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The inflammatory inception of gallbladder cancer

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Abstract

Gallbladder cancer is a lethal disease with notable geographical variations worldwide and a predilection towards women. Its main risk factor is prolonged exposure to gallstones, although bacterial infections and other inflammatory conditions are also associated. The recurrent cycles of gallbladder epithelium damage and repair enable a chronic inflammatory environment that promotes progressive morphological impairment through a metaplasia–dysplasia–carcinoma, along with cumulative genome instability. Inactivation of *TP53*, which is mutated in over 50% of GBC cases, seems to be the earliest and one of the most important carcinogenic pathways involved. Increased cell turnover and oxidative stress promote early alteration of *TP53*, cell cycle deregulation, apoptosis and replicative senescence. In this review, we will discuss evidence for the role of inflammation in gallbladder carcinogenesis obtained through epidemiological studies, genome-wide association studies, experimental carcinogenesis, morphogenetic studies and comparative studies with other inflammation-driven malignancies. The evidence strongly supports chronic, unresolved inflammation as the main carcinogenic mechanism of gallbladder cancer,

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Author contribution statement

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regardless of the initial etiologic trigger. Given this central role of inflammation, evaluation of the potential for GBC prevention removing causes of inflammation or using anti-inflammatory drugs in high-risk populations may be warranted.

Keywords

Gallbladder cancer; Gallstones; Chronic inflammation; Metaplasia; *Salmonella* Typhi; TP53

1. Introduction

Gallbladder cancer (GBC) is the most common malignancy of the biliary tree. Globally, GBC rates exhibit marked regional variability, reaching epidemic levels for some regions and ethnicities, especially in countries such as Chile, Bolivia, Peru, Ecuador, India and Poland. The basis for this variation likely resides in differences in environmental exposures interacting with genetic predisposition for modulating carcinogenesis. GBC risk increases with age, and women are affected two to six times more often than men [1,2].

The main risk factor for GBC is gallstone disease (GSD), which leads to a constant inflammatory state stimulated by recurrent cycles of cell death and regeneration of the epithelial layer [3,4]. The association between GSD and GBC is supported by Level II evidence (multiple-cohort studies) [5]. Subjects with GSD have a 21 to 57-fold increase in the risk of developing GBC [6]. Often, incidence of GSD correlates with GBC incidences geographically, and both conditions share common risk factors such as age, female gender, parity and ethnicity [1], factors that may accelerate gallstone formation [7]. Another important risk factor for GBC is the chronic carriage of *Salmonella* Typhi (OR 4.0), particularly for endemic regions such as south-central Asia and south-east Asia [8] where both infection and cancer correlate. Importantly, recent experimental evidence delineates a mechanism for *Salmonella*-induced GBC [9], supporting the role of this bacteria in the etiology of a proportion of GBC cases worldwide. In addition, other pathological conditions, such as primary sclerosing cholangitis, anomalous arrangement of the pancreaticobiliary duct (AAPBD) and bacterial infections, are related to the occurrence of GSD and GBC, and they all share a strong chronic inflammatory component [1].

GBC has long been of interest as a model for understanding the link between chronic inflammation and cancer. In the 20s, Archibald Leitch chose to study GBC because of its association with a "... particular endogenous irritant," the gallstone. Leitch implanted human gallstones, pebbles and pitch pellets in the gallbladders of guinea pigs and observed a progression from epithelial desquamation to increasingly severe lesions and eventually invasive adenocarcinomas, according to the length of time the animals survived. In contrast, melted lanoline, a soft material incapable of causing repeated cell damage, did not produce any alteration in the gallbladder. Leitch theorized that it was the damage caused by the foreign body, rather than its composition, that created a "... pathological condition of the tissues" suitable for the development of cancer [10]. Today we know that chronic inflammation is the "pathological condition" that links damage and cancer, leading to the recognition that "the biliary tract is the consummate example of inflammation-associated

carcinoma” [11]. Here we review the evidence linking inflammation to the generation of GBC in order to shed light on a little-studied phenomenon and to delineate the implications for carcinogenesis and cancer prevention.

2. Inflammation and cancer

The physiological aim of inflammation is the containment and eradication of infection, followed by a resolution process that seeks the restoration of the function of the affected areas. In the context of tissue damage with enhanced cell death, inflammation mediates a tissue-repair response in order to stimulate proliferation and regeneration [12]. When the initiating stimulus persists in time, however, the process progresses to chronic, nonresolving inflammation. Although inflammation is a necessary process for restoring homeostasis, its persistence promotes damage of normal tissues and, in turn, cell death stimulates more inflammation. Several pathologies share a common component of nonresolving inflammation, among which are obesity, asthma, chronic obstructive pulmonary disease, multiple sclerosis, inflammatory bowel disease, rheumatoid arthritis and cancer [13].

