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Gallstone and Gallbladder Disease: Biliary Tract and Cholangiopathies

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Abstract:	Cholestatic liver diseases are named primarily due to the blockage of bile flow and buildup of bile acids in the liver. Cholestasis can occur in cholangiopathies, fatty liver diseases and during COVID-19 infection. Most literature evaluates damage occurring to the intrahepatic biliary tree during cholestasis; however, there may be associations between liver damage and gallbladder damage. Gallbladder damage can manifest as acute or chronic inflammation, perforation, polyps, cancer and most commonly gallstones. Considering the gallbladder is an extension of the intrahepatic biliary network, and both tissues are lined by biliary epithelial cells that share common mechanisms and properties, it is worth further evaluation to understand the association between bile duct and gallbladder damage. In this comprehensive review, we discuss background information of the biliary tree and gallbladder, from function, damage, and therapeutic approaches. We then discuss published findings that identify gallbladder disorders in various liver diseases. Lastly, we provide the clinical aspect of gallbladder disorders in liver diseases and ways to enhance diagnostic and therapeutic approaches for congruent diagnosis.

February 6, 2023

RE: Resubmission of manuscript CPHY-22-0028 entitled "Gallstone and Gallbladder Disease: Biliary Tract and Cholangiopathies"

Dear Dr. Yatrik Shah,

We would like to thank the Reviewers and Editor for allowing us to resubmit our comprehensive review article (CPHY-22-0028) entitled "Gallstone and Gallbladder Disease: Biliary Tract and Cholangiopathies" to Comprehensive Physiology. This article has not been submitted in whole or in part to any other journals. We thank the Reviewers and Editor for their overall appreciation of our work and have attempted to address the minor issues stated. Any changes made to the manuscript have been marked in red and are also discussed below in a point-by-point response.

Reviewer 1:
This is a very well prepared, very thorough review manuscripts with a focus on the link between biliary disorders to gall stone abnormalities. The review gives basic physical and pathological overview, with subsequent elaboration on how common liver diseases affect gallbladder disease development. This review provides an unmet need to cover an area with significant diseases affecting population globally. This review should be received well for both basic scientists and clinicians in hepatology.

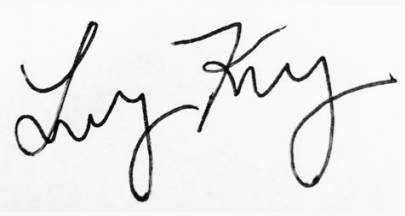
Response to Reviewer 1:
We thank the Reviewer for their overall positive response to our comprehensive review and hope that it is well-received within the field.

Reviewer 2:
In this article, the authors overviewed essential topics related to the biliary tract and cholangiopathies. The manuscript is nicely written and easy to follow. The Figures and tables are appropriate. A vast literature on these topics is comprehensively and succinctly covered and discussed. Aside from a few distracting typos identified in the manuscript, no significant concerns were raised. For example, errors on page 21, line 52; and page 17, line 31, and others should be corrected.

Response to Reviewer 2:
We thank the Reviewer for their positive comments regarding our manuscript. We have addressed the specified errors and have also read through the manuscript carefully in order to detect and correct other typographical and grammatical errors.

We would again like to thank the Reviewers and Editor for their time put forth in reviewing our manuscript. We believe the suggested changes improved the readability of our manuscript and are hopeful for a positive outcome following resubmission.

Sincerely,
Lindsey Kennedy



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Gallstone and Gallbladder Disease: Biliary Tract and Cholangiopathies

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ABSTRACT

Cholestatic liver diseases are named primarily due to the blockage of bile flow and buildup of bile acids in the liver. Cholestasis can occur in cholangiopathies, fatty liver diseases and during COVID-19 infection. Most literature evaluates damage occurring to the intrahepatic biliary tree during cholestasis; however, there may be associations between liver damage and gallbladder damage. Gallbladder damage can manifest as acute or chronic inflammation, perforation, polyps, cancer and most commonly gallstones. Considering the gallbladder is an extension of the intrahepatic biliary network, and both tissues are lined by biliary epithelial cells that share common mechanisms and properties, it is worth further evaluation to understand the association between bile duct and gallbladder damage. In this comprehensive review, we discuss background information of the biliary tree and gallbladder, from function, damage, and therapeutic approaches. We then discuss published findings that identify gallbladder disorders in various liver diseases. Lastly, we provide the clinical aspect of gallbladder disorders in liver diseases and ways to enhance diagnostic and therapeutic approaches for congruent diagnosis.

DIDACTIC SYNPOSIS:

Major teaching points:

- The gallbladder is a specialized organ that plays roles in bile modification and digestion of fats.
- Gallbladder damage can manifest as acute or chronic inflammation (cholecystitis), perforation, polyps, cancer, and more commonly gallstones (cholelithiasis).

- The gallbladder epithelial cells closely resemble those of the intrahepatic biliary tree, but distinct differences may account for specialized functions.
- Bile duct damage characterized by inflammation, fibrosis and ductular reaction can be found in primary sclerosing cholangitis (PSC), primary biliary cholangitis (PBC), alcohol-related liver disease (ARLD), non-alcoholic fatty liver disease (NAFLD), cholangiocarcinoma (CCA) and COVID-19.
- There is an association between gallbladder disorders and bile duct damage, but direct links are unknown.
- In some liver diseases, having congruent gallbladder damage increases morbidity and mortality in patients.
- Current work is underway evaluating different modalities that may be beneficial for the diagnosis or treatment of gallbladder disorders, specifically in the setting of liver disease.

DIDACTIC FIGURE LEGENDS:

- **Figure 1:** This figure labels the different parts of the gallbladder and the connected extrahepatic bile duct.
- **Figure 2:** This figure illustrates the different layers of the gallbladder wall and highlights some key receptors and transporters that maintain gallbladder functions.
- **Figure 3:** This figure illustrates some differences and similarities between acute and chronic cholecystitis.
- **Figure 4:** This figure illustrates the main gallbladder disorders discussed in this review and the main characteristics associated with them.

- **Figure 5:** This figure labels the human and mouse biliary tree and stem cell niches.
- **Figure 6:** This image shows an enlarged gallbladder in a PSC patient versus control.
- **Figure 7:** This photomicrograph shows a gallbladder stone and its needle-like crystals found in the gallbladder of *Mdr2*$^{-/-}$ mice.
- **Figure 8:** This image shows the layers of the gallbladder wall with corresponding tumor stage for gallbladder cancer.
- **Figure 9:** These graphs show changes in fasting gallbladder wall thickness and ejection fractions in control, steatosis and NASH patients.
- **Figure 10:** These images show low and high magnification of chronic cholecystitis in a patient with ARLD.
- **Figure 11:** This figure shows an inflamed liver in a patient with COVID-19 and qRT-PCR analysis confirming SARS-CoV-2 expression in the gallbladder with positive controls run as well.

INTRODUCTION ON THE GALLBLADDER

I. Gallbladder anatomy and function

Anatomically, in humans the gallbladder is in the upper abdomen beneath the liver, and in mice, it is attached with the diaphragm via connective tissue and is situated between the left and right medial lobes of the rodent liver (1). Cholangiocytes are ciliated epithelial cells that line the biliary tree and line the lumen of the hollow gallbladder in both humans and rodents. Bile is synthesized by hepatocytes and is drained into the biliary tree which acts as a conduit for bile flow. Bile flows through the intrahepatic biliary network and is stored in the gallbladder until its eventual drainage into the common bile duct, that is connected to the gallbladder. The fundus, the widest

part of the gallbladder, gradually narrows and tapers to form the infundibulum which eventually connects with the cystic duct that joins the common hepatic duct to form the common bile duct (Figure 1) (1). Bile, after being secreted from the gallbladder, travels to the duodenum via the hepatopancreatic ampulla where the common bile duct and pancreatic duct merge to make entry into the duodenum. Bile secretion from the gallbladder, known as gallbladder emptying, is regulated by the gastric hormone, cholecystokinin (CKK). CKK regulates the contractility of the gallbladder thereby regulating the emptying process (2). Apart from the contribution of cholesterol, gallbladder contractility or gallbladder emptying can be another cause for gallstone formation. Gallbladder contractility (emptying and filling) is regulated by the entero-hormone, CCK, and fibroblast growth factor (FGF)15 (in mice) and FGF19 (in human) respectively. CCK receptors are predominantly present in the muscularis (smooth muscle) of the gallbladder and are affected by high cholesterol levels. High circulating and membranous cholesterol induces hypomotility in the gallbladder (3). CCK-1 receptors were found to be sequestered by elevated cholesterol levels in a caveolin-3 dependent pathway (4). Sequestration of CCK-1 receptors would result in reduced gallbladder emptying and can result in increased risk of gallstone formation. Small and large cholangiocytes, which are distinct in structure and function, line the small and large bile ducts of the intrahepatic biliary tree in mice, which will be discussed in detail below. Cholangiocytes that line the gallbladder bear more resemblance to large cholangiocytes in mice.

Besides storage of bile, the gallbladder also functions to concentrate the composition of bile by reabsorption of water and various biliary constituents, such as bile acids (BAs) (5). This procedure of altering bile composition requires the intricate functioning of membrane transport across the biliary epithelium which have been the

focus of several early studies. One of the earliest studies by Diamond *et al.* in 1964 showed that the gallbladder regulates the concentration of bile by modulating isotonic reabsorption of water and sodium chloride through an active process (6). There are thirteen aquaporin (AQP) channels responsible for water absorption throughout the biliary tract, including the gallbladder (7). Among these channels, AQP1 and AQP8 are the two most widely expressed channels in the gallbladder epithelium (8); however, there are conflicting reports regarding the localization of AQP1 and AQP8 in the gallbladder. One study emphasizes profuse expression of AQP1 on the apical membrane of the gallbladder epithelia (9), another study reports that AQP1 is expressed on both apical and basolateral membranes with AQP8 being expressed mainly in the apical membrane of the gallbladder epithelial (10). AQP1 knockout (*AQP1*$^{-/-}$) mice have similar sized gallbladders as their wild-type (WT) controls, but had a significant difference in water permeability (9). Similarly, AQP8 may be involved in water absorption from the gallbladder, yet *AQP8*$^{-/-}$ mice didn't have significant physiological defects compared to WT controls (11). Defects in other AQPs can lead to dysfunctional water absorption and clinical conditions including cholestasis, obesity, and insulin resistance (12, 13). From the existing genetic knockout studies, it can be surmised that AQPs have far reaching effects in the liver and gallbladder.

The gallbladder also secretes mucin and bicarbonate. Mucin secretion occurs because of calcium-dependent pathway and bicarbonate secretion is mediated by adenosine 3',5'-cyclic monophosphate (cAMP)-dependent pathway. Both constituents are essential to exert cytoprotective effects on the gallbladder epithelia against toxic BAs. An electrogenic anion secretion study in isolated human gallbladder mucosa from normal and cystic fibrosis patients revealed that anion secretion in the gallbladder is facilitated by extracellular adenosine triphosphate (ATP) via purinergic receptor Y2

($P2Y_2$). This mechanism explains the altered and more toxic biliary composition during cystic fibrosis thereby contributing to hepatobiliary complications (14). Cystic fibrosis transmembrane conductance regulator protein (CFTR), the gene impaired in Cystic Fibrosis, regulates ion transport in the biliary epithelia. CFTR is a chloride channel regulated by the intracellular and extracellular concentration of cAMP. Its profuse localization in the apical membrane of biliary epithelia, including the gallbladder, is an indication of its significant role in regulating other ion channels. Ether-a-go-go-related gene 1 protein potassium channel is a voltage gated ion channel located in gallbladder smooth muscle which regulates contractility by modulating membrane potential (15). Taken together, the gallbladder physiology is mainly maintained by these ion channels that regulate transepithelial ion transport.

Just like the rest of gastrointestinal tract, the gallbladder is profusely innervated from both the central nervous system and enteric nervous system and primarily regulated by a ganglionic plexus located on the wall of the gallbladder fundus. An early study on guinea pig gallbladder suggests that the organ is constituted of four main layers of tissues: (i) the mucosa, (ii) the muscularis, (iii) the perimuscular fibrous tissue and (iv) serosa which is the layer of subperitoneal connective tissue (16). Each of these layers are highly innervated by the cholinergic neurons, these neurons also express neuroendocrine factors like substance P, neuropeptide Y and somatostatin. In addition to the presence of cholinergic neurons, the gallbladder was also found to express purinoreceptors (P2X), P2X2 and P2X3, that mainly signal via ATP (17). By immunohistochemistry, it was found that in guinea pigs the P2X2 and P2X3 receptors were expressed in the ganglia of the nerve fibers in the gallbladder. Moreover, this study highlights that nerves that stained positive for alpha calcitonin gene related peptide were also positive for P2X2 and P2X3 receptors (9). The role of these

neuropeptides in modulating gallbladder physiology is not well studied; however, it can be surmised from the existing studies that complex neuropeptide signaling in the highly innervated gallbladder plays an important role in gallbladder emptying and transepithelial ion channel transport that can influence the composition of bile. The gallbladder is a dynamic contributor to bile flow, physiology, and composition due to its expression of these different transporters and receptors (Figure 2).

II. *Gallbladder disease and gallstones*

Most gallbladder diseases occur because of dysfunctional bile secretion, including the malabsorption of ions and water in both the intra- and extra-hepatic cholangiocytes. However, inflammation and epithelial overgrowth can lead to various gallbladder disorders as well. Another widely prevalent cause of gallbladder diseases is a poor diet, which mainly manifests as gallstones, or cholelithiasis. Gallbladder-related diseases will be discussed in the following sections.

a. Gallbladder inflammation (cholecystitis)

Cholecystitis (i.e., gallbladder inflammation) is a multifactorial disorder, and one of the main causes of gallstone formation. Most gallstone cases lead to blockage of the cystic duct, resulting in bile accumulation that promotes inflammation (18); however, other biliary tract disorders, such as tumors and certain infections can promote cholecystitis (19, 20). In this section, we will focus on pathophysiology, diagnosis, and treatment of the most common gallbladder diseases, such as acute cholecystitis, chronic cholecystitis, and gallbladder perforation.

i. Acute cholecystitis

Acute cholecystitis is acute inflammation of the gallbladder due to obstruction of the cystic duct (21). The cystic duct can be blocked from gallstones or biliary sludge formation. Other less common causes can be due to the presence of a mass (primary

tumor or gallbladder polyp), parasites, or foreign bodies (22-24). Once the cystic duct is blocked, the gallbladder mucosa continues to produce mucus that is not drained, and the intraluminal pressure inside the gallbladder increases leading to an acute inflammatory response. Additionally, the secretion of prostaglandins, I_2 and E_2, can promote an inflammatory response (25). The pathophysiology of acute cholecystitis is characterized by three processes: (i) mechanical stimulus (gallbladder duct obstruction); (ii) bacterial infection; and (iii) irritation that promotes inflammation (18). There are two theories attempting to explain the pathogenesis of acute cholecystitis: (i) cystic duct obstruction and gallbladder artery occlusion (18), and (ii) cystic duct obstruction and perpetual lithogenic bile (26). In 2006, Yokoe *et al.* developed the Tokyo Guidelines for the management of acute cholangitis and cholecystitis (27) that were approved as worldwide criteria. Specifically, patients with acute cholecystitis have right upper quadrant or epigastric abdominal pain, Murphy's sign, and tenderness. If gallbladder inflammation persists, patients show fever, high levels of C-reactive protein, and abnormal white blood cell count. Finally, different imaging approaches can be used to diagnose acute cholecystitis, such as transabdominal ultrasonography (US), cholescintigraphy, and magnetic resonance imaging (MRI); however, US and cholescintigraphy are used most frequently (27). One approach to manage acute cholecystitis is reduction of gallstones in the gallbladder that move into the cystic duct. If there is not proper breakdown of the gallstones, complications may occur; such as, advanced cholecystitis or gallbladder perforation (25). Acute cholecystitis management includes (i) fasting to reduce the stress of inflamed gallbladder, (ii) rehydration with intravenous fluids, (iii) antibiotics to counteract the infections, (iv) administration of analgesic for pain, (v) procedures to remove gallstones through medication (indomethacin (28) and diclofenac (29)) and/or removal

of the gallbladder (cholecystectomy, laparoscopic cholecystectomy), which is the gold standard approach (30).

ii. Chronic cholecystitis

Chronic cholecystitis is characterized by continual inflammation of the gallbladder that drives mechanical and physiological dysfunction (31). Over 90% of chronic cholecystitis cases are associated with gallstone blockage in the cystic duct, leading to abdominal pain (biliary colic), episodic waves of epigastric pain, and discomfort (21). Studies show that lithogenic bile may promote gallbladder damage through free radical formation from hydrophobic BAs that, together with the reduction of the mucosa protection, induce a continuous inflammatory state (32, 33). Furthermore, the reduction in CCK receptor expression in the smooth muscle impairs gallbladder contraction leading to stasis and damaging lithogenic bile formation (31). Histological analysis showed that the gallbladder from patients with chronic cholecystitis has increased subepithelial and subserosal fibrosis, followed by mononuclear cell infiltration (21). Patients with chronic cholecystitis have continuous right upper abdominal pain that can extend into the back. Other symptoms include nausea, vomiting and anorexia (31). Hepatobiliary scintigraphy (34) or a hepatobiliary iminodiacetic acid scan with CCK (31) are the major imaging procedures used to confirm the presence of chronic cholecystitis. The gold standard procedure to treat this disorder is laparoscopic cholecystectomy, which is characterized by low morbidity and invasiveness (21, 31). Differences and similarities in acute versus chronic cholecystitis are shown in Figure 3.

iii. Gallbladder perforation

Gallbladder perforation is characterized by a hole or an opening in the gallbladder wall usually as a complication of acute cholecystitis. Gallbladder

perforation has high morbidity and mortality rates due to delays in diagnosis (21, 35, 36). Usually, a calculus is formed which blocks the drainage of bile from the cystic duct which increases intra-cholecystic pressure, epithelial injury, secretion of phospholipases, degradation of cell membranes, and intense inflammatory reaction, resulting in gallbladder perforation (37). Several studies observed that the most frequent site of perforation is the fundus (35, 38). Niemeier (1934) classified gallbladder perforation into three types: Type I, acute perforation into the free peritoneal cavity; Type II, subacute perforation where the perforated peritoneal cavity of the gallbladder is surrounded by an abscess; and Type III, chronic perforation with the presence of fistulous communication between the gallbladder and some other viscus (39). This classification was based on clinicopathological findings and was criticized by different studies. For instance, Anderson *et al*. reported a case series of cholecystobiliary fistulae and classified them as Type IV gallbladder perforation (40). The difficulty in diagnosing gallbladder perforation and distinguishing it from acute cholecystitis are documented (36, 41). Morbidity and mortality rates of gallbladder perforation are high due to delays in both diagnosis and treatment. Gallbladder perforation treatment includes cholecystectomy, drainage of abscess, if present, and abdominal lavage (35). In sum, an earlier diagnosis and immediate surgical intervention may reduce morbidity and mortality rates.

b. Gallbladder polyps

Gallbladder polyps are an elevation of the gallbladder mucosa that extends into the lumen (42, 43). Polyps may be classified between “true” and “pseudopolyps” based on earlier pathological descriptions (42). True gallbladder polyps are adenomas of the gallbladder wall that can progress into malignant phenotypes. Indeed, they can be

categorized as benign (fibromas, lipomas, and leiomyomas) or malignant (mesenchymal neoplasms, lymphoma, or metastases). Pseudopolyps do not have malignant potential and are categorized as cholesterol pseudopolyps, focal adenomyomatosis, and inflammatory pseudopolyps (42, 44). The progression of non-malignant gallbladder polyps to malignancy is characterized by different risk factors, including polyp size, Primary Sclerosing Cholangitis (PSC), Indian ethnicity, sessile polyps, gallstones, and gallbladder wall thickening (44, 45). The diagnosis of gallbladder polyps mostly occurs on accident during imaging (transabdominal ultrasound, multiparametric ultrasound, and endoscopic ultrasound) for diagnosis of intermittent right upper quadrant pain, nausea, and vomiting (46). According to the size of the polyps and the medical history of the patient, the management of gallstone polyps may be different. Briefly, if the polyps are 6-9 mm in a patient with the risk factors descried above, cholecystectomy is recommended; however, if the patient has 6-9 mm gallbladder polyps and do not have any risk factors, serial US examinations are required at 6 months, 1 year and then early up to 5 years to monitor size (44, 47).

c. Gallbladder cancer

Gallbladder cancer is the most common malignancy of the biliary tract with poor diagnosis and variation in incidence across the world (48, 49). Epidemiological studies observed that Native Americans and Southeast Asians are at a higher risk to develop gallbladder cancer, followed by Eastern European including Polish, Czech, Slovakian, and Asian. On the other hand, South Americans of Indian descent, Israeli and Japanese persons have shown moderate risk of gallbladder cancer development (48, 50, 51). This variability on the onset of gallbladder cancer is due to the combination of environmental and genetic factors. Indeed, women have a higher risk to develop gallbladder cancer compared to men (female:male ratio ~2.6:1), especially over 50

years of age (51). The enhanced incidence of gallbladder cancer in women is likely due to higher estrogen levels, which promotes the formation of gallstones through increasing cholesterol saturation in bile (52). Furthermore, there are other risk factors that can increase gallbladder cancer incidence, including body mass index (BMI), family history, cholelithiasis or other benign gallbladder pathologies, chronic infection with *Salmonella* or *Helicobacter pylori*, anomalous pancreatobiliary duct junction, porcelain gallbladder, gallbladder polyps, and obesity. Lastly, secondary risks factors including tobacco consumption, chemical exposure (benzene), high carbohydrate intake, and chronic diarrhea can influence gallbladder cancer risk (50, 51). The symptoms of gallbladder cancer are very vague and mimic biliary colic, making it difficult to diagnose; however, the advanced stage of gallbladder cancer is characterized by weight loss and jaundice, and imaging approaches can help in the identification of the tumor mass (49, 51). According to the American Joint Committee on Cancer's 8th edition, the staging of gallbladder cancer is divided into tumor (T) and lymph node (N) categories (53). Specifically, the T categories describe the tumor penetration levels within the gallbladder wall and the N categories describe the number of metastases in the lymph nodes (51, 53). Gallbladder cancer can be treated by chemotherapy, targeted therapy, and surgery (54). Early-stage gallbladder cancer patients can undergo surgical resection, but most of the diagnosis occurs when the cancer is advanced. In this case, gallbladder cancer patients undergo chemotherapy and a series of surgical procedures to improve their lifespan (49, 51, 54).

d. Gallstones (cholelithiasis)

Cholelithiasis is the clinical manifestation of concreted bile salts, bilirubin and sterols in the gallbladder or common bile ducts popularly known as gallstones or bile duct stones, respectively. Cholelithiasis is a disorder involved in many liver diseases,

and thus most of this chapter will be spent discussing the intricacies of this injury. Over time, cholelithiasis leads to multiple compactions resulting in an inflamed gallbladder, or cholecystitis (described above). Gallstones are formed in the gallbladder and/or intrahepatic bile ducts and sporadically move into the common bile duct or the intestines (55, 56). The presence of gallstone disease has an incidence rate of about 10% to 20% in the adult population (56, 57). Cholelithiasis can be symptomatic or asymptomatic depending on the lithiation or stone formation stage (58). The major factors leading to the formation of gallstones include defective gallbladder motility, metabolism and secretion of cholesterol and BAs (59). The gut microbiota is also involved in the regulation of BA metabolism and composition of the BA pool, contributing to gallstone formation (60, 61).

i. Types of gallstones (cholelithiasis) and formation

According to the composition of major constituents, gallstones are categorized into three types: pure cholesterol stones, pure pigment stones and mixed stones (62). Cholesterol gallstones are estimated to account for more than 80% of gallstones diagnoses (63). Several studies analyzing the composition of surgically removed gallstones found that cholesterol gallstones are the dominating cause of clinical gallstone disease (64). In a German study, cholesterol was observed to be the main constituent in 93.3% of gallstones, and pigment was in 5.5% of gallstones (65).

The origin of cholesterol gallstones has common pathogenic links with broad metabolic abnormalities characterized by altered cholesterol homeostasis, such as obesity, dyslipidemia, type 2 diabetes, NAFLD and the metabolic syndrome (56, 66, 67). In fact, many of these metabolic disorders have been associated with an elevated occurrence of cholesterol gallstones (68, 69).

Pigment stones are mainly constituted by calcium bilirubinate and can further be classified into black and brown stones (70). Black pigment stones are often related to physiological and pathophysiologic conditions including increased production of unconjugated bilirubin and hemolysis, and clinical conditions such as cirrhosis, spherocytosis, thalassemia, sickle cell disease, and malaria (70, 71). There is a higher incidence of black pigment stones than cholesterol gallstones in developing and Asian countries (72-74); however, the prevalence of cholesterol gallstones is increasing in Asia due to an increase in Westernized lifestyle (73). Brown pigment stones, which contain more cholesterol and fatty acids (FAs) than black pigment stones (75), are observed in the hepatic ducts and believed to be caused by cholangitis, biliary stasis (76, 77), or parasitic infestations (71). Brown pigment stones are not as common in Western countries as they are in Asia (78, 79). A figure summarizing the main gallbladder diseases can be found in Figure 4.

ii. Genetic risk factors of cholelithiasis

Just like other gastrointestinal disorders, risk factors for gallstone formation include both genetic and environmental components. Cholelithiasis is a complex polygenetic disease since the association between some gene variants and gallstone formation have been verified. For example, the single nucleotide polymorphisms of the genes HHEX (rs1111875), MC4R (rs17782313), MAP2K5 (rs2241423) and NRXN3 (rs10146997), were positively associated, but FAIM2 (rs7138803) was negatively associated with the occurrence of gallstone disease (80).

Extensive genetic analysis also identified a gallstone (*Lith*) gene map that is essential for the formation of gallstones. *Lith1* is one such gene that affects cholesterol-induced gallstones in mice (81). By using gallstone-susceptible mice (C57BL/J) and gallstone-resistant mice (AKR/J), it has been identified that *Lith1* and

Lith2 are related to gallstone formation. *Lith1* is involved in the regulation of liver cholesterol hypersecretion, and *Lith2* plays a role in the bile salt-dependent bile flow (82). In human species, ATP-binding cassette subfamily G 5 (*ABCG5*) and *ABCG8* are homologous to murine *Lith1* and *Lith2. ABCG5* and *ABCG8* are expressed in hepatocytes and intestinal cells and can be transported from the endoplasmic reticulum to the apical membrane as heterodimers (83). ABCG5/G8 can transport neutral sterols into bile in hepatocytes or promote cholesterol efflux from the enterocyte back to the intestinal lumen for fecal excretion (84). When ABCG5/G8 is inactivated, reduced efflux of cholesterol into bile results in increases cholesterol levels in plasma and liver. While knockdown of *ABCG5/8* may be a deterrent to gallstone formation by attenuation of cholesterol secretion, overexpression of *ABCG5/G8* may increase cholesterol levels in the gallbladder, thus enhancing the likelihood of cholesterol crystal formation (85). Furthermore, *ABCG5/G8* was observed to be related to cholesterol gallstone prevalence in patients, and the gallstone associated variants in *ABCG5/G8* (*ABCG5-R50C* and *ABCG8-D19H*) were found in German, Chinese, Chilean and Indian populations. Overall, these findings show that these two genes influence gallstone disease.

Even though ATP-binding cassette subfamily B member 11 (*ABCB11*) and liver X receptor alpha (*LXRA*) are in the interval of the *Lith* locus, no genetic susceptibility of gallstone formation was associated with these two genes in the German samples tested (86). *Lith6* is another locus in the gene map which has two functional candidate genes associated with it, apolipoprotein B mRNA-editing protein (*APOBEC1*) and peroxisome proliferator-activated receptor gamma (*PPARG*) (87, 88). Like the previous study, analysis of German patient samples did not find an association of *APOBEC1* or *PPARG* with gallstone susceptibility. More analysis and mapping of *Lith1*

and *Lith6* loci are needed to identify more variants of gallstone susceptibility in humans (88).

The apolipoprotein E4 allele is related to the prevalence of gallstone disease. The E4 allele was found to be positively associated with gallstone disease in a meta-analysis of Chinese Han populations (89). Another study showed no correlation between apolipoprotein E genotypes and gallstone disease in a Danish population (90). No significant associations for E4 allele carriers were found in mixed ethnic populations or in white populations by meta-analysis (90). Meanwhile, conflicting results were reported for the E4 association in Hispanic and Spanish populations (91, 92). In fact, the apolipoprotein E plays an important role in the regulation of the response to dietary cholesterol and cholesterol excretion into bile as evidenced in knockout mice (93). However, no influence on bile cholesterol excretion was found due to the E4 carrier state in Caucasians with gallstones (94).

