1

Introduction to the Physiology, Immunology and Pathology of the Liver and Biliary Tree

Marco Carbone[1] *and Mario Strazzabosco*[2]

[1] *Division of Gastroenterology, University of Milan Bicocca, Milan, Italy*
[2] *Digestive Disease Section, Yale Liver Center, Yale University School of Medicine, New Haven, CT, USA*

Abstract

The liver has many functions including metabolic homeostasis, disposal of endotoxins and xenotoxins, metabolism of bilirubin and urea, and bile formation and secretion. The process of bile formation depends on the liver synthesis and the canalicular secretion of bile acids. Besides their roles in dietary lipid absorption and cholesterol homeostasis, bile acids also play a key role as signaling molecules in the regulation of hepatic metabolism and energy homeostasis. The regenerative ability of the liver lies in the multiple niches of biliary tree stem cells.

Keywords *bile formation; carbohydrate metabolism; hepatocyte; cholangiocytes lipid metabolism; metabolic zonation; protein metabolism; bile acid.*

Key Points

- The liver is largely composed of hepatocytes and biliary epithelial cells or cholangiocytes; both hepatocytes and intrahepatic cholangiocytes differentiate from bipotent liver progenitors, the hepatoblasts.
- The liver has many functions, among which metabolic homeostasis, disposal of endotoxins and xenotoxins, metabolism of bilirubin and urea, and bile formation and secretion are just examples. The liver also produces fundamental circulating proteins and clotting factors and hormones. In addition, the liver receives and processes the blood coming from the intestine and has a fundamental role in innate immunity.
- Besides their roles in dietary lipid absorption and cholesterol homeostasis, bile acids (BAs) also play a key role as signaling molecules in the regulation of hepatic metabolism and energy homeostasis.
- BAs also have hormonal signaling functions and interact with dedicated receptors such as the nuclear receptor farnesoid X receptor and G protein-coupled receptors, which regulate BA homeostasis and BA-induced injury and/or inflammation.
- Multiple niches of biliary tree stem/progenitor cells reside in different locations along the human biliary tree and within the

Autoimmune Liver Disease: Management and Clinical Practice, First Edition. Edited by James Neuberger and Gideon M. Hirschfield.

liver parenchyma and have a key role in regeneration of the liver.
- Cholangiocytes modify the primary bile by secretion of chloride and bicarbonate fluid. This is a major protective mechanism for the biliary tree.
- Cholangiocytes, a barrier and secretory epithelium in normal conditions, activate and/or proliferate following a liver insult and give rise to the ductular reaction, a major driver of the progression of hepatic fibrosis.
- Cholangiocytes also contribute to the immune response through antigen presentation to immune cells, being a target of immune-mediated aggression or being the initiators of an inflammatory reaction that then progresses to adaptive immune activation.

Liver Cell Types and Organization

Liver cells can be classified into the following groups:

- parenchymal cells, which include hepatocytes and biliary epithelial cells (BECs);
- sinusoidal cells, which include hepatic sinusoidal endothelial cells and Kupffer cells; and
- perisinusoidal cells, which include the hepatic stellate cells (HSCs) and the pit cells [1].

The *hepatocytes*, which comprise approximately 60% of the liver cell mass, are epithelial cells with two distinct domains on their plasma membrane: (i) the sinusoidal (or basolateral) surface, facing the sinusoids, in contact with plasma through the fenestrated endothelium of the sinusoids, which allows a bidirectional flow of liquids and solutes though the space of Disse; and (ii) the canalicular (or apical) surface, which encloses the bile ductules and represents the beginning of the biliary drainage system.

The *BECs* (or *cholangiocytes)* are the epithelial cells lining the biliary tree. The biliary epithelium shows a morphologic heterogeneity that is associated with a variety of functions performed at the different levels of the biliary tree. Other than funneling bile into the intestine, BECs are actively involved in bile production by performing both absorptive and secretory functions via various membrane transport mechanisms including channels (e.g. water channels and chloride channels), transporters (e.g. ileal BA transporter), and exchangers (e.g. $Cl^-/HCO3^-$ or Na^+/H^+ exchangers). The large cholangiocytes are located at the level of interlobular and major bile ducts and they express several different ion channels and transporters at the basolateral or apical domain; they are believed to be mostly involved in secretin/cyclic AMP-regulated bile secretion. Smaller bile duct branches include terminal cholangioles and ductules or canals of Hering; the latter is a channel located at the ductular–hepatocellular junction, lined partly by hepatocytes and partly by cholangiocytes, and represents the physiologic link between the biliary tree and the hepatocyte canalicular system extended within the lobule. Their secretory function is believed to be mostly regulated by intracellular calcium. More recently, other important biological properties restricted to cholangiocytes lining the smaller bile ducts have been reported, especially with regard to their plasticity (the ability to undergo limited phenotypic changes), reactivity (the ability to participate in the inflammatory reaction to liver damage), and ability to behave as liver progenitor cells.

The *hepatic sinusoidal endothelial cells* (*HSECs*) represent 20% of the total liver cells. Unlike capillary endothelial cells, HSECs do not form intracellular junctions and simply overlap one another. They can secrete prostaglandins and cytokines [2]. They are also responsible for maintaining a cell niche that favors the quiescence of HSCs.

The *Kupffer cells* are specialized tissue macrophages and account for up to 90% of

the total population of fixed macrophages in the body. These are macrophages attached to the endothelial lining of the sinusoid, in greater numbers in the periportal areas. They are responsible for removing old and damaged blood cells or cellular debris, bacteria, viruses, parasites, and tumor cells.

The *HSCs*, also known as Ito cells or lipocytes, are mesenchymal cells and represent the major storage site of retinoids in healthy livers. They lie within the subendothelial space, and their long cytoplasmic extensions have close contact with parenchymal cells and sinusoids, where they may regulate blood flow and hence influence portal hypertension. HSC activation is the central event in hepatic fibrosis. During hepatocyte injury, HSCs proliferate, migrate to zone three of the acinus, change to a myofibroblast-like phenotype, and produce collagen and laminin.

Finally, the *pit cells* represent the natural killer (NK) cells of the liver and are located within the sinusoids. Pit cells show spontaneous cytotoxicity against tumor- and virus-infected hepatocytes.

Hepatic Metabolism

The liver is the site of many metabolic pathways. It stores and makes available many nutrients as energy sources. In turns, the metabolic function of the liver is regulated by hormones secreted by the pancreas, adrenal gland, and thyroid.

Bilirubin Metabolism and Transport

Bilirubin is an end-product of heme catabolism. Two enzymes are involved in bilirubin formation: the microsomal heme oxygenase converts heme to biliverdin and a cytosolic reductase subsequently reduces biliverdin to bilirubin.

