CHARLES UNIVERSITY IN PRAGUE
1st FACULTY OF MEDICINE
Institute of Clinical Biochemistry and Laboratory Diagnostics
Laboratory of Hepatology

LIVER GANGLIOSIDES IN ESTROGEN-INDUCED CHOLESTASIS

Doctoral Thesis

Filip MAJER

Advisor: Doc. RNDr. František Šmíd, CSc.
Charles University in Prague
1st Faculty of Medicine
Institute of Clinical Biochemistry and Laboratory Diagnostics

Co-advisor: Prof. MUDr. Libor Vítek, PhD, MBA
Charles University in Prague
1st Faculty of Medicine
Institute of Clinical Biochemistry and Laboratory Diagnostics

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1 INTRODUCTION AND REVIEW OF THE LITERATURE

Cholestasis, a common pathological condition where bile cannot flow from the liver to the duodenum, can be caused by various etiologies. Understanding of the pathogenesis and mechanisms regulating the pathological the pathological processes during cholestasis can lead to development of novel treatments with improvement of life quality of affected individuals.

1.1 Cholestasis

Secretion of bile is physiologically regulated by several membrane transport systems in the liver cells of the bile duct, cholangiocytes. Tissue and cell functional integrity are necessary for the proper function of the bile-secretory apparatus. Cholestasis is classified as a state when bile cannot flow from the liver to the duodenum or when its formation is reduced (Trauner, Meier et al. 1999; Zollner and Trauner 2006; O'Leary and Pratt 2007; Zollner and Trauner 2008). The cause of cholestasis may be a failure in the secretory transport system of hepatocytes or bile canaliculi cholangiocytes, or a block in the bile ductules blocking the flow from excretory pathway outside the liver. These two forenamed causes are called "intrahepatic" and "extrahepatic", in dependence on localization of the impairment of bile flow. If cholestasis is caused by impairment of hepatocytes, cholangiocytes or the intrahepatic small bile ducts then is considered as intrahepatic. Extrahepatic cholestasis occurs outside the liver due to obstruction of large bile ducts such as from a stone obstructing the pathway or stricture caused by abdominal mass of benign or malignant processes. Cholestasis can be various etiology: gallstones (Wang, Portincasa et al. 2008), abdominal mass (e.g. cancer), primary sclerosing cholangitis (Portincasa, Vacca et al. 2005; Michaels and Levy 2008), secondary to inflammatory bowel disease (Saich and Chapman 2008), primary biliary cirrhosis (Kumagi and Heathcote 2008) or other autoimmune disorders, congenital anomalies of the biliary tract, biliary trauma, drug-induced cholestasis, sepsis-induced cholestasis, biliary atresia (de Carvalho, Ivantes et al. 2007) and intrahepatic cholestasis of pregnancy (also known as ICP) (Pusl and Beuers 2007) and contraceptive-induced cholestasis (CIC).

Cholestasis may be also caused by drug ingestion. The most common drug inducers are antibiotics and chlorpromazine (Simon 1998). Drug induction of cholestasis by various drugs is exploited in experimental animal models allowing us to research into pathophysiological

mechanisms of cholestasis and comparison with clinical findings. The best known model of extrahepatic cholestasis due to bile duct obstruction is bile duct ligation (abbreviated BDL). Intrahepatic cholestasis is commonly induced by estrogens (contraceptive-induced cholestasis and intrahepatic cholestasis of pregnancy), endotoxins and other drugs (Rodriguez-Garay 2003).

Cholestasis is distinguished clinically by variable symptoms as jaundice, pruritus and failure to thrive. Biochemical changes include elevated serum levels of "cholestatic markers" reflecting impaired liver functions. These cholestatic enzymes include alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), 5'-nucleotidase (5'-NT), and elevation of total bile acids, cholesterol and bilirubin in serum (Rodriguez-Garay 2003). Bile salts are steroid monocarboxylic acids. Accumulation of hydrophobic bile acids may impair cell membranes since they possess strong detergent characteristics (Heuman, Pandak et al. 1991). Contemporaneously, accumulated bile acids down regulate their own biosynthesis through nuclear receptors. Moreover, cholestasis causes accumulation of the precursor of the biosynthesis, cholesterol, which decrease membrane fluidity and affects its function (Cooper 1978; Zollner, Marschall et al. 2006).

1.1.1 Intrahepatic cholestasis of pregnancy and contraceptive-induced cholestasis

Intrahepatic cholestasis of pregnancy (ICP), also referred as obstetric cholestasis, is a cholestatic condition during pregnancy. ICP adversely affects well-being of the mother and in particular can also impact neonatal outcome. ICP is characterized by pruritus of various degrees in the mother which commonly occurs in the second or third trimester of pregnancy. Blood tests can reveal elevated aminotransferases and total bile acid concentration (>10mmol/l), severe enough to affect quality of life; jaundice is uncommon in ICP, but may be present. All these symptoms and signs spontaneously relief till three weeks after delivery. Consequences of ICP can be premature delivery, fetal distress, preterm delivery and fetal loss in the infant through negative effects of retained bile acids (Heinonen and Kirkinen 1999; Kondrackiene and Kupcinskas 2008; Reyes 2008).

ICP affects 0.4-1% of pregnancies in major part of Western and Central Europe and North America. In the Czech Republic the reported incidence is 0.9% (Binder, Zima et al. 2007). ICP is more common in South America in Chile and Bolivia 5-15% and 1-2% in

Scandinavia and the Baltic states. Increased risk of preterm delivery (19-60%), meconium staining of amniotic fluid (27%), fetal bradycardia (14%), fetal distress (22-41%), and fetal loss (0.4-4.1%) was observed in ICP, especially when fasting serum bile acid levels exceed concentration 40 μmol/l. Intrahepatic cholestasis of pregnancy is used to be treated by administration of hydrophilic bile acid - ursodeoxycholic acid (10-20 mg/kg/d). (Kondrackiene, Beuers et al. 2007; Pusl and Beuers 2007). It is found that administration of UDCA is safe in late pregnancy (Palma, Reyes et al. 1992; Palma, Reyes et al. 1997). Surprisingly, no side effects have been reported in the mother or fetus, though, UDCA was administrated in high doses 1.5-2 g/d (Mazzella, Rizzo et al. 2001). Delivery after 38 week of pregnancy is recommended for outcome improvement, because fetal lungs are already mature enough for extra-uterine life (Fisk and Storey 1988).

ICP and contraceptive-induced cholestasis (CIC) are classified as acquired forms of cholestasis. ICP and CIC can affect women with normal findings in medical history. ICP and CIC symptoms normally disappear when levels of female sex hormones decrease at their normal concentration suggesting their important role in the ICP and CIC development (Bacq, Sapey et al. 1997). In addition, onset of ICP symptoms usually occurs in the third trimester of pregnancy when serum levels of female sex hormones, estrogens and progesterone, reach their maxima. Moreover, contraceptive induced cholestasis were found to associate with occurrence of ICP in patient's medical history or ICP in any other female family member making such a patient more susceptible to CIC. The recurrence rate of ICP in subsequent pregnant patients is less than 70% (Gonzalez, Reyes et al. 1989; Meier, Zodan et al. 2008).

ICP and CIC differ in levels of metabolites of bile acids. ICP is associated with increased levels of conjugated bile acids in serum and urine. Levels of unconjugated bile acids are normal as found in healthy pregnancies as well as in CIC. The changes in metabolites in ICP include predominant hydroxylated and sulfated bile acids in serum and urine. This finding of urinary pattern changes may indicate elevated alternative elimination of redundant retained serum bile acids by hydroxylations and sulfation. Likewise in serum and urine predominate sulfated progesterones while glucuronides were decreased or unchanged (Meng, Reyes et al. 1997).

Both hereditary and environmental factors may play a role in ICP development, however its complete pathogenesis remain to be explained (O'Leary and Pratt 2007). Among other findings, alterations in bile salt transport have been found associated with ICP.

Impartment of membrane bile salt transporters was found to associate with ICP and probably CIC. Carriers of impaired *BSEP* (Meier, Zodan et al. 2008), *MRP2* (Sookoian,

Castano et al. 2008) and *MDR3* gene (Floreani, Carderi et al. 2006) were found to be more susceptible for ICP.

1.1.2 Hepatobiliary transport system

Hepatocytes are polarized parenchymal epithelial cells of the liver with basolateral (sinusoidal) and apical (canalicular) plasma membrane pole. Hepatocytes produce bile acids from cholesterol. Levels of bile acids are also regulated through several membrane transporters (Figure 1). Bile salts are secreted across canalicular membrane to bile by active transport. Alteration in transporters can lead to the accumulation of bile acids and consequent liver injury. Hepatocytes take up biliary precursors and consituents at the basolateral membrane from sinusoidal blood through the fenestraes of the sinusoidal endothelial cells and Disse space. In hepatocytes bile salts reach the canalicular pole by diffusion (intracellular bile salt-binding proteins) or within intracellular lipid membranes (Trauner and Boyer 2003).

Several disorders are identified that associate with defective membrane transporters (Zollner and Trauner 2006). Dubin-Johnson syndrome is a rare autosomal recessive disorder characterized by chronic predominantly conjugated hyperbilirubinemia and by a defective transport of non-bile salt organic anions. The cause of the Dubin-Johnson syndrome was found to be a dysfunction of cMOAT protein (ABCC2). This protein transports a broad range of endogenous as well as xenobiotic compounds to the bile using ATP (adenosine triphosphate) (Kajihara, Hisatomi et al. 1998).

Levels of transporters and control of biosynthesis of bile salts is regulated by multiple nuclear receptors binding specific ligands. Some ligands of FXR, PXR, and CAR activate transcription of MRP2 gene through ER-8 response element in order to adjust excretion of conjugated toxic agents from the hepatocyte into the bile (Kast, Goodwin et al. 2002).

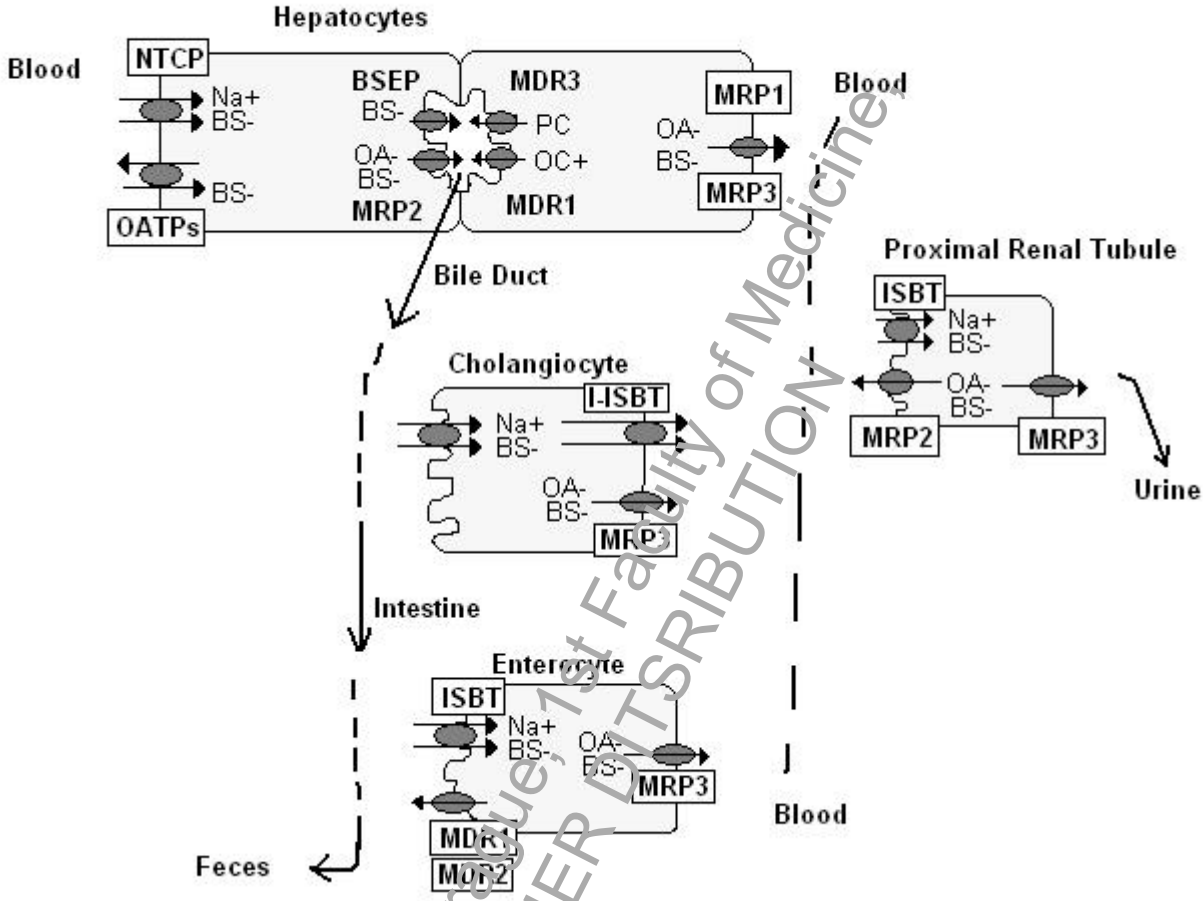

Figure 1-1. Transport of bile salts in the liver and extrahepatic tissues in humans. Bile salts (BS-) are taken up by transporters (NTCP - basolateral Na+/taurocholate cotransporter, OATPs - organic anion transporting proteins) into hepatocytes. Monovalent BS- are excreted through canalicular pole by BSEP (ABCB11; bile salt export pump). Divalent BS and organic anions (OA-) are excreted through MRP2 (ABCC2; multidrug resistance-associated protein 2). The export pump MDR3 (ABCB4; multidrug resistance protein 3) excrete phosphatidylcholine (PC), then in bile PC forms mixed micelles and help solubilization of hydrophobic BS and cholesterol. MDR1 (ABCB1, multidrug export pump) mediates excretion of organic cations (OC+). Alternative elimination of BS- is via MRP1 (ABCC1) and MRP3 (ABCC3) (multidrug resistance-associated proteins 1 and 3, respectively) into the systemic circulation and then into urine. These alternatives compromise ISBT (ileal Na+-dependent bile salt transporter, SLC10A2), MRP3 and truncated isoform (t-ISBT) (Trauner and Boyer 2003).

1.1.2 Bile acid homeostasis

Bile acid homeostasis is tightly regulated by several nuclear receptors including FXR (farnesoid X receptor) (Forman, Goode et al. 1995), PXR (pregnane X receptor), and CAR (constitutive androstane receptor). FXR and PXR facilitate homeostasis of bile acids but also cholesterol and lipids (Figure 2). It was found that FXR, PXR, and CAR act complementary to protect livers against bile acid induced injury. Thus they may constitute distinct layers of protection to defend the liver against damage by bile acids during cholestasis and modulate cholestatic state (Guo, Lambert et al. 2003; Fiorucci, Rizzo et al. 2007).

The enzymatic oxidation of cholesterol to bile acids is regulated via feed-forward activation by oxysterols. This activation is mediated by LXR (liver X receptor), a nuclear receptor binding oxysterols (e.g. oxysterols 24(S),25-epoxycholesterol and 24(S)-hydroxycholesterol) (Lehmann, Kliewer et al. 1997). The feedback repression is regulated by bile acid levels via activation of FXR. The main role of FXR is to be a sensor of bile acid level in enterohepatic tissues. FXR is activated by individual bile acids with the rank order of potency: CDCA > DCA > LCA > cholic acid (Makishima, Okamoto et al. 1999; Parks, Blanchard et al. 1999). PXR is activated by many compounds including naturally occurring steroids such as pregnenolone and progesterone and also by many xenobiotics as are synthetic glucocorticoids and anti-glucocorticoids. PXR has two isoforms PXR.1 and PXR.2 with distinct promoters and different activation by steroids (Kliewer, Moore et al. 1998). PXR is also activated by a range of drugs known to induce CYP3A4 expression (Lehmann, McKee et al. 1998).

The important role of FXR in bile acid homeostasis is reflected by its involvement in control of bile acids excretion and biosynthesis. However, FXR knockout mice were protected from obstructive cholestasis. Moreover, parallel deletion of FXR and PXR ameliorated liver dysfunction in knockout mice with cholestasis. Proposed mechanisms include the lowering of concentration of bile acids and changes in expression of bile salt transporters. FXR knockout mice also exhibit an alteration of lipid profile after induction of cholestasis bile duct ligation. It supports involvement of FXR in lipid metabolism. In this model, significant increment of physiological values was observed for high-density lipoprotein cholesterol and triglycerides by day 6 (Stedman, Liddle et al. 2006).