Young human adults with ATP binding cassette subfamily B member 4 (*ABCB4*) gene mutations present with low phospholipid levels in bile, which is associated with cholelithiasis (95). Mutations in mucin (*MUC*)-related genes have been extensively studied to elucidate the role of mucin in the development of gallstones. For example, *MUC5AC* encodes for a gel forming mucin that, when in excess, can promote gallstone concretion that is heavily influenced by interleukin (IL)-1β (96, 97). Tumor necrosis factor alpha (TNF-α) was also found to be induced by prostaglandin 2 which, in turn, induced the over expression of *MUC2* gene that is involved in gallstone formation (97).

iii. Lifestyle and cholelithiasis

An increase in alcohol consumption was inversely related to occurrence of gallstone disease in females (98). The negative correlation between alcohol

consumption and cardiovascular disease may explain the protective effects of alcohol consumption on cholesterol homeostasis (99). These benefits are attributed to increased cardio-protective blood levels of high density lipoprotein cholesterol and an increase in BAs (100). Other preventive mechanisms of alcohol consumption on gallstone formation include enhanced gallbladder motor function together with stimulation of contractions, thus reducing bile stasis and gallstone formation (101). Interestingly, a higher daily alcohol consumption was related to faster self-reported gut transit (102) and acute administration of alcohol was shown to stimulate propulsive pressure waves in the ileum but suppress impeding pressure waves in the jejunum (103). Therefore, the protective effects of alcohol consumption on gallstone disease may be due to the inhibition of secondary BA entry in the enterohepatic circulation.

Physical activity seems insignificant to gallstone disease. In a randomized controlled trial, an intervention of moderate or vigorous physical activity in pregnant women showed no influence on gallstone formation (104). Further, in the subgroup diagnosed with gallstones while being unaware of their status, physical activity was negatively related to clinical gallstone disease hospitalization when compared to a sedentary lifestyle (105). Furthermore, gallstone disease was inversely associated with physical activity in cohort studies (106). However, physical activity increases plasma CCK that enhance gallbladder contractions (107). These mechanisms may explain how physical activity exhibits beneficial impacts on pain related to gallstone disease.

iv. Obesity, weight loss and cholelithiasis

It was observed that gallstone disease is associated with certain body fat tissue (except BMI), such as: waist-to-hip circumference ratio with screen-detected gallstone disease, and computed tomography that measured visceral or subcutaneous fat with

clinical gallstone disease (108, 109). However, many other studies demonstrated the association between elevated BMI and gallstone formation, indicate BMI as an independent risk factor for the development of gallstone disease (110, 111). It has been estimated that a rise of more than 5 points of the BMI value increases the risk of gallstone disease by 1.63-fold (112). This correlation has been positive for females, but for males there is a lower association (113). This kind of variability may be attributed to the greater part of lean mass in men compared with women (113). It must be considered that there are other predominant factors such as estrogen levels in females, which can increase the synthesis and secretion of hepatic cholesterol, along with greater cholesterol saturation index and crystals formation, which make gallstone disease more prevalent in female patients (58).

On the other hand, excessive weight loss due to calorie restriction is also related to gallstone disease (114). There is more risk for incident screen-detected gallstone disease in patients undergoing bariatric surgery followed by rapid weight loss (115). The underlying mechanisms for gallstone disease prevalence during rapid weight loss may include an initial increase of bile cholesterol saturation, as well as impaired gallbladder motor function (116).

v. Estrogen and cholelithiasis

It has been reported that females are more predisposed to gallstone disease (98). This may be due to the binding of 17β-estradiol to intracellular estrogen receptors in the liver stimulating the excretion of cholesterol into bile, resulting in increased bile cholesterol saturation (117). Estrogen also promotes the activity of β-Hydroxy β-methylglutaryl-CoA (HMG-CoA) reductase to facilitate endogenous cholesterol synthesis (117). In one study, women with higher urinary estrone levels had a higher risk of gallstones disease (118). Similarly, hormone-replacement therapy promotes

increased bile cholesterol saturation in postmenopausal women (119). Overall, bile cholesterol saturation may play a key role in female gallstone disease.

vi. Microbiome influence on cholelithiasis

An increasing number of studies have shown the important role of the gut microbiome on cholelithiasis (61, 120). These complex microorganisms also exist in bile and the prevalence of gallstones is closely associated with abnormalities in bile duct flora. The microbiota of the gastrointestinal and biliary tracts are involved in almost all stages of bile formation, such as the regulation of cholesterol metabolism, lipid metabolism, biotransformation and enterohepatic circulation of BAs (121).

Studies have demonstrated the existence of living bacteria in gallstones. Microorganisms can enter the bile duct system from the duodenum via migration through the sphincter of Oddi, and they can also spread through the blood to the liver and next into bile (122). Microorganisms play a critical role in bile as nucleating factors, resulting in the formation of cholesterol and pigment gallstones (123). Gallstone formation can be regulated by bacteria properties in the gallbladder. For example, bacteria producing β-glucuronidase and phospholipase promoted pigment gallstones, while bacteria causing mucus abnormalities promoted cholesterol stone formation (124). Biofilm-forming bacteria in the bile, gallbladder, and gallstones are closely related to gallstone formation (125, 126). By comparing cholesterol gallstones with pigment gallstones, gram-positive bacteria were common in most of cholesterol gallstones, but not observed in pigment stones. Furthermore, *Helicobacter pylori*, a Gram-negative, motile bacteria was found in patients with symptomatic gallstone disease (127). However, this finding is still controversial, and more research is necessary to elucidate the role of the microbiota in gallstone disease. There are a

variety of risk factors that are associated with gallstone disease (Table 1) that need to be considered.

vii. Mouse models of cholelithiasis

The role of diet and ion channels have been well studied in cholelithiasis, and diet-induced models of cholelithiasis have widely been used to explore the effects and contributions of different ion channels to the concentration of bile. A lithogenic diet, which is constituted of 15% dairy fat, 50% sucrose, 20% casein and 1% cholesterol, is fed to mice for 18 weeks to induce cholelithiasis; however, various mouse strains respond differently where 100% of the C57BL/J and A/J strain were susceptible to and developed gallstones (81). Even though mucin has been highlighted to form a protective barrier in the gallbladder, studies in hamsters have reported that over secretion of mucin precedes gallstone formation in a lithogenic diet-induced model of gallstone formation (128). From other existing studies on animal models, it can be concluded that mucin is an important constituent of the gallstone matrix. In highly concentrated bile, gallbladder mucin can accelerate cholesterol monohydrate nucleation, a process that constitutes gallstone formation (129-131). There are several genes related to mucin expression such as *MUC1* and *MUC2* in the gallbladder that pose a genetic risk factor for gallstone initiation, as discussed above (132, 133).

Impaired lipid metabolism in the liver can translate to gallstone formation. A murine model with genetic knockout of liver-specific fatty acid binding protein 1 (*L-Fabp*$^{-/-}$ mice) fed with lithogenic diet for 2 weeks became significantly hypercholesterolemic along with developing more gallstones compared to the WT mice fed with lithogenic diet (134). *L-Fabp*$^{-/-}$ mice fed with chow diet also had increased fecal BA excretion and decreased ileal apical sodium-dependent bile acid transporter (*Asbt*) expression compared to the *L-Fabp*$^{-/-}$ mice fed with lithogenic diet, indicating

that enterohepatic shunting of BAs contributed to gallstone formation in this model (134). Knockdown of fatty acid transporter 2 (*Fatp2*$^{-/-}$ mice), which is also expressed in the gallbladder and the liver, showed reduced triglyceride content in the gallbladder and improved contractile strength in mice exposed to lithogenic diet (135). *Fatp2* is encoded by the solute carrier family 27-member 2 gene and knockdown by adeno associated virus (AAV) reduced gallstone formation in mice fed with lithogenic diet for 8 weeks (84). Interestingly, *Fatp2* knockdown did not affect cholesterol concentration and solubility in bile, but instead increased FA content in bile [83]. Although the authors did not elucidate the involvement of a specific pathway for Fatp2 mediated effects, they did highlight the role of prostaglandins in mediating gallbladder contractility [83].

CLINICAL ASPECTS OF GALLBLADDER DISEASE

I. Background

Gallstones represent the most prevalent disease of the biliary tract in the Western world, affecting 10-15% of the general population (136, 137). Changes in prevalence are observed according to gender and ethnicity (138) with Pima Indians exhibiting a historically higher rate of the gallstones with ~50% of adults affected (139). The economic burden of gallstone treatment is also significant (>$5 billion per year in the U.S.) and seems to be increasing (136). Gallstone-related mortality is declining and is relatively low (approximately 0.6%) but given the frequency of the disease, as reported in a 1979-2004 U.S. analysis, more than 1,000 patients per year die due to gallstone disease (140).

II. Symptomatic gallstones

Symptomatic gallstones are generally regarded as a condition requiring treatment since they have an increased risk of developing complications. As reported

previously, symptoms may be vague and not directly drawing attention to gallstones; however, prompt recognition and diagnosis may prevent conditions with significant morbidity and mortality, as reported in the following paragraphs.

III. Asymptomatic gallstones

Despite the difficulties in defining asymptomatic or symptomatic gallstones, the differences in the natural history of these two classes has been an argument for some time (141). In early studies on cholelithiasis, the estimated risk to develop symptoms was 1-2% yearly (142, 143). Onset of complications was ten times lower in asymptomatic patients (0.1-0.3% yearly) in comparison with symptomatic cases (144). In asymptomatic populations, the risk of treatment (typically surgical) is reportedly higher than the benefits (145, 146) and current guidelines do not suggest an operative approach for this subset of patients. Generally, observation of patients for onset of symptoms is advised (144, 147); however, exceptions may exist to this strategy. The most important exception in general practice is represented by porcelain gallbladder (148). This condition was historically linked to a significant risk in developing gallbladder cancer. Porcelain gallbladder consists of calcium deposition on the gallbladder wall (easily detected on US or computed tomography [CT] scan) that may present as complete or selective, with the latter form preferentially associated with malignancy. The high rate of cancer reported for this condition in early studies (12-33%), has been challenged by more recent data observing a lower rate of malignancy (≤6%) (148). Systematic gallbladder removal in patients with porcelain gallbladder remains controversial and consideration on a case-by-case evaluation seems wiser.

IV. Diagnosis

a. Symptoms and manifestations

Knowing the symptoms of gallstones in patients is of paramount importance to help distinguish between the two main clinical presentations, asymptomatic and symptomatic gallstone disease. For the past century, it is understood that the majority (nearly 70%) of gallstones are asymptomatic in nature, thus patients that complain of gastrointestinal issues are usually considered for treatment (141). However, the specific symptoms related to gallstone disease are not completely defined. A large cross-sectional Italian study, enrolling nearly 30,000 patients and focusing on gallstone symptoms concluded that right hypochondrium and/or epigastric pain (i.e., biliary colic), together with scarce tolerance to fatty meal, were among the more specific complaints (149). When these signs were present in the lack of gastro-esophageal reflux disease, they were far more specific for the diagnosis of gallstones. Cholelithiasis may induce biliary colic (150), that includes pain radiation to the back (right scapula), can last for hours and is associated with vomiting and other gastrointestinal symptoms, due to stone impaction in the cystic duct. Another sign noted during physical examination is the exacerbation of pain when the medical examiner has their hand firmly kept under the costal margin of the right chest (i.e., Murphy maneuver). Despite these definitions, the ability to detect symptoms of cholelithiasis differs in geographic location leading to heterogenous rates of treatment, definition of relevant signs and guidelines (151).

b. Blood biochemistry and imaging

There are no specific blood markers for the diagnosis of symptomatic cholelithiasis. Common liver function tests (alkaline phosphatase) and/or general inflammation indexes (C reactive protein levels and white blood cell counts) may be increased based on complications and the site of gallstone impaction. Some tests may

help in identifying specific complications, and these will be described in the corresponding paragraphs.

Beginning in the early 1980s, US emerged as an easy and specific imaging system for identifying gallstone disease (152). This technique has also been instrumental in identifying the natural history of gallstone formation in both asymptomatic and symptomatic forms. Typical stone US findings are iperechoic wall with a posterior shadow and, despite technical advancement, this technique remains superior in comparison with CT (153). MRI and cholangio-MRI have also had important applications for imaging gallstones. In fact, cholangio-MRI replaced diagnostic retrograde cholangio-pancreatography for gallstone detection since it accurately reproduces the anatomical picture of the biliary tree without safety issues. MRI is usually used as an integrative imaging approach when symptomatic gallstones are ruled out by US, but the potential presence of biliary stones need to be examined.

V. The clinical picture

The clinical picture of cholelithiasis may change widely ranging from asymptomatic forms to life-threatening conditions. The historical division of patients in two main classes (asymptomatic and symptomatic), even if it does not recapitulate the entire clinical horizon, is considered helpful in giving a general indication in selecting subjects needing treatment. Symptomatic patients may present with several complications and require closer monitoring or intervention.

a. Acute cholecystitis

As reported by Friedman *et al.* (141), acute cholecystitis appears to be the most frequent complication of gallstones, involving approximately one out of ten symptomatic patients. While the exact combination of clinical, biochemical and imaging features unequivocally leading to acute cholecystitis diagnosis is not yet

defined, the presence of fever, right hypochondrium pain, increased inflammatory markers and finding of gallbladder thickening and stones at US usually lead to the diagnosis (154). In the absence of stone migration to the common bile duct (described in the next paragraph) surgical resection of gallbladder (cholecystectomy) is generally indicated. Contraindications to cholecystectomy include those of general surgery such as septic shock or severely impaired clinical conditions. Conservative management of acute cholecystitis in patients with limited symptoms, even if sometimes successful, is generally not advised since ~60% of these patients would later require surgery and approximately one third will experience complications (155, 156). Timing for surgery depends on patient symptoms and risk of complications; however, a Cochrane Review comparing early (within 7 days from symptoms) and delayed (>6 weeks from symptoms) cholecystectomy for acute cholecystitis did not find significant differences in patient outcomes (157). A shorter hospital stay has been suggested when early cholecystectomy is performed.

b. Gallstones in the biliary tract and related complications

Even if stone migration to the biliary tract is not canonically considered a complication, this condition, associated with cholelithiasis in 10-20% of cases, is responsible for the most serious adverse events (158, 159). Analyzing the Swedish GallRisks registry, it was found that ~25% of patients with common bile duct stones may experience complications (160) while spontaneous expulsion from the biliary tract into the intestines is also possible. Common bile duct stone diagnosis is generally ruled out by the increase in liver function tests (usually normal if stones are retained in the gallbladder and/or cystic duct) and imaging (either US or MRI). Since common bile duct stones may determine relevant sequelae including obstructive jaundice, cholangitis and pancreatitis, bile tract cleansing is generally advised by current

guidelines (158, 159). The most relevant adverse conditions determined by stone impaction in the biliary tract are reported below.

Gallstones are the most frequent benign cause of obstructive jaundice, which impairs the liver and other physiological functions (161). Regarding the kidneys, in a study including 20 patients with obstructive jaundice (duration ~2 weeks), signs of acute tubular necrosis were observed at histology despite normal renal tests (162). Obstructive jaundice may also impair hemodynamic stability, immune fitness and the intestinal barrier leading to possible endotoxemia (161). Finally, obstructive jaundice may lead to bacterial overgrowth in the biliary tract, thus determining cholangitis.

Cholangitis diagnosis has been generally related to the presence of fever with spikes in pain in the right hypochondrium and jaundice (Charcot's triad); however, these signs were found to be present in just 22% of patients with cholangitis (163). Mortality of this condition remains significant, approaching 5% of cases (164). Broad spectrum antibiotics and, in severe cases, prompt biliary decompression is advised.

Gallstones are regarded as the most important cause of pancreatitis being responsible for more than one third of cases (165). Also, small stones/cholesterol crystals may sometimes give rise to acute pancreatitis (166). Epigastric pain increased pancreatic enzymes, and demonstration of stones at imaging may rule out the diagnosis. Mortality may occur in ~30% of severe cases (167).

There is an apparent association between gallbladder disorders, gallstones and bile duct damage. The role and occurrence of gallbladder disorders in cholestatic liver disease will be described in the following sections.

INTRODUCTION ON THE BILIARY TREE

I. Biliary tree structure, function and physiology

a. Background

The biliary tree, named so due to the resemblance the structure has with the branches of a tree, refers to the network of ducts that transport bile from the hepatocytes to the gallbladder and intestines. This system is involved in metabolism, waste disposal, and the movement and recycling of nutrients in the body (168-170). Bile plays a crucial role in the digestion and absorption of FAs, it emulsifies FAs and allows the hydrophobic molecules to be absorbed and transported for use or storage (168). A small percentage of the bile is lost in feces, allowing for larger molecules that cannot be excreted through the kidneys to also be disposed (168). The remainder of bile is reabsorbed and sent back to the liver through a cyclic process called enterohepatic circulation (171). Finally, selected vitamins and minerals use the biliary excretory system as a shuttle to connect to tissues where they are needed (168). The gallbladder is a protrusion extending from the biliary tree, indicating close anatomical relationships, and 10-15% of gallstone patients also present with bile duct stones (172); therefore, it is important to understand the biliary system and related diseases and how they may intersect with cholelithiasis.

b. Anatomy of the biliary tree

The branches of the biliary tree start in the liver, joining with other branches over and over until the whole network combines to form a single duct. The total length of the branches of the biliary tree in humans would be about 2 km (173). Different zones of the biliary tree can be separated by their area, diameter, morphology or physiology (174); however, in this review we will use luminal diameter to separate the different regions. The smallest sized bile ducts that make up the biliary tree begin at the canals of Hering, starting at just a few nanometers in diameter and lined by hepatic progenitor cells (HPCs) (171, 173). These canals separate canicular bile secreting

hepatocytes from the epithelial cholangiocytes that line the rest of the bile ducts. HPCs play a role in liver regeneration following injury, thus their presence in the canals of Hering is advantageous for hepatic recovery (175). The canals of Hering meet to form ductules, which come together as interlobular ducts, then septal ducts, each of which have consecutively larger diameters (170, 176). At this point, area ducts measure 300-400 μm in diameter and connect to the larger segmental ducts (400-800 μm) (171). This is where the left and right hepatic ducts, named for the liver lobes they branch into, finally come together to form the single common hepatic duct, collecting all the bile fluid the liver secretes (176). These measurements are for humans, and it is important to note that in rodents, cholangiocytes are more simply divided into small and large subsets, named for their anatomical location on either the small (<15 μm in diameter) or large (≥15 μm in diameter) ducts (177).

The common hepatic bile duct exits the liver then either diverts to the gallbladder through the cystic duct or continues from the liver as the common bile duct (171). The common bile duct meets the pancreatic duct after passing through the wall of the upper small intestine, to make the hepatopancreatic ampulla (i.e., the ampulla of Vater) (170, 176, 178). The ampulla of Vater consists of the conjoining pancreatic and common bile ducts, the sphincter of Oddi, and an extrusion of papilla where bile is released into the duodenum (168, 170, 178).

Along the murine intrahepatic large ducts and the human large segmental ducts, small peribiliary glands sporadically line the luminal wall (170, 171). The peribiliary glands are defined by their location, their mucinous secretions and their own stem cell niche that is separate from the HPCs (170). Secreting directly into the lumen of the bile ducts, intramural peribiliary glands have a mucosal epithelium and line the duct walls (170). Conversely, extramural peribiliary glands, located in the periductal

connective tissue, have their own conduits that transport their seromucosal secretions to the large bile duct lumen (170). Peribiliary glands have also been identified in the crypts of the gallbladder epithelium (179), indicating similar yet heterogenous cholangiocyte functions in the biliary tree and gallbladder. Branching of the biliary tree and its specific stem cell niches are shown in Figure 5.

While the inner walls of the ducts are lined by epithelial cholangiocytes and scattered peribiliary glands, a fibromuscular layer of tissue lays beneath (170, 178). This layer is made up of fibrous tissue and smooth muscle fibers (178). Where the ducts meet with the duodenum, the muscles form the sphincter of Oddi, which controls the release of the contents into the intestine (170, 176, 178). Additionally, the blood supply for the ducts comes from a network of vessels stemming from the hepatic artery (173). This network of vessels surrounds the bile ducts and is termed the peribiliary plexus (PBP) (173, 180). The PBP provides nutrients to the bile ducts to allow for growth, but it also allows for an alternative enterohepatic circulation route for BAs to be recycled back to hepatocytes via cholangiocytes in a process called cholehepatic shunting (169, 173). The normal route of enterohepatic circulation and recycling of BAs is through intestinal absorption, and then delivery to hepatocytes where they are secreted again into the ducts (168, 169). Interestingly, there is a concept of a cholecystohepatic shunt whereby the gallbladder coordinates BA uptake from bile to the liver (181).

c. Cholangiocytes

The differing physiologies of the cholangiocytes allow for a high level of control to alter the flow and composition of bile. Cholangiocytes, much like other epithelial cells, are polarized, have a multitude of transport proteins, and have distinct basolateral and apical membranes (174, 182). On the basolateral side, they connect

to basement membranes (170, 174) and on the apical side of cholangiocytes, microvilli and cilia line the lumen, and between these cells, tight junctions maintain cell polarity. Certain disease states can result in an interruption in tight junctions, interrupting the flow of bile (171). While all cholangiocytes have diverse physiologies, the size and location of the cells influence their form and function.

Starting just after the canals of Hering, narrow canalicular ducts (about 10 μm) are lined by small cuboidal epithelial cholangiocytes, which have little resorptive and secretory abilities (174). The properties of small cholangiocytes rely heavily on altering intracellular levels of Ca^{2+}, where large cholangiocyte activities are more dependent on cAMP levels (174, 183). Large cholangiocytes are longer, have less microvilli and cilia on their apical membrane, and have a lower cytoplasm to organelle ratio. Most of the larger cells' intracellular space is taken up by rough endoplasmic reticulum, suggesting that large cholangiocytes play a more specialized, less variable role than their small counterpart (174, 183). Conversely, small cholangiocytes resemble progenitor cells, with a higher nuclei to cytoplasm ratio (183). Like bile ducts, the gallbladder is lined with specialized epithelial cells. As small columnar cells with moderate cilia present on the apical membrane, the morphology of the epithelial cells that line the gallbladder resemble an intermediate between small and large cholangiocytes (184).

All cholangiocytes have a primary cilium, a thin peninsula-like extension of the cell to maximize the surface area of the membrane (173, 174). These cilia sample the passing fluid, allowing cholangiocytes to act as mechano-, osmo-, and chemosensors, recognizing and responding to changes in bile (174). Further, cholangiocyte action can be spurred by a variety of molecules, including hormones, BAs, neuropeptides, and alterations in luminal pressure, the action being the alteration of intracellular Ca^{2+}

and/or cAMP, with downstream effects altering the composition of bile, initiating cholangiocyte proliferation, or even signaling the activation of immune responses (173). Interestingly, while gallbladder epithelial cells are not noted to have primary cilium, they are similarly sensitive to the contents of bile, with a focus on water and ion manipulation (5).

d. Bile formation and flow

Hepatocyte secretions generate the bulk of bile, with cholangiocytes only accounting for about 40% of the daily production (168, 174). Bile production is prompted due to a series of reactions initiated at the beginning of a meal, especially one high in FAs. As an emulsifier, bile is a critical facilitator of the absorption of hydrophobic FAs (171). Once delivered, micelles are created to enclose and transport the lipids through the body (168). Between the delivery of bile to the duodenum and being secreted by canalicular hepatocytes, bile composition, flow, and pH is monitored and altered through a variety of mechanisms, including alterations controlled by gallbladder epithelial cells (185).

Previous cholehepatic research has defined two types of bile flow: BA-dependent flow and BA-independent flow (186). As previously stated, hepatocytes are the main facilitators of BA-dependent flow as the main producers and recyclers of BAs (187). For instance, hypercholeretic bile salts, such as the conjugated secondary bile salt nor-ursodeoxycholic acid (nor-UDCA), increase bile flow (171). This is especially noteworthy, as the composition of BAs has been noted to be linked to gallbladder motility (185). It is unknown if gallbladder hypomotility, or an increase in secondary BAs resulting in decreased biliary flow is the primary action, but the two have been highly correlated (185). Conversely, cholangiocytes support BA-independent flow (171, 186, 188).

Bile mostly consists of water, with only about 5% of the volume being attributed to other materials (171). At any time, bile can be composed of BAs, cholesterol, amino acids, glucose, steroids, enzymes, vitamins, and even heavy metals (168, 171, 187). Xenobiotics and toxins can also be present in bile (168, 171, 186). The biliary tract also acts as direct transport to the gut, where immunoglobulin A secreted in bile can protect against pathogens and promote symbiotic microorganisms (171, 189, 190). Other substances that use the biliary tract for transport elsewhere in the body include hormones and pheromones, as well as a number of vitamins (171). Even with all the other constituents within bile, BAs are the most abundant component (187). While the main function of the gallbladder is to pull water out and concentrate bile, the composition of BAs also influences the motility of the gallbladder (185).

BAs are mainly synthesized and secreted by hepatocytes (171, 173, 187, 191). The farnesoid X receptor (FXR) is the main regulator of the synthesis and secretion of BAs, and ASBT expressed by cholangiocytes regulates cholehepatic shunting (171, 187, 191, 192). ASBT is not only expressed by intrahepatic cholangiocytes, but by gallbladder epithelial cells, as well (193-195). It has been demonstrated that the gallbladder is able to uptake BAs in bile via ASBT, setting up the concept of a cholecystohepatic shunt (193-195). Primary BAs are generated from cholesterol and can be modified by additional side chains of taurine or glycine to become secondary BAs, which makes them a stronger acid and also decreases the chances of reabsorption (171, 187). Hypomotility of the gallbladder is linked to higher concentrations of secondary BAs, which is associated with an increased risk of developing cholelithiasis or cholangiocarcinoma (CCA) (196).

Once created, BAs are actively secreted from hepatocytes into bile mainly through the bile salt export pump (BSEP) (187). BAs are 100-1000X more

concentrated in bile than in plasma; therefore, they must be actively transported against this gradient (187). Most other components of bile maintain nearly the same concentration within bile fluid that exists in plasma, kept relatively standard through gradients found in the PBP (171, 173, 187). The regulation of BAs within plasma is also tightly controlled; however, certain biliary diseases alter this, spurring researchers to investigate the number of BAs detected in plasma of individuals with different liver and biliary pathologies (192, 197). So far, these studies have elucidated expected trends, such as the use of UDCA (the unconjugated form of nor-UDCA) for cholestasis treatment resulting in altered plasma BA concentrations (192). Additionally, recent research by Farhat et al. noted new trends, specifically that high levels of conjugated BAs in plasma link to increased risk for liver cancer or other progressive liver diseases (197). Additionally, higher levels of secondary BAs in plasma are associated with cholecystolithiasis and non-neoplastic polyps in the gallbladder (198, 199). Beyond the synthesis of BAs, bile pH and osmolarity are controlled by cholangiocyte activities (173). Interestingly, gallstone formation is not due to lower pH values directly, but is instead attributed to increased Ca^{2+} concentrations in the bile that subsequently lower the pH (200).

e. Bicarbonate Secretion

Chloride is exchanged for bicarbonate, making bile alkaline, and the BAs within are thus polar, de-pronated, and membrane impermeable (173, 201). This protective alkaline constitution of bile, termed the 'biliary bicarbonate umbrella,' shields cholangiocytes from BA-induced injury, and once secreted in the duodenum, it neutralizes the acidic gastric output, protecting the intestinal epithelium and bolstering the absorption of nutrients (168, 173). The initiation of chloride/bicarbonate exchange is stimulated by increased intracellular levels of cAMP (173, 183). This internal

increase in cAMP incites a rise in protein kinase A (PKA) activation, which results in the increased transportation of intracellular chloride to the apical membrane via vesicles with three specific proteins: CFTR, anion exchange protein 2 (AE2) and water channel AQP1 (173, 183, 190). CFTR is also expressed by gallbladder cells, and loss of CFTR leads to defects in gallbladder emptying and BA circulation (195). In response to CFTR loss, concentrations of secondary BAs (that are conjugated in the ileum) are reduced, and this is reversed with cholecystectomy, further indicating a cholecystohepatic shunt (195). Both CFTR and AE2 are highly expressed in the gallbladder compared to the intrahepatic ducts (181), and in the gallbladder epithelia CFTR is required for cAMP-dependent, AE2-mediated bicarbonate secretion (202). In patients with gallstones, bile bicarbonate levels are reduced, and thus bicarbonate is hypothesized to be the main buffer of bile similar to intrahepatic bile ducts (200).

