1 cells transfected with rat MRP3 the involvement of MRP3 in bile salt transport could be demonstrated (Hirohashi et al., 2000). A recent publication showed that the organic solute transporters (Ost)-α and Ost-β are localized in the intestine predominantly on the basolateral membrane of ileocytes. These transporters form a heterodimer and mediate apical efflux of taurocholate in transfected canine kidney cells and seems to be

responsible for the transport of bile salts across the basolateral membrane of enterocytes into portal blood (Dawson et al., 2005).

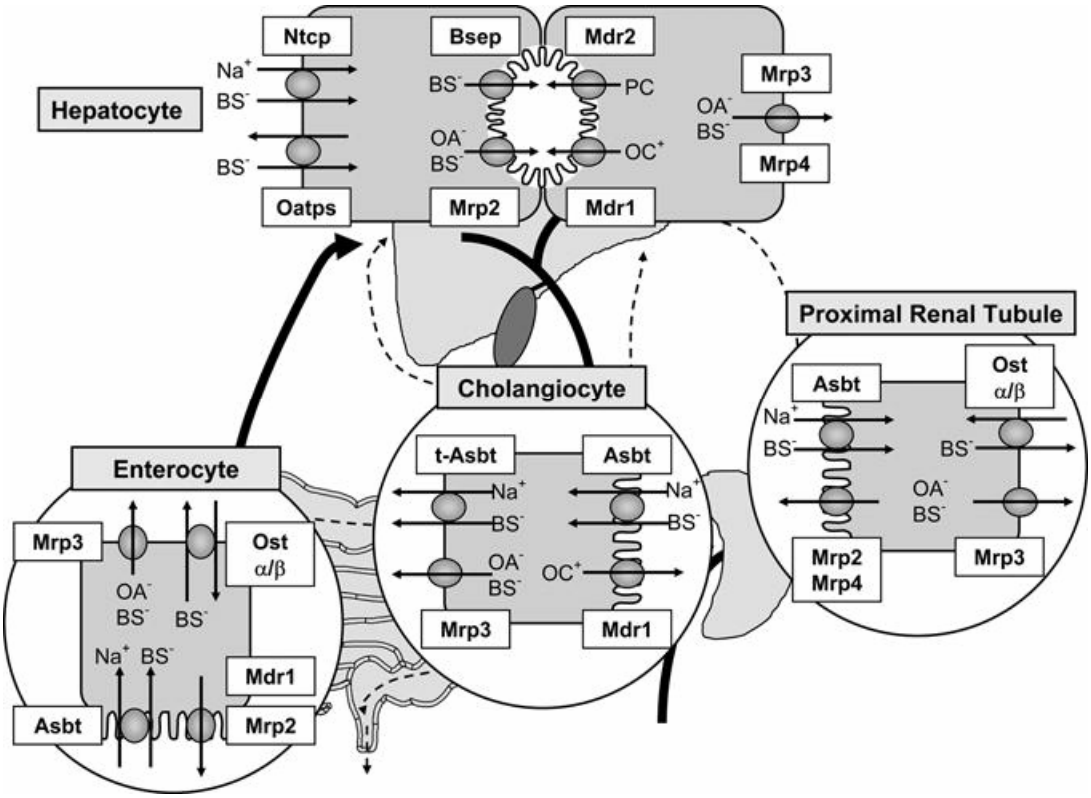

Figure: Trauner M et al., J Clin Gastroenterol 2005;39: S111-24

Hepatobiliary transport systems in liver and extrahepatic tissues in humans. Bile salts (BS^-) are taken up by hepatocytes via the basolateral Na^+ /taurocholate cotransporter (NTCP) and organic anion transporting proteins (OATPs). Monovalent BS^- are excreted via the canalicular bile salt export pump (BSEP) while divalent BS^- together with anionic conjugates (OA^-) are excreted via the canalicular conjugate export pump (MRP2). The phospholipids export pump (Mdr2/MDR3) facilitates excretion of phosphatidylcholine (PC), which forms mixed micells in bile together with BS^- and cholesterol. Cationic drugs (OC^+) are excreted by the multidrug resistance export pump (Mdr1). Other canalicular export pumps include the two-half transporter Abcg5/g8 for cholesterol and the breast cancer resistance protein (Bcrp) for OA^- (not shown). Basolateral isoforms of the multidrug resistance-associated protein (MRP3 and MRP4) provide an alternative route for the elimination of BS^- and nonbile salt OA^- from hepatocytes into systemic circulation. BS^- are reabsorbed in the terminal ileum via ileal Na^+ -dependent bile salt transporter (ASBT) and effluxed by the recently identified heterodimeric organic solute transporter Ost α/β. Similar mechanisms exist in proximal renal tubules and cholangiocytes where an additional, truncated isoform (t-ASBT) or MRP3 may be involved in BS^- efflux from cholangiocytes. In addition to ASBT and MRPs, proximal renal tubules

express Oatp1 (not shown). MRP2 is also present in the apical membrane of enterocytes and proximal renal tubules, while MDR1 is also found in intestine and bile ducts.

1.3 Transcriptional regulation of transporters involved in bile salt transport

The functional expression of membrane transport proteins can be regulated at several levels, including gene transcription and posttranscriptional activity. Although the mechanism that control gene transcription of membrane transporters are still incompletely understood, bile salt uptake and efflux systems might be regulated by the following nuclear hormone receptors as well as other transcription factors. Bile salts, sterols and fatty acids are natural ligands of nuclear hormone receptors expressed in liver and intestine. The requirement for a regulatory network for maintaining a bile salt homeostasis in the human body is evident from the fact that the intracellular accumulation of bile salts leads to cholestasis, hepatocyte apoptosis and parenchymal damage (Faubion et al., 1999).

1.3.1 Farnesoid X receptor (FXR) and retinoid X receptor (RXR)

FXR, which is highly expressed in the liver and intestine, but also in the adrenal gland and kidney, plays a dominant role in the regulation of bile acid synthesis and bile salt transport. FXR belongs to the NR1 family of nuclear receptors. Studies where transfection of expression plasmids containing murine and human FXR into monkey kidney CV-1 or human hepatoma HepG2 cells was performed, demonstrated that CDCA is the most effective activator of FXR (Makishima et al., 1999). The other bile acids, such as lithocholic acid and deoxycholic acid were found to be less effective. FXR together with its heterodimeric partner RXR acts as a transcription factor for several bile salt transporters, such as the hepatic bile salt export pump Bsep (Ananthanarayanan et al., 2001), the ileal bile acid binding protein I-Babp (Grober et al., 1999) and MRP2 (Kast et al., 2002). Recently, it was shown that human OSTα/OSTβ expression is induced by bile acids through ligand-dependent transactivation of both OST genes by FXR (Landrier et al., 2005).

In addition, the expression of short heterodimeric protein (SHP-1), which acts as a transcriptional repressor, is itself regulated by FXR and can downregulate the expression of several genes including Ntcp (Denson et al., 2001) and cholesterol 7α- hydroxylase CYP7A1, the rate limiting enzyme in bile salt synthesis from cholesterol. Repression of the rat Ntcp gene occurred via SHP-mediated inhibition of retinoid activation of the RARα:RXRα element. Bile acid feeding of mice markedly upregulated the expression of SHP-1 mRNA while reducing the expression of CYP7a1 (Lu et al., 2000). Activation of bile acid synthesis is suppressed by the

nuclear receptor SHP-1 by binding and repressing the transcriptional activity of the nuclear receptor liver receptor homolog (LRH-1) (Goodwin et al., 2000) and hepatocyte nuclear factor (HNF) 4α (Lee et al., 2000b), which are essential activators of the bile acid synthetic enzymes CYP7A1 and CYP8B1. However, partial maintenance of negative feedback regulation of bile salt synthesis in SHP null mice indicates the existence of SHP independent pathways (Kerr et al., 2002).

1.3.2 Pregnane X receptor (PXR) and Constitutive androstane receptor (CAR)

The pregnane X receptor (PXR) also known as steroid X receptor (SXR) in humans and the constitutive androstane receptor (CAR) play besides FXR an important role in the regulation of bile salt transporters. Furthermore they act as activators of detoxifying proteins (e.g. cytochrome P450 enzymes or transporters) and they enhance metabolism of potentially toxic xenobiotics and other compounds. They promote the metabolism and excretion of lipophilic substances from the body. Ligands of PXR include rifampicin, RU486, St. Johns wort extract, clotrimazol, steroids, statins, phenobarbital, bile salts and bile acid precursors and for CAR xenobiotics and phenobarbital, respectively (Kullak-Ublick et al., 2004). After interaction between the receptor and a specific ligand (Kliewer et al., 1999), ligand binding induces a conformational change within the receptor that facilitates binding of co-activator proteins (e.g. RXR). This heterodimer regulates the transcription of the target gene by binding to specific DNA response elements (Renaud and Moras, 2000). Concretely, PXR binds with RXR as a heterodimer to a “xenobiotic response element” in the promoter of the human CYP3A4 gene (Blumberg et al., 1998).

Targeted disruption of the mouse PXR gene abolishes the ability of xenobiotics to induce CYP3A. The importance of PXR for the regulation of CYP3A was demonstrated by an abolished protective effect of pregnolone-16-α-carbonitrile (a potent inducer of CYP3A) in PXR null mice after feeding with lithocholic acid which produces severe hepatic necrosis in mice (Staudinger et al., 2001; Xie et al., 2001).

Interestingly, rifampicin which is another potent ligand for SXR and inducer of CYP3A has been shown to be effective in the treatment of symptoms of pruritus in cholestatic disease (Cancado et al., 1998). It has been speculated that rifampicin may stimulate 6-α-hydroxylation of bile acids, leading to glucuronidation by UDP-glucuronosyl transferases and excretion bile salts by alternative pathways in the urine (Bremmelgaard and Sjovall, 1979; Wietholtz et al., 1996; Araya and Wikvall, 1999).

PXR is mainly expressed in the liver and intestine and other target genes that are activated by these receptors include Pgp, MRP2, MRP3, and OATP2 (Kast et al., 2002; Staudinger et al., 2003; Wang and LeCluyse, 2003).

CAR which is constitutively and highly expressed in the liver binds DNA as heterodimer with RXRα. Upon stimulation by a ligand CAR translocates from the cytoplasma to the nucleus. CAR stimulates the expression of MRP2 (Kast et al., 2002), MRP3 (Cherrington et al., 2002) and CYP2B1 (Xiong et al., 2002).

Although PXR and CAR are involved in signalling pathways that protect the body from xenobiotics, their activation by drugs represents the molecular basis for an important class of drug-drug interactions. Assays that detect PXR activation during drug development are used to predict and prevent these drug-drug interactions (Moore and Kliewer, 2000). Most cases of such interactions are related to elevated P-gp expression that lead to a decrease of the plasma level of concomitantly administered P-gp substrates. Whereas paclitaxel activation of PXR was leading to enhanced P-gp mediated drug clearance, docetaxel did not activate PXR. In contrast, ET-743, another potent antineoplastic agent, suppressed MDR1 transcription by acting as an inhibitor of PXR (Synold et al., 2001). These examples demonstrate how the molecular activities of nuclear receptors can control drug clearance.

1.3.3 Peroxisome proliferator activated receptor α (PPAR α)

The nuclear receptor PPARα, a ligand activated transcription factor that regulates the expression of a number of genes involved in peroxisomal and mitochondrial β-oxidation of fatty acids, activates the apical sodium-dependent bile salt transporter (ASBT/SLC10A2) (Jung et al., 2002) and the hepatocyte canalicular phospholipids flippase Mdr2/MDR3 (ABCB4) (Kok et al., 2003). The ligands include fatty acids, fibrates, eicosanoids and NSAIDS (Desvergne and Wahli, 1999). Upon activation, PPARα binds as a heterodimer with the retinoid X receptor (RXR) to a peroxisome proliferator-response element (PPRE) located in the promoter region of target genes.

The observation that patients with type IV hypertriglyceridemia exhibited decreased intestinal bile salt absorption and reduced ileal expression of ASBT mRNA postulated a possible link between intestinal bile salt absorption via ASBT and hepatic fatty acid catabolism (Duane et al., 2000). PPARα was shown to play a critical role in the adaptive response to fasting in mice (Kroetz et al., 1998; Kersten et al., 1999; Leone et al., 1999) and PPARα also influences bile

acid composition by induction of the sterol 12α-hydroxylase, which acts at a branch-point in the bile acid synthetic pathway by catalizing the conversion of 7α-hydroxy-4-cholesten-3-one to 7α,12α-dihydroxy-4-cholesten-3-one. This reaction determines the ratio of cholic acid to chenodeoxycholic acid. (Hunt et al., 2000) In Hepa 1c1c7 cells, which were transiently transfected with an expression plasmid for PPARα and RXRα and a luciferase reporter construct containing copies of the rat PPRE, an inhibition of PPARα reporter gene expression was shown with increasing concentrations of chenodeoxycholic acid (CDCA) in the presence or absence of Wy-14,643 (=PPARα ligand) (Sinal et al., 2001). It was concluded that during certain pathophysiological states, where intracellular bile acid concentrations might be elevated, effects on PPARα-dependent target gene regulation are possible (Sinal et al., 2001). In addition, PPARα was shown to transactivate the human ASBT gene (Jung et al., 2002). Incubation of human hepatoma HepG2 cells with CDCA resulted in a significant induction of PPARα mRNA levels and hPPARα gene expression was upregulated by taurocholic acid in human primary hepatocytes (Pineda Torra et al., 2003).

In addition to these ligand-activated nuclear receptors, other factors such as the hepatocate nuclear factor (HNF) family of liver enriched transcription factors including HNF1α, HNF3β and CCAAT/enhancer binding protein (C/EBP), as well as sterol responsive element binding protein (SREBP) and nuclear factor kappa B (NF-κB) also appear to play an important role in the regulation of bile salt transporters (Trauner and Boyer, 2003).

Another interesting inductor of intestinal Na^+ dependent bile salt transport seems to be the glucocorticoid receptor (GR), a nuclear steroid receptor. The human ASBT gene has been shown to be transactivated by the GR and its ligands dexamethasone and budesonide (Jung et al., 2004).

1.3.4 Cholestasis

Cholestasis is an impairment of bile secretion which may result either from a functional defect in bile formation at the level of hepatocytes (hepatocellular cholestasis: autoimmune, metabolic, infectious, genetic disorders) or from an impairment in bile secretion (transmembrane transport systems in hepatocytes and cholangiocytes) or from disturbed structural and/or functional integrity of the bile secretory pathway (Trauner et al., 1998). Under cholestatic conditions with impaired bile secretion, the enterohepatic circulation is disrupted. In animal models, rats with common bile duct ligation (CBDL) showed an impaired expression and function of the hepatic uptake (Ntcp/Slc10a1, Oatps/SLC21a) and excretory systems (Bsep/Abcb11, Mrp2/Abcc2)

(Trauner et al., 2005). In obstructive cholestasis, an increased cholehepatic shunting, as shown by increased expression of cholangiocellular Asbt, removes stagnant bile salts from the obstructed ducts returning them to the systemic circulation. The impaired expression and function of hepatic uptake systems, reduced expression of Asbt in the kidney, a change associated with a diminished capacity to reabsorb bile salts from the glomerular filtrate as well as an up-regulation of Mrp2 protein expression on the apical membrane of the rat renal proximal tubule (Lee et al., 2001) facilitates the excretion of bile salts by this alternative excretory route. MRP2 is associated with an increased ability to excrete divalent organic anions such as bile salt sulfates and glucuronides (bilirubin) that accumulate during cholestasis and would therefore facilitate extrahepatic pathways for bile salt and bilirubin excretion during cholestasis. In the intestine of bile duct-ligated rats down-regulation of ASBT expression in the terminal ileum was shown. The intestinal absorption rate of taurocholate was lower and the absorption rate was inversely correlated to serum bile salt concentrations when compared to sham operated rats (Sauer et al., 2000).

Apart from species differences in bile acid composition and transporter gene regulation, a major obstacle for a direct extrapolation of rodent data to human data is the different duration of cholestasis in animal models (day to weeks) versus human cholestatic disorders (weeks to months/years). Findings from animal experimental models can not be unequivocally applied to human cholestatic diseases. Therefore, in this thesis the effect of an obstructed bile secretory pathway on the intestinal expression of bile salt transporters and on regulatory proteins was investigated in human subjects.

1.4 ABC transporters

The ATP-binding cassette (ABC) transporters represent a large and diverse superfamily of transmembrane proteins which bind ATP and use the energy to drive the transport of various molecules across cell membranes. A complete ABC transporter consist of transmembrane domains which anchor the transporter in the lipid bilayer and two ATP-binding domains, also known as nucleotide binding folds (NBFs). Proteins are classified based on the sequence and organization of their nucleotide binding folds (NBFs). The transporters share extensive sequence homology and domain organisation including the characteristic ATP-binding cassette. They are classified into seven subfamilies (ABCA to ABCG). Their main function is the unidirectional, energy dependent translocation of compounds from the cytoplasm to the outside of the cell or into an intracellular compartment (endoplasmic reticulum (ER), mitochondria, peroxisome) against a concentration gradient.

The first member discovered in 1976 (Juliano and Ling, 1976) was P-glycoprotein (MDR1; ABCC1). This protein appeared to be overexpressed in tumour cells with a multidrug resistance phenotype where it conferred resistance to many unrelated cytotoxic drugs. Later the existence of the multi-drug resistance associated proteins (MRPs; ABCC) was revealed. Some of these transporters are relevant for drug transport, as well as the recently discovered ABC transporter breast cancer resistance protein (BCRP; ABCG2).

In this thesis MDR1 (ABCB1), MRP1-5 (ABCC1-5) as well as BCRP (ABCG2) were of particular interest, since these proteins can have a major impact on drug absorption and disposition, extrusion of toxic compounds to the outside of the cell and are involved in the transport of endogenous substrates, among others bile acids, bilirubin or cholesterol (Gottesman et al., 2002).

1.4.1 MDR1 (ABCB1)

MDR1 is the best characterized ABC transporter. P-glycoprotein (P-gp), the gene product of MDR1 has a molecular weight of 170 kDa and consists of 12 transmembrane domains and two nucleotide-binding sites. P-gp is expressed on the apical membrane of normal tissues such as intestine, kidney, liver, adrenal gland and blood-brain barrier. By limiting absorption and enhancing the excretion of toxic compounds/metabolites P-gp is assumed to function as gatekeeper against toxic xenobiotics in the gut or in the blood-brain barrier (Tanigawara, 2000). P-gp is a transporter with extreme wide substrate specificity and many unrelated substances were identified as P-gp substrates. However, a tendency towards organic compounds with cationic or amphiphatic nature could be determined (Schinkel and Jonker, 2003). The high expression in solid tumours indicated the pivotal role of Pgp in clinical resistance to chemotherapy. Individual differences in expression and/or activity of MDR1 (P-gp) were shown to lead to changes in drug bioavailability (Lown et al., 1997).

Moreover, genetic variants (single nucleotide polymorphisms, SNPs) can alter P-gp expression and function, as well as may also predispose to certain diseases. To date 28 SNPs have been identified in the MDR1 gene, whereas 11 SNPs resulted in an amino acid exchange (Schwab et al., 2003). The most important is the C3435T polymorphism, for which it was found that about 25% of Caucasian subjects were homozygous for this polymorphism (Cascorbi et al., 2001). This polymorphism does not influence the amino acid sequence but is associated with an altered P-gp expression and function. On average, the TT homozygotes have a lower level of intestinal Pgp resulting in an increase of digoxin plasma levels, compared to the CC genotype group (Hoffmeyer et al., 2000). C3435T is also reported to be a risk factor for certain class of diseases

including inflammatory bowel disease, Parkinson's disease and renal epithelial tumour (Siegsmund et al., 2002; Schwab et al., 2003; Sakaeda et al., 2004).

Interactions are likely to occur in multidrug therapy as P-gp transports a wide range of structurally diverse drugs and a large number of drugs potentially influence MDR activity and even some drugs alter the expression of MDR1. It has been reported that P-gp inhibitors such as verapamil, itraconazole, ritonavir, and talinolol increased the plasma concentrations of the P-gp substrate digoxin due to inhibition of P-gp mediated efflux (Verschraagen et al., 1999; Westphal et al., 2000a; Angirasa and Koch, 2002; Ding et al., 2004). Additionally, P-gp has also been shown to be inducible *in vitro* and *in vivo* by xenobiotics such as rifampicin (Westphal et al., 2000b), Phenobarbital (Lu et al., 2004), dexamethasone (Fardel et al., 1993), and herbal extracts from St. John's wort (Zhou et al., 2004). Interestingly even components of our daily nutrition, e.g. grapefruit juice have been shown to influence MDR1 activity (Soldner et al., 1999; Wang et al., 2001a). Increased P-gp expression can therefore lead to subtherapeutic concentrations of concomitantly administered substrates.

MDR1 inhibitors are evaluated in clinical trials of chemotherapy to reduce multidrug resistance (Kornblau et al., 1997).

1.4.2 MRP1-5 (ABCC1-5)

The family of multi-drug resistance associated proteins (MRPs) is another important group of human ABC transporters that are relevant for drug transport. All of them possess the characteristic ATP binding cassette motive but they vary in the number of their transmembrane domains. So far, this subfamily includes nine members (MRP1-9). In contrast to P-gp, MRPs work mainly as transporters of amphiphatic organic anions. Therefore, they are capable to extrude drug conjugates, such as glucuronide-, glutathione-, and sulphate-conjugates out of cells.

MRP1 (ABCC1) is ubiquitously expressed in the body and is localised on the basolateral membrane of epithelial cell layers as well as in the ER and post-Golgi vesicles. Physiological important substrates for MRP1 include glutathione S-conjugates such as leukotriene C4, as well as glucuronate and sulphate conjugates, e.g. bilirubin glucuronides (Keppler et al., 1998). In addition, anionic drugs and drugs like methotrexate or arsenite are also transported by MRP1 (Bakos et al., 2000). MRP1 drug resistance phenotype overlaps with that of Pgp and is associated with resistance to anthracyclines, etoposide and vinca alkaloids.

MRP2 (ABCC2) is expressed in the liver, intestine, kidney, placenta and blood- brain barrier. It mediates the transport of drugs and conjugated compounds into bile, intestinal lumen and urine, respectively and therefore out of the body (Schaub et al., 1997; Kusuhara and Sugiyama, 2002). The substrate specificity of MRP2 is similar to that of MRP1, and includes glutathion conjugates, billirubin glucuronides, and a number of drugs and their conjugated drug metabolites (Jedlitschky et al., 1997; Kawabe et al., 1999). These drugs include temocaprilat, irinotecan, SN-38, arsenite, cisplatin, methotrexate, vincristine, saquinavir, and ceftriaxone (Kusuhara and Sugiyama, 2002; Dietrich et al., 2003). Similar to MDR1, MRP2 seems to be inducible by rifampicin treatment (Fromm et al., 2000), which indicates possible interactions in multidrug therapy. Influence on the MRP2 protein expression was also shown for tamoxifen (Kauffmann et al., 1998). In addition, MDR1 and MRP2 share some substrates as well as inhibitors, which may lead to interactions and influence the oral bioavailability of certain drugs. Similarly to MDR 1 some components of our daily diet, such as the flavonoid epicatechin in tea (Vaidyanathan and Walle, 2001), chrysin and its metabolites (Walle et al., 1999) were shown to be substrates of MRP2.

As MRP2 is expressed in the tips of the intestinal villi, which are atrophic in celiac sprue, the reduction of MRP2 protein expression might be associated as consequence with impaired clearing of MRP2 substrates (Dietrich et al., 2003).

Polymorphisms, as described for MDR1, have also been shown for MRP2 (Itoda et al., 2002a; Itoda et al., 2002b), but neither frequency nor influence on transporter activity or expression have yet been defined. In patients with the Dubin-Johnson syndrome MRP2 is completely absent in canalicular membranes of hepatocytes and apical membranes of enterocytes due to a nonsense mutation in the MRP2 gene resulting in truncation and degradation of the protein. The absence of this transporter in the hepatocyte canicular membrane leads to impaired biliary secretion of glutathione, glutathione conjugates, and bilirubin glucuronides (Paulusma et al., 1997). If individual differences in MRP2 gene and protein expression might have influence on the prevalence of certain intestinal diseases, such as Crohns disease and celiac sprue is currently under inverstigation.

MRP3 (ABCC3), like MRP1, is present on the basolateral membrane of polarized cells, mainly in liver, intestine and kidney (Scheffer et al., 2000). Substrates of MRP3 include glucuronate conjugates. MRP3 transports a wide range of bile salts and seems to be involved in their reabsorption to the portal blood on the basolateral membrane of the intestinal epithelial cells

(Hirohashi et al., 2000). MRP3-transfection of cell lines conferred resistance to epipodophyllotoxins, vincristine and methotrexate (Kool et al., 1999). Therefore, MRP3 may also contribute to a protective function by excreting a range of toxic substances from various epithelial cell types.

MRP4 (ABCC4) and MRP5 (ABCC5) are 2 structurally similar members of the MRP family and are both capable of transporting therapeutic nucleoside based compounds (Schuetz et al., 1999; Lee et al., 2000a; Chen et al., 2001).

For **MRP4**, there are no definite data concerning cellular localization or tissue distribution. For instance, it has been reported that MRP4 is located on the basolateral membrane of prostate cells (Lee et al., 2000a), whereas others showed MRP4 expression on the apical membrane of kidney cells (van Aubel et al., 2002). The significance of MRP4 in drug transport is at present unclear as well. However, an over-expression of MRP4 severely impaired the antiviral efficacy of adefovir, azidothymidine, 9-(2-phosphonylmethoxyethyl)adenine (PMEA) and of other nucleoside analogs in PMEA-resistant lymphoblastoid cell line (Schuetz et al., 1999). This finding has two potential clinical significances and may affect therapeutic response. First, MRP4 can decrease the intracellular concentration of the respective antiretroviral drug, which leads to impaired suppression of HIV replication. Second MRP4 overexpression in cells lines can protect from the cytotoxic effects of antiretroviral drugs.