The immune system has evolved to sense necrotic cell death as a way to indirectly detect the dissemination of infection. For this reason, the immune system can respond to both infectious (viruses, bacteria) and non-infectious (tumors, injuries) scenarios where necrosis occurs [14]. During necrosis, which often results from nonphysiological damage, dying cells release endogenous molecules called damage-associated molecular patterns, which are able to stimulate T and dendritic cells and enable an inflammatory response, dilatating arterioles and venules to leak fluid and recruit leukocytes from the blood into the tissue, which results in heat and swelling, classical signs of an inflammatory response [15]. It has been proposed that chronic injury to tissues can result in an aberrant healing and regenerative response that ultimately promotes the expansion and progression of initiated cells via mechanisms closely related to inflammatory processes. If inflammation is chronically provoked by repetitive injury, cell death or other factors, the resulting process can promote cancer formation [16]. As described by Harold Dvorak, “... tumors appear to the host in the guise of wounds or, more correctly, of an unending series of wounds that continually initiate healing but never heal completely” [17]. Under this scenario, the inflammatory response does not eradicate the primary stimulus, as would normally occur in most cases of infection or injury, and thus a chronic form of inflammation ensues which ultimately contributes to tissue damage.

Evidence for the role of inflammation in tumor promotion has accumulated for some time [18], and today, a large body of data supports the hypothesis that inflammation is the link between tissue injury and cancer origin. This pro-cancer inflammation can be separated from the tumor-elicited inflammation, which occurs when the invasive tumor is already established and drives invasion and metastatic processes [16]. There is strong evidence that chemical and physical insults promote cancers of the gastrointestinal tract and liver by inducing chronic inflammation [19], and examples of inflammatory conditions that favor the development of cancer can be found in many organs including ovary, pancreas, esophagus, stomach, liver, bladder, colon, lung and endometrium [20–22].

Inflammation may promote early alteration of *TP53*, possibly through increased cell turnover and oxidative stress, although the precise mechanisms are unknown. Inactivation of the *TP53* gene, either by deletion or mutation, is the most common genetic alteration observed across cancers at different anatomic sites [23], including GBC [24,25]. *TP53* alterations are observed even in histologically normal epithelia from GDS patients with chronic cholecystitis, and the frequency of *TP53* alterations increases as impairment of epithelial architecture progresses from metaplasia to invasive carcinoma [26,27]. Environmental exposures can lead to *TP53* mutations and affect inflammatory and other immune responses. For example, aflatoxin B1 (AFB1) exposure leads to specific somatic mutations in *TP53*, with a high frequency of transversions at codon 249 [28], and animal studies have found that AFB1 exposure leads to increased pro-inflammatory cytokine and regulatory cytokine expression while decreasing lymphocyte proliferation [29,30]. A recent short report found that GBC cases were 13 times more likely to have detectable circulating aflatoxin-albumin adducts than normal controls (OR, 13.2; 95% CI, 4.3–47.9) [31]. While the finding needs to be replicated in other studies, it is of interest because of the link between aflatoxin, *TP53* mutations, and inflammation.

Loss of function of *TP53* is observed in other preneoplasias associated with inflammation-generating conditions and tissue damage. For example, *TP53* loss of function is common in Barrett's esophagus, a metaplasia that arises in response to chronic gastric reflux with chronic esophagitis and is the precursor to esophageal adenocarcinoma [32]. Similarly, *TP53* alteration is considered an early event for ulcerative colitis-associated (i.e., inflammation-related) colorectal cancer [33], while it is a late event for sporadic colorectal cancer. The histological progression of colitis-associated versus sporadic colorectal cancer is also different; while the sporadic colorectal cancer presents with progression from polyp to carcinoma, ulcerative colitis-associated cancer involves increasing histological grades of dysplasia that culminate in an invasive carcinoma [34], with a morphogenetic progression similar to GBC. In addition, *Helicobacter pylori* infection and multi-atrophic gastritis associated with intestinal metaplasia of the stomach, the precursor to intestinal-type gastric cancers, often presents with mutation, deletion and IHC overexpression of *TP53* [35–37]. In the liver, the highest rates of *TP53* mutations are found in hepatocellular carcinomas [38] and intrahepatic cholangiocarcinomas associated with hepatitis B [39]. Taken together, these observations suggest that regardless of the specific etiologic agents involved in the development of the aforementioned cancers, chronic inflammation and persistent tissue damage contribute to carcinogenesis initially through the inactivation of *TP53*.