Other factors can affect bicarbonate secretion, including autonomic neurotransmitters (173, 174). Acetylcholine and phenylephrine upregulate biliary bicarbonate secretion, while gastrin-releasing peptide and vasoactive intestinal peptide (VIP) mediates a consistent baseline of bicarbonate (171, 173). Further, hormones such as somatostatin, endothelin, dopamine, and gastrin inhibit the rise of intracellular cAMP (171, 173, 201). Bile also contains nucleotides and nucleosides that, when interacting with P2Y receptors on the apical membrane, can result in increased bicarbonate secretion (171). It is interesting that many of these processes can be recapitulated in some fashion in the gallbladder. Acetylcholine promotes mucin release in the gallbladder as a defensive mechanism (203) which potentially aids in bicarbonate secretion since this process is found on intrahepatic bile ducts (204). Additionally, VIP is a potent stimulator of cAMP production in the human gallbladder epithelial cells that regulates fluid secretion, and VIP expression is higher in the

gallbladder than the intrahepatic bile ducts (181). Somatostatin decreases gallbladder motility (205), and endothelin is overproduced in acute cholecystitis and increases gallbladder tone (3,4). Lastly, P2Y2 is expressed on isolated gallbladder epithelial cells (34) and stimulates mucin secretion (49).

f. Biliary immune function

While cholangiocytes, including those of the gallbladder epithelium, play a crucial role in bile flow and composition, they also play a role in both the innate and adaptive immune systems (173, 174). Cholangiocytes and gallbladder epithelial cells have receptors to identify pathogen- and damage-associated molecular patterns, including some of the same proteins that B and T lymphocytes possess such as toll-like receptors (206). Further, rather than being limited to downstream actions, cholangiocytes can proliferate and actively recruit immune cells to areas of injury (171, 183, 201). Cholangiocyte proliferation is tightly regulated by paracrine and endocrine factors, including growth factors like transforming growth factor (TGF) and TNF, cytokines, neuropeptides, and hormones (173). For instance, progesterone and estrogen have been linked to increased proliferation, where anti-progesterone/estrogen or a drop in levels of these hormones results in limited cholangiocyte growth, and even increased risk of disease states (173, 207, 208).

Cholangiocytes are attributed to the initiation of immune responses within the biliary tract due to their high level of intra- and extracellular communication (173), and following damage they secrete pro-inflammatory cytokines and chemokines, which communicate the location and type of injury to neighboring and immune cells (209).

While gallbladder epithelial cells have similar immune receptors and responses to those of cholangiocytes, they are located further down the biliary tract, and thus play a delayed, but still important immune role (210). One study found that gallbladder

epithelial cells express mRNA for a variety of cytokines and chemokines, as well as directly secrete TNF (210). Another study using donated human gallbladders, found the presence of multipotent endodermal stem cells within the gallbladder epithelium increased in pathologic gallbladders versus comparatively healthy gallbladders (211). Research on the potential immune functions of gallbladder epithelial cells is still ongoing and evolving.

g. Cholangiocyte-dependent fibrosis

Profibrotic factors can be released to incite downstream effects that promote fibrogenesis (212, 213). One study has shown that silencing one TGF-β isoform may be an effective treatment for fibrotic biliary and liver diseases, limiting the expression of pro-fibrotic genes and conversely promoting antifibrotic PPAR expression (212). Further, chronic activation of cholangiocytes can result in the development of biliary fibrosis, damage, or cancer (212). Overly active fibrogenesis results in a buildup of scar tissue can result in decreased functionality of the biliary tract, eventually leading to biliary cirrhosis (201, 214). The gallbladder epithelial cells react similarly, with prolonged inflammation and immune response potentially resulting in severe fibrosis, perforation of the gallbladder, or even gallbladder cancer (215-217).

h. *Cholestasis*

Cholestasis refers to a decrease or halt in bile flow anywhere along the biliary tree. While there are a number of hereditary cholestatic disorders caused by genetic mutations, the most common forms of cholestasis are presented through PSC, primary biliary cholangitis (PBC), CCA, and cholelithiasis (218, 219). No matter the cause of cholestasis, there are few treatments available. The main treatment is to supplement with BA analogues, UDCA or obeticholic acid (OCA) that work to reduce BA synthesis. If UDCA or OCA treatment fails, a liver transplant is the last option (218, 220). UDCA,

when recognized by the biliary tract, increases bile flow, lessens toxicity, and encourages the recycling of nontoxic over toxic bile salts (221). Unfortunately, only about 40% of patients with cholestasis respond to UDCA treatment, highlighting the need for alternative therapies (192, 220). OCA works to reduce toxic BA levels by reducing BA synthesis and enhancing hepatic BA efflux (222). Clinical trials on OCA use in PBC, PSC and fatty liver diseases have proved promising, but more work regarding efficacy is necessary (222).

LINKS BETWEEN THE GALLBLADDER AND CHOLESTATIC LIVER DISEASES

VI. Primary sclerosing cholangitis (PSC)

a. Background

PSC is a rare cholangiopathy that firstly targets the bile ducts in the liver leading to inflammation, fibrosis, stricturing and eventual cirrhosis and liver cancer (223). The majority of PSC patients have extrahepatic and intrahepatic bile duct involvement, while a small proportion of diagnoses having intrahepatic only PSC (223). PSC affects more males than females, and the median age at diagnosis is 40 years (218, 224). Due to the initial unspecific manner of PSC symptoms at onset, PSC is not typically diagnosed until the disease has progressed (218). Currently, there are no approved therapies for the treatment of PSC, with BA therapeutics including UDCA and OCA being tested as potential therapeutics (218). PSC patients have a high risk of developing CCA and the only curative treatment for PSC is liver transplantation; however, recurrence rates are high demonstrating that this approach is not viable (218). While PSC primarily targets the biliary tree, the fibroinflammatory nature of PSC can lead to chronic inflammation which can subsequently affect the gallbladder.

b. PSC, cholelithiasis and cholecystitis

An early study from 1988 interrogated the incidence of gallbladder disease in PSC and found that 89% of PSC patients had abnormal gallbladders, and after excluding patients who had thickened gallbladder wall due to end-stage liver disease, 41% of the remaining PSC patients presented with gallbladder abnormalities (225). PSC patients with abnormal gallbladders presented with gallstones, gallbladder dysfunction associated with PSC and neoplasms, indicating that gallbladder abnormalities are frequent among PSC patients (225). These findings were verified in a large study from 2008 that found that 41% of PSC patients present with gallbladder abnormalities, 25% have gallstones and 25% have cholecystitis (226). PSC patients also have papillary hyperplasia, pseudo gland formation, inflammation, smooth muscle hypertrophy and fibrosis in the gallbladder, but these abnormalities were found to a similar degree in chronic cholecystitis patients (227). PSC patients and chronic cholecystitis patients both presented with mononuclear cell infiltration of the epithelium, and although the incidence was higher in PSC it was not significant (227). Therefore, there may not be a distinct gallbladder signature in PSC patients compared to chronic cholecystitis. A separate study found that PSC-related cholecystitis showed diffuse infiltrate, predominantly plasma cells, within the lamina propria which was not significantly noted in chronic cholecystitis alone; therefore, the authors suggest that diffuse lymphoplasmacytic acalculous cholecystitis is a distinct form of PSC-associated cholecystitis (228). Incidence of cholecystitis is significantly higher (30%) in patients with extrahepatic PSC when compared to intrahepatic only PSC (9%) (226). These findings slightly differ from a Japanese cohort where ~12% of PSC patients were concomitantly diagnosed with gallstones (229), although this study did not distinguish between intra- and extra-hepatic PSC.

Transabdominal US is used to identify bile duct wall thickening and dilatations in PSC, but in one study this approach also identified that up to 41% of PSC patients presented with an enlarged gallbladder (Figure 6), gallstones, cholecystitis or mass lesions (230). The small study found that all PSC patients presented with irregularly thick gallbladder wall (230). This study further found that while PSC patients had enlarged gallbladders their rates of gallbladder emptying were normal (230).

The gut influence on cholelithiasis was previously discussed, and it is also known that ~80% of PSC patients have concomitant inflammatory bowel disease (IBD) (231). Interestingly, around 50% of IBD patients present with hepatobiliary manifestations, including PSC, cholestasis and gallstones (232). Patients with Crohn's Disease, severe ileitis or ileal resection have bile malabsorption leading to gallstone formation (232), further indicating the gut-liver axis in cholelithiasis.

Multidrug resistance 2 gene knockout (*Mdr2*$^{-/-}$) mice are used as a model of PSC, and these mice spontaneously form cholecystolithiasis (233). The gallbladder in *Mdr2*$^{-/-}$ mice has needle-like cholesterol stones as early as 12 weeks of age (Figure 7) (233). The highly pro-inflammatory hepatobiliary environment might be contributing to the concretion of gallstones and aiding in cholecystitis. Moreover, the ability of *Mdr2*$^{-/-}$ mice to spontaneously generate gallstones without the induction from lithogenic diet makes it a versatile model to study the intricate signaling mechanisms involved in the concretion and crystallization of gallstones. Female *Mdr2*$^{-/-}$ mice developed 50% more gallstones than male *Mdr2*$^{-/-}$ mice indicating a sexual dimorphic effect (233), but this dichotomous effect has not been published in humans with PSC. *Abcb11* encodes BSEP that is responsible for the export of BAs from the hepatocyte to the bile canaliculus, and *Abcb11* colocalizes with the *Lith1* (responsible for cholesterol-induced gallstone formation) quantitative trait locus (234). To understand if *Abcb11* is

responsible for gallstone formation, the authors generated mice with overexpression of *Abcb11* and subsequently fed them a lithogenic diet (234). It was found that *Abcb11* overexpression induced biliary BA secretion and bile flow but did not affect cholelithogenesis (234).

c. Gallbladder cancer in PSC

Aside from cholelithiasis and cholecystitis, there is an increased rate of gallbladder cancer in patients with PSC (235). Some patients present with gallbladder lesions, which more than half of the time represent adenocarcinoma, and as such cholecystectomy is recommended in all instances of gallbladder lesions regardless of size (236). Gallbladder carcinoma was associated with intrahepatic bile duct dysplasia, CCA and IBD in PSC patients, and gallbladder dysplasia was associated with hilar/intrahepatic bile duct dysplasia, CCA, IBD and older age at transplant; however, similar associations were not found for sex or PSC duration (235). From this study, the authors conclude that PSC patients have a neoplastic "field effect" along the intra- and extra-hepatic bile ducts in PSC, including the gallbladder (235). Importantly, in 40-50% of PSC patients with gallbladder neoplasms, these polyps are malignant (237). From these studies, one would consider cholecystectomy to be an important intervention for PSC patients presenting with gallbladder polyps. However, one study found that 40% of PSC patients that underwent cholecystectomy due to gallbladder polyp or mass presence had early postoperative complications (238).

VII. *Primary biliary cholangitis (PBC)*

PBC is an autoimmune-mediated cholangiopathy that targets the interlobular (i.e., small) bile ducts of the biliary tree (239). Risk factors for PBC include being female, over 50 years old, and living in a Western country (218, 224). In early stages

(stage I/II) of PBC, there is a large degree immune cell influx to the peribiliary location, compensatory proliferation of the bile ducts, reduced presence of ductulo-canalicular junctions (necessary for bile outflow) and loss of the protective bicarbonate umbrella (240). As PBC progresses to later stages (stage III/IV) cytotoxic T cell mediated destruction of the bile ducts leads to ductopenia via apoptosis of the small cholangiocytes (239). Ductopenia has also been attributed to enhanced senescence and toxic BA-mediated cell death due to loss of the bicarbonate umbrella and ductulo-canalicular junctions (240). These surmounting injuries lead to peribiliary fibrosis and cirrhosis if left untreated (239). UDCA and OCA are first-line therapies approved for the treatment of PBC, but a number of patients are non-responders to these approaches (241). While PBC is an autoimmune liver disease, patients do not respond to traditional immunosuppressants, making treatment of the inflammatory cascade challenging (241). Due to the pan-inflammatory presence in PBC, it is unsurprising that 73% of patients with PBC present with extrahepatic manifestations of autoimmune disease, including Sjogren's syndrome, thyroid disease and systemic sclerosis involving the skin, lungs, gastrointestinal tract, heart or musculoskeletal system (241).

a. *Gallbladder disorders and cholelithiasis in PBC*

There are few studies that identify if changes in the gallbladder or gallbladder disease occur in patients with PBC. A case study found that a 70-year-old Hispanic woman with PBC/autoimmune hepatitis overlap syndrome and associated cirrhosis had multiple gallstones and bile duct stones, and a periampullary mass (242), but this may have been associated with cirrhosis and not driven by PBC. In one study, it was noted that patients with PBC did not have a significant difference in gallbladder size, wall thickness or emptying compared to controls (230). Another study conversely found that the gallbladders of PBC patients had epithelial hyperplasia, pseudo gland

formation, inflammation, fibrosis, smooth muscle hypertrophy and mononuclear cell infiltrate, but the degree is like what is found in chronic cholecystitis and PSC patients (227) indicating that gallbladder abnormalities may be non-specific in cholangiopathies. As well, it is unclear if these patients presented with cirrhosis, which in and of itself increases the risk of gallbladder disease regardless of etiology (243).

A national hospital-based study in Italy looked at hospitalized PBC patients and found that this cohort had an increased incidence of malignant neoplasms of the gallbladder, and this occurrence was higher in women than in men (244). In another study, cholelithiasis in PBC was significantly associated with intra- and extra-hepatic CCA (245). However, these are the only studies identifying associations between PBC and gallbladder cancer, thus more work is necessary.

b. Microbiota in PBC

PBC patients have decreased diversity of microbes and higher levels of genera associated with inflammation, but this dysbiosis is partially reversed by UDCA (246). As stated above, BAs and the microbiota can play a role in cholelithiasis; therefore, this association in PBC may be attractive. Interestingly, 75% of the bacterial clones isolated from gallbladder bile from PBC patients were gram-positive cocci, with only 5% of gram-positive cocci found in gallbladder bile from patients with cholecystolithiasis (Table 2 and Table 3) (247). *Staphylococcus aureus* was the predominant gram-positive bacteria in PBC gallbladder bile (247). However, this study did not indicate if the PBC patients presented with gallbladder abnormalities, and thus the correlative or causative effect of dysbiosis in PBC on gallbladder disease is unknown.

There is a lack of understanding on the association of PBC and gallbladder diseases. While some abnormalities and cancer were noted, this may be a

consequence of cirrhosis and not etiology dependent. Furthermore, no studies have reported on gallbladder abnormalities or cholelithiasis in mouse models of PBC. Therefore, more investigation is key to answering this question.

VIII. Cholangiocarcinoma (CCA)

Cancer cells and the tumor microenvironment (TME) interact with each other to form multicellular systems, called tumors. The composition of the TME is characterized by extracellular matrix (ECM), and various cell types such as immune cells, endothelial cells, pericytes, and fibroblasts (248). CCA is cancer of the bile ducts and is the second largest primary liver malignancy, after hepatocellular carcinoma (HCC). CCA tends to escape immune surveillance, and for this reason it is associated with a poor prognosis and poorly defined symptoms (249). Most CCA cases are defined as an incurable malignancy, and the 5-year survival rate for CCA is abysmally low (250). CCA can be defined by the following subtypes: intrahepatic (iCCA), perihilar (pCCA), and distal (dCCA) (251). The last two groups of CCA, pCCA and dCCA, are regrouped under the term of extrahepatic CCA (eCCA) and can include gallbladder cancer (252). Many risk factors such as NAFLD, non-alcoholic steatohepatitis (NASH), alcohol-related liver disease (ARLD), and biliary fibroinflammatory response can contribute to CCA development (253, 254). MicroRNAs (miRNAs) are small non-coding RNAs that play various roles in the modulation of CCA (255). Various studies have shown that alteration of miRNAs may act as oncogenic or onco-suppressing in CCA. Furthermore, in gallstone disease, there is upregulation of miR-210 that reduces the expression of its target, ATPase phospholipid transporting 11A gene, in human gallbladder epithelial cells (256). miR-130b inhibits the expression of the specific protein 1, and consequently there is decrease of MUC5AC expression. It is well known that

hepatolithiasis is strongly related to chronic inflammation and overexpression of MUC5AC as well, which can be a contributor to liver cancer initiation (257).

a. Cholangiocarcinoma, cholelithiasis and gallbladder cancer

On occasion, gallstones can migrate into the bile ducts and induce complications. The presence of bile duct stones is considered a significant risk factor for the development of CCA due to repeated mechanical injury and inflammation of the intrahepatic biliary tract epithelium (258, 259). The size, presence and number of gallstones are significantly associated with increased risk of CCA (260). Cholecystectomy reduced the risk of gallstones associated with CCA, with a greater risk reduction seen in eCCA than iCCA (261). This was mirrored in another study where gallstones increased the risk of iCCA and eCCA with a decline in risk following cholecystectomy (262). Another study contrarily found that dilation of the bile ducts is frequent following cholecystectomy and can cause inflammation and increase the risk of CCA (263); however, this was in a cohort of patients with normal bile ducts whereas the former was in a population of CCA patients. The biliary microbiome can regulate various damages within the liver, including cholelithiasis as discussed above. One study found that the relative abundance of Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria was similar in patients with dCCA and new onset bile duct stones (264) identifying that shared microbial communities may be a link between gallstone formation and CCA development. In a rare case report, a 65-year-old woman presented with jaundice and concomitant cholecystitis due to an impacted gallstone (265). Following pancreaticoduodenectomy, histopathological analysis revealed that the patient had primary gallbladder malignancy along with CCA (265). While the link between gallstones and CCA risk is known, the incidence of concomitant CCA and gallbladder cancer appears to be rare. The incidence of other gallbladder disorders in

CCA seems unreported in the literature; thus, more work may be required in this area. Histological imaging of gallbladder cancer can be found in Figure 8.

IX. Non-alcoholic fatty liver disease (NAFLD)

NAFLD, also known as metabolic-associated fatty liver disease, is the most common chronic liver disorder globally (266). As the obesity epidemic continues to grow, the incidence of NAFLD is increasing worldwide. Approximately 24% of U.S. adults have NAFLD and about 10% of this population has an advanced form of NAFLD termed NASH. The incidence of NAFLD in children is also rising with about 10% of U.S. children aged 2-19 years having NAFLD (267). NAFLD also shows ethnic disparities, with the highest incidence in Hispanic populations (268). The risk factors for NAFLD includes obesity, type 2 diabetes mellitus, hypertriglyceridemia, Western diet and sedentary lifestyle (269). Interestingly, a large scale study using the U.S. National Health and Nutrition Examination Survey revealed the positive correlation between glucose intolerance, plasma insulin levels and C-peptide content with gallstone incidence (270)

The pathogenesis of NAFLD was first explained by the 'two-hit' theory (271, 272), and later referred to as "muti-hit hypothesis". The first 'hit' starts with insulin resistance caused by excessive FA accumulation in hepatocytes, a state known as hepatic steatosis (273, 274). A number of secondary 'hits' come after the exposure to chronic fat accumulation (272), including oxidative stress-induced mitochondria dysfunction, endoplasmic reticulum (ER) stress (275), apoptosis induced-regeneration (276), gut-derived endotoxin-induced inflammation (277), and cholestatic-induced lipid metabolism dysregulation (278). These multiple secondary hits synergistically, but not sequentially, happen during the progression of NAFLD. These events eventually lead

to chronic inflammation and fibrosis, resulting in NASH (279). NASH is characterized by hepatic ballooning, lobular inflammation, and macro steatosis. About 20% of NASH patients will develop cirrhosis, with potential risk of liver failure or hepatocellular carcinoma (280).

A longitudinal cohort study showed increased risk of gallstone formation in NAFLD patients, especially in females (281). Further studies showed association between NAFLD and gallstones with a higher NAFLD incidence in women with gallstones (282-284). Additionally, gallbladder wall thickness and gallbladder dysfunction can occur in NAFLD patients that do not present with gallstones (Figure 9) (285). It has also been shown that NASH prevalence in patients with gallbladder disease is 18% in the morbidly obese population, but mechanisms linking these factors is unknown (286). Lastly, cholelithiasis was not associated with advanced fibrosis or definite NASH in a NAFLD cohort, further complicating potential associations between gallbladder disease and NAFLD (287).

Human genome-wide association studies (GWAS) have revealed several genes that may explain the vulnerability and increased risk of NAFLD observed in some subpopulations. The most confirmed and studied genetic variant that is associated with NAFLD is PNPLA3 (288-290). The Rs738409 [G] I148M allele of PNPL3 correlated to increased risk of NAFLD and is most found in Hispanic populations. Furthermore, the Rs738409 [G] I148M mutation increased NAFLD risk and body weight gain (291), and an increased risk of higher steatosis, portal inflammation, fibrosis and oxidative stress (291-294). Conversely, rs6006460[T] is enriched in African American populations and shows protective effects against the development of NAFLD as the population shows a lower risk of NAFLD and lower hepatic fat content (289). However, a study did not find increased risk of gallstone

formation in patients with I148M mutation *per se* (295). Nevertheless, another genetic study showed that the polyunsaturated FAs were much higher in individuals with PNPLA3^{148M} variants when compared to non-carriers. Other genetic variants with moderate effect sizes were shown in transmembrane 6 superfamily member 2, glucokinase regulator (GCKR), and membrane bound O-acyltransferase domain-containing 7 (296). Another GWAS study also found GCKR variant showed increased risk of gallstone diseases (297). The DNA methylation of PPARG is associated with fibrosis severeness in NAFLD (298). Interestingly, activation of PPARG prevents cholesterol gallstone formation by increasing bile salt synthesis and enterohepatic circulation in lithogenic mice models (299). The same study also noticed that PPARG activation alleviated hepatic steatosis and obesity symptoms (299). This indicates that both NAFLD and gallstone formation share some common mechanisms.

a. Fatty acid (FA) uptake, storage and signaling

The rate of hepatic FA uptake is determined not just by the circulating concentrations that comes from the adipose tissue and gut, but also relies on FATP and caveolin (300-304). Meanwhile, vaveolin-1 depletion increased cholesterol crystallization in lithogenic diet-induced mice by inhibition of hepatic cholesterol levels and bile salts transportation (305). Cluster differentiation 36 (CD36), as the most studied lipid transporter, facilitates hepatocyte FA update and trafficking (306). Hepatocyte specific depletion of CD36 improved steatosis by decreasing the triglyceride, diacylglycerol, and cholesterol in a NAFLD genetic mouse model and diet induced model (307). In fact, oxidation is increased in *CD36*$^{-/-}$ mice via inhibition of sterol regulatory element-binding protein 1 (SREBP1) in diet-induced NAFLD (308). Further, circulating CD36, a soluble form of CD36, was found to be strongly associated with insulin resistance (309) in type 2 diabetes and advanced steatosis in NAFLD

(310). Depletion of CD36 also showed resistance to lithogenic diet induced gallstones in mice by altering the lipid composition in the biliary tract and enhanced gallbladder contractility (311).

Besides FA uptake from exogenous sources, hepatic FA comes directly from *de novo* lipogenesis, that is converted from monocarbohydrates and proteins. In this process, acetyl-CoA is converted to malonyl-CoA and fatty acyl-CoA. This process adds FAs to hepatocytes and causes triglyceride accumulation in the cells by inhibiting fatty oxidation (312). SREBP1c and carbohydrate-responsive element-binding protein (ChREBP) also regulates *de novo* lipogenesis. Interestingly, both SREBP1c and ChREBP can be stimulated through activation of LXR which is regulated by insulin (313). Further, insulin could directly activate SREBP1c though translocation from the Golgi to the nucleus (314). LXR activation increased the susceptibility of gallstone formation in lithogenic-diet induced mice by elevated cholesterol and phospholipids concentration and decreased bile salt concentration (315).

b. Bile acid metabolism

As previously mentioned, NAFLD starts with simple steatosis followed by multiple secondary insults. One of the offenses is the dysregulation of BA metabolism, which is mediated by the liver-gut axis (316). About 95% of BAs are recycled through the hepatic portal system, and BAs can regulate glucose and lipid homeostasis via nuclear receptor activation, including FXR (317). Interestingly, *FXR*$^{-/-}$ mice showed dysregulated lipid metabolism, enhanced serum BAs, cholesterol, and serum lipoprotein profile (318). While another study showed increased bile salt hydrophobicity and cholesterol crystallization in *FXR*$^{-/-}$ mice, which is an indication of gallstone formation. Further, the reactivation of FXR in these knockout mice prevented gallstone formation (319).

c. NAFLD, cardiovascular disease and cholelithiasis

As stated, the NAFLD spectrum varies from simple steatosis to metabolic steatohepatitis, and it can further progress to liver fibrosis, cirrhosis, and hepatocellular carcinoma. The coexistence of NAFLD and gallstone disease has been found, mainly due to several shared risk factors such as age, ethnicity, obesity, insulin resistance, and metabolic syndrome (320). A study has indicated an increased incidence of gallstone formation in patients with NAFLD (47%) versus patients without NAFLD (26%) (321).

Recent studies have indicated that gallstone disease is closely associated with the occurrence of cardiovascular disease, and the occurrence of gallstone disease increases the incidence of cardiovascular disease (322). Based on a meta-analysis of 10 published studies, patients with gallstone disease had a higher risk of diabetes, hypertension, coronary heart disease, atrial fibrillation, and hyperlipidemia. In addition, gallstone disease was found to be related to a 1.23-fold increase in the incidence of cardiovascular and cerebrovascular diseases. In another study of 5,928 subjects by Daniel *et al*., gallstone disease was closely related to many kinds of cardiovascular diseases (323). Gallstone disease, cardiovascular disease and NAFLD also share common risk factors such as obesity, age, sex, and disorders of lipid metabolism, and these factors are major risk factors for metabolic syndrome. Metabolic syndrome is closely related to cardiovascular disease and gallstones may be considered a characteristic of this (324). Cholesterol is transported into plasma by lipoproteins, micelles, and vesicles in bile. If excess cholesterol were accumulated in the arterial wall, atherosclerosis may occur. The excess cholesterol that is not dissolved by bile salts or phospholipids will precipitate as solid cholesterol monohydrate crystals, which can lead to the formation of cholesterol gallstones (84).

d. NAFLD, cholestasis and cholelithiasis

Gallstones are one of the causes of extrahepatic cholestasis, while cholestasis is linked to NAFLD progression in various studies. A case-control study conducted in 2015 revealed more severe histological damage in NAFLD with cholestasis compared to NAFLD patients without cholestasis (325). This is further highlighted in a study conducted in 2018, where about 30% of NAFLD patients showed cholestasis (326). It is suggested that NAFLD patients with cholestasis show more pronounced hepatic inflammation, unbalanced carbohydrate and lipid metabolism, apoptosis, and fibrosis (326). Another study conducted with 90 NASH patients showed a positive association between centrilobular ductular reaction and fibrosis stage (327). Although this study did not provide direct evidence of cholestasis influence on the homeostasis of lipid and carbohydrates in the liver, a number of animal studies using an *Mdr2*$^{-/-}$ mouse model connect cholestatic liver injury and impaired liver function (278) to dysregulation of lipid metabolism and steatosis (328). Specifically, the genes that control lipid synthesis, storage, and oxidation is dysregulated. Interestingly, the same study found that the supplementation of nor-UDCA or high-fat diet showed a protective role in *Mdr2*$^{-/-}$ mice and reversed the fibrosis (328).

Patients with NAFLD showed increased plasma BAs, specifically elevated primary and secondary BAs. Bacteria that metabolize taurine and glycine, two critical components in producing secondary BAs are increased (329). Furthermore, elevated primary BAs are also found in gallstone patients (198). On the other hand, intrahepatic cholestasis of pregnancy (IPC) showed significantly higher incidence in NAFLD patients when compared to other chronic diseases or pregnancies without chronic liver disease (330). Further, the incidence of gallstones in IPC is much higher in women who do not present IPC (331). Taken together, cholestasis and elevated BAs increase

the risk of NAFLD and gallstones. However, further work needs to be done in human association studies and molecular mechanisms underlying the BA metabolism, gallstone formation and NAFLD.