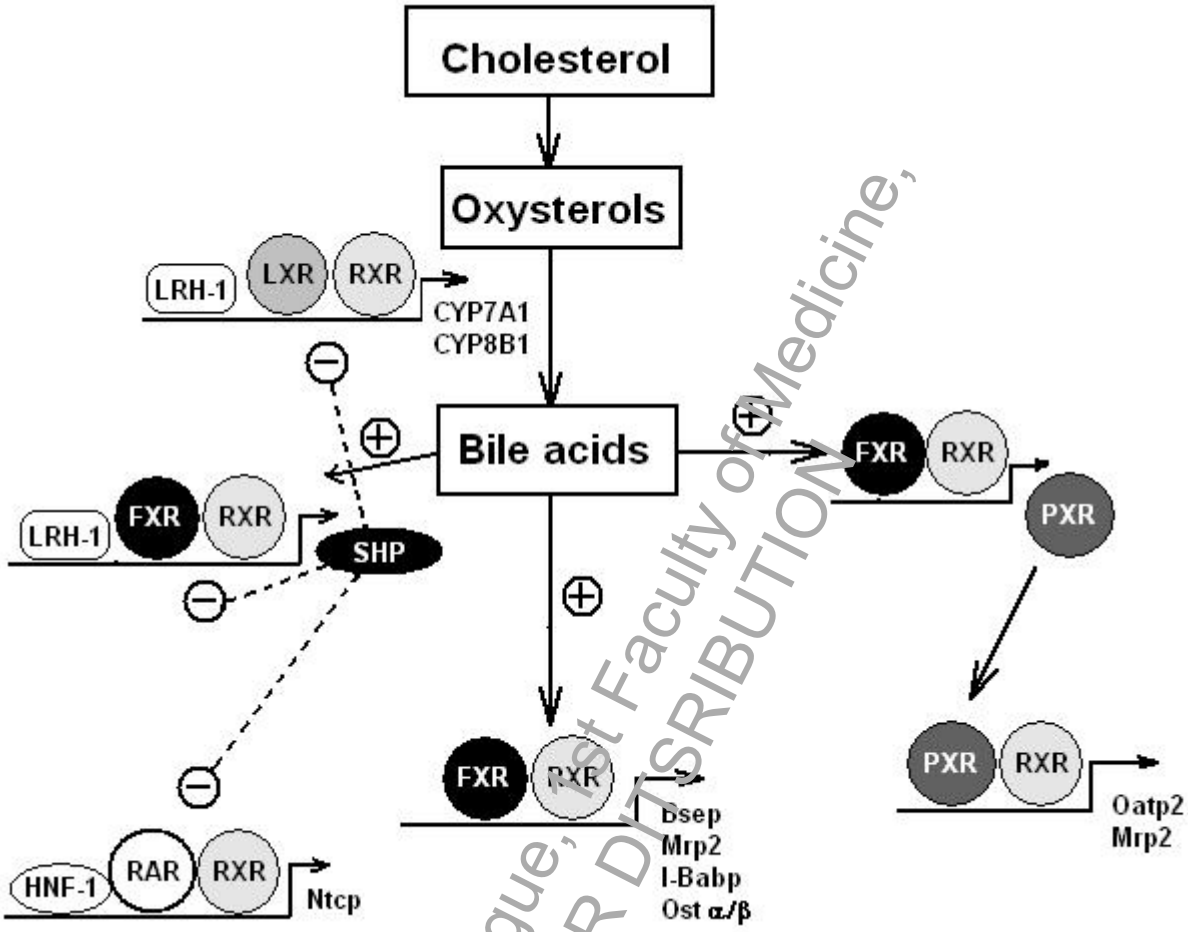

Figure 1-2. Schematic diagram of regulation of homeostasis of bile acids in the hepatocyte by FXR and other involved nuclear receptors. Increase in concentration of bile acids induce activation of FXR. Then the FXR/RXR heterodimer activates SHP (small heterodimer partner), which is repressed by LRH-1. LRH-1 is displaced as soon as SHP is activated and it results in inhibition of synthesis of new bile acids from cholesterol. Moreover, SHP binds to NTCP promoter in place of HNF-1 inhibiting bile salt basolateral uptake. Activated FXR promote expression of canalicular export pumps such as Bsep and Mrp2 and also basolateral efflux pumps Mrp3, Mrp4 and OSTa and b. (Trauner and Boyer 2003; Fiorucci, Rizzo et al. 2007). FXR also activates expression of PXR, the receptor activated by many drugs. PXR promote expression of basolateral transporters Oatp2 and Mrp2.

1.1.3 Nonparenchymal liver cell populations

The huge amount of liver functions is carried out by liver parenchymal cells (hepatocytes) and nonparenchymal cells (Kupffer cells, hepatic stellate cells, cholangiocytes and sinusoidal endothelial cells).

Kupffer cells, specialized macrophages located in the liver, play an important role in maintenance of homeostasis and normal physiology that comprise defense against pathogens, removal of apoptotic, damaged or unwanted cells (e.g. damaged red cells) via their scavenger function (Terpstra and van Berkel 2000). Moreover, Kupffer cells also participate in pathological processes such as acute and chronic responses to drugs, immune response via phagocytosis of invading microorganisms. Activated Kupffer cells release a large number of inflammatory mediators, growth factors, and reactive oxygen species. Released mediators act on other cell types which can cause their activation or even cell death (Decker 1990; Smedsrod, De Bleser et al. 1994; Kharbanda, Rogers et al. 2004; Roberts, Ganey et al. 2007).

Liver sinusoidal endothelial cells (SEC, LSEC or HSEC for hepatic) completely line the interior surface of sinusoidal wall. The liver sinusoids differ from other blood capillaries in the body. They are fenestrated, lack a diaphragm and a basal lamina underneath the endothelium (Braet and Wisse 2002). LSEC are more fenestrated in zone 3 than zone 1 of the liver acinus (Wack, Ross et al. 2001; Lee, Semela et al. 2007). Differentiated LSEC do not express platelet endothelial cell adhesion molecule (PECAM-1, CD31) and laminin-1 (extracellular matrix glycoprotein of basement membrane) which are markers of continuous endothelium (Couvelard, Scoazec et al. 1996). However, during angiogenesis LSEC are not fenestrated and express these markers of continuous endothelium. Switching over LSEC's phenotypes affects metabolic activity of neighboring liver cells and consequently through body fluids further distant cells. The reason is that defenestration decrease LSEC permeability and thus blocks metabolism of neighboring liver cells. This process play an important role in many diseases (Cogger, Muller et al. 2004). LSEC posses high scavenger activity (Smedsrod, Pertoft et al. 1990). Seternes et al. demonstrated that *in vivo* clearing cells are predominantly LSEC (Seternes, Sorensen et al. 2002; Kogelberg, Tolner et al. 2007). Moreover, LSEC have an important role in hepatic immunity (Warren, Bertolino et al. 2007; Lee and Kubes 2008).

Stellate cells (SC) are another important liver cell population. SC are in space of Disse in close contatct with LSEC. SC wrap around endothelial cells providing anatomic evidence of the concept of SC as liver specific pericytes (Lee, Semela et al. 2007). SC display histological and morphological characteristics similar to pericytes. SC respond to local vasoconstrictors thrombin and angiotensin-II similarly to other perivascular contractile cells (Pinzani, Failli et al. 1992) supporting their pericytic function. SC are located along the sinusoids at relatively fixed distances. An obvious feature of SC is the presence of cytoplasmic lipid droplets. Phenotype of SC is intermediate phenotype of vascular smooth muscle cells and fibroblasts with a possibility to differentiate into a myofibroblast phenotype with diverse functions. These

phenotypic changes are mediated by paracrine interplay with LSEC and are involved in various disease processes (Lee, Semela et al. 2007).

1.1.4 Zonation of liver metabolism

In 1980 Rappaport introduced concept of the liver acinus as the hepatic microcirculation unit (Rappaport 1980). A large number of histochemical studies showed that the hepatocytes in the liver differ in morphological, histochemical, and biochemical characteristics. There were set three metabolic zones without sharp boundaries: a periportal zone, a perivenous (or pericentral) zone, and an intermediate zone in between (Rappaport 1980). In the past, cells from these zones were mainly isolated by microdissection (Guder and Schmidt 1976). Later, isolation of periportal and perivenous hepatocytes by liver perfusion was described. Since the direction of perfusion was portal vein to cava or cava to portal vein (Quistorff 1985; Quistorff, Grunnet et al. 1985). Isolated liver cells were biochemically characterized.

Hepatocytes of the periportal and perivenous zones markedly differ in their contents and activities of many subcellular structures, enzymes and other proteins. The liver cells are surrounded by different regulatory mediators caused by the gradients established during blood and metabolic flow of oxygen, metabolites, substrates and hormones. The surrounding heterogeneity is important for short-term and also for long-term regulation of metabolism. The zonal heterogeneity can response to longer lasting physiological and pathological alterations such as starvation, diabetes or regeneration after partial hepatectomy. The zonation develops gradually during the first weeks of postnatal life. The model proposes a functional specialization for the two zones periportal and perivenous. The predominant activities in the periportal zone are oxidative energy metabolism with β-oxidation and amino acid metabolism, ureagenesis, gluconeogenesis, cholesterol synthesis, bile formation and oxidation protection. In the perivenous zone predominate glycolysis, liponeogenesis, ketogenesis, glutamine formation and biotransformation of xenobiotics (Fig. 1-3) (Jungermann 1986; Tosh, Alberti et al. 1988; Jungermann and Kietzmann 1996). Many studies found an important role of b-catenin-dependent signaling in the regulation of expression of genes of preferentially perivenously localized proteins. Whereas, signaling through Ras-dependent pathways may switch to a periportal phenotype. (Braeuning, Menzel et al. 2007). However, what keep zonation is not yet

fully understood (Ohtake, Maruko et al. 2004; Braeuning, Ittrich et al. 2006; Hailfinger, Jaworski et al. 2006).

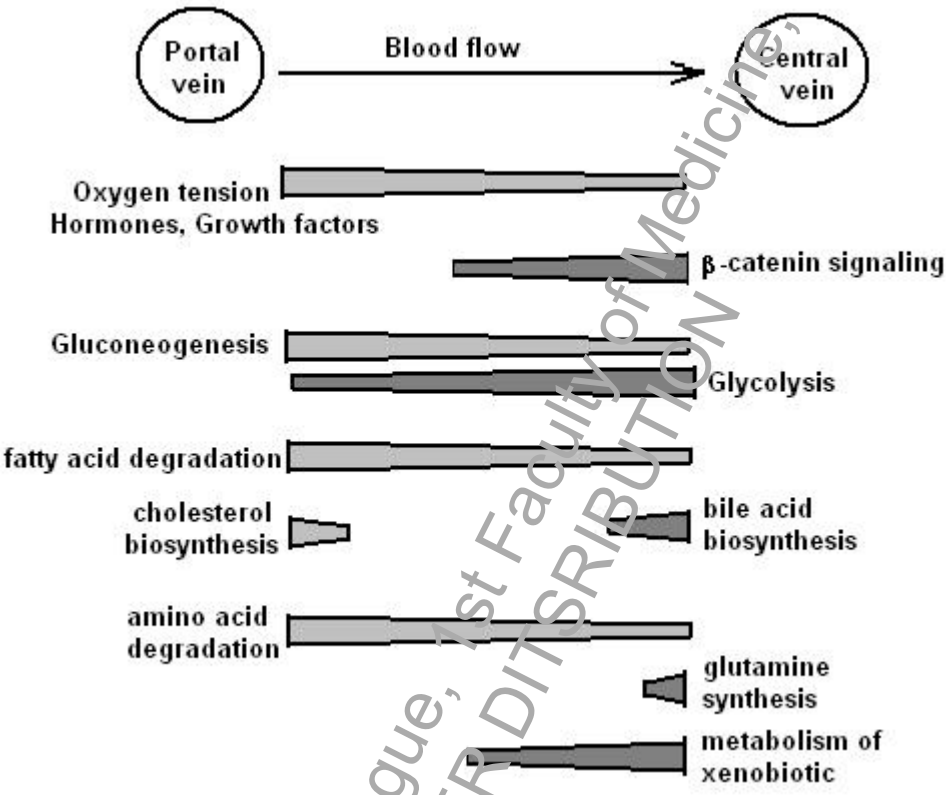

Figure 1-3. Schematic representation of zonation of the liver acinus. The figure shows the gradients of metabolic processes taking place in different hepatocyte subpopulations along the portocentral axis: oxygen tension, hormones / growth factors, and b-catenin signaling that have been involved in regulation of perivenous gene expression (Jungermann and Kietzmann 2000; Braeuning, Ittrich et al. 2006).

1.1.5 Mechanism of cholestasis induction by estrogens

Estrogens strongly affect gene expression in the liver as shown by hepatic gene expression profiling using Genechips in zebrafish exposed to 17α-ethynylestradiol where authors observed 1622 genes that were significantly affected ($p<0.001$) (Hoffmann, Torontali et al. 2006). It is well established that administration of exogenous estrogens over a period of days impair hepatic excretory capacity, decreases bile flow in the rat and impairs hepatic excretory function in rats as well as in humans (Mueller and Kappas 1964; Kreek, Peterson et al. 1969).

Both metabolism and excretion of drugs is mediated by the liver. Molecules are taken up by hepatocytes where they are biotransformed and further transported. Molecules enter via basolateral/sinusoidal membrane transporters or by passive diffusion. In the cell, the molecules are biotransformed by heterogeneous Phase I and Phase II enzymes. Finally, metabolites and precursors of drugs are either secreted into bile by canalicular membrane transporters or effluxed back into the blood stream (Sun, Liu et al. 2006).

The bulk of estrogens are metabolized. Urinary excretion of unchanged EE is very low (Abdel-Aziz and Williams 1970). Estrogens are metabolized in various ways (Rebbeck, Troxel et al. 2006). 2-hydroxylation, 3-sulfation and 3-glucuronidation are the main metabolic pathways. 3-sulfation is the major metabolic pathway of EE in human liver under physiological conditions but with large interindividual variations (Shiraga, Niwa et al. 2004). EE-3-sulfate and EE-3-glucuronide are eliminated by secretion into the gastrointestinal tract, hydrolyzed by gut bacteria to EE, and EE can be subsequently reabsorbed. The 2-hydroxylation of EE is mainly catalyzed at a much higher rate by cytochromes P450 CYP2C proteins and less by CYP2E. EE is also metabolized by the most abundant class of cytochromes P-450 CYP3A (Ball, Forrester et al. 1990).

Among EE and its metabolites the metabolite EE-17G (EE-glucuronide) was found to exhibit the cholestatic effect. The administration of EE-17G causes an immediate, profound and reversible inhibition of bile flow. Similarly metabolites of the natural estrogen E2 (estradiol) estradiol-17β, estradiol-3β-D-glucuronide or estradiol-3-sulfate-17β-D-glucuronide do not induce any cholestatic effects. It may be emphasized that glucuronidation at the 17β-position is required for the cholestatic effect (Meyers, Slikker et al. 1980). The enzyme responsible for glucuronidation, UDP-glucuronosyltransferase UGT2B7, catalyzes exclusively conversion of estriol and estradiol to glucuronidated metabolites at the 17β-OH position, yielding the cholestatic steroid D-ring glucuronides (Gall, Zawada et al. 1999). Furthermore, sulfation of the phenolic hydroxyl group of EE-17G abolishes its cholestatic effects. EE-17G to EE-3S-17G are secreted in the bile as shown by analysis of the bile (Musey, Green et al. 1972). EE-3S-17G is also deconjugated by arylsulfatase C (estrogen sulfatase) to reform cholestatic EE-17G and sulfate (Sun, Liu et al. 2006).

EE-cholestasis is associated with alterations in canalicular membrane transporters (Bossard, Stieger et al. 1993) and with changes in bile salt biosynthesis and content (Senafi, Clarke et al. 1994; Koopen, Post et al. 1999). The model of ICP and CIC is administration of EE-17G or E2-17G, a physiological estrogen metabolite associated with cholestasis during

pregnancy. EE-17G administration is also used as a model of acute estrogen-induced cholestasis (Utili, Tripodi et al. 1990; Gosland, Tsuboi et al. 1993). E2-17G is cholestatic at high concentrations. Single bolus dose of estradiol-17β-D-glucuronide (E2-17G) induces immediate, profound but transient cholestasis in rats. Cholestatic effect is in part mediated by the endocytic retrieval of the canalicular Mrp2 and Bsep transporters and by cis-inhibition of canalicular transport of glutathione. In estrogen cholestasis, increased permeability of the tight junction is also observed. In sustained cholestasis, a significant changes of localization of zonnula occludens-1 and occludin and Mrp2 was found (Moumo, Veggi et al. 2003). Western blotting analysis showed decreased Mrp2 (-41%) and increased Mrp3 (+200%) expression levels in EE-induced cholestasis. These alterations indicate that up-regulation of Mrp3 leads to predominance of detoxification via basolateral excretion. Thus the induction of basolateral transporter Mrp3 may prevent the cells against accumulation of Mrp endogenous or exogenous substrates in response to reduced expression and activity of apical Mrp2 (Ruiz, Villanueva et al. 2006).

In another experimental model of ICP, in the pregnant rats, similar alterations of membrane transporters were observed. Protein level of Oatp2 was decreased, whereas Oatp1 did not change. Similarly as in previous study canalicular Mrp2 protein was decreased whereas mRNA expression was found to be stable. mRNA level of MRP6 was decreased whereas Mrp1 was not altered. In contrast, basolateral protein MRP3 was decreased suggesting different pathogenesis of ICP and EE-induced cholestasis (Cao, Stieger et al. 2002).

EE binds to nuclear estrogen receptor alpha (ER-α), enters the nucleus, activating transcription of EE-regulated genes. ER-α is expressed in hepatocytes alone, whereas cholangiocytes express both ER-α and ER-β (Barkhem, Carlsson et al. 1998; Alvaro, Alpini et al. 2000). ER-α is significantly expressed in liver, whereas the level of ER-β mRNA in liver is very low (Couse, Lindzey et al. 1997).

It is generally believed that glucuronidation blocks activity of estrogens (Raftogianis, Creveling et al. 2000). It is important to note that there is no data on possible interaction among E2-17G and ER-α. Despite cholestatic activity of E2-17G was determined (Vore, Montgomery et al. 1983; Sánchez Pozzi, Crocenzi et al. 2003), ER-α knockout mice were resistant to EE induction of cholestasis and did not develop any of the hepatotoxic phenotypes such as hepatomegaly, elevation in serum bile acids, increase of alkaline phosphatase activity, liver degeneration, and inflammation. When wild type mice were EE-treated, ER-α decreased levels of the expression of bile acid and cholesterol transporters (bile salt export pump (BSEP), Na+/taurocholate cotransporting polypeptide (NTCP), OATP1, OATP2, ABCG5, and ABCG8)

in the rat liver. Accordingly, secretion of bile acids and cholesterol into bile markedly decreased in EE-treated wild-type mice but not in the EE-treated ER-α knockout mice. Moreover, ER-α increased the expression of CYP7B1 and decreased the CYP7A1 and CYP8B1, which change the bile acid content toward increment of muricholic acid in the serum. ER-β FXR, PXR, and CAR were not found that participate in regulation of the expression of bile acid transporter and biosynthesis enzyme genes following EE exposure (Yamamoto, Moore et al. 2006).