The majority (up to 85%) of heme is derived from hemoglobin and only a small fraction from other heme-containing proteins such as cytochrome P450, myoglobin and immature bone marrow cells. Bilirubin formed in the monocytic–macrophage cell system of liver, spleen and bone marrow and some of the bilirubin formed in the hepatocytes from hepatic heme are released into plasma where bilirubin is bound to albumin at high-affinity binding sites. In normal conditions, only a minimal amount of bilirubin is unbound in plasma. An increase in free bilirubin would allow the pigment to enter tissues where it can have toxic effects; this is what is observed in neonates with defective conjugation and in Crigler–Najjar syndrome, when diffusion of unbound bilirubin into the brain can cause kernicterus.

In normal conditions, bilirubin is efficiently taken up by the liver whereas the albumin remains in plasma. In the liver, bilirubin is bound initially to glutathione-*S*-transferase, then glucuronidated and excreted into bile. Microsomal bilirubin uridine diphosphate glucuronosyltransferase (UGT) is the enzyme that converts unconjugated bilirubin to conjugated bilirubin monoglucuronide and diglucuronide. Biliary excretion of the glucuronide is mediated by the adenosine triphosphate (ATP)-dependent multidrug resistance protein (MRP)-2 and this is the rate-limiting factor in the transport of bilirubin from plasma to bile. Bilirubin diglucuronide is not reabsorbed from the small intestine; in the colon it may be hydrolyzed by bacterial β-glucuronidases, producing urobilinogens and urobilin, which are excreted in the stool or urine.

Carbohydrate Metabolism

The liver plays a key role in carbohydrate metabolism. It maintains carbohydrate stores by synthesizing glycogen and generating glucose from precursors such as lactate, pyruvate, and amino acids. Glycogen stored in the liver is the main source of rapidly available glucose for the whole organism. In the fed state, glycogen synthesis occurs preferentially in the perivenous hepatocytes whereas in the fasting state, glucose release via glycogenolysis

and gluconeogenesis initially occurs in periportal hepatocytes. The liver glycogen stores contain up to a 2-day supply of glucose, after which glucogenesis occurs mainly from lactate. In acute liver failure, the blood glucose level may drop whereas this is infrequent in chronic liver disease, where it is more common to observe hyperglycemia and insulin resistance. This may be related to decreased glucose uptake by muscle and reduced glycogen storage in the liver and muscle.

Lipid Metabolism

A major role of the liver in lipid metabolism is to synthesize large quantities of cholesterol and phospholipids, many of which are packaged with lipoproteins and made available to the rest of the body. Free cholesterol also derives from the uptake of chylomicron remnants and lipoproteins from the circulation. BAs are synthesized from free cholesterol, and both BA and cholesterol are secreted into bile. Bile provides a major route for cholesterol excretion.

The rate-limiting step of cholesterol synthesis is the conversion of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) to mevalonate by the enzyme HMG-CoA reductase, which is located almost exclusively in periportal cells. Synthesis is increased in biliary duct obstruction, terminal ileal resection, biliary or intestinal lymph fistula, and with medications such as colestyramine, corticosteroids and thyroid hormones. Cholesterol synthesis is inhibited by BAs, cholesterol feeding, fasting, and medications such as fibrates, nicotinic acid, and statins.

During chronic cholestasis, such as in primary biliary cholangitis and primary sclerosing cholangitis (PSC) but also in acute cholestasis, cholesterol serum levels are increased. This is mainly secondary to retention of cholesterol normally excreted in bile but also to increased hepatic synthesis of cholesterol, reduced plasma lecithin-cholesterol acyltransferase activity, and regurgitation of biliary lecithin, which produces a shift of cholesterol from pre-existing tissue cholesterol into the plasma. In patients with severe chronic cholestasis who are however malnourished, for example in premature ductopenic variant of primary biliary cholangitis (PBC) or in carcinomatous biliary obstruction, the serum cholesterol may be lower. Elevated cholesterol levels are clinically associated with skin xanthomas and xanthelasma. Hypercholesterolemia is not consistently associated with subclinical atherosclerosis in PBC, but should be treated if other risk factors for cardiovascular disease are also present.

In addition, the liver has other roles in lipid metabolism such as oxidizing triglycerides to produce energy; synthesizing lipoproteins; and converting excess carbohydrates and proteins into fatty acids and triglyceride, which are then exported and stored in adipose tissue.

Protein Metabolism

Amino acids are derived from the diet and from tissue breakdown and they reach the liver via the portal vein. Many critical aspects of protein metabolism occur in the liver, such as the deamination and transamination of amino acids, followed by conversion of the non-nitrogenous parts of these molecules to glucose or lipids. Several of the enzymes used in these pathways, e.g. alanine and aspartate aminotransferases, are commonly measured in serum to assess liver damage.

The liver is also responsible for a number of vital metabolic processes, including the removal of ammonia (an important factor in the development of hepatic encephalopathy) via the synthesis of urea; the synthesis of non-essential amino acids; and the synthesis of most plasma proteins such as albumin (the major plasma protein), fibrinogen, α_1-antitrypsin, haptoglobin, ceruloplasmin, transferrin, and several coagulation factors.

Metabolic Zonation

The multiple functions of the liver are facilitated by a functional specialization

of the liver parenchyma, known as metabolic zonation, where hepatocytes show different functional and structural characteristics according to their location in the liver acinus. Within each acinus, the functional unit in terms of blood flow, blood rich in nutrients and hormones enters at the portal triad through the portal vein, mixes with oxygen-rich blood from the hepatic artery, flows through the sinusoids and eventually exits the lobule through the central vein. As the blood flows through the sinusoids there is free exchange of nutrients and metabolites between blood and hepatocytes. Functional variation is observed in hepatocytes based on their location along the portal–central axis. Hepatocytes exhibit a distinct gene expression based on their location within the acinus, which manifests as diverse availability of substrates and concentration of enzymes in different parts of the acinus. Based on this organization and heterogeneity of hepatocytes, the acinus comprises three geographical areas or zones: periportal or zone 1, midzonal or zone 2, and perivenous or zone 3. Hepatocytes in zone 3 contain the drug-metabolizing P450 enzymes, have a reduced oxygen supply, receive a higher concentration of any toxic product of drug metabolism, and have a reduced glutathione concentration compared with zone 1. This makes them particularly susceptible to drug-induced liver injury. Also, hepatocytes in zone 1 receive blood with a high bile salt concentration and are therefore particularly important in bile salt-dependent bile formation, whereas hepatocytes in zone 3 are important in non-bile salt-dependent bile formation. Functions such as gluconeogenesis, glycolysis, and ketogenesis appear to be dependent on the direction of blood flow along the sinusoid. For others, such as cytochrome P450 activity, the gene transcription rate differs between perivenular and periportal hepatocytes. This functional zonation is often lost in liver diseases.