X. Alcohol-related liver disease (ARLD)

ARLD has been the main cause of liver-associated mortality worldwide (332). This chronic liver disease is the most common and can progress from alcoholic fatty liver to alcoholic steatohepatitis (ASH) (333). Chronic ASH can eventually develop liver fibrosis and cirrhosis, which may lead to HCC. In addition, severe ASH (with or without cirrhosis) can cause alcoholic hepatitis (AH), which is an acute clinical presentation of ARLD that is associated with liver failure and high mortality (334).

Most ARLD patients are diagnosed with jaundice or complications of cirrhosis when they reach the medical care (335). Screening of ARLD in the primary-care setting at an early stage and subsequent behavioral interventions should be encouraged. Abstinence from alcohol is the best treatment for all stages of ARLD (336, 337). Unfortunately, ARLD patients in advanced stages who do not respond to medical therapy have a very low life expectancy, and the only therapeutic option associated with a survival benefit is liver transplantation (338). At 1-year post-transplantation, the survival rate has steadily improved to 80-85% in 2010 (339). In addition, transplant recipients with ARLD are at high risk of cardiovascular diseases, infections, and cancers (340, 341). Overall, more effective, and safer therapies are urgently needed to ultimately reduce the burden, morbidity, and mortality of ARLD.

a. Alcohol consumption and cholelithiasis

Almost forty years ago, a case-control study first reported that alcohol consumption was associated with a decreased risk of developing gallstones, whereas

increased intake of sugars was associated with an increased risk (342). Interestingly, the association of alcohol with reduced risk of gallstones was found in both males and females (342). However, women have been regarded to have a higher risk of gallstone formation due to sex hormone signaling (118). In this regard, the relation between alcohol intake and cholecystectomy were observed by Leitzmann *et al.* in a large cohort of women (343). Their study also revealed that the intake of all alcoholic beverages is inversely associated with the risk of cholecystectomy in women (343). In another large prospective study of over 1 million women that consume alcohol (patients were excluded if they had a clinical history of either liver cirrhosis or gallbladder disease before recruitment), Liu *et al.* further confirmed that alcohol consumption is associated with an increase in the risk of liver cirrhosis but a decrease in the risk of gallbladder disease (344).

b. Thickening of the gallbladder wall in alcoholic hepatitis

Thickening of the gallbladder wall is often seen with US in patients with ARLD. In a retrospective evaluation of 125 consecutive gallbladder sonograms, it was reported that gallbladder wall thickening was associated with hypoalbuminemia in the absence of chronic cholecystitis in a cohort of chronic alcoholics (345). However, another US evidence-based study suggested that portal hypertension, not hypoalbuminemia, is the dominant factor causing gallbladder wall thickening in cirrhotic patients (346). Therefore, more research may be required in this area to better understand the comorbidity of gallbladder wall thickening.

c. Gallbladder perforation and gallbladder variceal hemorrhage in ARLD

Gallbladder perforation is a relatively uncommon complication of ARLD-related cirrhosis and may happen with or without gallstones. The diagnosis of gallbladder perforation is challenging due to the lack of classical symptoms and signs of

perforation (347). Chu *et al.* reported the first case of a 41-year-old man with alcoholic cirrhosis who developed fatal spontaneous gallbladder variceal bleeding (348). Unfortunately, the diagnosis of gallbladder varices eluded conventional imaging and was made only at autopsy; therefore, direct causation of spontaneous gallbladder variceal bleeding with ARLD is unknown. One case study reported gallbladder perforation with alcoholic liver cirrhosis and asymptomatic gallstones (347). The patient was initially diagnosed as HCC-associated rupture based on CT scan images and the patient's clinical history of alcohol-related liver cirrhosis; however, further laparotomy examination revealed that the patient has gangrenous cholecystitis with perforation, suggesting that gallbladder perforation should be taken into consideration as a potential cause of acute abdominal pain (Figure 10) (347). Furthermore, it was observed that a Child-Pugh A alcohol-related liver cirrhosis patient had developed acute gallbladder perforation with spillage of stones into the peritoneal cavity (349). Gallbladder perforation is a rare complication in ARLD and alcohol-related liver cirrhosis, but caution should be taken for those with specific risk factors.

d. ARLD and cholelithiasis

Since alcohol-related cirrhosis is the advanced stage of ARLD, many studies evaluated prevalence and incidence of cholelithiasis with cirrhotic patients (350). Acalovschi *et al.* assessed the risk factors for gallstone formation and the characteristics of liver cirrhosis in 140 patients with multivariate analysis. Similar to what was discussed previously, they reported that alcohol-related cirrhosis and male gender (not female) were inversely correlated with cholelithiasis symptom presence (351). In cirrhotic female patients, the risk of developing cholelithiasis was significantly greater (351). However, another multivariate study shows that cholelithiasis was

significantly more frequent in cirrhotic patients with previous alcohol abuse with no difference in relation to sex (352).

e. Animal studies on alcohol consumption and cholelithiasis

Animal studies are key for identifying molecular mechanisms regulating disease processes. Unfortunately, little work has been done to investigate ARLD and gallbladder diseases in murine models. One study evaluated the effect of alcohol consumption on BA profiles in a chronic gavage mouse model (353). Interestingly, ethanol intake significantly increased BA profiles (mainly free BAs and taurine-conjugated BAs) in the gallbladder of 50% ethanol fed mice (353). The total BAs in the gallbladder were also significantly increased in the 50% ethanol treated groups (353). The authors also demonstrated that 50% ethanol increased the expression of BA-related enzymes and transporters, including BSEP and ASBT in the liver (353). The close association with BAs, BA transporters and gallstone formation may indicate that very high alcohol consumption can contribute to cholelithiasis. However, this percent of ethanol intake is not physiologically relevant, and thus findings should be considered with caution.

XI. SARS-CoV-2-related liver disease

SARS-CoV-2, the virus responsible for COVID-19, has been under an intense lens of investigation since the identification of the highly contagious infection. At first, it was uncertain if patients with chronic liver or biliary disorders were more at risk for severe COVID-19 than others, with the American Association for the Study of Liver Diseases making a statement in 2020 that higher risk was probable due to the observed mechanistic interactions of the virus with angiotensin-converting enzyme 2 (ACE-2) (354). ACE-2 acts as a functional transporter, allowing the virus entry into the

cell, making hepatocytes and cholangiocytes, which express ACE-2, targets for potential infection (354, 355). Over the past two years, research has begun to identify comorbidities that correlate to higher risk of fatality, as well as disease states and damage caused by fighting the infection. Further, COVID-19 patients with evidence of liver dysfunction account for about half of those infected (354, 356). Of importance, one case report found 3 adults that developed prolonged and severe cholestasis following COVID-19 infection, leading to the notion that there may be a rare COVID-19-related cholangiopathy (357). Another study found that biomarkers of liver injury were elevated in 23.4% of Delta-infected and 18.8% of Omicron-infected COVID-19 patients, with the predominant marker being identifiers of cholangiocyte damage (358). Interestingly, liver and cholangiocyte injury biomarkers did not differ between patients with or without pre-existing liver injury (358). This work is supported by another study indicating that 32.7% of COVID-19 infected patients had elevated markers of cholangiocyte damage, which correlated with longer hospital stays (359). The full impact of COVID-19 on cholestasis and biliary damage will likely not be determined until long into the future since the disease is relatively new.

a. SARS-CoV-2 related gallbladder disease

Several COVID-19 patients have presented with severe cholecystitis. Like cholangiocytes, gallbladder epithelial cells present with high levels of ACE-2, which is thought to explain the presence of viral RNA present in the gallbladder epithelial cells of affected patients (Figure 11) (354, 355). As with hepatobiliary dysfunction, the severity of COVID-19 infection appears to directly influence the severity of cholecystitis, with over half the case studies identifying those patients with complicated or severe COVID-19 as having acalculous or gangrenous cholecystitis (354-356, 360). Conversely, some cholecystic COVID-19 patients had less severe COVID-19, but still

presented with acute cholecystitis (361-363). In one case report of a patient with COVID-19 and gangrenous cholecystitis, immune cell infiltration and blood vessel involvement can be seen in the gallbladder. This disparity between critically ill and non-critically ill COVID-19 patients with similar cholecystic presentations suggests that underlying risk factors may account for progression of the diseased state, including similar risk factors to cholestasis, genetic proclivity, and co-morbidities. Additionally, COVID-19-linked cholecystitis cases have been seen around the world, suggesting there may not be a strong connection to lifestyle or ethnicity. As more individuals recover from COVID-19, it is important to explore any lasting damage induced by the virus.

CLINICAL ASPECTS OF GALLBLADDER DISEASE IN LIVER DISEASE

XII. Prevention and treatment

a. Prevention

Pigmented stones are less frequently observed and represent <10% of cases worldwide. Specific risk factors, such as parasitic biliary infection or blood diseases (hemolytic anemia) may attenuate brown stone prevalence (172). The burden of cholesterol gallstones seems worldwide, but prevention may not be an easy target since there is a complex interplay between genetic, metabolic, dietary, environmental and gender related factors contributing to stone formation (364). Among modifiable cholelithiasis risk factors, those related to lifestyle (diet and physical activity) have captured more attention. Reduced physical exercise (365) and obesity (366, 367) were consistently reported in association with increased risk of cholesterol stones. Regarding diet type and habits: i) reduction of carbohydrates, meat, and fats in favor of vegetables as well as; ii) avoidance of long fasting periods, seem protective for cholesterol stone formation (368). In this setting, alcohol consumption has been

suggested to be inversely correlated with gallstones (369); however, it is important to note that studies on diet or general physical activity are largely based on self-reported data and possibly altered by other personal and environmental factors thus justifying discrepancy between different studies. Finally, a condition in which gallstone prevention may be feasible and beneficial is related to rapid weight loss. A weight decrease >1.5 kg/week has been associated with an increased risk of gallstones (370) and similarly after bariatric surgery (particularly when Roux-en-Y gastric by-pass is performed) stone formation may be expected (371). In these situations, UDCA prophylactic therapy is advised (144, 372).

b. Pharmacological treatment

UDCA consistently demonstrates gallstone dissolution capabilities. This effect was evident when UDCA was administered at a dose of 7 mg/kg with radio-transparent, non-calcified stones ≤1 cm in size and in patients with a functional gallbladder (373). UDCA inhibition of cholesterol intestinal uptake and secretion in bile may explain its stone dissolution properties (374). Therapeutic application of UDCA, however, is hindered by high stone recurrence, accounting for more than 50% of cases on 10-year follow-up (375). This negative aspect is in part compensated by the observation that long-term treatment (up to 18 years) is associated with a decrease in biliary pain and acute cholecystitis in patients with symptomatic gallstones at baseline (376). In practice, UDCA dissolution therapy may be considered in symptomatic patients with elevated surgical risk or denying surgery. In acute symptomatic gallstones, use of non-steroidal anti-inflammatory drugs is generally indicated. In acute cholecystitis, antibiotic therapy remains controversial while it remains useful in cases of concurrent biliary tract infection, such as cholangitis or abscess formation (144).

c. Surgical approaches

An extensive examination of the operative procedures regarding the management of gallstones and their complications is behind the scope of this review since several publications and guidelines have focused on this issue (154, 159, 377). In this paragraph just the most relevant concepts on operative strategies for gallstones will be reported.

Surgical removal of the gallbladder (cholecystectomy) remains the advised approach in symptomatic gallstone disease (144, 378). Cholecystectomy, in fact, is a measure to block stone recurrence since gallbladder dysfunction (dysmotility and changes in bile reabsorption/concentration process) contributes to cholesterol nucleation (57, 194). Starting from 1985 laparoscopic (mini-invasive) cholecystectomy has been a major advancement in gallbladder surgery reducing hospital stay and allowing a faster post-surgical recovery, in comparison with open access (379). More than 90% of cholecystectomies are approached with the mini-invasive procedure presently; however, conversion or direct start with open surgery may be considered in difficult or complicated cases (144). For common bile duct stones, a specific mini-invasive approach based on endoscopic-retrograde-cholangiopancreatography (ERCP) technique has been consistently suggested and adopted (158, 159). ERCP is successful for common bile duct stone extraction in approximately 90% of cases and is also able to solve other gallstone complications such as acute cholangitis or biliary pancreatitis (380, 381). Finally, percutaneous cholecystostomy may be considered to prevent complications of acute cholecystitis in less fit patients (377).

XIII. Gallstones in cholestatic liver disease

a. Prevalence

Several studies converge in demonstrating an increased prevalence of gallstones in patients with liver diseases. In a cross-sectional and longitudinal study,

involving patients with liver cirrhosis, a gallstones prevalence of 29.5% was reported (382). The presence of stones was more prevalent according to age and severity of cirrhosis while it did not change according to gender or cause of disease. In the same study, a cumulative incidence of 40.8% at eight years was reported, similar to that observed in a previous study (383). Data from patients undergoing liver transplantation also confirm this trend (384). Interestingly, and differently from the general population, the majority of gallstones in cirrhotic patients is represented by pigmented stones, possibly as a consequence of the unbalance between mono-conjugated (less water soluble) and di-conjugated bilirubin in bile (385). Regarding chronic cholestatic adult liver diseases, a significant increase in cholecystectomy (27%) was reported in comparison with control (17%) in PBC patients (386). In another study, PSC patients were examined demonstrating a similar prevalence of gallstone and cholecystitis accounting for 25% of cases (226). Finally, regarding non-cirrhotic liver diseases, interest is gaining in the relationship between fatty liver and gallstones. In a study on patients with type 2 diabetes it was found that prevalence of gallstones was similar regardless of NAFLD presence (25.5% NAFLD vs. 23.6% control) even if this condition was more associated to symptoms and cholecystectomy (387). However, the possible relationship between fatty liver and gallstones remains complex due to the presence of several confounding factors (type 2 diabetes, obesity, etc.) and considering that gallstones may be an early indicator of the metabolic derangement leading to NASH (388).

b. Treatment

Since definitive therapy of symptomatic gallstones largely requires surgical and/or invasive procedures, and cirrhotic patients are considered extremely fragile in this regard, clinical management of these patients remains difficult. Portal

hypertension and reduced liver functions are factors setting cirrhotic patients in a class of high surgical risk. Gallbladder surgical removal (open cholecystectomy) was defined as “hazardous” in an early study reporting 83% mortality in patients with liver diseases and impaired prothrombin time (389). A more recent Danish study also confirmed a ten-fold increase in 30 days mortality after open cholecystectomy in cirrhotic patients in comparison with control (390). Providentially, this tragic picture had a relevant improvement due to the advent of laparoscopic approaches in recent decades (391, 392). In a meta-analysis comparing open or laparoscopic gallbladder removal in cirrhosis, the latter was associated with a significant decrease in complications and hospital stay (393). However, a crucial point is represented by the stratification of risk in each single patient. Child-Turcotte-Pugh score has been historically developed to evaluate the surgical risk of cirrhotic patients (394). According to Child-Turcotte-Pugh evaluation and severity of liver disease, the patient may belong to class A, B or C. It is agreed that A or B patients may undergo laparoscopic cholecystectomy while those in C class are usually not considered for surgery due to poor conditions (144). More recently another scoring system has gained interest in the assessment of cirrhotic patient prognosis and their priority for liver transplant: the so-called model-(for)-end-stage-liver–disease (MELD) (395). Even though a study demonstrated a preoperative MELD score >13 to be associated with cholecystectomy complications in cirrhotic patients (396), the cut-off for a safe procedure has not been identified so far.

In conclusion, while the prevalence of gallstones increases in patients with liver impairment, the usual therapeutic approaches are risky in a significant percentage of them, and other effective strategies are lacking. The evidence that stones are more frequent in advanced liver impairment (382) is also of concern demonstrating that those more in need of treatment are, at the same time, the ones with increased

contraindications. In this setting, medical therapy also seems of marginal help. In fact, cirrhotic patients are usually affected by pigmented stones and UDCA does not have significant effects on them.

Extensive research is needed to find alternative (non-invasive/medical) approaches to gallstone treatment in patients with liver disease. Regarding this issue, it should also be considered that NAFLD is a rising pathological liver condition affecting more than one third of adult western populations (269) and is unfortunately associated with both liver cirrhosis and gallstone disease.

CONCLUSION

Gallbladder disorders and gallstones are significant occurrences that can impact quality of life and mortality in humans. The association of gallbladder diseases, specifically gallstones, with cholestatic disorders highlights an important association between the gallbladder and the intrahepatic biliary tree (Table 4). It is intuitive that these two tissues would be interlinked in both normal and pathological states considering that the gallbladder is an extension of the biliary tree, and they are lined by a similar epithelial cell type; however, research generally looks at either gallbladder disease or intrahepatic biliary disease separately. The fact that gallbladder damage, gallstones and even gallbladder cancer have been shown to be associated with different liver disorders highlights the notion that we should look closer into the mechanisms and crosstalk mediating these paracrine injuries during various cholestatic liver diseases. Research that better understands the occurrence of gallbladder injury in cholestasis and whether they feedback on each other to promote damage in one another is necessary to better define whether congruent damage in these tissues can be treated separately or if it highlights a different issue or necessary intervention.

It is largely known that gallbladder damage and gallstones are highly regulated by cholesterol, BAs, lithogenic bile and bile stasis. These findings are not surprising since these components are found in high concentrations in bile and can remain in the gallbladder for an increased amount of time while waiting for the physiological signal that induces gallbladder emptying. This finding is also important to note since bile flow and BA circulation and conjugation can be regulated by intrahepatic cholangiocytes. This mechanism shows that processes mediated by the intrahepatic bile ducts may, in turn, regulate gallbladder damage or stone formation as a downstream consequence. This is also highlighted by the finding that both the intrahepatic and gallbladder cholangiocytes express transporters important for the transport of BAs. A similar expression profile was also noted for receptors and transporters necessary for water and bicarbonate secretion. Considering similar mechanism are found in these different biliary populations, it is unsurprising that damage in these two compartments may be linked; however, it is important to note expression discrepancies between the intrahepatic and gallbladder cholangiocytes, with higher expression profiles potentially noted in the gallbladder epithelia. Therefore, the gallbladder may play an important role in in bile modification that can in turn impact pathophysiology, which is something to be considered when discussing cholecystectomy.

One of the major treatments for gallbladder disorders is cholecystectomy; however, this may not always be feasible or desired by the patient. If we can better evaluate the link between cholestasis, biliary damage, and gallbladder disorders we could potentially find therapeutics to target these that do not include surgical intervention. In line with this, a better understanding of the intricacies linking the intrahepatic biliary tree and gallbladder can help to identify modalities or biomarker that can indicate gallbladder damage early on to better detect injury at earlier stages.

As discussed in the last part of this comprehensive review, much work is being done to identify new diagnostic and therapeutic approaches to counteract gallbladder disorders. It is necessary that future work, both in clinical trials, meta-analyses, and pre-clinical models, better evaluate the gallbladder during liver disease to better understand these issues and identify improved approaches for patients.

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FIGURE LEGENDS:

Figure 1: Image of the gallbladder and associated anatomical parts. The gallbladder can be divided into the fundus, body and neck and it then branches to the cystic duct that connects with the common bile duct. The common bile duct can further branch into the common hepatic duct, which further branch into left hepatic duct and right hepatic duct. Image made with BioRender.

Figure 2: Image of the layers of the gallbladder wall with various transporters and receptors important for gallbladder physiology. The gallbladder wall is divided into the following layers: mucosa, muscularis, perimuscular fibrous tissue and serosa. The epithelial in the mucosa layer modulate water, chloride, and bicarbonate secretion with aquaporin channels, cystic fibrosis transmembrane conductance regulator, and the purinergic Y2 receptor. The muscularis is involved with neuropeptide signaling and potassium release by ether-a-go-go related 1 potassium channel. Image made with BioRender.

Figure 3: Comparison of acute and chronic cholecystitis. Acute cholecystitis is an acute inflammatory response and can be due to cystic duct obstruction, overproduction of mucus, and/or lithogenic bile. Chronic cholecystitis is due to ongoing inflammation and is primarily associated with cystic duct blockage and lithogenic bile. Image made with BioRender.

Figure 4: Diagram of the main gallbladder disorders. Cholelithiasis is gallstone formation (either cholesterol, brown or black stones) and can complicate issues by becoming lodged in the cystic duct. Polyps are generally benign but can rarely be cancerous. Cholecystitis can be either acute or chronic, is mostly brought on by gallstones, is associated with abdominal pain and can result in gallbladder perforation.

Gallbladder cancer is a rare condition and is usually labeled as adenocarcinoma. Image made with BioRender.

Figure 5: Diagram of the different portions of the biliary tree in humans and mice. In humans, the biliary tree is separated from the most distal to the most proximal end as follows: canals of Hering, ductules, interlobular ducts, septal duct, area ducts, segmental ducts, left and right hepatic duct, and common hepatic duct. The mouse biliary tree is divided into two parts: the small ducts and the large ducts. Stem cell niches termed hepatic progenitor cells (HPCs) and the peribiliary glands can be found at the ends of small ducts or in the larger duct walls, respectively. Image made with BioRender.

Figure 6: Ultrasonography of the gallbladder (longitudinal and transversal scans) in a PSC patient (top and middle panels; length=12.3 cm; width=6.6 cm; height=6.0 cm; volume=253.0 mL) and a healthy control gallbladder (bottom panel; length=7.2 cm; width=2.5 cm; height=2.8 cm; volume=26.2 mL). Reprinted with permission from *Gut*. 1996 Oct; 39(4):594-599.

Figure 7: Photomicrograph images of gallbladder stones in *Mdr2*$^{-/-}$ mice (magnification=400X). (A) Needle-like crystals (arrows) found on the edges of a yellow-colored stone. Needle-like crystals are short, straight, filamentous cholesterol crystals. (B) Radial crystal pattern of a stones core showing needle-like crystals (arrow). Reprinted with permission from *Hepatology.* 2004 Jan; 39(1):117-128.

Figure 8: Histological image of the layers of the gallbladder wall in gallbladder cancer, corresponding to T stage. HA=hepatic artery; PV=portal vein. Reprinted with permission from *Gastroenterology Clinics of North America.* 2010; 39:333.

Figure 9: (A) Fasting gallbladder wall thickness in healthy controls, steatotic patients and NASH patients. (B) Gallbladder ejection fractions in healthy controls, steatotic

patients and NASH patients. Reprinted with permission from *Journal of Neurogastroenterology and Motility.* 2016 Jul; 22(3):470-476.

Figure 10: Pathological imaging of hematoxylin and eosin (H&E) staining of the gallbladder from an ARLD patient. (A) 10X imaging of H&E staining and (B) 40X imaging of H&E staining showing chronic cholecystitis with suppurative inflammation (arrows). Reprinted with permission from *Medicine (Baltimore).* 2018 May; 97(18): e0414.

Figure 11: Radiological findings of the gallbladder and SARS-CoV2 qRT-PCR from a COVID-19 infected patient. (A) Abdominal CT scan showing cholecystitis. qRT-PCR was performed on gallbladder samples to assess SARS-CoV-2 presence and (B) shows 3 samples from the gallbladder that were positive for SARS-CoV-2, and (C) the RNA control was consistently positive. Reprinted with permission from *Journal of Hepatology.* 2020 Dec; 73(6):1566-1568.

Gallstone and Gallbladder Disease: Biliary Tract and Cholangiopathies

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ABSTRACT

Cholestatic liver diseases are named primarily due to the blockage of bile flow and buildup of bile acids in the liver. Cholestasis can occur in cholangiopathies, fatty liver diseases and during COVID-19 infection. Most literature evaluates damage occurring to the intrahepatic biliary tree during cholestasis; however, there may be associations between liver damage and gallbladder damage. Gallbladder damage can manifest as acute or chronic inflammation, perforation, polyps, cancer and most commonly gallstones. Considering the gallbladder is an extension of the intrahepatic biliary network, and both tissues are lined by biliary epithelial cells that share common mechanisms and properties, it is worth further evaluation to understand the association between bile duct and gallbladder damage. In this comprehensive review, we discuss background information of the biliary tree and gallbladder, from function, damage, and therapeutic approaches. We then discuss published findings that identify gallbladder disorders in various liver diseases. Lastly, we provide the clinical aspect of gallbladder disorders in liver diseases and ways to enhance diagnostic and therapeutic approaches for congruent diagnosis.

DIDACTIC SYNPOSIS:

Major teaching points:

- The gallbladder is a specialized organ that plays roles in bile modification and digestion of fats.
- Gallbladder damage can manifest as acute or chronic inflammation (cholecystitis), perforation, polyps, cancer, and more commonly gallstones (cholelithiasis).

- The gallbladder epithelial cells closely resemble those of the intrahepatic biliary tree, but distinct differences may account for specialized functions.
- Bile duct damage characterized by inflammation, fibrosis and ductular reaction can be found in primary sclerosing cholangitis (PSC), primary biliary cholangitis (PBC), alcohol-related liver disease (ARLD), non-alcoholic fatty liver disease (NAFLD), cholangiocarcinoma (CCA) and COVID-19.
- There is an association between gallbladder disorders and bile duct damage, but direct links are unknown.
- In some liver diseases, having congruent gallbladder damage increases morbidity and mortality in patients.
- Current work is underway evaluating different modalities that may be beneficial for the diagnosis or treatment of gallbladder disorders, specifically in the setting of liver disease.

DIDACTIC FIGURE LEGENDS:

- **Figure 1:** This figure labels the different parts of the gallbladder and the connected extrahepatic bile duct.
- **Figure 2:** This figure illustrates the different layers of the gallbladder wall and highlights some key receptors and transporters that maintain gallbladder functions.
- **Figure 3:** This figure illustrates some differences and similarities between acute and chronic cholecystitis.
- **Figure 4:** This figure illustrates the main gallbladder disorders discussed in this review and the main characteristics associated with them.

- **Figure 5:** This figure labels the human and mouse biliary tree and stem cell niches.
- **Figure 6:** This image shows an enlarged gallbladder in a PSC patient versus control.
- **Figure 7:** This photomicrograph shows a gallbladder stone and its needle-like crystals found in the gallbladder of *Mdr2*$^{-/-}$ mice.
- **Figure 8:** This image shows the layers of the gallbladder wall with corresponding tumor stage for gallbladder cancer.
- **Figure 9:** These graphs show changes in fasting gallbladder wall thickness and ejection fractions in control, steatosis and NASH patients.
- **Figure 10:** These images show low and high magnification of chronic cholecystitis in a patient with ARLD.
- **Figure 11:** This figure shows an inflamed liver in a patient with COVID-19 and qRT-PCR analysis confirming SARS-CoV-2 expression in the gallbladder with positive controls run as well.

INTRODUCTION ON THE GALLBLADDER

I. Gallbladder anatomy and function

Anatomically, in humans the gallbladder is in the upper abdomen beneath the liver, and in mice, it is attached with the diaphragm via connective tissue and is situated between the left and right medial lobes of the rodent liver (1). Cholangiocytes are ciliated epithelial cells that line the biliary tree and line the lumen of the hollow gallbladder in both humans and rodents. Bile is synthesized by hepatocytes and is drained into the biliary tree which acts as a conduit for bile flow. Bile flows through the intrahepatic biliary network and is stored in the gallbladder until its eventual drainage into the common bile duct, that is connected to the gallbladder. The fundus, the widest

part of the gallbladder, gradually narrows and tapers to form the infundibulum which eventually connects with the cystic duct that joins the common hepatic duct to form the common bile duct (Figure 1) (1). Bile, after being secreted from the gallbladder, travels to the duodenum via the hepatopancreatic ampulla where the common bile duct and pancreatic duct merge to make entry into the duodenum. Bile secretion from the gallbladder, known as gallbladder emptying, is regulated by the gastric hormone, cholecystokinin (CKK). CKK regulates the contractility of the gallbladder thereby regulating the emptying process (2). Apart from the contribution of cholesterol, gallbladder contractility or gallbladder emptying can be another cause for gallstone formation. Gallbladder contractility (emptying and filling) is regulated by the entero-hormone, CCK, and fibroblast growth factor (FGF)15 (in mice) and FGF19 (in human) respectively. CCK receptors are predominantly present in the muscularis (smooth muscle) of the gallbladder and are affected by high cholesterol levels. High circulating and membranous cholesterol induces hypomotility in the gallbladder (3). CCK-1 receptors were found to be sequestered by elevated cholesterol levels in a caveolin-3 dependent pathway (4). Sequestration of CCK-1 receptors would result in reduced gallbladder emptying and can result in increased risk of gallstone formation. Small and large cholangiocytes, which are distinct in structure and function, line the small and large bile ducts of the intrahepatic biliary tree in mice, which will be discussed in detail below. Cholangiocytes that line the gallbladder bear more resemblance to large cholangiocytes in mice.

