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REVIEW

# Intra-hepatic and extra-hepatic cholangiocarcinoma: New insight into epidemiology and risk factors

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## Abstract

Cholangiocarcinoma (CCA) is a malignant tumour that arises from biliary epithelium at any portion of the biliary tree. CCA is currently classified as intra-hepatic or extra-hepatic CCA (EH-CCA). Recent evidences suggest that intra-hepatic CCA (IH-CCA) and EH-CCA are biologically different cancers, giving further support to a number of recent epidemiological studies showing large differences in terms of incidence, mortality and risk factors. The purpose of this manuscript is to review recent literature dealing with the descriptive epidemiology and risk factors of CCA with a special effort to compare IH- with EH-CCA.



**Key words:** Liver flukes; Viral hepatitis; Liver stem cells; Peribiliary glands; Epidemiology; Extra-hepatic cholangiocarcinoma; Risk factors; Primary sclerosing cholangitis; Intra-hepatic cholangiocarcinoma; Liver cirrhosis

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## INTRODUCTION

Cholangiocarcinoma (CCA) is a malignant tumour that arises from biliary epithelium at any portion of the bile duct system, from bile ductules to the ampulla of Vater[1,2]. Intra-hepatic cholangiocarcinoma (IH-CCA) develops within the liver parenchyma while extra-hepatic cholangiocarcinoma (EH-CCA) involves the biliary tree within the hepatoduodenal ligament. EH-CCAs are further divided into hilar or distal tumours. Hilar CCA, also called Klatskin tumour, is located within 2 cm from the bifurcation of the common duct[3]. Distal EH-CCA may affect the middle or the distal portion of main bile duct. A number of recent studies have focused on the role of stem cells in the origin of cancer. It has been elucidated that stem cells, due to their biologic features, are particularly prone to be involved in the carcinogenic process[4]. When a liver stem/progenitor cell on its way to differentiation develops into cancer (maturation arrest), this can give rise to tumours with a whole range of phenotypes with varying hepatocellular and cholangiocellular differentiation characteristics[5]. Stem cell niches have been identified in the canals of Hering/bile ductules[6-8]. They can differentiate into hepatocytes and cholangiocytes and, as postulated by different authors, can give rise to hepatocarcinoma (HCC), IH-CCA and combined hepato-cholangiocarcinoma (cHCC-CCA), a rare form of intrahepatic cancer formed by cancer cells sharing

features of both HCC and CCA[5]. More recently, we have found convincing evidence of the presence of additional stem cell niches located at the base of peribiliary glands (PBGs), distributed along the wall of the biliary tree[9]. PBGs start intrahepatically[10] at the level of the segmental ducts and are particularly dense in the extrahepatic biliary tree at the level of cystic duct, hilum and periampullary region[9], the sites where most EH-CCAs emerge. As intrahepatic and extra-hepatic PBGs are indistinguishable from one another[10], we assume that these stem cell niches are the source of cells for cell turnover in all bile ducts distal to the interlobular bile ducts as well as sites vulnerable to oncogenic transformation. This allows us to hypothesize that IH-CCA could arise from two distinct cellular lineages: one lineage originating from liver stem cells residing in the canals of Hering and one originating from the stem cells within the PBGs (Figure 1). These advances in stem cells location and other recent evidences suggest that IH-CCA and EH-CCA are biologically different cancers, giving further support to a number of recent epidemiological studies showing large differences in terms of incidence, mortality and risk factors. The purpose of this manuscript is to review recent literature dealing with the descriptive epidemiology and risk factors of CCA with a special effort to compare IH- with EH-CCA.

## CLASSIFICATION OF CHOLANGIOCARCINOMA IN EPIDEMIOLOGIC STUDIES

CCA is classified by the International Classification of Diseases for Oncology (ICD-O) according to their anatomic site (topography) and histology (morphology)[11]. Welzel *et al*[12] elegantly highlighted that until 2006 hilar CCA, Klatskin tumour, may have been mistakenly classified as IH-CCA under all versions of ICD-O. Before this report, some authors described the epidemiology and the risk factors for CCA, clearly considering the Klatskin tumours (8162) as IH-CCA. The conclusions of these studies[13,14] must be considered with caution in evaluating the differences in the epidemiology and risk factors of the IH- and EH-CCA. Pathological examination is generally considered the "gold standard" for the diagnosis and, consequently, a high degree of confidence is placed in microscopically verified cancer registrations[15]. In an Italian series of clinically diagnosed CCA, tissue-proven diagnosis was obtained in 78.45% of IH-CCAs and in 80.40% of EH-CCAs[16]. As described by West *et al*[15] in England and Wales, the proportion of IH-CCAs verified by microscopy has declined dramatically from 80.5% in the period 1968-1972 to 37.6% in the period 1993-1997, relatively more than what observed in the case of the EH-CCA (from 51.0% to 44.1%). An additional bias in CCA classification is cHCC-CCA, a biologically different subtype, which is considered to represent 1.0%-3.5% of primary liver cancers[17]. However, in a population of clinically diagnosed (ordinary or enhanced CT, ultrasonography, and hematological marker detection) cases of IH-CCA, the proportion of the cHCC-CCA has been reported to be 30%, as definitively demonstrated in surgically resected cases[18]. Donato *et al*[19], in a case–control study investigating several risk factors for IH-CCA (hepatitis C and B virus infection, alcohol intake, and hepatolithiasis), identified 2 cases of cHCC-CCAs among the 26 histologically verified IH-CCAs (7.6%), suggesting a bias in the interpretation of the results relative to the risk factors of IH-CCA. On this basis, non-histologically verified cases of IH-CCA have to be considered to be a mixed population of CCAs and cHCC-CCAs. The true proportion of the two cancers, remains to be evaluated. The lack of histological verification could affect the ability to evaluate the relative weight of a risk factor. In addition, some reports evaluating the epidemiology[20-22], the risk factors[23-25] and the mortality rate[26-28] are based exclusively on topographic classification (ICD-9; ICD-10 or others classification). In contrast, in more recent studies, the criteria used to identify IH-CCA or EH-CCA are those described by the European RARECARE[29] project where, IH-CCA has been identified by specific topographical (C22.0, C22.1) and morphological codes (8000–8005, 8010, 8011, 8020-8022, 8050-8084, 8140-8141, 8160, 8161, 8480-8500, 8550, 8560, 8570-8572) while EH-CCA has been identified by the topographical code C24.0 and the morphological codes 8000-8005, 8010, 8011, 8020-8022, 8140-8141, 8144, 8160-8161, 8162 (Klatskin tumour), 8190, 8230-8231, 8260, 8310, 8480-8500, 8550, 8560, 8570-8573, 8575-8576. CCA with not otherwise specified (NOS) localization is identified by topographical code 24.9, irrespective of histology.