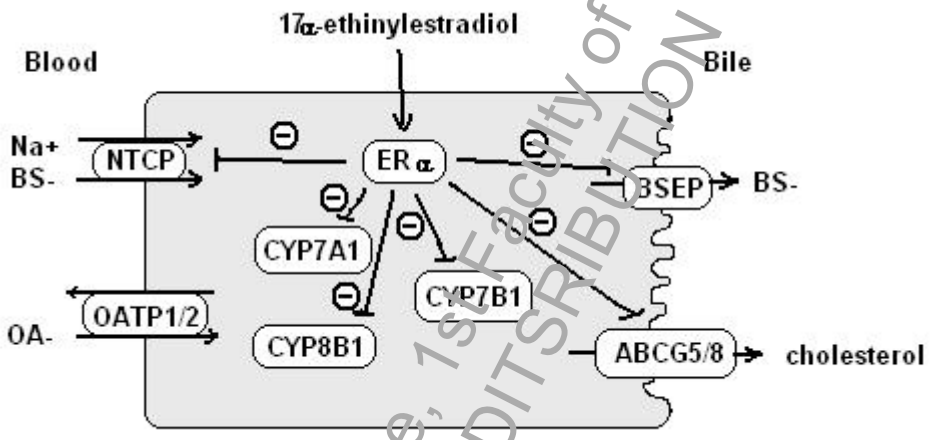

Figure 1-4. Estrogen receptor α (ER-α) mediated hepatotoxicity. ER-α is activated by EE (17α-ethinylestradiol), estrogen receptor agonist. It leads to down-regulation of bile acid transporters (NTCP, OATP1/2, and BSEP), cholesterol transporters (ABCG5/8), and alterations in biosynthesis of bile acid (CYP7A1, CYP7B1, and CYP8B1). This leads to down-regulation of secretion of biliary lipids and alteration of bile acid content (Yamamoto, Moore et al. 2006).

Estrogen induced cholestasis can be treated by UDCA (ursodeoxycholic acid). Administration of UDCA in EE-rats increased bile acid secretion and bile flow. However, it did not improve glutathione and bile acid-independent bile flow. NTCP mRNA and protein were decreased in EE-rats and was not restored by UDCA administration. Contrary, BSEP protein expression was decreased in EE-rats and restored to levels of control group after UDCA administration. This restoration is possibly effect of a normalization of the BSEP expression (Bouchard, Yousef et al. 1993; Micheline, Emmanuel et al. 2002).

Among other drugs diosgenin was tested for its promising action on bile flow. Diosgenin was shown that has the strong ability to replace physiological estrogen estradiol bound to ER-α with IC50 10 nM (Yen, Su et al. 2005). In contrast, diosgenin reduced

cholestatic effect of E2-17G and prevented some cholestatic effects of EE, such as decreased taurocholate bile secretion, increased ALP, canalicular membrane cholesterol/phospholipids ratio and biliary lipid lamellae excretion (Accatino, Pizarro et al. 1998).

1.1.6 Plasmatic membrane fluidity in cholestasis

In this connection it should be noted, that it was described more than two decades ago that erythrocytes from patients with cholestasis exhibited decreased membrane fluidity (Balistreri, Leslie et al. 1981).

Next, Smith and Gordon analyzed rat livers with EE-induced cholestasis and found an increase in cholesterol ester and sphingomyelin content, as well as an increase in the cholesterol/phospholipid ratio, but an unaltered fatty acids composition (Smith and Gordon 1988). The decreased fluidity persisted not only in liposomes prepared from total liver extract of treated rats but also in liposomes of the phospholipid (sphingomyelin) fraction which was prepared by precipitation with cold acetone. In this phospholipid/sphingomyelin fraction glycosphingolipids (GSL) were no doubt present but they were not analyzed, though a body of evidence that GSL influence membrane fluidity exists (Bertoli, Masserini et al. 1981; Uchida, Nagai et al. 1981; Masserini, Palestini et al. 1989).

Furthermore, Rosario et al. (Rosario, Sutherland et al. 1988) examined the effect of EE administration on protein and lipid composition as well as membrane fluidity using purified sinusoidal and canalicular membrane fractions and found that EE administration selectively decreased sinusoidal membrane fluidity. Lipid analyses showed only minor changes in phospholipid/sphingomyelin/phosphatidylcholine ratio, cholesterol/phospholipid ratio and fatty acid distribution, which could not explain a decrease of membrane fluidity. Again, complex GSL were not analyzed (Amigo, Mendoza et al. 1999). In addition, altered membrane fluidity was also described in cholestasis induced with lithocholate (Vu, Tuchweber et al. 1992), as well as bile duct ligation, or phalloidin infusion (Hyogo, Tazuma et al. 1999; Hyogo, Tazuma et al. 2000).

1.2.1 Gangliosides: Function and biosynthesis

The main lipid components of animal cell membranes are glycerophospholipids, sphingolipids, and cholesterol. Among these, sphingolipids are less abundant (Feizi 1985). Gangliosides are sialic acid-containing glycosphingolipids (GSL), highly concentrated in the outer lipid bilayer of animal cells and are particularly abundant in the plasma membranes of neurons. They show a strong amphiphilic character due to their constitution. They constitute of a hydrophobic ceramide part and a structurally variable hydrophilic oligosaccharide part protruding toward the extracellular environment (Figure 1-5).

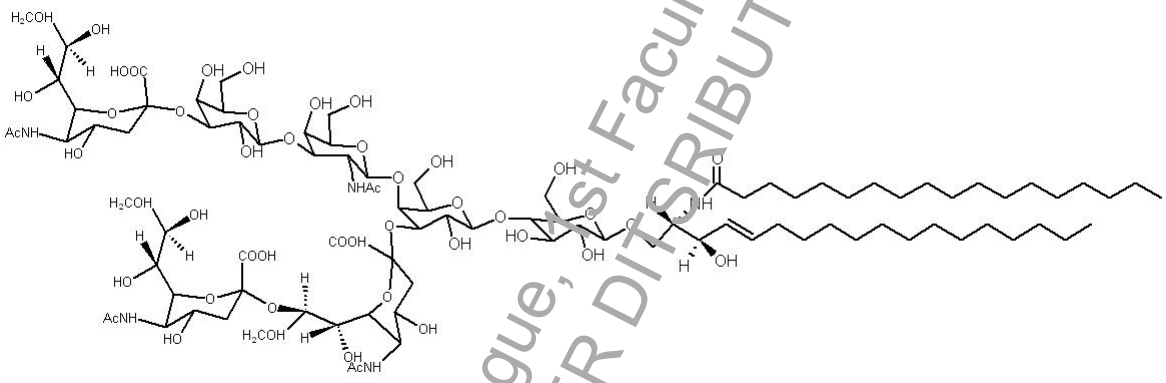

Figure 1-5. Chemical structure of GT1b ganglioside occurring in the brain of vertebrates, chosen in order to display the amphiphilic character. The hydrophobic membrane ceramide group can be seen on the right and the variable oligosaccharide part with sialic acid residues is on the left (Tettamanti, Bassi et al. 2003).

The main lipids of the plasma membrane, phospholipids contain mostly unsaturated alkyl chains, a requirement for membrane fluid properties, whereas glycosphingolipids contain mostly saturated alkyl chains in their hydrophobic part. The high transition temperatures of saturated chains exclude them from unsaturated chains with a lower transition temperature (Sonnino, Mauri et al. 2007). Ceramide part is a more rigid and the addition of glycosphingolipids to cells reduced the cell membrane fluidity (Bertoli, Masserini et al. 1981).

In glycosphingolipids at the interface of water and membrane lipids are heteroatoms of the ceramide part. This rigid system comprises planar amide group and double bound, and hydroxyl group at position 2. The amide group and hydroxyl group constituting of more electronegative atoms are able to form stable hydrogen bonds. At the same time they can act as hydrogen bond donors and acceptors. This property enables sphingolipids to form a stable net

of hydrogen bonds. Thus the headgroups by themselves promote the clustering process of glycosphingolipids. In contrast, the more abundant other membrane lipids, glycerophospholipids do not posses this property due to lack of hydroxyl as well as amide group. Therefore they can act only as acceptors of hydrogen bonds and also cholesterol posses very limited capacity to form hydrogen bonds by its one hydroxyl group. The van der Waals forces between saturated and unsaturated alkyl chains have been estimated being about 2–3kcal per a hydrocarbon chain. The clustering of hydrogen bonds at the interface can contribute with 3–10 kcal to the van der Waals interaction of hydrocarbon chains. It results in lateral orientation of molecules forming membranes with the hydrogen bond donor-acceptor groups of sphingolipids promoting the formation of a stable membrane rigid zone with a network of hydrogen-bonds together with cholesterol (Sonnino, Prinetti et al. 2006). In other words, gangliosides are not homogeneously distributed on the cell surface. The driving forces form the membrane areas with a distinct lipid composition leading to a liquid-ordered phase environment with attached interacting membrane proteins. This segregated membrane microdomains "lipid rafts" can consisted of cholesterol, gangliosides and glycosylphosphatidyl inositol-anchored proteins, and other sphingolipids (van Meer and Lisman 2002; Hakomori 2003; Sonnino, Prinetti et al. 2006; Sonnino, Mauri et al. 2007).

Gangliosides have various functions. They act as receptor molecules and, together with the other GSL, are generally believed to protect cells against harmful environmental factors by forming a mechanically stable and chemically resistant outer leaflet of the cell membrane (van Meer and Lisman 2002). Besides stabilizing cell membranes, gangliosides also seem to be involved in cell-to-cell interactions and recognition (Hakomori and Igarashi 1995), endocytosis and trafficking (Parton 1994), as well as an important role in signal transduction the regulation of various receptor tyrosine kinases in the plasma membrane including the receptors of epidermal growth factor (Zhou, Hakomori et al. 1994), nerve growth factor (Mutoh, Tokuda et al. 1995), insulin (Nojiri, Stroud et al. 1991) and even the modulation of memory protection (Rahmann 1995).

The biosynthesis of gangliosides starts with synthesis of the ceramide part. The ceramide part of ganglioside consists of a sphingoid base and a fatty acid. Sphingosine is biosynthesized by serine palrnitoyltransferase (SPT) by the condensation reaction of serine and palmitoyl-CoA to 3-ketosphinganine. This resulting base may be modified or used to ceramide synthesis. Ceramide is then glycosylated to glycosphingolipids or used for synthesis of sphingomyelin (Radin 1984; Merrill 2002). Complex oligosaccharide part originate from lactosylceramide and its sialylated derivatives GM3, GD3, GT3 which serve in distal Golgi as

precursors of 0-, a-, b- and c- pathways of biosynthesis (Figure 1-6) (Kolter, Proia et al. 2002; van Echten-Deckert and Herget 2006).

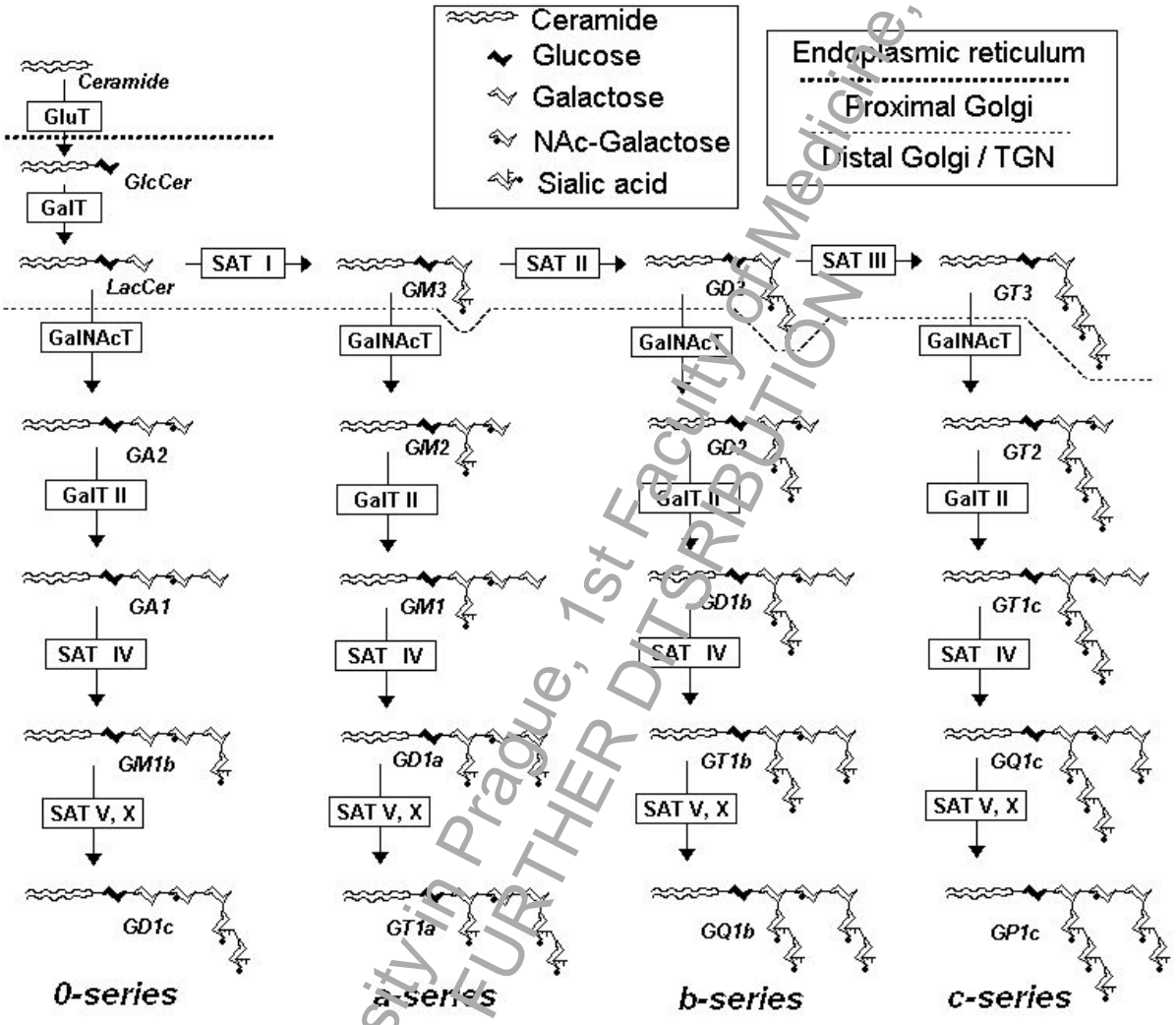

Figure 1-6. Scheme of the *de novo* biosynthesis of the oligosaccharide moieties of gangliosides of *0, a, b* and *c* series. Abbreviations of enzymes catalyzing indicated reactions are as follows: SAT I, II, and III - sialyl(NeuAc)-transferases I, II, and III; GalTII - galactosyl(Gal)-transferase; GalNAcT - *N*-acetylgalactosaminyl(GalNAc)-transferase; SAT IV, V, and X - sialyl(NeuAc)-transferase IV, V and X. (Kolter, Proia et al. 2002; Tettamanti 2004; van Echten-Deckert and Herget 2006). Abbreviations of gangliosides according to Svennerholm (Svennerholm 1963; Svennerholm 1994).

The biosynthesis of gangliosides takes place on intracellular membranes (endoplasmic reticulum and Golgi apparatus). The biosynthesis is catalysed by membrane-bound enzymes. Transport of *de novo* synthesized gangliosides to the plasma membrane is carried out by

sorting vesicles toward the membrane and consequential fusion. Degradation of gangliosides take place in secondary lysosomes by lysosomal acidic hydrolytic enzymes (predominantly soluble) (Figure 1-7). The hydrolysis is associated with binding to activator proteins (e.g. GM2 activator protein) and negatively charged lipids (e.g. dolichol phosphate) (Tettamanti, Bassi et al. 2003).

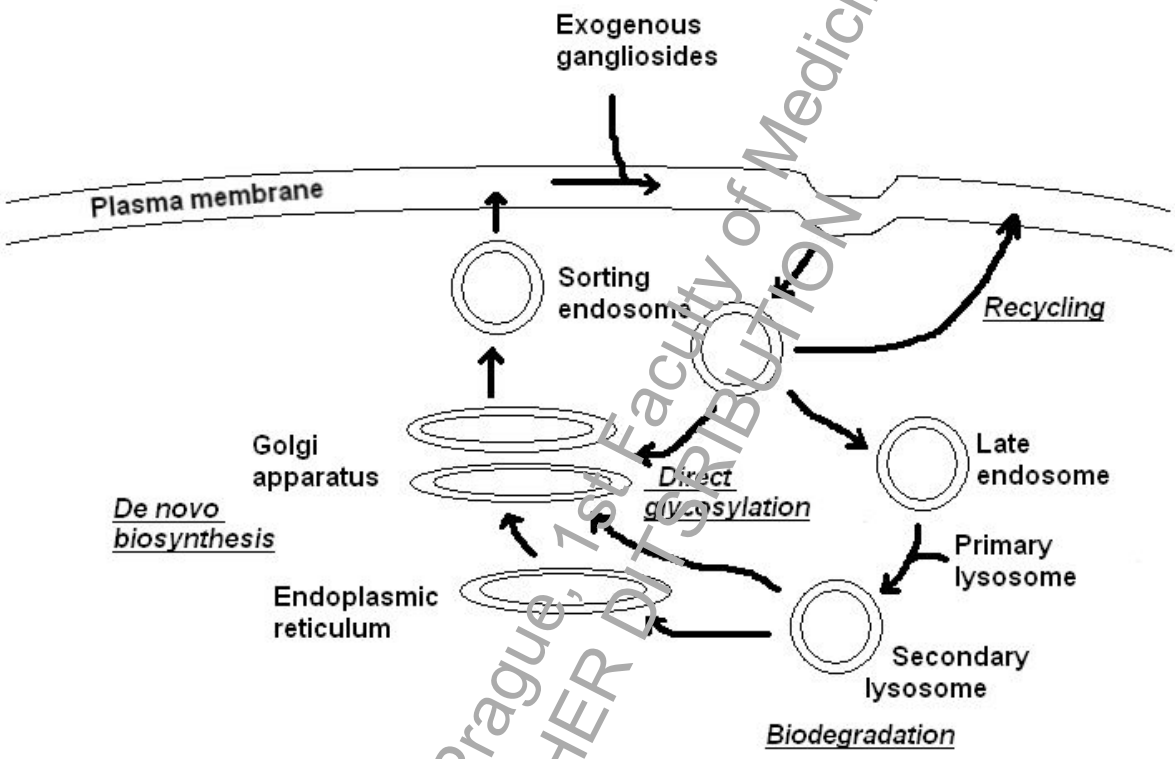

Figure 1-7. Subcellular metabolism of gangliosides on intracellular membranes. Scheme shows intracellular traffic of gangliosides: recycling, *de novo* biosynthesis, direct glycosylation, biodegradation, and salvage pathway routing from secondary lysosome to Golgi aparatus (Tettamanti, Bassi et al. 2003).

The composition of gangliosides in the cellular membranes varies, depending on different cell types, and also changes with cell differentiation during both embryonic development (Yu 1994) and malignant transformation (Hakomori 1985; Hakomori 2001). The formation of the GSL variety is controlled at the transcriptional and post-transcriptional levels (Kolter, Proia et al. 2002).