Besides storage of bile, the gallbladder also functions to concentrate the composition of bile by reabsorption of water and various biliary constituents, such as bile acids (BAs) (5). This procedure of altering bile composition requires the intricate functioning of membrane transport across the biliary epithelium which have been the

focus of several early studies. One of the earliest studies by Diamond *et al.* in 1964 showed that the gallbladder regulates the concentration of bile by modulating isotonic reabsorption of water and sodium chloride through an active process (6). There are thirteen aquaporin (AQP) channels responsible for water absorption throughout the biliary tract, including the gallbladder (7). Among these channels, AQP1 and AQP8 are the two most widely expressed channels in the gallbladder epithelium (8); however, there are conflicting reports regarding the localization of AQP1 and AQP8 in the gallbladder. One study emphasizes profuse expression of AQP1 on the apical membrane of the gallbladder epithelia (9), another study reports that AQP1 is expressed on both apical and basolateral membranes with AQP8 being expressed mainly in the apical membrane of the gallbladder epithelial (10). AQP1 knockout (*AQP1*$^{-/-}$) mice have similar sized gallbladders as their wild-type (WT) controls, but had a significant difference in water permeability (9). Similarly, AQP8 may be involved in water absorption from the gallbladder, yet *AQP8*$^{-/-}$ mice didn't have significant physiological defects compared to WT controls (11). Defects in other AQPs can lead to dysfunctional water absorption and clinical conditions including cholestasis, obesity, and insulin resistance (12, 13). From the existing genetic knockout studies, it can be surmised that AQPs have far reaching effects in the liver and gallbladder.

The gallbladder also secretes mucin and bicarbonate. Mucin secretion occurs because of calcium-dependent pathway and bicarbonate secretion is mediated by adenosine 3',5'-cyclic monophosphate (cAMP)-dependent pathway. Both constituents are essential to exert cytoprotective effects on the gallbladder epithelia against toxic BAs. An electrogenic anion secretion study in isolated human gallbladder mucosa from normal and cystic fibrosis patients revealed that anion secretion in the gallbladder is facilitated by extracellular adenosine triphosphate (ATP) via purinergic receptor Y2

($P2Y_2$). This mechanism explains the altered and more toxic biliary composition during cystic fibrosis thereby contributing to hepatobiliary complications (14). Cystic fibrosis transmembrane conductance regulator protein (CFTR), the gene impaired in Cystic Fibrosis, regulates ion transport in the biliary epithelia. CFTR is a chloride channel regulated by the intracellular and extracellular concentration of cAMP. Its profuse localization in the apical membrane of biliary epithelia, including the gallbladder, is an indication of its significant role in regulating other ion channels. Ether-a-go-go-related gene 1 protein potassium channel is a voltage gated ion channel located in gallbladder smooth muscle which regulates contractility by modulating membrane potential (15). Taken together, the gallbladder physiology is mainly maintained by these ion channels that regulate transepithelial ion transport.

Just like the rest of gastrointestinal tract, the gallbladder is profusely innervated from both the central nervous system and enteric nervous system and primarily regulated by a ganglionic plexus located on the wall of the gallbladder fundus. An early study on guinea pig gallbladder suggests that the organ is constituted of four main layers of tissues: (i) the mucosa, (ii) the muscularis, (iii) the perimuscular fibrous tissue and (iv) serosa which is the layer of subperitoneal connective tissue (16). Each of these layers are highly innervated by the cholinergic neurons, these neurons also express neuroendocrine factors like substance P, neuropeptide Y and somatostatin. In addition to the presence of cholinergic neurons, the gallbladder was also found to express purinoreceptors (P2X), P2X2 and P2X3, that mainly signal via ATP (17). By immunohistochemistry, it was found that in guinea pigs the P2X2 and P2X3 receptors were expressed in the ganglia of the nerve fibers in the gallbladder. Moreover, this study highlights that nerves that stained positive for alpha calcitonin gene related peptide were also positive for P2X2 and P2X3 receptors (9). The role of these

neuropeptides in modulating gallbladder physiology is not well studied; however, it can be surmised from the existing studies that complex neuropeptide signaling in the highly innervated gallbladder plays an important role in gallbladder emptying and transepithelial ion channel transport that can influence the composition of bile. The gallbladder is a dynamic contributor to bile flow, physiology, and composition due to its expression of these different transporters and receptors (Figure 2).

II. *Gallbladder disease and gallstones*

Most gallbladder diseases occur because of dysfunctional bile secretion, including the malabsorption of ions and water in both the intra- and extra-hepatic cholangiocytes. However, inflammation and epithelial overgrowth can lead to various gallbladder disorders as well. Another widely prevalent cause of gallbladder diseases is a poor diet, which mainly manifests as gallstones, or cholelithiasis. Gallbladder-related diseases will be discussed in the following sections.

a. Gallbladder inflammation (cholecystitis)

Cholecystitis (i.e., gallbladder inflammation) is a multifactorial disorder, and one of the main causes of gallstone formation. Most gallstone cases lead to blockage of the cystic duct, resulting in bile accumulation that promotes inflammation (18); however, other biliary tract disorders, such as tumors and certain infections can promote cholecystitis (19, 20). In this section, we will focus on pathophysiology, diagnosis, and treatment of the most common gallbladder diseases, such as acute cholecystitis, chronic cholecystitis, and gallbladder perforation.

i. Acute cholecystitis

Acute cholecystitis is acute inflammation of the gallbladder due to obstruction of the cystic duct (21). The cystic duct can be blocked from gallstones or biliary sludge formation. Other less common causes can be due to the presence of a mass (primary

tumor or gallbladder polyp), parasites, or foreign bodies (22-24). Once the cystic duct is blocked, the gallbladder mucosa continues to produce mucus that is not drained, and the intraluminal pressure inside the gallbladder increases leading to an acute inflammatory response. Additionally, the secretion of prostaglandins, I_2 and E_2, can promote an inflammatory response (25). The pathophysiology of acute cholecystitis is characterized by three processes: (i) mechanical stimulus (gallbladder duct obstruction); (ii) bacterial infection; and (iii) irritation that promotes inflammation (18). There are two theories attempting to explain the pathogenesis of acute cholecystitis: (i) cystic duct obstruction and gallbladder artery occlusion (18), and (ii) cystic duct obstruction and perpetual lithogenic bile (26). In 2006, Yokoe *et al.* developed the Tokyo Guidelines for the management of acute cholangitis and cholecystitis (27) that were approved as worldwide criteria. Specifically, patients with acute cholecystitis have right upper quadrant or epigastric abdominal pain, Murphy's sign, and tenderness. If gallbladder inflammation persists, patients show fever, high levels of C-reactive protein, and abnormal white blood cell count. Finally, different imaging approaches can be used to diagnose acute cholecystitis, such as transabdominal ultrasonography (US), cholescintigraphy, and magnetic resonance imaging (MRI); however, US and cholescintigraphy are used most frequently (27). One approach to manage acute cholecystitis is reduction of gallstones in the gallbladder that move into the cystic duct. If there is not proper breakdown of the gallstones, complications may occur; such as, advanced cholecystitis or gallbladder perforation (25). Acute cholecystitis management includes (i) fasting to reduce the stress of inflamed gallbladder, (ii) rehydration with intravenous fluids, (iii) antibiotics to counteract the infections, (iv) administration of analgesic for pain, (v) procedures to remove gallstones through medication (indomethacin (28) and diclofenac (29)) and/or removal

of the gallbladder (cholecystectomy, laparoscopic cholecystectomy), which is the gold standard approach (30).

ii. Chronic cholecystitis

Chronic cholecystitis is characterized by continual inflammation of the gallbladder that drives mechanical and physiological dysfunction (31). Over 90% of chronic cholecystitis cases are associated with gallstone blockage in the cystic duct, leading to abdominal pain (biliary colic), episodic waves of epigastric pain, and discomfort (21). Studies show that lithogenic bile may promote gallbladder damage through free radical formation from hydrophobic BAs that, together with the reduction of the mucosa protection, induce a continuous inflammatory state (32, 33). Furthermore, the reduction in CCK receptor expression in the smooth muscle impairs gallbladder contraction leading to stasis and damaging lithogenic bile formation (31). Histological analysis showed that the gallbladder from patients with chronic cholecystitis has increased subepithelial and subserosal fibrosis, followed by mononuclear cell infiltration (21). Patients with chronic cholecystitis have continuous right upper abdominal pain that can extend into the back. Other symptoms include nausea, vomiting and anorexia (31). Hepatobiliary scintigraphy (34) or a hepatobiliary iminodiacetic acid scan with CCK (31) are the major imaging procedures used to confirm the presence of chronic cholecystitis. The gold standard procedure to treat this disorder is laparoscopic cholecystectomy, which is characterized by low morbidity and invasiveness (21, 31). Differences and similarities in acute versus chronic cholecystitis are shown in Figure 3.

iii. Gallbladder perforation

Gallbladder perforation is characterized by a hole or an opening in the gallbladder wall usually as a complication of acute cholecystitis. Gallbladder

perforation has high morbidity and mortality rates due to delays in diagnosis (21, 35, 36). Usually, a calculus is formed which blocks the drainage of bile from the cystic duct which increases intra-cholecystic pressure, epithelial injury, secretion of phospholipases, degradation of cell membranes, and intense inflammatory reaction, resulting in gallbladder perforation (37). Several studies observed that the most frequent site of perforation is the fundus (35, 38). Niemeier (1934) classified gallbladder perforation into three types: Type I, acute perforation into the free peritoneal cavity; Type II, subacute perforation where the perforated peritoneal cavity of the gallbladder is surrounded by an abscess; and Type III, chronic perforation with the presence of fistulous communication between the gallbladder and some other viscus (39). This classification was based on clinicopathological findings and was criticized by different studies. For instance, Anderson *et al*. reported a case series of cholecystobiliary fistulae and classified them as Type IV gallbladder perforation (40). The difficulty in diagnosing gallbladder perforation and distinguishing it from acute cholecystitis are documented (36, 41). Morbidity and mortality rates of gallbladder perforation are high due to delays in both diagnosis and treatment. Gallbladder perforation treatment includes cholecystectomy, drainage of abscess, if present, and abdominal lavage (35). In sum, an earlier diagnosis and immediate surgical intervention may reduce morbidity and mortality rates.

b. Gallbladder polyps

Gallbladder polyps are an elevation of the gallbladder mucosa that extends into the lumen (42, 43). Polyps may be classified between “true” and “pseudopolyps” based on earlier pathological descriptions (42). True gallbladder polyps are adenomas of the gallbladder wall that can progress into malignant phenotypes. Indeed, they can be

categorized as benign (fibromas, lipomas, and leiomyomas) or malignant (mesenchymal neoplasms, lymphoma, or metastases). Pseudopolyps do not have malignant potential and are categorized as cholesterol pseudopolyps, focal adenomyomatosis, and inflammatory pseudopolyps (42, 44). The progression of non-malignant gallbladder polyps to malignancy is characterized by different risk factors, including polyp size, Primary Sclerosing Cholangitis (PSC), Indian ethnicity, sessile polyps, gallstones, and gallbladder wall thickening (44, 45). The diagnosis of gallbladder polyps mostly occurs on accident during imaging (transabdominal ultrasound, multiparametric ultrasound, and endoscopic ultrasound) for diagnosis of intermittent right upper quadrant pain, nausea, and vomiting (46). According to the size of the polyps and the medical history of the patient, the management of gallstone polyps may be different. Briefly, if the polyps are 6-9 mm in a patient with the risk factors descried above, cholecystectomy is recommended; however, if the patient has 6-9 mm gallbladder polyps and do not have any risk factors, serial US examinations are required at 6 months, 1 year and then early up to 5 years to monitor size (44, 47).

c. Gallbladder cancer

Gallbladder cancer is the most common malignancy of the biliary tract with poor diagnosis and variation in incidence across the world (48, 49). Epidemiological studies observed that Native Americans and Southeast Asians are at a higher risk to develop gallbladder cancer, followed by Eastern European including Polish, Czech, Slovakian, and Asian. On the other hand, South Americans of Indian descent, Israeli and Japanese persons have shown moderate risk of gallbladder cancer development (48, 50, 51). This variability on the onset of gallbladder cancer is due to the combination of environmental and genetic factors. Indeed, women have a higher risk to develop gallbladder cancer compared to men (female:male ratio ~2.6:1), especially over 50

years of age (51). The enhanced incidence of gallbladder cancer in women is likely due to higher estrogen levels, which promotes the formation of gallstones through increasing cholesterol saturation in bile (52). Furthermore, there are other risk factors that can increase gallbladder cancer incidence, including body mass index (BMI), family history, cholelithiasis or other benign gallbladder pathologies, chronic infection with *Salmonella* or *Helicobacter pylori*, anomalous pancreatobiliary duct junction, porcelain gallbladder, gallbladder polyps, and obesity. Lastly, secondary risks factors including tobacco consumption, chemical exposure (benzene), high carbohydrate intake, and chronic diarrhea can influence gallbladder cancer risk (50, 51). The symptoms of gallbladder cancer are very vague and mimic biliary colic, making it difficult to diagnose; however, the advanced stage of gallbladder cancer is characterized by weight loss and jaundice, and imaging approaches can help in the identification of the tumor mass (49, 51). According to the American Joint Committee on Cancer's 8th edition, the staging of gallbladder cancer is divided into tumor (T) and lymph node (N) categories (53). Specifically, the T categories describe the tumor penetration levels within the gallbladder wall and the N categories describe the number of metastases in the lymph nodes (51, 53). Gallbladder cancer can be treated by chemotherapy, targeted therapy, and surgery (54). Early-stage gallbladder cancer patients can undergo surgical resection, but most of the diagnosis occurs when the cancer is advanced. In this case, gallbladder cancer patients undergo chemotherapy and a series of surgical procedures to improve their lifespan (49, 51, 54).

d. Gallstones (cholelithiasis)

Cholelithiasis is the clinical manifestation of concreted bile salts, bilirubin and sterols in the gallbladder or common bile ducts popularly known as gallstones or bile duct stones, respectively. Cholelithiasis is a disorder involved in many liver diseases,

and thus most of this chapter will be spent discussing the intricacies of this injury. Over time, cholelithiasis leads to multiple compactions resulting in an inflamed gallbladder, or cholecystitis (described above). Gallstones are formed in the gallbladder and/or intrahepatic bile ducts and sporadically move into the common bile duct or the intestines (55, 56). The presence of gallstone disease has an incidence rate of about 10% to 20% in the adult population (56, 57). Cholelithiasis can be symptomatic or asymptomatic depending on the lithiation or stone formation stage (58). The major factors leading to the formation of gallstones include defective gallbladder motility, metabolism and secretion of cholesterol and BAs (59). The gut microbiota is also involved in the regulation of BA metabolism and composition of the BA pool, contributing to gallstone formation (60, 61).

i. Types of gallstones (cholelithiasis) and formation

According to the composition of major constituents, gallstones are categorized into three types: pure cholesterol stones, pure pigment stones and mixed stones (62). Cholesterol gallstones are estimated to account for more than 80% of gallstones diagnoses (63). Several studies analyzing the composition of surgically removed gallstones found that cholesterol gallstones are the dominating cause of clinical gallstone disease (64). In a German study, cholesterol was observed to be the main constituent in 93.3% of gallstones, and pigment was in 5.5% of gallstones (65).

The origin of cholesterol gallstones has common pathogenic links with broad metabolic abnormalities characterized by altered cholesterol homeostasis, such as obesity, dyslipidemia, type 2 diabetes, NAFLD and the metabolic syndrome (56, 66, 67). In fact, many of these metabolic disorders have been associated with an elevated occurrence of cholesterol gallstones (68, 69).

Pigment stones are mainly constituted by calcium bilirubinate and can further be classified into black and brown stones (70). Black pigment stones are often related to physiological and pathophysiologic conditions including increased production of unconjugated bilirubin and hemolysis, and clinical conditions such as cirrhosis, spherocytosis, thalassemia, sickle cell disease, and malaria (70, 71). There is a higher incidence of black pigment stones than cholesterol gallstones in developing and Asian countries (72-74); however, the prevalence of cholesterol gallstones is increasing in Asia due to an increase in Westernized lifestyle (73). Brown pigment stones, which contain more cholesterol and fatty acids (FAs) than black pigment stones (75), are observed in the hepatic ducts and believed to be caused by cholangitis, biliary stasis (76, 77), or parasitic infestations (71). Brown pigment stones are not as common in Western countries as they are in Asia (78, 79). A figure summarizing the main gallbladder diseases can be found in Figure 4.

ii. Genetic risk factors of cholelithiasis

Just like other gastrointestinal disorders, risk factors for gallstone formation include both genetic and environmental components. Cholelithiasis is a complex polygenetic disease since the association between some gene variants and gallstone formation have been verified. For example, the single nucleotide polymorphisms of the genes HHEX (rs1111875), MC4R (rs17782313), MAP2K5 (rs2241423) and NRXN3 (rs10146997), were positively associated, but FAIM2 (rs7138803) was negatively associated with the occurrence of gallstone disease (80).

Extensive genetic analysis also identified a gallstone (*Lith*) gene map that is essential for the formation of gallstones. *Lith1* is one such gene that affects cholesterol-induced gallstones in mice (81). By using gallstone-susceptible mice (C57BL/J) and gallstone-resistant mice (AKR/J), it has been identified that *Lith1* and

Lith2 are related to gallstone formation. *Lith1* is involved in the regulation of liver cholesterol hypersecretion, and *Lith2* plays a role in the bile salt-dependent bile flow (82). In human species, ATP-binding cassette subfamily G 5 (*ABCG5*) and *ABCG8* are homologous to murine *Lith1* and *Lith2. ABCG5* and *ABCG8* are expressed in hepatocytes and intestinal cells and can be transported from the endoplasmic reticulum to the apical membrane as heterodimers (83). ABCG5/G8 can transport neutral sterols into bile in hepatocytes or promote cholesterol efflux from the enterocyte back to the intestinal lumen for fecal excretion (84). When ABCG5/G8 is inactivated, reduced efflux of cholesterol into bile results in increases cholesterol levels in plasma and liver. While knockdown of *ABCG5/8* may be a deterrent to gallstone formation by attenuation of cholesterol secretion, overexpression of *ABCG5/G8* may increase cholesterol levels in the gallbladder, thus enhancing the likelihood of cholesterol crystal formation (85). Furthermore, *ABCG5/G8* was observed to be related to cholesterol gallstone prevalence in patients, and the gallstone associated variants in *ABCG5/G8* (*ABCG5-R50C* and *ABCG8-D19H*) were found in German, Chinese, Chilean and Indian populations. Overall, these findings show that these two genes influence gallstone disease.

Even though ATP-binding cassette subfamily B member 11 (*ABCB11*) and liver X receptor alpha (*LXRA*) are in the interval of the *Lith* locus, no genetic susceptibility of gallstone formation was associated with these two genes in the German samples tested (86). *Lith6* is another locus in the gene map which has two functional candidate genes associated with it, apolipoprotein B mRNA-editing protein (*APOBEC1*) and peroxisome proliferator-activated receptor gamma (*PPARG*) (87, 88). Like the previous study, analysis of German patient samples did not find an association of *APOBEC1* or *PPARG* with gallstone susceptibility. More analysis and mapping of *Lith1*

and *Lith6* loci are needed to identify more variants of gallstone susceptibility in humans (88).

The apolipoprotein E4 allele is related to the prevalence of gallstone disease. The E4 allele was found to be positively associated with gallstone disease in a meta-analysis of Chinese Han populations (89). Another study showed no correlation between apolipoprotein E genotypes and gallstone disease in a Danish population (90). No significant associations for E4 allele carriers were found in mixed ethnic populations or in white populations by meta-analysis (90). Meanwhile, conflicting results were reported for the E4 association in Hispanic and Spanish populations (91, 92). In fact, the apolipoprotein E plays an important role in the regulation of the response to dietary cholesterol and cholesterol excretion into bile as evidenced in knockout mice (93). However, no influence on bile cholesterol excretion was found due to the E4 carrier state in Caucasians with gallstones (94).

Young human adults with ATP binding cassette subfamily B member 4 (*ABCB4*) gene mutations present with low phospholipid levels in bile, which is associated with cholelithiasis (95). Mutations in mucin (*MUC*)-related genes have been extensively studied to elucidate the role of mucin in the development of gallstones. For example, *MUC5AC* encodes for a gel forming mucin that, when in excess, can promote gallstone concretion that is heavily influenced by interleukin (IL)-1β (96, 97). Tumor necrosis factor alpha (TNF-α) was also found to be induced by prostaglandin 2 which, in turn, induced the over expression of *MUC2* gene that is involved in gallstone formation (97).

iii. Lifestyle and cholelithiasis

An increase in alcohol consumption was inversely related to occurrence of gallstone disease in females (98). The negative correlation between alcohol

consumption and cardiovascular disease may explain the protective effects of alcohol consumption on cholesterol homeostasis (99). These benefits are attributed to increased cardio-protective blood levels of high density lipoprotein cholesterol and an increase in BAs (100). Other preventive mechanisms of alcohol consumption on gallstone formation include enhanced gallbladder motor function together with stimulation of contractions, thus reducing bile stasis and gallstone formation (101). Interestingly, a higher daily alcohol consumption was related to faster self-reported gut transit (102) and acute administration of alcohol was shown to stimulate propulsive pressure waves in the ileum but suppress impeding pressure waves in the jejunum (103). Therefore, the protective effects of alcohol consumption on gallstone disease may be due to the inhibition of secondary BA entry in the enterohepatic circulation.

Physical activity seems insignificant to gallstone disease. In a randomized controlled trial, an intervention of moderate or vigorous physical activity in pregnant women showed no influence on gallstone formation (104). Further, in the subgroup diagnosed with gallstones while being unaware of their status, physical activity was negatively related to clinical gallstone disease hospitalization when compared to a sedentary lifestyle (105). Furthermore, gallstone disease was inversely associated with physical activity in cohort studies (106). However, physical activity increases plasma CCK that enhance gallbladder contractions (107). These mechanisms may explain how physical activity exhibits beneficial impacts on pain related to gallstone disease.

iv. Obesity, weight loss and cholelithiasis

It was observed that gallstone disease is associated with certain body fat tissue (except BMI), such as: waist-to-hip circumference ratio with screen-detected gallstone disease, and computed tomography that measured visceral or subcutaneous fat with

clinical gallstone disease (108, 109). However, many other studies demonstrated the association between elevated BMI and gallstone formation, indicate BMI as an independent risk factor for the development of gallstone disease (110, 111). It has been estimated that a rise of more than 5 points of the BMI value increases the risk of gallstone disease by 1.63-fold (112). This correlation has been positive for females, but for males there is a lower association (113). This kind of variability may be attributed to the greater part of lean mass in men compared with women (113). It must be considered that there are other predominant factors such as estrogen levels in females, which can increase the synthesis and secretion of hepatic cholesterol, along with greater cholesterol saturation index and crystals formation, which make gallstone disease more prevalent in female patients (58).

On the other hand, excessive weight loss due to calorie restriction is also related to gallstone disease (114). There is more risk for incident screen-detected gallstone disease in patients undergoing bariatric surgery followed by rapid weight loss (115). The underlying mechanisms for gallstone disease prevalence during rapid weight loss may include an initial increase of bile cholesterol saturation, as well as impaired gallbladder motor function (116).

v. Estrogen and cholelithiasis

It has been reported that females are more predisposed to gallstone disease (98). This may be due to the binding of 17β-estradiol to intracellular estrogen receptors in the liver stimulating the excretion of cholesterol into bile, resulting in increased bile cholesterol saturation (117). Estrogen also promotes the activity of β-Hydroxy β-methylglutaryl-CoA (HMG-CoA) reductase to facilitate endogenous cholesterol synthesis (117). In one study, women with higher urinary estrone levels had a higher risk of gallstones disease (118). Similarly, hormone-replacement therapy promotes

increased bile cholesterol saturation in postmenopausal women (119). Overall, bile cholesterol saturation may play a key role in female gallstone disease.

vi. Microbiome influence on cholelithiasis

An increasing number of studies have shown the important role of the gut microbiome on cholelithiasis (61, 120). These complex microorganisms also exist in bile and the prevalence of gallstones is closely associated with abnormalities in bile duct flora. The microbiota of the gastrointestinal and biliary tracts are involved in almost all stages of bile formation, such as the regulation of cholesterol metabolism, lipid metabolism, biotransformation and enterohepatic circulation of BAs (121).

Studies have demonstrated the existence of living bacteria in gallstones. Microorganisms can enter the bile duct system from the duodenum via migration through the sphincter of Oddi, and they can also spread through the blood to the liver and next into bile (122). Microorganisms play a critical role in bile as nucleating factors, resulting in the formation of cholesterol and pigment gallstones (123). Gallstone formation can be regulated by bacteria properties in the gallbladder. For example, bacteria producing β-glucuronidase and phospholipase promoted pigment gallstones, while bacteria causing mucus abnormalities promoted cholesterol stone formation (124). Biofilm-forming bacteria in the bile, gallbladder, and gallstones are closely related to gallstone formation (125, 126). By comparing cholesterol gallstones with pigment gallstones, gram-positive bacteria were common in most of cholesterol gallstones, but not observed in pigment stones. Furthermore, *Helicobacter pylori*, a Gram-negative, motile bacteria was found in patients with symptomatic gallstone disease (127). However, this finding is still controversial, and more research is necessary to elucidate the role of the microbiota in gallstone disease. There are a

variety of risk factors that are associated with gallstone disease (Table 1) that need to be considered.

vii. Mouse models of cholelithiasis

The role of diet and ion channels have been well studied in cholelithiasis, and diet-induced models of cholelithiasis have widely been used to explore the effects and contributions of different ion channels to the concentration of bile. A lithogenic diet, which is constituted of 15% dairy fat, 50% sucrose, 20% casein and 1% cholesterol, is fed to mice for 18 weeks to induce cholelithiasis; however, various mouse strains respond differently where 100% of the C57BL/J and A/J strain were susceptible to and developed gallstones (81). Even though mucin has been highlighted to form a protective barrier in the gallbladder, studies in hamsters have reported that over secretion of mucin precedes gallstone formation in a lithogenic diet-induced model of gallstone formation (128). From other existing studies on animal models, it can be concluded that mucin is an important constituent of the gallstone matrix. In highly concentrated bile, gallbladder mucin can accelerate cholesterol monohydrate nucleation, a process that constitutes gallstone formation (129-131). There are several genes related to mucin expression such as *MUC1* and *MUC2* in the gallbladder that pose a genetic risk factor for gallstone initiation, as discussed above (132, 133).

Impaired lipid metabolism in the liver can translate to gallstone formation. A murine model with genetic knockout of liver-specific fatty acid binding protein 1 (*L-Fabp*$^{-/-}$ mice) fed with lithogenic diet for 2 weeks became significantly hypercholesterolemic along with developing more gallstones compared to the WT mice fed with lithogenic diet (134). *L-Fabp*$^{-/-}$ mice fed with chow diet also had increased fecal BA excretion and decreased ileal apical sodium-dependent bile acid transporter (*Asbt*) expression compared to the *L-Fabp*$^{-/-}$ mice fed with lithogenic diet, indicating

that enterohepatic shunting of BAs contributed to gallstone formation in this model (134). Knockdown of fatty acid transporter 2 (*Fatp2*$^{-/-}$ mice), which is also expressed in the gallbladder and the liver, showed reduced triglyceride content in the gallbladder and improved contractile strength in mice exposed to lithogenic diet (135). *Fatp2* is encoded by the solute carrier family 27-member 2 gene and knockdown by adeno associated virus (AAV) reduced gallstone formation in mice fed with lithogenic diet for 8 weeks (84). Interestingly, *Fatp2* knockdown did not affect cholesterol concentration and solubility in bile, but instead increased FA content in bile [83]. Although the authors did not elucidate the involvement of a specific pathway for Fatp2 mediated effects, they did highlight the role of prostaglandins in mediating gallbladder contractility [83].