## INCIDENCE

We searched the literature for case series of IH-CCA and EH-CCA diagnosed according the currently recognized criteria, described by the European RARECARE[29]. Welzel *et al*[12] reported how the incidence of IH-CCA (excluding hilar CCA) in the USA over the period from 1992 to 2000, showed a 4% annual increase. The incidence of EH-CCA (including code 8162/3) remained constant (annual percent changes = 1%), as did the incidence of hilar CCA reported under the code 8162/3. Between 1973 and 1991, the increase in incidence of IH-CCA was similar to that for the 1992 to 2000 period (annual percent changes = 5%) while, in contrast, there was a 1%/year decrease in EH-CCA incidence[12]. Consistent with the data from US registers, the AISF "Cholangiocarcinoma" committee reporting comprehensive national data from Italian National Cancer Registries[30], described 5517 histologically confirmed CCAs. For EH-CCA, an increasing trend of age standardised incidence rates from 11.5 to 13.5 cases per million was observed between 1988 and 2002. In the same period, a consistently increasing trend was also observed for IH-CCA: from 5 to 12 cases per million (average increase = 6% per year)[30]. Between 1995 and 2006 a progressive increase in incidence with age was seen for EH-CCA, IH-CCA and NOS. EH-CCA incidence rates were similar to those for IH-CCA for ages 45-54 years (6.5 *vs*