Interestingly, in fluke-related cholangiocarcinoma, a cancer mainly caused by the biliary colonization of *Opisthorchis viverrini* or *Clonorchis sinensis* flukes, *TP53* is mutated in 40–44% of cases, compared to ~10% in non-infection-related cholangiocarcinoma [40,41]. This high frequency of *TP53* mutations, together with the low frequency of oncogene activation summarized elsewhere [42], is similar to that observed in GBC [24]. This similarity between these two types of biliary cancers may be explained in part by the tissue damage and inflammatory process triggered by specific factors (parasite or gallstone) inside the biliary tract. This notion supports the idea that chronic inflammation, rather than the specific etiologic factor, is the driving force behind biliary carcinogenesis.

The relationship between inflammation and *TP53* has been also studied in non-neoplastic pathologies. *TP53* mutations and chromosomal alterations are found in the atherosclerotic plaques and synovia of rheumatoid arthritis patients, both conditions strongly related to chronic inflammation [43]. This observation supports the idea that deregulation of p53 is promoted in the context of tissue damage and inflammation, as seems to occur during gallbladder carcinogenesis. Although the mechanisms that link *TP53* to chronic inflammation and carcinogenesis are not clear, *TP53* is activated under acute DNA damage, hyperproliferative signals, oxidative stress and ribonucleotide depletion. Activation of *TP53* enables cell cycle arrest, allowing cells to repair the genome damage before proceeding through the cell cycle and thereby limiting propagation of potentially oncogenic mutations [44]. If reparation is not fulfilled, the cell may die through apoptosis or enter into replicative senescence, the most important mechanisms to eliminate damaged cells. Both of these processes are often orchestrated through activation of *TP53* [45]. In this context, it has been found that human preneoplastic lesions have a widespread activation of DNA damage signaling (even before the occurrence of p53 mutations), providing a barrier against tumorigenesis through the induction of apoptosis or senescence [46,47]. Therefore, in an aged tissue exposed for years to damage and chronic inflammation, the barrier imposed by wild-type p53 seems to be perhaps the greatest obstacle to overcome, which may be why the mutated clones are selected so efficiently in the context of GBC.

3. Gallstone disease, chronic inflammation and gallbladder cancer risk

GSD is the main risk factor for GBC, and its complex pathogenesis has been reviewed in detail elsewhere [48]. The *ABCG8-DH19* polymorphism at the *ABCG5/G8* heterodimer partner was the first genetic risk factor of cholesterol GSD discovered by a GWAS study and subsequently replicated in different populations [49]. This lithogenic polymorphism generates a gain of function of the protein allowing an increased biliary sterol secretion from the canalicular membrane of hepatocytes into the biliary tree, contributing to cholesterol hypersaturation and thereby promoting gallstone formation [50]. Interestingly, beyond its role as a risk factor for GSD, this polymorphism has also been associated with an increased risk of developing GBC in different ethnic populations [51,52]. The mechanism by which a gain of function in the *ABCG5/G8* gene may also enhance the risk for GBC has not been elucidated, although it may be related to a higher risk of developing GSD early in life [53] and/or the capacity to transport other substrates into the biliary tree, such as plant sterols or other chemicals with potentially carcinogenic effects [54–57]. Besides the strong influence of this gallstone-modulating polymorphism in the development of GBC, polymorphisms in genes related to the immune system, inflammation and oxidative stress have been associated with increased risk of GBC, namely *PTGS2* [58], *TLR2*, *TLR4* [59], *IL1RN*, *IL1B* [60], *IL10* [61], *IL8* [62], *CCR5* [63], *LXRβ* [64] and *OGG1* [65]. Thus, variants in inflammation-related genes may, under the stimulus of gallstones or other insults, accelerate the development of GBC.