CLINICAL ASPECTS OF GALLBLADDER DISEASE

I. Background

Gallstones represent the most prevalent disease of the biliary tract in the Western world, affecting 10-15% of the general population (136, 137). Changes in prevalence are observed according to gender and ethnicity (138) with Pima Indians exhibiting a historically higher rate of the gallstones with ~50% of adults affected (139). The economic burden of gallstone treatment is also significant (>$5 billion per year in the U.S.) and seems to be increasing (136). Gallstone-related mortality is declining and is relatively low (approximately 0.6%) but given the frequency of the disease, as reported in a 1979-2004 U.S. analysis, more than 1,000 patients per year die due to gallstone disease (140).

II. Symptomatic gallstones

Symptomatic gallstones are generally regarded as a condition requiring treatment since they have an increased risk of developing complications. As reported

previously, symptoms may be vague and not directly drawing attention to gallstones; however, prompt recognition and diagnosis may prevent conditions with significant morbidity and mortality, as reported in the following paragraphs.

III. Asymptomatic gallstones

Despite the difficulties in defining asymptomatic or symptomatic gallstones, the differences in the natural history of these two classes has been an argument for some time (141). In early studies on cholelithiasis, the estimated risk to develop symptoms was 1-2% yearly (142, 143). Onset of complications was ten times lower in asymptomatic patients (0.1-0.3% yearly) in comparison with symptomatic cases (144). In asymptomatic populations, the risk of treatment (typically surgical) is reportedly higher than the benefits (145, 146) and current guidelines do not suggest an operative approach for this subset of patients. Generally, observation of patients for onset of symptoms is advised (144, 147); however, exceptions may exist to this strategy. The most important exception in general practice is represented by porcelain gallbladder (148). This condition was historically linked to a significant risk in developing gallbladder cancer. Porcelain gallbladder consists of calcium deposition on the gallbladder wall (easily detected on US or computed tomography [CT] scan) that may present as complete or selective, with the latter form preferentially associated with malignancy. The high rate of cancer reported for this condition in early studies (12-33%), has been challenged by more recent data observing a lower rate of malignancy (≤6%) (148). Systematic gallbladder removal in patients with porcelain gallbladder remains controversial and consideration on a case-by-case evaluation seems wiser.

IV. Diagnosis

a. Symptoms and manifestations

Knowing the symptoms of gallstones in patients is of paramount importance to help distinguish between the two main clinical presentations, asymptomatic and symptomatic gallstone disease. For the past century, it is understood that the majority (nearly 70%) of gallstones are asymptomatic in nature, thus patients that complain of gastrointestinal issues are usually considered for treatment (141). However, the specific symptoms related to gallstone disease are not completely defined. A large cross-sectional Italian study, enrolling nearly 30,000 patients and focusing on gallstone symptoms concluded that right hypochondrium and/or epigastric pain (i.e., biliary colic), together with scarce tolerance to fatty meal, were among the more specific complaints (149). When these signs were present in the lack of gastro-esophageal reflux disease, they were far more specific for the diagnosis of gallstones. Cholelithiasis may induce biliary colic (150), that includes pain radiation to the back (right scapula), can last for hours and is associated with vomiting and other gastrointestinal symptoms, due to stone impaction in the cystic duct. Another sign noted during physical examination is the exacerbation of pain when the medical examiner has their hand firmly kept under the costal margin of the right chest (i.e., Murphy maneuver). Despite these definitions, the ability to detect symptoms of cholelithiasis differs in geographic location leading to heterogenous rates of treatment, definition of relevant signs and guidelines (151).

b. Blood biochemistry and imaging

There are no specific blood markers for the diagnosis of symptomatic cholelithiasis. Common liver function tests (alkaline phosphatase) and/or general inflammation indexes (C reactive protein levels and white blood cell counts) may be increased based on complications and the site of gallstone impaction. Some tests may

help in identifying specific complications, and these will be described in the corresponding paragraphs.

Beginning in the early 1980s, US emerged as an easy and specific imaging system for identifying gallstone disease (152). This technique has also been instrumental in identifying the natural history of gallstone formation in both asymptomatic and symptomatic forms. Typical stone US findings are iperechoic wall with a posterior shadow and, despite technical advancement, this technique remains superior in comparison with CT (153). MRI and cholangio-MRI have also had important applications for imaging gallstones. In fact, cholangio-MRI replaced diagnostic retrograde cholangio-pancreatography for gallstone detection since it accurately reproduces the anatomical picture of the biliary tree without safety issues. MRI is usually used as an integrative imaging approach when symptomatic gallstones are ruled out by US, but the potential presence of biliary stones need to be examined.

V. The clinical picture

The clinical picture of cholelithiasis may change widely ranging from asymptomatic forms to life-threatening conditions. The historical division of patients in two main classes (asymptomatic and symptomatic), even if it does not recapitulate the entire clinical horizon, is considered helpful in giving a general indication in selecting subjects needing treatment. Symptomatic patients may present with several complications and require closer monitoring or intervention.

a. Acute cholecystitis

As reported by Friedman *et al.* (141), acute cholecystitis appears to be the most frequent complication of gallstones, involving approximately one out of ten symptomatic patients. While the exact combination of clinical, biochemical and imaging features unequivocally leading to acute cholecystitis diagnosis is not yet

defined, the presence of fever, right hypochondrium pain, increased inflammatory markers and finding of gallbladder thickening and stones at US usually lead to the diagnosis (154). In the absence of stone migration to the common bile duct (described in the next paragraph) surgical resection of gallbladder (cholecystectomy) is generally indicated. Contraindications to cholecystectomy include those of general surgery such as septic shock or severely impaired clinical conditions. Conservative management of acute cholecystitis in patients with limited symptoms, even if sometimes successful, is generally not advised since ~60% of these patients would later require surgery and approximately one third will experience complications (155, 156). Timing for surgery depends on patient symptoms and risk of complications; however, a Cochrane Review comparing early (within 7 days from symptoms) and delayed (>6 weeks from symptoms) cholecystectomy for acute cholecystitis did not find significant differences in patient outcomes (157). A shorter hospital stay has been suggested when early cholecystectomy is performed.

b. Gallstones in the biliary tract and related complications

Even if stone migration to the biliary tract is not canonically considered a complication, this condition, associated with cholelithiasis in 10-20% of cases, is responsible for the most serious adverse events (158, 159). Analyzing the Swedish GallRisks registry, it was found that ~25% of patients with common bile duct stones may experience complications (160) while spontaneous expulsion from the biliary tract into the intestines is also possible. Common bile duct stone diagnosis is generally ruled out by the increase in liver function tests (usually normal if stones are retained in the gallbladder and/or cystic duct) and imaging (either US or MRI). Since common bile duct stones may determine relevant sequelae including obstructive jaundice, cholangitis and pancreatitis, bile tract cleansing is generally advised by current

guidelines (158, 159). The most relevant adverse conditions determined by stone impaction in the biliary tract are reported below.

Gallstones are the most frequent benign cause of obstructive jaundice, which impairs the liver and other physiological functions (161). Regarding the kidneys, in a study including 20 patients with obstructive jaundice (duration ~2 weeks), signs of acute tubular necrosis were observed at histology despite normal renal tests (162). Obstructive jaundice may also impair hemodynamic stability, immune fitness and the intestinal barrier leading to possible endotoxemia (161). Finally, obstructive jaundice may lead to bacterial overgrowth in the biliary tract, thus determining cholangitis.

Cholangitis diagnosis has been generally related to the presence of fever with spikes in pain in the right hypochondrium and jaundice (Charcot's triad); however, these signs were found to be present in just 22% of patients with cholangitis (163). Mortality of this condition remains significant, approaching 5% of cases (164). Broad spectrum antibiotics and, in severe cases, prompt biliary decompression is advised.

Gallstones are regarded as the most important cause of pancreatitis being responsible for more than one third of cases (165). Also, small stones/cholesterol crystals may sometimes give rise to acute pancreatitis (166). Epigastric pain increased pancreatic enzymes, and demonstration of stones at imaging may rule out the diagnosis. Mortality may occur in ~30% of severe cases (167).

There is an apparent association between gallbladder disorders, gallstones and bile duct damage. The role and occurrence of gallbladder disorders in cholestatic liver disease will be described in the following sections.

INTRODUCTION ON THE BILIARY TREE

I. Biliary tree structure, function and physiology

a. Background

The biliary tree, named so due to the resemblance the structure has with the branches of a tree, refers to the network of ducts that transport bile from the hepatocytes to the gallbladder and intestines. This system is involved in metabolism, waste disposal, and the movement and recycling of nutrients in the body (168-170). Bile plays a crucial role in the digestion and absorption of FAs, it emulsifies FAs and allows the hydrophobic molecules to be absorbed and transported for use or storage (168). A small percentage of the bile is lost in feces, allowing for larger molecules that cannot be excreted through the kidneys to also be disposed (168). The remainder of bile is reabsorbed and sent back to the liver through a cyclic process called enterohepatic circulation (171). Finally, selected vitamins and minerals use the biliary excretory system as a shuttle to connect to tissues where they are needed (168). The gallbladder is a protrusion extending from the biliary tree, indicating close anatomical relationships, and 10-15% of gallstone patients also present with bile duct stones (172); therefore, it is important to understand the biliary system and related diseases and how they may intersect with cholelithiasis.

b. Anatomy of the biliary tree

The branches of the biliary tree start in the liver, joining with other branches over and over until the whole network combines to form a single duct. The total length of the branches of the biliary tree in humans would be about 2 km (173). Different zones of the biliary tree can be separated by their area, diameter, morphology or physiology (174); however, in this review we will use luminal diameter to separate the different regions. The smallest sized bile ducts that make up the biliary tree begin at the canals of Hering, starting at just a few nanometers in diameter and lined by hepatic progenitor cells (HPCs) (171, 173). These canals separate canicular bile secreting

hepatocytes from the epithelial cholangiocytes that line the rest of the bile ducts. HPCs play a role in liver regeneration following injury, thus their presence in the canals of Hering is advantageous for hepatic recovery (175). The canals of Hering meet to form ductules, which come together as interlobular ducts, then septal ducts, each of which have consecutively larger diameters (170, 176). At this point, area ducts measure 300-400 μm in diameter and connect to the larger segmental ducts (400-800 μm) (171). This is where the left and right hepatic ducts, named for the liver lobes they branch into, finally come together to form the single common hepatic duct, collecting all the bile fluid the liver secretes (176). These measurements are for humans, and it is important to note that in rodents, cholangiocytes are more simply divided into small and large subsets, named for their anatomical location on either the small (<15 μm in diameter) or large (≥15 μm in diameter) ducts (177).

The common hepatic bile duct exits the liver then either diverts to the gallbladder through the cystic duct or continues from the liver as the common bile duct (171). The common bile duct meets the pancreatic duct after passing through the wall of the upper small intestine, to make the hepatopancreatic ampulla (i.e., the ampulla of Vater) (170, 176, 178). The ampulla of Vater consists of the conjoining pancreatic and common bile ducts, the sphincter of Oddi, and an extrusion of papilla where bile is released into the duodenum (168, 170, 178).

Along the murine intrahepatic large ducts and the human large segmental ducts, small peribiliary glands sporadically line the luminal wall (170, 171). The peribiliary glands are defined by their location, their mucinous secretions and their own stem cell niche that is separate from the HPCs (170). Secreting directly into the lumen of the bile ducts, intramural peribiliary glands have a mucosal epithelium and line the duct walls (170). Conversely, extramural peribiliary glands, located in the periductal

connective tissue, have their own conduits that transport their seromucosal secretions to the large bile duct lumen (170). Peribiliary glands have also been identified in the crypts of the gallbladder epithelium (179), indicating similar yet heterogenous cholangiocyte functions in the biliary tree and gallbladder. Branching of the biliary tree and its specific stem cell niches are shown in Figure 5.

While the inner walls of the ducts are lined by epithelial cholangiocytes and scattered peribiliary glands, a fibromuscular layer of tissue lays beneath (170, 178). This layer is made up of fibrous tissue and smooth muscle fibers (178). Where the ducts meet with the duodenum, the muscles form the sphincter of Oddi, which controls the release of the contents into the intestine (170, 176, 178). Additionally, the blood supply for the ducts comes from a network of vessels stemming from the hepatic artery (173). This network of vessels surrounds the bile ducts and is termed the peribiliary plexus (PBP) (173, 180). The PBP provides nutrients to the bile ducts to allow for growth, but it also allows for an alternative enterohepatic circulation route for BAs to be recycled back to hepatocytes via cholangiocytes in a process called cholehepatic shunting (169, 173). The normal route of enterohepatic circulation and recycling of BAs is through intestinal absorption, and then delivery to hepatocytes where they are secreted again into the ducts (168, 169). Interestingly, there is a concept of a cholecystohepatic shunt whereby the gallbladder coordinates BA uptake from bile to the liver (181).

c. Cholangiocytes

The differing physiologies of the cholangiocytes allow for a high level of control to alter the flow and composition of bile. Cholangiocytes, much like other epithelial cells, are polarized, have a multitude of transport proteins, and have distinct basolateral and apical membranes (174, 182). On the basolateral side, they connect

to basement membranes (170, 174) and on the apical side of cholangiocytes, microvilli and cilia line the lumen, and between these cells, tight junctions maintain cell polarity. Certain disease states can result in an interruption in tight junctions, interrupting the flow of bile (171). While all cholangiocytes have diverse physiologies, the size and location of the cells influence their form and function.

Starting just after the canals of Hering, narrow canalicular ducts (about 10 μm) are lined by small cuboidal epithelial cholangiocytes, which have little resorptive and secretory abilities (174). The properties of small cholangiocytes rely heavily on altering intracellular levels of Ca^{2+}, where large cholangiocyte activities are more dependent on cAMP levels (174, 183). Large cholangiocytes are longer, have less microvilli and cilia on their apical membrane, and have a lower cytoplasm to organelle ratio. Most of the larger cells' intracellular space is taken up by rough endoplasmic reticulum, suggesting that large cholangiocytes play a more specialized, less variable role than their small counterpart (174, 183). Conversely, small cholangiocytes resemble progenitor cells, with a higher nuclei to cytoplasm ratio (183). Like bile ducts, the gallbladder is lined with specialized epithelial cells. As small columnar cells with moderate cilia present on the apical membrane, the morphology of the epithelial cells that line the gallbladder resemble an intermediate between small and large cholangiocytes (184).

All cholangiocytes have a primary cilium, a thin peninsula-like extension of the cell to maximize the surface area of the membrane (173, 174). These cilia sample the passing fluid, allowing cholangiocytes to act as mechano-, osmo-, and chemosensors, recognizing and responding to changes in bile (174). Further, cholangiocyte action can be spurred by a variety of molecules, including hormones, BAs, neuropeptides, and alterations in luminal pressure, the action being the alteration of intracellular Ca^{2+}

and/or cAMP, with downstream effects altering the composition of bile, initiating cholangiocyte proliferation, or even signaling the activation of immune responses (173). Interestingly, while gallbladder epithelial cells are not noted to have primary cilium, they are similarly sensitive to the contents of bile, with a focus on water and ion manipulation (5).

d. Bile formation and flow

Hepatocyte secretions generate the bulk of bile, with cholangiocytes only accounting for about 40% of the daily production (168, 174). Bile production is prompted due to a series of reactions initiated at the beginning of a meal, especially one high in FAs. As an emulsifier, bile is a critical facilitator of the absorption of hydrophobic FAs (171). Once delivered, micelles are created to enclose and transport the lipids through the body (168). Between the delivery of bile to the duodenum and being secreted by canalicular hepatocytes, bile composition, flow, and pH is monitored and altered through a variety of mechanisms, including alterations controlled by gallbladder epithelial cells (185).

Previous cholehepatic research has defined two types of bile flow: BA-dependent flow and BA-independent flow (186). As previously stated, hepatocytes are the main facilitators of BA-dependent flow as the main producers and recyclers of BAs (187). For instance, hypercholeretic bile salts, such as the conjugated secondary bile salt nor-ursodeoxycholic acid (nor-UDCA), increase bile flow (171). This is especially noteworthy, as the composition of BAs has been noted to be linked to gallbladder motility (185). It is unknown if gallbladder hypomotility, or an increase in secondary BAs resulting in decreased biliary flow is the primary action, but the two have been highly correlated (185). Conversely, cholangiocytes support BA-independent flow (171, 186, 188).

Bile mostly consists of water, with only about 5% of the volume being attributed to other materials (171). At any time, bile can be composed of BAs, cholesterol, amino acids, glucose, steroids, enzymes, vitamins, and even heavy metals (168, 171, 187). Xenobiotics and toxins can also be present in bile (168, 171, 186). The biliary tract also acts as direct transport to the gut, where immunoglobulin A secreted in bile can protect against pathogens and promote symbiotic microorganisms (171, 189, 190). Other substances that use the biliary tract for transport elsewhere in the body include hormones and pheromones, as well as a number of vitamins (171). Even with all the other constituents within bile, BAs are the most abundant component (187). While the main function of the gallbladder is to pull water out and concentrate bile, the composition of BAs also influences the motility of the gallbladder (185).

BAs are mainly synthesized and secreted by hepatocytes (171, 173, 187, 191). The farnesoid X receptor (FXR) is the main regulator of the synthesis and secretion of BAs, and ASBT expressed by cholangiocytes regulates cholehepatic shunting (171, 187, 191, 192). ASBT is not only expressed by intrahepatic cholangiocytes, but by gallbladder epithelial cells, as well (193-195). It has been demonstrated that the gallbladder is able to uptake BAs in bile via ASBT, setting up the concept of a cholecystohepatic shunt (193-195). Primary BAs are generated from cholesterol and can be modified by additional side chains of taurine or glycine to become secondary BAs, which makes them a stronger acid and also decreases the chances of reabsorption (171, 187). Hypomotility of the gallbladder is linked to higher concentrations of secondary BAs, which is associated with an increased risk of developing cholelithiasis or cholangiocarcinoma (CCA) (196).

Once created, BAs are actively secreted from hepatocytes into bile mainly through the bile salt export pump (BSEP) (187). BAs are 100-1000X more

concentrated in bile than in plasma; therefore, they must be actively transported against this gradient (187). Most other components of bile maintain nearly the same concentration within bile fluid that exists in plasma, kept relatively standard through gradients found in the PBP (171, 173, 187). The regulation of BAs within plasma is also tightly controlled; however, certain biliary diseases alter this, spurring researchers to investigate the number of BAs detected in plasma of individuals with different liver and biliary pathologies (192, 197). So far, these studies have elucidated expected trends, such as the use of UDCA (the unconjugated form of nor-UDCA) for cholestasis treatment resulting in altered plasma BA concentrations (192). Additionally, recent research by Farhat et al. noted new trends, specifically that high levels of conjugated BAs in plasma link to increased risk for liver cancer or other progressive liver diseases (197). Additionally, higher levels of secondary BAs in plasma are associated with cholecystolithiasis and non-neoplastic polyps in the gallbladder (198, 199). Beyond the synthesis of BAs, bile pH and osmolarity are controlled by cholangiocyte activities (173). Interestingly, gallstone formation is not due to lower pH values directly, but is instead attributed to increased Ca^{2+} concentrations in the bile that subsequently lower the pH (200).

e. Bicarbonate Secretion

Chloride is exchanged for bicarbonate, making bile alkaline, and the BAs within are thus polar, de-pronated, and membrane impermeable (173, 201). This protective alkaline constitution of bile, termed the 'biliary bicarbonate umbrella,' shields cholangiocytes from BA-induced injury, and once secreted in the duodenum, it neutralizes the acidic gastric output, protecting the intestinal epithelium and bolstering the absorption of nutrients (168, 173). The initiation of chloride/bicarbonate exchange is stimulated by increased intracellular levels of cAMP (173, 183). This internal

increase in cAMP incites a rise in protein kinase A (PKA) activation, which results in the increased transportation of intracellular chloride to the apical membrane via vesicles with three specific proteins: CFTR, anion exchange protein 2 (AE2) and water channel AQP1 (173, 183, 190). CFTR is also expressed by gallbladder cells, and loss of CFTR leads to defects in gallbladder emptying and BA circulation (195). In response to CFTR loss, concentrations of secondary BAs (that are conjugated in the ileum) are reduced, and this is reversed with cholecystectomy, further indicating a cholecystohepatic shunt (195). Both CFTR and AE2 are highly expressed in the gallbladder compared to the intrahepatic ducts (181), and in the gallbladder epithelia CFTR is required for cAMP-dependent, AE2-mediated bicarbonate secretion (202). In patients with gallstones, bile bicarbonate levels are reduced, and thus bicarbonate is hypothesized to be the main buffer of bile similar to intrahepatic bile ducts (200).

Other factors can affect bicarbonate secretion, including autonomic neurotransmitters (173, 174). Acetylcholine and phenylephrine upregulate biliary bicarbonate secretion, while gastrin-releasing peptide and vasoactive intestinal peptide (VIP) mediates a consistent baseline of bicarbonate (171, 173). Further, hormones such as somatostatin, endothelin, dopamine, and gastrin inhibit the rise of intracellular cAMP (171, 173, 201). Bile also contains nucleotides and nucleosides that, when interacting with P2Y receptors on the apical membrane, can result in increased bicarbonate secretion (171). It is interesting that many of these processes can be recapitulated in some fashion in the gallbladder. Acetylcholine promotes mucin release in the gallbladder as a defensive mechanism (203) which potentially aids in bicarbonate secretion since this process is found on intrahepatic bile ducts (204). Additionally, VIP is a potent stimulator of cAMP production in the human gallbladder epithelial cells that regulates fluid secretion, and VIP expression is higher in the

gallbladder than the intrahepatic bile ducts (181). Somatostatin decreases gallbladder motility (205), and endothelin is overproduced in acute cholecystitis and increases gallbladder tone (3,4). Lastly, P2Y2 is expressed on isolated gallbladder epithelial cells (34) and stimulates mucin secretion (49).

f. Biliary immune function

While cholangiocytes, including those of the gallbladder epithelium, play a crucial role in bile flow and composition, they also play a role in both the innate and adaptive immune systems (173, 174). Cholangiocytes and gallbladder epithelial cells have receptors to identify pathogen- and damage-associated molecular patterns, including some of the same proteins that B and T lymphocytes possess such as toll-like receptors (206). Further, rather than being limited to downstream actions, cholangiocytes can proliferate and actively recruit immune cells to areas of injury (171, 183, 201). Cholangiocyte proliferation is tightly regulated by paracrine and endocrine factors, including growth factors like transforming growth factor (TGF) and TNF, cytokines, neuropeptides, and hormones (173). For instance, progesterone and estrogen have been linked to increased proliferation, where anti-progesterone/estrogen or a drop in levels of these hormones results in limited cholangiocyte growth, and even increased risk of disease states (173, 207, 208).

Cholangiocytes are attributed to the initiation of immune responses within the biliary tract due to their high level of intra- and extracellular communication (173), and following damage they secrete pro-inflammatory cytokines and chemokines, which communicate the location and type of injury to neighboring and immune cells (209).

While gallbladder epithelial cells have similar immune receptors and responses to those of cholangiocytes, they are located further down the biliary tract, and thus play a delayed, but still important immune role (210). One study found that gallbladder

epithelial cells express mRNA for a variety of cytokines and chemokines, as well as directly secrete TNF (210). Another study using donated human gallbladders, found the presence of multipotent endodermal stem cells within the gallbladder epithelium increased in pathologic gallbladders versus comparatively healthy gallbladders (211). Research on the potential immune functions of gallbladder epithelial cells is still ongoing and evolving.

g. Cholangiocyte-dependent fibrosis

Profibrotic factors can be released to incite downstream effects that promote fibrogenesis (212, 213). One study has shown that silencing one TGF-β isoform may be an effective treatment for fibrotic biliary and liver diseases, limiting the expression of pro-fibrotic genes and conversely promoting antifibrotic PPAR expression (212). Further, chronic activation of cholangiocytes can result in the development of biliary fibrosis, damage, or cancer (212). Overly active fibrogenesis results in a buildup of scar tissue can result in decreased functionality of the biliary tract, eventually leading to biliary cirrhosis (201, 214). The gallbladder epithelial cells react similarly, with prolonged inflammation and immune response potentially resulting in severe fibrosis, perforation of the gallbladder, or even gallbladder cancer (215-217).

h. *Cholestasis*

Cholestasis refers to a decrease or halt in bile flow anywhere along the biliary tree. While there are a number of hereditary cholestatic disorders caused by genetic mutations, the most common forms of cholestasis are presented through PSC, primary biliary cholangitis (PBC), CCA, and cholelithiasis (218, 219). No matter the cause of cholestasis, there are few treatments available. The main treatment is to supplement with BA analogues, UDCA or obeticholic acid (OCA) that work to reduce BA synthesis. If UDCA or OCA treatment fails, a liver transplant is the last option (218, 220). UDCA,

when recognized by the biliary tract, increases bile flow, lessens toxicity, and encourages the recycling of nontoxic over toxic bile salts (221). Unfortunately, only about 40% of patients with cholestasis respond to UDCA treatment, highlighting the need for alternative therapies (192, 220). OCA works to reduce toxic BA levels by reducing BA synthesis and enhancing hepatic BA efflux (222). Clinical trials on OCA use in PBC, PSC and fatty liver diseases have proved promising, but more work regarding efficacy is necessary (222).

LINKS BETWEEN THE GALLBLADDER AND CHOLESTATIC LIVER DISEASES

VI. Primary sclerosing cholangitis (PSC)

a. Background

PSC is a rare cholangiopathy that firstly targets the bile ducts in the liver leading to inflammation, fibrosis, stricturing and eventual cirrhosis and liver cancer (223). The majority of PSC patients have extrahepatic and intrahepatic bile duct involvement, while a small proportion of diagnoses having intrahepatic only PSC (223). PSC affects more males than females, and the median age at diagnosis is 40 years (218, 224). Due to the initial unspecific manner of PSC symptoms at onset, PSC is not typically diagnosed until the disease has progressed (218). Currently, there are no approved therapies for the treatment of PSC, with BA therapeutics including UDCA and OCA being tested as potential therapeutics (218). PSC patients have a high risk of developing CCA and the only curative treatment for PSC is liver transplantation; however, recurrence rates are high demonstrating that this approach is not viable (218). While PSC primarily targets the biliary tree, the fibroinflammatory nature of PSC can lead to chronic inflammation which can subsequently affect the gallbladder.

b. PSC, cholelithiasis and cholecystitis

An early study from 1988 interrogated the incidence of gallbladder disease in PSC and found that 89% of PSC patients had abnormal gallbladders, and after excluding patients who had thickened gallbladder wall due to end-stage liver disease, 41% of the remaining PSC patients presented with gallbladder abnormalities (225). PSC patients with abnormal gallbladders presented with gallstones, gallbladder dysfunction associated with PSC and neoplasms, indicating that gallbladder abnormalities are frequent among PSC patients (225). These findings were verified in a large study from 2008 that found that 41% of PSC patients present with gallbladder abnormalities, 25% have gallstones and 25% have cholecystitis (226). PSC patients also have papillary hyperplasia, pseudo gland formation, inflammation, smooth muscle hypertrophy and fibrosis in the gallbladder, but these abnormalities were found to a similar degree in chronic cholecystitis patients (227). PSC patients and chronic cholecystitis patients both presented with mononuclear cell infiltration of the epithelium, and although the incidence was higher in PSC it was not significant (227). Therefore, there may not be a distinct gallbladder signature in PSC patients compared to chronic cholecystitis. A separate study found that PSC-related cholecystitis showed diffuse infiltrate, predominantly plasma cells, within the lamina propria which was not significantly noted in chronic cholecystitis alone; therefore, the authors suggest that diffuse lymphoplasmacytic acalculous cholecystitis is a distinct form of PSC-associated cholecystitis (228). Incidence of cholecystitis is significantly higher (30%) in patients with extrahepatic PSC when compared to intrahepatic only PSC (9%) (226). These findings slightly differ from a Japanese cohort where ~12% of PSC patients were concomitantly diagnosed with gallstones (229), although this study did not distinguish between intra- and extra-hepatic PSC.

Transabdominal US is used to identify bile duct wall thickening and dilatations in PSC, but in one study this approach also identified that up to 41% of PSC patients presented with an enlarged gallbladder (Figure 6), gallstones, cholecystitis or mass lesions (230). The small study found that all PSC patients presented with irregularly thick gallbladder wall (230). This study further found that while PSC patients had enlarged gallbladders their rates of gallbladder emptying were normal (230).

The gut influence on cholelithiasis was previously discussed, and it is also known that ~80% of PSC patients have concomitant inflammatory bowel disease (IBD) (231). Interestingly, around 50% of IBD patients present with hepatobiliary manifestations, including PSC, cholestasis and gallstones (232). Patients with Crohn's Disease, severe ileitis or ileal resection have bile malabsorption leading to gallstone formation (232), further indicating the gut-liver axis in cholelithiasis.