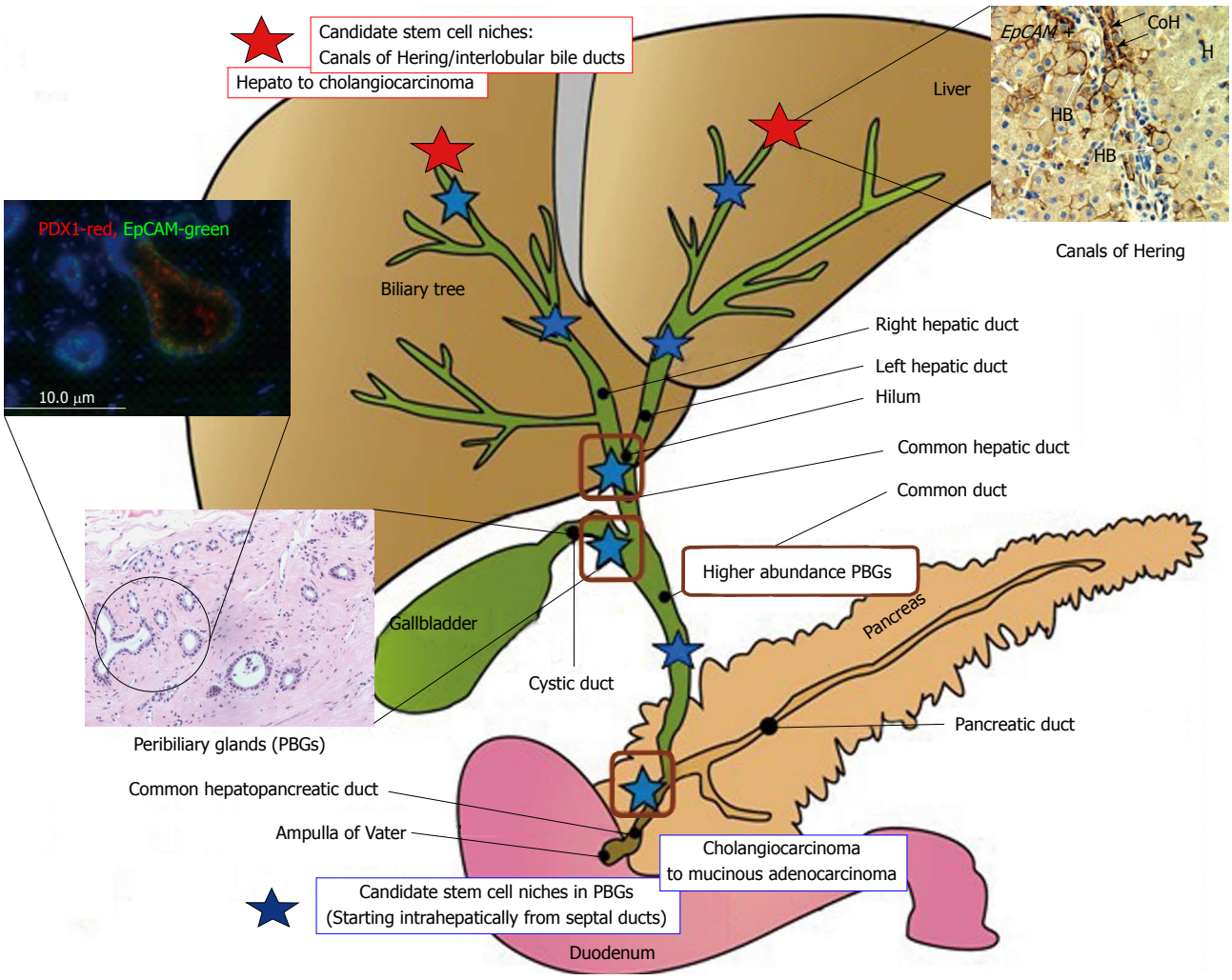


**Figure 1 Hypothesized origin of intra-hepatic cholangiocarcinoma and extra-hepatic cholangiocarcinoma.** Stem cell niches have been identified within the liver in the canals of Hering (red stars). Hepatic stem cells can differentiate into hepatocytes and cholangiocytes and can give rise to hepatocarcinoma (HCC), intra-hepatic cholangiocarcinoma (IH-CCA) and combined hepato-cholangiocarcinoma (cHCC-CCA). Convincing evidences are achieved on the presence of additional stem cell niches located at the bottom of peribiliary glands (PBGs) (blue stars). PBGs are mucous tubulo-alveolar glands, found deep within the ducts wall distributed along the biliary tree, starting intra-hepatic from the septal ducts. Proliferation of cells within PBGs, observed in chronic diseases or associated with malignancies, follow a pattern similar to that with typical stem cell compartment activation. PBGs are particularly dense in cystic duct, hilum and periampullary region, which are sites where extra-hepatic cholangiocarcinoma (EH-CCAs) typically emerge (encircled blue stars). As the intra-hepatic PBGs are indistinguishable from the extra-hepatic ones, we assume that these stem cells niches are sources for cell turnover of entire bile ducts distal to the interlobular bile ducts as well as sites vulnerable to oncogenic transformation. When a liver or biliary stem/progenitor cell on its way to differentiation develops into cancer, this can give rise to tumours with a whole range of phenotypes. This allows us to hypothesize that IH-CCA could arise from two distinct cellular lineages: the lineage originating from the liver stem cells residing in the canal of Hering, giving rise to a range of phenotypes from HCC to CCA comprising cHCC-CCA and the one originating from stem cells within the PBGs, giving rise to a range of phenotypes from CCA to mucinous adenocarcinoma. CCA risk factors associated to IH-CCA rather than EH-CCA are demonstrate to involve differently and specifically each of the two lineages, with the chronic parenchimal liver diseases affect the one from canals of Hering and chronic biliary disease (excluded PBC) and liver fluke affect the one from the PBGs.

6.8), while EH-CCA showed a more pronouncing increase of incidence rate in the subsequent age classes[30]. For all ages and for both sub-sites, incidence was more than 50% higher in men than in women. Interestingly, the proportion of NOS cases also increased with age[30]. Jepsen *et al*[31] identified, in Denmark, a cases series of 1335 IH-CCAs and 1269 EH-CCAs and reported, in contrast to observations in the US and Italy, an incidence rate decrease of 3.7% (95% CI: 3.0%-4.4%) and 2.7% (95% CI: 1.9%-3.4%) per year for IH- and EH-CCAs, respectively. The incidence rates for IH- and EH-CCAs in 1978 were 1.27 (95%CI: 0.96-1.58) and 1.05 (95% CI: 0.77-1.34) per 100 000 people, respectively[31]. From the mid-1980s, incidence rates started to decrease and by 2002 the incidence rates for IH- and EH-CCAs were 0.46 (95%CI: 0.29-0.62) and 0.74 (95% CI: 0.53-0.95) per 100 000 people, respectively[31]. The decrease in incidence was observed in both sexes and in all three arbitrarily defined age-groups (i.e. < 60, 60-79 and ≥ 80 years)[31].

In summary, with the exception of data from Denmark, studies investigating CCA epidemiology indicate a progressive worldwide increase of incidence and mortality for IH-CCA, whereas EH-CCA seems to be stable or slightly decreasing[12,13,21,30,32]. Large differences in incidence and mortality between IH-CCA and EH-CCA observed in these studies suggest that these two types of CCA emerge from pathological conditions (risk factors) which are largely different.