MRP4 can be considered to be an organic anion transporter, as is expected for an MRP family member. Other substrates include folic acid, bile acids, methotrexate and 6-mercaptopurine (Wielinga et al., 2002). A physiological role of MRP4 might be the release of prostaglandins from cells (Reid et al., 2003).

MRP5 (ABCC5) is widely expressed throughout most tissues. Like MRP4, it has an affinity to nucleotidebased substrates. A study demonstrated that MRP5 transports the cyclic nucleotides cAMP and cGMP (Jedlitschky et al., 2000), but the physiological function of this transporter remains to be elucidated. There are no reports at present, which could suggest a role for MRP5 in drug disposition. Experiments with transfected cells showed enhanced efflux of DNP-SG (2,4-dinitrophenyl-S-glutathione), adefovir, and the purine analogues 6-mercaptopurine and thioguanine (Wijnholds et al., 2000).

1.4.3 ABCG2 (BCRP)

Human breast cancer resistance protein (BCRP, ABCG2), which belongs to the ABC transporter family, was discovered and cloned by Doyle et al. from a doxorubicin-resistant MCF7 breast

cancer cell line (MCF7/AdrVp) (Doyle and Ross, 2003). Structurally, BCRP is a half-transporter (one nucleotide-binding domain, 6 transmembrane domains) and it seems very likely that it functions as a homodimer (Ozvegy et al., 2001). Whether BCRP can also function as a heterodimer with other halftransporters of the ABCG class is not known. BCRP is expressed in different tissues, among others in the bile canalicular membrane of hepatocytes and in the apical membrane of intestinal epithelial cells (Doyle and Ross, 2003). It is a transporter with a wide substrate specificity recognizing molecules of either negative or positive charge, organic anions and sulphate conjugates. Antitumor agents have been widely examined and BCRP can render tumor cells resistant to the anticancer drugs topotecan, mitoxantrone, doxorubicin, and daunorubicin (Jonker et al., 2000). Also, BCRP mediates apically directed drug transport, appears to reduce drug bioavailability, and protects fetuses against drugs (Jonker et al., 2000). Recently, BCRP induction in human T-cells was observed after prolonged exposure to sulfasalazine. In the same study enhanced TNFα release and an insufficient inhibition of TNFα production by sulfasalazine was demonstrated, suggesting that drug resistance might also be induced by anti-inflammatory agents such as sulfasalazine (van der Heijden et al., 2004a; van der Heijden et al., 2004b).

1.5 Solute carrier (SLC)

The SLC (Solute Carrier) family includes ion coupled transporters, facilitated transporters, and exchangers. The genes encoding these transporters are divided into 43 gene families (SLC1-43, according to the HUGO Gene Nomenclature Committee) and include 298 transporter genes at present (Hediger et al., 2004). These SLC membrane proteins use cellular chemical and/or electrical gradients to move molecules across cell membranes. Physiologically, they transport many endogenous substances such as amino acids, glucose, bicarbonate, bile acids, ascorbic acid, urea or fatty acids. However, members of this superfamily can also be involved in drug transport and play a role in drug disposition. Many of them are expressed in important organs for drug disposition such as kidney, liver, and intestine. Here, the expression and regulation of the apical sodium dependent bile salt transporter (ASBT, SLC10A2) was investigated, which represents a critical component of the enterohepatic circulation of bile salts.

1.5.1 Na^+/ taurocholate cotransporting polypeptide (NTCP; SLC10A1), Apical sodium dependent bile salt transporter (ASBT, SLC10A2)

NTCP and ASBT belong to the same family of solute carriers. By reabsorbing bile salts from the blood, bile, glomerular filtrate as well as intestinal lumen they are critical determinants of the

enterohepatic circulation of bile salts. Both are cotransporters that mediate sodium-dependent bile salt uptake into hepatocytes (NTCP), cholangiocytes, enterocytes and renal proximal tubular cells (ASBT), respectively.

ASBT is a membrane glycoprotein which consists of 348 amino acids and is expressed on the apical membrane of enterocytes in the terminal ileum, of proximal renal tubular cells and of cholangiocytes. Human ASBT transports conjugated and unconjugated bile salts with a higher affinity for CDCA and DCA than for taurocholate (Craddock et al., 1998). Subjects with mutations in the ASBT gene suffer from congenital diarrhea and steatorrhea, due to bile salt malabsorption (Oelkers et al., 1997) and pharmacological inhibition of the transport activity leads to interruption of enterohepatic circulation of bile salts with changes in the cholesterol and bile acid homeostasis (Lewis et al., 1995; Huff et al., 2002; West et al., 2002; Bhat et al., 2003; Telford et al., 2003). Adaptive induction of ASBT mRNA expression, transporter protein and transport activity was observed in cholic acid fed rats (Stravitz et al., 1997), whereas decreased ASBT expression was found in the ileum of bile duct ligated rats (Sauer et al., 2000).

The transcriptional regulation of bile salt transporters, as already described in Chapter 3 is very complex, and the intracellular factors that influence ASBT gene expression remain largely unknown. One such potential factor might be the nuclear receptor PPARα, a ligand activated transcription factor that regulates the expression of a number of genes involved in peroxisomal and mitochondrial β-oxidation of fatty acids. PPARα binds as a heterodimer with the retinoid X receptor (RXR) to a peroxisome proliferator-response element (PPRE) located in the promoter region of target genes. PPARα was shown to play a critical role in the adaptive response to fasting in mice (Kroetz et al., 1998; Kersten et al., 1999; Leone et al., 1999) and PPARα also influences bile acid composition by induction of the sterol 12α-hydroxylase, which determines the ratio of cholic acid to chenodeoxycholic acid (Hunt et al., 2000). Recently, PPARα was shown to transactivate the human ASBT gene (Jung et al., 2002). In addition, hPPARα gene expression was upregulated by taurocholic acid in human primary hepatocytes (Pineda Torra et al., 2003).

NTCP is a membrane glycoprotein which consists of 349 amino acids and shares about 35% amino acid identity with the human ASBT. The human NTCP is expressed on the basolateral membrane (sinusoidal membrane) of human hepatocytes and promotes by its high affinity for conjugated bile salts the extraction of bile salts from portal blood into the hepatocytes. NTCP was not investigated in this thesis. Additional informations are provided in a review by Hagenbuch et al. (Hagenbuch and Dawson, 2004).

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2. Material and Methods

2.1 Absolute quantification of transporter mRNA expression (quantitative real-time RT-PCR)

2.1.1 Introduction

Reverse transcription polymerase chain reaction (RT-PCR) is the most common method for analysing mRNA expression patterns and comparing mRNA levels in different samples. In this thesis, real time RT-PCR (TaqMan®) was used to measure gene expression in human tissue or cell lines. With this method the specific gene products generated during each cycle of the PCR can be reliably measured and are directly proportionate to the amount of template prior to the start of the PCR. Prior to PCR amplification the isolated cellular mRNA was reverse transcribed into cDNA which subsequently was quantified with TaqMan® analysis using the standard curve method. By using external standards that comprise known amounts of specific cDNA fragments of the gene of interest, the unknown amount of cDNA in the analysed samples could be expressed as absolute transcript numbers of the corresponding gene.

2.1.2 Real-time PCR (TaqMan® assay)

The 5`nuclease assay or TaqMan® assay is a highly sensitive method to determine mRNA levels quantitatively. This method uses a target specific oligonucleotide, the TaqMan probe, which anneals between the forward and reverse primer sites. The probe carries a reporter dye on the 5`end (6-carboxy-fluorescein) and a quencher dye on the 3` end (6-carboxy-tetramethyl-rhodamine). As long as the probe is intact the fluorescence of the reporter dye is suppressed by the quencher dye. However, during the PCR the DNA polymerase (Taq polymerase) cleaves the probe due to its 5`-3` exonuclease activity. Now, a fluorescent signal is generated because the reporter dye is separated from the quencher dye. Consequently, there is an increase of fluorescence after each PCR cycle. With the ability to measure the PCR products as they are accumulating, in "real time," it is possible to measure the amount of PCR product at a point in which the reaction is still in the exponential range. It is only during this exponential phase of the PCR reaction that it is possible to extrapolate back to determine the starting amount of template. During the exponential phase in real-time PCR experiments, a fluorescence signal threshold is determined at which point all samples can be compared. Therefore, the number of PCR cycles required to generate enough fluorescent signal to reach this threshold is defined as the *cycle threshold*, or Ct. These Ct values are directly proportionate to the amount of starting template

and are the basis for calculating mRNA expression levels. The *baseline* is defined as the PCR cycles in which a signal is accumulating but is beneath the limits of detection of the instrument.
For each study, TaqMan experiments were carried out either on a Gene Amp 5700 Sequence Detector using 96 well plates with total reaction volumes of 25 µL, or on a 7900HT Sequence Detection System using 384 well plates with total reaction volumes of 10 µL (all Applied Biosystems, Rotkreuz, Switzerland). PCR conditions were throughout 10 min 95℃ followed by 40 cycles of 15 s 95℃ and 1 min 60℃. TaqMan Unive rsal PCR Mastermix (Applied Biosystems) was used. Each reaction contained 1 ng/µL cDNA and the concentrations of primers and probes were 900 nM and 225 nM, respectively.

Gene	Probe	start	length	Tm
MDR1	5`-AAGCTGTCAAGGAAGCCAATGCCTATGACTT-3`	1929	31 bp	69.0 ℃
MRP1	5`-CCTCCACTTTGTCCATCTCAGCCAAGAG-3`	2267	28 bp	69.0 ℃
MRP2	5`-CTCAATATCACACAAACCCTGAACTGGCTG-3`	3773	30 bp	68.0 ℃
MRP3	5`-CCAACCGGTGGCTGAGCATCG-3`	3608	21 bp	69.0 ℃
MRP4	5`-CAAACCGAAGACTCTGAGAAGGTACGATTCCT-3`	2094	32 bp	68.4 ℃
MRP5	5`-CTGACGGAAATCGTGCGGTCTTGG-3`	804	24 bp	69.0 ℃
Villin	5`-TCATCAAAGCCAAGCAGTACCCACCAAG-3`	977	28 bp	69.2 ℃
GAPDH	5`-CGCCTGGTCACCAGGGCTGC-3`	79	20 bp	69.0 ℃
BCRP	5`-CCATTGCATCTTGGCTGTCATGGCTT-3`	1883	26 bp	69.4 ℃
ASBT	5`-TTCAGCTCTCCTTCACTCCTGAGGAGCTC-3`	1419	29 bp	69.0 ℃
PPARα	5`-AGGCTGCAAGGGCTTCTTTCGGC-3`	570	23 bp	69.0 ℃

Gene	Forward Primer	start	length	Tm
MDR1	5`-CTGTATTGTTTGCCACCACGA-3`	1854	21 bp	58.0 ℃
MRP1	5`-GGGCTGCGGAAAGTCGT-3`	2236	17 bp	58.0 ℃
MRP2	5`-ACTGTTGGCTTTGTTCTGTCCA-3`	3746	22 bp	58.4 °C
MRP3	5`-GGTGGATGCCAACCAGAGAA-3`	3567	20 bp	59.0 ℃
MRP4	5`-AAGTGAACAACCTCCAGTTCCAG-3`	2026	23 bp	58.3 ℃
MRP5	5`-CTGCAGTACAGCTTGTTGTTAGTGC-3`	768	25 bp	59.0 ℃
Villin	5`-CATGAGCCATGCGCTGAAC-3`	957	19 bp	59.9 ℃
GAPDH	5`-GGTGAAGGTCGGAGTCAACG-3`	42	20 bp	59.0 ℃
BCRP	5`-CAGGTCTGTTGGTCAATCTCACA-3`	1859	23 bp	58.7 ℃
ASBT	5`-ACGCAGCTATGTTCCACCATC-3`	1397	21 bp	59.0 ℃
PPARα	5`-CATTACGGAGTCCACGCGT-3`	547	19 bp	58.0 ℃

Gene	Reverse Primer	start	length	Tm
MDR1	5`-AGGGTGTCAAATTTATGAGGCAGT-3`	1992	24 bp	59.0 ℃
MRP1	5`-AGCCCTTGATAGCCACGTG-3`	2315	19 bp	57.0 ℃
MRP2	5`-CAACAGCCACAATGTTGGTCTCTA-3`	3845	24 bp	60.0 ℃
MRP3	5`-GCAGTTCCCCACGAACTCC-3`	3651	19 bp	59.0 ℃
MRP4	5`-GGCTCTCCAGAGCACCATCT-3`	2144	20 bp	58.0 ℃
MRP5	5`-TCGGTAATTCAATGCCCAAGTC-3`	860	22 bp	59.8 ℃
Villin	5`-TCATTCTGCACCTCCACCTGT-3`	1028	21 bp	59.2 ℃
GAPDH	5`-ACCATGTAGTTGAGGTCAATGAAGG-3`	164	25 bp	59.0 ℃
BCRP	5`-TCCATATCGTGGAATGCTGAAG-3`	1936	22 bp	58.7 °C
ASBT	5`-GCGGGAAGGTGAATACGACA-3`	1469	20 bp	60.0 ℃
PPARα	5`-ACACCAGCTTGAGTCGAATCG-3`	616	21 bp	59.0 ℃

Table 2.1: Sequence, starting position, length (base pairs), and melting temperature of the primers and probes that were used for TaqMan analysis.

Primers and probes (Table 2.1) were designed according to the guidelines of Applied Biosystems with help of the Primer Express 2.0 software. Primers were synthesized by Invitrogen (Basel, Switzerland), probes by Eurogentec (Seraing, Belgium).
All samples in this thesis were run in triplicates. Not reverse transcribed RNA served as negative control. No significant amplification was observed in these samples.
In Chapter 3.1 to 3.4 for each sample, the number of gene transcripts (ASBT, BCRP, MDR1, MRP1-5, PPARα) and the number of villin transcripts were determined. By calculating the ratio of gene/villin mRNA, the gene expression was normalized to the enterocyte content. Determination of villin, an enterocyte specific, constitutively expressed protein can be used to control for variation of enterocyte content in biopsies (Lown et al 1997, Taipalensuu et al 2001).
In Chapter 5 gene expression of MDR1, BCRP, MRP1-5 was normalized to GAPDH, a gene which occurs ubiquitary in human tissues.

2.1.3 Generation of cDNA standards for absolute mRNA quantification

In order to generate standard curves gene-specific cDNA fragments with known concentrations as standards were used. These standards serve as a template during the real-time PCR because they cover the TaqMan primer/probe area and therefore they are amplified similar to the cellular reverse transcribed mRNA of the appropriate gene. Standards were obtained by PCR amplification using primers that anneal outside the area where the TaqMan primers anneal on the template. Since MDR1, MRP1-5, Villin, and ASBT are expressed in Caco-2 cells and BCRP is expressed in BB19 cells, reverse transcribed RNA of these cell lines as a template for PCR amplification was used. Each reaction contained 25 ng cDNA and 300nm of each primer in a total reaction volume of 25µL. The primers (Table 2.2) were designed using the primer express software 2.0 (Applied Biosystems) and were manufactured by Invitrogen (Basel, Switzerland). The components of the PCR reaction (AmpliTaq Gold, 10x PCR buffer, dNTPs, MgCl2) were purchased form Applied Biosystems. Thermal cycling was conducted using a Mastercycler personal from Eppendorf (Hamburg, Germany) and an annealing temperature of 55℃ was used. The PCR products were purified by running a 1.5% agarose gel (TAE buffer, 100V, 50 min) and by a subsequent gel extraction (gel extraction kit, Qiagen).
The obtained standards were quantified using the PicoGreen® dsDNA quantitation kit according to the manufacturers protocol (Molecular Probes, Eugene, OR). The PicoGreen® reagent is an ultrasensitive fluorescent nucleic acid stain for quantitating double-stranded DNA using bacteriophage lambda DNA as a standard. The amount of cDNA in the sample was expressed as ng DNA per mL.

Additionally, the purified and quantified PCR products were analysed by sequencing (Microsynth GmbH, Balgach, Switzerland). The received sequences were aligned to the genes of interest using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) in order to confirm the identity of the PCR products. For further calculations the molecular weights of the cDNA fragments (Table 2.3) were determined on the basis of the corresponding sequence with the help of a biopolymer calculator (http://paris.chem.yale.edu/extinct.frames.html).

primer	sequence	start	length	Tm
MDR1 forward	5`-ACAGTCCAGCTGATGCAGAGG-3`	1730	21 bp	59.1 ℃
MDR1 reverse	5`-CCTTATCCAGAGCCACCTGAAC-3`	2150	22 bp	58.7 ℃
MRP1 forward	5`-CACACTGAATGGCATCACCTTC-3`	2173	22 bp	59.1 ℃
MRP1 reverse	5`-CCTTCTCGCCAATCTCTGTCC-3`	2489	21 bp	59.8 ℃
MRP2 forward	5`-CCAATCTACTCTCACTTCAGCGAGA-3`	3509	25 bp	60.0 ℃
MRP2 reverse	5`-AGATCCAGCTCAGGTCGGTACC-3`	3981	22 bp	60.5 ℃
MRP3 forward	5`-TCTATGCAGCCACATCACGG-3`	3419	20 bp	59.3 ℃
MRP3 reverse	5`-GTCACCTGCAAGGAGTAGGACAC-3`	3746	23 bp	58.8 ℃
MRP4 forward	5`-AAGTGAACAACCTCCAGTTCCA-3`	2026	22 bp	57.3 ℃
MRP4 reverse	5`-CCGGAGCTTTCAGAATTGAC-3`	2543	20 bp	56.1 ℃
MRP5 forward	5`-CTAGAGAGACTGTGGCAAGAAGAGC-3`	570	25 bp	59.0 ℃
MRP5 reverse	5`-AAATGCCATGGTTAGGATGGC-3`	902	21 bp	59.6 ℃
Villin forward	5`-AGAAAGCCAATGAGCAGGAGAA-3`	926	22 bp	59.1 ℃
Villin reverse	5`-ATGGATGTGGCATCGAACTTC-3`	1163	21 bp	58.5 ℃
GAPDH forward	5`-ACATCGCTCAGAACACCTATGG-3`	16	22 bp	58.0 ℃
GAPDH reverse	5`-GCATGGACTGTGGTCATGAGTC-3`	572	22 bp	59.0 ℃
BCRP forward	5'-TTTCAGCCGTGGAACTCTTT-3'	1529	20 bp	56.2 ℃
BCRP reverse	5'-TGAGTCCTGGGCAGAAGTTT-3'	1990	20 bp	56.0 ℃
ASBT forward	5`-CATCTCTGGTTGCTCTCGTTGTTC-3`	1098	24 bp	61.1 ℃
ASBT reverse	5`-TGATGTCTACTTTTCGTCAGGTTGAA-3`	1651	26 bp	60.0 ℃
PPARα forward	5`-AGGAAGCTGTCCTGGCTCAG-3`	381	20 bp	58.2 ℃
PPARα reverse	5`-CGTCCAAAACGAATCGCG-3`	734	18 bp	59.8 ℃

Table 2.2: Sequence, starting position, length (base pairs), and melting temperature of the primers that were used for the generation of gene-specific cDNA standards.

amplicon	gene name	accession number	length	molecular weight
MDR1	ABCB1	NM_000927	421 bp	130377.2 g/mol
MRP1	ABCC1	NM_004996	317 bp	97797.4 g/mol
MRP2	ABCC2	NM_000392	473 bp	145684.6 g/mol
MRP3	ABCC3	NM_020038	328 bp	100912.6 g/mol
MRP4	ABCC4	NM_005845	518 bp	159304.6 g/mol
MRP5	ABCC5	NM_005688	333 bp	102594.6 g/mol
Villin	VIL1	NM_007127	238 bp	73486.6 g/mol
GAPDH	GAPDH	M17851	557 bp	171314.4 g/mol
BCRP	ABCG2	NM_004827	462 bp	142064.4 g/mol

ASBT	SLC10A2	NM_000452	554 bp	170455.8 g/mol
PPARα	PPARA	BC000052	353 bp	109082.8 g/mol

Table 2.3: Gene name, gene bank accession number, length (base pairs) and molecular weight of the PCR amplicons that were used as standards for TaqMan analysis.

2.1.4 Standard curve method

A standard curve for each gene on each plate is essential for accurate quantification of mRNA transcript numbers. The standard curves were generated by a serial dilution of cDNA standard solutions with known amount of PCR template. To obtain standard curves that span the range above and below the amount of the unknown samples, the quantified standard solutions were first analysed in TaqMan assays and adapted by further dilutions (= **standard dilution** in equation 1) so that the obtained curves were adequate.

Linear standard curves were composed by plotting the Ct values of the standards against the log of their corresponding serial dilution factor. Slope and Y-intercept of the standard curve line were then calculated by linear regression. By measuring the Ct value of the unknown sample under the same conditions, its corresponding serial dilution factor (= **X** in equation 1) could then be determined.

Based on this serial dilution factor (X) the number of cDNA molecules of the analysed gene in the sample (transcript number) could be estimated. Therefore, the number of cDNA fragments in the applied standard solution (standard 1) was calculated and subsequently multiplied with the serial dilution factor (X) of the sample. Usually, the transcript number is normalised to 1 µg RNA. The following equation (equation 1) shows how the transcript number per µg RNA was calculated.

$$\textbf{Transcript number µg total RNA} = \frac{\textbf{C x V x N x X}}{\textbf{standard dilution x MW x 1x1012}}$$

Equation 1: C (ng/mL) is the concentration of the standard determined with the PicoGreen® assay. **V** (µL) is the volume of sample cDNA that contains 1 µg of reverse transcribed RNA. This is 100 µL for the common cDNA concentration of 10 ng/µL. **N** is Avogadro`s number (6.022x1023 molecules per mol). **X** is the serial dilution factor of the sample determined with the standard curve. The **standard dilution** describes how-fold the standard 1 has been diluted for adapting the standard curve. **MW** (g/mol) is the molecular weight of the standard. **1x1012** accounts for conversions of units.

2.2 Immunohistochemical assessment of BCRP and ASBT in human intestinal biopsies

Immunohistochemistry was used to evaluate the expression of the apical sodium-dependent bile acid transporter (ASBT/SLC10A2) and of breast cancer resistance protein (BCRP/ABCG2) in human intestinal tissue.

For immunohistochemical assessment of ASBT expression in human intestinal tissue a polyclonal rabbit anti-human ASBT (generous gift from Dr. P.A. Dawson, Wake Forest University Baptist Medical Center, Winston-Salem, NC) 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.

BCRP monoclonal antibody BXP-21 was purchased from Alexis Biochemicals (Lausen, Switzerland).

Human intestinal tissue was mounted in OCT compound (Sakura Finetek, Zooterwoude, The Netherlands), frozen in liquid nitrogen and stored at -70℃. Sections (5 µm) of human intestine were air dried overnight and a periodate-lysine-paraformaldehyde solution (3%) was used for post-fixation. Then the sections were washed with washing solution (TBS/NaCl, Tween 0.05%) and incubated with normal horse serum (for BCRP incubation) or with goat serum (for ASBT incubation) for 30 min at room temperature as blocking solution. Then, the tissue sections were incubated either with a 1:40 dilution of the BCRP monoclonal antibody BXP-21 (Alexis Biochemicals, Lausen, Switzerland) or with a 1:400 dilution of the polyclonal rabbit anti-human ASBT overnight at 4℃.

Samples were washed three times with washing solution and incubated with the horse antimouse IgG secondary antibody (for BCRP incubation) or with the goat anti-rabbit IgG secondary antibody (for ASBT incubation) for 30 min at room temperature, respectively. After three washes with the washing solution, a perhydral solution (H_2O_2 (0.3%), sodium azide (0.1%) in PBS) was used to destroy the endogenous peroxidase activity. The staining was performed with the avidin/biotinylated enzyme complex (ABC method) according to the manufacturer`s instructions (Vectastain, Elite kit, Vector Laboratories, Burlingane, CA, USA). For detection 3-amino-9-ethylcarbazole (AEC), which forms a red end product, was used (Biogenex, San Ramon, CA, USA).

Sections, which served as negative controls were incubated only with the horse antimouse IgG or with the goat anti-rabbit IgG secondary antibody, respectively. Biopsies from normal terminal ileum were used as positive control.

2.3 Determination of bilirubin and bile acid plasma concentrations

Blood samples from subjects were obtained shortly before 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 extracted with Bond-Elut C18 cartridges (Analytichem International, San Diego, CA), solvolysis was performed to cleave sulfate 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 x 0.33 mm, Chrompack, Middelburg, The Netherlands). Hydrogen was the carrier gas (P=0.6 kg/cm^2). A temperature program from 140°C to 270°C with 8°/min was started after on-column injection. Eluting bile acid derivates were detected by a flame ionization detector. Fasting plasma samples were stored at - 20°C until analyzed.

3. Expression of bile acid transporters in the gut and their adaptive regulation in patients with obstructive cholestasis

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

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3.1.1 Abstract

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 an adaptive regulation of the human intestinal ASBT exists during obstructive cholestasis is not known.

Human ASBT mRNA expression along the intestinal tract was analyzed by real time PCR in biopsies of 14 control subjects undergoing both gastroscopy and colonoscopy. Their duodenal ASBT mRNA expression was compared to 20 patients with obstructive cholestasis. Additionally, in 4 patients with obstructive cholestasis, duodenal ASBT mRNA expression was measured after reconstitution of bile flow.

Normalized ASBT expression in control subjects was highest (mean arbitrary units± SEM) in the terminal ileum 1010 ± 330. Low ASBT expression was found in the colonic segments (8.3±5, 4.9±0.9, 4.8±1.7 and 1.1±0.2, ascending, transverse, descending, and sigmoid colon, respectively). Duodenal ASBT expression of control subjects was found with 171.8±20.3 at about four fold higher levels when compared to 37.9±6.5 ($p<0.0001$) in patients with obstructive cholestasis. Individual ASBT mRNA expression was inversely correlated with bile acid and bilirubin plasma concentrations. In 4 cholestatic patients average ASBT mRNA increased from 76±18 before to 113±18 after relief of cholestasis (NS). Immunohistochemical assessment indicates that ASBT protein is expressed on the apical surface of the duodenal epithelial cells.