1.2.2 Sorting of proteins and sphingolipids in polarized cells

The membrane of polarized epithelial cells consists of functionally different apical and basolateral domains displaying specific protein and lipid composition. These proteins and lipids are sorted in the trans-Golgi network and then directly transported to the apical membrane (Rodriguez-Boulan and Powell 1992; van Meer and Lisman 2002). Like proteins, lipids also show polarized distribution as evidenced by studies with hepatocyte-derived HEP-G2 cells incubated with fluorescent dye-labeled glucosylceramide and sphingomyelin (van IJzendoorn, Zegers et al. 1997). In these experiments, the fluorescent analog of glucosylceramide displayed a preferential localization in canalicular membrane while the fluorescent analog of sphingomyelin was sorted into basolateral pole via the reverse transcytotic route (van IJzendoorn, Zegers et al. 1997). Some evidence for direct transport of proteins into the apical plasma membrane in hepatocytes also exists (Polishchuk, Di Pentima et al. 2004), but numerous apical membrane proteins are indirectly sorted and lipid rafts seem to be instrumental in targeting of some apical proteins (Slimane, Trugnan et al. 2003). Furthermore, association of microdomains and GPI-anchored proteins (Brown and Rose 1992) promote oligomerization of GPI-anchored proteins and it could further stabilize GPI-proteins raft complex, needed for apical sorting (Paladino, Sarnataro et al. 2004). Delivery of apical proteins and lipids to the cell surface and their recycling is regulated by cellular levels of a second messenger cAMP (cyclic adenosine monophosphate) (Snyder 2000).

The key role of microdomains in trafficking was proved by depletion of cholesterol using β-methyl-cyclodextrin and by depletion of glycosphingolipids by inhibitor of ceramide synthase. Both agents blocked translocation of apical proteins from basolateral early endosomes to SAC (SubApical Compartment – sorting compartment) (Nyasae, Hubbard et al. 2003). Likewise, in studies of HEPG2 cells, treatment with glucosylceramide synthase inhibitor delayed translocation from Golgi apparatus to the apical surface of apical membrane transporter MDR1 (Wojtal, de Vries et al. 2006). In addition, Thy-1 (CD90) and PrPc (Prion Protein Cellular) are two GPI-proteins enriched in the axon and in the cell body of neurons associating with microdomains of different composition. The different compositions of microdomains are in keeping with trafficking dynamics and basolateral or apical routing (Brugger, Graham et al. 2004; Tivodar, Paladino et al. 2006). Moreover, the destination of GPI-anchored proteins in endosomes of mammalian cells depends on sphingolipid and cholesterol concentrations. It further support the crucial role of microdomains in cellular trafficking (Mayor, Sabharanjak et al. 1998; Chatterjee, Smith et al. 2001). Usage of toxin

aerolysin for detection of GPI-anchored proteins provided the time of association with microdomains showing that the time might also be important for the final destination (Abrami, Fivaz et al. 1998; Fivaz, Vilbois et al. 2002). Nevertheless, the distribution of GSL microdomains in the canalicular and basolateral membranes of hepatocytes has not been investigated *in situ.*

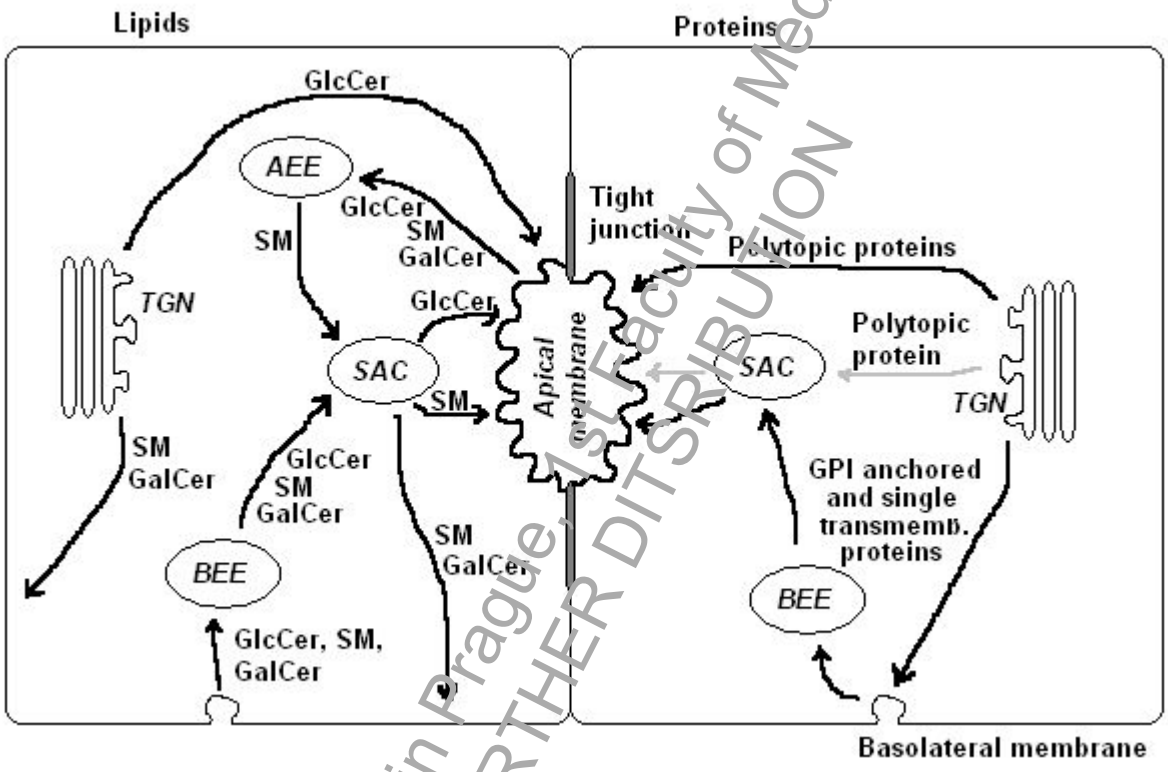

Figure 1-8. Sorting of sphingolipids and proteins in polarized hepatocytes. (**Left**) Sorting of sphingolipids. *De novo* biosynthesized GlcCer, GalCer and SM(sphingomyelin) are directly translocated from the TGN(trans Golgi network) to apical membrane and basolateral plasma membrane. Then, the sphingolipids are delivered to distinct AEE(apical early endosomes) or BEE(basolateral early endosomes) and transported to the SAC(subapical compartment) (Hoekstra, Tyteca et al. 2004). In the SAC sorting and segregation of internalized sphingolipids occurs. GlcCer is transported to the apical membrane, whereas SM and GalCer are transported to the basolateral membrane. (**Right**) Trafficking of apical proteins. GPI and single transmembrane proteins are transported via the transcytotic pathway, thorough SAC. Polytopic proteins (e.g. ABC transporter MDR1) are directly transported to the apical membrane. One member of the ABC transporter family has been shown to be transported via SAC (Ait Slimane and Hoekstra 2002).

1.2.3 Toxins binding gangliosides – detection of gangliosides

Some bacterial toxin binds to glycosphingolipids (GM2 (Jolivet-Reynaud and Alouf 1983), GM1 (Cuatrecasas 1973), GD1a (Holmgren 1973), GD1b and GT1b (Angstrom, Teneberg et al. 1994), GT1b (Takeda, Takeda et al. 1976), GQ1b and GT1b and GD1a (Takamizawa, Iwamori et al. 1986)) with high binding specificity (Lencer, Hirst et al. 1999). Together with low molecular weight and non-toxicity (usage of fragments or toxin subunits retaining GSL binding activity) they are predetermined being used for specific detection of gangliosides like antibodies.

The mostly used toxin for detection is cholera toxin. Cholera toxin consist of one 27kDa A-subunit and five 12kDa B-subunits. The B-subunit (ChtB) retains the GM1 ganglioside binding ability and is widely used for detection of lipid rafts. ChtB forms pentamers in dependence on its concentration. ChtB posses affinity to the GM1 ganglioside like antibodies to their epitops (Holmgren and Lonnroth 1975; Holmgren, Lonnroth et al. 1975).

Tetanus toxin is known to bind b-series gangliosides with the highest affinity to GD1b and GT1b gangliosides. The presence of GT1b and GD1b has been shown essential for toxin binding, furthermore, holotoxin is much less toxic in the knockout mice lacking b-series gangliosides (Kitamura, Igimi et al. 2005). However, tetanus toxin binding is sensitive to proteases suggesting membrane protein component (Lazarovici and Yavin 1986). The best pH for tetanus toxin binding is pH 7.4 and the optimum pH for toxin membrane activity is acidic pH (Deinhardt, Berninghausen et al. 2006; Slade, Schoeniger et al. 2006). Tetanus toxin exhibits saturation between 0.1 and 1 μg/ml. Authors Lazarovici and Yavin also showed 3-4 fold lower binding at 4°C than 37°C and preference of low ionic strength when investigated human erythrocytes supplemented with disialo- and trisialo-gangliosides (Lazarovici and Yavin 1985). The holotoxin consists of a 100kDa heavy and a 50kDa light chain. The ganglioside binding part was found to be C-end of the heavy chain (tetanus toxin C-fragment= TTC; in the literature sometimes called TeNT HcC). TTC is 460 amino acids long. TTC fragment was used for tracing fiber connections in the CNS being rich in b-series gangliosides. Many SCLC (Small Cell Lung Carcinoma) cell lines showed up-regulation of the GD3 synthase gene and enrichment in b-series of gangliosides (Yoshida, Fukumoto et al. 2001) and labeling by tetanus toxin C-fragment (Heymanns, Neumann et al. 1989).

The affinity of chtB to GM1 assayed by ELISA is almost 50x higher than affinity of tetanus toxin to GD1b an GT1b gangliosides (Angstrom, Teneberg et al. 1994).

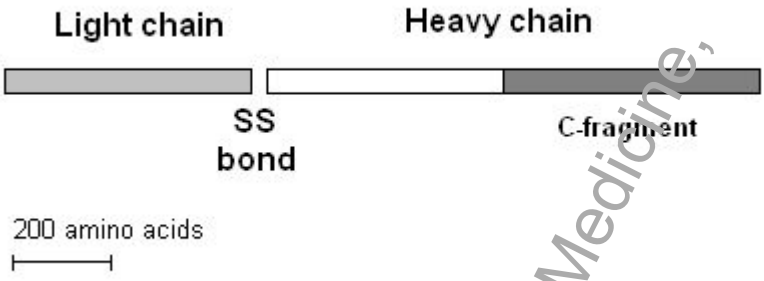

Figure 1-9. Tetanus toxin consists of two polypeptide chains of the same origin, a light chain and a heavy chain. The chains are bound together via an interchain disulfide bond. The heavy chain can be cleaved to N-fragment and C-fragment of the heavy chain (Montecucco and Schiavo 1995; Herreros, Ng et al. 2001).

Another question is whether gangliosides are accessible enough for the detection agents. Since the lipid bilayer consists of much bigger molecules such as glycoproteins. It also undoubtedly depends on cell cycle, cell type and shape of involved molecules (Table 1-1).

Cholera toxin (A-subunit + B-subunit pentamer)	~87 kDa
Cholera toxin B-subunit	~12 kDa
Tetanus toxin	~150 kDa
Tetanus toxin C-fragment	~50 kDa
Concavalin A	~56 kDa
Wheat germ agglutinin	~36 kDa
IgG antibodies	~120 kDa
IgM antibodies	~160 kDa

Table 1-1. Comparison of the Molecular Weights of carbohydrate binding molecules: antibodies, toxins and others used for detection (Schrevel, Gros et al. 1981).

2 SCOPE OF THE THESES

The aim of this study was to reveal whether ganglioside composition is changed in EE-induced cholestasis (Smith and Gordon 1988).

Consequent aims were:

1. Analysis of ganglioside content and their changes after cholestasis induction by EE-treatment in the liver

2. To determine changes in ganglioside localization in the rat liver after estrogen induction of cholestasis.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and other materials

Bovine Serum Albumin Fraction V (BSA)	Sigma, (USA)
Chloroform, methanol, xylen and other solvents	Penta, (Czech Republic)
Tween 20, Triton X-100, Saponin	Sigma, (USA)
Wistar strain of rats	Anlab, (Germany)
Ethinylestradiol	Sigma, (USA)
1,2-propanediol	Fluka, (Switzerland)
Neuraminidase from Clostridium perfringens type V	Sigma, (USA)
Cholera toxin B-subunit biotin conjugate	List laboratories, (USA)
Primary and secondary antibodies	Calbiochem, Pierce, Sigma, Molecular Probes, Santa Cruz, (USA), Sevapharm (CZ)
ABC kit	Vector Laboratories, (USA)
Streptavidin peroxidase polymer (S2438)	Sigma (USA)
Orcinol, Resorcinol	Merck, (Germany)
Silica-gel 60	Merck, (Germany)
HPTLC Alufolien Kieselgel 60	Merck, (Germany)
HPTLC glass plates Kieselgel 60	Merck, (Germany)
Polyisobutylmethacrylate	Aldrich, (DE)
4-chloro-1-naphtol	Aldrich, (Germany)
HPLC column HEMA BIO-300 DEAE	Tessek, (Czech Republic)

3.1.2 Reagents and Media

Resorcinol reagent: 10 ml of a stock solution prepared from 2 g of resorcinol (benzene-1,3-diol) and 100 ml of water (stored at 4°C) was added to 80 ml of concentrated hydrochloric acid and 0.25 ml of 0.1M $CuSO_4$ and filled with water to 100ml. Stored at 4°C for up to 3 weeks.

Orcinol reagent: 2% orcinol (5-methylbenzene-1,3-diol) 2M H_2SO_4 in ethanol. Stored at 4°C and protected from light.

Formaldehyde (freshly prepared from paraformaldehyde): The temperature of 4g of paraformaldehyde suspended in 85ml of water was raised to around 70°C with stirring, and added a little bit of NaOH to raise the pH so that the paraformaldehyde was depolymerized into formaldehyde (monomer). Then 10ml of 10x concentrated PBS is added. Finally, pH is adjusted to 7.4 and the volume filled in with water to final volume of 100ml. Formaldehyde must be handled in a fume hood!

Anhydrous acetone (dried with $CaCl_2$): Dried acetone is prepared by adding 30 g of anhydrous calcium chloride to 100 ml of a commercially available acetone. This way prepared suspension is allowed stand overnight. The next day the drying agent is removed by filtration and yielded the dried acetone by distillation by collecting the main fraction at around 56°C after several drops of fore shots.

Phosphate buffered saline (10x concentrated): A 10 liter stock of 10x PBS can be prepared by dissolving 100g NaCl, 2.5g KCl, 18g $Na_2HPO_4{\cdot}2H_2O$ and 3g KH_2PO_4 in 1 L of distilled water, and topping up to 10 L. The pH is ~6.8, but when diluted to 1x PBS it should change to 7.4. On dilution, the resultant 1x PBS should have a final concentration of 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, and a pH of 7.4.

4-chloro-1-naphthol. Stock soulution is prepared by dissolution of 6 mg 4-chloro-1-naphthol in 2 ml of methanol. Before reaction 2 ml of the stock solution is added to 10 ml of Tris-HCl buffer pH 7.6 and 6.7 µl of 30% H_2O_2.

Primuline: acetone with water (80/20 v/v), and primuline to 0.05% (w/v). TLC is sprayed with this solution for visualization of lipid spots under UV light.

DAB: 1mg of 3,3'-Diaminobenzidine tetrahydrochloride (stored at -20°C) is dissolved in 100 µl of dimethylformamide. For color development the 100 µl is added to 1ml 10mM Tris-HCl pH 7.2-7.4 and 1ml of water with 6.7 µl 30% H_2O_2.

Alkaline Phosphatase development solution: Sections are delipidated in chloroform/acetone (1/1 v/v) for 5 min at 4°C. The dried sections are incubated in naphtol AS-MX phosphate (4 mg in 0.5 ml of DMF), and 10 ml of Tris-HCl buffer pH 9.2 containing 3.5 mg of Fast Blue BB hydrochloride salt for 30 minutes with the resulting pH being 8.5.

3.2 Methods

3.2.1 Animals and treatments, induction of cholestasis

Female Wistar rats were housed under controlled temperature and a natural 12 h light–dark cycle. Groups of age- and weight-matched rats were used in all studies and the animals had free access to food and water throughout the experiments, but were fasted overnight before experimentation. All aspects of the study met the accepted criteria for the humane care and the experimental use of laboratory animals and all protocols were approved by the Animal Research Committee of the 1st Medical Faculty, Charles University of Prague.

In study of histological changes the cholestasis was induced in the experimental group (n=11) by subcutaneous injections of EE (Sigma, St Louis, MO, USA) in a dose 5 mg/kg b.wt. dissolved in 0.2 ml of 1,2-propanediol (PD) (Fluka, Switzerland) applied daily for 5 or 18 consecutive days. The control group of animals consisted of a subgroup (n=7) without any treatment and a subgroup (n=8) treated with 0.2 ml of PD daily.

In study providing data on changes in ganglioside content were three groups of animals: experimental (n = 6), control group (n=6) and a PD subgroup (n = 3).

3.2.2 Collection of samples

After laparotomy under ether anaesthesia, the inferior vena cava was cannulated, blood samples were collected and livers were flushed blood-free with heparin (100.000 IU/l) in saline for 2 min and weighed. For further histological analyses, the livers were weighed, cut and then rapidly frozen in petroleum ether in acetone-dry-ice bath and stored at -80°C For quantitative histochemical analysis of GM1 ganglioside, the liver specimens were collected using systematic uniform random sampling method. For Real-Time PCR analysis liver pieces were submerged in RNAlater (Qiagen, USA) and stored at -80°C. The pieces of liver tissues were stored at -20°C until processed biochemical analysis of ganglioside composition.

3.2.3 Analysis of serum markers of cholestasis

In order to determine the degree of cholestasis and liver injury, the following serum levels were assessed: total bile acids (TBA), total bilirubin, alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT).

TBA were determined enzymatically (3α-OH-bile acids, spectrophotometric kit BI-1605, Randox, UK), while all other markers were quantified on an automatic analyzer (model 717; Hitachi, Tokyo, Japan). Total bilirubin was analyzed by the diazo reaction according to Jendrassik and Gróf, the activities of serum transaminases and ALP were determined enzymatically using routine commercial chemicals.