Hepatic Transport Systems

Hepatic uptake and efflux of electrolytes and solutes involved in bile formation are maintained by distinct transport systems expressed at the two surface domains of hepatocytes. After canalicular secretion, bile composition undergoes further modification in the bile canaliculi, involving reabsorption and secretion processes maintained by the apical and basolateral transport systems in cholangiocytes (Figure 1.1).

Basolateral (Sinusoidal) Transporters

The uptake of exogenous and endogenous compounds from the portal circulation is facilitated by a number of basolaterally located, sodium-dependent and sodium-independent transporters. The sodium-dependent transporters include the sodium taurocholate cotransporting polypeptide (NTCP), specific to conjugated bile salts and certain sulfated steroids. NTCP accounts for more than 80% of conjugated but less than half of unconjugated bile salt uptake. The sodium-independent transporters include several members of the superfamily of organic anion-transporting polypeptides (OATPs). With the exception of OATP2B1, the other OATPs exhibit overlapping transport activities for conjugated and unconjugated bile salts, neutral steroids, steroid sulfates and glucuronides, selected organic cations, and drugs including the antihistamine fexofenadine, opioid peptides, digoxin, the HMG-CoA reductase inhibitor pravastatin, the angiotensin-converting enzyme inhibitor enalapril, and the antimetabolite methotrexate. In addition, the basolateral hepatocyte membrane also localizes several ATP-dependent efflux pumps belonging to the family of MRPs, multispecific transporters for different organic anions. These have been implicated in the cellular efflux of drugs conjugated with glutathione, glucuronic acid, and sulfate (MRP1); the efflux of bile salts (MRP3); and the transport of nucleoside analog drugs (MRP4).

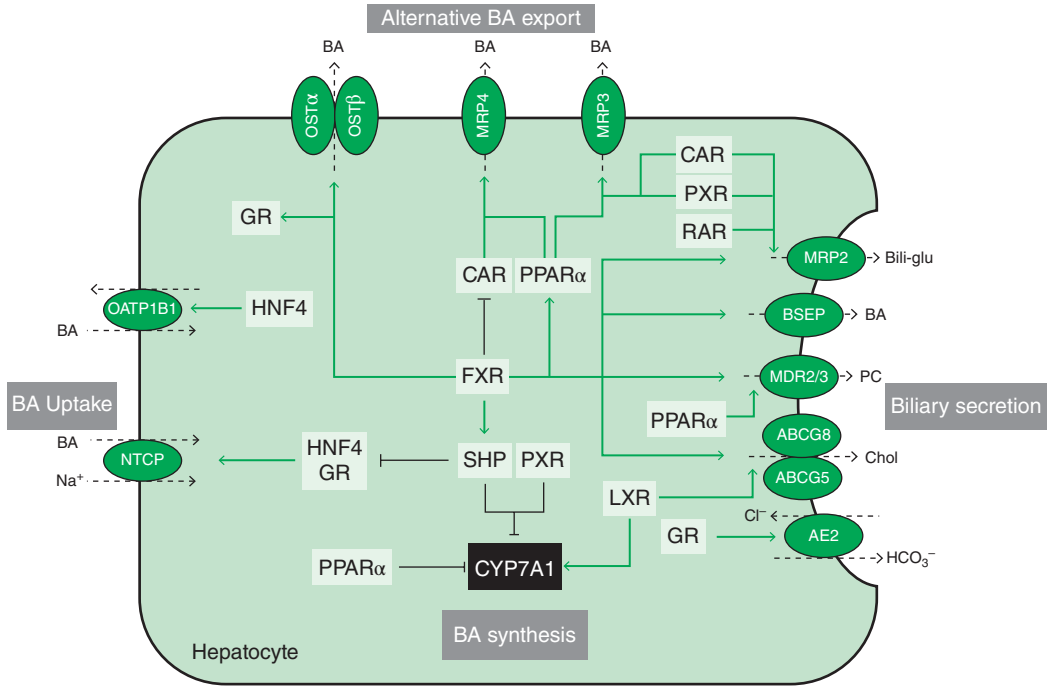

Figure 1.1 Transcriptional regulation of hepatocellular bile formation. Expression of hepatobiliary transporters in hepatocytes determines hepatic bile acid (BA) flux and hepatocellular concentrations of these potentially toxic metabolites. To ensure the balance between synthesis, uptake and excretion, expression of hepatobiliary transporters is tightly regulated by nuclear receptors (NRs). NRs provide a network of negative feedback and positive feed-forward mechanisms for the control of intracellular concentration of biliary constituents, which are often also ligands for these NRs. BA-activated FXR is a central player in this network, that represses hepatic BA uptake (NTCP) and (via SHP) synthesis (CYP7A1), promotes bile secretion via induction of canalicular transporters (BSEP, MRP2, ABCG5/8, MDR3), and induces BA elimination via alternative export systems at the hepatic basolateral (sinusoidal) membrane (OSTα/β). Several NR pathways converge at the level of CYP7A1 as the rate-limiting enzyme in BA synthesis. CAR and PXR facilitate adaptation to increased intracellular BA concentrations by upregulation of alternative hepatic export routes (MRP3 and MRP4) and induction of detoxification enzymes (not shown). Together with RAR, these xenobiotic receptors also regulate the canalicular expression of MRP2. Cholesterol sensor LXR promotes biliary cholesterol excretion via ABCG5/8. Stimulation of AE2 expression by GR stimulates biliary bicarbonate secretion thus reducing bile toxicity. Green arrows indicate stimulatory and red lines suppressive effects on target genes. Bili-glu, bilirubin glucuronide; BSEP, bile salt export pump; CAR, constitutive androstane receptor; CYP7A1, cholesterol 7α-hydroxylase; FXR, farnesoid X receptor; GR, glucocorticoid receptor; HNF4, hepatocyte nuclear factor 4; LXR, liver X receptor; MDR3, multidrug resistance protein 3, phospholipid flippase; MRP2, multidrug resistance-associated protein 2; MRP3, multidrug resistance-associated protein 3; MRP4, multidrug resistance-associated protein 4; NTCP, sodium taurocholate cotransporting polypeptide; OSTα/β, organic solute transporter alpha and beta; PC, phosphatidylcholine; PXR, pregnane X receptor; PPARα, peroxisome proliferator-activated receptor alpha; RAR, retinoic acid receptor; SHP, small heterodimer partner. *Source:* Halilbasic *et al*. [3]. Reproduced with permission of Elsevier.