Obstructive cholestasis in humans leads to down-regulation of ASBT mRNA expression in the distal part of the human duodenum.

3.1.2 Introduction

Bile acids are amphipathic steroidal compounds derived from the enzymatic catabolism of cholesterol in the liver by cytochrom 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 the release of gastrointestinal peptides (Koop et al., 1996). Through a 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 (Meier and Stieger, 2002). One of these transport proteins, the apical sodium-dependent bile acid transporter ASBT (SLC10A2) has been detected in the ileum, cecum, and kidney (Craddock et al., 1998) 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 (Craddock et al., 1998). Subjects with mutations in the ASBT gene suffer from congenital diarrhea and steatorrhea, due to bile acid malabsorption (Oelkers et al., 1997). Pharmacological inhibition of ASBT leads to interruption of enterohepatic circulation of bile acids with changes in the cholesterol and bile acid homeostasis (Lewis et al., 1995; Huff et al., 2002; West et al., 2002; Bhat et al., 2003; Telford et al., 2003). Adaptive induction of ASBT mRNA expression, transporter protein and transport activity was observed in cholic acid fed rats (Stravitz et al., 1997), whereas decreased ASBT expression was found in the ileum of bile duct ligated rats.(Sauer et al., 2000) In addition in Wistar rats, a marked reduction of ASBT protein expression was observed in microsomal membrane fractions from whole kidney after ligation of the common bile duct (CBDL) with the consequence of increased urinary bile acid excretion. These results indicate that ASBT is adaptively regulated in different tissues during obstructive cholestasis in the rat.

Lanzini et al. (Lanzini et al., 2003) studied the effects of cholestasis on intestinal bile acid transport in 14 subjects with chronic cholestasis due to primary biliary cirrhosis (PBC) before and during ursodeoxycholic acid (UDCA) administration. Prolonged retention of the bile acid analogue ^{75}Se-homocholic acid taurine (^{75}SeHCAT) in patients with PBC was observed compared to healthy controls and patients with Crohn`s disease. The retention of ^{75}SeHCAT decreased with UDCA treatment inferring that luminal bile acid levels might 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 the expression of ASBT in the duodenum of healthy subjects and compare the results to patients with obstructive cholestasis. Because direct analysis of the ASBT gene expression in samples from the terminal ileum can not be performed in humans with obstructive cholestasis due to obvious ethical reasons, we analyzed in the first part of this study ASBT mRNA expression in healthy subjects in different segments of the human intestine. We found that ASBT mRNA is also expressed in the duodenum, however to a lesser extent than in the terminal ileum.

Additionally, we analyzed the gene expression of multidrug resistance associated protein 3 (MRP3), which is expressed on the basolateral membrane of the enterocyte (Rost et al., 2002) and which is capable of transporting bile salts (Hirohashi et al., 2000), as well as the expression

of multidrug resistance associated protein 2 (MRP2). Human duodenal MRP2 protein expression was downregulated during cholestasis (Dietrich et al., 2004). Biopsies of the duodenum of patients with obstructive cholestasis were obtained during a therapeutic endoscopic retrograde cholangiopancreatography (ERCP).

3.1.3 Patients and Methods

Patients

14 healthy subjects (7 males, 7 females) were enrolled into the first part of the study after giving informed consent. The indication for combined upper and lower GI tract endoscopy was a cancer screening programme. Biopsies were obtained from the duodenum, the 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 carcinoma of the pancreatic head, 4 had cholangiocarcinoma (Klatskin tumour) and two had metastatic diseases. 10 patients had benign diseases (8 patients with choledocholithiasis and two patients with a benign stenosis of the common bile duct). Obstructive jaundice was defined 1) on the basis of chemical parameters (bilirubin, γ-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 cited parameters. Patients were only included, if the subject was not taking any medication known to affect ASBT, MRP2 as well as MRP3 expression. Demographic details are given in Table 1.

Table 1: Patient characteristics of the two study groups

Characteristics	**Icteric patients** (n=20)	**Controls** (n=14)	**p-value**
Age mean (±SEM)	67.4 (±2.8)	59.8 (±2.7)	NS
BMI (kg/m2) (±SEM)	24.1 (±0.8)	28.4 (±1.6)	p= 0.015
Gender	10 males and 10 females	7 males and 7 females	NS
Diagnosis	10 T/10 BS	3G/ 3E/ 8U	
Bilirubin (µmol/l)	235 (42-640)	11 (5-17)	P< 0.001
Bile acids (µmol/l)	122.6 (20.5-401)	1.7 (0.5-3.2)	P< 0.001

T = tumor, BS = biliary stone; G = mild gastritis, E = mild esophagitis, U = macroscopically unaffected mucosa

Material

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

Real-time polymerase chain reaction analysis of human ASBT, MRP2, MRP3, PPAR-α and Villin (VIL1) mRNA (TaqMan assay) were shown in Chapter 2

Primers and Probes for TaqMan Analysis see Table 2.1

Statistical analysis

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

3.1.4 Results

3.1.4.1 Expression pattern of ASBT mRNA in the human intestine

Human ASBT mRNA expression was studied in 14 control subjects (7 women and 7 men), who were undergoing a combined gastroscopy and colonoscopy. The results were normalized by calculation of the ASBT/villin ratio. The normalized ASBT expression ± 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 (Figure 1).

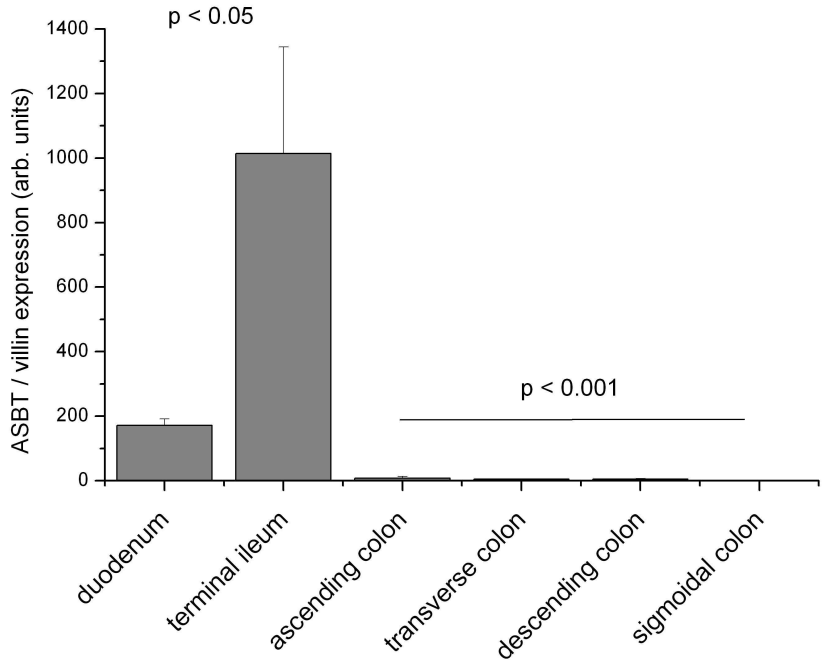


Figure 1: mRNA expression of ASBT in different gut segments. Data represents means (±SEM) of biopsies from 14 healthy subjects

3.1.4.2 Duodenal expression of ASBT mRNA and PPARα mRNA

An 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 to the levels obtained in 14 control subjects. As shown in Figure 2, ASBT mRNA expression (ASBT/villin ratio ± SEM) was about four-fold lower in patients with obstructive cholestasis (37.9 ± 6.5) when compared with control subjects (171.8 ± 20.3) ($p < 0.001$).

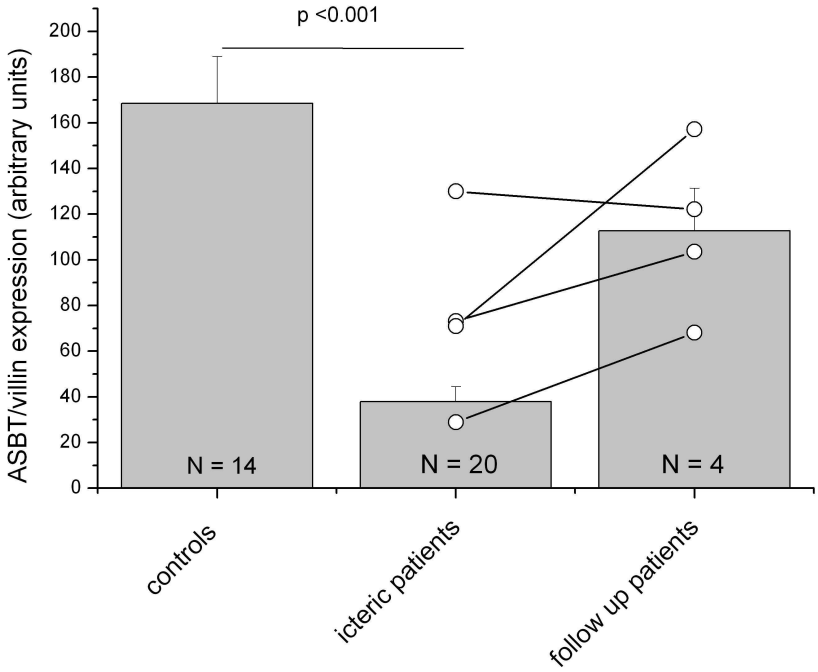


Figure 2: Comparison of ASBT mRNA expression in patients with obstructive cholestasis (N = 20) vs. control subjects (N = 14) and follow up patients (N = 4). Data represent means ± SEM. Scatter plot: lines connect individual ASBT mRNA expression pre- and post reconstitution of bile flow in 4 follow-up patients.

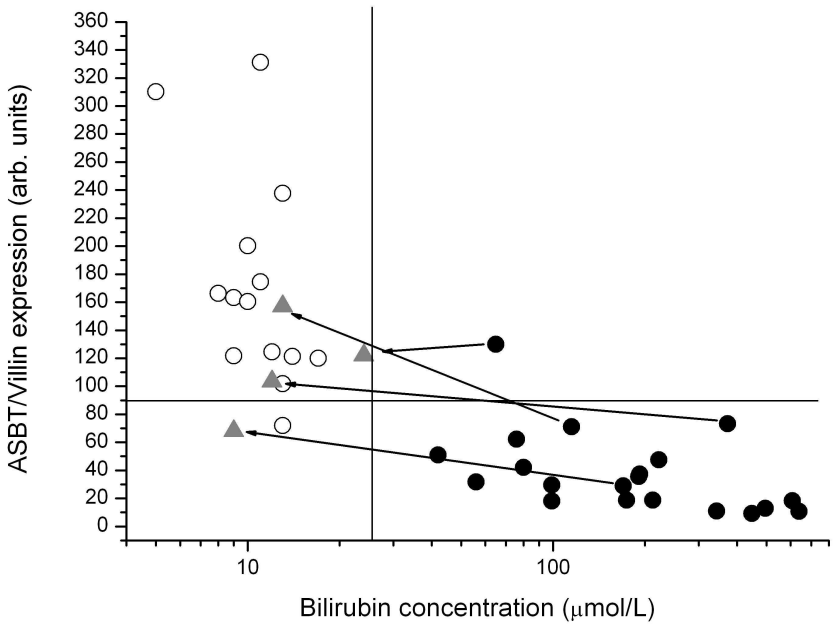
Patients with obstructive cholestasis due to a tumour showed a trend for lower ASBT mRNA expression when compared to patients with a benign aetiology of obstructive cholestasis (31.2 ± 7.3 for tumour induced and 44.6 ± 10.8 benign obstruction); the difference did, however, not reach statistical significance. The 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 μmol/L (NS) for patients with and without tumours, respectively.

PPARα mRNA expression was not significantly different between cholestatic patients and controls.

3.1.4.3 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 (Figure 3A and B).



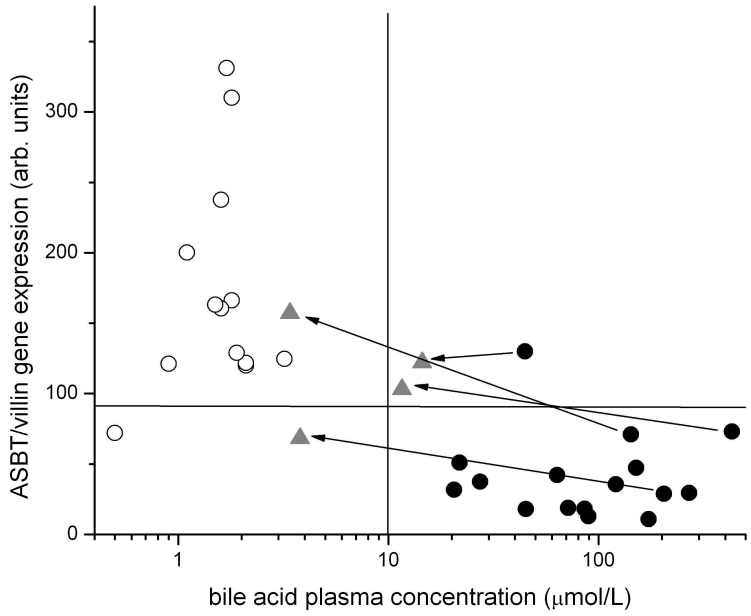


Figure 3A and 3B: Individual correlation of bilirubin (**3A**) and bile acid (**3B**) plasma concentrations with ASBT mRNA expression in patients with obstructive cholestasis (•) and control subjects (O). Data represent means ± SEM. Arrows connect individual values pre- and post reconstitution of bile flow in 4 follow-up patients.

3.1.4.4 Effect of reconstitution of bile flow on ASBT mRNA expression

In 5 of the included 20 icteric patients a follow-up gastroscopy could be 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, 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 with Whipple operation due to a tumor in the head of the pancreas could not be included in the follow-up analysis, because duodenal biopsies could not be obtained after a complete duodenopancreatectomy. In the patient with cholangiocarcinoma, only a partial duodenopancreatectomy was performed, so duodenal biopsies could be obtained in the follow-up endoscopy.

At follow-up endoscopy the bilirubin and bile acid plasma concentrations had normalized in all patients and 3 of 4 patients showed an increased expression of ASBT mRNA compared to the baseline value obtained. These three follow-up patients with bilirubin (bile acid) plasma concentrations of 373 (431), 170 (204) and 115 (170) μmol/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) μmol/l before intervention and 24 (14.5) μmol/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 the ASBT expression before 76 ± 18 and after reconstitution 113 ± 18 of bile flow for these four follow up patients was not significant (paired t-test; p = 0.15).

3.1.4.5 Immunohistochemistry of ASBT

Immunohistochemistry analysis in healthy subjects revealed a clear staining of ASBT in ileal and duodenal mucosa (Figure 4A and B, respectively). Staining of duodenal mucosa was clearly less intense than that from ileal tissue. In the duodenal mucosa of icteric patients, staining for ASBT was almost completely abolished (Figure 4C). After reconstitution of bile flow a more intense staining was found in the same tissue (Figure 4D).

3.1.4.6 Duodenal expression of MRP2 mRNA and MRP3 mRNA

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

Patients with obstructive cholestasis showed a significantly lower MRP2 mRNA expression (0.21 ± 0.02) compared to control subjects (0.32 ± 0.03, p= 0.004). After reconstitution of bile flow in 4 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 the MRP3 expression (data not shown).

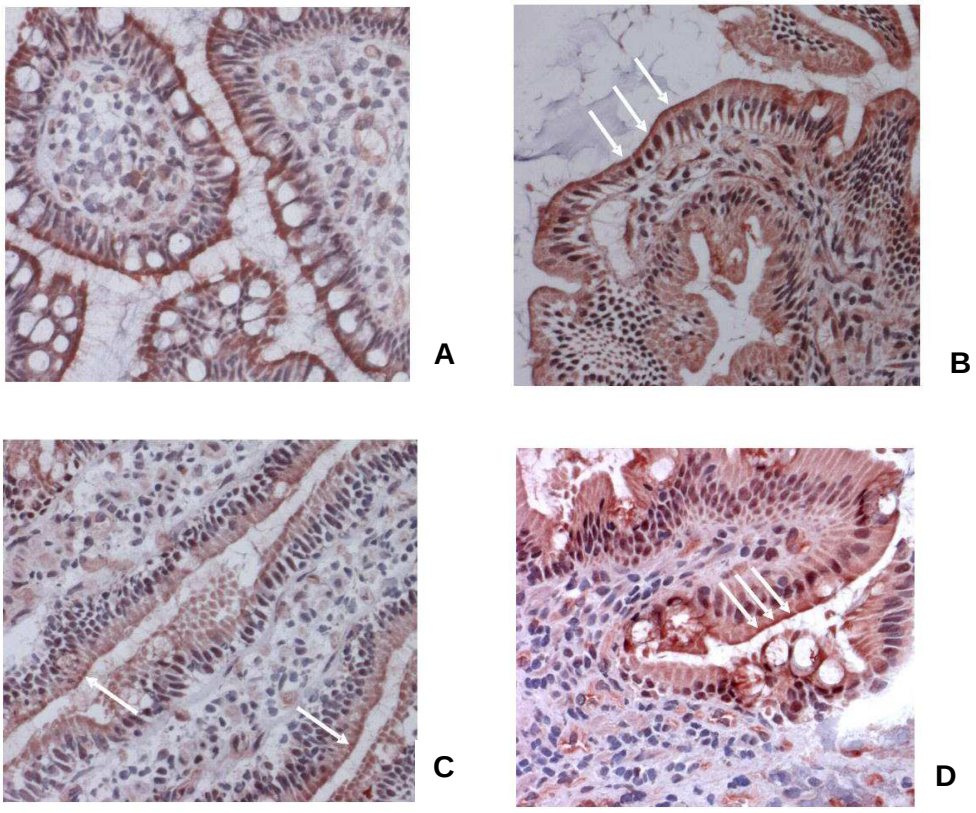


Figure 4 A-D: Immunohistochemical localization of ASBT protein on the apical membrane of the ileum and duodenum of humans using a polyclonal rabbit anti-human ASBT antibody: clear staining of ileal (**4A**) and duodenal epithelial cells (**4B**) of control subjects. Almost abolished staining of duodenal epithelial cells in a patient with obstructive cholestasis before (**4C**) and restored duodenal staining after (**4D**) reconstitution of bile flow (all pictures at 200-fold magnification).

3.1.5 Discussion

Bile acids undergo extensive enterohepatic and cholehepatic circulation through a coordinated action of several transport proteins in the hepatocytes, cholangiocytes and enterocytes (Trauner and Boyer, 2003). 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 the enterohepatic circulation of bile acids. Human ASBT is an efficient transport system for conjugated and unconjugated bile acids (Craddock et al., 1998). 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, a significant down-regulation of duodenal ASBT mRNA expression could be demonstrated in patients with complete or near complete obstruction of the bile duct when compared to control subjects. Finally, restoring bile flow to the duodenum increased ASBT mRNA expression in a subpopulation of patients with bile duct obstruction.

The down-regulation 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 (Sauer et al., 2000; Lee et al., 2001). In addition to the down-regulation 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 when compared to sham operated rats (Sauer et al., 2000). Patients with mutations in the ASBT gene can suffer from congenital diarrhoea and steatorrhea, which is explained by an interrupted enterohepatic bile acid circulation (Oelkers et al., 1997). Further clinical data indicate that in patients with primary sclerosing cholangitis, biliary enrichment of ursodeoxycholic acid (UDCA) decreases with increasing cholestasis (Stiehl et al., 1995). Moreover, in patients with bile duct obstruction and external biliary drainage intestinal absorption of UDCA was decreased, before relief of cholestasis (Sauer et al., 1999). These data suggest that in humans intestinal bile acid absorption is reduced during obstructive cholestasis.

What factors influence ASBT gene expression in the enterocyte is largely unknown. Animal studies suggest that luminal bile acids might be one such regulatory factor. Reduction of intestinal bile acid concentration during fasting (Dumaswala et al., 1994), after biliary diversion (Higgins et al., 1994) or bile duct ligation (Dumaswala et al., 1996) is accompanied by a decrease in ileal bile acid transport. Stravitz et al. could demonstrate that increasing intestinal bile acid concentrations by cholic acid feeding leads to an increase in the sodium dependent transport rate (Stravitz et al., 1997). Furthermore, adaptive induction of Asbt mRNA levels and transporter protein as well as increased Asbt transport activity by cholic acid fed rats was documented (Stravitz et al., 1997).

In contrast to 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 to sham-operated animals (Sauer et al., 2000). 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 the urine increased progressively in both cholestatic animal models but also in clinical cholestatic disorders (Stiehl et al., 1985; Lee et al., 2001). In

Wistar rats, a marked reduction in Asbt protein expression in microsomal membrane fractions from whole kidney after a common bile duct ligation (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 (Lee et al., 2001). In the same study, an up-regulation 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 sulfates 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 could show that ASBT mRNA expression correlated inversely with bilirubin as well as with bile acid plasma concentrations, which were both used as a marker for obstructive cholestasis.

Bile acids are synthesized from cholesterol in the liver; their production is a major mechanism of cholesterol elimination and important for the maintenance of cholesterol homeostasis (Vlahcevic et al., 1999). The down-regulation of ASBT expression in obstructive cholestasis might therefore be of clinical interest. In a previous publication, it was shown that inhibition of the 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 the cholesterol 7α-hydroxylase (CYP7A1) (Li et al., 2004). 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 of serum 7α-hydroxycholesterol was observed after biliary drainage (Okamoto et al., 1994). The 7α-hydroxylation rate was significantly lower for patients with obstructive cholestasis when compared to healthy subjects (Bertolotti et al., 2001). These data suggest that in case of a substantial bile acid malabsorption the activity of 7α-hydroxylase in the liver and the synthesis of bile acids is increased. Other authors suggest that inhibition of ASBT with SC-435 reduces LDL cholesterol and ApoB by enhanced plasma clearance of LDL ApoB (Huff et al., 2002; Telford et al., 2003). Treatment of Caco-2 cell monolayers with 25-hydroxycholesterol significantly inhibited Na^+-dependent ^{3}H-taurocholate uptake. The inhibition in hASBT activity was associated with reduction in both the level of hASBT mRNA and its promoter activity (Alrefai et al., 2004). ASBT protein expression did not change in rats but rose by 31% in rabbits when feeding them with cholesterol (cholest-5-en-3β-ol) (Xu et al., 2000). Administration of SC-435 to apo $E^{-/-}$ 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 hypercholesterolemia resulting in decreased risk for atherosclerosis (Bhat et al., 2003). Taken together all these data suggest that the expression of ASBT in the enterocyte seems to be an important component in the cholesterol homeostasis and lipid metabolism in humans.

The intracellular factors that influence ASBT gene expression remain largely unknown. One potential factor might be the activation of nuclear receptor PPARα, a ligand activated transcription factor that regulates the expression of a number of genes involved in peroxisomal and mitochondrial β-oxidation of fatty acids. PPARα binds as a heterodimer with the retinoid X receptor (RXR) to a peroxisome proliferator-response element (PPRE) located in the promoter region of target genes. PPARα was shown to play a critical role in the adaptive response to fasting in mice (Kroetz et al., 1998; Kersten et al., 1999; Leone et al., 1999). PPARα also influences bile acid composition by induction of the sterol 12α-hydroxylase, which acts at a branch-point in the bile acid synthetic pathway by catalizing the conversion of 7α-hydroxy-4-cholesten-3-one to 7α,12α-dihydroxy-4-cholesten-3-one. This reaction determines the ratio of cholic acid to chenodeoxycholic acid (Hunt et al., 2000). In Hepa 1c1c7 cells, which were transiently tranfected with an expression plasmid for PPARα and RXRα and a luciferase reporter construct containing copies of the PPRE from the rat ACOX gene, an inhibition of PPARα reporter gene expression was shown with increasing concentrations of chenodeoxycholic acid (CDCA) in the presence or absence of Wy-14,643 (=PPARα ligand) (Sinal et al., 2001). It was concluded that during certain pathophysiological states, where intracellular bile acid concentrations might be elevated, effects on PPARα-dependent target gene regulation are possible (Sinal et al., 2001). Recently, PPARα was shown to transactivate the human ASBT gene (Jung et al., 2002). Incubation of human hepatoma HepG2 cells with CDCA resulted in a significant induction of PPARα mRNA levels. In addition, hPPARα gene expression was upregulated by taurocholic acid in human primary hepatocytes (Pineda Torra et al., 2003). However, no difference between patients with obstructive cholestasis and control subjects was observed with regard to duodenal PPARα mRNA expression in our study.

In a previous study, Dietrich et al demonstrated that obstructive cholestasis promotes down-regulation of intestinal MRP2 protein expression in rats and humans (Dietrich et al., 2004). The reduction was correlated with the duration of cholestasis and was reversible after reconstitution of bile flow. In the same study in patients with obstructive cholestasis a non significant decrease of MRP2 mRNA expression was detected when compared to control subjects. However, the

authors observed that decline of intestinal rat Mrp2 mRNA occurs more slowly and gradually than down-regulation of its protein expression. Our results showed a significant down-regulation of MRP2 mRNA expression when compared to control subjects and a non significant increase after reconstitution of bile flow in 4 patients. One explanation for the down-regulation of MRP2 gene expression in our study compared to Dietrich et al. (Dietrich et al., 2004) might be higher plasma bilirubin concentrations in our patients with obstructive cholestasis, another might be duration of cholestasis, which was not clearly stated in both studies and therefore might have been different. But also other factors such as food derived compounds or substrates might be involved in the regulation of gene expression. For example, it was shown that grapefruit and orange juice inhibited the transport by MRP2 (Honda et al., 2004); components of our daily diet, such as the flavonoid epicatechin in tea (Vaidyanathan and Walle, 2001), chrysin and its metabolites are substrates of MRP2 (Walle et al., 1999). Drugs are unlikely to have contributed to the different MRP2 mRNA expression in the present study and the study performed by Dietrich et al. (Dietrich et al., 2004) because patients with drugs, which are known to affect MRP2 expression, were excluded from both studies. Finally, our data support the results of Dietrich et al. (Dietrich et al., 2004), which demonstrate an adaptive down-regulation of duodenal MRP2 in patients with obstructive cholestasis.