Studies in murine cholesterol GSD models exposed to lithogenic diets (containing high amounts of cholesterol and cholic acid) have provided a glimpse into the inflammatory changes that occur during the process of gallstone formation. In one study, mice with cholesterol crystals, an early stage of gallstone formation, developed local changes in the

gallbladder characterized by increased mucus layer thickness, interleukin-1 and myeloperoxidase activity in the wall of the gallbladder [66]. In another study using the same model, morphological changes including epithelial hyperplasia, muscularis hypertrophy and increased wall thickness were observed as early as four weeks after the mice began the lithogenic diet. These changes were accompanied by inflammatory infiltrate composed of eosinophils, macrophages, neutrophils and lymphocytes within the lamina propria [67]. Moderate granulocyte infiltrate was also observed in the gallbladder with progressive impairment of gallbladder emptying [68]. Maurer and colleagues have shown that functional T-cells are crucial in the development of GSD since Rag2^{-/-} mice, which are B- and T-cell deficient, were resistant to cholesterol gallstone formation when fed a lithogenic diet for 8 weeks [69]. Thus, at least in murine models of GSD, chronic gallbladder inflammation occurs at early stages as a local response to the presence of a lithogenic bile (i.e. cholesterol supersaturated bile) even before macroscopic gallstones are seen [68]. In addition, gallbladder tissue from patients with GSD has been observed to contain higher levels of infiltration of COX-2/iNOS-positive macrophages, iNOS-positive granulocytes and mast cells than controls without GSD. These levels decreased when patients were treated with ursodeoxycholic acid, a hydrophilic bile acid that reduces biliary cholesterol content (i.e., reduces biliary cholesterol saturation index) and improves gallbladder muscle contractility by decreasing the cholesterol content in the plasma membrane of smooth muscle cells [70]. Interestingly, a cross-ethnic study comparing the lithogenic properties of bile from cholesterol GSD patients from Chile (Latinos) and The Netherlands (Dutch) clearly showed significant differences between bile samples from these two countries. Bile from Chileans showed significantly faster cholesterol nucleation time and higher protein content (mainly IgA and probably also mucin), in spite of lower cholesterol saturation index [71]. Even though histological analysis was not performed, this study suggests that the lithogenic process and degree of gallbladder inflammatory response differs between ethnic groups with different risks of GSD GBC [71]. Of note, Native Americans and Latinos with high Native American ancestry tend to develop GSD early in life and are more likely to develop symptoms or complications and present with multiple gallstones rather than solitary gallstones [72]. Indeed, larger or higher volume of gallstones (in the case of solitary or multiple stones, respectively) correlates with higher risk of GBC, reflecting most probably a history of prolonged carrier stage of gallstones and/or a higher lithogenic state [3]. Finally, chronic inflammatory infiltration, along with other histological changes, is almost universally observed in the gallbladder of GSD patients [73]. Although the current paradigm states that these histological changes are induced by an aseptic chemical stimulus (i.e. lithogenic bile), in the era of the explosive knowledge related to the intestinal microbiota, this paradigm could change. Recent evidences suggest that the gallbladder milieu of GSD patients is not aseptic; one recent study identified microbiota of intestinal origin almost universally in the bile of most patients with GSD [74]. Taken together, the evidence shows that chronic inflammatory infiltrate is present in the gallbladders of GSD patients and that these changes can occur during the early stages of GSD formation and seem to differ in intensity between higher and lower risk populations, suggesting genetic susceptibility.

Two models of malignant transformation are recognized in the gallbladder: the metaplasia–dysplasia–carcinoma and the adenoma–carcinoma sequence [75–80]. However, gallstone–

related gallbladder carcinogenesis occurs mainly through the metaplasia–dysplasia–carcinoma pathway rather than through the transformation of a pre-existing benign tumor lesion [81]. Epithelial metaplasia is defined as a transformation of a differentiated epithelium into another differentiated epithelium and is associated with tissue damage and chronic inflammation [32]. Metaplasia is a common finding in gallbladder tissues exposed to gallstones, with frequencies ranging between 59.5–95.0% for pseudopyloric and 9.5%–58.1% for intestinal metaplasia [82,83]. Metaplasia is also found in 66% of gallbladders with infiltrating carcinoma [73]. In addition, the severity of the lesions found in the gallbladder epithelium worsens as the weight, volume and size of the gallstones increases [84]. Given the high frequency of metaplasia in chronic cholecystitis together with the evidence of genetic and epigenetic alterations already present in metaplasia [42], metaplasia is considered the site of initiation of gallbladder cancer, analogous to tumors at other anatomic sites (e.g., squamous metaplasia of the lung associated with long-term exposure to cigarette smoke, Barrett’s esophagus with gastric acid reflux, intestinal metaplasia of the stomach with *Helicobacter pylori* infection and acinar-to-ductal metaplasia with pancreatitis) [85]. In addition, in the stomach, the expression of human interleukin-1 β (IL-1 β) in transgenic mice leads to spontaneous gastric inflammation in the absence of *Helicobacter* infection. Overexpression of IL-1 β leads to chronic gastritis, metaplasia and high-grade dysplasia/carcinoma [86], which suggests that chronic inflammation signaling in the absence of insult may be sufficient to trigger metaplasia.