Hepatocellular transporters are subject to extensive transcriptional and posttranscriptional regulation, allowing for adaptational changes in response to the intracellular accumulation of bile salts. During cholestasis, the NTCP is suppressed through farnesoid X receptor (FXR)-mediated signaling, thereby preventing the hepatocyte from further accumulating toxic bile salts. Likewise, the expression of OATP1B1 is downregulated. In contrast, cholestasis leads to FXR-mediated activation of hepatic OATP1B3, which might constitute an escape mechanism

promoting the hepatocellular clearance of xenobiotics during cholestasis.

In addition to its role in the uptake of conjugated BAs, NTCP also plays a key role in hepatitis B and hepatitis D virus entry into hepatocytes; and, recently, NTCP has also been shown to modulate hepatitis C virus infection of hepatocytes by regulating innate antiviral immune responses in the liver. As such NTCP has been established as a novel antiviral target.

Apical (Canalicular) Transporters

Various ATP-binding cassette (ABC) transporters mediate the secretion of bile salts and xenobiotics across the canalicular membrane of hepatocytes. These include members of the family of multidrug resistance (MDR) P-glycoproteins such as MDR1 (ABCB1), MDR3 (ABCB4), and the bile salt export pump (BSEP or ABCB11). Within the family of MDR proteins, BSEP and MDR3 are two highly conserved members that are involved in the secretion of cholephilic compounds from the liver cell into the bile canaliculus.

BSEP constitutes the predominant bile salt efflux system of hepatocytes and mediates the cellular excretion of numerous conjugated bile salts such as taurine- or glycine-conjugated cholate, chenodeoxycholate, and deoxycholate. MDR3 works as an ATP-dependent phospholipid flippase, translocating phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane. Canalicular phospholipids are then solubilized by canalicular bile salts to form mixed micelles, thereby protecting cholangiocytes from the detergent properties of bile salts.

In addition to these processes, MRP2, the only canalicular member of the MDR-associated protein family, mediates the canalicular transport of glucuronidated and sulfated bile salts. MRP2 is the main driving force for bile salt-independent bile flow through canalicular excretion of reduced glutathione. Furthermore, MRP2 transports a wide spectrum of organic anions, including bilirubin diglucuronide, glutathione conjugates, leukotriene C4, and divalent bile salt conjugates, as well as drug substrates such as chemotherapeutic agents and antibiotics.

MDR1 contributes to the canalicular excretion of drugs and other xenobiotics into bile, although its exact contribution has yet to be established. Its broad substrate specificity and its physiologic expression in various tissues with excretory and protective functions make MDR1 one of the major determinants of drug disposition and toxicity. Substrates are neutral and positively charged organic compounds and include various chemotherapeutic and immunosuppressant agents, antiarrhythmic drugs, HIV protease inhibitors, and antifungals.

Transcriptional regulation of BSEP and MDR3 is mediated by FXR and their activation leads to increased bile salt efflux and the formation of mixed micelles in the biliary tree during cholestatic episodes, thereby preventing the toxic effects of bile salts on hepatocytes and cholangiocytes. In addition, FXR has been shown to induce MRP2 expression, which might constitute another compensatory mechanism during cholestasis. In contrast, MDR1 is upregulated via the pregnane X receptor (PXR), which in addition to endogenous ligands is activated by different xenobiotics. This pathway is part of a general cellular mechanism of detoxification, because MDR1 is the key transporter protein involved in the cellular efflux of numerous drugs and xenobiotics.

Drug Metabolism

The liver has a major role in drug metabolism. The main hepatocyte enzymes involved in metabolism belong to the cytochrome P450 group, a large family of related enzymes housed in the smooth endoplasmic reticulum of the hepatocyte. Metabolism is often divided into two phases of biochemical reaction. Phase 1 involves reduction, hydrolysis or oxidation of the drug, the latter being the most common process. After phase 1 reactions, the resulting drug metabolite is still often chemically active. Phase 2 metabolism involves

conjugation with glutathione, methyl or acetyl groups, which usually occurs in the cytoplasm of the hepatocyte and makes the metabolite more hydrosoluble. This facilitates excretion as well as decreasing the pharmacologic activity. Some drugs may undergo just phase 1 or just phase 2 metabolism, but more often the drug will undergo phase 1 and then phase 2 sequentially.

Many factors can affect liver metabolism of drugs. The numbers of hepatocytes and enzyme activity can decline, with a reduction in the metabolic potential of the liver, following aging, acute and chronic liver disease, and conditions that affect hepatic blood flow. Metabolism can also be altered due to genetic deficiency of a particular enzyme and secondary to the use of other drugs as well as dietary and environmental factors. Capillarization of sinusoids during chronic liver disease increases the bioavailability of drugs at high hepatic extraction, possibly increasing the side effects. Drug-induced liver injury is a major clinical problem, is often favored by exposure to a combination of drugs and, at times, may be mediated by immunologic mechanisms.

Bile Formation, Secretion and the Enterohepatic Circulation

Bile is a complex secretion that originates from hepatocytes and is modified distally by absorptive and secretory transport systems in the bile duct epithelium. Bile formation by the hepatocytes involves secretion of osmotically active inorganic and organic anions into the canalicular lumen, followed by passive water movement. Bile then enters the gallbladder where it is concentrated or is delivered directly to the bowel.

Bile comprises about 95% water in which are dissolved a number of endogenous constituents, including bile salts, bilirubin, phospholipid, cholesterol, amino acids, steroids, enzymes, porphyrins, vitamins, and heavy metals, as well as exogenous drugs, xenobiotics and environmental toxins [4]. Lipophilic constituents are in solution in mixed micelle composed of BAs, phospholipids, and cholesterol.

Bile is essential for several important functions:

- the excretion of potentially harmful exogenous lipophilic substances, as well as the excretion of endogenous substrates such as bilirubin and bile salts;
- the digestion and absorption of lipid in the gut by bile salts;
- cholesterol homeostasis, by facilitating intestinal cholesterol absorption and, on the other hand, promoting cholesterol elimination;
- the excretion of immunoglobulin A (IgA) and inflammatory cytokines, thus protecting the organism from enteric infections;
- signaling properties of the BAs in the liver and the intestine, which are mediated by nuclear BA receptors such as FXR, PXR and vitamin D receptor (VDR), as well as by membrane a5b1 integrin, epidermal growth factor receptor, and sphingosine-1-phosphate receptor 2.

Our understanding of cholestatic liver diseases has been profoundly advanced by the discovery of nuclear receptors for BA signaling and their role in hepatobiliary excretory function and the adaptive changes counteracting the liver injury caused by retained, potentially toxic, and proinflammatory BAs. Ligand-activated nuclear receptors such as FXR control a broad range of metabolic processes, including hepatic BA transport and metabolism, lipid and glucose metabolism, drug disposition, as well as liver regeneration, inflammation, fibrosis, cell differentiation, and tumor formation. Moreover, FXR has anti-inflammatory and immunomodulatory actions and controls intestinal integrity and permeability, as well as gut microbiota. Conversely, the gut microbiota metabolizes BAs, with formation of secondary BAs that, in turn, modulate BA signaling. Based on these

broad physiologic effects in the liver and intestine, drugs targeting FXR and TGR5 therefore open important perspectives for pharmacotherapy of cholestatic and metabolic liver disorders, including the complications of liver cirrhosis such as portal hypertension and hepatocellular carcinoma (HCC).