MRP3 is expressed on the basolateral membrane in the proximal small bowel (Rost et al., 2002) and is capable of transporting bile salts, including taurocholate, glycocholate, taurochenodeoxycholate-3-sulfate, taurolithocholate-3-sulfate (Hirohashi et al., 2000). 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 of human duodenal MRP3 gene expression in patients with obstructive cholestasis when compared to control subjects. Similarly, no compensatory up-regulation of human duodenal MRP3 expression in cholestatic patients was previously described (Dietrich et al., 2004).

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 down-regulation of ASBT and MRP2 mRNA in the duodenum can be observed in patients with obstructive cholestasis, when compared to control subjects. Furthermore, the duodenal ASBT mRNA expression levels inversely correlate with the 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 of ASBT mRNA was observed in 3 out 4 patients.

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aza-1-azoniabicyclo[2.2. 2]octane methanesulfonate (SC-435), an ileal apical sodium-codependent bile acid transporter inhibitor alters hepatic cholesterol metabolism and lowers plasma low-density lipoprotein-cholesterol concentrations in guinea pigs. *J Pharmacol Exp Ther* **303:**293-299.

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3.2 Distribution of breast cancer resistance protein (BCRP/ABCG2) mRNA expression along the human GI tract

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3.2.1 Abstract

Human breast cancer resistance protein (BCRP/ABCG2) is an ABC-transporter that is present on the luminal membrane of intestinal epithelial cells and restricts absorption of anticancer drugs such as methotrexate, topotecan, mitoxantrone, and doxorubicin. The exact anatomic distribution of BCRP along the gastrointestinal (GI) tract has, however, not been determined before. The aim of this study was, therefore to investigate BCRP mRNA expression pattern along the GI tract in 14 healthy subjects. Furthermore, BCRP duodenal mRNA expression was compared with MDR1/ABCB1 mRNA. Additionally, BCRP mRNA expression was investigated in two human intestinal cell lines (Caco-2 and LS180). Since previous animal studies have suggested sex specific differences in BCRP expression, we analyzed intestinal BCRP expression with respect to sex. Biopsies were taken from different gut segments (duodenum, terminal ileum and ascending, transverse, descending and sigmoid colon). Gene expression was assessed by quantitative real-time PCR (Taqman). BCRP mRNA expression was maximal in the duodenum and decreased continuously down to the rectum (terminal ileum 93.7 percent, ascending colon 75.8 percent, transverse colon 66.6 percent, descending colon 62.8 percent, and sigmoid colon 50.1 percent compared to duodenum, respectively). BCRP expression in the duodenum was comparable to MDR1/ABCB1 gene expression. Caco-2 cells showed a comparable expression of BCRP as human duodenal tissue. Gender specific differences in BCRP expression were not observed. These findings represent the first systematic site-specific analysis of BCRP expression along the GI tract. This information might be helpful to develop target strategies for orally administered anticancer drugs.

3.2.2 Introduction

BCRP/ABCG2 is a half-transporter that belongs to the *white* subfamily of ATP-binding cassette (ABC) transporters. BCRP was originally cloned from multidrug resistant tumour cells (Allikmets et al., 1998; Doyle et al., 1998; Miyake et al., 1999) and displays a wide substrate specificity. It mediates the energy dependent translocation of various anticancer drugs such as methotrexate (Volk et al., 2002), topoisomerase inhibitors (such as topotecan (Kruijtzer et al., 2002)), mitoxantrone, and doxorubicin (Doyle et al., 1998) across cellular membranes. BCRP knock-out mice were found to be healthy and showed no major pathological alterations. When fed with a chlorophyll rich diet containing the chloprophyll degradation product, the phototoxic phenophorbide a, the BCRP knock-out mice developed phototoxic skin lesions (Jonker et al.,

2000). Its localization indicates an important role in the protection of tissues against xenobiotics. BCRP expression was detected mainly in excretory organs e.g. in canalicular membranes of the liver, in epithelial cells of the small intestine, colon, kidney and lung, as well as in the blood-brain barrier and the placenta (Scheffer et al., 2000; Maliepaard et al., 2001).

The expression of BCRP in epithelial cells of the intestine implies, that BCRP might be an important transporter limiting the absorption of orally administered anticancer drugs and ingested toxins (Maliepaard et al., 2001; Jonker et al., 2002; Pavek et al., 2005). Due to its broad substrate specificity, BCRP might influence the pharmacokinetics of many unrelated substances including anticancer drugs, HIV drugs, and endogenous compounds (van Herwaarden et al., 2003; Polli et al., 2004). Up to now, there is little knowledge about the expression pattern of BCRP along the human intestine. This information however might be helpful for the development of specific galenical targeting approaches, which may be utilized to improve intestinal absorption of anticancer drugs. Therefore, the expression of BCRP was investigated in the human intestine of 14 healthy subjects and its duodenal expression was compared with that of MDR1. *In vitro* screening of drug absorption is commonly done in human intestinal cell lines (such as Caco-2 (Hilgers et al., 1990; Pfrunder et al., 2003) and LS180 (Thummel et al., 2001)). Therefore, it is of interest to compare the expression level of BCRP in these cell lines with the human duodenal BCRP mRNA expression.

In addition, membrane transport differences of endogenous and xenobiotic compounds associated with sex have been reported previously for several transport proteins (Piquette-Miller et al., 1998; Salphati and Benet, 1998; Urakami et al., 1999; Urakami et al., 2000; Buist et al., 2002; Kobayashi et al., 2002; Buist and Klaassen, 2004). Recently, sex associated differences for Bcrp, the BCRP analogue in rat and mice has been described by Tanaka et al (Tanaka et al., 2005). They observed a higher expression of Bcrp mRNA of male rats in the kidney and of male mice in the liver compared to females. These sex differences were attributed to the suppressive effect of estradiol in rats and to the inductive effect of testosterone in mice, respectively. Intestinal expression of rat and mouse Bcrp seems not be influenced by sex. However data about intestinal rat and mouse Bcrp expression exhibited high interspecies differences and were restricted to duodenum, jejunum and ileum. We therefore wanted to determine, whether there are sex-related differences in human BCRP mRNA expression along the intestinal tract that might lead to pharmacokinetic variations in drug absorption.

3.2.3 Material and Methods

Cell cultures

Caco-2 cells (passage 42) and LS180 cell line (passage 36) were purchased from ATCC (Manassas, USA). Both cell lines were cultured in Dulbecco's MEM with Glutamax-I, supplemented with 10% (v/v) fetal calf serum, 1% non essential amino acids, 1% sodium pyruvate, 50µg/ml gentamycin. All cultures were maintained in a humified 37℃ incubator with a 5% carbon dioxide in air atmosphere.

Biopsies

Intestinal biopsies were obtained from 14 healthy subjects (7 female, 7 male, age 43-75 years, average age 59.8 years, no medication), which served as a control group in a clinical study. This study systematically investigated the regional expression of different genes in patients with inflammatory bowel disease. The study protocol included specifically the investigation of drug transporting proteins and it was approved by the local Ethical Committee. Informed consent was obtained from subjects prior to inclusion. Indications for a combined gastroscopy and colonoscopy in these control patients were cancer screening, irritable bowel syndrome and unspecific abdominal pain. No macroscopically pathological findings were observed during endoscopies in these subjects. Three to four biopsies each were obtained from duodenum, terminal ileum, ascending colon, transverse colon, descending colon and sigmoid colon.

Preparation of samples and Taqman assay see Chapter 2

Statistical Analysis

BCRP gene expression was compared between the different intestinal segments by analysis of variance. In the case of significant differences between intestinal segments, all segments were compared with the expression in duodenum using Dunnett's *t*-test using sex as a covariate. Comparison of BCRP and/or MDR1 mRNA expression was performed by unpaired two-sided t-test. The level of significance was P =0.05. All statistical comparisons were performed using SPSS for Windows software (version 12.0).

3.2.4 Results

The expression pattern of BCRP from the duodenum to the sigmoid colon is shown in Figure 1. Maximal BCRP mRNA expression was found in the duodenum. In the colonic segments BCRP mRNA expression is continuously decreasing from proximal to distal. In ascending colon the

BCRP level is significantly reduced to 75.8 percent of the duodenum, in transverse colon to 66.6 percent, in descending colon to 62.8 percent, and in sigmoid colon to 50.1 percent, respectively. In the terminal ileum BCRP mRNA expression is slightly but not significantly reduced compared to duodenum (93.7 percent). The expression of BCRP mRNA was not significantly different between males and females, neither in the duodenum and the terminal ileum, nor in the different colonic segments of the human GI tract (Figure 1). BCRP mRNA expression was normalized to villin to account for variation in enterocyte content (ratio of BCRP/villin mRNA) as suggested in the literature (Lown et al., 1997; Taipalensuu et al., 2001). This was justified, since the mRNA expression of villin was not significantly different between the different parts of the intestine. The variability of BCRP mRNA measurement was determined by repetitive determination (N = 10) and amounted to 4.3 % (coefficient of variation).

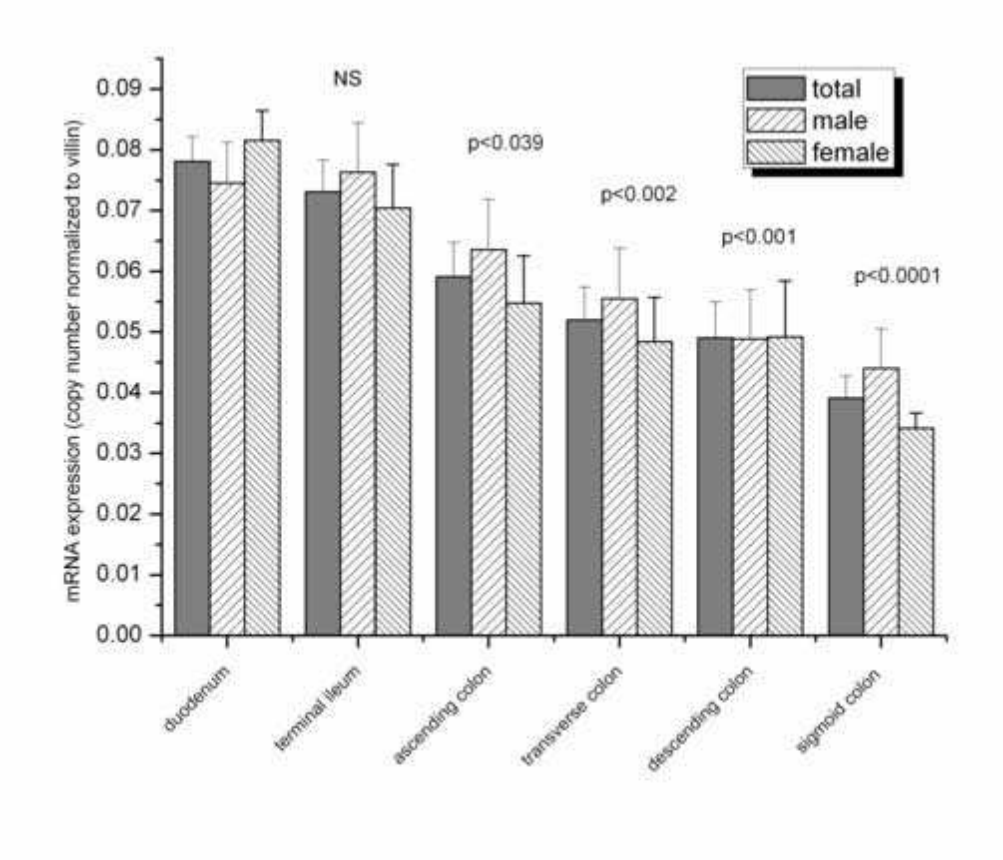

Figure 1: Expression of BCRP/ABCG2 mRNA in different gut segments. Data represent means (± SEM) of biopsies from 14 healthy subjects (7 males, 7 females), except terminal ileum, where biopsies from 13 subjects (6 males, 7 females) were used.

Since MDR1 and BCRP share some of their substrates, duodenal mRNA expression of these genes was compared. MDR1 mRNA and BCRP mRNA expression was in the same range in the duodenum, with a slightly but significantly ($p<0.05$) lower expression of BCRP (Figure 2).

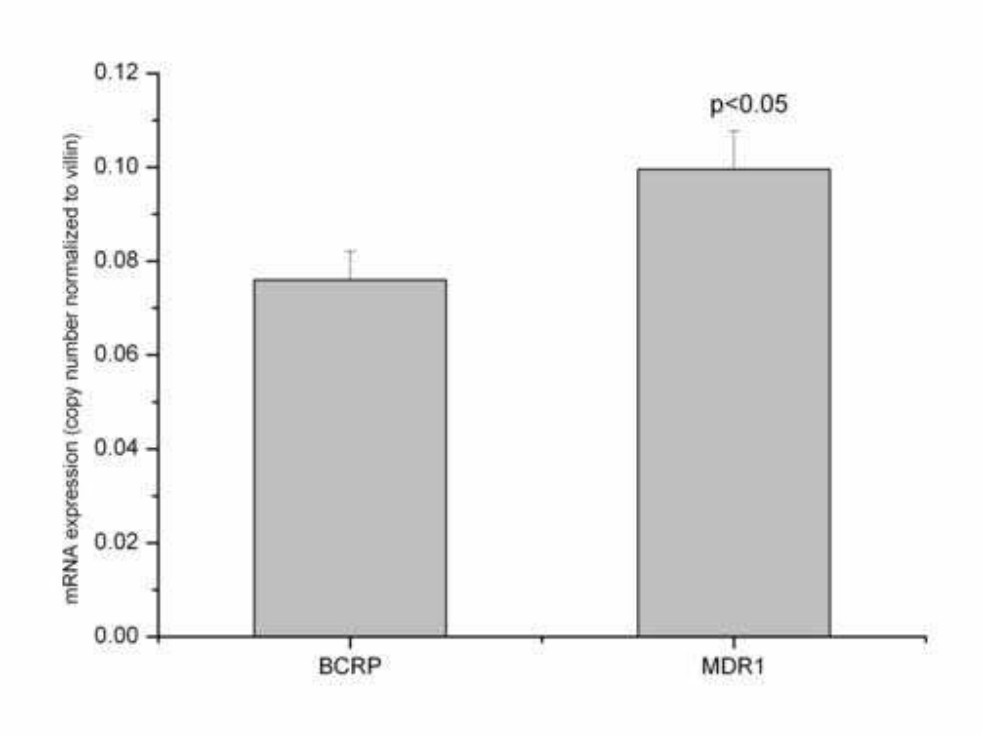

Figure 2: Expression of BCRP/ABCG2 mRNA and MDR1/ABCB1 mRNA in human duodenum normalized to the expression of villin. Data represent means (± SEM) of biopsies from 14 healthy subjects.

The duodenal mRNA expression of BRCP was comparable to the expression in Caco-2 cells, which are reported to exhibit duodenal-like transporter expression (Pfrunder et al., 2003). However, the mRNA expression of BCRP in LS180 cells was almost 100-fold lower ($p < 0.001$; Figure 3).

3.2.5 Discussion

Previous studies had reported, that cellular BCRP is localized in the apical membranes of small intestinal and colonic epithelia (Maliepaard et al., 2001; Pavek et al., 2005), where it limits the bioavailability of toxic compounds. There is some information about tissue distribution of BCRP in animal species such as rat and mice or BCRP expression in isolated parts of the intestine (Taipalensuu et al., 2001; Tanaka et al., 2005). However, only limited information is available about the site-specific localization of BCRP along the GI tract in humans, which might be

important for the development of specific galenic formulations of anticancer drugs. Here, we present for the first time a systematic analysis of the site-specific expression of BCRP along the GI tract. BCRP mRNA expression decreased continuously from the duodenum to the sigmoid colon. In human jejunum, a part which was not investigated in our study due to ethical reasons, Taipalensuu et al. (Taipalensuu et al., 2001) found a high level of BCRP mRNA expression. The BCRP gene expression exhibited even a 3.4-fold higher expression than the MDR1 gene. Data in rat (Tanaka et al., 2005) have shown that the level of Bcrp gene expression is higher in the jejunum compared to duodenum. However, it is not trivial to extrapolate animal data to humans, because species differences have been described even between rodents. Whereas rats expressed high levels of Bcrp in the ileum, the ileal level of Bcrp mRNA in mice was rather low. Nevertheless one is tempted to speculate, that BCRP expression levels might be maximal in the jejunum. Since expression of BCRP is still high in the terminal ileum, which is close to the jejunum, these data are not in contrast to our findings.

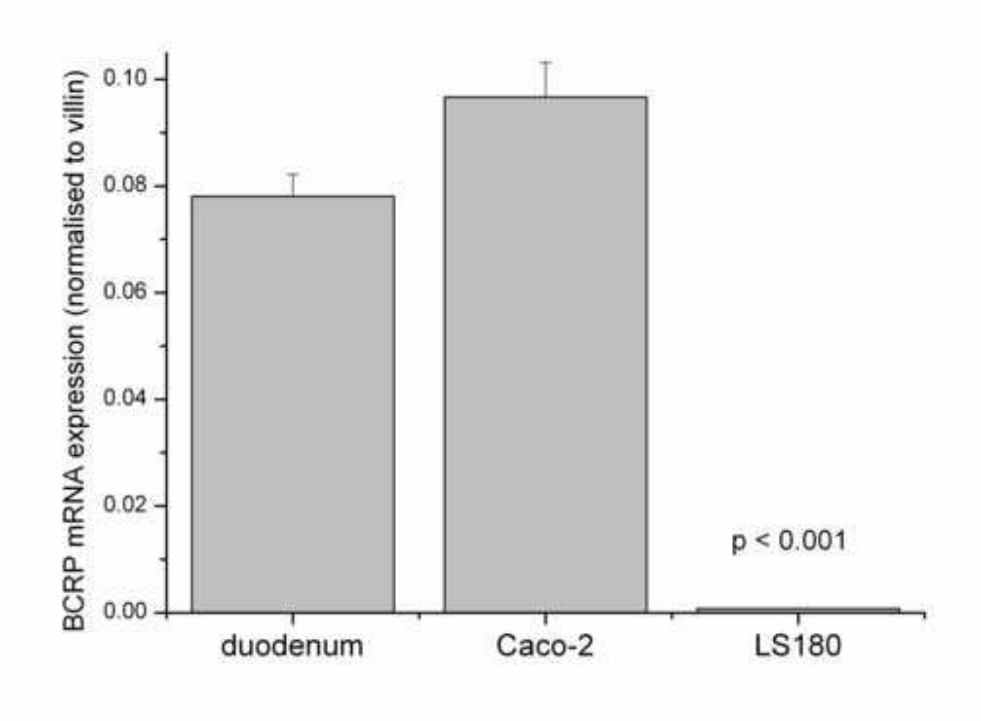

Figure 3: Expression of BCRP/ABCG2 mRNA in human duodenum (N=14); Caco-2 cells (N=3) and LS180 cells (N=3) normalized to the expression of villin. BCRP mRNA expression was significantly lower in LS180 cells than in Caco-2 cells and duodenal tissue ($P < 0.001$). Data represent means (± SEM).

Differences in the membrane transport of xenobiotics and endogenous compounds caused by different levels of sexual hormones such as testosterone and estradiol have been previously described in several studies (Lu et al., 1996; Cerrutti et al., 2002). This sex related differences in membrane transport includes several membrane transporters such as organic cation transporters (Urakami et al., 1999; Urakami et al., 2000), organic anion transporters (Buist et al., 2002; Kobayashi et al., 2002; Buist and Klaassen, 2004), and multidrug resistance proteins Mdr1a, Mdr1b and Mdr2 (Piquette-Miller et al., 1998; Salphati and Benet, 1998). Recently, Tanaka et al. reported sex-related differences of BCRP expression levels in rodents (Tanaka et al., 2005). We found no significant differences in the level of BCRP mRNA expression between males and females, neither in the duodenum and the terminal ileum, nor in the different colonic segments of the human GI tract. We therefore conclude, that sexual hormones have most probably no effect on the expression pattern of BCRP in the adult human intestine. The importance of MDR1 and MRPs for the protection from enteral absorption of potentially toxic xenobiotics and their limiting effects on enteral drug absorption has become more and more aware. BCRP shows some degree of substrate overlapping with these transporters and is also expressed in the small and large intestine (Allen and Schinkel, 2002). To estimate the potential impact of BCRP for detoxification and drug absorption, we compared the level of BCRP mRNA expression in the duodenum with the level of MDR1 mRNA, another important ABC-transporter of xenobiotics in the intestine. We showed comparable mRNA expression of MDR1 and BCRP, with a slightly but significantly ($p<0.05$) lower expression of BCRP. In jejunum, BCRP mRNA expression was found to be even higher as MDR1 mRNA expression (Taipalensuu et al., 2001). Taken together these findings indicate that BCRP might play an important role for limiting the absorption of orally administered anticancer drugs and ingested toxins.

The comparable mRNA expression of BRCP in human duodenum and the colonic carcinoma derived cell line Caco-2 may indicate its usefulness for *in vitro* studies of BRCP mediated transport of drugs. In addition, another colonic adenocarcinoma-derived cell line, LS180, was investigated for BCRP mRNA expression. This cell line is commonly used for the assessment of gene induction (Thummel et al., 2001; Pfrunder et al., 2003; Collett et al., 2004). However, due to their low expression of BRCP mRNA, LS180 cells do not seem to be suitable for investigation of BRCP function.

We have to admit that our study results represent only mRNA expression, which may not correlate with protein expression and/or function. However, due to ethical reasons we were limited with the number and volumes of tissue biopsies taken from our patients. Therefore, for

this mapping study only mRNA expression experiments could be performed. To assess functional expression of BRCP in different gut segments, further dedicated studies are needed.

3.2.6 Conclusion

These findings represent the first systematic site-specific analysis of BCRP expression along the GI tract and shows that its expression significantly decreased in all colonic segments compared with the small intestine. This knowledge might be important to develop target strategies for orally administered anticancer drugs.

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3.3. Decreased expression of breast cancer resistance protein (BCRP) in the intestine of patients with obstructive cholestatis

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Journal of Hepatology

3.3.1 Abstract

Objective: Human breast cancer resistance protein (BCRP/ABCG2) is present on the apical membranes of the liver, in the epithelial cells of the small and large intestine and in kidney cells; the transporter mediates energy dependent efflux of drugs and potentially toxic compounds, and of various endogenous substrates such as bile acids. During obstructive cholestasis with a disrupted enterohepatic circulation, an adaptive regulation of several transporters for bile acids, bilirubin and cholesterol has been documented. An adaptive regulation of human intestinal BCRP during obstructive cholestasis has, however, not been described before.

Methods: BCRP mRNA was quantified by real time PCR (Taqman) in duodenal biopsies of 14 control subjects and compared to the duodenal BCRP mRNA expression of 19 patients with obstructive cholestasis. In addition, duodenal BCRP mRNA expression was measured in 4 patients with obstructive cholestasis after reconstitution of bile flow. BCRP protein levels were determined in 6 cholestatic and 6 healthy subjects by immunohistochemistry. Finally, fasting bile acid and bilirubin concentrations were determined by specific assay systems.

Results: Normalized duodenal BCRP mRNA expression (mean BCRP/Villin ± SEM) was 0.51 (± 0.03) in controls and 0.27 (± 0.02) in cholestatic patients ($p<0.001$). In a semiquantitative analysis of immunohistochemical protein assessment (0 = no expression, 1 = low, 2 = intermediate, 3 = high expression) mean BCRP protein expression (mean±SEM) were significantly reduced in 6 patients with obstructive cholestasis (1.67 ± 0.38) when compared to 6 healthy subjects 2.91 (± 0.08) ($p< 0.005$). After reconstitution of bile flow bile acid and bilirubin plasma levels returned to normal and BCRP mRNA expression increased by 1.44-, 1.71-, 1.05-, and 1.90-fold, respectively (N=4).

Conclusion: BCRP is down-regulated in the human duodenum during obstructive cholestasis. We infer that a reduced intestinal BCRP expression during cholestasis influences the accumulation of bile acids, food-derived carcinogens and the pharmacokinetics of various drugs that are transported by BCRP.

3.3.2 Introduction

The breast cancer resistance protein (BCRP/ABCG2) is a half-transporter that belongs to the G subfamily of ATP-binding cassette (ABC) transporters. Similar to P-glycoprotein, BCRP was detected and cloned from multi-drug resistant tumour cells (Doyle et al., 1998; Miyake et al., 1999). Both efflux transporters are highly expressed in organs that play a protective role against toxic substances such as the intestine, the kidney, the liver, the blood brain barrier, and the placenta (Maliepaard et al., 2001). In human jejunum, BCRP is expressed in the apical membrane of enterocytes along with other efflux pumps MRP2 (multidrug resistance associated protein 2) and P-glycoprotein, the gene product of MDR1/ABCB1 (multidrug resistance protein 1) (Taipalensuu et al., 2001). BCRP mediates the translocation of various drugs like methotrexate, mitoxantrone, anthracyclines, SN-38, and topotecan (Doyle et al., 1998; Schellens et al., 2000; Volk and Schneider, 2003). It has further an essential role in extruding metabolites such as glucuronide and sulfate conjugates formed in enterocytes into the intestinal lumen (Adachi et al., 2005). Moreover, BCRP protects the body from toxic constituents of food such as the carcinogen PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) and the chlorophyll-derived phototoxin pheophorbide a (Jonker et al., 2002; van Herwaarden et al., 2003). In summary, its localization in the apical membrane of intestinal cells and its wide substrate specificity suggests that BCRP is an important transporter limiting the absorption of orally administered drugs and ingested toxins.

Most members of the ABCG subfamily are involved in the translocation of endogenous compounds. ABCG1 is supposed to be a regulator of cholesterol and phospholipid transport (Klucken et al., 2000), whereas the heterodimeric proteins ABCG5 / ABCG8 promote biliary excretion and limit intestinal absorption of neutral sterols (Yu et al., 2002). BCRP can also transport endogenous substrates such as sulphated steroids (Imai et al., 2003; Suzuki et al., 2003) and primary bile acids (Janvilisri et al., 2005). The precise physiological role of BCRP remains, however, to be defined.