**Table 1 Summary of risk factors significantly associated to intra-hepatic cholangiocarcinoma[a] and/or extra-hepatic cholangiocarcinoma[b] as assessed by case control studies (Odd ratios by multivariate analyses)**

| Risk factors for IH-CCA (Ref.) | Odds ratios for increased risk | Risk factors for EH-CCA (Ref.) | Odds ratios for increased risk |
|---|---|---|---|
| Bile duct diseases and conditions | | | |
| Choledochal cysts[35,36] | 10.7-36.9 | Choledochal cysts[35] | 47.1 |
| Cholangitis/PSC[35] | 64.2 | Cholangitis/PSC[35] | 45.7 |
| Biliary cirrhosis/PBC[35] | 19.8 | Biliary cirrhosis/PBC[35] | 11.8 |
| Cholelithiasis[34,39] | 13.5 | Cholelithiasis[34,35] | 2.6-11 |
| Choledocholithiasis[34,41] | 22.5-34 | Choledocholithiasis[35] | 34 |
| Cholecystitis[35] | 8.5 | Cholecystitis[35] | 5.9 |
| Cholecystectomy[35,38] | 3.6-5.4 | Cholecystectomy[34,35] | 5.8-12 |
| Hepatolithiasis[19]c,[36,38,39,41] | 50.0-4.8; 6.7[c] | | |
| Liver flukes | | | |
| Clonorchis sinensis infection[36,40] | 8.6-13.6 | Clonorchis sinensis infection[40] | 6.5 |
| Digestive diseases | | | |
| IBD[35] | 4 | IBD[35] | 2.1 |
| Crohn's disease[35] | 2.4 | Crohn's disease[35] | 2.8 |
| Ulcerative colitis[35] | 4.5 | | |
| Duodenal ulcer[35] | 3.4 | Duodenal ulcer[35] | 1.9 |
| Chronic pancreatitis[35] | 5.9 | Chronic pancreatitis[35] | 9.3 |
| Endocrine disorders | | | |
| Diabetes mellitus type II [36-38] | 1.8-3.2 | Diabetes mellitus type II [34,35] | 1.5-3.2 |
| Thyrotoxicosis[35] | 1.5 | Thyrotoxicosis[35] | 1.7 |
| Miscellaneous conditions | | | |
| Alcohol intake > 80 g/d[36,37] | 5.9-6.6 | Alcohol intake > 80 g/d e[37] | 3.6 |
| Obesity[35] | 1.7 | | |
| Smoking[35] | 1.8 | | |
| Chronic liver diseases | | | |
| Alcoholic liver disease[35] | 3.1 | Alcoholic liver disease[35] | 4.5 |
| Non specific cirrhosis[35,36,41] | 10-16.5 | Non specific cirrhosis[35] | 5.4 |
| Hemochromatosis[35] | 2.6 | | |
| Hepatic schistosomiasis[41] | 11 | | |
| Non alcoholic liver disease[35] | 3 | | |
| HCV infection[19]c,[35-39] | 4.4-7.9; 9.7[c] | | |
| HCV infection plus cirrhosis[39] | 8.53 | | |
| HBsAg positive[34,36-39] | 2.3-9.7 | | |
| HBsAg positive plus cirrhosis[34,39,41] | 13-18 | | |

[a]Histological verified cases; [b]Histological verified cases comprise Klatskin tumour; [c]IH-CCA cases comprise 2 cases of cHCC-CCA. The table is prepared summarizing findings by case control studies investigating risk factors associated to IH-CCA and/or EH-CCA as assessed by multivariate analyses. The case-control studies are selected from the papers individuated by the follow terms searched on PubMed: ["cholangiocarcinoma"(MeSH Terms) OR "cholangiocarcinoma"(All Fields)] AND ["risk factors"(MeSH Terms) OR ["risk"(All Fields) and "factors"(All Fields)] OR "risk factors"(All Fields) OR ["risk"(All Fields) AND "factor"(All Fields)] OR "risk factor"(All Fields)]] NOT ["review"(Publication Type) OR "review literature as topic"(MeSH Terms) OR "review"(All Fields)] AND English(lang). The criteria selections of the works comprise moreover the case definition of CCA: Histological verified cases series of IH-CCA and/or EH-CCA with appropriate topographic classification (Klatskin tumours classified as EH-CCA and excluded from the IH-CCAs). In the 10 case control studies selected, several putative risk factors have been evaluated. The risk factors are collected together based on the patho-physiologic mechanisms lead to CCA (cells primarily targeted or activated by the diseases and therefore involved in the carcinogenic process) and on the homogeneity of the risk factors. IH-CCA: Intra-hepatic cholangiocarcinoma; EH-CCA: Extra-hepatic cholangiocarcinoma.

Some final considerations are needed. CCA is still considered a rare cancer. However, considering the hepatobiliary system as a whole, cancers of the gallbladder, intrahepatic and extra-hepatic biliary tree together represent approximately 30% of the total primary liver cancers with incidence rates approaching that of HCC[16]. Moreover, among patients transplanted for liver cirrhosis of various aetiologies ($n$ = 496), an incidental CCA was diagnosed in 10/82 (12.2%) explanted livers[33]. This means that CCA remains frequently undiagnosed.