Furthermore, metaplastic lesions arise under the expression of key transcription factors that redirect the epithelial phenotype to a different type, and gallbladder metaplasia is often correlated with the expression of CDX2 [87], a homeobox transcription factor involved in normal development of the intestine that is also commonly found in the intestinal metaplasia of the esophagus and stomach [85]. Normal gallbladder epithelia do not express CDX2 and the surrounding leucocytes are mainly composed by T cell lymphocytes (CD3+; CD4+ and CD8+) and macrophage populations (CD14+ ; CD68+ and CD163+), with low or null levels of B cells (CD20+) (Fig. 1). In contrast, CDX2-positive metaplastic gallbladder epithelium it is often infiltrated with denser populations of T and B lymphocytes and macrophages (Fig. 2). In addition, the presence of metaplastic changes is correlated with an increase in the average gallbladder wall thickness [88]. Diffuse gallbladder wall thickening, defined as an enlargement of >3 mm measured by ultrasound, can be seen in primary gallbladder inflammatory processes such as acute, chronic, and acalculous cholecystitis [89].

PTGS2 (also referred to as cyclooxygenase-2 COX-2) is an enzyme that is involved in prostaglandin biosynthesis and thereby participates in the promotion and resolution of inflammation. PTGS2 is not usually expressed in normal gallbladder epithelium but is apparent in dysplastic lesions, with increased expression in high-grade compared to low-grade lesions. Increased COX-2 expression is particularly common in samples with p53 overexpression [90,91]. Aspirin, a cyclooxygenase inhibitor, has been associated with a reduced risk of GBC [92], similar to what has been observed for colorectal cancer [93]. It has been hypothesized that this reduction in the risk of GBC may be due to attenuation in gallstone formation associated with nonsteroidal anti-inflammatory drug (NSAID) use [94–96]; however, many studies have failed to show a decreased risk of gallstones associated with NSAID use [97–103]. In fact, a population-based case-control study in Shanghai,

China, that included over 350 GBC cases, over 1000 biliary stone patients, and over 1000 healthy adults observed an inverse association between aspirin use and GBC [odds ratio (OR), 0.37; 95% confidence interval (CI), 0.17–0.88 compared to healthy controls and OR, 0.44; 95% CI, 0.19–0.99 compared to patients with biliary stones], but no association between aspirin and biliary stones (OR, 0.92; 95% CI, 0.59–1.44 compared to healthy controls) [92]. These findings suggest that NSAIDs do not affect risk of gallstones but may help reduce the risk of GBC, even in the context of GSD. Additional studies are needed to evaluate the potential for NSAID use to contribute to GBC prevention.

The acute-phase reactant C-reactive protein (CRP) has long been recognized as a marker of inflammation and has gained attention as a prognostic marker of future cardiovascular events [104]. A nested case-control study within the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort revealed that high levels of CRP are associated with higher risk of developing GBC. Although this study did not report history of GSD, it is likely that the majority of the GBC cases were related to GSD in this cohort, which suggests that GSD may induce an inflammatory state that can be measured in plasma several years prior to the onset of the neoplasia [105]. In support of this hypothesis, increasing CRP levels in the bile have been associated with increasing number of gallstones present [106]. In addition, a recent study of GBC cases and gallstone controls from China and Chile found strong associations between inflammation-related markers and GBC (Koshiol, submitted). In particular, CCL20, CRP, CXCL8, CXCL10, resistin, and serum amyloid A were strongly associated in both study populations, with ORs ranging from 7.2 (95% CI: 2.8–18.4) for CXCL10 to 58.2 (12.4–273.0) for CXCL8 (Koshiol, submitted). Chemokines were frequently associated with GBC versus gallstones, suggesting that chemokine systems may be important to gallbladder carcinogenesis. Mechanistic characterization of chemokine systems may help identify targets for treatment [107].