Multidrug resistance 2 gene knockout (*Mdr2*$^{-/-}$) mice are used as a model of PSC, and these mice spontaneously form cholecystolithiasis (233). The gallbladder in *Mdr2*$^{-/-}$ mice has needle-like cholesterol stones as early as 12 weeks of age (Figure 7) (233). The highly pro-inflammatory hepatobiliary environment might be contributing to the concretion of gallstones and aiding in cholecystitis. Moreover, the ability of *Mdr2*$^{-/-}$ mice to spontaneously generate gallstones without the induction from lithogenic diet makes it a versatile model to study the intricate signaling mechanisms involved in the concretion and crystallization of gallstones. Female *Mdr2*$^{-/-}$ mice developed 50% more gallstones than male *Mdr2*$^{-/-}$ mice indicating a sexual dimorphic effect (233), but this dichotomous effect has not been published in humans with PSC. *Abcb11* encodes BSEP that is responsible for the export of BAs from the hepatocyte to the bile canaliculus, and *Abcb11* colocalizes with the *Lith1* (responsible for cholesterol-induced gallstone formation) quantitative trait locus (234). To understand if *Abcb11* is

responsible for gallstone formation, the authors generated mice with overexpression of *Abcb11* and subsequently fed them a lithogenic diet (234). It was found that *Abcb11* overexpression induced biliary BA secretion and bile flow but did not affect cholelithogenesis (234).

c. Gallbladder cancer in PSC

Aside from cholelithiasis and cholecystitis, there is an increased rate of gallbladder cancer in patients with PSC (235). Some patients present with gallbladder lesions, which more than half of the time represent adenocarcinoma, and as such cholecystectomy is recommended in all instances of gallbladder lesions regardless of size (236). Gallbladder carcinoma was associated with intrahepatic bile duct dysplasia, CCA and IBD in PSC patients, and gallbladder dysplasia was associated with hilar/intrahepatic bile duct dysplasia, CCA, IBD and older age at transplant; however, similar associations were not found for sex or PSC duration (235). From this study, the authors conclude that PSC patients have a neoplastic "field effect" along the intra- and extra-hepatic bile ducts in PSC, including the gallbladder (235). Importantly, in 40-50% of PSC patients with gallbladder neoplasms, these polyps are malignant (237). From these studies, one would consider cholecystectomy to be an important intervention for PSC patients presenting with gallbladder polyps. However, one study found that 40% of PSC patients that underwent cholecystectomy due to gallbladder polyp or mass presence had early postoperative complications (238).

VII. *Primary biliary cholangitis (PBC)*

PBC is an autoimmune-mediated cholangiopathy that targets the interlobular (i.e., small) bile ducts of the biliary tree (239). Risk factors for PBC include being female, over 50 years old, and living in a Western country (218, 224). In early stages

(stage I/II) of PBC, there is a large degree immune cell influx to the peribiliary location, compensatory proliferation of the bile ducts, reduced presence of ductulo-canalicular junctions (necessary for bile outflow) and loss of the protective bicarbonate umbrella (240). As PBC progresses to later stages (stage III/IV) cytotoxic T cell mediated destruction of the bile ducts leads to ductopenia via apoptosis of the small cholangiocytes (239). Ductopenia has also been attributed to enhanced senescence and toxic BA-mediated cell death due to loss of the bicarbonate umbrella and ductulo-canalicular junctions (240). These surmounting injuries lead to peribiliary fibrosis and cirrhosis if left untreated (239). UDCA and OCA are first-line therapies approved for the treatment of PBC, but a number of patients are non-responders to these approaches (241). While PBC is an autoimmune liver disease, patients do not respond to traditional immunosuppressants, making treatment of the inflammatory cascade challenging (241). Due to the pan-inflammatory presence in PBC, it is unsurprising that 73% of patients with PBC present with extrahepatic manifestations of autoimmune disease, including Sjogren's syndrome, thyroid disease and systemic sclerosis involving the skin, lungs, gastrointestinal tract, heart or musculoskeletal system (241).

a. *Gallbladder disorders and cholelithiasis in PBC*

There are few studies that identify if changes in the gallbladder or gallbladder disease occur in patients with PBC. A case study found that a 70-year-old Hispanic woman with PBC/autoimmune hepatitis overlap syndrome and associated cirrhosis had multiple gallstones and bile duct stones, and a periampullary mass (242), but this may have been associated with cirrhosis and not driven by PBC. In one study, it was noted that patients with PBC did not have a significant difference in gallbladder size, wall thickness or emptying compared to controls (230). Another study conversely found that the gallbladders of PBC patients had epithelial hyperplasia, pseudo gland

formation, inflammation, fibrosis, smooth muscle hypertrophy and mononuclear cell infiltrate, but the degree is like what is found in chronic cholecystitis and PSC patients (227) indicating that gallbladder abnormalities may be non-specific in cholangiopathies. As well, it is unclear if these patients presented with cirrhosis, which in and of itself increases the risk of gallbladder disease regardless of etiology (243).

A national hospital-based study in Italy looked at hospitalized PBC patients and found that this cohort had an increased incidence of malignant neoplasms of the gallbladder, and this occurrence was higher in women than in men (244). In another study, cholelithiasis in PBC was significantly associated with intra- and extra-hepatic CCA (245). However, these are the only studies identifying associations between PBC and gallbladder cancer, thus more work is necessary.

b. Microbiota in PBC

PBC patients have decreased diversity of microbes and higher levels of genera associated with inflammation, but this dysbiosis is partially reversed by UDCA (246). As stated above, BAs and the microbiota can play a role in cholelithiasis; therefore, this association in PBC may be attractive. Interestingly, 75% of the bacterial clones isolated from gallbladder bile from PBC patients were gram-positive cocci, with only 5% of gram-positive cocci found in gallbladder bile from patients with cholecystolithiasis (Table 2 and Table 3) (247). *Staphylococcus aureus* was the predominant gram-positive bacteria in PBC gallbladder bile (247). However, this study did not indicate if the PBC patients presented with gallbladder abnormalities, and thus the correlative or causative effect of dysbiosis in PBC on gallbladder disease is unknown.

There is a lack of understanding on the association of PBC and gallbladder diseases. While some abnormalities and cancer were noted, this may be a

consequence of cirrhosis and not etiology dependent. Furthermore, no studies have reported on gallbladder abnormalities or cholelithiasis in mouse models of PBC. Therefore, more investigation is key to answering this question.

VIII. Cholangiocarcinoma (CCA)

Cancer cells and the tumor microenvironment (TME) interact with each other to form multicellular systems, called tumors. The composition of the TME is characterized by extracellular matrix (ECM), and various cell types such as immune cells, endothelial cells, pericytes, and fibroblasts (248). CCA is cancer of the bile ducts and is the second largest primary liver malignancy, after hepatocellular carcinoma (HCC). CCA tends to escape immune surveillance, and for this reason it is associated with a poor prognosis and poorly defined symptoms (249). Most CCA cases are defined as an incurable malignancy, and the 5-year survival rate for CCA is abysmally low (250). CCA can be defined by the following subtypes: intrahepatic (iCCA), perihilar (pCCA), and distal (dCCA) (251). The last two groups of CCA, pCCA and dCCA, are regrouped under the term of extrahepatic CCA (eCCA) and can include gallbladder cancer (252). Many risk factors such as NAFLD, non-alcoholic steatohepatitis (NASH), alcohol-related liver disease (ARLD), and biliary fibroinflammatory response can contribute to CCA development (253, 254). MicroRNAs (miRNAs) are small non-coding RNAs that play various roles in the modulation of CCA (255). Various studies have shown that alteration of miRNAs may act as oncogenic or onco-suppressing in CCA. Furthermore, in gallstone disease, there is upregulation of miR-210 that reduces the expression of its target, ATPase phospholipid transporting 11A gene, in human gallbladder epithelial cells (256). miR-130b inhibits the expression of the specific protein 1, and consequently there is decrease of MUC5AC expression. It is well known that

hepatolithiasis is strongly related to chronic inflammation and overexpression of MUC5AC as well, which can be a contributor to liver cancer initiation (257).

a. Cholangiocarcinoma, cholelithiasis and gallbladder cancer

On occasion, gallstones can migrate into the bile ducts and induce complications. The presence of bile duct stones is considered a significant risk factor for the development of CCA due to repeated mechanical injury and inflammation of the intrahepatic biliary tract epithelium (258, 259). The size, presence and number of gallstones are significantly associated with increased risk of CCA (260). Cholecystectomy reduced the risk of gallstones associated with CCA, with a greater risk reduction seen in eCCA than iCCA (261). This was mirrored in another study where gallstones increased the risk of iCCA and eCCA with a decline in risk following cholecystectomy (262). Another study contrarily found that dilation of the bile ducts is frequent following cholecystectomy and can cause inflammation and increase the risk of CCA (263); however, this was in a cohort of patients with normal bile ducts whereas the former was in a population of CCA patients. The biliary microbiome can regulate various damages within the liver, including cholelithiasis as discussed above. One study found that the relative abundance of Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria was similar in patients with dCCA and new onset bile duct stones (264) identifying that shared microbial communities may be a link between gallstone formation and CCA development. In a rare case report, a 65-year-old woman presented with jaundice and concomitant cholecystitis due to an impacted gallstone (265). Following pancreaticoduodenectomy, histopathological analysis revealed that the patient had primary gallbladder malignancy along with CCA (265). While the link between gallstones and CCA risk is known, the incidence of concomitant CCA and gallbladder cancer appears to be rare. The incidence of other gallbladder disorders in

CCA seems unreported in the literature; thus, more work may be required in this area. Histological imaging of gallbladder cancer can be found in Figure 8.

IX. Non-alcoholic fatty liver disease (NAFLD)

NAFLD, also known as metabolic-associated fatty liver disease, is the most common chronic liver disorder globally (266). As the obesity epidemic continues to grow, the incidence of NAFLD is increasing worldwide. Approximately 24% of U.S. adults have NAFLD and about 10% of this population has an advanced form of NAFLD termed NASH. The incidence of NAFLD in children is also rising with about 10% of U.S. children aged 2-19 years having NAFLD (267). NAFLD also shows ethnic disparities, with the highest incidence in Hispanic populations (268). The risk factors for NAFLD includes obesity, type 2 diabetes mellitus, hypertriglyceridemia, Western diet and sedentary lifestyle (269). Interestingly, a large scale study using the U.S. National Health and Nutrition Examination Survey revealed the positive correlation between glucose intolerance, plasma insulin levels and C-peptide content with gallstone incidence (270)

The pathogenesis of NAFLD was first explained by the ‘two-hit’ theory (271, 272), and later referred to as “muti-hit hypothesis”. The first ‘hit’ starts with insulin resistance caused by excessive FA accumulation in hepatocytes, a state known as hepatic steatosis (273, 274). A number of secondary ‘hits’ come after the exposure to chronic fat accumulation (272), including oxidative stress-induced mitochondria dysfunction, endoplasmic reticulum (ER) stress (275), apoptosis induced-regeneration (276), gut-derived endotoxin-induced inflammation (277), and cholestatic-induced lipid metabolism dysregulation (278). These multiple secondary hits synergistically, but not sequentially, happen during the progression of NAFLD. These events eventually lead

to chronic inflammation and fibrosis, resulting in NASH (279). NASH is characterized by hepatic ballooning, lobular inflammation, and macro steatosis. About 20% of NASH patients will develop cirrhosis, with potential risk of liver failure or hepatocellular carcinoma (280).

A longitudinal cohort study showed increased risk of gallstone formation in NAFLD patients, especially in females (281). Further studies showed association between NAFLD and gallstones with a higher NAFLD incidence in women with gallstones (282-284). Additionally, gallbladder wall thickness and gallbladder dysfunction can occur in NAFLD patients that do not present with gallstones (Figure 9) (285). It has also been shown that NASH prevalence in patients with gallbladder disease is 18% in the morbidly obese population, but mechanisms linking these factors is unknown (286). Lastly, cholelithiasis was not associated with advanced fibrosis or definite NASH in a NAFLD cohort, further complicating potential associations between gallbladder disease and NAFLD (287).

Human genome-wide association studies (GWAS) have revealed several genes that may explain the vulnerability and increased risk of NAFLD observed in some subpopulations. The most confirmed and studied genetic variant that is associated with NAFLD is PNPLA3 (288-290). The Rs738409 [G] I148M allele of PNPL3 correlated to increased risk of NAFLD and is most found in Hispanic populations. Furthermore, the Rs738409 [G] I148M mutation increased NAFLD risk and body weight gain (291), and an increased risk of higher steatosis, portal inflammation, fibrosis and oxidative stress (291-294). Conversely, rs6006460[T] is enriched in African American populations and shows protective effects against the development of NAFLD as the population shows a lower risk of NAFLD and lower hepatic fat content (289). However, a study did not find increased risk of gallstone

formation in patients with I148M mutation *per se* (295). Nevertheless, another genetic study showed that the polyunsaturated FAs were much higher in individuals with PNPLA3^{148M} variants when compared to non-carriers. Other genetic variants with moderate effect sizes were shown in transmembrane 6 superfamily member 2, glucokinase regulator (GCKR), and membrane bound O-acyltransferase domain-containing 7 (296). Another GWAS study also found GCKR variant showed increased risk of gallstone diseases (297). The DNA methylation of PPARG is associated with fibrosis severeness in NAFLD (298). Interestingly, activation of PPARG prevents cholesterol gallstone formation by increasing bile salt synthesis and enterohepatic circulation in lithogenic mice models (299). The same study also noticed that PPARG activation alleviated hepatic steatosis and obesity symptoms (299). This indicates that both NAFLD and gallstone formation share some common mechanisms.

a. Fatty acid (FA) uptake, storage and signaling

The rate of hepatic FA uptake is determined not just by the circulating concentrations that comes from the adipose tissue and gut, but also relies on FATP and caveolin (300-304). Meanwhile, vaveolin-1 depletion increased cholesterol crystallization in lithogenic diet-induced mice by inhibition of hepatic cholesterol levels and bile salts transportation (305). Cluster differentiation 36 (CD36), as the most studied lipid transporter, facilitates hepatocyte FA update and trafficking (306). Hepatocyte specific depletion of CD36 improved steatosis by decreasing the triglyceride, diacylglycerol, and cholesterol in a NAFLD genetic mouse model and diet induced model (307). In fact, oxidation is increased in *CD36*$^{-/-}$ mice via inhibition of sterol regulatory element-binding protein 1 (SREBP1) in diet-induced NAFLD (308). Further, circulating CD36, a soluble form of CD36, was found to be strongly associated with insulin resistance (309) in type 2 diabetes and advanced steatosis in NAFLD

(310). Depletion of CD36 also showed resistance to lithogenic diet induced gallstones in mice by altering the lipid composition in the biliary tract and enhanced gallbladder contractility (311).

Besides FA uptake from exogenous sources, hepatic FA comes directly from *de novo* lipogenesis, that is converted from monocarbohydrates and proteins. In this process, acetyl-CoA is converted to malonyl-CoA and fatty acyl-CoA. This process adds FAs to hepatocytes and causes triglyceride accumulation in the cells by inhibiting fatty oxidation (312). SREBP1c and carbohydrate-responsive element-binding protein (ChREBP) also regulates *de novo* lipogenesis. Interestingly, both SREBP1c and ChREBP can be stimulated through activation of LXR which is regulated by insulin (313). Further, insulin could directly activate SREBP1c though translocation from the Golgi to the nucleus (314). LXR activation increased the susceptibility of gallstone formation in lithogenic-diet induced mice by elevated cholesterol and phospholipids concentration and decreased bile salt concentration (315).

b. Bile acid metabolism

As previously mentioned, NAFLD starts with simple steatosis followed by multiple secondary insults. One of the offenses is the dysregulation of BA metabolism, which is mediated by the liver-gut axis (316). About 95% of BAs are recycled through the hepatic portal system, and BAs can regulate glucose and lipid homeostasis via nuclear receptor activation, including FXR (317). Interestingly, $FXR^{-/-}$ mice showed dysregulated lipid metabolism, enhanced serum BAs, cholesterol, and serum lipoprotein profile (318). While another study showed increased bile salt hydrophobicity and cholesterol crystallization in $FXR^{-/-}$ mice, which is an indication of gallstone formation. Further, the reactivation of FXR in these knockout mice prevented gallstone formation (319).

c. NAFLD, cardiovascular disease and cholelithiasis

As stated, the NAFLD spectrum varies from simple steatosis to metabolic steatohepatitis, and it can further progress to liver fibrosis, cirrhosis, and hepatocellular carcinoma. The coexistence of NAFLD and gallstone disease has been found, mainly due to several shared risk factors such as age, ethnicity, obesity, insulin resistance, and metabolic syndrome (320). A study has indicated an increased incidence of gallstone formation in patients with NAFLD (47%) versus patients without NAFLD (26%) (321).

Recent studies have indicated that gallstone disease is closely associated with the occurrence of cardiovascular disease, and the occurrence of gallstone disease increases the incidence of cardiovascular disease (322). Based on a meta-analysis of 10 published studies, patients with gallstone disease had a higher risk of diabetes, hypertension, coronary heart disease, atrial fibrillation, and hyperlipidemia. In addition, gallstone disease was found to be related to a 1.23-fold increase in the incidence of cardiovascular and cerebrovascular diseases. In another study of 5,928 subjects by Daniel *et al*., gallstone disease was closely related to many kinds of cardiovascular diseases (323). Gallstone disease, cardiovascular disease and NAFLD also share common risk factors such as obesity, age, sex, and disorders of lipid metabolism, and these factors are major risk factors for metabolic syndrome. Metabolic syndrome is closely related to cardiovascular disease and gallstones may be considered a characteristic of this (324). Cholesterol is transported into plasma by lipoproteins, micelles, and vesicles in bile. If excess cholesterol were accumulated in the arterial wall, atherosclerosis may occur. The excess cholesterol that is not dissolved by bile salts or phospholipids will precipitate as solid cholesterol monohydrate crystals, which can lead to the formation of cholesterol gallstones (84).

d. NAFLD, cholestasis and cholelithiasis

Gallstones are one of the causes of extrahepatic cholestasis, while cholestasis is linked to NAFLD progression in various studies. A case-control study conducted in 2015 revealed more severe histological damage in NAFLD with cholestasis compared to NAFLD patients without cholestasis (325). This is further highlighted in a study conducted in 2018, where about 30% of NAFLD patients showed cholestasis (326). It is suggested that NAFLD patients with cholestasis show more pronounced hepatic inflammation, unbalanced carbohydrate and lipid metabolism, apoptosis, and fibrosis (326). Another study conducted with 90 NASH patients showed a positive association between centrilobular ductular reaction and fibrosis stage (327). Although this study did not provide direct evidence of cholestasis influence on the homeostasis of lipid and carbohydrates in the liver, a number of animal studies using an *Mdr2*$^{-/-}$ mouse model connect cholestatic liver injury and impaired liver function (278) to dysregulation of lipid metabolism and steatosis (328). Specifically, the genes that control lipid synthesis, storage, and oxidation is dysregulated. Interestingly, the same study found that the supplementation of nor-UDCA or high-fat diet showed a protective role in *Mdr2*$^{-/-}$ mice and reversed the fibrosis (328).

Patients with NAFLD showed increased plasma BAs, specifically elevated primary and secondary BAs. Bacteria that metabolize taurine and glycine, two critical components in producing secondary BAs are increased (329). Furthermore, elevated primary BAs are also found in gallstone patients (198). On the other hand, intrahepatic cholestasis of pregnancy (IPC) showed significantly higher incidence in NAFLD patients when compared to other chronic diseases or pregnancies without chronic liver disease (330). Further, the incidence of gallstones in IPC is much higher in women who do not present IPC (331). Taken together, cholestasis and elevated BAs increase

the risk of NAFLD and gallstones. However, further work needs to be done in human association studies and molecular mechanisms underlying the BA metabolism, gallstone formation and NAFLD.

X. Alcohol-related liver disease (ARLD)

ARLD has been the main cause of liver-associated mortality worldwide (332). This chronic liver disease is the most common and can progress from alcoholic fatty liver to alcoholic steatohepatitis (ASH) (333). Chronic ASH can eventually develop liver fibrosis and cirrhosis, which may lead to HCC. In addition, severe ASH (with or without cirrhosis) can cause alcoholic hepatitis (AH), which is an acute clinical presentation of ARLD that is associated with liver failure and high mortality (334).

Most ARLD patients are diagnosed with jaundice or complications of cirrhosis when they reach the medical care (335). Screening of ARLD in the primary-care setting at an early stage and subsequent behavioral interventions should be encouraged. Abstinence from alcohol is the best treatment for all stages of ARLD (336, 337). Unfortunately, ARLD patients in advanced stages who do not respond to medical therapy have a very low life expectancy, and the only therapeutic option associated with a survival benefit is liver transplantation (338). At 1-year post-transplantation, the survival rate has steadily improved to 80-85% in 2010 (339). In addition, transplant recipients with ARLD are at high risk of cardiovascular diseases, infections, and cancers (340, 341). Overall, more effective, and safer therapies are urgently needed to ultimately reduce the burden, morbidity, and mortality of ARLD.

a. Alcohol consumption and cholelithiasis

Almost forty years ago, a case-control study first reported that alcohol consumption was associated with a decreased risk of developing gallstones, whereas

increased intake of sugars was associated with an increased risk (342). Interestingly, the association of alcohol with reduced risk of gallstones was found in both males and females (342). However, women have been regarded to have a higher risk of gallstone formation due to sex hormone signaling (118). In this regard, the relation between alcohol intake and cholecystectomy were observed by Leitzmann *et al.* in a large cohort of women (343). Their study also revealed that the intake of all alcoholic beverages is inversely associated with the risk of cholecystectomy in women (343). In another large prospective study of over 1 million women that consume alcohol (patients were excluded if they had a clinical history of either liver cirrhosis or gallbladder disease before recruitment), Liu *et al.* further confirmed that alcohol consumption is associated with an increase in the risk of liver cirrhosis but a decrease in the risk of gallbladder disease (344).

b. Thickening of the gallbladder wall in alcoholic hepatitis

Thickening of the gallbladder wall is often seen with US in patients with ARLD. In a retrospective evaluation of 125 consecutive gallbladder sonograms, it was reported that gallbladder wall thickening was associated with hypoalbuminemia in the absence of chronic cholecystitis in a cohort of chronic alcoholics (345). However, another US evidence-based study suggested that portal hypertension, not hypoalbuminemia, is the dominant factor causing gallbladder wall thickening in cirrhotic patients (346). Therefore, more research may be required in this area to better understand the comorbidity of gallbladder wall thickening.

c. Gallbladder perforation and gallbladder variceal hemorrhage in ARLD

Gallbladder perforation is a relatively uncommon complication of ARLD-related cirrhosis and may happen with or without gallstones. The diagnosis of gallbladder perforation is challenging due to the lack of classical symptoms and signs of

perforation (347). Chu *et al.* reported the first case of a 41-year-old man with alcoholic cirrhosis who developed fatal spontaneous gallbladder variceal bleeding (348). Unfortunately, the diagnosis of gallbladder varices eluded conventional imaging and was made only at autopsy; therefore, direct causation of spontaneous gallbladder variceal bleeding with ARLD is unknown. One case study reported gallbladder perforation with alcoholic liver cirrhosis and asymptomatic gallstones (347). The patient was initially diagnosed as HCC-associated rupture based on CT scan images and the patient's clinical history of alcohol-related liver cirrhosis; however, further laparotomy examination revealed that the patient has gangrenous cholecystitis with perforation, suggesting that gallbladder perforation should be taken into consideration as a potential cause of acute abdominal pain (Figure 10) (347). Furthermore, it was observed that a Child-Pugh A alcohol-related liver cirrhosis patient had developed acute gallbladder perforation with spillage of stones into the peritoneal cavity (349). Gallbladder perforation is a rare complication in ARLD and alcohol-related liver cirrhosis, but caution should be taken for those with specific risk factors.

d. ARLD and cholelithiasis

Since alcohol-related cirrhosis is the advanced stage of ARLD, many studies evaluated prevalence and incidence of cholelithiasis with cirrhotic patients (350). Acalovschi *et al.* assessed the risk factors for gallstone formation and the characteristics of liver cirrhosis in 140 patients with multivariate analysis. Similar to what was discussed previously, they reported that alcohol-related cirrhosis and male gender (not female) were inversely correlated with cholelithiasis symptom presence (351). In cirrhotic female patients, the risk of developing cholelithiasis was significantly greater (351). However, another multivariate study shows that cholelithiasis was

significantly more frequent in cirrhotic patients with previous alcohol abuse with no difference in relation to sex (352).

e. Animal studies on alcohol consumption and cholelithiasis

Animal studies are key for identifying molecular mechanisms regulating disease processes. Unfortunately, little work has been done to investigate ARLD and gallbladder diseases in murine models. One study evaluated the effect of alcohol consumption on BA profiles in a chronic gavage mouse model (353). Interestingly, ethanol intake significantly increased BA profiles (mainly free BAs and taurine-conjugated BAs) in the gallbladder of 50% ethanol fed mice (353). The total BAs in the gallbladder were also significantly increased in the 50% ethanol treated groups (353). The authors also demonstrated that 50% ethanol increased the expression of BA-related enzymes and transporters, including BSEP and ASBT in the liver (353). The close association with BAs, BA transporters and gallstone formation may indicate that very high alcohol consumption can contribute to cholelithiasis. However, this percent of ethanol intake is not physiologically relevant, and thus findings should be considered with caution.

XI. SARS-CoV-2-related liver disease

SARS-CoV-2, the virus responsible for COVID-19, has been under an intense lens of investigation since the identification of the highly contagious infection. At first, it was uncertain if patients with chronic liver or biliary disorders were more at risk for severe COVID-19 than others, with the American Association for the Study of Liver Diseases making a statement in 2020 that higher risk was probable due to the observed mechanistic interactions of the virus with angiotensin-converting enzyme 2 (ACE-2) (354). ACE-2 acts as a functional transporter, allowing the virus entry into the

cell, making hepatocytes and cholangiocytes, which express ACE-2, targets for potential infection (354, 355). Over the past two years, research has begun to identify comorbidities that correlate to higher risk of fatality, as well as disease states and damage caused by fighting the infection. Further, COVID-19 patients with evidence of liver dysfunction account for about half of those infected (354, 356). Of importance, one case report found 3 adults that developed prolonged and severe cholestasis following COVID-19 infection, leading to the notion that there may be a rare COVID-19-related cholangiopathy (357). Another study found that biomarkers of liver injury were elevated in 23.4% of Delta-infected and 18.8% of Omicron-infected COVID-19 patients, with the predominant marker being identifiers of cholangiocyte damage (358). Interestingly, liver and cholangiocyte injury biomarkers did not differ between patients with or without pre-existing liver injury (358). This work is supported by another study indicating that 32.7% of COVID-19 infected patients had elevated markers of cholangiocyte damage, which correlated with longer hospital stays (359). The full impact of COVID-19 on cholestasis and biliary damage will likely not be determined until long into the future since the disease is relatively new.

a. SARS-CoV-2 related gallbladder disease

Several COVID-19 patients have presented with severe cholecystitis. Like cholangiocytes, gallbladder epithelial cells present with high levels of ACE-2, which is thought to explain the presence of viral RNA present in the gallbladder epithelial cells of affected patients (Figure 11) (354, 355). As with hepatobiliary dysfunction, the severity of COVID-19 infection appears to directly influence the severity of cholecystitis, with over half the case studies identifying those patients with complicated or severe COVID-19 as having acalculous or gangrenous cholecystitis (354-356, 360). Conversely, some cholecystic COVID-19 patients had less severe COVID-19, but still

presented with acute cholecystitis (361-363). In one case report of a patient with COVID-19 and gangrenous cholecystitis, immune cell infiltration and blood vessel involvement can be seen in the gallbladder. This disparity between critically ill and non-critically ill COVID-19 patients with similar cholecystic presentations suggests that underlying risk factors may account for progression of the diseased state, including similar risk factors to cholestasis, genetic proclivity, and co-morbidities. Additionally, COVID-19-linked cholecystitis cases have been seen around the world, suggesting there may not be a strong connection to lifestyle or ethnicity. As more individuals recover from COVID-19, it is important to explore any lasting damage induced by the virus.