## RISK FACTORS

We searched the literature for case series of IH-CCA and EH-CCA diagnosed according the currently recognized criteria (i.e. European RARECARE)[29], or case series with appropriate topographic classification of histologically verified CCA. Most series are case-control studies with multivariate analysis. The results of the 10 studies selected on the basis of these criteria are summarized in Table 1[19,34-42]. Several putative risk factors have been evaluated including, biliary diseases [choledochal cysts, cholangitis, primitive sclerosing cholangitis (PSC), biliary cirrhosis, primary biliary cirrhosis (PBC), cholelithiasias, choledocholithiasis, hepatolithiasis, cholecystitis], surgery (cholecystectomy), liver flukes, gastrointestinal diseases [inflammatory bowel diseases (IBD), duodenal ulcer, chronic pancreatitis], type II diabetes, cigarette smoking, alcohol intake, obesity and parenchymal liver diseases (chronic liver diseases, alcoholic liver disease, nonspecific cirrhosis, hemochromatosis, chronic non alcoholic liver disease, HCV infection, HBV infection). Of course, the

geographic distribution of the risk factors had a strong influence on the study design, with liver flukes almost exclusively investigated in the endemic area such as Far East and South East Asia. Conversely, the studies performed in the US, Europe, Japan and China mainly focused on viral hepatitis, cirrhosis, PSC and other biliary disease (with exclusion of liver flukes), type II diabetes mellitus, alcohol intake and smoking. Moreover, through the years, IH-CCA has been more extensively studied than EH-CCA. The investigated risk factors could be classified on the basis of the cells primarily targeted or activated by the diseases and therefore involved in the carcinogenic process. Biliary diseases such as choledochal cysts, cholangitis/PSC, secondary biliary cirrhosis, choledocholithiasis, hepatolithiasis, cholecystitis, and liver flukes are pathologic conditions primarily affecting large intra-hepatic bile ducts and/or extra-hepatic bile ducts. As might be expected, these pathological conditions are risk factors for both IH- and EH-CCA, as shown in Table 1. Cholelithiasias and cholecystectomy primarily affect extra-hepatic bile ducts. Consequently, these conditions are recognized risk factors mainly for EH-CCA (Table 1). Parenchymal liver diseases including, chronic viral and non-viral liver diseases, have the interlobular bile ducts, bile ductules and the canals of Hering as their primary targets. In fact, interlobular bile ducts, bile ductules and the canals of Hering are the main players involved in ductular reaction, a phenomenon shared by all these pathologies. Consequently, these conditions are recognized risk factors mainly for IH-CCA (Table 1). Type II diabetes and alcohol intake, are probably general cancer risks and, therefore, don't affect IH- and EH-CCA differently, as shown in Table 1.

The evidence for a possible association between liver flukes and CCA has been evaluated by the International Agency for Research on Cancer (IARC) since 1994[43]. Opisthorchis viverrini infestation, endemic in South East Asia, was considered definitively carcinogenic. Three studies (in Thailand) allow estimation of the relative risk associated with infection although none of them evaluates the discrete risk of IH-CCA and/or EH-CCA correlated to Opisthorchis viverrini[44-46]. Clonorchis sinensis infection revealed by radiologic evidence or stool examination was shown as an independent risk factor both for IH-CCA and for EH-CCA as assessed by multivariate analysis (Table 1). Other case-control studies from Korea[43,47] allow estimation of the role of Clonorchis sinensis in CCA development, however there was no evaluation of the discrete risk of IH-CCA and/or EH-CCA[47]. Liver flukes inhabit mainly the intra- and extra-hepatic bile ducts and, rarely, in the gallbladder and pancreatic duct. The infection is associated with a number of hepatobiliary diseases, including cholangitis, cholecystitis and hepato-cholelithiasis and is associated with several pathological changes in the liver, gallbladder and extra-hepatic bile ducts[48]. Microscopically, the intra-hepatic lesions are confined to the biliary tree, particularly to the large and medium-sized bile ducts where the flukes are harboured[48]. During liver fluke infection, inflammation, periductal fibrosis, and proliferative responses, may represent predisposing lesions that enhance the susceptibility of DNA to carcinogens including several *N*-nitroso compounds and their precursors[48,49].

Biliary diseases (cholangitis, PSC, choledochal cysts, biliary cirrhosis, choledocholithiasis and cholecystitis) are also indicated to be independent strong risk factors for both IH-CCA and EH-CCA as assessed by multivariate analysis (Table 1).