3.2.4 Isolation and TLC analysis of liver gangliosides

Liver gangliosides were analyzed by a densitometric method after TLC separation and detection with resorcinol-HCl reagent, as previously described (Smid and Reinisova 1973). Homogenization of the rat livers (3 g of the total ~10 g of liver tissue) was performed using knife homogenizer (HOMOGENIZER type 302, Mechanika precyzyjna, Polsko) in methanol then was added chloroform to ratio 1:2 (Folch, Lees et al. 1957), the extract was filtrated, filtrate kept and filter cake re-extracted with chloroform/methanol/water 1:2:0.05 by volume increasing yield of polysialogangliosides (Suzuki 1964). The extracts were pooled and proportion of chloroform and methanol adjusted to the final proportion 2:1, added 0.2 volume of 0.88% KCl in water and gangliosides were partitioned into a water–methanol phase. Then, gangliosides were twice more extracted from the lower phase with 0.4 volume of 0.88% KCl. The lower organic phase were used for determination of cholesterol and phospholipids as lipid inorganic phosphate (see below) (Rouser, Fkeischer et al. 1970; Cohen, Hertz et al. 1980; Ellerbe, Meiselman et al. 1989). From the pool of upper water-methanolic phases methanol was removed under reduced pressure in a rotating evaporator. The yielded solution of gangliosides was dialyzed against distilled water for 5 days, and lyophilized to dryness. Finally, gangliosides were purified on a small silica-gel column with methanol-chloroform solvent system (Yu and Ledeen 1972). Total lipid sialic acid was determined in samples by spectrophotometry with resorcinol–HCl reagent (Svennerholm 1957).

Samples of isolated gangliosides were dissolved in 0.1 ml of chloroform–methanol 1:1 (v/v), and 10 µl of the solution was applied on an HPTLC silica-gel plate (Merck, Darmstadt, Germany) using a CAMAG LINOMAT IV sampler (Camag, Switzerland). Gangliosides were separated in a solvent system (chloroform–methanol–0.2% aqueous $CaCl_2$, 55:45:10, v/v/v),

and detected with resorcinol–HCl reagent after spraying and heating the plate as violet spots. For the selective detection of sialic acid the lowering of the heating temperature down to 100°C was required with a prolongation of the heating time to 30–35 minutes. Other sugars did not react under these conditions. The chromatograms were evaluated by densitometry in reflectance mode at 580 nm, using a TLC Scanner II, CAMAG (Muttenz, Switzerland) and 'Cats' 3.20 software. At the beginning of evaluation, standard mixture of gangliosides was prepared within the range of their concentrations in samples. Four dilutions of this mixture were applied on corresponding starting lines in increasing amounts and concentrations were calculated from calibration graphs for each individual ganglioside. Alternatively, the sialic acid content corresponding to individual ganglioside fractions was calculated on the basis of the calibration of the GM1 ganglioside standard (Sigma, USA) separated on the same plate as used previously by Chigorno et al. (1982). This GM1 calculation gave compatible results and was used for final evaluation.

3.2.5 Lipid phosphorus and total cholesterol

Lipid phosphorus for determination of phospholipids was assayed in the lower organic phases yielded from Folch extraction procedure accordingly to Rouser (Rouser, Fkeischer et al. 1970) to determine changes in phospholipids to cholesterol ratio. The procedure for an aliquot of hepatic lipids was as follows. In a glass tube washed with 5% nitric acid was evaporated an aliquot of the lower phase. Then concentrated perchloric acid (70%) was added and the lipids were digested by heating for 20 minutes at 180°C in the heated metal block with the upper one half of each tube extending outside of the block so that perchloric acid fumes do not escape out. After 20 minutes the tubes were cooled, and further reagents were added in order: water, ammonium molybdate and 10% solution of ascorbic acid in water. Blue phosphomolybdate complex was developed by heating in a boiling water bath for 5 minutes. After short centrifugation is measured absorbance at 797nm.

Total cholesterol was assayed using gas chromatography-mass spectrometry accordingly to (Cohen, Hertz et al. 1980; Ellerbe, Meiselman et al. 1989). Cholesterols (cholesteryl esters) were alkali-hydrolyzed, extracted using hexane and directly analyzed by gas chromatography-mass spectrometry (6890N GC, Agilent, USA) with 95% fenyl/methyl silicone capillary column.

3.2.6 Isolation of monosialogangliosides and estimation of fucosyl-GM1 ganglioside

The amount of Fuc-GM1 was assayed in monosialoganglioside fractions of the liver homogenate from control and EE-treated groups of animals by TLC with resorcinol detection and by TLC immunostaining. Homogenization of the six rat livers (6 x 2g of liver tissue) was performed in methanol, the gangliosides were partitioned into water–methanol phase (see above) (Folch, Lees et al. 1957; Suzuki 1964) removed methanol, dialyzed for 5 days, and then lyophilized to dryness (concentrated to small volume). Samples of isolated gangliosides were dissolved in 0.1M NaOH in methanol and incubated 1 hour at 37°C to remove labile lipids by alkaline methanolysis followed by neutralization with 1M acetic acid in methanol, removing contaminants by extraction with hexane, addition of water to the upper methanolic non-hexane phase, evaporation of methanol under reduced pressure in a rotating evaporator, dialysis against water and lyofilization to dryness.

Crude gangliosides were separated on a column 4x250mm Sepharon HEMA BIO 300 DEAE macroporous mechanically stabile and hydrophilic hydroxymethylmethacrylate medium basic anion-exchanger (Smid, Bradova et al. 1986). Mono-, di-, tri- and tetra-sialogangliosides were eluted with linear gradient of ammonium acetate in methanol (from 0 to 0.15M). Acetate gradient was checked by conductometry in each third tube. Fractions were analyzed by thin-layer chromatography with resorcinol-HCl reagent, some of them were condensed under stream of nitrogen at 45°C. Then identified monosialoganglioside fractions were pooled, diluted with distilled water, then methanol was removed under reduced pressure at 45°C, water solution was dialyzed to remove ammonium acetate and dialyzed sample was freeze dried.

Thus obtained monosialogangliosides of control and EE-treated group were dissolved in 0.1 ml of chloroform–methanol 1:1 (v/v). In these samples total lipid sialic acid was determined by spectrophotometry with resorcinol-HCl detection and separated in a solvent system (chloroform–methanol–0.2% aqueous CaCl2, 55:45:10, v/v/v) on silica-gel HPTLC aluminum plates with the same sample amount and arrangement by thin-layer chromatography with a kidney bovine fucosyl-GM1 (A kind gift of Dr. Ledvinová and Dr. Befekadu) as a standard and detected by spraying the plate with orcinol-H_2SO_4 reagent. The chromatogram was densitometrically evaluated in reflectance mode at 550 nm.

3.2.7 Light microscopy

Morphological analysis: Small tissue blocks (about 1 mm^3) were fixed in 4% paraformaldehyde in Na-cacodylate buffer (pH 7.4) for 2 h, dehydrated subsequently in graded methanol at 4 °C and then at −20°C, and embedded in LR Gold resin (Sigma, USA). Polymerization was performed under UV light at −25°C overnight. Semi-thin sections were cut, stained with toluidine blue and examined for autofluorescence of pigment granules.

Alkaline phosphatase histochemistry: Tissue specimens were rapidly frozen in liquid nitrogen. The catalytic activity of ALP was demonstrated in thin 6 μm cryostat sections using simultaneous azo-coupling method (Lojda, Gossrau et al. 1979). Control and experimental sections were incubated together for 150 min at room temperature, counterstained with Mayer´s hematoxylin and mounted in glycerin jelly.

GM1 histochemistry: Frozen 6 μm sections were fixed first in dry cold acetone (−20°C) for 2 min and then in 4% freshly prepared paraformaldehyde for 20 min. Endogenous peroxidase activity was blocked by incubation for 15 min in PBS supplemented by 1% H_2O_2 and 0.1% dissolved sodium azide.

Endogenous biotin was blocked by means of the blocking kit with avidin 0,1% and biotin 0,01% (DakoCytomation, Denmark). In order to block nonspecific binding, sections were treated with 3% BSA in PBS for 15 min. Cholera toxin was used for GM1 ganglioside detection (Wu and Ledeen 1988). Sections were incubated for 60 min at room temperature with biotinylated cholera toxin-B-subunit (List Biological Laboratories, USA) diluted 1:250 in PBS plus 3% BSA. After washing in PBS, the sections were labeled with Streptavidin-peroxidase polymer Ultrasensitive (Sigma, USA) diluted 1:400 in PBS containing 0.05% Tween 20 at room temperature for 60 min. Peroxidase activity was visualized with DAB.

Sections were counterstained with Mayer´s hematoxylin and mounted in glycerine jelly. Two control tests were included in each series. First, fixed sections were extracted with chloroform–methanol mixture 2:1 at room temperature for 30 min, followed by histochemical staining. Second, the cholera toxin B-subunit was omitted in negative control tests.

Quantitative study on the distribution of GM1 ganglioside in the hepatic lobule: In this study, 11 animals from EE-treated and 7 from untreated control group were used. From each liver, 6 pieces of randomly sampled tissue blocks were taken. One frozen 6 μm thick section was used from each block, and all sections were stained simultaneously using the above described histochemical reaction. The hematoxylin counterstaining was omitted. The images of whole sections were photographed at the objective magnification 5× (NA=0.12) and stored using the IM 500 Image Manager (Leica). The quantity of reaction product was determined as

mean optical brightness of marked area using the image analysis program ACC 6.0 (SOFO, Czech Republic) and ImageJ (USA). Two ways were used for the quantification. First, whole section was marked and its mean optical brightness was evaluated. Second, areas of liver parenchyma in peripheral (periportal; zone I) intermediate (zone II) and central (zone III) zones of hepatic lobules were marked and their mean optical brightness was determined separately.

Densitometric analysis of the amount of GM1 ganglioside in areas of sinusoidal membrane and of adjacent cytoplasm: Ten animals from EE treated, eight animals from PD and six animals from the control group were taken to this analysis. Six liver specimens were used from each animal. One section from each specimen was used for GM1 ganglioside detection with cholera toxin B-subunit histochemical procedure described above. On each section, four hepatic lobules with clearly discernible central vein were selected. In each lobule, one measuring frame in central lobular zone III and one measuring frame in corresponding peripheral lobular zone I were selected for analysis. In each frame, 15 areas of sinusoidal surface and 15 areas of adjacent hepatocyte cytoplasm were selected by the stratified random sampling method (Hamilton and Allen 1995) and marked out. The reaction product was quantified as mean optical density of analyzed areas as determined by the densitometric program CUE 2 (Olympus) at objective magnification 40× (NA=0.7).

3.2.8 Statistical analysis

Data are presented as mean ± SD, or median and 25–75% range. The statistical significance of differences between variables was evaluated by t-test or Mann-Whitney Rank Sum test in cases where data were not normally distributed. Histochemical data are presented as the mean ± SD of optical brightness, or differences between the optical brightness in peripheral and central zones of hepatic lobules, or the ratios of optical densities measured in sinusoidal membranes and hepatocyte cytoplasm (sin/cyt) in peripheral and central lobular zones. Differences with p-value less than 0.05 were considered significant. The most significant results were correlated with the serum TBA concentrations using linear regression analysis. All calculations were performed using software EpiInfo 3.2.2 (Centers for Disease Control and Prevention, Atlanta, Georgia, USA).

3.2.9 RNA extraction, cDNA synthesis and quantitative real-time PCR

RNA was extracted from livers (~100mg) appropriately stored in RNAlater of control or EE-treated animals using acid guanidinium thiocyanate-phenol-chloroform mixture (RNA

Blue reagent, Top-Bio, Czech Republic). Purity and quantity of RNA was checked by Nanodrop spectrophotometer (NanoDrop Technologies, USA).

Singlestrand cDNA synthesis was performed using MMLV reverse transcriptase (Top-Bio, Czech Republic), according to manufacturer's instructions; a mixture of 10 μg of isolated RNA and 50 ng of random hexamers was used per reaction. Reverse transcription ran at 25°C for 10 min (priming) followed by 50 min at 50°C for cDNA synthesis, followed by heating the samples at 85°C for 5 min and chilling them at 10°C terminating the reaction.

Quantitative real-time PCR was performed using Mastercycler ep realplex (Eppendorf, Germany). Eight-fold diluted single-strand cDNA was used as template and mixed at 1:1 (vol/vol) with qPCR 2× SYBR green master mix containing 20 mM Tris-HCl, pH 8.8, 100 mM KCl, 0.2% Triton X-100, 3 mM MgCl2, 400 μM dATP, 400 μM dTTP, 400 μM dGTP, 400 μM dCTP, 50 U/ml Taq DNA polymerase, SYBR green and primers. Primers were added to the final concentration of 0.25 μM. The following primers were used:

actin forward: 5′ ACTCTTCCAGCCTTCCTTCC 3′;
reverse: 5′ ATCTCCTTCTGCATCCTGTC 3′;
GalTII (galactosyl(Gal)-transferase II, GM1-synthase)
forward: 5′ AACGCCATTCGGGGATCTT 3′;
reverse: 5′ CTCTGAGGCCAGCTCAGCAAA 3′;
SATII (sialyl(NeuAc)-transferase II, GD3-synthase)
forward: 5′ GTAATGAAAGCCTTAAGCACAGC 3′;
reverse: 5′ CTTCTCTGCATCCAGGAACTTT 3′.

The PCR reaction started by a 2-min hot-start DNA polymerase activation at 95°C followed by 40 cycles of target cDNA amplification (1 cycle = denaturation at 95°C for 15 s, annealing at 57°C for 15 s, elongation at 72°C for 20 s and acquisition of SYBR green fluorescence). The same protocol was used for amplification of all three genes.

To ensure that correct DNA fragments were amplified in PCR, a melting curve of PCR products was obtained by increasing the temperature from 70°C to 95°C with a temperature transition rate of 0.2°C/s. Furthermore, the size of all PCR products was controlled by agarose electrophoresis. The final real-time-PCR data were expressed as the C_T values of GalTII and SATII normalized with the C_T of β-actin of each sample (Livak and Schmittgen 2001).

4 RESULTS

4.1 Induction of cholestasis - Biochemical analyses

EE treatment for 18 days resulted in the development of severe cholestasis, as evidenced by a marked elevation of serum bilirubin and TBA (Table 4-1a and 4-1b), as well as pronounced histological changes in livers typical of severe cholestasis (see below). Besides these effects, as compared with the controls, a significant loss of body weight was recorded in EE-treated animals (293 ± 32 vs 212 ± 43 g, p = 0.004). Treatment with 1,2-propanediol, a vehicle for EE, did not result in any change in cholestatic markers (data not shown).

Table 4-1a. Serum levels of cholestatic markers in control and experimental group in study of changes in ganglioside content.

Marker	Controls (n = 6)	EE treated rats (n = 6)	p value
ALT-L [μkat/L]	0.87 ± 0.14	1.16 ± 0.20	0.026
AST-L [μkat/L]	1.41 ± 0.28	1.85 ± 0.96	0.374
ALP [μkat/L]	2.04 ± 0.40	2.75 ± 1.50	0.394
Bilirubin [μmol/L]	2.10 ± 0.20	26.0 ± 17.0	0.002
TBA [μmol/L]	39 ± 25	589 ± 186	0.002

Table 4-1b. Biochemical markers of control and EE-treated rats Controls EE-treated in study of histological localization.

	Controls (n = 7)	**EE-treated rats (n = 11)**	**p-value**
Weight of rats [g]	296 ± 26	219 ± 34	**0.00001**
Weight of liver [g]	10.7 (9.6 - 11.2)	9.6 (8.6 - 11.9)	0.63
TBA [μmol/l]	45 (21 - 53)	382 (256 - 619)	**0.00001**
Bilirubin [μmol/l]	2.0 (1.9 - 2.4)	7.5 (5.5 - 27.8)	**0.00001**
ALT [μkat/l]	0.81 (0.75 - 1.04)	0.92 (0.81 - 1.20)	0.22
AST [μkat/l]	1.28 (1.17 - 1.61)	1.18 (1.00 - 1.77)	0.76
ALP [μkat/l]	2.12 (1.68 - 2.44)	2.42 (1.78 - 3.13)	0.38

Data are presented as mean ± SD, or median (25%-75%) when not normally distributed

Changes in cholesterol and lipid phosphorus were also observed in the same sense as stated in the literature (Smith and Gordon 1988). Total cholesterol in control rats 1.24 ± 0.24 and EE-treated rats 1.57 ± 0.27 mg/g tissue (n = 6, p value = 0.064). Lipid phosphorus control 685 ± 28 and EE-treated rats 619 ± 66 ug/g tissue (n = 6, p value = 0.083).

4.2 Total lipid sialic acid and TLC analysis of ganglioside content

The mean total lipid sialic acid in livers of EE-treated rats was 79.1 ± 9.0 nmol/g of wet weight of liver tissue when compared with 44.3 ± 15.2 nmol/g in the control animals ($p < 0.01$), i.e. ratio 1.78:1. Hepatic ganglioside distribution in EE-treated and control samples obtained from TLC (Fig. 4-2) analysis and densitometry (Fig. 4-3) is shown in Table 4-4. Although induction of cholestasis leads to more than a 3-fold increase of GD1a ganglioside (a marker of the a-biosynthetic pathway) in this particular experiment, the most pronounced effect

was detected in gangliosides of the b-pathway, with 1–2 orders of magnitude higher amounts of GD3, GD1b and GT1b in the cholestatic livers. In total, all a-series gangliosides increased 1.3-fold in cholestatic livers, whereas b-series gangliosides were up-regulated 54-fold.

Fig. 4-2. Marked increase of b-pathway gangliosides GD3, GD1b and GT1b isolated from cholestatic (EE1–EE6) and control (C1–C6) rat livers. All ganglioside samples were applied on TLC in the amount corresponding to 0.225 g of liver tissue on HPTLC plate of silica-gel, separated in solvent system chloroform–methanol–0.2% aqueous CaCl2, 55:45:10, v/v/v, detected with resorcinol–HCl reagent and scanned using a TLC Scanner II, CAMAG (Muttenz, Switzerland). Densitometrical evaluation was based on the calibration with all gangliosides and with GM1 only. Both evaluations were found as compatible that is why GM1 was used for final evaluation (quantitative data are stated in Table 4-4. The amounts of sialic acid in track of standards is 2.2 nmol for GT1b 2.2, 1.7 nmol for GD1b, 0.8 nmol for GD2 nmol, 2.7 nmol for GD1a, 2.5 nmol for GD3, 4.3 nmol for GM1, 0.8 nmol for GM2 and 4.1 nmol for GM3.