BCRP is capable of transporting bile acids. This fact may indicate that this transporter is involved in bile acid homeostasis. As an efflux pump, it could protect the enterocytes from potential toxic bile acid concentrations. During cholestasis, in which the enterohepatic circulation is disrupted, an adaptive regulation of transporters for bile acids, bilirubin and cholesterol occurs (Tanaka et al., 2002; Zollner et al., 2003; Denk et al., 2004; Kamisako and Ogawa, 2005). These changes take place in the liver, the kidney, as well as in the intestine. We have recently shown that the responsible gene for bile acid reuptake in the intestine, the apical sodium dependent bile acid transporter (ASBT), is down-regulated in cholestatic patients (Hruz et al., 2006).

Here we have analyzed the intestinal expression of BCRP in patients with obstructive cholestasis. The disease is associated with elevated bile acid levels in serum, a lack of bile acids in the intestinal lumen, and increase in proinflammatory cytokines (Plebani et al., 1999). We hypothesized that BCRP, as a potential bile acid transporter, could show an altered gene expression during obstructive cholestasis. Indeed, we can show that cholestatic patients exhibit decreased BCRP expression in the intestine compared to healthy subjects. Further studies are necessary to characterize the impact of these results on the pharmacokinetics of drugs that are BCRP substrates.

3.3.3 Material and Methods

Patients

Fourteen healthy subjects (7 females, 7 males) and 19 cholestatic patients (10 females, 9 males) were enrolled in the study after giving informed consent. The group of control subjects had a mean age of 59.8 years and cholestatic patients of 67.5 years, whereas bile acid levels were 1.7 (0.5 - 3.2) and 122.6 (20.5 - 431.3) µmol/L, respectively.

Table 2: Biochemical Parameters (means ± SD)

Parameter	Control	Patients with cholestasis	P-Value
Bilirubin (5-26 µmol/L)	11.1 ± 2.9	235.1 ± 186.2	P < 0.001
ALAT (10-37 U/L)	24.2 ± 9.2	193.1 ± 163.1	P < 0.003
ASAT (11-36 U/L)	23.4 ± 7.9	122.1 ± 102.5	P < 0.002
γ-GT (11-66 U/L)	58.0 ± 7.6	578.3 ± 430.3	P < 0.001
AP (43-106 U/L)	65.5 ± 13.1	375.5 ± 416.5	P < 0.01
Total cholesterol (3-5.2 mmol/L)	5.5 ± 0.8	32.8 ± 101.1	NS
Bile acids (< 10 µmol/L)	1.7 ± 0.7	122.6 ± 108.8	P < 0.001

Note. Normal range in parenthesis; NS = statistically not significant

The control subjects had an indication for a gastrointestinal tract endoscopy within a cancer-screening program, whereas patients with obstructive cholestasis had an interventional endoscopic retrograde cholangiopancreatography (ERCP). Within the cholestatic group, nine

patients had obstructive tumors (3 carcinoma of the pancreatic head, 4 cholangiocarcinoma (Klatskin tumor) and 2 metastatic disease). 10 patients had benign diseases (8 patients with choledocholithiasis and two patients with a benign stenosis of the common bile duct). Obstructive jaundice was defined on the basis of chemical parameters (bilirubin, γ-glutamyltransferase, and alkaline phosphatase) and on imaging procedures (ultrasound and ERCP) demonstrating a dilated bile duct system. All control patients had normal values of the above mentioned parameters. During endoscopy four biopsy specimens were obtained from the distal part of the duodenum. Biopsies were immediately stored at –70℃ until further processing. The study was approved by the State Ethical Committee of Basel (*Ethische Kommission beider Basel*, EKBB).

Real-time RT-PCR analysis (TaqMan) described in Chapter 2

Sequences of PCR primers used for generating the standards and of TaqMan primers / probes used for real-time PCR (Table 2.1; Chapter 2)

Immunohistochemical assessment is described in Chapter 2

Determination of bile acid plasma concentrations is described inChapter 2

Statistics

All values were expressed as means ± SEM. Icteric patient's BCRP expression was compared to that of healthy controls by analysis of variance (ANOVA). All comparisons were performed as two-sided comparisons using the SPSS for Windows software (version 12.0). Level of significance was $p<0.05$.

3.3.4 Results

3.3.4.1 BCRP mRNA expression is down-regulated during cholestasis

Intestinal mRNA expression of BCRP and villin was analyzed in 14 healthy subjects and 19 patients with obstructive cholestasis. In cholestatic patients mean BCRP mRNA levels were reduced to 53.6 % when compared to healthy subjects. Relative expression (mean BCRP/villin ± SEM) was 0.51 (± 0.03) in controls and 0.27 (± 0.02) in cholestatic patients ($p<0.001$) (Figure 1A). Mean villin mRNA expression was not significantly different between the control and cholestatic group (data not shown).

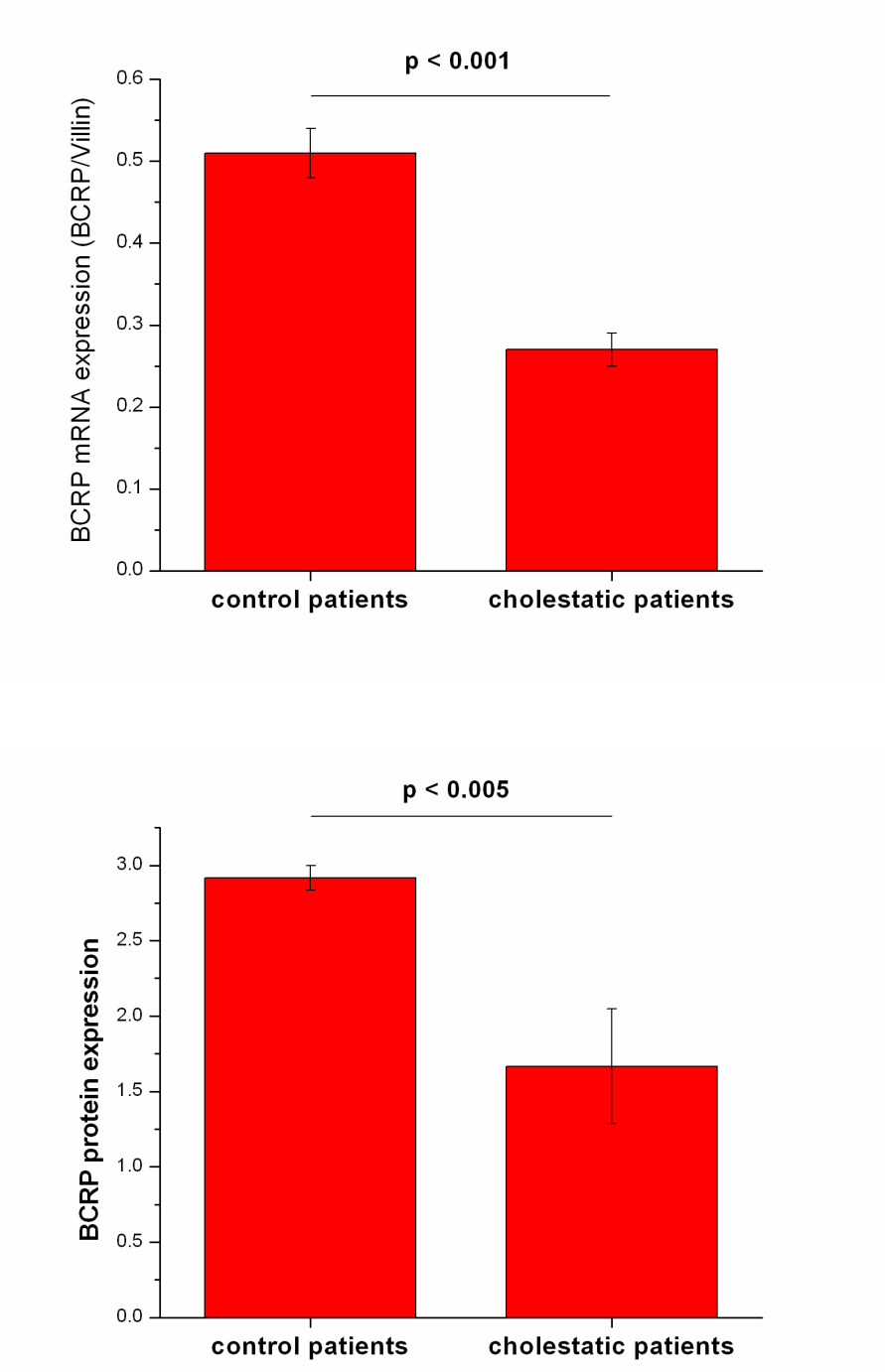

Figure 1A and 1B: Comparison of BCRP expression in healthy subjects versus cholestatic patients (mean ± SEM). 1A: Expression of BCRP mRNA relative to villin (14 controls versus 19 cholestatic patients). 1B: BCRP protein expression using immunohistochemistry with semiquantitative analysis (6 controls vs. 6 cholestatic patients).

Figure 2 displays the individual BCRP mRNA expression in correlation to bile acid serum concentration.

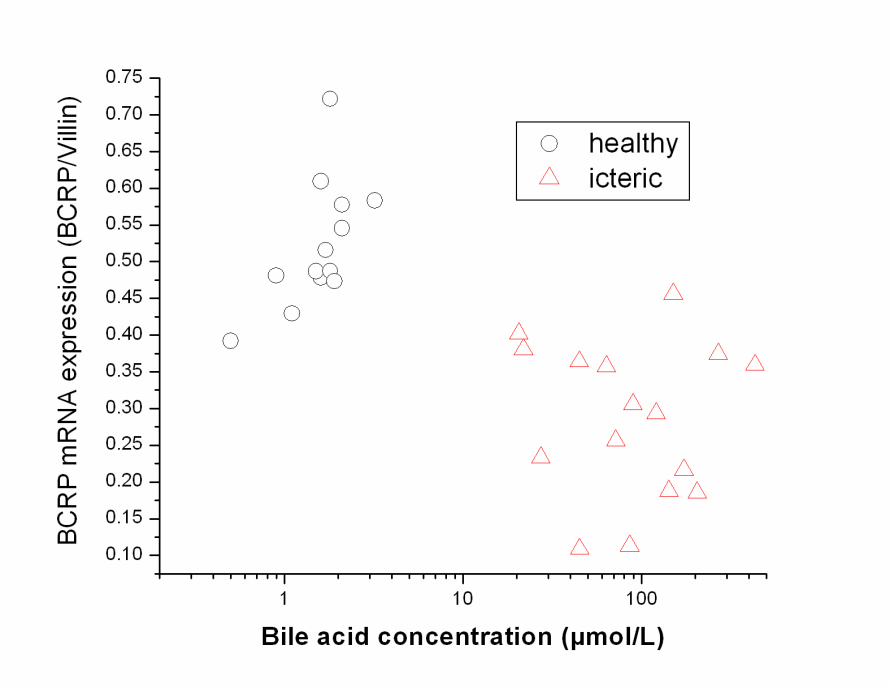

Figure 2: Individual BCRP mRNA expression (BCRP/villin) in correlation to bile acid plasma concentration in healthy subjects and cholestatic patients.

In 4 patients a follow-up gastroscopy could be performed after reconstitution of bile flow. These patients showed normalized bile acid and bilirubin plasma levels at the time when duodenal follow-up biopsies were taken. In all 4 patients the BCRP mRNA expression increased (1.44-, 1.71-, 1.05-, and 1.90-fold) when compared to the expression level of cholestasis (Figure 3).

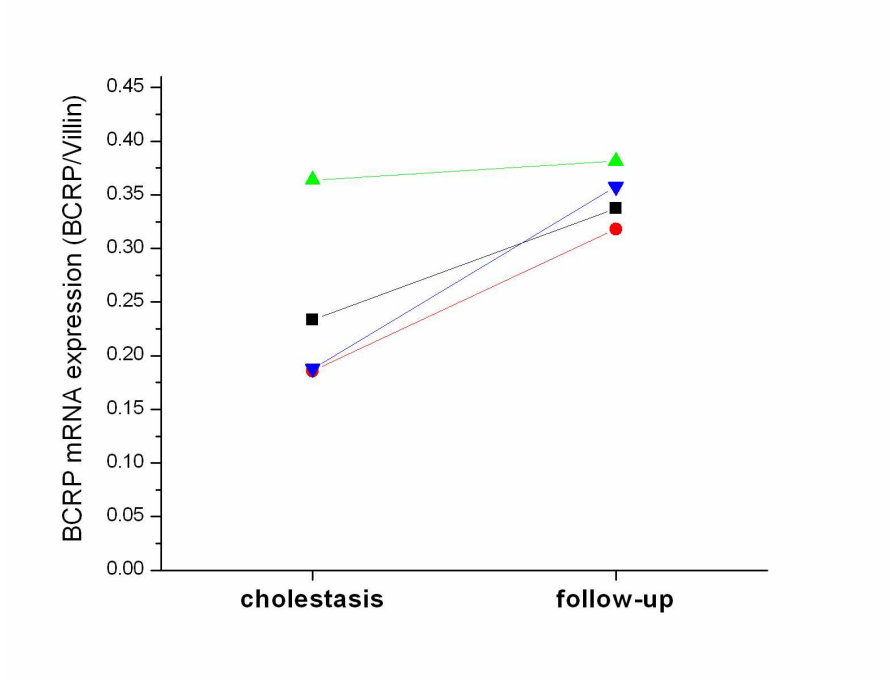

Figure 3: Change of relative BCRP mRNA expression (BCRP/villin) in 4 patients during cholestasis and after reconstitution of bile flow.

3.3.4.2 BCRP protein levels are decreased in cholestatic patients

BCRP protein expression was evaluated by immunohistochemistry. Semiquantitative analysis in biopsies of 6 control subjects and 6 cholestatic patients was done by a trained pathologist. BCRP protein was expressed on the apical membrane of the duodenal epithelial cells. Representative pictures of duodenal tissues are displayed in Figure 4A (healthy subject) and 4B (cholestatic patient). Expression levels were rated as follows: 0 = no expression, 1 = low, 2 = intermediate, 3 = high expression. In patients with obstructive cholestasis mean BCRP protein levels were reduced to 57.1 % when compared to healthy subjects. Protein expression (mean ± SEM) was 2.91 (± 0.08) in control subjects and 1.67 (± 0.38) in cholestatic patients ($p < 0.005$) (Figure 1B).

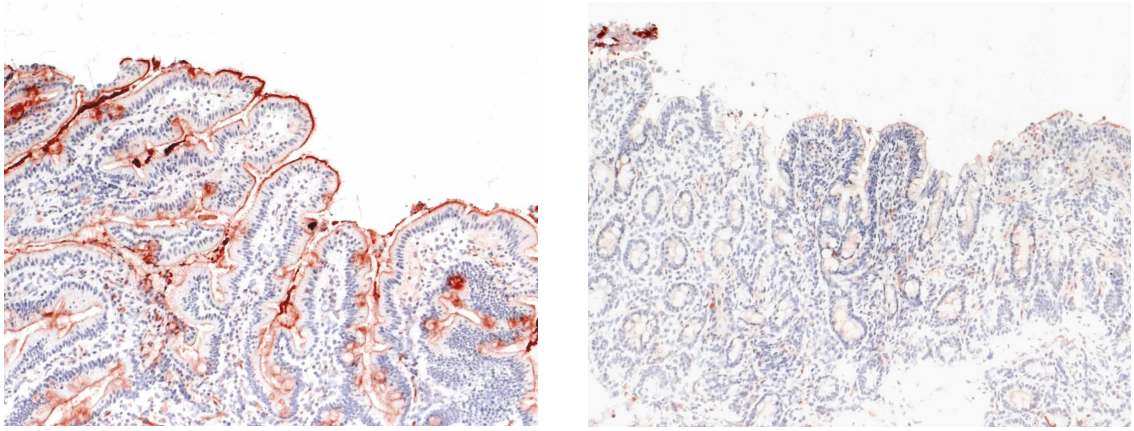

Figure 4A and 4B: Immunohistochemical localization of BCRP protein on the apical membrane of human duodenal epithelial cells using a polyclonal rabbit anti-human BCRP antibody (all pictures at 200-fold magnification). 4A: clear staining of BCRP protein in a duodenal sample from a healthy subject. 4B: reduced staining in a duodenal sample of a patient with obstructive cholestasis

3.3.5 Discussion

Our data demonstrate that during complete or near complete obstruction of the bile duct by a tumour or biliary stone cholestasis intestinal BCRP expression is down-regulated on the transcriptional level leading to reduced amount of BCRP protein. As a proof of principle, reconstitution of bile flow by endoscopic or surgical therapy initiated normalization of intestinal BCRP expression associated with normalization of serum bile acids and bilirubin levels.

At the moment, little is known about the transcriptional regulation of this transporter. Only recently, an estrogen response element in the BCRP promotor was discovered (Ee et al., 2004). Tanaka and coworkers observed, however, a higher BCRP expression in the kidney and liver of male rats and male mice, respectively (Tanaka et al., 2005). Furthermore, it has been reported that estrogens down-regulate BCRP protein expression by posttranscriptional mechanisms (Imai et al., 2005). Recently we have analyzed BCRP mRNA expression along the human gastrointestinal tract of healthy subjects with respect to sex (Gutmann et al., 2005). No sex-related differences were observed. The data of the present study again show no significant differences between women and men (data not shown). We infer from these data that sexual hormones have most probably no effect on the expression of BCRP in the human intestine.

This is the first study demonstrating that the expression of BCRP is altered in the human intestine during disease. Our results are in conflict with another study, where no differences in

duodenal BCRP protein levels were observed between icteric patients and healthy subjects (Dietrich et al., 2004a). The authors analyzed the expression of several ABC-transporters including BCRP in the human intestine and found only MRP2 to be down-regulated. A possible explanation for this discrepancy could be different durations and/or severity of cholestasis between the two patient groups in both studies.

During cholestasis, the expression of bile acid and bilirubin transporters such as the ABC-transporters MRP2, MRP3, MRP4, and organic anion transporting polypeptides (OATPs) is significantly changed (Tanaka et al., 2002; Wagner et al., 2003; Denk et al., 2004; Dietrich et al., 2004a). Alterations in transporter expression do not only occur in the liver but also in remote organs such as the kidney and intestine. The systemic mediators that regulate these events during cholestasis have not been fully discovered. Proinflammatory cytokines, bile salts, or hormones have been suggested to control transporter gene expression (Trauner et al., 2005).

IL-1 β has been identified as the central mediator for the down-regulation of intestinal MRP2 (Denson et al., 2002; Dietrich et al., 2004a). In liver, TNF-α and IL-1 β are responsible for the down-regulation of several hepatobiliary transporters (Geier et al., 2003). Cytokines mediate these effects by reducing the binding activity of nuclear receptors to the corresponding promoters (Denson et al., 2002; Geier et al., 2003; Li and Klaassen, 2004). Bile acids act through binding to their endogenous receptor farnesoid X receptor (FXR), which induces the expression of small heterodimer partner (SHP) (Lu et al., 2000). SHP in turn represses the expression of several genes involved in bile acid homeostasis (Jung and Kullak-Ublick, 2003; Popowski et al., 2005). PXR is a further transcription factor that can be activated by bile acids (Xie et al., 2001). PXR activation leads to the induction of multiple detoxification pathways including transporters (Stedman et al., 2005). Moreover, hormones like glucocorticoids can influence transporter expression, as they are able to transactivate the intestinal bile acid transporter ASBT via the glucocorticoid receptor (Jung et al., 2004). However, until now, there are no indications that intestinal BCRP is also regulated by these mechanisms. Members of the ABCG subfamily such as ABCG1 and ABCG5 / ABCG8 are repressed by bile acids via the FXR-SHP pathway in vitro (Brendel et al., 2002; Freeman et al., 2004). This demonstrates that further investigations of BCRP regulation are required.

In the state of obstructive cholestasis virtually no bile acids are present in the intestinal lumen. If BCRP mediates the efflux of bile salts that enter the enterocyte from the lumen, a down-regulation of this transporter would make sense. On the other hand, as BCRP is a potent efflux pump for a variety toxic compounds, a diminished expression could weaken the intestinal barrier

and the excretory function of the gut. Dietrich and coworkers determined the elimination of the dietary carcinogen PhIP in bile duct ligated rats (Dietrich et al., 2004b). They demonstrated that the excretion of this toxin was significantly reduced in parallel with a decreased expression of MRP2 and BCRP in liver. Furthermore, tissue binding of reactive metabolites was increased in liver and colon. A reduced intestinal BCRP expression could substantially contribute to the accumulation of carcinogens in the gut enterocytes. This might partly explain the observation that patients with primary sclerosing cholangitis, a chronic cholestatic disease, have a higher risk of developing colorectal carcinoma (Broome et al., 1995).

In conclusion, we showed that patients with obstructive cholestasis exhibit a decreased expression of BCRP on mRNA and protein level. After reconstitution of bile flow BCRP mRNA levels normalized in all follow-up patients. Therefore, mediators that are associated with cholestasis seem to influence BCRP expression on the transcriptional level. Importantly, a decreased expression of this efflux pump could increase the accumulation of food-derived carcinogens and influence the pharmacokinetics of various anticancer drugs. Whether these results have clinical implications for cancer therapy is beyond the scope of this study.

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3.4 Mapping of multidrug resistance gene 1 and multidrug resistance associated protein isoform 1 to 5 mRNA expression along the human intestinal tract

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3.4.1 Abstract

Efflux transporters such as P-glycoprotein and multidrug resistance-associated proteins (MRPs) in the intestinal wall restrict intestinal drug transport. To overcome this limitation for enteral drug absorption, galenical targeting approaches have been proposed for site-specific luminal drug release in segments of the gut, where expression of the respective absorption-limiting transporter is minimal. Therefore, expression of multidrug resistance gene 1 (MDR1) and MRP1-5 was systematically investigated in 10 healthy subjects. Biopsies were taken from different segments of the gastrointestinal tract (from duodenum, terminal ileum as well as ascending, transverse, descending, and sigmoid colon). Gene expression was investigated by quantitative real-time PCR (TaqMan). MRP3 appeared to be the most abundantly expressed transporter in the investigated parts of the human intestine, except for the terminal ileum, where MDR1 showed the highest expression. The ranking of transporter gene expression in the duodenum was MRP3>>MDR1>MRP2>MRP5>MRP4>MRP1. In the terminal ileum the ranking order was as follows: MDR1>MRP3>>MRP1≈MRP5≈MRP4>MRP2. In all segments of the colon (ascending, transverse, descending, and sigmoid colon), the transporter gene expression showed the following order: MRP3>>MDR1> MRP4≈MRP5>MRP1>>MRP2. We have shown, for the first time, systematic site-specific expression of MDR1 and MRPs along the gastrointestinal tract in humans. All transporters showed alterations in their expression levels from the duodenum to sigmoid colon. The most pronounced changes were observed for MRP2 with high levels in the small intestine and hardly any expression in colonic segments. This knowledge may be useful to develop new targeting strategies for enteral drug delivery.

3.4.2 Introduction

Efflux transporters in the intestinal wall form a barrier to cellular accumulation of toxins as well as to drug absorption (Schinkel, 1997). Important efflux proteins in the gut are P-glycoprotein [gene product of the multidrug resistance 1 (MDR1) gene] and multidrug resistance-associated protein (MRP) transporters. They belong to the superfamily of ATP-binding cassette (ABC) transporters. ABC transporters mediate the translocation of a wide variety of substances across cellular membranes using ATP hydrolysis (Horio et al., 1991; Senior et al., 1995). The expression of ABC transporter genes is widespread throughout many tissues, most notably in excretory sites such as the liver, kidney, blood-brain barrier, and intestine. Therefore, they play a critical role in absorption and tissue distribution of orally administered drugs (Schuetz et al.,

1998; Ambudkar et al., 1999). Due to their broad substrate specificity, they may influence the pharmacokinetics of many chemically unrelated substances (e.g., HIV drugs, anticancer drugs, endogenous compounds) (Lee et al., 1997; Schinkel, 1998; Schuetz et al., 1999; Borst et al., 2000). MDR1 preferentially extrudes large hydrophobic, positively charged molecules, whereas the members of the MRP family extrude both hydrophobic uncharged molecules and water-soluble anionic compounds.

There is little knowledge about the expression pattern of those ABC transporters along the human intestine. Taipalensuu investigated gene expression of 10 ABC transporters in jejunal biopsies from healthy subjects (Taipalensuu et al., 2001). The highest expression was shown for breast cancer resistance protein and MRP2. Nakamura investigated the expression of three ABC transporters in duodenal and colorectal tissues in humans (Nakamura et al., 2002). In comparison to duodenum, in colon they found a decrease in MDR1 expression, equal levels of MRP1, and a strong decrease in MRP2 expression. However, this comparison was not obtained in the same subjects. Therefore, the intraindividual expression differences between these transporters could not be assessed.

Knowledge of the topographical distribution may be important for the development of specific galenical targeting approaches, which may be utilized to improve intestinal absorption of drugs. Therefore, in this study, the expression of MDR1 and MRP1-5 genes was investigated in the human intestine of 10 healthy subjects.

3.4.3 Material and methods

Intestinal biopsies

Intestinal biopsies were obtained from a group of 10 healthy subjects (5 female, 5 male, aged 50–76 years, average age 62 years, no medication), which served as a control group in a clinical study designed to investigate the regional expression of different genes in patients with inflammatory bowel disease. The study protocol included specifically the investigation of drugtransporting proteins and was approved by the local ethical committee (*Ethische Kommission beider Basel*, EKBB). Informed consent was obtained from all subjects prior to inclusion. No macroscopically pathological findings were observed during endoscopies in these subjects. Three to four biopsies were obtained from duodenum, terminal ileum, ascending colon, transverse colon, descending colon, and sigmoid colon (Figure 3.4.1). Due to low enterocyte content, duodenal biopsies from one subject had to be discarded, leading to nine duodenal samples.