PSC, a disease affecting both intra-hepatic and extra-hepatic bile ducts, represents the strongest independent risk factor both for IH-CCA and for EH-CCA as evaluated by multivariate analysis (Table 1). In addition, IBD possibly associated with or preceding PSC, may significantly influence this risk since IBD was indicated as an independent risk factor for both IH-CCA and EH-CCA (Table 1). Unfortunately, definitive conclusions on how the coexistence of IBD with PSC may increase CCA risk compared to IBD or PSC alone are still lacking. Several older studies have evaluated the cumulative risk of CCA in PSC patients but none of these studies evaluated separately the discrete risk of IH-CCA and/or EH-CCA correlated to PSC[25,50-53]. More recently, Erichsen *et al*[54] demonstrated that the risk of both IH-CCA and EH-CCA in IBD patients, 65% percent with ulcerative colitis (UC), 25% with Crohn's disease (CD), and 10% with ICD codes for both diseases (incidence rates of 7.6 per 100 000 person years), was 4 times higher than in IBD-free population controls, RR = 4.0 (95%CI: 2.5-6.4). CCA occurred nearly twice as frequently in UC patients as in CD patients; the incidence rate was 8.2 (95%CI: 5.4-12.5) per 100 000 person years among UC patients versus 4.3 (95%CI: 1.8-10.3) per 100 000 person years among CD patients[54]. Interestingly, the incidence of CCA was highest within the first year at risk but decreased with time[54]. Approximately 4% of IBD patients with colonic involvement develop PSC, which increased the risk of both IH-CCA and EH-CCA (Table 1). In the study by Erichsen *et al*[54], it was found that at least half of patients with IBD and CCA had been diagnosed with PSC and the authors discuss how the remaining patients may already have or later develop PSC. Several case-control studies were conducted to evaluate differences between PSC patients with or without CCA in order to identify risk factors which could differentiate the two groups and thus helping in identification of PSC patients at high risk of CCA development[50,51,55,56]. Unfortunately, no statistical difference in the frequency or duration of IBD between PSC patients with and without CCA was found. Finally, as assessed by Kornfeld *et al*[50], PSC patients who experienced a cumulative risk to develop CCA of 11.2% 10 years after diagnosis, when diagnosed with associated IBD had a somewhat better prognosis: 10-year survival of 71.8%, compared with 60% for patients without IBD. Duration of IBD, and colectomy had no influence on survival and CCA risk[50]. In summary PSC and IBD are currently considered risk factors for both IH- and EH-CCA. Whether or not, the association of IBD with PSC further increases the risk of IH- or EH-CCA with respect to IBD or PSC alone is still debated.

Inflammation and biliary stasis are common features shared by different pathologies that increase CCA risk.

These include PSC, cholangitis, choledochal cysts, secondary biliary cirrhosis, choledocholithiasis, hepatolithiasis and cholecystitis. Recently, the role of the inflammation in CCA pathogenesis has been elucidated. The activation of NF-κB *via* Toll-like receptor ligands (lipopolysaccharide, flagellin) or bile acids (deoxycholate, taurodeoxycholate, and glycodeoxycholate) and Sonic Hedgehog signalling have been considered of relevance in inducing neoplastic cholangiocyte transformation during chronic inflammation. Furthermore, it was highlighted how oncogenic signals (Hedgehog, myc), innate immune activation (TLR), and bile acids all converge to suppress mir-29 promoter activity and expression. Mir-29 is a down-regulator of prosurvival protein Mcl-1[57]. Multipotent stem/progenitors are located at the base of the peribiliary glands and differentiate into mature cholangiocytes during migration from the bottom to the top of the glands[9], recapitulating in the biliary tree the intestinal model where proliferation of multipotential stem cells within Lieberkuhn's crypts, cellular migration and differentiation along the crypt-villus axis, are critical for development and maintenance of intestinal architecture[58]. The development of PSC and UC appears to precede dysplasia respectively of the biliary and of the colonic mucosa and represents a marker of patients at risk for developing CCA and colorectal adenocarcinoma[59]. The common embryologic origin of biliary tree and intestine and the similarities in the stem cell niches residing in the two organs lead to the suggestion that the inflammatory immune-mediated processes underlining PSC and UC could target cells originating from biliary and intestinal lineage indistinguishably. This opens new perspectives on current knowledge of these pathologies and on the similarities between colorectal adenocarcinoma and CCA[60].

The association between PBC and EH-CCA, described by Welzel *et al*[35], deserves some comment. PBC does not affect extra-hepatic bile ducts and, therefore, the reasons for increased susceptibility of cancer transformation in these large ducts are unclear. The absence of diagnostic confirmation and the possibility that some secondary biliary cirrhosis were included in the investigated population must be considered. The same authors discussed how, because of the small numbers of PBC, the reported association should be confirmed by other studies[35].

Hepatolithiasis has been indicated as a strong independent predictor of IH-CCA development (OR range 50-4.8), whereas none of the case-control studies have investigated the role of hepatolithiasis in EH-CCA (Table 1).

Cholelithiasias was indicated as an independent predictor of EH-CCA development (OR range 2.6-11), but not of IH-CCA (Table 1). These data are in accordance with the finding that the risk of EH-CCA increases with size of gallstones, calcification of gallbladder epithelium ("porcelain gallbladder") and duration of disease[61]. Gallbladder cancer arises usually as a consequence of chronic inflammation associated with gallstones[61]. Persistent inflammation is thought to promote carcinogenesis by causing DNA damage, activating tissue reparative proliferation, and by creating a local environment that is enriched with cytokines, other growth factors and oxysterols[61,62].

Cholecystectomy was shown to be highly significant as a predictor of EH-CCA (OR range 12-5.8) and also IH-CCA (OR range 5.4-3.6) (Table 1). However, a single study failed to confirm the association for both EH-CCA and IH-CCA[38]. In different human pathologies including gallstone and chronic liver diseases[62,63], bile contains oxygenated derivatives of cholesterol (oxysterols) which possess diverse biological properties[64]. After cholecystectomy, changes in the profile of lipids circulating in bile along the gut-liver axis (enterohepatic circulation) have been described with increased levels of oxysterols[65]. It was proposed that enhanced COX-2 protein expression by oxysterols may participate in the genesis and progression of CCA[64]. Thus, the continued exposure of extrahepatic ducts to constituents, such as oxysterols, which are enriched in the bile of cholecystectomized patients, could represent the basic mechanism responsible for the association of this condition with EH-CCA. Recently, it was observed that obese patients have a stronger acute inflammatory response to cholecystectomy[66] where, increased biliary levels of oxysterols could play a role. As obesity represents a risk factor for gallstones as well as a risk factor for several inflammatory and neoplastic conditions, it could be hypothesized that, in the specific setting of cholecystectomized patients, the obesity sustains high levels of oxysterols in bile with increased risk of EH-CCA development.