Liver X Receptors (LXRs), nuclear receptors belonging to the ligand-activated transcription factor family, are involved in the control of lipid homeostasis, glucose metabolism, proliferation, and CNS development and has been proposed as drug target for several pathological conditions due to its link with inflammatory states [108]. Importantly, LXR- β genotypes were found to be significantly associated with females patients with GBC or gallstones, suggesting that LXR β polymorphisms influence gallbladder cancer susceptibility through estrogen and gallstone-dependent pathways [64]. In female mice in which the LXR β has been inactivated, preneoplastic lesions of the gallbladder developed and evolved to cancer in old animals. LXR β -/- female gallbladders show severe inflammation, with regions of dysplasia and high cell density, hyperchromasia, metaplasia, and adenomas at the age of 11 months, and with activation of components of the TGF- β signaling pathway. The elimination of estrogens in ovariectomized animals prevented any signs of inflammation, dysplasia, or metaplasia. The authors concluded that the gallbladder preneoplastic lesions in LXR β mice are due to a complex interaction between the absence of the anti-proliferative and anti-inflammatory action of LXR β and the hyperactivation of TGF- β signaling and estrogen action [109]. This mouse model is of particular interest because these mice develop cancerous lesions under an inflammatory background but without lithiasis or infection, suggesting that inflammation can lead to gallbladder carcinogenesis regardless of the agent that generates it.

4. Other inflammatory pathologies that predispose to gallbladder cancer

4.1. Anomalous arrangement of the pancreaticobiliary duct (AAPBD)

AAPBD is a congenital biliary anomaly associated with a high frequency of GBC, with relative risks between 167.2 and 419.6 times higher among AAPBD patients than in the general population [110]. Cumulative evidence indicates that carcinogenesis in gallbladder and biliary epithelia of patients with AAPBD is a multistage process that progresses through a metaplasia–dysplasia–carcinoma sequence driven by chronic inflammation, similar to GBC caused by gallstones. This process seems to be related to the mix of bile and pancreatic juice that stagnate in the gallbladder and the bile duct, especially in the dilated common bile duct. Under this chronic inflammation, pancreatic enzymes may activate, bile acid fraction may change, and mutagenic or occasionally carcinogenic substances may be yielded, leading to genetic and morphological changes [111]. Infiltration of mononuclear cells and fibrosis beneath the columnar lining epithelium and in the subserosal fibrous tissue is observed in most gallbladder specimens with AAPBD, similar to chronic cholecystitis, together with intense COX-2 immunoreactivity in epithelial cells, fibroblasts, endothelial cells and smooth muscle cells [112]. Interestingly, the prevalence of *KRAS* mutations is higher in GBC related to AAPBD than GBC not related to AAPBD [113,114]. This pattern suggests that the occurrence of *KRAS* mutations in patients with AAPBD is more related to the etiology of pancreatic cancer [111,115], where *KRAS* prevalence is very high [116], compared to gallstone-related GBC, where the frequency of *KRAS* mutations is low or nonexistent [117,118].

4.2. Autoimmune diseases

Autoimmune diseases have been associated with increased risk of cancers [119,120], including GBC [121]. Recently, five autoimmune conditions were associated with higher standardized incidence ratios for GBC: Celiac disease, Crohn's disease, pernicious anemia, ulcerative colitis and polymyositis/dermatomyositis [122]. However, this study was unable to evaluate primary sclerosing cholangitis (PSC), which is an established risk factor for GBC [1]. PSC is the classic hepatobiliary manifestation of inflammatory bowel disease and other immune-mediated diseases and is characterized by chronic bile duct destruction and progression to end-stage liver disease. Chronic injury occurs in small, medium, and large bile ducts with an inflammatory and obliterative concentric periductal fibrosis leading to biliary strictures [123]. Gallbladder metaplasia, dysplasia and carcinoma occur with high frequency in PSC patients. The metaplasia–dysplasia–carcinoma sequence observed in the setting of PSC is similar to that proposed for sporadic GBC. In a histological study of 72 gallbladders resected from patients with PSC, strong morphological alterations were found. Lymphoplasmacytic chronic cholecystitis was present in 49%, pseudopyloric metaplasia in 96%, intestinal metaplasia in 50%, dysplasia in 37% and adenocarcinoma in 14% of samples. The close association between gallbladder neoplasia and intrahepatic biliary neoplasia supports the concept of a neoplastic “field effect” in biliary tract of patients with PSC [124]. Around half of PSC patients have abnormal gallbladders, and bile duct stones are found in as many as 25% [125]. In another series of 102 patients with PSC who underwent a cholecystectomy, 13% had a gallbladder mass, and of these 7 had adenocarcinomas and the other 6 had benign masses. In those patients with benign masses, 33% had associated

epithelial dysplasia, while in patients with primary GBC, 57% had associated dysplasia [126]. In another study, gallbladder dysplasia/carcinoma was found in 30% of operative specimens, and evidence of inflammation and fibrosis of the gallbladder wall was found in nearly half of gallbladder dysplasias and carcinomas, respectively [127]. As exemplified by PSC, autoimmune diseases probably increase risk of GBC via exacerbation of biliary inflammation.