In addition, BAs stimulate glucagon-like peptide (GLP)-1 production via TGR5 activation. GLP-1 is known to promote insulin secretion and thus regulate glucose homeostasis. Because GLP-1 mimetics and receptor agonists are currently under clinical development and have shown promise in improving glucose homeostasis in diabetes, BA-based TGR5 agonists may be a potential therapeutic to stimulate GLP-1 secretion in diabetic patients.

Bile Acid Synthesis and Metabolism

BAs are synthesized from cholesterol. In humans, the "primary" BAs are cholic acid (CA) and chenodeoxycholic acid (CDCA). Before secretion into the bile, both CA and CDCA are conjugated to the amino group of taurine or glycine. Conjugation enhances the hydrophilicity of the BA, the major function of this being to decrease the passive diffusion of BAs across the cell membranes during their transit through the biliary tree and intestine. Therefore, conjugated BAs are absorbed only if a specific membrane carrier is present. The process of bile formation depends on the liver synthesis and the canalicular secretion of BAs. The active transport of BAs across the canalicular membranes of hepatocytes is a primary driving force for bile flow. The majority of the BAs in the intestine are absorbed intact. Approximately 15% are deconjugated by the bacterial flora in the distal small intestine, with the production of "secondary" BAs by the conversion of CA to deoxycholic acid and of CDCA to lithocholic acid. Most of the conjugated and deconjugated BAs are reabsorbed in the distal intestine and undergo enterohepatic circulation that maintains the BA pool. Thus, at least 12 major conjugated primary and secondary bile salt species are contained in human bile, although primary bile salts are usually predominant.

Enterohepatic Bile Acid Circulation

BAs undergo an enterohepatic circulation that depends on active transport systems in the liver and the intestine (Figure 1.2). More specifically, BAs are excreted from hepatocytes into bile through BSEP/ABCB11 at the bile canaliculus, reabsorbed in the ileum by the apical sodium-dependent bile salt transporter (ASBT/SLC10A2), and return through the portal blood to the liver, where they are taken up by hepatocytes via the basolateral transport systems NTCP/SLC10A1 for conjugated BAs and OATPs/SLCO/SLC21 family for unconjugated BAs, thus limiting the amount of BA spillover into the systemic circulation. BAs complete the enterohepatic circulation six to eight times a day and are highly efficiently conserved. BAs that escape ileal reabsorption reach the colon, where they are deconjugated and metabolized (e.g. dehydroxylated) by gut microbiota to secondary BAs, which can still be passively absorbed as unconjugated BAs in the colon. Unconjugated BAs are partially reconjugated (and rehydroxylated) during their passage through the liver before being excreted into bile again, which completes their enterohepatic cycle. In addition, BAs are filtered by the glomeruli and then reabsorbed in renal tubules, again limiting their renal loss.

BAs may also cycle between cholangiocytes and hepatocytes through a *cholehepatic shunt* pathway. Unconjugated BAs induce a greater degree of bile flow per BA molecule excreted in bile. To account for this hypercholeretic effect, it was proposed that unconjugated BAs may be passively absorbed by bile ducts, enter the peribiliary plexus adjacent to intrahepatic bile ducts, and then forwarded to the hepatic sinusoids to be returned to cholangiocytes by hepatocyte secretion. Cholehepatic shunting initiated by passive absorption of non-ionized bile salt results in the generation of HCO_3^--rich hypercholeresis [4,5].

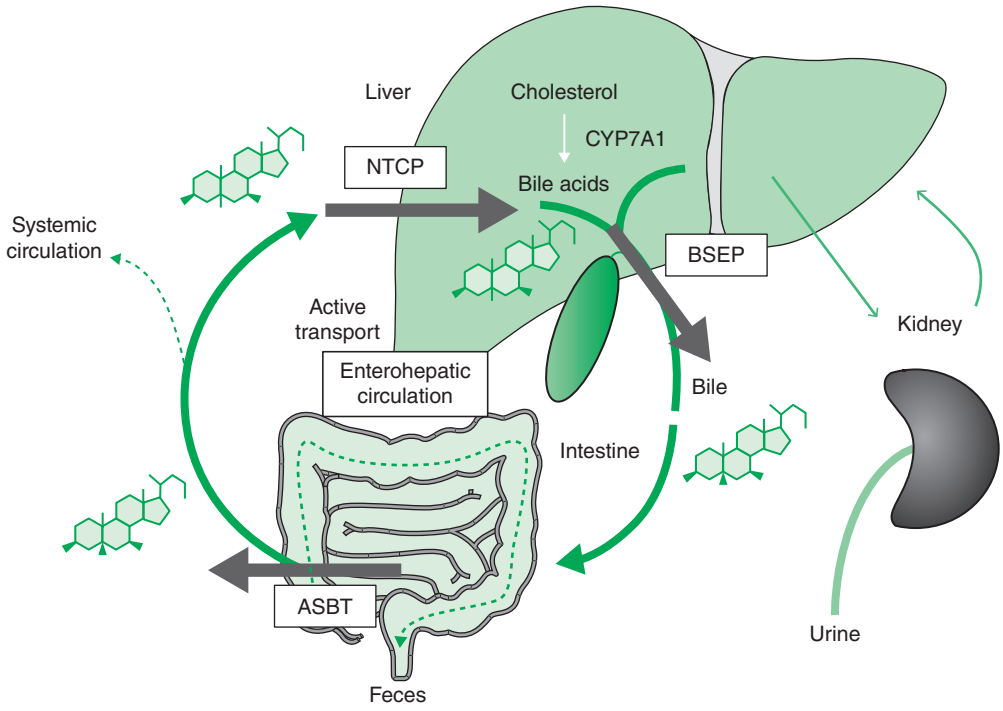

Figure 1.2 Enterohepatic circulation of bile acids (BAs). After hepatic synthesis and biliary secretion, BAs undergo enterohepatic circulation. The BSEP (ABCB11) is the main canalicular transporter for BAs. After reabsorbtion by ASBT/SLC10A1 in the ileum, BAs (and exit through OSTα/β from enterocytes; not shown) are transported back to the liver through the portal blood. Reuptake of conjugated BA from portal blood through NTCP/SLC10A1 (and OATPs for unconjugated BAs; not shown) into the hepatocytes completes the enterohepatic circulation. ASBT, apical sodium-dependent bile salt transporter; BSEP, bile salt export pump; NTCP, sodium taurocholate cotransporting polypeptide; OATPs, organic anion transporters; OST, organic solute transporter α/β. *Source:* Trauner *et al*. [5]. Reproduced with permission of John Wiley and Sons.