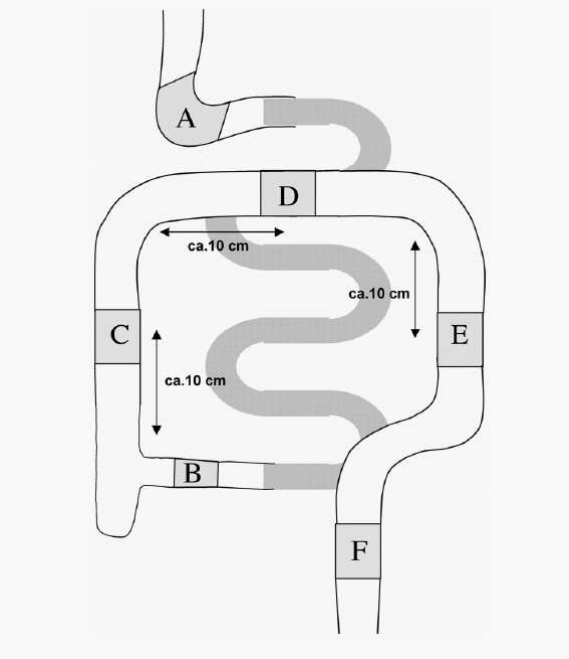

Figure 3.4.1 Schematic overview of biopsy sampling. Samples were taken from the duodenum (A), terminal ileum (B), ascending colon (C), transverse colon (D), descending colon (E), and sigmoid colon (F).

Preparation of Samples and Taqman analysis was described in chapter 2

Statistical analysis

Gene expression was compared between the different intestinal segments by analysis of variance. In the case of significant differences, all segments were compared with the expression in duodenum using two-sided Dunnett's multicomparison t test. The level of significance was $P<0.05$. Comparisons were performed using SPSS for Windows software (version 11.0; SPSS Inc., Chicago, IL).

3.4.4 Results

There was a considerable interindividual variability of transporter gene expression amounting on average to 34% (CV%). Figure 3.4.2 displays the expression and ranking of all transporters in the analyzed tissues normalized to villin. MRP3 appeared to be the most abundantly expressed transporter in the investigated parts of the human intestine, except for the terminal ileum where MDR1 showed the highest expression. The ranking of transporter gene expression in the duodenum was MRP3 >> MDR1 > MRP2 > MRP5 > MRP4 > MRP1. In the terminal ileum the

ranking order was as follows: MDR1 > MRP3 >> MRP1 ≈ MRP5 ≈ MRP4 > MRP2. In all segments of the colon (ascending, transverse, descending, and sigmoid colon), the transporter expression showed the following order: MRP3 >> MDR1 > MRP4 ≈ MRP5 > MRP1 >> MRP2.

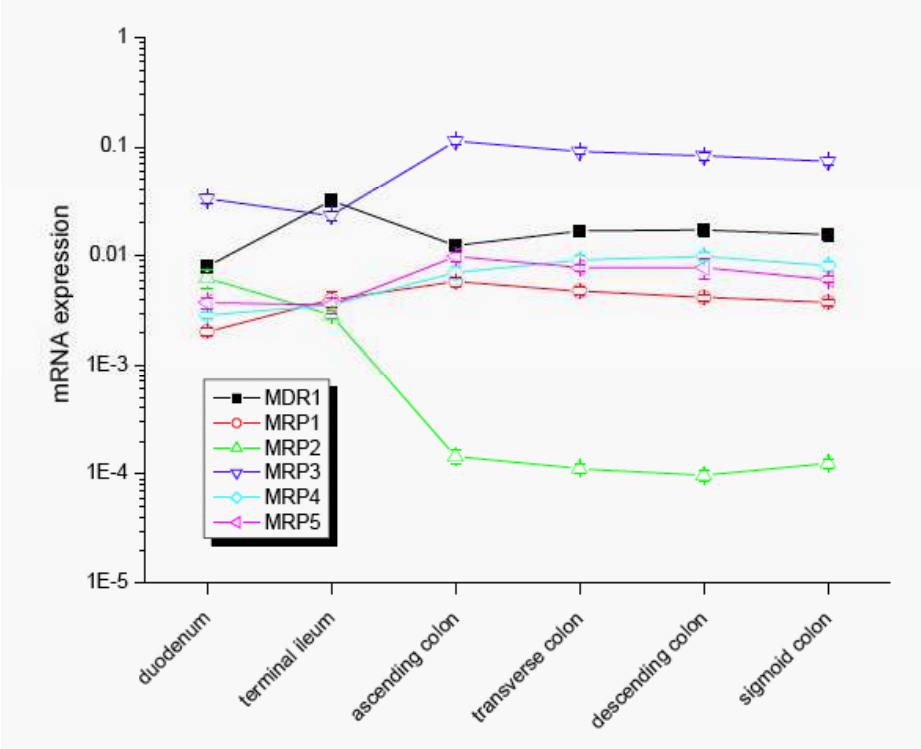

Figure 3.4.2: Expression of all investigated transporters in the analyzed tissues normalized to villin expression. Data represent means (± SEM) of biopsies from 10 health subjects, except duodenum, where biopsies from 9 subjects were used.

Figure 3.4.3 shows the expression pattern of each individual transporter from the duodenum to the sigmoid colon normalized to villin. Compared with the duodenum, the expression of MDR1 was 4- fold higher in the terminal ileum and approximately 2-fold higher in the colonic segments. MRP1 exhibited a 2- to 3-fold higher expression in both the terminal ileum and colon compared with duodenum. MRP2 showed highest expression in the duodenum, half-levels in the terminal ileum, and hardly any MRP2 transcripts in each colonic segment. MRP3, MRP4, and MRP5 exhibited a similar expression pattern with equal levels in the duodenum and terminal ileum, but a 2- to 3-fold increase in the colon. Within the colon, MRP1, MRP3, and MRP5 showed an expression pattern with decreasing levels from proximal to distal, whereas MDR1, MRP2, and MRP4 levels remained rather constant.

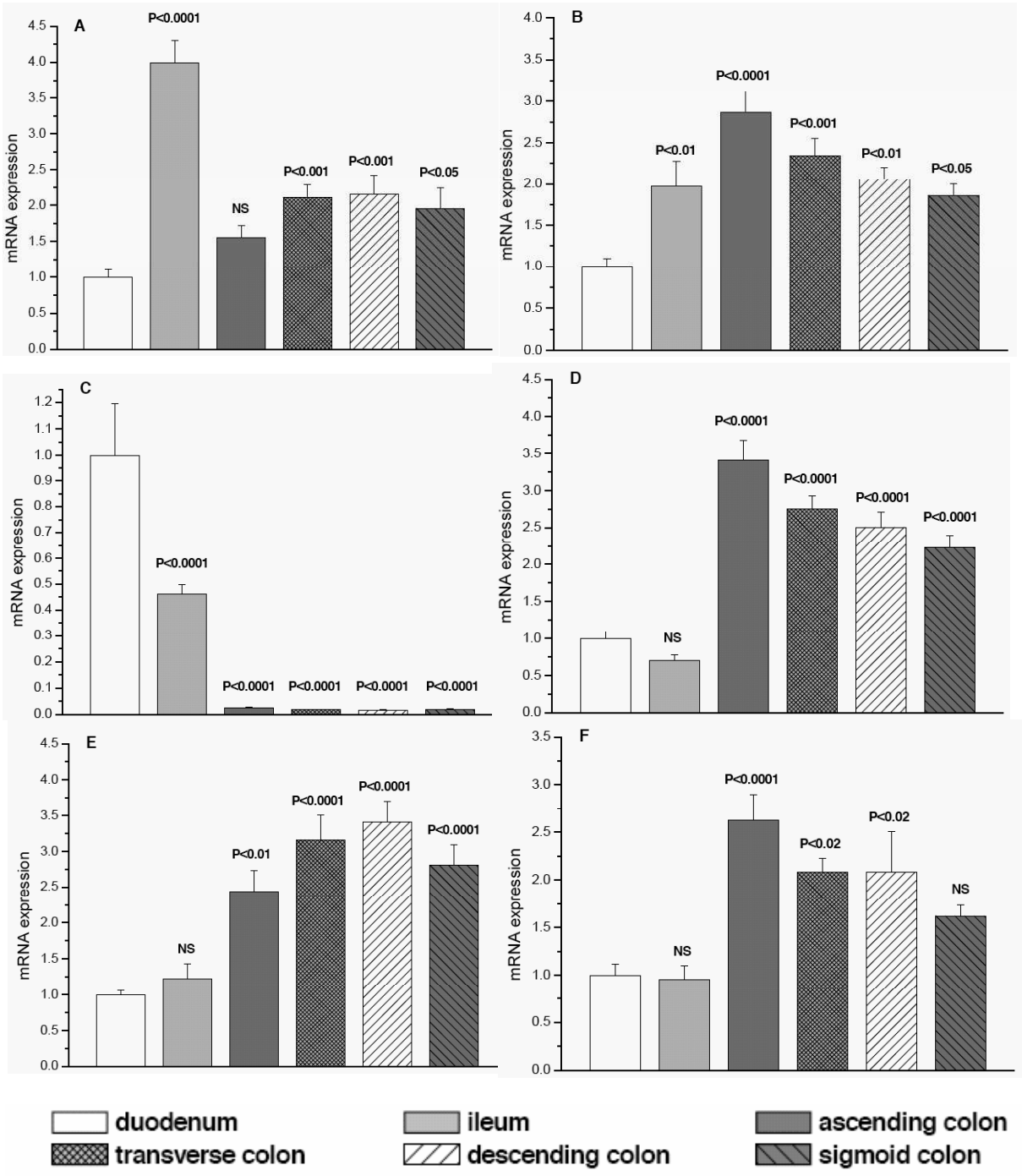

Figure 3.4.3 (A – F): Transporter specific gene expression in different gut segments normalized to the villin expression. A: MDR1, B: MRP1, C: MRP2, D: MRP3 E: MRP4 and F: MRP5. Data represent means

(± SEM) of biopsies from 10 healthy subjects, except duodenum, where biopsies from 9 subjects were used.

3.4.5 Discussion

Only little information is available about the expression of ABC transporters along the intestinal tract. Available information relates mainly to MDR1 and MRP2 expression (Dietrich et al., 2003; Lindell et al., 2003) and previous studies have usually focused on isolated parts of the intestine (Taipalensuu et al., 2001; Lindell et al., 2003), on animal models (Achira et al., 2002; Takara et al., 2003), or on cancer cells (Nakamura et al., 2002; Li et al., 2003; Pfrunder et al., 2003). In the present study, systematic site-specific expression of MDR1 and MRP isoforms along the gastrointestinal tract in humans was shown. All transporters showed alterations in their expression levels from the duodenum to the sigmoid colon. The most pronounced changes were observed for MRP2, with high levels in the small intestine and hardly any expression in colonic segments. One drawback of the present study is the lack of samples from the jejunum, an important site for drug absorption. Because the subjects in our study underwent combined gastroscopy and colonoscopy procedures for screening of gastrointestinal cancer, jejunoscopy was not indicated and could not be performed. However, Taipalensuu and co-workers focused on the human jejunum and found a transporter expression with the following ranking: MRP2 > MDR1 ≈ MRP3 > MRP5 ≈ MRP1 > MRP4 (Taipalensuu et al., 2001). This transporter expression pattern in the jejunum, besides the high MRP2 levels, shows strong similarity to the pattern we found in the terminal ileum, which is conclusive because of the proximity of these tissues.

It is suggested that MDR1 physiologically functions as a gatekeeper against xenobiotics in the gut. The bioavailability of many drugs is reduced due to MDR1 efflux. MDR1 shows extremely broad substrate specificity, including anticancer agents, antibiotics, antivirals, calcium channel blockers, and immunosuppressants. With respect to the expression of MDR1 in the human intestine, an increase from proximal to distal was stated, with the highest expression levels documented in the colon (Fricker et al., 1996; Dietrich et al., 2003; Chan et al., 2004). In mice, however, Chianale and co-workers found the highest levels of mdr3 mRNA in the ileum (Chianale et al., 1995). In the rat intestine, the P-glycoprotein-mediated drug efflux showed highest activity in the ileum as well (Stephens et al., 2001). We could also demonstrate, in humans, higher MDR1 mRNA levels in the terminal ileum compared with the duodenum. These results are consistent with human data from Mouly and Pain, who reported an increase in P-glycoprotein from duodenum to ileum (Mouly and Paine, 2003).

MRP1 showed the lowest variation in mRNA levels within the intestinal tract. This is in good agreement with the fact that MRP1 is expressed ubiquitously. Physiologically important substrates for MRP1 include glutathione *S*-conjugates such as leukotriene C4, as well as bilirubin glucuronides (Keppler et al., 1998). In addition, anionic drugs and drugs conjugated to glutathione, like methotrexate or arsenite, are also transported by MRP1 (Bakos et al., 2000; Vernhet et al., 2000).

Relatively low MRP2 mRNA levels were found in the human duodenum and even lower levels in the terminal ileum, but almost no MRP2 expression in the entire colon. These results were also found in the rat intestine (Mottino et al., 2000; Rost et al., 2002), but up to now, they were not confirmed in humans. Our results are also consistent with the expression pattern of glutathione *S*-transferase in the human gastrointestinal tract mucosa (Coles et al., 2002). This phase II metabolizing enzyme provides the conjugated compounds for subsequent export by MRP2 or MRP1. The substrate specificity of MRP2 is similar to that of MRP1, and includes glutathione conjugates, bilirubin glucuronides, and a number of drugs and their conjugated drug metabolites (Jedlitschky et al., 1997; Kawabe et al., 1999), including pravastatin, temocaprilat, irinotecan, SN-38, arsenite, cisplatin, methotrexate, vincristine, saquinavir, and ceftriaxone (Kusuhara and Sugiyama, 2002; Dietrich et al., 2003). Regarding the amount of drugs transported by MRP2, a drug targeting which circumvents absorption sites with high MRP2 expression would be of benefit, especially for drugs with low bioavailability. MRP3 transports a wide range of bile salts and seems to be involved in their reabsorption (Hirohashi et al., 2000). For MRP3, Rost and coworkers showed low expression in the rat duodenum and high expression in the ileum and colon (Rost et al., 2002). Here, low MRP3 expression in the duodenum as well as in the terminal ileum was shown. MRP3 expression increased in the colon but diminished slightly from proximal to distal segments. This reduction in transporter expression from ascending to sigmoid colon was observed for MRP1, MRP3, and MRP5. Interestingly, all of these transporters are located on the basolateral membrane. For MDR1, MRP2, and MRP4, probably located on the apical membrane (Chan et al., 2004) we observed rather constant expression levels throughout the entire colon.

With respect to MRP4, we found equal expression levels in the duodenum and the terminal ileum but a 3-fold increase in the colon. To our knowledge, there is no previous publication on the MRP4 expression in the colon. However, in lymphoblastoid cell line lines an overexpression of MRP4 severely impaired the antiviral efficacy of adefovir, azidothymidine, and other nucleoside analogs (Schuetz et al., 1999). Other substrates include folic acid, bile acids, methotrexate, and 6-mercaptopurine (Wielinga et al., 2002; Chan et al., 2004). A physiological

role of MRP4 might be the release of prostaglandins from cells (Reid et al., 2003). Similarly, MRP5 showed low expression in the small intestine, but a 2-fold increase in the different colon segments. Both, MRP4 and MRP5 have an affinity to nucleotide-based substrates. There are no reports, at present, which could suggest a role for MRP5 in intestinal drug disposition. Experiments with transfected cells showed enhanced efflux of 2,4-dinitrophenyl-*S*-glutathione, adefovir, and the purine analogs 6-mercaptopurine and thioguanine (Wijnholds et al., 2000). Jedlitschky and co-workers demonstrated that MRP5 transports the cyclic nucleotides cAMP and cGMP (Jedlitschky et al., 2000), but the physiological function of this transporter remains to be elucidated.

3.4.6 Conclusion

In this study, for the first time, systematic site-specific expression of MDR1 and MRP isoforms along the gastrointestinal tract in humans was shown. All transporters showed alterations in their expression levels from the duodenum to the sigmoid colon. The most pronounced changes were observed for MRP2, with high levels in the small intestine and hardly any expression in colonic segments. The knowledge of transporter expression along the intestinal tract may be useful to develop new targeting strategies for enteral drug delivery.

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4. MDR1, MRP1-5 and BCRP mRNA expression in human Monocyte Derived Macrophages

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4.1 Abstract

Introduction: Multidrug resistance transporters such as MDR1, MRP1-5 and BCRP are membrane efflux pumps that extrude a wide variety of drugs and endogenous compounds, thereby reducing their intracellular concentration. Therapy resistance in patients with an acute or chronic inflammatory disease treated with drugs which are substrates of MDR transporters is a relevant clinical problem. As macrophages play an important role in innate immune response, the knowledge of the transporter expression in the monocyte and in the monocyte derived macrophage might be of special value for pharmacological treatment and could be essential for understanding treatment failure in patients with acute or chronic inflammatory disease.

Methods: Peripheral blood mononuclear cells (PBMC`s) were isolated from venous blood of 5 healthy donors. The CD14+ PBMC subpopulation (monocytes) was purified by magnetic cell separation and cultivated in AB-serum containing medium for 7 days, to obtain monocyte derived macrophages (MDM). On day 6 part of the differentiated cells were stimulated with lipopolysaccharide (LPS) for 24h. Cells were phenotyped by staining with FITC-conjugated mouse monoclonal antibodies (mAb) to human HLA-DR, CD14, CD71 and CD 206. MDR1, MRP1-5 and BCRP mRNA expression was measured by real time PCR (Taqman).

Results: MDR1 and BCRP mRNA was not expressed in monocytes and only low expression was found in MDM for both transporters, which decreased insignificantly after incubation with LPS. MRP1 mRNA expression was lowest in monocytes and increased significantly with differentiation into MDM ($p < 0.01$) as well as after stimulation with LPS ($p < 0.001$). In contrast, highest MRP2 mRNA expression was found in monocytes, which decreased by six fold after differentiation into MDM ($p < 0.001$). MRP3-5 showed a similar expression pattern with the lowest gene expression in monocytes, which increased significantly during differentiation into MDM and decreased after LPS stimulation.

Conclusion: The investigated ABC transporters MDR1, MRP1-5 and BCRP showed different gene expression patterns during differentiation of monocytes into MDM and subsequent stimulation with LPS, indicating different physiological functions.

4.2 Introduction

ATP- binding cassette (ABC) transporters are transmembrane proteins that translocate a wide variety of substrates across extra- and intracellular membranes using ATP hydrolysis (Horio et al., 1988). Multidrug resistance transporter 1 (MDR1; ABCB1), multidrug resistance associated protein 1-5 (MRP1-5; ABCC1-5), as well as the breast cancer resistance protein (BCRP; ABCG2) are membrane efflux pumps which form a barrier to cellular accumulation of toxins as

well as to drug absorption, thereby lowering their intracellular concentration (Schinkel, 1997) and protecting tissue from xenobiotic accumulation and subsequent toxicity. Multidrug resistance is a phenomenon in which a cell exposed to a single drug, becomes cross-resistant to a large number of structurally unrelated compounds. Expression of P-glycoprotein (P-gp; gene product of the MDR1 gene) in different types of solid tumors and in leukemias has been associated with clinical resistance to chemotherapy, indicating that P-gp mediated multidrug resistance is an important clinical problem (Plaat et al., 2000; Sonneveld, 2000). Furthermore, there is increasing evidence that several multidrug resistance transporter play an important role in acute and chronic inflammatory processes (Wijnholds et al., 1997; Panwala et al., 1998) and might contribute to the development of therapy resistance in certain inflammatory diseases (Maillefert et al., 1996; Farrell et al., 2000). Spontaneous intestinal inflammation similar to that of human IBD was observed in mdr1a (-/-) mice (Panwala et al., 1998). Patients with UC and CD exhibited decreased Pgp expression in $CD3^+$ intestinal lymphocytes when compared to healthy controls (Yacyshyn et al., 1999). High MDR1 protein expression in $CD3^+$ circulating lymphocytes in IBD patients that required surgery several years earlier for failed medical therapy was demonstrated (Farrell et al., 2000) and a significant correlation between peripheral blood lymphocytes and colonic mucosal MDR expression among patients with IBD and controls was found. These results implicate that MDR expression might play a role in determining the response of IBD patients to therapy.

In IBD, besides lymphocytes, other cell types such as macrophages play a key role in orchestrating a specific mucosal immune response. Peripheral blood monocytes, which originate from bone marrow progenitor cells, mature to different types of histiocytes and macrophages when they migrate from the bloodstream into various tissue. Macrophages have been shown to express an activated phenotype (CD14+) in patients with IBD that is usually not expressed in normal intestine (Rogler et al., 1997). These activated macrophages can substantially enhance the immune response in patients with IBD by acting as an antigen presenting cell (APC) and by secreting proinflammatory cytokines such as IL-1, IL-6, IL-12, IL-18 and TNFα.

Several drugs used in the treatment of IBD patients are MDR, MRP or BCRP substrates. In vitro models with high MDR or MRP overexpressing cells have provided experimental evidence, that glucocorticoids (Pgp, MRP4), sulfasalazine (BCRP), cyclosporine (Pgp), methotrexate (MRP1-3) and the active metabolite of the inactive prodrug azathioprine, 6-mercaptopurine (MRP5) are actively transported out of these cells (Ueda et al., 1992; Schinkel et al., 1995; Borst et al., 1999; Borst et al., 2000; Keppler et al., 2000; van der Heijden et al., 2004).

Although, MDR1, MRP1-5 and BCRP are expressed in a variety of normal tissues very little is known about their expression and function in cells of the immune system. Therefore, MDR1, MRP1-5 and BCRP mRNA expression was investigated in human peripheral blood monocytes and monocyte derived macrophages (MDM). In addition, the effect of lipopolysaccharide (LPS) incubation on MDM on the gene expression of MDR transporters was studied. Knowledge of MDR transporter expression in the monocyte and in the monocyte derived macrophage might contribute to the improvement of pharmacological treatment of IBD patients and might help to understand better the molecular mechanisms leading to treatment failure in patients with inflammatory diseases.

4.3 Material and Methods

4.3.1 Reagents

All chemicals were of highest quality available and were obtained from commercial sources. LPS preparation (Salmonella enterica serovar typhimurium) was purchased from Sigma Chemical Co (St. Louis, Mo.).

4.3.2 Preparation and culture of peripheral blood monocytes

Peripheral blood mononuclear cells (PBMC`s) were isolated from venous blood of 5 healthy donors (5 male, average age 34.2 years, no medication) by Ficoll gradient centrifugation. CD14+ PBMC subpopulation (monocytes) was purified by magnetic cell separation (Miltenyi Biotech, Bergisch Gladbach, Germany) using $CD14^+$ magnetic beads according to producer`s protocol.
Monocytes then were cultured at 37℃ in RPMI medium supplemented with gentamycin 15 µg/ml, sodium pyruvate (0.5 mM), hepes (0.75 mM), glutamax (1mM), non essential amino acids (all from Gibco Paisley, Scotland), 5% fetal calf serum and 10% heat inactivated (30 min at 56°C, which does not destroy LBP bioactivity) human serum (pool of six samples from healthy individuals with blood group AB from the blood donor bank, Blutspendezentrum, Universitätsspital Basel, Switzerland). Cultivation was performed in hydrophobic quadriPERM Teflon wells (Vivascience, Göttingen, Germany) for 7 days with or without stimulation with LPS 100 ng/ml culture medium after 6 days (intermediate feeding of medium).

4.3.3 Phenotyping of cells

Cells were phenotyped by staining with FITC-conjugated mouse monoclonal antibodies (mAb) to human HLA-DR, CD14, CD71 and CD206 (Becton Dickinson, San Diego, USA). Cell acquisition

was done by a flow-cytometer (FACScalibur) equipped with Cell Quest software (Becton Dickinson, San Diego, USA).

Real time polymerase chain reaction analysis of human MDR1, MRP1-5, BCRP mRNA (TaqMan assay) as described in Chapter 2

Statistical analysis

Gene expression was studied in CD14+ PBMC`s (monocytes), monocyte derived macrophages with and without stimulation with LPS. For the comparison of the gene expression level the Tukey test was used. The level of significance was $P < 0.05$. Comparisons were performed using SPSS for windows software (version 12.0, SPSS Inc., Chicago, USA).

4.4 Results

For the analysis of ABC transporter mRNA of CD14+ PBMC`s (monocytes) during differentiation into monocyte derived macrophages (MDM) and subsequent activation with LPS, isolated monocytes from peripheral blood of healthy donors were cultured in human AB-serum containing medium. One part of the cultured cells was used for phenotyping by staining with FITC-conjugated mouse monoclonal antibodies (mAb) to human HLA-DR, CD14, CD71 and CD206. The remaining cells were then used for the analysis of ABC transporter mRNA expression using real time PCR (Taqman).