CLINICAL ASPECTS OF GALLBLADDER DISEASE IN LIVER DISEASE

XII. Prevention and treatment

a. Prevention

Pigmented stones are less frequently observed and represent <10% of cases worldwide. Specific risk factors, such as parasitic biliary infection or blood diseases (hemolytic anemia) may attenuate brown stone prevalence (172). The burden of cholesterol gallstones seems worldwide, but prevention may not be an easy target since there is a complex interplay between genetic, metabolic, dietary, environmental and gender related factors contributing to stone formation (364). Among modifiable cholelithiasis risk factors, those related to lifestyle (diet and physical activity) have captured more attention. Reduced physical exercise (365) and obesity (366, 367) were consistently reported in association with increased risk of cholesterol stones. Regarding diet type and habits: i) reduction of carbohydrates, meat, and fats in favor of vegetables as well as; ii) avoidance of long fasting periods, seem protective for cholesterol stone formation (368). In this setting, alcohol consumption has been

suggested to be inversely correlated with gallstones (369); however, it is important to note that studies on diet or general physical activity are largely based on self-reported data and possibly altered by other personal and environmental factors thus justifying discrepancy between different studies. Finally, a condition in which gallstone prevention may be feasible and beneficial is related to rapid weight loss. A weight decrease >1.5 kg/week has been associated with an increased risk of gallstones (370) and similarly after bariatric surgery (particularly when Roux-en-Y gastric by-pass is performed) stone formation may be expected (371). In these situations, UDCA prophylactic therapy is advised (144, 372).

b. Pharmacological treatment

UDCA consistently demonstrates gallstone dissolution capabilities. This effect was evident when UDCA was administered at a dose of 7 mg/kg with radio-transparent, non-calcified stones ≤1 cm in size and in patients with a functional gallbladder (373). UDCA inhibition of cholesterol intestinal uptake and secretion in bile may explain its stone dissolution properties (374). Therapeutic application of UDCA, however, is hindered by high stone recurrence, accounting for more than 50% of cases on 10-year follow-up (375). This negative aspect is in part compensated by the observation that long-term treatment (up to 18 years) is associated with a decrease in biliary pain and acute cholecystitis in patients with symptomatic gallstones at baseline (376). In practice, UDCA dissolution therapy may be considered in symptomatic patients with elevated surgical risk or denying surgery. In acute symptomatic gallstones, use of non-steroidal anti-inflammatory drugs is generally indicated. In acute cholecystitis, antibiotic therapy remains controversial while it remains useful in cases of concurrent biliary tract infection, such as cholangitis or abscess formation (144).

c. Surgical approaches

An extensive examination of the operative procedures regarding the management of gallstones and their complications is behind the scope of this review since several publications and guidelines have focused on this issue (154, 159, 377). In this paragraph just the most relevant concepts on operative strategies for gallstones will be reported.

Surgical removal of the gallbladder (cholecystectomy) remains the advised approach in symptomatic gallstone disease (144, 378). Cholecystectomy, in fact, is a measure to block stone recurrence since gallbladder dysfunction (dysmotility and changes in bile reabsorption/concentration process) contributes to cholesterol nucleation (57, 194). Starting from 1985 laparoscopic (mini-invasive) cholecystectomy has been a major advancement in gallbladder surgery reducing hospital stay and allowing a faster post-surgical recovery, in comparison with open access (379). More than 90% of cholecystectomies are approached with the mini-invasive procedure presently; however, conversion or direct start with open surgery may be considered in difficult or complicated cases (144). For common bile duct stones, a specific mini-invasive approach based on endoscopic-retrograde-cholangiopancreatography (ERCP) technique has been consistently suggested and adopted (158, 159). ERCP is successful for common bile duct stone extraction in approximately 90% of cases and is also able to solve other gallstone complications such as acute cholangitis or biliary pancreatitis (380, 381). Finally, percutaneous cholecystostomy may be considered to prevent complications of acute cholecystitis in less fit patients (377).

XIII. Gallstones in cholestatic liver disease

a. Prevalence

Several studies converge in demonstrating an increased prevalence of gallstones in patients with liver diseases. In a cross-sectional and longitudinal study,

involving patients with liver cirrhosis, a gallstones prevalence of 29.5% was reported (382). The presence of stones was more prevalent according to age and severity of cirrhosis while it did not change according to gender or cause of disease. In the same study, a cumulative incidence of 40.8% at eight years was reported, similar to that observed in a previous study (383). Data from patients undergoing liver transplantation also confirm this trend (384). Interestingly, and differently from the general population, the majority of gallstones in cirrhotic patients is represented by pigmented stones, possibly as a consequence of the unbalance between mono-conjugated (less water soluble) and di-conjugated bilirubin in bile (385). Regarding chronic cholestatic adult liver diseases, a significant increase in cholecystectomy (27%) was reported in comparison with control (17%) in PBC patients (386). In another study, PSC patients were examined demonstrating a similar prevalence of gallstone and cholecystitis accounting for 25% of cases (226). Finally, regarding non-cirrhotic liver diseases, interest is gaining in the relationship between fatty liver and gallstones. In a study on patients with type 2 diabetes it was found that prevalence of gallstones was similar regardless of NAFLD presence (25.5% NAFLD vs. 23.6% control) even if this condition was more associated to symptoms and cholecystectomy (387). However, the possible relationship between fatty liver and gallstones remains complex due to the presence of several confounding factors (type 2 diabetes, obesity, etc.) and considering that gallstones may be an early indicator of the metabolic derangement leading to NASH (388).

b. Treatment

Since definitive therapy of symptomatic gallstones largely requires surgical and/or invasive procedures, and cirrhotic patients are considered extremely fragile in this regard, clinical management of these patients remains difficult. Portal

hypertension and reduced liver functions are factors setting cirrhotic patients in a class of high surgical risk. Gallbladder surgical removal (open cholecystectomy) was defined as “hazardous” in an early study reporting 83% mortality in patients with liver diseases and impaired prothrombin time (389). A more recent Danish study also confirmed a ten-fold increase in 30 days mortality after open cholecystectomy in cirrhotic patients in comparison with control (390). Providentially, this tragic picture had a relevant improvement due to the advent of laparoscopic approaches in recent decades (391, 392). In a meta-analysis comparing open or laparoscopic gallbladder removal in cirrhosis, the latter was associated with a significant decrease in complications and hospital stay (393). However, a crucial point is represented by the stratification of risk in each single patient. Child-Turcotte-Pugh score has been historically developed to evaluate the surgical risk of cirrhotic patients (394). According to Child-Turcotte-Pugh evaluation and severity of liver disease, the patient may belong to class A, B or C. It is agreed that A or B patients may undergo laparoscopic cholecystectomy while those in C class are usually not considered for surgery due to poor conditions (144). More recently another scoring system has gained interest in the assessment of cirrhotic patient prognosis and their priority for liver transplant: the so-called model-(for)-end-stage-liver–disease (MELD) (395). Even though a study demonstrated a preoperative MELD score >13 to be associated with cholecystectomy complications in cirrhotic patients (396), the cut-off for a safe procedure has not been identified so far.

In conclusion, while the prevalence of gallstones increases in patients with liver impairment, the usual therapeutic approaches are risky in a significant percentage of them, and other effective strategies are lacking. The evidence that stones are more frequent in advanced liver impairment (382) is also of concern demonstrating that those more in need of treatment are, at the same time, the ones with increased

contraindications. In this setting, medical therapy also seems of marginal help. In fact, cirrhotic patients are usually affected by pigmented stones and UDCA does not have significant effects on them.

Extensive research is needed to find alternative (non-invasive/medical) approaches to gallstone treatment in patients with liver disease. Regarding this issue, it should also be considered that NAFLD is a rising pathological liver condition affecting more than one third of adult western populations (269) and is unfortunately associated with both liver cirrhosis and gallstone disease.

CONCLUSION

Gallbladder disorders and gallstones are significant occurrences that can impact quality of life and mortality in humans. The association of gallbladder diseases, specifically gallstones, with cholestatic disorders highlights an important association between the gallbladder and the intrahepatic biliary tree (Table 4). It is intuitive that these two tissues would be interlinked in both normal and pathological states considering that the gallbladder is an extension of the biliary tree, and they are lined by a similar epithelial cell type; however, research generally looks at either gallbladder disease or intrahepatic biliary disease separately. The fact that gallbladder damage, gallstones and even gallbladder cancer have been shown to be associated with different liver disorders highlights the notion that we should look closer into the mechanisms and crosstalk mediating these paracrine injuries during various cholestatic liver diseases. Research that better understands the occurrence of gallbladder injury in cholestasis and whether they feedback on each other to promote damage in one another is necessary to better define whether congruent damage in these tissues can be treated separately or if it highlights a different issue or necessary intervention.

It is largely known that gallbladder damage and gallstones are highly regulated by cholesterol, BAs, lithogenic bile and bile stasis. These findings are not surprising since these components are found in high concentrations in bile and can remain in the gallbladder for an increased amount of time while waiting for the physiological signal that induces gallbladder emptying. This finding is also important to note since bile flow and BA circulation and conjugation can be regulated by intrahepatic cholangiocytes. This mechanism shows that processes mediated by the intrahepatic bile ducts may, in turn, regulate gallbladder damage or stone formation as a downstream consequence. This is also highlighted by the finding that both the intrahepatic and gallbladder cholangiocytes express transporters important for the transport of BAs. A similar expression profile was also noted for receptors and transporters necessary for water and bicarbonate secretion. Considering similar mechanism are found in these different biliary populations, it is unsurprising that damage in these two compartments may be linked; however, it is important to note expression discrepancies between the intrahepatic and gallbladder cholangiocytes, with higher expression profiles potentially noted in the gallbladder epithelia. Therefore, the gallbladder may play an important role in in bile modification that can in turn impact pathophysiology, which is something to be considered when discussing cholecystectomy.

One of the major treatments for gallbladder disorders is cholecystectomy; however, this may not always be feasible or desired by the patient. If we can better evaluate the link between cholestasis, biliary damage, and gallbladder disorders we could potentially find therapeutics to target these that do not include surgical intervention. In line with this, a better understanding of the intricacies linking the intrahepatic biliary tree and gallbladder can help to identify modalities or biomarker that can indicate gallbladder damage early on to better detect injury at earlier stages.

As discussed in the last part of this comprehensive review, much work is being done to identify new diagnostic and therapeutic approaches to counteract gallbladder disorders. It is necessary that future work, both in clinical trials, meta-analyses, and pre-clinical models, better evaluate the gallbladder during liver disease to better understand these issues and identify improved approaches for patients.

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FIGURE LEGENDS:

Figure 1: Image of the gallbladder and associated anatomical parts. The gallbladder can be divided into the fundus, body and neck and it then branches to the cystic duct that connects with the common bile duct. The common bile duct can further branch into the common hepatic duct, which further branch into left hepatic duct and right hepatic duct. Image made with BioRender.

Figure 2: Image of the layers of the gallbladder wall with various transporters and receptors important for gallbladder physiology. The gallbladder wall is divided into the following layers: mucosa, muscularis, perimuscular fibrous tissue and serosa. The epithelial in the mucosa layer modulate water, chloride, and bicarbonate secretion with aquaporin channels, cystic fibrosis transmembrane conductance regulator, and the purinergic Y2 receptor. The muscularis is involved with neuropeptide signaling and potassium release by ether-a-go-go related 1 potassium channel. Image made with BioRender.

Figure 3: Comparison of acute and chronic cholecystitis. Acute cholecystitis is an acute inflammatory response and can be due to cystic duct obstruction, overproduction of mucus, and/or lithogenic bile. Chronic cholecystitis is due to ongoing inflammation and is primarily associated with cystic duct blockage and lithogenic bile. Image made with BioRender.

Figure 4: Diagram of the main gallbladder disorders. Cholelithiasis is gallstone formation (either cholesterol, brown or black stones) and can complicate issues by becoming lodged in the cystic duct. Polyps are generally benign but can rarely be cancerous. Cholecystitis can be either acute or chronic, is mostly brought on by gallstones, is associated with abdominal pain and can result in gallbladder perforation.

Gallbladder cancer is a rare condition and is usually labeled as adenocarcinoma. Image made with BioRender.

Figure 5: Diagram of the different portions of the biliary tree in humans and mice. In humans, the biliary tree is separated from the most distal to the most proximal end as follows: canals of Hering, ductules, interlobular ducts, septal duct, area ducts, segmental ducts, left and right hepatic duct, and common hepatic duct. The mouse biliary tree is divided into two parts: the small ducts and the large ducts. Stem cell niches termed hepatic progenitor cells (HPCs) and the peribiliary glands can be found at the ends of small ducts or in the larger duct walls, respectively. Image made with BioRender.

Figure 6: Ultrasonography of the gallbladder (longitudinal and transversal scans) in a PSC patient (top and middle panels; length=12.3 cm; width=6.6 cm; height=6.0 cm; volume=253.0 mL) and a healthy control gallbladder (bottom panel; length=7.2 cm; width=2.5 cm; height=2.8 cm; volume=26.2 mL). Reprinted with permission from *Gut*. 1996 Oct; 39(4):594-599.

Figure 7: Photomicrograph images of gallbladder stones in *Mdr2*$^{-/-}$ mice (magnification=400X). (A) Needle-like crystals (arrows) found on the edges of a yellow-colored stone. Needle-like crystals are short, straight, filamentous cholesterol crystals. (B) Radial crystal pattern of a stones core showing needle-like crystals (arrow). Reprinted with permission from *Hepatology.* 2004 Jan; 39(1):117-128.

Figure 8: Histological image of the layers of the gallbladder wall in gallbladder cancer, corresponding to T stage. HA=hepatic artery; PV=portal vein. Reprinted with permission from *Gastroenterology Clinics of North America.* 2010; 39:333.

Figure 9: (A) Fasting gallbladder wall thickness in healthy controls, steatotic patients and NASH patients. (B) Gallbladder ejection fractions in healthy controls, steatotic

patients and NASH patients. Reprinted with permission from *Journal of Neurogastroenterology and Motility.* 2016 Jul; 22(3):470-476.

Figure 10: Pathological imaging of hematoxylin and eosin (H&E) staining of the gallbladder from an ARLD patient. (A) 10X imaging of H&E staining and (B) 40X imaging of H&E staining showing chronic cholecystitis with suppurative inflammation (arrows). Reprinted with permission from *Medicine (Baltimore).* 2018 May; 97(18): e0414.

Figure 11: Radiological findings of the gallbladder and SARS-CoV2 qRT-PCR from a COVID-19 infected patient. (A) Abdominal CT scan showing cholecystitis. qRT-PCR was performed on gallbladder samples to assess SARS-CoV-2 presence and (B) shows 3 samples from the gallbladder that were positive for SARS-CoV-2, and (C) the RNA control was consistently positive. Reprinted with permission from *Journal of Hepatology.* 2020 Dec; 73(6):1566-1568.

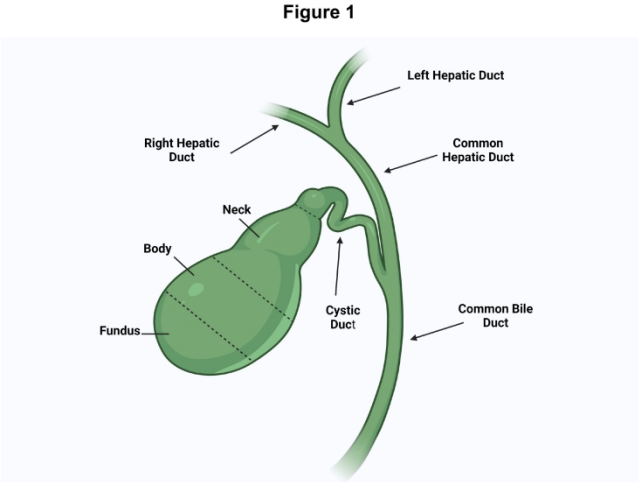

Figure 1

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Figure 2

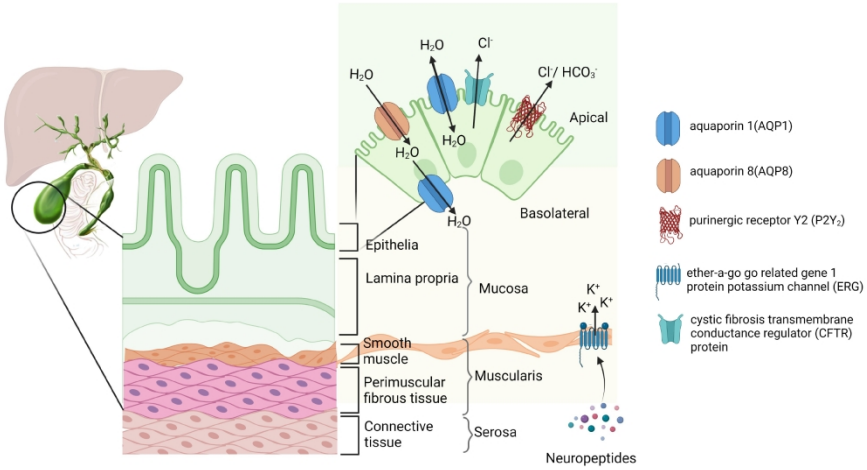

figure 2

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Figure 3

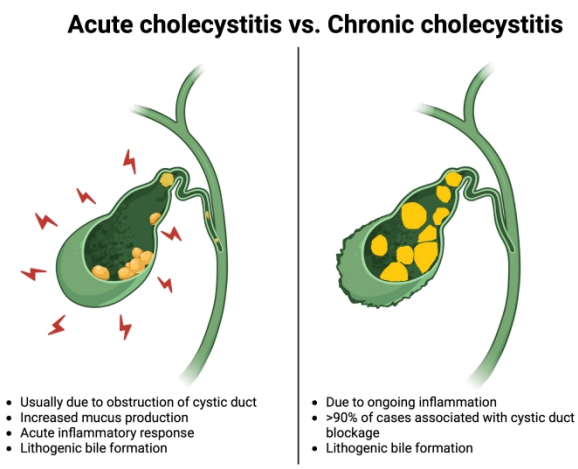

figure 3

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Figure 4

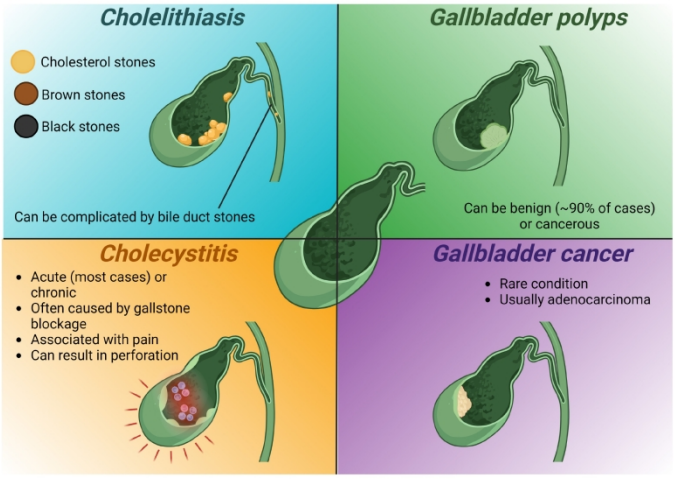

figure 4

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Figure 5

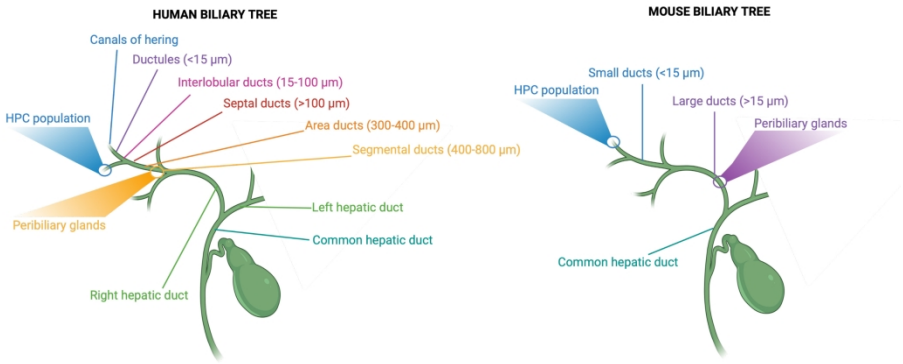

figure 5

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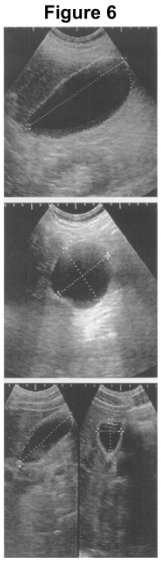

figure 6

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Figure 7

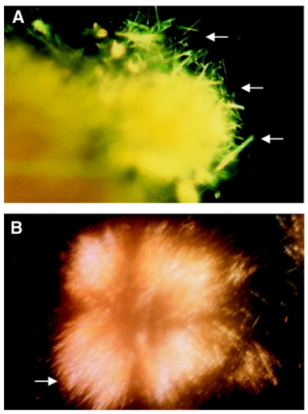

figure 7

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Figure 8

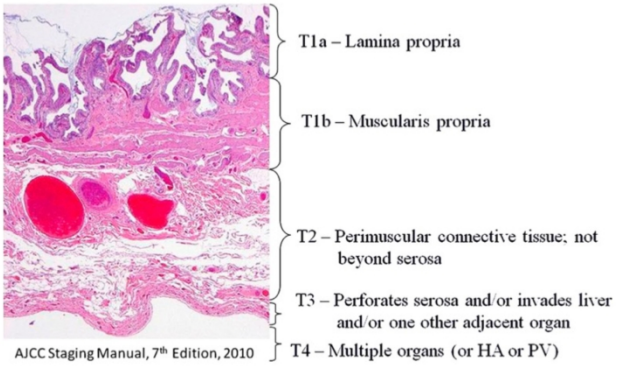

figure 8

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Figure 9

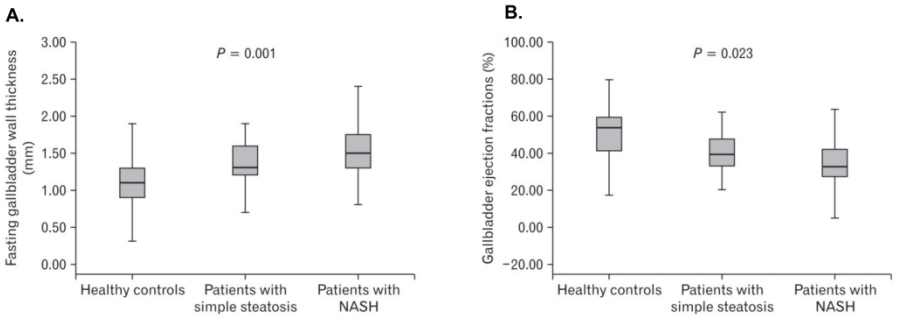

figure 9

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Figure 10

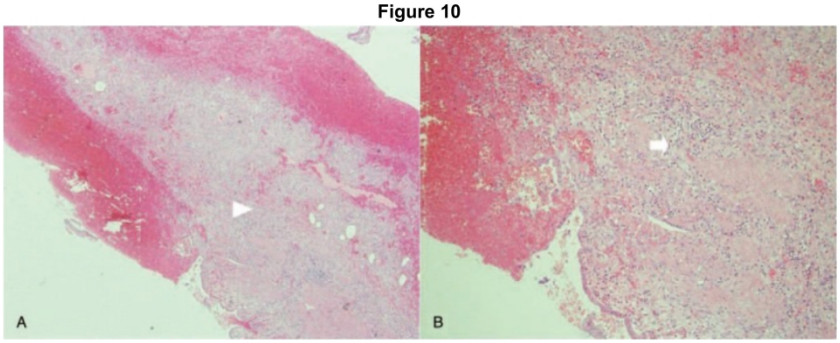

figure 10

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Figure 11

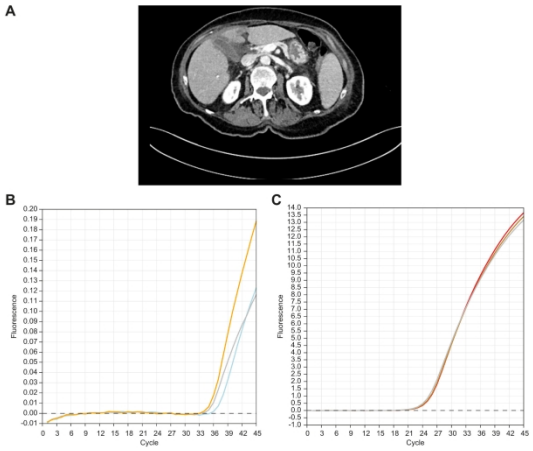

figure 11

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Table 1: Risk factors associated with cholelithiasis

Risk factors	Cholelithiasis
Aging	+
Indian ethnicity	+
Genetic:	
- SNPs:	
- *HHEX, MC4R, MAP2K5* and *NRXN3*;	+
- *FAIM2*;	-
- *Lith 1/2* genes (mouse);	+
- *ABCG5*/8 genes (human);	+
- Apolipoprotein E4 allele;	+
- Mutation in *ABCB4;*	+
- Mucin related genes;	+
Lifestyle:	
- Alcohol consumption;	-
- Low physical activity;	+
Female sex;	+
Obesity;	+
Rapid weight lost;	+
Microbiome:	
- Bacteria producing β-glucuronidase and phospholipase;	+
- Bacteria causing mucus abnormalities	+
- Helicobacter pylori	+

Positive (+) risk association for the development of cholelithiasis; Negative (-) risk association for the development of cholelithiasis. HHEX=Hematopoietically expressed homeobox; MC4R=Melanocortin-4-receptor; MAP2K5=Mitogen-activated protein kinase kinase 5; NRXN3=Neurexin-3; FAIM2=Fas apoptotic inhibitory molecule 2; Lith=Lithogenic gene; ABCG=ATP-binding cassette subfamily G; ABCB=ATP-binding cassette subfamily B

Table 2: Bacterial species detected in the bile of PBC patients.

Bacterial species	Sequenced colonies	Detected case
*Staphylococcus aureus**	40 (40%)	1, 2, 4, 5
*Enterococcus faecium**	20 (20%)	3, 6
Streptococcus pneumoniae or other *streptococci**	15 (15%)	9, 10
Lactobacillus plantarum	8 (8%)	7
Helicobacter pylori	4 (4%)	10
Propionibacterium acnes	5 (5%)	1, 8
Lactobacillus gasseri	2 (2%)	7
Corynebacterium otitidis	2 (2%)	8
Agrobacterium tumefaciens	1 (1%)	8
Flavobacterium breve	1 (1%)	8
Clostridium sordellii	1 (1%)	8
Micrococcus luteus	1 (1%)	8
	100 colonies	10 cases

Gram-positive cocci are marked*
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Table 3: Bacterial species detected in the bile of cholecystolithiasis patients.

Bacterial species	Sequenced colonies	Detected case
*Pseudomonas aeruginosa***	23 (28.8%)	28, 29, 30, 32
*Escherichia coli***	20 (25%)	26, 31
Clostridium perfringens	18 (22.5%)	27, 29, 30, 33
Sutterella wadsworthia	8 (10%)	27, 28, 29
Propionibacterium acnes	7 (8.8%)	33
*Enterococcus faecium**	4 (5%)	27
	80 colonies	8 cases

Gram-positive cocci are marked*; gram-negative cocci are marked**
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Table 4: Gallbladder disorders found in different liver diseases

Liver Disease	Gallbladder Disorder(s)
Primary Sclerosing Cholangitis (PSC)	- Gallbladder abnormalities - Gallstones - Cholecystitis - Gallbladder polyps - Cancer
Extrahepatic PSC	- 30% have cholecystitis
Intrahepatic PSC	- 9% have cholecystitis
PSC-IBD	- Gallbladder cancer
Cholangiocarcinoma (CCA)	- Gallbladder cancer
Primary Biliary Cholangitis (PBC)	- Neoplastic phenotype of gallbladder - Cholelithiasis
Non-alcoholic fatty liver disease (NAFLD)	- Cholesterol gallstone formation - Cholelithiasis
Alcohol-related liver disease (ARLD)	- Gallstone formation - Gallbladder wall thickening - Gallbladder perforation - Gallbladder variceal hemorrhage - Cholelithiasis
SARS-CoV-2-related liver disease	- Acute cholecystitis - Gangrenous cholecystitis

PSC=Primary sclerosing cholangitis; IBD=Inflammatory bowel disease; CCA=Cholangiocarcinoma; PBC=Primary biliary cholangitis; NAFLD=Non-alcoholic fatty liver disease; ARLD=Alcohol-related liver disease