Type II diabetes has been indentified as an independent predictor of IH-CCA in 2/4 studies and of EH-CCA in 2/3 studies (Table 1).

Heavy alcohol intake (> 80 g/d) has been identified by multivariate analysis as an independent risk factor associated with both IH-CCA (range of OR 5.9-6.6) and EH-CCA (OR 3.6), as showed in Table 1, while 12 or more g/d of alcohol was not significant[34,39].

Type II diabetes and heavy alcohol intake, they probably reflect a general cancer risk.

Obesity was considered to represent an independent predictor of IH-CCA (Table 1), whereas the same study failed to demonstrate an association with EH-CCA[35].

Smoking was suggested as an independent predictor of IH-CCA in 1 of 4 studies (Table 1), while 3 different studies failed to demonstrate an association with EH-CCA[34,35,38].

Hemochromatosis and non-alcoholic fatty liver disease were identified as independent predictors of IH-CCA development, even if with a lower strength of association than other risk factors (viral hepatitis, cirrhosis) and they failed to predict EH-CCA (Table 1).

Cirrhosis of unspecified aetiology and alcoholic liver disease are independent risk factors significantly associated, according to multivariate analysis, with both IH-CCA and EH-CCA (Table 1). Specifically, cirrhosis showed an OR significantly higher for IH-CCA than EH-CCA (10-16.5 *vs* OR 5.4). However, it was not possible to determine whether the group of non specific cirrhosis included a fraction of biliary cirrhosis secondary to undetected pathologies of the extra hepatic biliary tree. This introduces

a potential bias which may possibly invalidate the apparent association between non specific cirrhosis and EH-CCA. Many biliary diseases leading to secondary biliary cirrhosis are associated with EH-CCA development, as showed in Table 1. However, a possible physiopathological basis for the apparent association could be the high level of oxysterols in cirrhotic patients, compared to both control and viral hepatitis patients[63], which could affect the extra hepatic biliary tree *via* bile or *via* peribiliary plexus blood flow.

In the last few years, hepatitis viruses (HCV, HBV) have been extensively investigated and debated because of the recognized global health burden of infection-associated cancer[43]. So far, there is no evidence which actually supports a role for viral hepatitis in EH-CCA development (Table 1). In contrast, many well conceived case-report studies and one cohort study have demonstrated the association between hepatitis viruses and IH-CCA, with ranges of OR from 2.7 to 9.7 (Table 1). It is interesting to note that the highest OR relative to HCV (i.e. 9.7) resulted from a study incorporating a few cases of cHCC-CCA in the IH-CCA group[19] corroborating the suggestion that the mixed subtype of primary liver cancer is induced by hepatitis virus[18]. In addition, it is important to note confounding factors that could affect the results of different studies including age of investigated population and the degree of liver damage (i.e. chronic hepatitis, cirrhosis *etc*.). A few authors have investigated the role of HBV and HCV infection as CCA risk factors independent of liver cirrhosis. Excluding the IH-CCA associated with cirrhosis Tao *et al*[34] showed that HBsAg was independently associated with IH-CCA (OR: 7.3; 95%CI: 3.1-17.2). Also, in a cohort study of asymptomatic subjects, those who tested HBsAg-positive had a significantly higher risk for IH-CCA (histological and/or clinical diagnosed) than those who tested negative for both HBsAg and anti-HCV. The risk was further increased in HBsAg positive patients with ALT levels of 40 KU or higher[67]. Yamamoto *et al*[36] revealed by multivariate analysis that during chronic hepatitis C infection without cirrhosis the adjusted OR for ICC was 2.32. Several other case-control studies evaluated viral related liver disease by investigating HBV[37,39] or HCV[38] infection without clarifying the stage of the associated liver disease. It was demonstrated, as showed in Table 1, that HBsAg seropositivity and anti-HCV seropositivity with unspecified liver disease represent independent risk factors for development of IH-CCA. Recently, a large cohort study (follow-up 8 years) demonstrated that HCV infection significantly increased the risk of clinically diagnosed HCC (155.0) and IH-CCA (155.1 ICD-9), with OR for HCC more than 5 fold higher than for IH-CCA. Results indicated that EH-CCA (156.1, 156.2, 156.8 and 156.9) and pancreatic adenocarcinoma (157.0, 157.1, 157.2, 157.3, 157.8 and 157.9), were not associated with HCV infection[68]. In relation to HBV infection (HBsAg seropositivity), the range of the OR, in the positive study ranged from 2.3 to 9.7 (Table 1). However, there is not conclusive agreement since further two studies failed to demonstrate a definitive association between anti-HCV seropositivity and IH-CCA[37,39]. Similarly, two studies on HBV failed to demonstrate HBsAg seropositivity as a risk factor for IH-CCA[19,38]. It is interesting to note that the two negative studies on HCV patients were conducted on populations of IH-CCA patients with an average age of 53.2 and 60.7 years respectively[37,39], whereas Lee *et al*[40] recently reported that the age at the diagnosis of the HCV related IH-CCA was 64.9 years[40]. In contrast, a strong agreement exists that viral cirrhosis is a significant risk factor for IH-CCA. Lee *et al*[40] demonstrated that cirrhosis increased the risk for IH-CCA by 3.2 fold in HCV patients. Yamamoto *et al*[36] revealed by multivariate analysis that as liver status worsened during HCV infection, the adjusted OR for IH-CCA tended to increase (chronic hepatitis, 2.32 *vs* cirrhosis, 5.03). As for HCV, it was observed that the presence of HBV-related cirrhosis increases the OR for IH-CCA development from 7.3 to 18 according to Tao *et al*[34], and from 9.7 to 13, according to Zhou *et al*[42]. Consistent with this evidence Lee *et al*[40] demonstrated that cirrhosis increased the risk for IH-CCA by 3.2 fold in HBV patients. These authors reported that the mean age of IH-CCA patients with hepatitis B (56.4 ± 11.1 years) was 9 years younger than that of IH-CCA patients with hepatitis C (65.6 ± 9.17 years), similar to results observed in HCC. Taken together the current literature suggests that the association between IH-CCA and hepatitis viruses (HBV, HCV) is markedly stronger when cirrhosis is present, suggesting that the background of enhanced proliferation and cell cycle acceleration, together with chronic inflammation, represents a significant pathogenic determinant. The occurrence of HCC, IH-CCA and cHCC-CCA in chronic liver diseases, exemplifies the postulated (see Roskams) wide spectrum of histological presentation of stem cell derived liver cancer. Liver stem cell activation accompanies many forms of liver damage irrespective of aetiology, making such cells very probable carcinogen targets[69]. HBV[70] and HCV[71] components have been demonstrated in bile duct epithelial cells during chronic infection. The infection of resident hepatic stem cells by HBV and HCV could explain the subsequent transmission of the viruses both to hepatocytes and to cholangiocytes. However, the hypotheses of a direct mutagenic role of HBV and HCV on liver stem cells is not actually sustained by the existing literature. The role of hepatitis viruses as risk factors for IH-CCA, independently of liver cirrhosis, needs to be further addressed together with the possible pathogenic mechanism responsible for this association.