Phenotyping of monocytes showed a high CD14 and HLA-DR expression, whereas the differentiation markers CD71 and CD206 were low. During differentiation into MDM CD14 and HLA-DR decreased and CD71 and CD 206 increased. Stimulation of MDM with LPS was followed by a slight increase of CD14 and HLA-DR. The differentiation marker CD71 and CD 206 remained unchanged (Table1).

	monocytes	**macrophages**	**LPS stimulated macrophages**
CD14	521.9 ± 70.7	14.8 ± 3.6	35.7 ± 7.1
CD71	6.9 ± 1.5	67.6 ± 13.8	69.9 ± 18.2
CD206	4.4 ± 0.5	15.9 ± 4.5	12.6 ± 3.2
HLA-DR	226.6 ± 12	120 ± 24.3	150 ± 26

Table1: FACS Analysis of monocytes, macrophages and LPS stimulated macrophages (mean FI ± SEM)

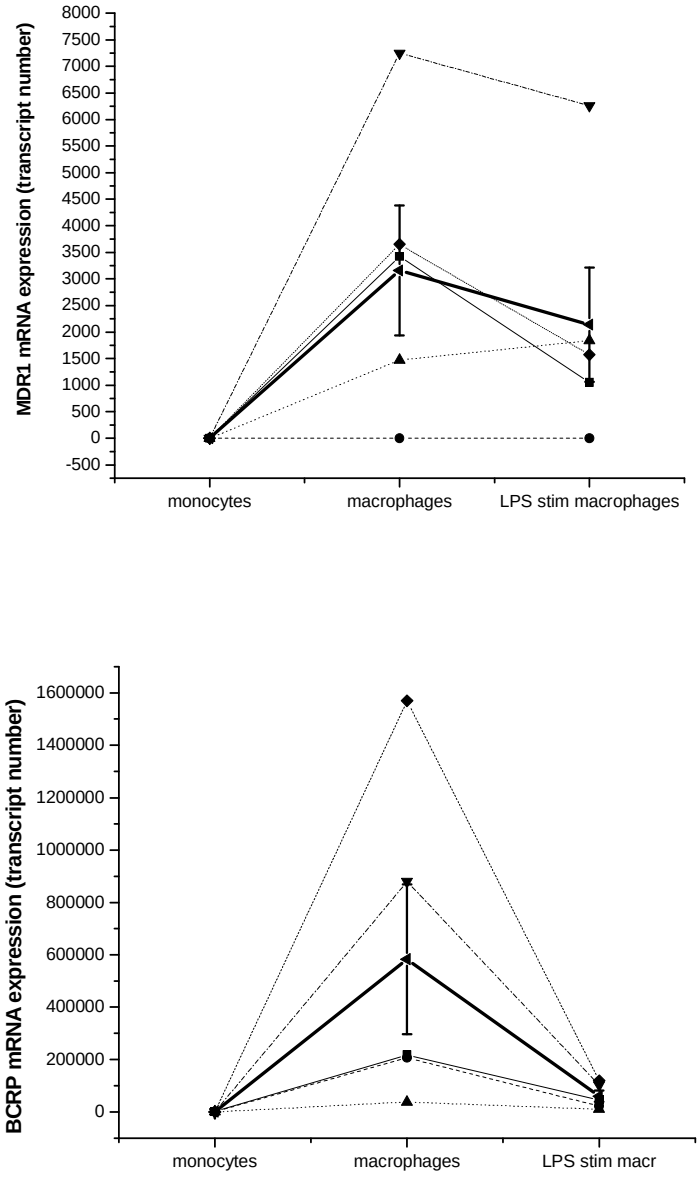


Figure 1: MDR1 and BCRP mRNA expression (mean arbitrary units ± SEM) in monocytes, macrophages and LPS stimulated macrophages (N=5)

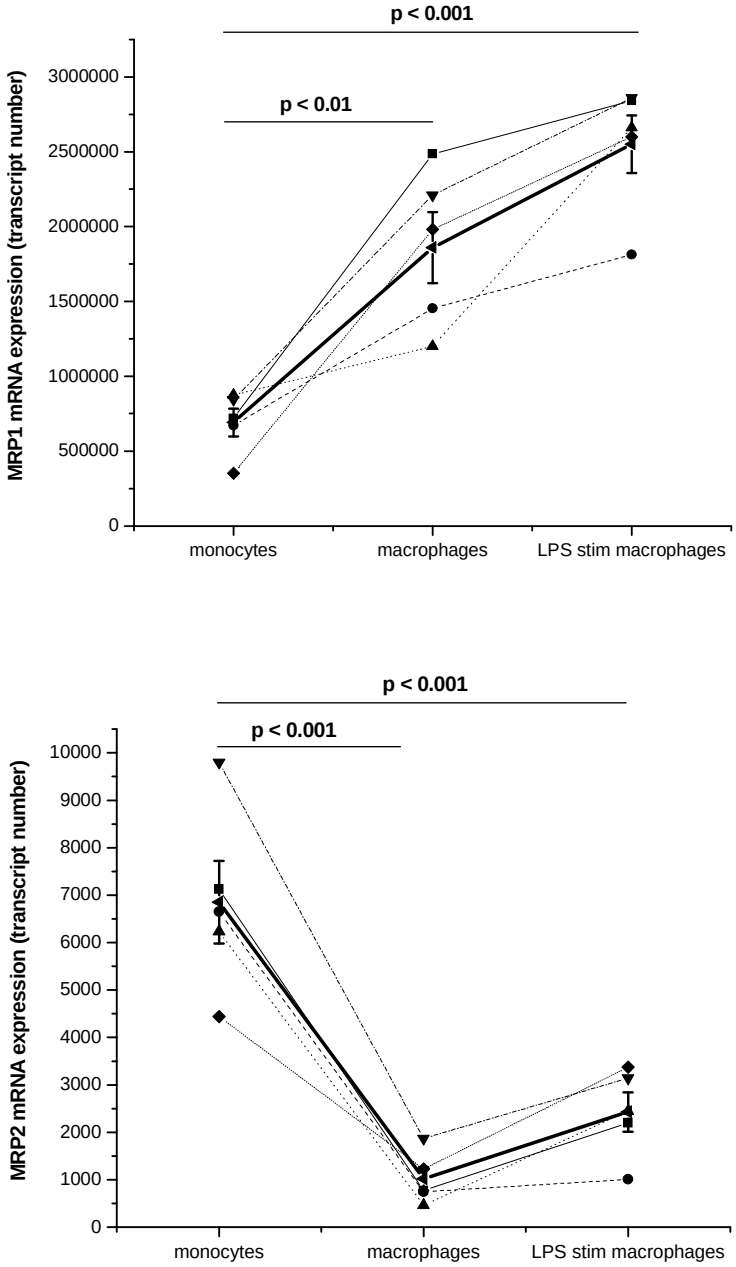
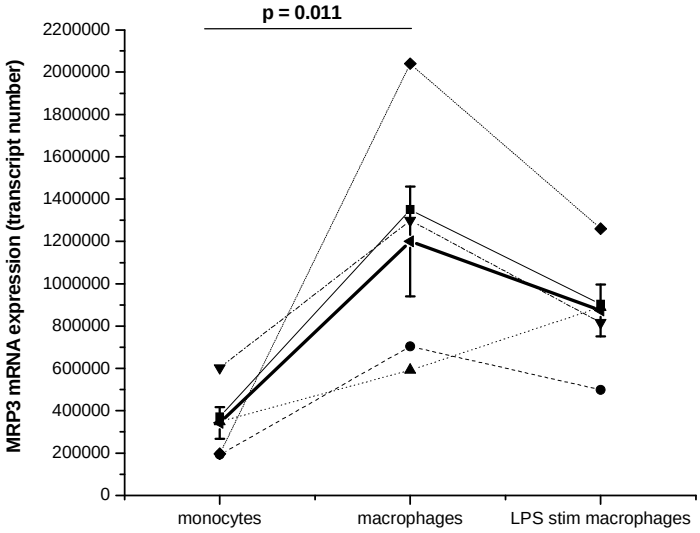


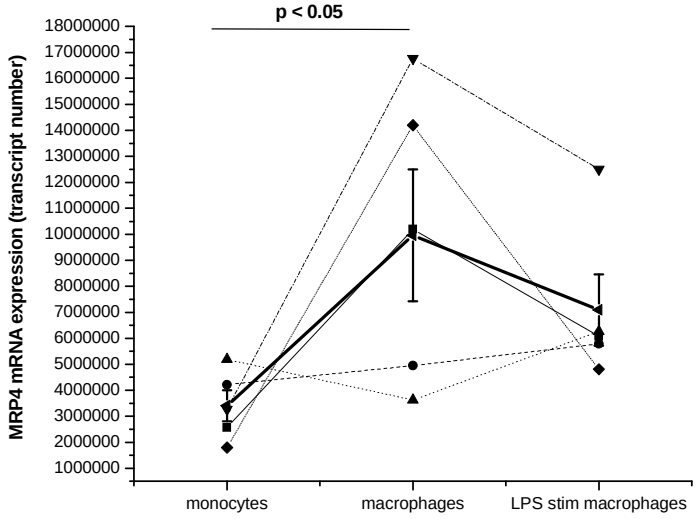
Figure 2A and 2B: MRP1 and MRP2 mRNA expression (mean arbitrary units ± SEM) in monocytes, macrophages and LPS stimulated macrophages (N=5)

MDR1 mRNA and BCRP mRNA were not expressed in monocytes. Both transporters showed low expression after differentiation into MDM, which decreased insignificantly by activation with LPS (Figure1). MRP1 mRNA expression was lowest in monocytes, which increased significantly

with differentiation into MDM ($p < 0.01$) and upon stimulation with LPS ($p < 0.001$) (Figure 2A). In contrast, highest MRP2 mRNA expression was found in monocytes, and was about six fold lower after differentiation into MDM ($p < 0.001$) (Figure 2B).

A similar expression pattern could be shown for MRP3, MRP4 and MRP5 with the lowest gene expression in monocytes and increased gene expression in MDM by 4-fold ($p= 0.011$), 3-fold ($p< 0.05$) and 2.5-fold ($p< 0.01$), respectively. Gene expression decreased for all three transporters in LPS stimulated macrophages (Figure 2C, 2D, 2E). The decrease reached significance only for MRP5 ($p< 0.01$).





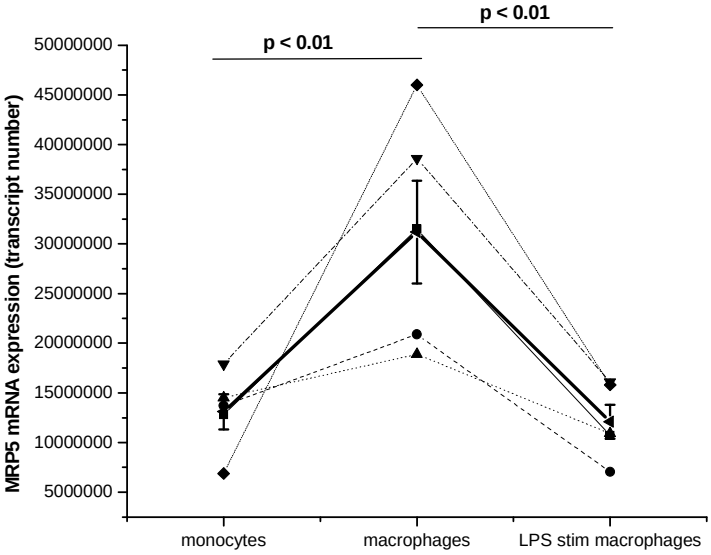


Figure 2C-E: MRP3-5 mRNA expression (mean arbitrary units ± SEM) in monocytes, macrophages and LPS stimulated macrophages (N=5)

4.5 Discussion

Here, we present an analysis of MDR1, MRP1-5 and BCRP mRNA expression in human peripheral blood monocytes and their change after differentiation into monocyte-derived macrophages (MDM). Furthermore we investigated the influence of the incubation of MDM with lipopolysaccharide (LPS) on the expression level of these transporters.

In the present study, MDR1 and BCRP mRNA was not expressed in peripheral blood monocytes of healthy male donors. After differentiation into MDM for both ABC transporters only low mRNA expression could be demonstrated. Comparable results with any expression of P-glycoprotein (P-gp) were reported in monocytes and monocyte derived dendritic cells using immunolabeling experiments (Laupeze et al., 2001a). Furthermore, neither verapamil nor probenecid, which are known inhibitors of P-gp, altered intracellular rhodamine 123 levels in CD14+ monocytes (Laupeze et al., 2001b) indicating no functional activity of P-gp in these cells. In contrast, intracellular staining of P-gp with monoclonal antibodies was detected in CD14+ monocytes by confocal microscopy and in the same study P-gp staining in CD14+ cells was found unaltered in blood samples of HIV patients compared to controls (Malorni et al., 1998). MDR1 expression was also studied in other cell types of the human peripheral blood. The highest expression was found in CD56+ cells, followed by CD8+ > CD4+ > CD19+ cells (Oselin et al., 2003). Puddu et al. reported that P-gp expression was upregulated in a dose- and time-dependent manner in

MDM by treatment with IFN-γ (Puddu et al., 1999). It was shown that this upregulation was a specific response of primary macrophages, as IFN-γ treatment of primary lymphocytes and monocytic cell lines did not result in any increase of P-gp expression. Recently, it was suggested that P-gp mediated therapy resistance might be a common problem in chronic inflammatory processes (Farrell et al., 2000). For example, it was shown that peripheral blood mononuclear cells (PBMC) of patients with ulcerative colitis with previous glucocorticoid therapy had significant higher levels of MDR1 mRNA compared to patients without history of glucocorticoid administration (Hirano et al., 2004). Patients with UC and CD exhibited decreased Pgp expression in $CD3^+$ intestinal lymphocytes when compared to healthy controls (Yacyshyn et al., 1999) and high MDR1 protein expression in $CD3^+$ circulating lymphocytes in IBD patients that required surgery several years earlier for failed medical therapy was demonstrated .(Farrell et al., 2000) Significantly elevated MDR expression in PBL has been shown also in patients with rheumatoid arthritis (RA) that require glucocorticoids (Maillefert et al., 1996).

BCRP expression was detected mainly in excretory organs, e.g in canalicular membranes of the liver, in epithelial cells of the small intestine, colon, kidney and lung as well as in the blood brain barrier and the placenta (Scheffer et al., 2000; Maliepaard et al., 2001). It was found to be expressed in primitive hematopoietic stem cells (Zhou et al., 2005). However, there are no data about BCRP expression in peripheral blood cells. Interestingly, *in vitro* long term exposure of human T-cells to sulfasalazine (SSZ) was leading to an induction of BCRP protein expression (van der Heijden et al., 2004). Furthermore, an increase in TNFα release was observed, although the anti-inflammatory properties of SSZ, as a disease modifying antirheumatic drug (DMARD), have been attributed to diminished production of proinflammatory cytokines such as TNFα. Therefore the authors concluded that SSZ can lead to development of resistance by induction of BCRP and to a less effective inhibition of nuclear NFκB activation by SSZ in the resistant cells. In the same study P-gp was unaffected, while MRP1 expression was downregulated.

In the present study we could demonstrate that the level of MRP1 mRNA and MRP3-5 mRNA is significantly lower in monocytes than in MDM. In contrast, the level of MRP2 mRNA expression was significantly higher in monocytes than in MDM.

There are only rare data about expression of MRP1-5 in human peripheral blood cells. It was found that normal peripheral blood cells, regardless of cell lineage, expressed a similar basal level of MRP mRNA (Abbaszadegan et al., 1994). In another study, MRP1 and MRP2 mRNA expression was measured in human peripheral lymphocytes and showed highest expression in CD4+ cells, followed by CD8+ > CD19+ > CD56+ cells (Oselin et al., 2003). MRP1 as well as

MRP4 and MRP5 mRNA expression was described to be low in MDM cells and *in vitro* infection of MDMs with HIV-1/Ba-L increased the mRNA expression level of MRP1, MRP4 and MRP5 (Jorajuria et al., 2004b). Incubation of HIV infected MDM with zidovudine, a nucleoside reverse transcriptase inhibitors, which is transported by MRP4 and MRP5 (Schuetz et al., 1999; Reid et al., 2003), increased MDR1, MRP4 and MRP5 mRNA expression. Also in infected MDM, PSC833 (Pgp inhibitor) as well as probenecide (unspecific MRP1 inhibitor) increased the anti-HIV activity of zidovudine and indinavir, a protease inhibitor, which is transported by MRP1 (Srinivas et al., 1998), substantially (Jorajuria et al., 2004a). Indinavir (10nM) showed a 14% inhibition without and an 81% inhibition in combination with inhibitors.

Based on these results with zidovudine and indinavir MDM seems to be a suitable model system investigating the regulation of expression of multidrug resistance transporters by drugs. These drugs should include glucocorticoids (MDR1), methotrexate (MRP1-3), cyclosporine (MDR1), 6-mercaptopurine (MRP4) and sulfasalazine (BCRP), which all are substrates of MDR transporters and are used in treatment of patients with IBD.

In addition, in the present study, the effect on multidrug resistance tranporter expression after LPS incubation of MDM was investigated and a significant increase in MRP1 mRNA expression as well as a significant decrease in MRP5 mRNA expression was found. Increased expression after LPS administration was also found for MRP2 mRNA and decreased expression was observed for BCRP, MDR1, MRP3 and MRP4 mRNA. Lipopolysaccharide (LPS), a component of gram negative bacterial cell wall, activates macrophages via binding to the CD14 receptor and the signal is mediated by the Toll like Receptor pathway. This initial response to a stimulus is followed by production of proinflammatory cytokines leading to a cascade of reactions with activation of the NFκB pathway, production of reactive oxygen products and upregulation of inducible nitric oxide (NO) synthase (iNOS). To our knowledge, the effects of LPS on MDR transporter expression have been studied only in hepatocytes. LPS decreased mRNA levels of Mrp2 and increased Mrp1, Mrp3 and Mdr1b mRNA expression in rat hepatocytes, whereas Mrp5 mRNA was unchanged (Cherrington et al., 2004). Elferink et al studied the effect of endotoxin induced cholestasis (with LPS) in rat and human liver. They found that MRP2 mRNA remained unchanged in human liver during 24h of incubation with LPS. In contrast MRP protein was almost completely removed from the canalicular membrane in the presence of LPS, indicating posttranscriptional regulation of protein expression (Elferink et al., 2004). LPS incubation of rat liver was leading to downregulation of Mrp2 and to an induction of Mrp3 protein expression in periportal hepatocytes and also a strong induction of Mrp5 mRNA was found in the same study (Elferink et al., 2004).

Our FACS analysis showed that MDM had only low level of CD14 receptors and LPS stimulation of MDM was associated with only discrete changes in the expression pattern of MDR transporters. Interestingly, Smith et al. demonstrated that intestinal macrophages, which were low for CD14 and CD 89 (CD14- and CD89-), have been downregulated for LPS- and IgA mediated functions (Smith et al., 2001). So, low level of CD14 receptors with a possible downregulation for LPS mediated functions might be an explanation for the only discrete changes of expression levels of the investigated drug transporters in our study upon stimulation with LPS.

In conclusion, the mRNA expression level of MDR1, MRP1-5 and BCRP varies in monocytes and MDM. While MDR1 and BCRP are not expressed in monocytes and only low in MDM, MRP2 is highest expressed in monocytes with significantly lower expression in MDM. MRP1 and MRP3-5 show a significant increase when differentiated into MDM. Stimulation of MDM with LPS showed a significant increase of MRP1 mRNA and a significant decrease of MRP5 mRNA expression. Our results indicate that ABC transporters are adaptively regulated during differentiation into MDM and might therefore fulfill different physiological functions. Since many drugs that are used in the treatment of acute or chronic inflammatory processes are substrates of ABC transporters and macrophages play an important role in the innate immune response, MDM could be an interesting cell type studying MDR transporter regulation by anti-inflammatory and immunosuppressive drugs.

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5. Inflammation in prostate tissue does not influence MDR transporter expression

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5.1 Abstract

Objective: Multidrug resistance (MDR) transporters such as MDR1, MRP1-5 and BCRP are membrane efflux pumps that extrude drugs such as fluoroquinolones and α-adrenergic antagonists, which are used in the treatment of patients with chronic pelvic pain syndrome (CPPS), a syndrome caused by inflammatory and non-inflammatory factors. Therapy resistance in these patients is a relevant clinical problem. Since biopsies from patients with CPPS could not be obtained due to ethical reasons, the aim of the present study was to evaluate the influence of inflammation on MDR transporter expression in prostate tissue of patients with benign prostatic hyperplasia.

Methods: BCRP, MDR1 and MRP1-5 mRNA was quantified by real time PCR (Taqman) in prostate tissue of 50 patients with benign prostatic hyperplasia. Severity and localization of inflammation was assessed on hematoxylin and eosin stained sections. In addition, MDR transporter expression in prostate tissue of BPH patients was compared to LNcaP and PC3 prostate carcinoma cell lines.

Results: The ranking of transporter gene expression in the prostate tissue of patients with BPH was MRP4 = MRP1 >> MRP5 > MRP3 >> MDR1 = BCRP >> MRP2. Neither severity nor localization of inflammation in the prostate tissue influenced the expression of these MDR transporters. A significant decrease of BCRP mRNA expression (normalized to GAPDH mRNA) was observed for BPH patients treated with α-adrenergic antagonists (mean ± SEM) 0.0067 ± 0.0007 when compared to untreated patients 0.004 ± 0.0005 ($p < 0.05$) before undergoing TUR-P. Expression of MDR transporters did vary in different prostate carcinoma cell lines (LncaP, PC3), when compared to prostate tissue of BPH patients. This indicates that using prostate carcinoma cell lines as predictive in vitro models for MDR transporters, variable expression of these transporters must be considered.

Conclusion: Inflammation in the prostate tissue of patients with benign prostatic hyperplasia did not influence mRNA expression of BCRP, MDR1 and MRP1-5.

5.2 Introduction

Multidrug resistance transporter 1 (MDR1; ABCB1), multidrug resistance associated protein 1-5 (MRP1-5; ABCC1-5), as well as the breast cancer resistance protein (BCRP; ABCG2) are transmembrane efflux pumps that translocate a wide variety of substrates across extra- and intracellular membranes using ATP hydrolysis (Horio et al., 1988). They are protecting tissue from xenobiotic accumulation and subsequent toxicity by forming a barrier to cellular accumulation of toxins as well as to drug absorption (Schinkel, 1997). In consequence they

lower the intracellular concentration of these compounds. Multidrug resistance is a phenomenon in which a cell exposed to a single drug, becomes cross-resistant to a large number of structurally unrelated compounds. Expression of P-glycoprotein (P-gp; gene product of the MDR1 gene) in different types of solid tumors and in leukemias has been associated with clinical resistance to chemotherapy, indicating that P-gp mediated multidrug resistance is an important factor explaining chemotherapy failure in patients with cancer (Plaat et al., 2000; Sonneveld, 2000). Several studies focused on the expression of multidrug resistance transporters in prostate cancer and human prostate cancer cell lines. They found an upregulation of different multidrug associated proteins in disseminated, progressive prostate cancer and concluded that multidrug resistance transporters may influence response to chemotherapy (van Brussel et al., 1999; Van Brussel et al., 2001).

At present, except for P-glycoprotein (gene product of MDR1), which expression has been assessed in the healthy prostate (Thiebaut et al., 1987), little is known about the systematic expression of other multidrug resistance transporters, such as MRP1-5 and BCRP in prostate tissue.

Besides prostate cancer and benign prostatic hyperplasia (BPH), prostatitis is a challenging urological diagnostic entity. Chronic abacterial Prostatitis / Chronic Pelvic Pain Syndrome (CPPS, NIH Classification of prostatitis category III) is a common genitourinary diagnosis in the 18 to 65-year old men (Collins et al., 1998) and presents with discomfort or pain in the pelvic region, variable voiding and sexual symptoms. The quality of health of patients with chronic prostatitis is impaired (Nickel, 2000). The syndrome is thought to be caused by infectious and non-infectious prostatic inflammation as well as noninflammatory disease. The aetiology is not known, a microorganism based aetiology with several microbial pathogens, as well as cryptic non-cultureable organisms are discussed (Domingue and Hellstrom, 1998). Other possible aetiological factors include dysfunctional high-pressure voiding, intraprostatic ductal reflux, autoimmune etiology, chemical (urine and its metabolites, i.e. uric acid), neuromuscular and others. Antibiotic therapy with the fluoroquinolones ofloxacin and ciprofloxacin or trimethoprim/sulfamethoxazole is the recommended treatment of chronic prostatitis, if there is a clinical, bacteriological or supporting immunological evidence of prostate infection (Bjerklund Johansen et al., 1998). In a telephone survey among primary care physicians (PCP's) and urologists in Canada 1996 fluoroquinolones were thought to result in the successful treatment of prostatitis in only 64% of the cases (Nickel et al., 1998). When antibiotics fail other treatments are recommended, among others α-blockers as well as anti-inflammatories (NSAIDS) (Nickel,

2000). However, these drugs do ameliorate symptoms only in a small part of patients. At present, effective management strategies for chronic prostatitis are lacking.

Taking into account, that there is increasing evidence that several multidrug resistance transporter play an important role in acute and chronic inflammatory processes (Wijnholds et al., 1997; Panwala et al., 1998) and might contribute to the development of therapy resistance in certain inflammatory diseases (Maillefert et al., 1996; Farrell et al., 2000), the evaluation of the transporter expression in inflamed prostatic tissue might be of therapeutical relevance for patients with chronic prostatitis. Because antibiotics (e.g. fluoroquinolones), α-blockers as well as anti-inflammatories (NSAIDS) are substrates of multidrug transporters, altered expression of P-gp, MRP1-5 or BCRP in inflamed prostatic tissue may influence the intracellular concentration of these drugs and the efficacy of these treatments.

Therefore the aim of this study was to assess the expression of multidrug resistance transporters in prostatic tissue of patients with benign prostatic hyperplasia and to evaluate the impact of inflammation on the expression level of these transporters. Due to ethical reasons no biopsies could be obtained from patients diagnosed with CPPS category III.

5.3 Methods

Subjects

In the study 50 consecutive patients with no clinical or laboratory indication for prostate cancer that qualified for and underwent transurethral resection of prostate (TUR-P) for benign prostatic hyperplasia in the Urology Department of the University Clinic Kantonspital Liestal between April 2003 and January 2004 were included. Patients, who were treated with known inducers of drug metabolising enzymes or MDR transport proteins (such as barbiturates, rifampicin, rifabutin, antiepileptics (phenytoin, carbamazepine), HIV protease inhibitors (especially nelfinavir and ritonavir), St John's wort were excluded from the study. The patients were 72 ± 1.2 years (mean ± SEM) old. All were of Caucasian race and the BMI was 26.7 ± 0.5 kg/m^2.

The study was approved by the local ethic commission (Ethische Kommission beider Basel (EKBB)). Patients have signed a written informed consent.

Cell cultures

The human prostatic carcinoma cell lines LNCaP and PC3 were cultured in standard RPMI complete medium, supplemented with 10% (v/v) fetal calf serum, 1% sodium pyruvate, 50µg/ml gentamycin and 1% 1M HEPES (pH 7.0). Cells were seeded in 12- well plates (each cell line

N=4). Cultures were maintained in a humified 37℃ incubator with a 5% carbon dioxide in air atmosphere.