Death and Regeneration of Hepatocytes

Cell Death

Hepatocytes can die because of either necrosis or apoptosis. *Necrosis* is the loss of plasma membrane integrity with release of the cellular contents locally, which triggers an inflammatory response. *Apoptosis* is a highly regulated process in which cells that are damaged, senescent or deregulated self-destruct with a lower release of inflammatory products. Dying cells undergo morphologic modifications including chromatin condensation, nuclear fragmentation, and generation of apoptotic bodies. Furthermore, they express signals on the cell surface that allow macrophage recognition. Apoptosis is essential to avoid the outflow of intracellular contents and to limit the immunologic response against intracellular autoantigens. Nevertheless, apoptotic bodies and fragments can under some circumstances constitute a major source of immunogens in autoimmune diseases that involve the targeting of ubiquitous autoantigens. This has been described in PBC. In the BECs of patients with PBC there is increased DNA fragmentation, implying increased apoptosis, when compared with normal controls. While mitochondrial proteins are present in all nucleated cells, in PBC there is a highly specific multilineage immune response directed to the nominal mitochondrial autoantigenic epitope, the inner lipoyl domain of

the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2) of the BECs. Apoptosis of BECs has been proposed as a potential source of "neoantigens" that could be responsible for activation of autoreactive T lymphocytes or a target for effector cells or antibodies. PDC-E2 is not only immunologically intact during apoptosis in BECs, but it localizes in the apoptotic bodies of BECs where it is accessible to recognition by anti-mitochondrial antibodies.

Liver Regeneration

Liver possesses a unique capacity to replace its mass after tissue injury or loss. The regenerative process involves a cascade of events that moves cells from their resting G0 phase through G1, S phase (DNA synthesis), and G2 to M phase (mitotic cell division). A typical example is hepatic growth after partial hepatectomy. The majority of research on liver regeneration has focused on cytokine- and growth factor- mediated pathways involved in initiation and progression through the cell cycle. During more extensive acute liver injury, which far exceeds the capacity of remaining healthy hepatocytes to replicate and restore liver function, resident liver progenitor cells (i.e. oval cells) are activated to support or take over the role of regeneration. In adults, there are multiple niches of biliary tree stem/progenitor cells (BTSCs) residing in different locations along the human biliary tree and niches found within the liver parenchyma, with a key role in regeneration of the liver. Figure 1.3 shows the location of stem/progenitor cell niches in the human biliary tree. Canals of Hering harbor hepatic stem/progenitor cells (HpSCs), while peribiliary glands (PBGs) constitute the niche for BTSCs.

Those within the biliary tree are found in PBGs and contain especially primitive stem cell populations, expressing endodermal transcription factors relevant to both liver and pancreas, pluripotency genes, and even markers indicating a genetic signature overlapping with that of intestinal stem cells [7]. The distribution of PBGs is not uniform, varying along the biliary tree: PBGs are mostly found in the hepatopancreatic ampulla and

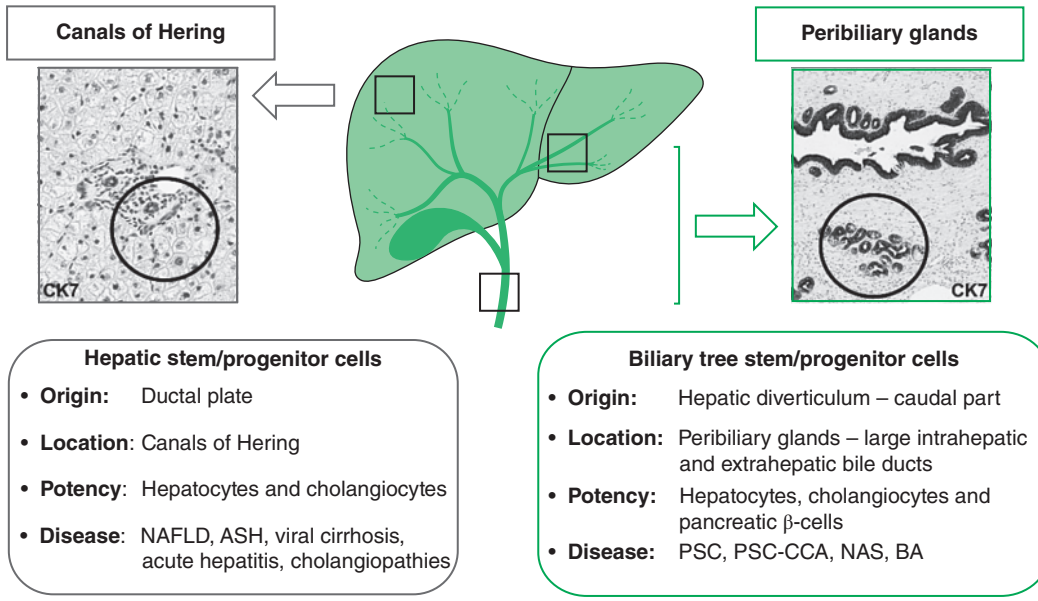

Figure 1.3 Stem/progenitor cell niches in the human biliary tree. Canals of Hering harbor hepatic stem/progenitor cells (HpSCs), while peribiliary glands (PBGs) constitute the niche for biliary tree stem/progenitor cells (BTSCs). ASH, alcoholic steatohepatitis; BA, bile acid; CCA, cholangiocarcinoma; NAFLD, non-alcoholic fatty liver disease; NAS, non-anastomotic strictures; PSC, primary sclerosing cholangitis. *Source:* Overi *et al*. [6].

are less numerous in the common bile duct; they are not present in the gallbladder, but a stem cell-like compartment is located in the epithelial crypts. Biliary progenitors support the renewal of large intrahepatic and extrahepatic bile ducts. Stem cells are present in the canals of Hering, and participate in the renewal of the small intrahepatic bile ducts and in the regeneration of liver parenchyma. Small hepatocytes located in pericentral positions are also believed to act as progenitor cells on certain occasions. Distinct subpopulations of mature hepatocytes and stem/progenitor cell compartments are differentially activated depending on the nature and duration of the liver damage versus different human pathologies.