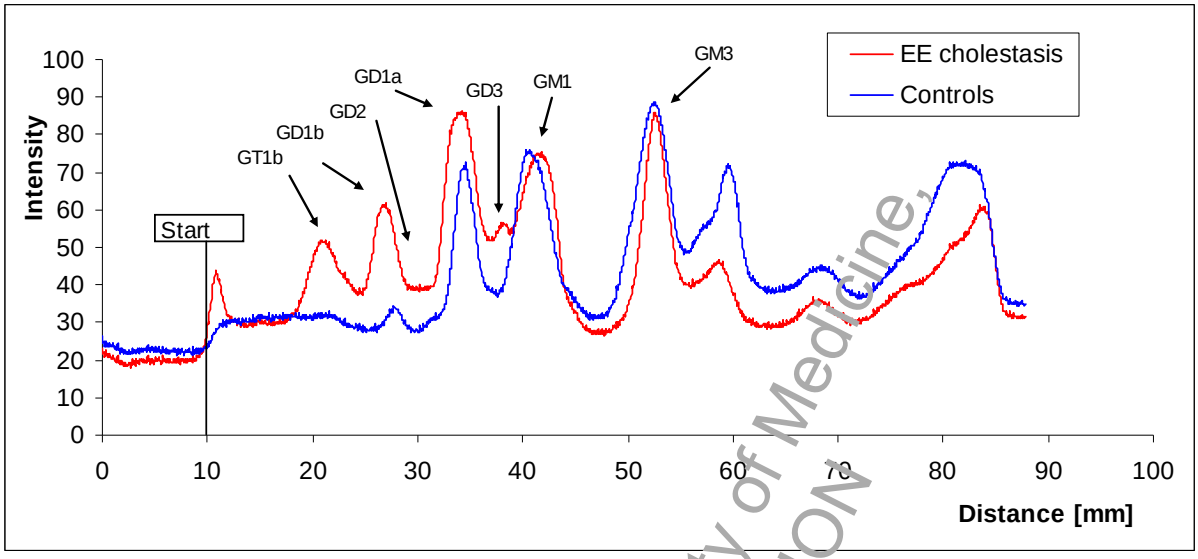

Fig. 4-3. Mean of scans of control and EE-treated group.

Table 4-4. Ganglioside distribution in liver tissue of control and EE-treated rats.

Ganglioside biosynthetic pathway	Ganglioside	Controls (n = 6) [nmol/g liver]	EE treated rats (n = 6) [nmol/g liver]	p value
a-series	GM3	11.4 ± 2.2	7.1 ± 0.6	0.001
	GM1	7.8 ± 2.4	10.5 ± 3.0	0.146
	GD1a	3.6 ± 1.0	11.8 ± 3.0	0.001
b-series	GD3	0.08 ± 0.03	2.0 ± 1.2	0.002
	GD2	0.02 ± 0.03	0.32 ± 0.34	0.041
	GD1b	0.10 ± 0.06	5.4 ± 1.6	0.002
	GT1b	0.06 ± 0.03	6.4 ± 2.6	0.002

The densitometric method used in our study gave comparable results to the photometric method published by Suzuki (1964); and an equal amount of mono-, di- and trisialogangliosides gave densitometric areas in the ratio of 1:2:3, according to the content of sialic acid in the molecule. The samples gave a linear calibration up to 0.4 µmol of ganglioside, (i.e. up to approximately 6 nmol of sialic acid; Smid and Reinisova, 1973). A similar linear response (in the range of 0.1–6 nmol of bound sialic acid) between densitometric responses and increasing sialic acid content of the different standard gangliosides was published by Chigorno

et al. (1982); however, using an alternative sialic acid detection method, with p-dimethylaminobenzaldehyde-HCl (Ehrlich reagent).

4.3 TLC Fuc-GM1

Cholera toxin B-subunit can bind also Fuc-GM1 ganglioside but with one half of the GM1 affinity (Masserini, Freire et al. 1992). Therefore, we needed to know if Fuc-GM1 is present. That is why we isolated monosialoganglioside fraction from the control rat liver pooled from 6 control animals (C) and cholestatic rat liver pooled from 6 EE-treated animals (EE). Both fractions C and EE were separated on TLC and the plate was evaluated by densitometry (Fig. 4-5). We assumed that the amount of FucGM1 is lower than 2ng/g tissue weight and much lower than GM1. Thus interference of Fuc-GM1 in GM1 histochemistry (see section 4.6. GM1 histochemistry) we concluded as not significant.

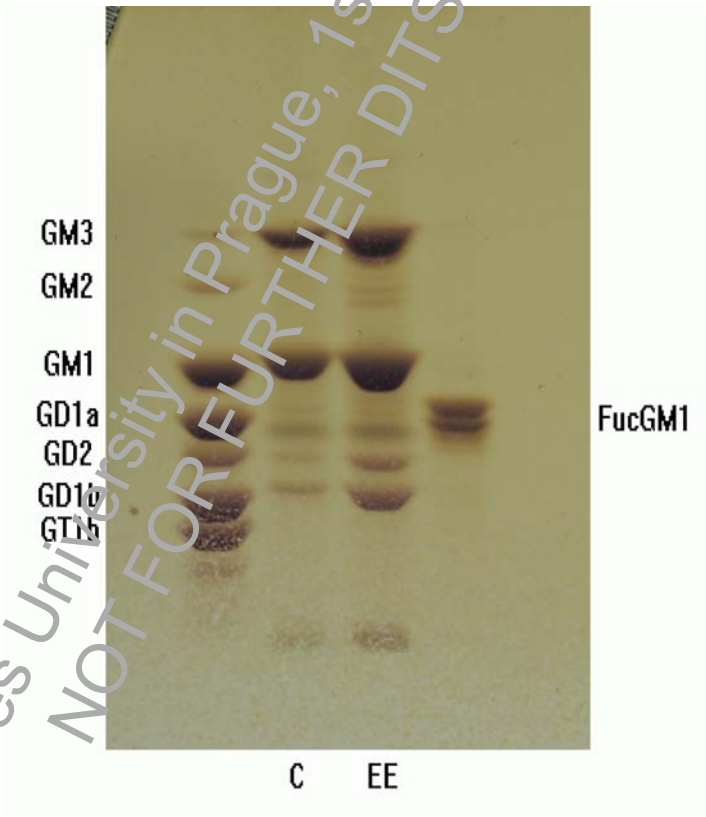

Fig.4-5. TLC of crude liver monosialogangliosides, orcinol-H_2SO_4 staining. From the right ~10ng brain gangliosides, pool of fraction enriched in monosialogangliosides from Control livers, monosialogangliosides from EE-livers, standard of FucGM1 (gift of Dr. Ledvinová).

4.4 Light microscopy – histopathological and morphological changes

Unlike the findings characteristic for control animals (Fig. 4-6a), morphological signs of cholestasis were found in the liver of EE-treated rats, even though individual liver samples differed in the degree of pathological changes. More or less conspicuous degree of feathery degeneration of hepatocytes occurred mainly in peripheral areas of lobules, though it was found occasionally in hepatocytes of all lobular zones (Fig. 4-6b, 4-6d). Liver samples of EE-treated animals displayed also variable amounts of intracellular bile pigments, while no pigment depositions were found in bile canaliculi (Fig. 4-6c). In some cases, fatty degeneration with numerous lipid droplets in hepatocytes was observable particularly in the zone I and zone II of the lobule (Fig. 4-6b). Liver cells displaying features of apoptosis or necrosis were evenly spread throughout the liver parenchyma (Fig. 4-6d).

4.5 Alkaline phosphatase histochemistry

EE-induced cholestasis was also reflected in ALP catalytic histochemical analysis. In the control group, ALP was confined just to the canalicular membrane of hepatocytes in peripheral zones (zone I) of hepatic lobules and the enzyme activity expressed by the amount of reaction product was very low (Fig. 4-6e). The sections from EE-influenced liver specimens showed a markedly higher amount of the reaction product lining the canalicular and basolateral hepatocyte membranes in all zones of hepatic lobules. Only those parts of hepatocyte membrane directly adjacent to blood sinusoids displayed no enzymatic activity (Fig. 4-6f).

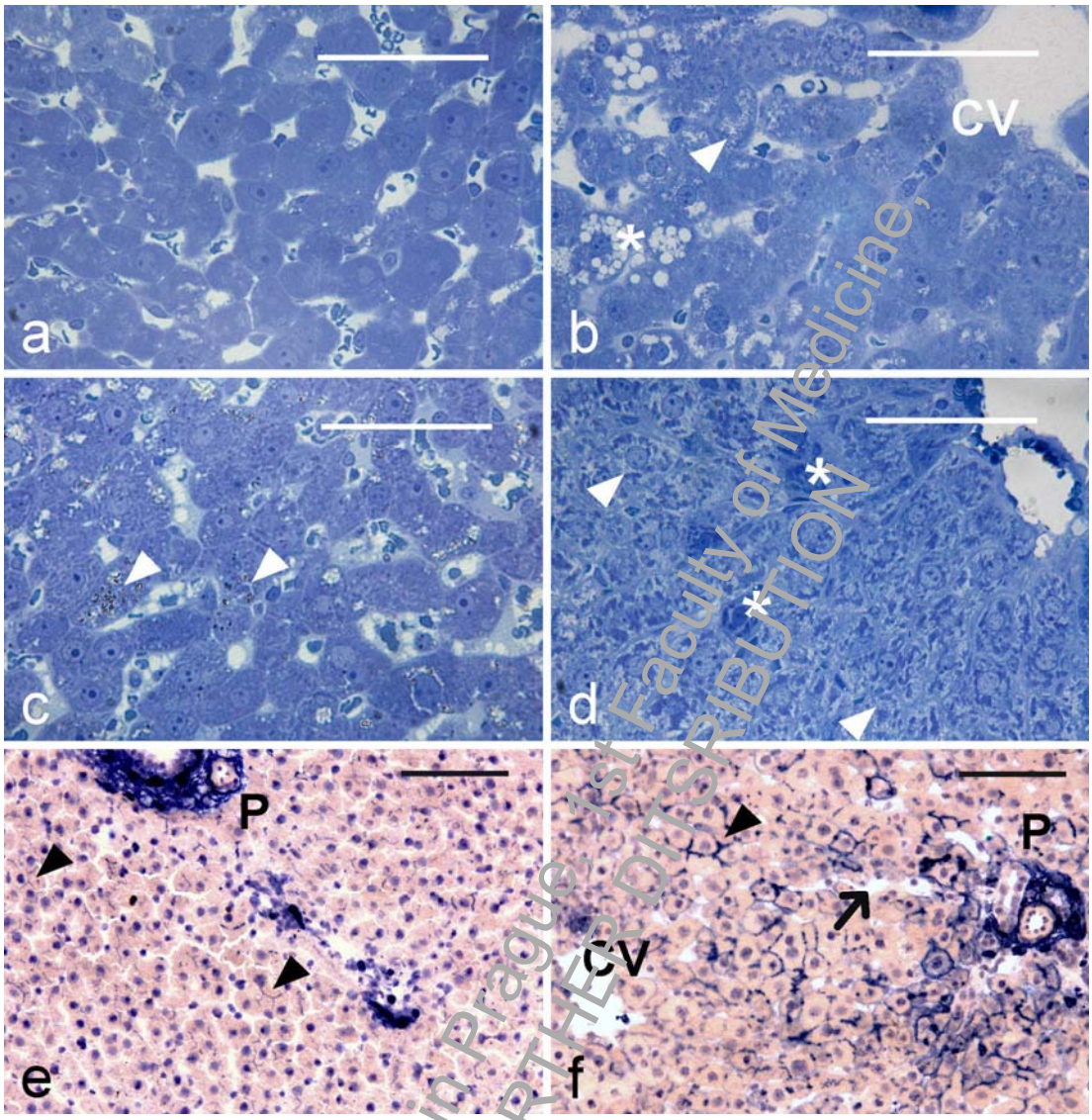

Fig. 4-6. Histopathological analysis of cholestatic rat livers. Light microscopy of the liver tissue of control and EE-treated animals. **(**a) Normal liver morphology, (b, c, d) EE-treated animals stained with toluidine blue. Pathological changes of hepatocytes were classified as follows: (b) steatosis (*) and some degree of feathery degeneration (arrowhead) in the central lobular zone (c) abundant intracellular localization of bile pigments (arrowheads), as revealed by light refraction. The pigment granules displayed no autofluorescence; (d) massive feathery degeneration (arrowheads) of hepatocytes and sparsely spread cells displaying some features of apoptosis and necrosis (*); (e, f) ALP histochemistry of liver tissue in EE-treated and control animals. (e) Control liver shows only mild ALP activity on the canalicular hepatocyte membrane at the periphery of hepatic lobule (arrowheads). (f) Except for the sinusoidal pole of hepatocyte membrane (arrow), the EE-treated liver displays ALP activity in canalicular and basolateral hepatocyte membranes in all zones of hepatic lobules (arrowhead); p = portal area, cv = central vein, bars = 100 μm.

4.6 GM1 histochemistry

In the liver specimens of all control animals, a small amount of reaction product of GM1 ganglioside histochemistry was distributed on both the sinusoidal and canalicular membranes except for the central lobular zone (zone III), where almost no reaction occurred (Fig. 4-7a). In all EE-treated animals, the GM1 ganglioside was nearly uniformly distributed in all lobular zones from the portal areas up to the central veins (Fig. 4-7b). Sinusoidal surfaces of hepatocytes were conspicuously lined with the reaction product, whereas the reaction on canalicular surfaces was rather weak.

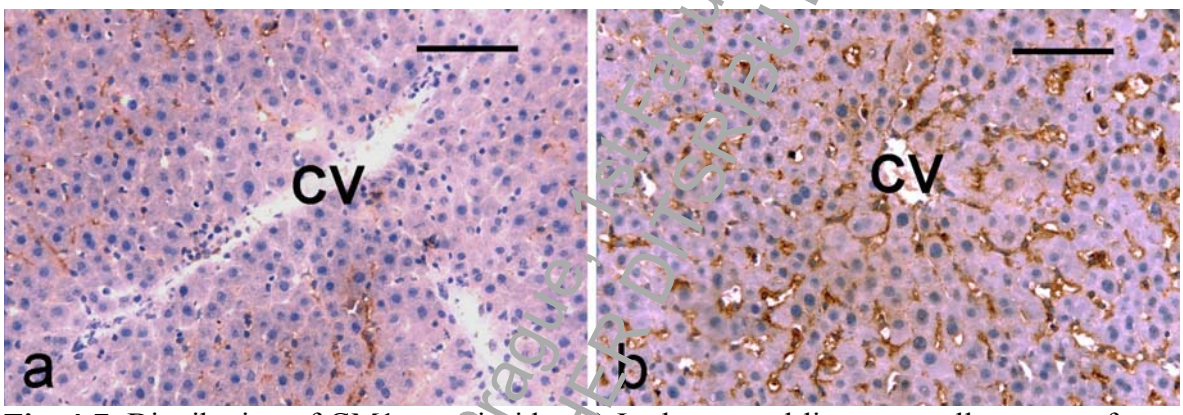

Fig. 4-7. Distribution of GM1 ganglioside. (a) In the control liver, a small amount of reaction product occurs in canalicular and sinusoidal hepatocyte membrane in peripheral and intermediate lobular zones (zone I and II) whereas the central zone is nearly free of it. (b) In the EE-treated liver, the reaction product is found in canalicular and sinusoidal hepatocyte membranes in all lobular zones; cv – central vein; bars = 100 μm.

No GM1 staining was observed in both control blank experiments in which either the GM1 was extracted or the cholera toxin was omitted in staining procedure.(Fig. 4-8).

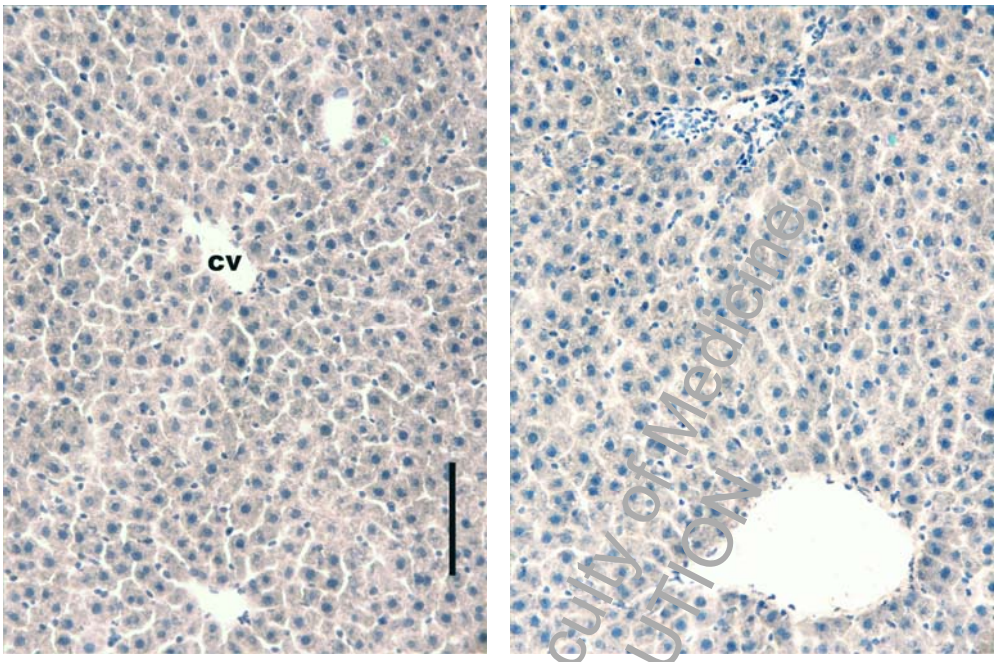

Fig. 4-8. Blanks for GM1 histochemistry: No cholera-toxin B-subunit conjugate (left) and after lipid extraction with chloroform + methanol (right). bars = 100 μm.

4.6.1 Quantiative study on the distribution of GM1 ganglioside in hepatic lobule

The distribution pattern of GM1 ganglioside in liver sections is demonstrated under low microscopic magnification (Fig. 4-10). The amount of GM1 ganglioside expressed as optical brightness of whole sections showed no significant difference between EE-treated and control groups (Table 4-9).

Table 4-9. Densitometric quantification of GM1 ganglioside in control and EE-treated livers.