Real-time polymerase chain reaction analysis of prostate tissue (see Chapter 2)

Sequence of the primers and probes that were used for TaqMan analysis see in Chapter 2.

Histological assessment

Histological assessment was done by two trained pathologists. First, frozen sections were fixed, sectioned and stained with hematoxylin and eosin. Then, the severity of inflammation was rated as follows: 0 = no inflammation, 1 = low, 2 = intermediate, 3 = high grade of inflammation. If prostate tissue was found to be inflamed, assessment for localization of inflammation acinar/periacinar versus interstitial inflammation was performed.

Data analysis and statistics

Gene expression was studied in prostate tissue of patients undergoing TUR-P. For the comparison of the gene expression level in prostate tissue with different grade of inflammation the two-sided Dunnett's multicomparison t test was used. The level of significance was $P < 0.05$. Comparisons were performed using SPSS for windows software (version 12.0, SPSS Inc., Chicago, USA).

5.4 Results

Expression of multidrug resistance transporters in prostate tissue

The expression and ranking of all transporters in the prostate tissue normalized to GAPDH is shown in Figure 1. MRP4 and MRP1 appeared to be the most abundantly expressed transporter in prostate tissue of patients with benign prostatic hyperplasia (BPH) undergoing transurethral resection of prostate (TUR-P). MRP3 and MRP5 were found approximately 2 times and MDR1 and BCRP 6-7 times less expressed than MRP4. Only very low expression was found for MRP2. The ranking of transporter gene expression in the prostate tissue of patients with BPH was MRP4 = MRP1 >> MRP5 > MRP3 >> MDR1 = BCRP >> MRP2.

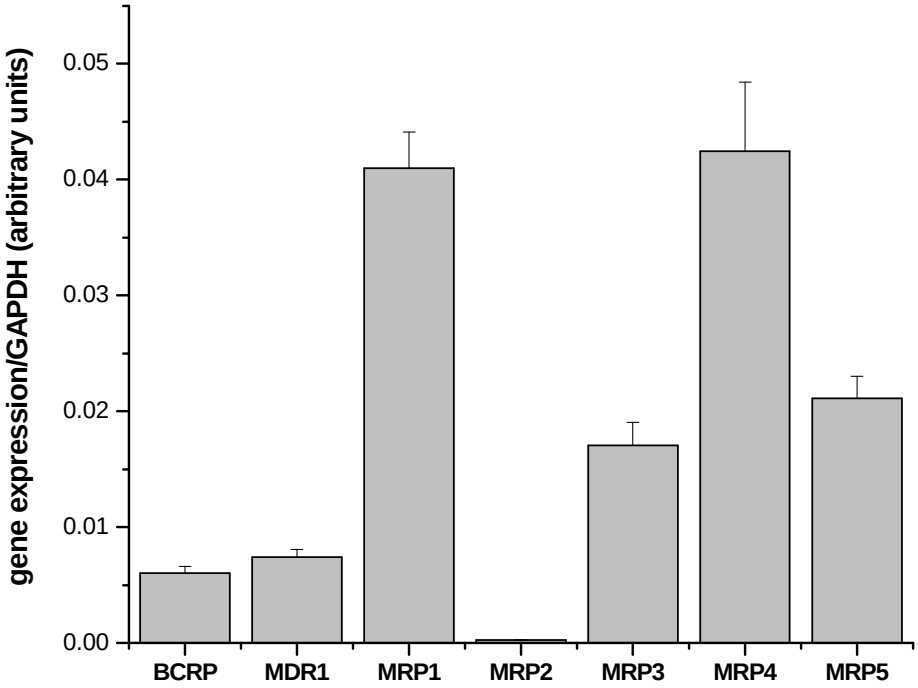


Figure 1: Expression of MDR1, BCRP and MRP1-5 in the prostate tissue of patients with benign prostatic hyperplasia (N=50) normalized to GAPDH expression. Data represent means (± SEM).

Inflammation did not alter the expression of multidrug resistance transporters in prostate tissue

In the histologic assessment, which was done on hematoxylin and eosin stained sections, 13 patients were found with no inflammation, 10 with low grade, 17 with intermediate and 13 with high grade of inflammation in the prostate tissue. Inflammatory cells were found in 6 patients predominantly periacinar/acinar, in 24 patients interstitial and in 7 patients both patterns were observed. Gene expression of multidrug resistance transporters was not altered neither by severity of inflammation nor localization of inflammation (data not shown).

BCRP expression and α-blocker

BCRP/GAPDH (mean ± SEM) gene expression was significantly decreased in patients with BPH treated with α-blocker (0.0067 ± 0.0007; N=12) when compared to α-blocker untreated patients (0.004 ± 0.0005; N=38) before TUR-P was performed ($p < 0.05$) (Figure 2).

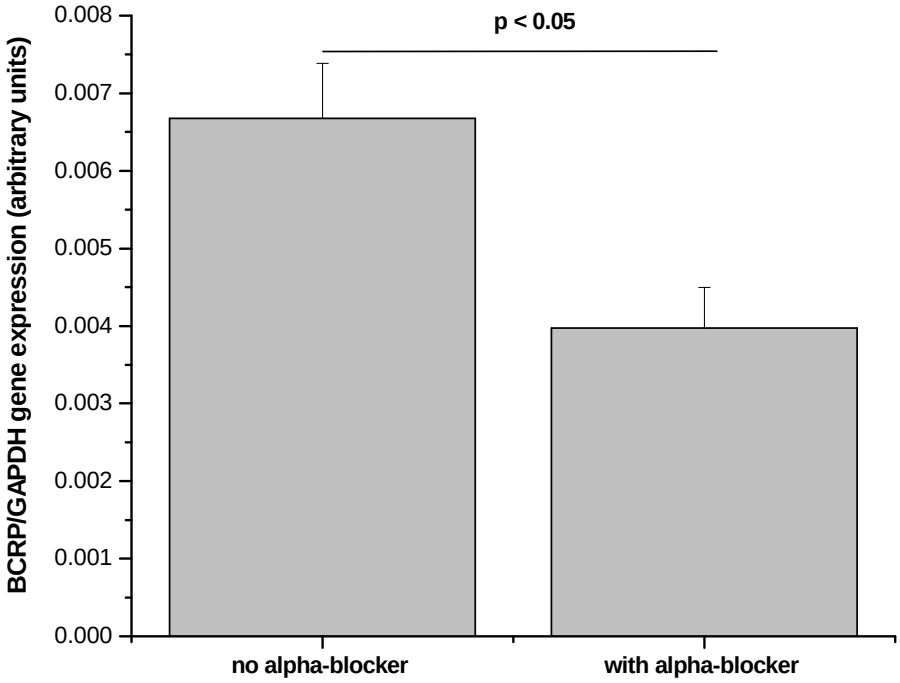


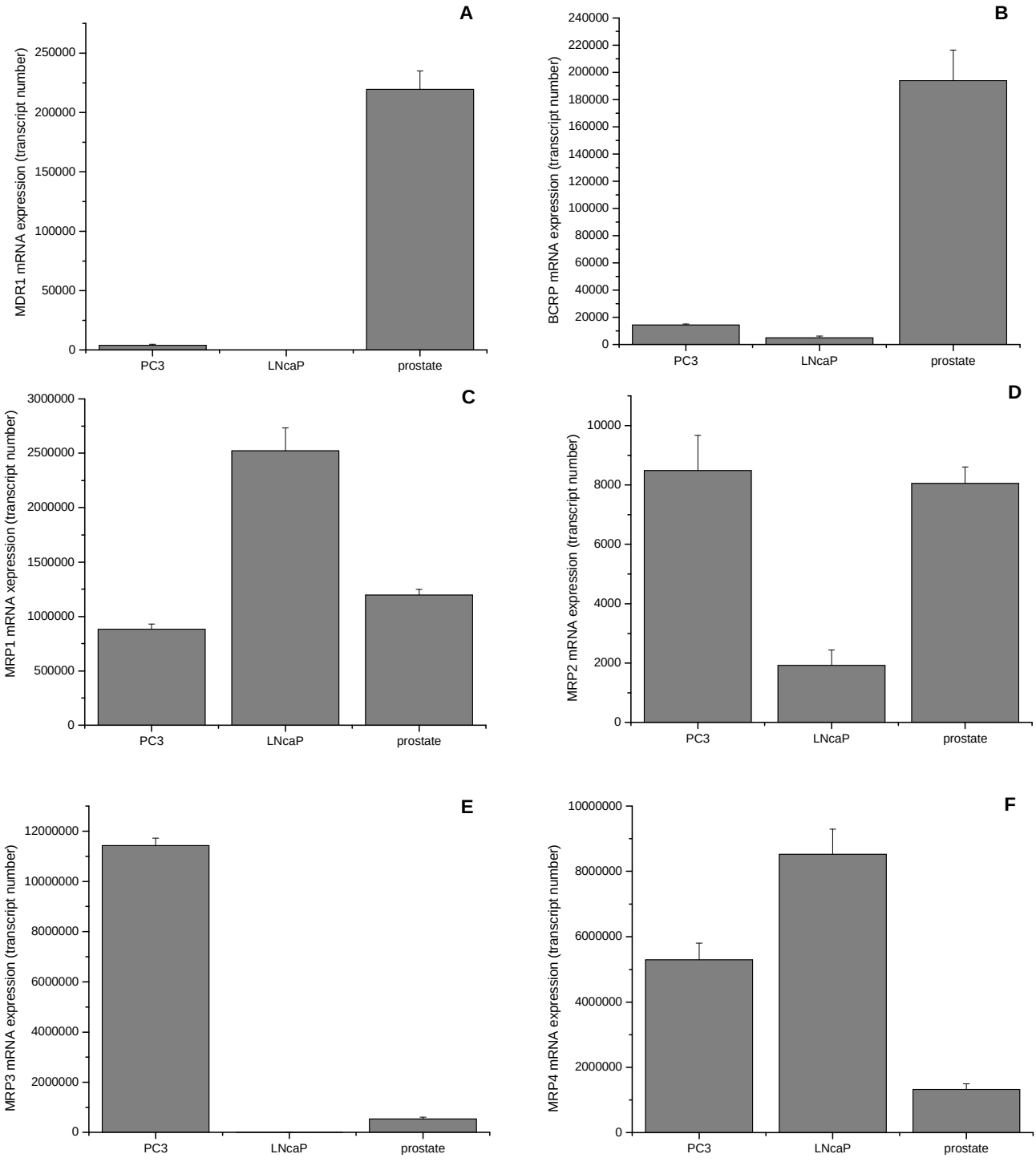
Figure 2: Comparison of BCRP mRNA expression in patients with benign prostatic hyperplasia treated with α-adrenergic antagonist (N = 12) vs. untreated patients (N = 38) before undergoing TUR-P normalized to the expression of GAPDH. Data represent means (± SEM).

Expression of multidrug resistance transporters in human prostate carcinoma cell lines

For evaluation of the multidrug resistance gene expression two prostate carcinoma cell lines (LNcaP and PC3) were used and compared to the expression in prostate tissue of BPH patients. Almost no BCRP and MDR1 gene expression was found in both prostate cell lines when compared to the prostate tissue of BPH patients (Figure 3A and 3B). MRP1 expression in BPH patients and in PC3 carcinoma cell line was comparable. MRP1 expression in LNcaP carcinoma cell line was 2-3times higher (Figure 3C). As already shown MRP2 expression was very low in BPH patients, which is comparable to the PC3 carcinoma cell line. Even lower MRP2 expression was found for the LNcaP cell line (Figure 3D).

MRP3 expression was 20-25times higher in the PC3 cell line compared to prostate tissue of BPH patients. No expression of MRP3 was found in the LNcaP cell line (Figure 3E). MRP4 expression was highest in the LNcaP cell line, followed by PC3 cell line and prostate tissue of

BPH patients (Figure 3F). Both cell lines and prostate tissue of BPH patients show an almost similar expression level for MRP5 expression (Figure 3G).



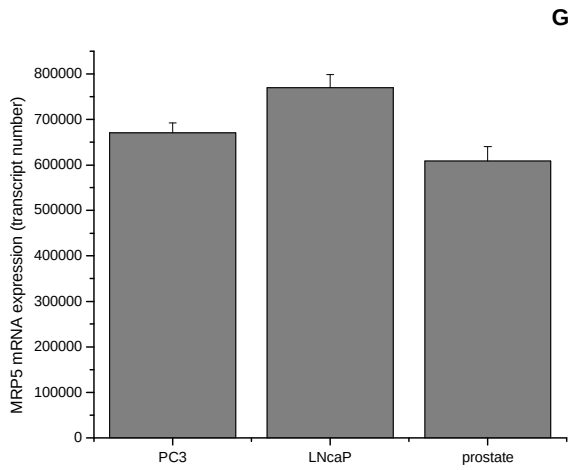


Figure 3: Expression of MDR1, BCRP and MRP1-5 mRNA in prostate tissue of patients with benign prostatic hyperplasia and two prostate carcinoma cell lines (LNcaP, PC3). Data represent mean transcript number (± SEM).

5.5 Discussion

Expression of several multidrug resistance (MDR) transporters in patients with BPH was evaluated in this study. It could be shown that MRP4 and MRP1 were the two most abundantly expressed transporters in the prostate tissue, followed by MRP3 and MRP5. MDR1 and BCRP were markedly less expressed, whereas no expression was observed for MRP2. At present there is only little information in the literature about the expression of these MDR transporters in the human prostate.

Compared to other tissues such as adrenal gland, kidney, liver, lung as well as the intestine MDR1 mRNA expression in prostate tissue was found to be low (Fojo et al., 1987). When compared to the liver Uchiumi et al reported minimal expression of MRP3 as well as MRP2 in prostate tissue (Uchiumi et al., 1998). In contrast MRP4, which was examined in a range of human tissues by hybridization analysis, transcript levels were highest in the prostate (Lee et al., 1998). MRP4 was localized on the basolateral membrane of tubuloacinar cells and it was suggested that one function of MRP4 might be the efflux of xenobiotics and toxins out of prostatic epithelial cells (Lee et al., 1998).

MDR1 was detected in both benign and malignant prostate cells (Kawai et al., 2000). In non-malignant prostate tissue P-glycoprotein (Pgp; the gene product of MDR1) was detected on the apical membrane of the epithelial cells with higher staining intensity towards the inner zone,

whereas in malignant specimens Pgp showed lower staining intensities and was found to be expressed more heterogeneously in the cancer tissue (Kawai et al., 2000). Almost all clinical based studies investigating the role of MDR transporters in human prostate were focusing on prostate cancer. Although MDR1 was not expressed in diverse prostate carcinoma cell lines (van Brussel et al., 1999) a direct correlate was found between tumor grade, stage and prostate specific antigen levels in a retrospective analysis of paraffin-embedded tissue of patients with prostate cancers and controls with BPH (Bhangal et al., 2000).

MRP1 is consistently expressed in prostate cancer cell lines (van Brussel et al., 1999) and was found to be expressed even in early stages of prostate cancer (Sullivan et al., 1998) with an increase in late disease stages (Van Brussel et al., 2001). Drug resistance phenotype due to incubation with doxorubicin was reported for various prostate carcinoma cell lines (David-Beabes et al., 2000).

Chronic abacterial Prostatitis / Chronic Pelvic Pain Syndrome (CPPS, NIH Classification of prostatitis category III) is thought to be caused by infectious and non-infectious prostatic inflammation as well as noninflammatory disease. Patients with CPPS often do not respond to first line treatment with antibiotics and subsequently also fail to benefit from second line treatments such as α-blockers as well as anti-inflammatories (NSAIDs). Fluoroquinolones are the recommended antibiotics in the treatment of chronic prostatitis (Bjerklund Johansen et al., 1998). Interestingly, several fluoroquinolones are substrates of different MDR transporters as shown in transport assays performed in different tissue types and cell lines (Tamai et al., 2000; Terashi et al., 2000; Lowes and Simmons, 2002; Michot et al., 2004; Sasabe et al., 2004). α-Adrenergic antagonists such as Prazosin and Doxazosin are substrates of BCRP (Ozvegy et al., 2001) and of MDR1 (Takara et al., 2002), respectively. Aspirin was shown to enhance Pgp expression in a human lymphoma cell line. MRP4 acts as a prostaglandin transporter and nonsteroidal anti-inflammatory drugs were shown to inhibit this transport (Reid et al., 2003). As several MDR transporters play an important role in acute and chronic inflammatory processes (Wijnholds et al., 1997; Panwala et al., 1998) and might contribute to the development of therapy resistance in certain inflammatory diseases (Maillefert et al., 1996; Farrell et al., 2000), the aim of the present study was to evaluate the influence of inflammation in prostate tissue of BPH patients on MDR transporter expression. Due to ethical reasons, tissue of patients with CPPS could not be obtained.

Here, the grade of inflammation and localization of inflammation (acinar/periacinar versus interstitial) was assessed on hematoxylin and eosin stained sections on prostate tissue of patients with BPH. Neither severity nor localization of inflammation changed the expression of

the investigated MDR transporters. The influence of inflammation on MDR transporter expression in prostate tissue of patients with BPH or of patients with chronic prostatitis has not been reported in the literature before. However, in a rat prostatitis model poor success rate of treatment with norfloxacin was not due to altered pharmacokinetics in the inflamed prostate (Nickel et al., 1995). This could implicate that transporters did not influence the transport of norfloxacin in this model.
Patients treated with an α-adrenergic antagonist before undergoing resection of prostate showed a decreased BCRP expression when compared to the untreated group. Of these 12 patients, 8 were taking tamsulosin, 2 terazosin and 2 alfuzosin. These patients were taking the medication until TUR-P was performed. Tamsulosin is a potent α-blocker which is metabolised by CYP3A4 and CYP2D6 (Kamimura et al., 1998). If tamsulosin is transported by an MDR transporter is currently not known. As the influence of α-adrenergic antagonists on MDR expression was not a primary question of this study the result here is not conclusive and needs to be confirmed.

In addition, the expression of MDR transporters in prostate tissue of BPH patients as well as in two prostate carcinoma cell lines (LNcaP and PC3) was evaluated. It could be shown, that BCRP and MDR1 mRNA expression was very low in both prostate carcinoma cell lines when compared to the prostate tissue of BPH patients. MRP1 expression in BPH patients and in PC3 carcinoma cell line was comparable, but MRP1 expression in the LNcaP carcinoma cell line was 2-3times higher. MRP2 expression was very low in BPH patients with a comparable expression level in PC3 carcinoma cell line. MRP3 expression was 20-25times higher in the PC3 cell line when compared to prostate tissue of BPH patients. No expression of MRP3 was found in the LNcaP cell line. MRP4 expression was highest in the LNcaP cell line, followed by PC3 cell line and prostate tissue of BPH patients. Both prostate carcinoma cell lines and prostate tissue of BPH patients show an almost similar expression level concerning MRP5 expression. In general, expression of MDR transporters showed variable expression levels in the prostate carcinoma cell lines and in prostate tissue of BPH patients. In consequence, when prostate carcinoma cell lines are used as predictive in-vitro models for MDR variable expression of these transporters must be considered.

In conclusion, the ranking of transporter gene expression in the prostate tissue of patients with BPH was MRP4 = MRP1 >> MRP5 > MRP3 >> MDR1 = BCRP >> MRP2. Neither severity nor localization of inflammation in the prostate tissue influenced the expression of these MDR transporters. A significant decrease of BCRP mRNA expression was observed for BPH patients

treated with α-adrenergic antagonists before undergoing TUR-P when compared to untreated patients. However, this result needs to be confirmed on the posttranscriptional level. Expression of MDR transporters did vary in different prostate carcinoma cell lines (LncaP, PC3), when compared to prostate tissue of BPH patients and indicates that choice of carcinoma cell lines for in vitro studies with MDR transporters has to be done carefully.

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Conclusion and Outlook

The aim of this thesis was to investigate the role of transmembrane transporters in healthy intestine and gastrointestinal diseases. In healthy subjects, systematic site-specific analysis of various transmembrane transporters along the intestinal tract was presented here (Chapter 3.1, 3.2, 3.4). All investigated transporters showed alterations in their mRNA expression levels from the duodenum to the sigmoid colon. As many of the investigated tranporters were efflux transporters such as MDR1, BCRP, MRP1-5 which are localized in the intestinal wall and restrict intestinal drug transport, this knowledge might be important to develop drug targeting strategies for orally administered drugs.

During obstructive cholestasis, expression and regulation of ASBT, which is a critical determinant of the reuptake of bile acids from the intestine and of BCRP, which mediates among others energy dependent efflux of bile acids, were investigated. Both transporters were downregulated during obstructive cholestasis. We concluded that for ASBT adaptive gene regulation may represent a mechanism preventing the accumulation of hepatotoxic bile acids during cholestasis. Reduced intestinal BCRP expression during cholestasis might influence the accumulation of bile acids, food-derived carcinogens and the pharmacokinetics of various drugs that are transported by BCRP. However, the molecular mechanisms explaining this observed adaptive regulation, which might be mediated by bile acids, drugs, inflammatory cytokines or other factors, are not known. In the literature several nuclear receptors have been described to be involved in the regulation of ASBT (Chapter 1.3). PPARα, which is one of these nuclear receptors, was not significantly different between cholestatic patients and controls. In conclusion, additional studies are needed to investigate the molecular factors which are involved in the regulation of gastrointestinal transporters involved in bile acid transport during obstructive cholestasis.

To understand the molecular mechanisms for insufficient or failed therapy in the treatment of patients with Inflammatory Bowel Disease (IBD) is another key project of our laboratory. Intestinal macrophages play a central role in the orchestration of innate immune response reactions in the gut and anti-inflammatory and/or immunosuppressive drugs such as glucocorticoids (MDR1), methotrexate (MRP1-3), cyclosporine (MDR1), 6-mercaptopurine (MRP4) and sulfasalazine (BCRP), which all are used in the treatment of IBD patients, are substrates of MDR transporters.

Therefore, a method for isolation of CD14+ peripheral blood cells (monocytes), their *ex vivo* cultivation and differentiation into macrophages was established. First, the influence of the differentiation process of monocytes into monocyte derived macrophages (MDM) and the stimulation of bacterial products (LPS) of MDM on MDR transporter expression was investigated, as demonstrated. At present, the effect of budesonide, methotrexate, 6-mercaptopurine and sulfasalazine on the transporter expression in *ex vivo* cultivated human MDM is evaluated.
In a next step, we will isolate CD14+ monocytes from peripheral blood mononuclear cells from healthy controls, UC patients, and CD patients, differentiate them into MDM, and characterize them regarding MDR transporter expression patterns.

In addition, it is planned to extract *lamina propria* mononuclear cells and intraepithelial lymphocytes from intestinal mucosa. This method will be established using larger samples from porcine intestine, with the aim to try to down-scale the method to smaller tissue sample sizes (as for human biopsies). Subsequently, the method will be transferred to biopsies of human intestinal tissues. Alternatively, the use of human surgically resected intestinal tissue is foreseen. Primary cell cultures of these cellular subtypes will be investigated by means of quantitative real-time PCR analysis, Western blot, and fluorescence activated cell sorting (FACS) to study the expression pattern of MDR transporters.
At present, effects of IBD drugs, e.g. budesonide on MDR mRNA and protein expression in different intestinal epithelial cell lines (LS180, Caco-2 and HT29) are systematically evaluated. In our *in vitro* studies we are focusing on nuclear receptors such as the glucocorticoid receptor (GR) and pregnane X receptor (PXR) with the goal to gain more insight in the molecular mechanisms underlying glucocorticoid resistance or dependence often seen in IBD patients.
The human ASBT gene has been shown to be transactivated by the GR and its ligands dexamethasone and budesonide. Induction of ASBT expression in the intestine of Crohn patients may improve the chologenic component of diarrhea. In a subproject we plan to evaluate the expression of ASBT in Crohn patients which are not responding to treatment with steroids.

Another important task of our group and of my present work was to initiate a prospective clinical study in IBD patients, which should investigate MDR transporter gene expression in intestinal biopsies (intestinal epithelial cells, intestinal macrophages) and blood samples (lymphocytes) in newly diagnosed IBD patients compared to treatment refractory IBD patients, patients in remission and disease free controls. The aim of the study is to investigate the expression of

MDR transporter in intestinal tissue biopsies and blood samples to demonstrate, if the expression levels of MDR transporters vary due to:

- inter-individual differences
- inflammatory processes
- pharmacological treatment

For analysis of MDR transporter expression TaqMan analysis, Western blot and immunostaining will be applied.

For the analysis 5 patient groups of 60 patients each are needed: A) new, untreated patients (aged 18-60 years) with Crohn's disease, B) new untreated patients with ulcerative colitis , C) therapy refractory patients with IBD, D) IBD patients in remission and E) patients with irritable bowel syndrome (control group, see below) undergoing colonoscopy. In patients with macroscopical signs of inflammation, biopsies from the inflammed and when possible also from the non-inflammed part are taken. Up to now, a total number of 130 patients have been included into the study and biopsies have been collected. Unfortunately, no preliminary analysis could be presented in this thesis.

In collaboration with external gastroenterologists blood samples of IBD patients will be collected for the analysis of "Single nucleotide polymorphisms (SNP)" of different MDR transporter genes. It will be evaluated, if the SNP´s can be used as predictors for susceptibility and/or treatment responsiveness. Allelic discrimination assays for other SNPs of ABC-transporters such as a 5'-nuclease assay for the MDR1G2677T and the MDR1G2677A polymorphism have already been established by Heike Gutmann in our group, whereas allelic discrimination assays for other multidrug transporters such as BCRPG34A, BCRPC421A, BCRPA616C, and BCRPA1768T are in development currently.

The "prostate study" was performed to evaluate the influence of inflammation on expression of MDR tranporters in patients with BPH. Neither severity nor localization of inflammation altered the expression of the investigated MDR transporters. This project was designed as an isolated project and will not be investigated further.

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Published Abstracts

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