Cholangiocyte Reaction to Biliary Damage

BECs are usually quiescent, but following a liver insult they activate and/or proliferate. A typical element of the repair response to liver damage is the ductular reaction (DR), a stereotyped histopathologic lesion of the biliary epithelium that plays a fundamental role in the progression of hepatic fibrosis. The DR is characterized by a marked proliferation of cholangiocytes, leading to formation of reactive ductular cells (RDCs), with poor cytoplasm and arranged in cell cords without a lumen or in richly anastomosed small-diameter ducts (<10 μm) with almost unrecognizable lumens. RDCs are activated epithelial cells that secrete a vast array of factors, including cytokines, chemokines, growth factors, and angiogenic factors. They may derive from hepatocytes undergoing a process of ductular metaplasia, or from activation of the hepatic progenitor cell compartment and/or from proliferation and dedifferentiation of preexisting cholangiocytes. The increase in RDCs is generally associated with a significant increase in inflammatory infiltrate and portal fibrosis.

RDCs are considered the major driver of portal fibrosis during parenchymal and/or biliary injury. The deposition of fibrosis follows paracrine cross-talk mediated by the ability of RDCs to secrete profibrotic and proinflammatory growth factors, and cross-talk with cells of mesenchymal origin, in particular Kupffer cells and portal fibroblasts, which are the main effectors of fibrosis, as stimulators of the deposition of extracellular matrix by activated myofibroblasts. In addition, RDCs also establish paracrine communications with endothelial cells that provide the vascular support necessary for the growth and arborization of the ductal structures themselves [8].

Protective Role of Biliary HCO_3^- Secretion

The cholangiocyte is exposed to millimolar concentrations of hydrophobic bile salts, which are toxic to other cells such as hepatocytes at micromolar levels. Resistance against these noxious compounds and their cytolytic potential is therefore essential. One of the strategies that cholangiocytes have developed to survive is the biliary HCO_3^- umbrella.

Biliary HCO_3^- secretion sustains bile flow and confers its appropriate viscosity, generates part of the alkaline tide necessary for optimal digestion of various nutrients within the intestine, and protects the apical surface of cholangiocytes against protonated apolar hydrophobic BA monomers by maintaining an alkaline pH above the apical membrane. Isoforms of the Cl^-/HCO_3^- exchanger, AE2, are responsible for the vast majority of biliary HCO_3^- secretion. Dysfunction of any of the elements involved in HCO_3^- formation might weaken the biliary HCO_3^- umbrella and contribute to the development of chronic cholestatic liver disease such as sclerosing cholangitis.

Cholangiocytes and Immunity

BECs are a first line of defense in liver innate immunity: they can present antigen to immune cells, be a target of immune-mediated

aggression, or be the initiators of an inflammatory reaction that then progresses to adaptive immune activation [9]. The contribution of BECs to liver immune responses was initially believed to be limited to the secretion of immunoglobulin A (IgA) into the bile, but it is now clear that their role in the immune response is far more complex. The biliary epithelium stands as a first line of defense against bacteria, fungi and other pathogens by secreting antimicrobial peptides such as defensin and cathelicidin. A major role in epithelial innate immunity in BECs is played by Toll-like receptors (TLRs) and by nuclear receptors. TLRs can recognize pathogen-associated molecular patterns (PAMPs), i.e. bacterial elements such as lipopolysaccharide (LPS), DNA and RNA fragments, but also respond to endogenous components or damage-associated molecular patterns (DAMPs), such as hyaluronan and high mobility group box 1 (HMGB1) that are released from damaged cells. TLR4-mediated signaling is the better studied in cholangiocytes. Once activated by LPS or other ligands, TLR4 activates two different pathways, one mediated by NF-κB, which stimulate the expression of a number of pro-inflammatory cytokines and chemokines, and one mediated by mitogen-activated protein kinase (MAPK)/activator protein 1 (AP-1), which requires the nuclearization of the AP-1 complex. In normal cholangiocytes, TLR4 signaling is repressed by protective mechanisms aimed at maintaining LPS tolerance. Since the biliary epithelium is continuously in contact with bacterial products of intestinal origin, changes in one or more regulatory checkpoints may trigger an exaggerated inflammatory response in the liver. For example in cystic fibrosis-related liver disease, a decrease in LPS tolerance plays a major role in the development of the disease. A continuous stress in the absence of a correct modulation of the TLR-mediated responses could be the trigger for a chronic inflammatory or autoimmune response.

Nuclear receptors, particularly the retinoid X receptor (RXR), have recently been involved in the immune response of BECs. This is a receptor superfamily that includes the glucocorticoid receptor, the retinoic acid receptor, the VDR, the liver X receptors, and the peroxisome proliferator-activated receptors (PPARs). Nuclear receptors control several cell functions including cell proliferation and apoptosis, cell metabolism, cell–cell interaction, detoxification from BAs, and bile secretion.

In addition, continuous exposure to DAMPs and PAMPs could promote cellular senescence. Cell senescence is a mechanism of irreversible cell arrest in G1 stage induced by different stimuli. The main causes responsible for the onset of senescence are DNA damage (particularly but not exclusively) to the telomeres, the activation of mitogenic signals induced by oncogene activation, epigenetic modifications, and expression of tumor suppressor genes. All these signals lead to different physiologic responses generally leading to tumor suppression; however, in some cases it could promote cancer development or induce a fibrosing response and mediate age-related degenerative diseases. Once senescent, cells not only cease proliferation but assume a senescence-associated secretory phenotype (SASP) characterized by the secretion of a plethora of peptides with profibrogenic, proinflammatory, and tumorigenic properties. This indicates that senescence could not only act as a barrier to tumor growth, but also paracrinally stimulate the activation of aberrant reparative/regenerative responses. In chronic biliary diseases, cholangiocyte senescence is likely the result of ongoing inflammation, a sort of "exhaustion" of the activated cholangiocytes. This is particularly important in PSC, given the association with cholangiocarcinoma.

Biochemical Markers and Patterns of Hepatic Injury

Contrary to the kidney, no single test can be used to assess liver function. The liver biochemical tests, or liver function tests (LFTs), provide indirect evidence of hepatobiliary

disease. LFTs that more accurately reflect liver synthesis include serum albumin, serum bilirubin, and prothrombin time, which is standardized to the international normalized ratio (INR). The enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are markers of hepatocellular disease, whereas alkaline phosphatase (ALP) and gamma-glutamyltranspeptidase (GGT) are markers of cholestasis. The clinical evaluation of patients with abnormal LFTs involves an accurate interpretation of the pattern of liver damage in the context of an accurately collected medical history and physical examination. The severity and pattern of LFT abnormality, assessed by serial measurements, can be distinctive or aspecific. Different patterns of damage can be observed: hepatocellular damage, with predominant elevations in AST and ALT; cholestatic damage, with predominant increases in ALP, GGT, and bilirubin; and infiltrative damage with ALP and GGT increased disproportionately to bilirubin. These patterns are valuable in directing specific serologic tests, imaging, and liver biopsy. However, they are not diagnostic for a specific cause, nor are they able to distinguish whether cholestasis is intrahepatic or extrahepatic.