	Controls (n = 7)	**EE-treated rats (n = 11)**	**p value**
Mean optical brightness measured in whole sections	0.61±0.04	0.61±0.04	0.923
Differences of optical brightness between lobular zones I and III	0.097 (0.079-0.107)	0.055 (0.034-0.058)	**0.0035**

This finding corresponds to the insignificant difference of GM1 concentration determined by biochemical analysis. In contrast to the results obtained in whole sections, the differences of the GM1 content between peripheral and central lobular zones were significantly higher in controls (Table 4-9). The linear regression analysis demonstrated that the higher degree of cholestasis, measured by TBA concentration, correlated well with the lower difference of the GM1 content between lobular zone I and III.

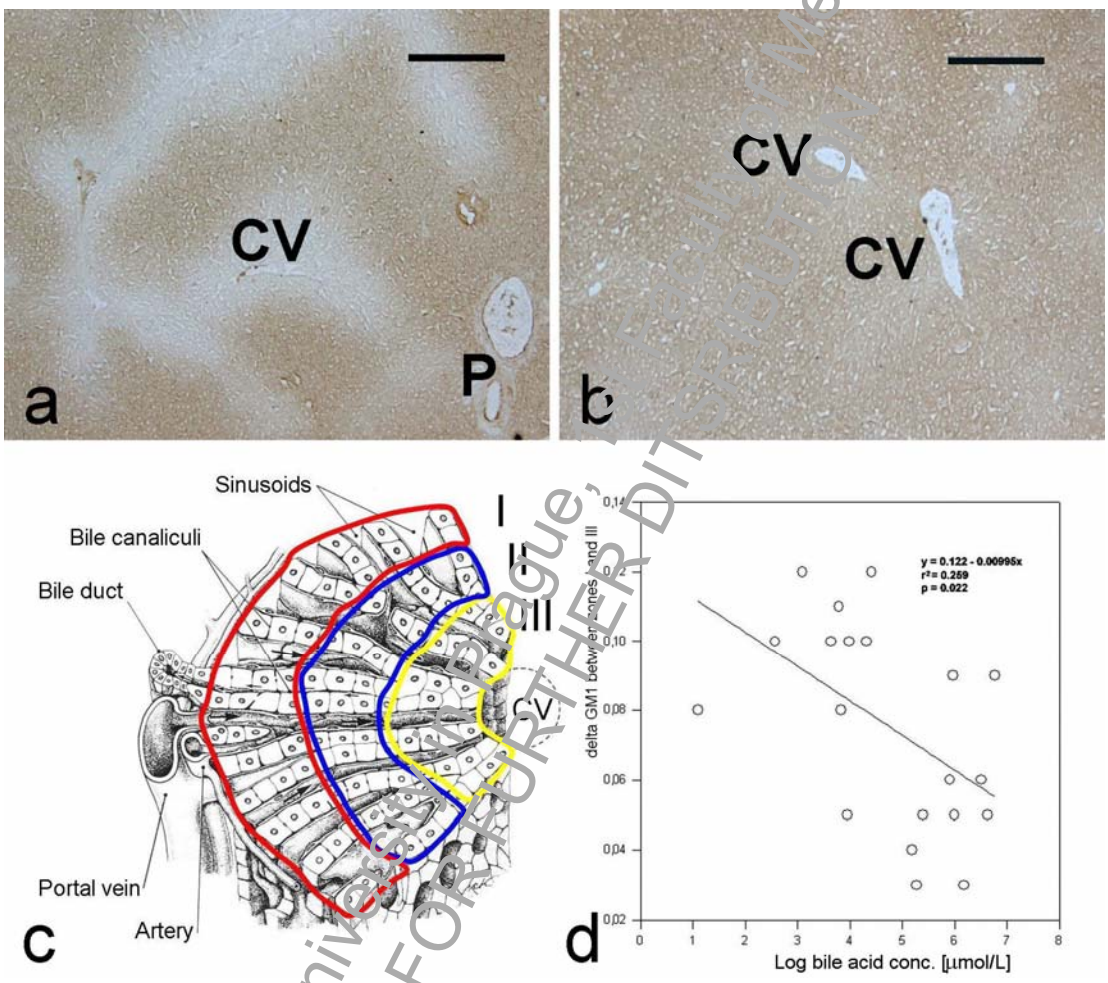

Fig. 4-10. Histochemical localization of GM1 ganglioside in rat liver as seen at low magnification. (a) In control liver, the reaction product occurs in lobular zones I and II and is nearly or completely absent in zone III. (b) In EE-treated liver, the difference in the amount of reaction product between peripheral and central areas of the lobule is hardly observable. cv = central vein, p = portal area; bars = 500 µm. (c) Schematic representation of the liver lobule with marked areas of measurement. Red = peripheral zone (zone I), blue = intermediate zone (zone II), yellow = central zone (zone III). d) Linear regression analysis between serum TBA concentration and the difference in GM1 ganglioside content between lobular zones I and III.

4.6.2 Comparison of the amount of GM1 ganglioside in areas of sinusoidal membrane and of adjacent cytoplasm

The effect of EE on localization of GM1 was analyzed under high magnification. The results are summarized in Table 4-11.

Differences in GM1 staining between samples obtained from untreated and PD-treated animals

A possible influence of PD on GM1 staining was investigated by comparing the PD-treated animals and those without treatment. A slight increase of sinusoidal GM1 staining in zone III was observed (Table 3a; p=0.02).

Table 4-11. Results of densitometric quantification of GM1 ganglioside in areas of sinusoidal surface (sin) and of adjacent cytoplasm (cyt). C = untreated controls.

				Mean ± SD	Mean ± SD	p value
a	zone III sin PD	vs.	zone III sin C	0.184 ± 0.012	0.164 ± 0.015	**0.02**
b	zone I sin PD	vs.	zone I sin C	0.283 ± 0.019	0.296 ± 0.036	0.57
c	zone III cyt PD	vs.	zone III cyt C	0.141 ± 0.013	0.135 ± 0.012	0.47
d	zone I cyt PD	vs.	zone I cyt C	0.189 ± 0.016	0.200 ± 0.019	0.30
e	zone I sin C	vs.	zone III sin C	0.296 ± 0.036	0.164 ± 0.013	**0.003**
f	zone III sin EE	vs.	zone III sin PD	0.228 ± 0.023	0.184 ± 0.012	**0.0002**
g	zone III cyt EE	vs.	zone III cyt PD	0.138 ± 0.020	0.141 ± 0.014	0.75
h	zone III sin/cyt EE	vs.	zone III sin/cyt PD	1.675 ± 0.144	1.313 ± 0.108	**0.00002**
i	zone I sin EE	vs.	zone I sin PD	0.264 ± 0.028	0.283 ± 0.020	0.13
j	zone I cyt EE	vs.	zone I cyt PD	0.149 ± 0.023	0.189 ± 0.017	**0.0008**
k	zone I sin/cyt EE	vs.	zone I sin/cyt PD	1.803 ± 0.204	1.511 ± 0.074	**0.002**
l	zone I sin EE	vs.	zone III sin EE	0.264 ± 0.028	0.228 ± 0.023	**0.007**
m	zone I sin PD	vs.	zone III sin PD	0.283 ± 0.019	0.184 ± 0.011	**0.0002**

For this reason, PD-treated animals were used as controls in the study of EE influence on GM1 staining. Significant changes were found neither in sinusoidal GM1 staining in zone I (Table 4-11b, p=0.57) nor in cytoplasm of both zones I and III (Table 4-11c, d). It should be noted that in untreated controls, significantly higher GM1 staining on sinusoidal surfaces was found in zone I compared to zone III (Table 4-11e, p=0.03).

The effect of EE-treatment on GM1 staining in areas of sinusoidal surface of central lobular zone III

Data in Table 4-11f show a significant increase of GM1 centrolobular sinusoidal surface areas of EE-treated rats when compared with those treated with PD (p=0.0002). No significant difference in GM1 staining was found between EE- and PD-treated animals in cytoplasmic areas in zone III (Table 4-11g). Therefore, the increase of sin/cyt ratio seems to be due to increased sinusoidal staining in EEtreated animals (Table 4-11h, p=0.00002) at a stable level of staining in sub-sinusoidal areas of cytoplasm. Linear regression analysis confirmed that widening difference between sin and cyt staining correlated well (p=0.00001) with higher degree of cholestasis as indicated by higher serum TBA concentration (see Fig. 4-14d).

The effect of EE-treatment on GM1 staining in peripheral lobular zone I

Data presented in Table 4-11i show no significant difference in GM1 staining in sinusoidal surfaces areas in zone I between EE- and PD-treated animals (p=0.12). In contrast, the decrease of GM1 staining in the adjacent cytoplasmic area was found highly significant (Table 4-11j, p=0.0008). Consequently, the difference in ratios sin/cyt EE to sin/cyt PD is also highly significant (Table 4-11k, p=0.002). Examples of staining in cytoplasm of hepatocytes in periportal zone PD and EE-treated livers are shown in Fig. 4-13b and d.

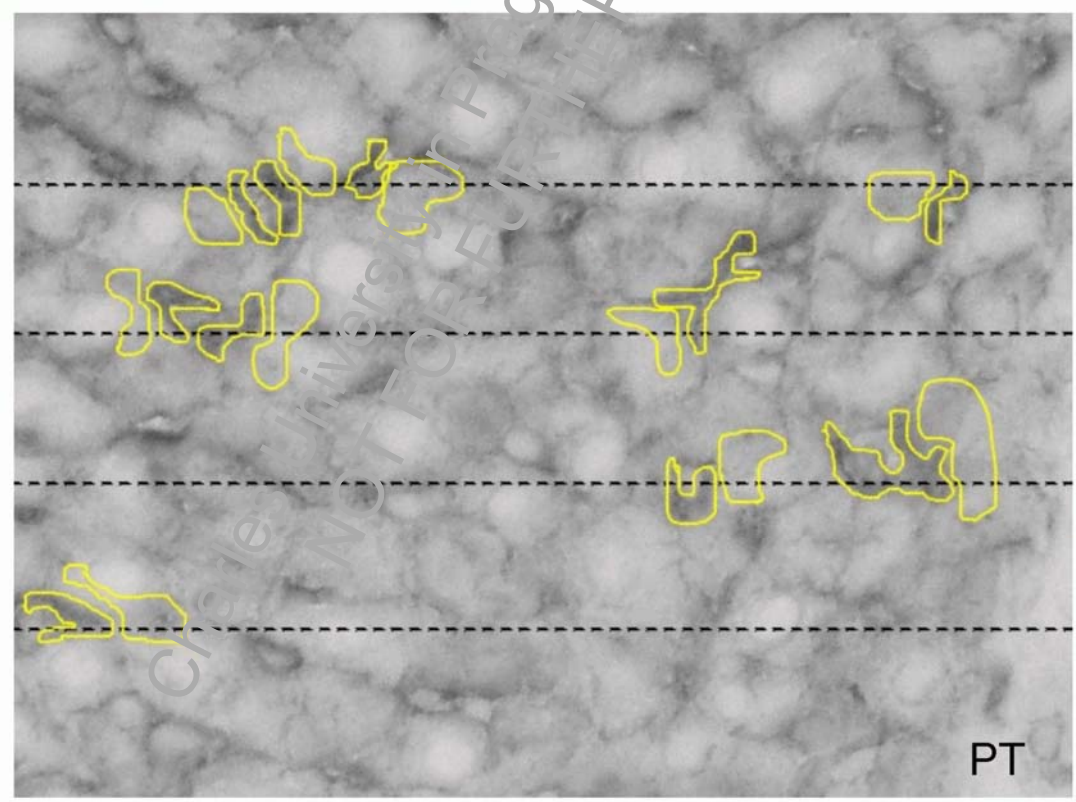

Fig. 4-12. Image analysis of GM1 in rat liver examined at high magnification (40x, NA=0.7). The demonstration of selection of areas in individual measuring frame. Usually several

measuring frames are necessary in each field to select 15 areas of sinusoidal membranes and 15 sub-sinusoidal areas of cytoplasm by stratified random sampling method in central zone III and the same was done for neighboring peripheral areas.

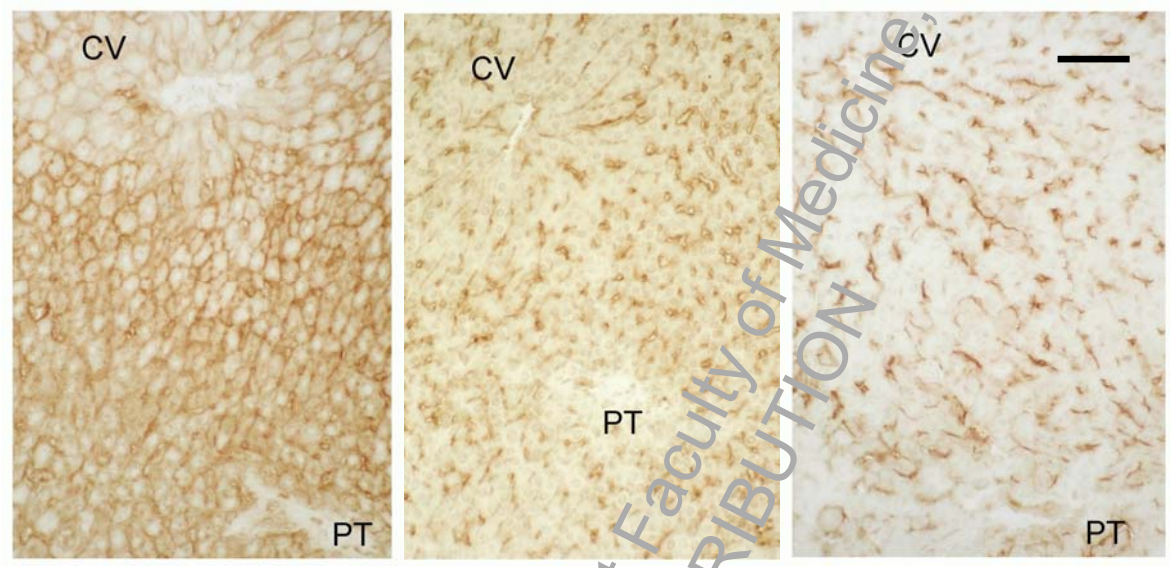

Fig. 4-13. (left) GM1 staining in cytoplasm of PD treated rats decreased rapidly in cytoplasm of EE treated rats (middle and right) especially of peripheral zone I. Serum concentration of TBA were 46, 366 and 868 μmol/l. Bar = 50 μm. Objective magnification in photography 20x (NA=0.5). CV = central vein. PT = portal area.

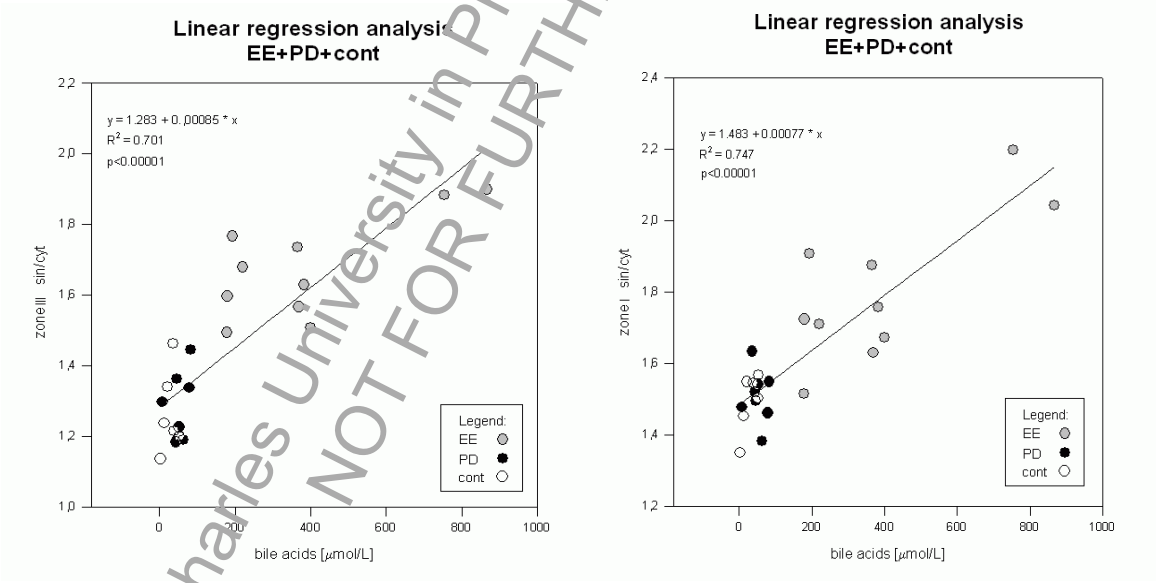

Fig. 4-14. (left) Linear regression analysis between serum TBA concentration and difference in GM1 ganglioside content between sinusoidal membrane and subsinusoidal area of cytoplasm in **zone III**. (right) Linear regression analysis between serum TBA concentration and

difference in GM1 ganglioside content between sinusoidal membrane and subsinusoidal area of cytoplasm in **zone I** of hepatic lobules.

Linear regression analysis confirmed that higher degree of cholestasis expressed as TBA concentration correlate with increasing difference between sinecyt staining (Fig. 4-14e) in zone I. Finally, an increase of sinusoidal GM1 was found between zone III and zone I in both EE-treated (Table 4-11k, p=0.007) and PD-treated rats (Table 4-11m, p=0.0002).

4.7 Analysis of gangliosides and expression levels of mRNA of GM1-synthase and GD3-synthase

To clarify the biochemical basis of the observed differences between EE- and PD-treated animals we further analyzed the content of gangliosides in whole liver. Data presented in Fig. 4-15a shows a general scheme of ganglioside biosynthesis, consisting of 0, a, b and c branch.