4.3. Bacterial infections

Of the multiple inflammatory causes of cancer susceptibility, infection has gradually been accepted as a major driver of inflammation-induced tumorigenesis, with up to 20% of all cases of cancer worldwide associated with microbial infection [21]. The best evidence for causal bacterial involvement in inflammation-induced cancer comes from infections with *H. pylori*, a known risk factor for gastric adenocarcinoma [128]. Several bacterial genera have been identified by culture or by PCR in the gallbladders of patients with cholecystitis and cholelithiasis, including *Salmonella*, *Escherichia*, *Klebsiella* and *Helicobacter*, among others [129]. Although the role of these microorganisms in gallbladder carcinogenesis is still unknown, the relationship between *S. Typhi* and GBC is among the best characterized.

Early evidence linking *S. Typhi* with gallbladder complications decades after primary infection was reported by Botsford in 1941 [130], who described acute typhoid cholecystitis and cholelithiasis occurring 43 years after typhoid fever, and by Axelrod et al. in 1971 [131], who described cholecystitis and GBC in a 80-year-old woman who had typhoid fever at age 13 years. In 1979, Welton et al. showed that chronic typhoid carriers die of hepatobiliary cancer six times more often than controls [132]. Similar conclusions were obtained later by others studies finding an epidemiological link between chronic typhoid carrier state and hepatobiliary cancer risk [133–136]. Studies performed in North India, an area of high endemicity for both typhoid fever and GBC, have reported ORs for GBC of 8.5 [137], 9.2 [138], 14 [139] and 22.8 [140] among chronic typhoid carriers, supporting the strong association between these pathologies. In other regions such as Shanghai, no association was found between *S. Typhi* and biliary cancer due to the very low prevalence of chronic carriers in this population [141]. However, in this same study population, lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria, and two LPS-pathway proteins were associated with GBC compared to gallstones, suggesting that bacterial infections more broadly may be relevant for the transition from gallstones to GBC (Van Dyke, manuscript in preparation). In areas with a higher prevalence of *S. Typhi*, either historically or currently, the association with GBC is remarkably consistent (Koshiol, manuscript in preparation).

Dongol and co-authors performed a clinical analysis of microbiological culture of bile and antisera profiling, categorizing patients as positive for *S. Typhi* and Paratyphi (N = 48), positive for non-*Salmonella* bacteria (N = 226), or negative for *Salmonella* (N = 1103). Compared to controls, they found that *Salmonella*-positive gallbladders exhibited a greater frequency of distension, inflammation, and empyema (pus within the gallbladder cavity) and more polymorphonuclear infiltration than lymphocytic infiltration, with 13% of the *Salmonella*-positive gallbladder specimens having massive neutrophil infiltrate near the

lumen, compared to 4% of negative cultures ($P < 0.05$) and 5% of the non-*Salmonella* culture positives ($P < 0.05$), respectively. Furthermore, an additional 15% of the *Salmonella*-positive gallbladder specimens had acute-on-chronic cholecystitis (neutrophil infiltrate near the lumen with lymphocyte infiltrate and dysplasia in the mucosa) compared to 5% ($P < 0.05$) and 7% ($P < 0.05$) of the culture negatives and the non-*Salmonella* culture positives, respectively [142]. Infected gallbladders show histopathological damage characterized by destruction of the epithelium and massive infiltration of neutrophils, accompanied by a local increase of proinflammatory cytokines [143].

Epidemiological studies conducted in endemic regions showed a strong link between the chronic typhoid carrier state and gallstones; approximately 90% of chronically infected carriers have gallstones [144]. *Salmonella*, particularly *S. Typhi*, is particularly efficient in producing biofilms [145]. In the mouse model $129 \times 1/\text{SvJ}$, mice fed with a lithogenic diet consistently formed gallstones that enhanced colonization and persistence of serovar Typhimurium in gallbladder tissue and bile following infection. The presence of this gallstone material was associated with significant numbers of *Salmonellae* that increased over time, and these gallstone-containing mice had a 3-log increase in fecal shedding of serovar Typhimurium compared with infected controls lacking gallstones [146]. In addition, *Salmonella* O-antigen capsule genes are bile-induced and specifically required for biofilm formation on cholesterol gallstones [147]. These results suggest that *Salmonella* biofilms on cholesterol gallstones significantly facilitate gallbladder colonization during persistent, asymptomatic infection.