Hepatocellular Necrosis

Elevation of ALT and AST indicates hepatocellular necrosis. The interpretation of these increases should consider the rate of rise, the severity (peak level), the AST/ALT ratio, and coexisting abnormalities in other LFTs and other investigations. ALT and AST are enzymes that catalyze the transfer of amino groups from alanine or aspartic acid to ketoglutaric acid to form pyruvic acid and oxaloacetic acid, respectively, during gluconeogenesis. ALT is localized primarily in the liver and confined to the cytoplasm, while AST can be released by the liver, myocardium, skeletal muscle, kidney, pancreas, and blood cells, and can be found in the cytoplasm and mitochondria. During hepatocellular injury they are released into the bloodstream. However, their increase is not always pathologic: they can be raised by vigorous physical activity, and rarely an isolated AST can be the result of the binding of the enzyme with an immunoglobulin forming a macro-enzyme (macro-AST) complex. The diagnostic specificity of mild-to-moderate increases in aminotransferases is poor, with many differential diagnoses being possible, whereas the spectrum of liver conditions indicated by markedly elevated aminotransferase levels (>2000 IU/l) narrows to viral (mostly hepatitis A and hepatitis B virus), ischemic (shock liver), and drugs. Autoimmune hepatitis can sometimes have an acute outset with striking elevation of aminotransferases. Rarely, bile duct stones can manifest as marked rise in aminotransferase, although this is followed by a rapid fall within 48 hours.

The AST/ALT ratio can often provide a clue to the diagnosis. In the majority of cases of hepatitis, the AST/ALT ratio is less or equal to 1. The AST/ALT ratio is typically greater than 2 during alcoholic hepatitis. This occurs because damage is primarily mitochondrial (thus more AST is released systemically) and ALT synthesis is more sensitive than AST to pyridoxal 5-phosphate deficiency, a common finding in alcoholics, leading to lower serum ALT levels. Supplementation with pyridoxine in patients with alcoholic hepatitis results in a rise in the level of ALT. An AST/ALT ratio greater than 1 in patients without a history of alcoholism is suggestive of advanced fibrosis or cirrhosis. An AST/ALT ratio greater than 4 is observed in patients with fulminant Wilson disease.

Cholestasis

Cholestasis is an overarching term applied to conditions in which there is impairment of bile formation and/or bile flow. It occurs where there is a failure at any point along the biliary tree, between the basolateral (sinusoidal) membrane of the hepatocyte and the ampulla of Vater, as a result of congenital or acquired injuries, that leads to impaired secretion of bile such that biliary constituents spill into blood. It

may result from (i) hepatocellular and/or cholangiocellular secretory defects or (ii) obstruction of bile ducts by bile duct lesions, stones or tumors, but may also be related to mixed mechanisms in conditions such as PBC or PSC.

ALP and GGT are markers of cholestasis. ALP is a ubiquitous membrane-bound glycoprotein that catalyzes the hydrolysis of phosphate monoesters at basic pH values. Liver and bone are the major source of serum ALP. The liver isoenzyme is located on the canalicular side of the hepatocyte plasma membrane and the luminal surface of bile duct epithelium. Serum ALP elevation more than three times normal strongly suggests cholestasis if bone disease is absent and GGT is elevated. In patients with cholestasis, the ALP elevation is triggered by increased synthesis and release of the enzyme into serum rather than impaired biliary secretion. BAs build up in hepatocytes and solubilize the plasma membrane, thereby resulting in release of ALP. The half-life of serum ALP is 5–7 days, and therefore serum ALP remains elevated for several days after resolution of the biliary obstruction. ALP is not used as a marker of cholestasis in adolescent and pregnant women since ALP in these conditions can be raised as a consequence of rapid bone growth and placental growth. Chronic renal failure can result in elevation of the intestinal ALP isoenzyme. In patients with raised ALP, hyperthyroidism should be ruled out. Rarely, ALP can be identified in patients with underlying malignancy not involving either liver or bones. This is the Regan isoenzyme, biochemically different from the liver isoenzyme, that has been described in lung cancer, Hodgkin disease, and renal cell carcinoma. Finally, ALP should be tested after fasting since its level can rise after a fatty meal.

GGT is an enzyme that can be induced by several stimuli such as drugs and alcohol. It is mainly localized in hepatocytes and biliary epithelia, and is also present in extrahepatic tissues such as kidney, spleen, pancreas, heart, lung, and brain, but not bone. The lack of GGT in bone can be used to distinguish a liver source from a bone source of a raised ALP. GGT is more liver specific than ALP, although during cholestasis is less specific since it can be influenced by other factors such as alcohol, fat, and drugs. A GGT/ALP ratio over 2.5 may point to alcohol abuse, although up to one-third of those who abuse alcohol (>80 g/day) have a normal GGT. A normal GGT in patients with elevated liver ALP isoenzyme should raise the suspicion of benign recurrent intrahepatic cholestasis.

References

1 Ober, E.A. and Lemaigre, F.P. (2018). Development of the liver: insights into organ and tissue morphogenesis. *J. Hepatol.* 68: 1049–1062.

2 Roskams, T.A., Theise, N.D., Balabaud, C. et al. (2004). Nomenclature of the finer branches of the biliary tree: canals, ductules, and ductular reactions in human livers. *Hepatology* 39: 1739–1745.

3 Halilbasic, E., Claudel, T., and Trauner, M. (2013). Bile acid transporters and regulatory nuclear receptors in the liver and beyond. *J. Hepatol.* 58 (1): 155–168.

4 Boyer, J.L. (2013). Bile formation and secretion. *Compr. Physiol.* 3: 1035–1078.

5 Trauner, M., Fuchs, C.D., Halilbasic, E., and Paumgartner, G. (2017). New therapeutic concepts in bile acid transport and signaling for management of cholestasis. *Hepatology* 65 (4): 1393–1404.

6 Overi, D., Carpino, G., Cardinale, V. et al. (2018). Contribution of resident stem cells to liver and biliary tree regeneration in human diseases. *Int. J. Mol. Sci.* 19 (10): 2917. https://doi.org/10.3390/ijms19102917.

7 Cardinale, V., Wang, Y., Carpino, G. et al. (2011). Multipotent stem/progenitor cells in human biliary tree give rise to hepatocytes, cholangiocytes, and pancreatic islets. *Hepatology* 54: 2159–2172.

8 Fabris, L., Spirli, C., Cadamuro, M. et al. (2017). Emerging concepts in biliary repair and fibrosis. *Am. J. Physiol. Gastrointest. Liver Physiol.* 313: G102–G116.

9 Strazzabosco, M., Fiorotto, R., Cadamuro, M. et al. (2018). Pathophysiologic implications of innate immunity and autoinflammation in the biliary epithelium. *Biochim. Biophys. Acta* 1864: 1374–1379.