The magnitude of the association between HBV and HCV positivity (with or without liver diseases) with IH-CCA is markedly lower than for the association with HCC[72,73] and the same is evident for hemochromatosis[74]. While HCC arises from cells directly infected by HCV or HBV (resident stem cells or adult hepatocytes), IH-CCA histogenesis is more heterogeneous, originating from resident liver stem cells or from cells not primarily affected by the hepatitis viruses but secondarily involved in the course of chronic liver diseases, such as cholangiocytes located in medium-large ducts (septal duct) or cells located in PBGs[1,2]. Therefore, when the association of HBV or

HCV with IH-CCA is factored in when calculating total cases of IH-CCA irrespective of their histogenesis, the magnitude of the association is certainly attenuated.

It should be noted that the mortality rates of all types of liver cancer (including liver cancers of poorly specified morphology) has increased in recent decades and that the improved diagnostic tools are not the only possible explanation for this epidemiological observation[32]. Taking into consideration the HCC increases in Japan, which are mostly HCV-related, it has been suggested that also US might experience a similar epidemic increase of HCV related primary tumours in the near future[75]. Analyzing the deaths relating to liver disease occurring from 1990 through 1998 in the US population[76], the age-specific and age-adjusted mortality rates for hepatitis C-related diseases increased by 220% from 1993 to 1998 (0.57 to 1.67/100 000), the rate for primary biliary cirrhosis increased overall by 12.8%, while mortality rate for alcohol-related chronic liver disease declined and remained unchanged for HBV-related liver diseases. As described by Welzel *et al*[12], the incidence of IH-CCA in the USA increased 4% annually between 1992 and 2000. Thus, the increase in IH-CCA incidence overlaps geographically and temporally with the increase in the mortality rates for HCV-related liver diseases. At the same time, it is unlikely that HBV infection could play a role in the increased mortality rates for IH-CC, as HBV infection rates and mortality remained stable or slightly decreased in the U.S. population in recent decades[76,77]. As far as other risk factors concerned, liver flukes are virtually absent in western countries and PSC-IBD incidence seems to be stable. Therefore, the reported progressive and impressive increase in incidence and mortality for IH-CCA reported worldwide could be reasonably ascribed to the burden of HCV infection.

Recently, we demonstrated that PBGs are stem cell niches in human extra hepatic bile ducts and that the sites for high abundance of PGBs overlap with the sites where malignancies occur with higher frequency in extra-hepatic bile ducts[9] (Figure 1). PBGs are strongly involved in the inflammatory diseases of the large intra-hepatic and extra-hepatic bile ducts, such as liver flukes, cholangitis, PSC, choledochal cysts, secondary biliary cirrhosis, choledocholithiasis, hepatolithiasis, cholecystitis and in CCA[78-84]. In these pathological conditions, PBG cells proliferate and acquire the expression of stem cell and neuroendocrine markers (C-met, c-erbB-2, argyrophil granules, chromogranin A)[81,82,84,85]. On the basis of these recent observations, we hypothesize that early cells within PBGs are the sites of origin of malignancies associated with chronic diseases or pathologic conditions of the biliary tree. Further studies are needed to corroborate this hypothesis which could explain the large different epidemiologic profile of IH- and EH-CCA.

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