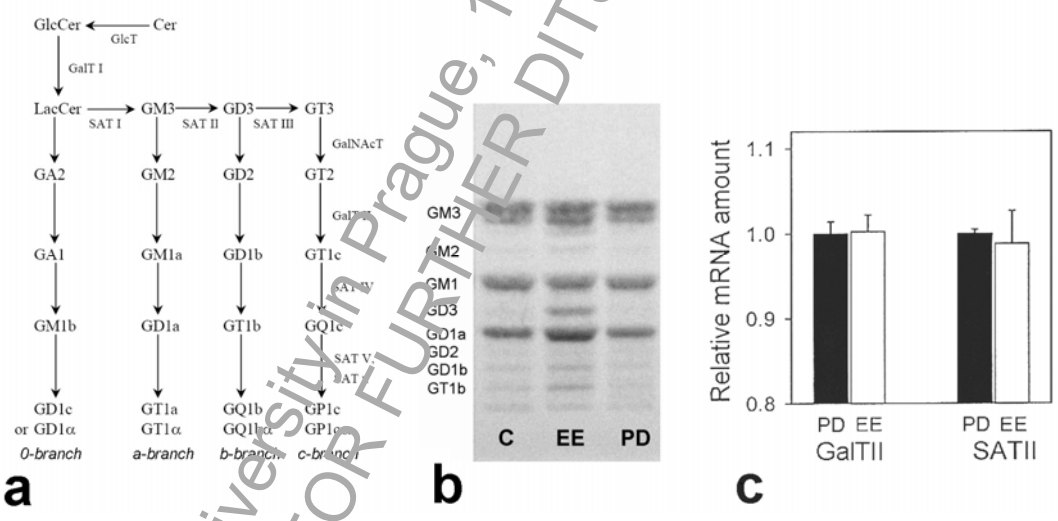

Fig. 4-15. (a) Scheme of ganglioside biosynthesis with branches *0, a, b* and *c*. Abbreviations of enzymes catalyzing the indicated reactions: SAT I, II, and III - sialyl(NeuAc)-transferases I, II, and III; GalTII - galactosyl(Gal)-transferase; GalNAcT - *N*-acetylgalactosaminyl (GalNAc)-transferase; SAT IV, V, and X - sialyl(NeuAc)-transferase IV, V and X. (b) TLC of gangliosides in untreated control (C), EE-treated (EE) and propanediol-treated (PD) rat liver shows an increase in *b*-branch gangliosides and GD1a *a* branch , but GM1 is not significantly increased. (c) Real-time PCR analysis of GalTII and SATII mRNA in liver cells. Total RNA was isolated from liver cells of control and EE-treated animals and subjected to real-time-PCR

using primers for actin, GalTII and SATII. Results shown are representative of three independent experiments performed in triplicates.

TLC shows that in a-branch of gangliosides, there is no significant difference in the amount of GM1 ganglioside and a slight increase in the amount of GD1a in EE-treated animals compared to PD-treated and untreated animals. There is a significant increase in GD3 and b-branch gangliosides. To determine whether there are any differences in the expression of enzymes involved in synthesis of GM1 or b-branch gangliosides, expression levels of mRNA for GalTII (galactosyl(Gal)-transferase II, GM1-synthase) and SATII (sialyl(NeuAc)-transferase II , GD3-synthase) were estimated using real-time PCR. Data presented in Fig. 4-15c reveal that there are no differences in the expression levels of GalTII mRNA and SATII mRNA between liver cells isolated from control or EE-treated animals.

5 DISCUSSION

Our study on changes of ganglioside content in the rat liver in Biomedical chromatography (Majer, Trnka et al. 2007) is the first report on changes of the ganglioside pattern in rat liver in EE-induced cholestasis, an experimental model of cholestasis of pregnancy and oral contraceptive-induced. The control Wister rats, used in the experiment, showed significant changes in liver ganglioside content. The total lipid sialic acid was almost twice as high as compared to control rats (p=0.002). The TLC analysis of GM1 ganglioside in rat livers showed only slight insignificant increase in EE-treated rats when compared with controls in total liver extract GM1 is not enhanced in EE-treated animals. However, gangliosides of b-branch of biosynthesis GD3, GD1b and GT1b are increased significantly (Fig.4-2) when in control livers high predominance of a-pathway gangliosides occurred. To conclude changes in liver ganglioside content, in cholestatic livers, we detected significant increases of concentrations of total lipid sialic acid, a-biosynthetic pathway ganglioside GD1a, and particularly the b-pathway gangliosides (GD3, GD1b and GT1b). These changes in ganglioside content and composition were much more highly expressed in this model of cholestasis compared with the bile duct ligation-induced cholestasis, published by Senn (Senn, Geiser et al. 1991). The observations in changes of ganglioside content are consistent with the results reported by Mesaric and Decker (Mesaric and Decker 1990), who studied the influence of sex steroid hormones on the activities of GM3 and GD3 synthases in isolated hepatocytes. Progesterone, β-estradiol and testosterone all activated GD3 (but not GM3) synthase when added directly to hepatocytes cultured in modified William's E medium.

The increase of the b-pathway in EE-treated liver might be due to GD3 synthase (sialyltransferase II) overexpression/activation, leading to the increased synthesis of GD3 from GM3. Other mechanisms, however, might also contribute. Senn et al. (Senn, Geiser et al. 1991) observed a marked increase of GD3 content in bile duct ligation-induced cholestasis, despite low hepatic activities of GD3 synthase. As the majority of gangliosides are concentrated in cell membranes, the histological findings suggest that b-series gangliosides might contribute to a cytoprotective resistance of hepatocytes, against the deleterious effects of bile acids intracellularly accumulated during cholestasis. This assumption was corroborated with data by Kasai et al. (Kasai, Kamimura et al. 1993) on LEC rats almost completely lacking b-series gangliosides. These rats regularly develop severe hepatic pathology, suggesting the importance

of b-series gangliosides for protection against various harmful factors. In addition, it was demonstrated that experimental cholestasis is commonly associated with the activation of NF-κB (Schoemaker, Gommans et al. 2003; Yang, Uchiyama et al. 2004). In the study of hepatic content of GD3 ganglioside was found to be substantially increased in cholestatic rats. It is capable of inactivation of NF-κB, indicating another possible explanation for ganglioside-mediated hepatocyte cytoprotection. In fact, GD3 has been demonstrated to selectively repress NF-κB dependent gene expression by preventing the translocation of active NF-κB complexes to the nuclei (Colell, Garcia-Ruiz et al. 2001), and thus regulating substantially cell apoptosis. The dramatic increase of b-series gangliosides might contribute to the protection of hepatocytes from the deleterious effects of cholestasis.

Several approaches may be used to study GSL in cholestatic liver membranes. Biochemical approach may be appropriate for analysis of GSL in membranes isolated by gradient ultracentrifugation; however, it is difficult to obtain canalicular and basolateral membrane fractions in sufficient quantity and purity. Considering that ALP activity serves as a marker of purity of canalicular membrane fraction isolated by gradient ultracentrifugation, the changes in ALP activity localization observed in EE-administered rats question the possibility to achieve a satisfactory and reliable purity of membrane fractions.

Unlike in normal rat liver, where weak ALP activity was restricted just to the canalicular hepatocyte membrane of peripheral lobular zone, the ALP activity in EE-administered animals was spread over the whole hepatocyte cell membrane except the sinusoidal part in all lobular zones. These results are in agreement with the study showing that the ALP activity in EE-treated rats extends to the central lobular zone (Arrese, Pizarro et al. 1995), whereas a weak activity is confined only to the canalicular hepatocyte membrane at the periphery of the lobule in normal rat (Koudstaal, Runsink et al. 1975). The spreading of ALP from canalicular to lateral membranes explains why ALP is an unreliable marker of canalicular membranes in their isolation from cholestatic livers.

Due to the above mentioned problems in isolation of membranes by ultracentifugation, selective histochemical approach based on specific binding of cholera toxin B-subunit to GM1 was used in the histological study. The specificity of this binding has been carefully tested on several occasions. Wu and Ledeen (Wu and Ledeen 1988) confirmed that, among different gangliosides, only GM1 binds cholera toxin B-subunit with very high affinity. In another study, Parton (Parton 1994) demonstrated that the only components capable of binding cholera toxin B-subunit are glycolipids and not glycoproteins. The histological study is the first *in vivo*

study demonstrating the localization of gangliosides in normal and cholestatic liver. The findings in normal rat liver tissue clearly detects GM1 localized on both the canalicular and sinusoidal surfaces in lobular zones I and II, and its nearly complete absence in liver parenchyma nearby the central vein. In the liver tissue after EE treatment, where the product of histochemical reaction was typically found in all lobular zones, an apparent change of GM1 ganglioside distribution in hepatic lobule was observed. Differences in optical brightness in separate lobular zones III and I, also supported by linear regression analysis, confirmed link between GM1 ganglioside redistribution and cholestasis (Fig. 4-10). These findings might be associated with lobular blood flow and liver zonation.

As blood flows in liver sinusoids from the periphery to the center of the lobule, numerous substances including bile acids are eliminated by hepatocytes. Considering the well known fact that the higher presence of GSL increases membrane rigidity, we conclude that the shift of GM1 ganglioside localized in the central zone of the liver lobule of EE-treated animals can be a response of the hepatocytes to the harmful effects of bile acids accumulated in sinusoidal blood during cholestasis.

The data on mRNA analysis of GalTII are in accordance with biochemical analysis of ganglioside content by TLC. The different distribution of GM1 gangliosides in zones I and III in the EE-treated animals, compared to control samples, and the same level (content) of GM1 and mRNA for GalTII (GM1-synthase) in both groups suggest that not higher biosynthesis in the whole liver but rather locally confined differences in biosynthesis in the central zone might be responsible for the observed differences in increased GM1 expression in sinusoidal surface of central zone in EE-treated animals. It should also be noted that the increase in GD3, observed by TLC does not seem to be due to an increased expression of SATII, as can be inferred from the results of RT-PCR analysis. Further studies, e.g. determination of sialyltransferase activity, are necessary for explanation of the increase in b-branch gangliosides. Detailed densitometry at high magnification of the sinusoidal membrane in peripheral zone I did not show any significant increase of GM1 staining in EE-treated animals when compared with those treated with PD or those with no treatment (see data in Table 4-11b and i). These data suggest that GM1 concentration in sinusoidal membrane in peripheral lobular zone is present to a limited extent even in control animals. It thus seems that hepatocytes in peripheral zone I are attacked by harmful molecules even in untreated animals. Very surprising is observation of decreased GM1 staining in subsinusoidal area of peripheral zone cytoplasm. One possible explanation might be that the GM1 is rapidly transported by endosomes from cytoplasm into membranes at limited GM1 biosynthesis. This conclusion is supported by TLC

densitometry data (Majer, Trnka et al. 2007) and the results of RT-PCR. The two main findings of densitometric analysis under high magnification, (a) namely the increase of sin/cyt ratio due to increased staining of sinusoidal surface in zone III, and (b) the increase of sin/cyt ratio in zone I due to a decrease in GM1 staining in cytoplasm, correspond with the increased TBA blood concentration in EE-treated animals. According to previous reports, a shift in lipid content occurs within hepatocyte membranes during cholestasis contributing to the modulation of membrane fluidity (Smith and Gordon 1988; Vu, Tuchweber et al. 1992). The results of this study prove that GSL redistribution might be responsible for these changes. This conclusion is also corroborated by the known fact that ganglioside molecules are more rigid due to the conformation of ceramide and oligosaccharide parts of the molecule. The ceramide portion adopts a rigid conformation with two closely packed, parallel hydrocarbon chains (Pascher 1976; Pascher, Lundmark et al. 1992), while the oligosaccharide portion is conformationally dependent on the high stability of sialic acid (Czarniecki and Thornton 1977) and some other factors (Tettamanti, Masserini et al. 1988). It is well established that the presence of gangliosides reduces the membrane fluidity (Bertoli, Masserini et al. 1981). The study on histological changes documents for the first time that GM1 ganglioside appears in enhanced levels in sinusoidal membranes of hepatocytes during cholestasis. It further demonstrates that microscopic methods remain an important analytical tool, especially in those situations where biochemical and genetical approaches reach their limits.

6 CONCLUSIONS – SUMMARY

The thesis focuses on changes in ganglioside content and localization in the rat liver in 17α-ehtinylestradiol induced cholestasis. In this study we analyzed ganglioside content in the liver homogenate by TLC. We aimed to undertake changes in localization of gangliosides in cholestatic rat liver with ChtB as a specific probe.

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6.1 Changes in ganglioside composition in 17α-ehtinylestradiol induced cholestasis.

(1) The total amount of gangliosides was found higher in cholestatic livers as evidenced by increase in total lipid sialic acid.
(2) The distribution of gangliosides of b-series significantly increased in cholestatic livers.
(3) There are also changes in amount of GQ1a and GM1 ganglioside when compared to controls.

6.2 Changes in localization of gangliosides.

(1) Zonation of GM1 in the liver acinus has not been described so far.
(2) We observed significant GM1 positivity in sinusoids of the liver lobule.
(3) Even thought we did not manage to reveal the changes in sinusoidal surface in detail.

Thought we did not exactly identify exact localization of gangliosides at the cellular level, this study brought new findings regarding GM1 histochemistry. Association of changes in localization of liver gangliosides requires further investigation to testify protective hypothesis.

7 ABBREVIATIONS

ALP	alkaline phosphatase
BSA	bovine serum albumin
EE	17α-ethinylestradiol
PD	1,2-propanediol
GSL	glycosphingolipids
PBS	phosphate buffered saline
TBA	total bile acids
DEAE	diethylaminoethyl
EDTA	ethylendiamintetraacetic acid
GSL	glycosphingolipides
HPLC	high performance liquid chromatography
HPTLC	high performance thin layer chromatography
Cer	ceramide
ChtB	choleratoxin B-subunit

Ganglioside symbols, according to Svennerholm (Svennerholm 1963; Svennerholm 1994), with IUPAC-IUB (1977) nomenclature in parentheses

GM3	(II^3NeuAc-LacCer)
GM2	(II^3NeuAc-$GgOse_3$Cer)
GM1	(II^3NeuAc-$GgOse_4$Cer)
GD3	($II^3(NeuAc)_2$-Lac-Cer)
GD2	($II^3(NeuAc)_2$-$GgOse_3$Cer)
GD1a	(IV^3NeuAc,II^3NeuAc$GgOse_4$Cer)
GD1b	($II^3(NeuAc)_2$-$GgOse_4$Cer)
GT1b	(IV^3NeuAc,$II^3(NeuAc)_2$-$GgOse_4$Cer)

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9 LIST OF PUBLICATIONS AND ABSTRACTS

9.1.1 Publications on the topics of the doctoral thesis

Changes in GM1 ganglioside content and localisation in cholestatic rat liver
Jirkovská Marie, Majer Filip, Šmídová Jaroslava, Stříteský Jan, Shaik Gouse M, Dráber Petr, Vítek Libor, Mareček Zdenek, Šmíd František
Glycoconjugate Journal, Vol 24, Issue 4-5, Jul 2007, 231-41. Epub 2007 Feb 27

Estrogen–induced cholestasis results in a dramatic increase of b-series gangliosides in the rat liver
Majer Filip, Trnka Ladislav, Vítek Libor, Jirkovská Marie, Mareček Zdeněk, Šmíd František
Biomedical Chromatography, Vol 21, Issue 5, May 2007, 446-50.

9.1.2 Other publications

Structure-based specificity mapping of secreted aspartic proteases of *Candida parapsilosis, Candida albicans,* and *Candida tropicalis* using peptidomimetic inhibitors and homology modeling
Majer Filip, Pavlíčková Libuše, Majer Pavel, Hradilek Martin, Dolejší Elena, Hrušková-Heidingsfeldová Olga, Pichová Iva
Biological Chemistry, Vol 387, Issue 9, Sept 2006, 1247-1254.

Identification of bilirubin reduction products formed by *Clostridium perfringens* isolated from human neonatal fecal flora
Vítek Libor, Majer Filip, Muchová Lucie, Zelenka Jaroslav, Jirásková Alena, Branný Pavel, Malina Jiří, Karel Ubik
Journal of Chromatography B, Vol 833, Issue 2, 3 Apr 2006, 149-157.

Photodynamic therapy of non-melanoma skin cancer with topical Hypericum perforatum extract: A pilot study
Kacerovská Denisa, Pizinger Karel, Majer Filip, Šmíd František
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Two aspartic proteinases secreted by the pathogenic yeast Candida parapsilosis differ in expression pattern and catalytic properties
Hrušková-Heidingsfeldová Olga, Dostál Jiří, Majer Filip, Havlíková Jana, Hradilek Martin, Pichová Iva
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9.2 Abstracts on the topics of the doctoral thesis

14th United European Gastroenterology Week, Oct 21-25, 2006, Berlin, Germany
Changes in the spektrum of gangliosides in the hepatocyte sinusoidal membrane in experimental cholestatis.
Mareček Zdeněk, Jirkovská Marie, Šmídová Jaroslava, Marečková Olga, Majer Filip, Trnka Ladislav, Vítek Libor, Šmíd František
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Morphology 2006, 43rd International Congress on Anatomy, 43rd Lojda Symposium on Histochemistry, Sept 3-6, 2006, Prague, Czech Republic,
The content and localization of GM1 ganglioside in normal and cholestatic rat liver.
Jirkovská Marie, Šmídová Jaroslava, Šmíd František, Majer Filip, Vítek Libor, Mareček Zdeněk

31st FEBS Congress, June 24-29, 2006, Istanbul, Turkey
Estrogen induced cholestasis leads to increase of b-series gangliosides and a redistibution of GM1 in the rat liver
Majer Filip, Jirkovská Marie, Vítek Libor, Mareček Zdeněk, Šmíd František
The FEBS Journal, Vol 273, Suppl 1, June 2006, page 264

ESCI 2006. 40th Annual Scientific Meeting of the European Society for Clinical Investigation March 15 – 18, 2006, Prague, Czech Republic
Localization of GMI ganglioside in hepatocyte membrane of normal liver and in experimental cholestasis
Jirkovska Marie, Šmídová Jaroslava, Majer Filip, Vítek Libor, Mareček Zdeněk, Šmíd František
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Mareček Zdeněk, Jirkovská Marie, Šmídová Jaroslava, Marečková Olga, Majer Filip, Trnka Ladislav, Vítek Libor, Šmíd František
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Šmíd František, Jirkovská Marie, Vítek Libor, Šmídová Jaroslava, Majer Filip, Mareček Zdeněk
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XXXIII. Májové hepatologické dny, May 4-6, 2005, Karlovy Vary, Czech Republic
1) The change of ganglioside spectrum in the rat liver in ethinylestradiol-induced cholestasis.
Majer Filip, Trnka Ladislav, Šmíd František, Jirkovská Marie, Vítek Libor, Mareček Zdeněk
2) Increased GM1 ganglioside content in the hepatocyte's sinusoidal membrane in experimental cholestasis
Jirkovská Marie, Majer Filip, Šmídová Jaroslava, Vítek Libor, Mareček Zdeněk, Šmíd František
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