Recently, molecular evidence linked *Salmonella* infection with GBC development. *Salmonella enterica* induced malignant transformation in predisposed mice, gallbladder organoids and fibroblasts with *TP53* mutations and MYC amplification. This process depended on activation of MAPK and AKT pathways to sustain transformation. Post-infection, *Salmonella* stably upregulates a series of interferon-response genes that suppress host inflammatory responses, evidencing an imprint of a normal host cell response to bacterial infection [9]. In addition, recent studies suggest that different *Salmonella* serovars elicit different immune responses. For example, the capsular polysaccharide of *S. Typhi* seems to suppress mucosa inflammation, while non-typhoidal *Salmonella* serovars (e.g., *S. Typhimurium*, *Salmonella* Enteritidis) cause strong inflammatory responses [148,149], suggesting that *S. Typhi* may facilitate chronic carriage by suppressing acute inflammation, potentially leading to chronic altered immune response.

Although little is known at the population level about the true carcinogenic effect of *Salmonella* in the absence of gallstones, the epidemiological evidence supporting chronic *Salmonella* infection as a risk factor for GBC is consistent [8]. We know that *Salmonella* infection is prominently efficient in the presence of gallstones. Both gallstones and bacteria are capable of inducing an inflammatory response, thus it is plausible to hypothesize that co-occurrence of these agents would increase the risk of developing GBC.

5. Conclusions and future prospects

Evidence gathered on GBC and comparisons with better-studied neoplasms strongly support the hypothesis that sustained chronic inflammation enables carcinogenesis in the gallbladder, where lithogenic bile, gallstones, toxins and probably bacterial infections trigger an inflammatory process that is not resolved and persists for decades (Fig. 3). This scenario opens up new avenues for research. First, GBC is an ideal model to study the role of inflammation in carcinogenesis. The generation of animal models that recapitulate human gallbladder carcinogenesis process is still an area that needs development. Such models would foster research on carcinogenesis, prevention and diagnosis of GBC. Second, although cholecystectomy is thought to contribute to the decreasing incidence of GBC in Western countries [150,151] and is currently used as a means to reduce the burden of GBC in Chile [152], there is still room to explore the implementation of strategies to diminish the occurrence of gallstones and/or attenuate chronic inflammation, for example by using pharmacological intervention. Finally, while GBC is relatively rare worldwide and has been less studied than other solid tumors, including other hepatobiliary malignancies, it represents a public health problem for several countries. There is a need for mechanistic research and sophisticated experimental models of gallbladder carcinogenesis in order to gain knowledge to help to improve prevention and clinical management of high-risk populations.

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Abbreviations:

GBC	gallbladder cancer
GSD	gallstone disease
PTGS2 (COX-2)	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
AAPBD	anomalous arrangement of the pancreaticobiliary duct
PSC	primary sclerosing cholangitis
LXRs	Liver X Receptors
NSAID	nonsteroidal anti-inflammatory drug

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Fig. 1. Leukocyte populations on non-metaplastic gallbladder epithelium. T cell populations (CD3, CD4 and CD8) are normally observed in non-metaplastic epithelium (CDX2 negative) and submucosa. As well, low infiltration of B cells (CD20) and macrophages (CD14, CD68 and CD163) may be observed in gallbladder submucosa.

Fig. 2.

Leukocyte subpopulations in gallbladder lamina propria with epithelial atypia associated to intestinal metaplasia. Metaplastic epithelium (CDX2-positive) is often infiltrated with T cells, B cells and macrophage populations at higher densities than those observed in normal epithelium, a sign of chronic inflammation.

Fig. 3.

Chronic inflammation and promotion of gallbladder cancer. The persistent damage done by lithogenic bile, gallstones, and possibly infections and autoimmune diseases, enables recurrent cycles of epithelium destruction and regeneration. The alteration of the epithelial structure progresses from inflamed tissue to an accumulation of metaplastic and dysplastic lesions, ultimately culminating with the establishment of invasive carcinoma. The release of inflammatory mediators induces the recruitment of leukocyte populations (particularly lymphocytes and macrophages), with an increase of reactive oxygen species (ROS) and COX-2 overexpression. In this context, *TP53* mutation is preferentially selected at early stages, fostering further DNA damage and tumoral progression. ROS = reactive oxygen species; COX-2 = cyclooxygenase 2; IM = intestinal metaplasia; and PM = pseudopyloric metaplasia.