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TITOLO DELLA TESI: Advanced biliary tract cancer: assessment of risk and prognostic factors, and analysis of treatment intensification for relapsed disease.

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1. Background: biliary tract cancer

Biliary tract cancer (BTC) is an umbrella term comprising a variety of epithelial malignancies arising from the biliary tree: intrahepatic cholangiocarcinoma (ICC) originates from the intrahepatic bile ducts; extrahepatic cholangiocarcinoma (ECC) originates from the extrahepatic bile ducts, and is further categorized as proximal (pECC when arising from the biliary carrefour (Klatskin-Altemeier tumor), and distal when arising from the medium or distal choledocum (dECC); gallbladder cancer (GC) originates from the colecyst. The composition as of primary sites varies according to the geographic region[1]: among the advanced forms (aBTC), ICC accounts for half of the cases, the other half being split among ECC and GC[2]. Ampullary cancer (AC) is often regarded as a rare, additional primary site of BTC[3,4].

1.1. Epidemiology

A higher incidence of BTC is observed in Southern Europe than Northern European countries. Italy, where BTC accounts for 1% of new cancer diagnoses, and is regarded as the second most common primary hepatic malignancy after hepatocellular carcinoma (HCC), lies among the intermediate-incidence countries[1,5]. Crude incidence rates of BTC in Italy are 7.8 and 8.0 cases/100.000 inhabitants/year, for women and men, respectively, which translates into a whole-life risk of contracting the disease of 1/144 and 1/132, respectively[6]. Interestingly, compared to all other gastrointestinal malignancies, BTC is associated with a reverted geographical gradient, as higher incidences are observed in Southern Italy than in Northern regions [Figure 1, re-elaborated from "I numeri del cancro in Italia 2016"[7]), which suggests a territorial co-segregation of risk factors rather than a differential coverage of regional tumor registers or differences in epidemiological collections.

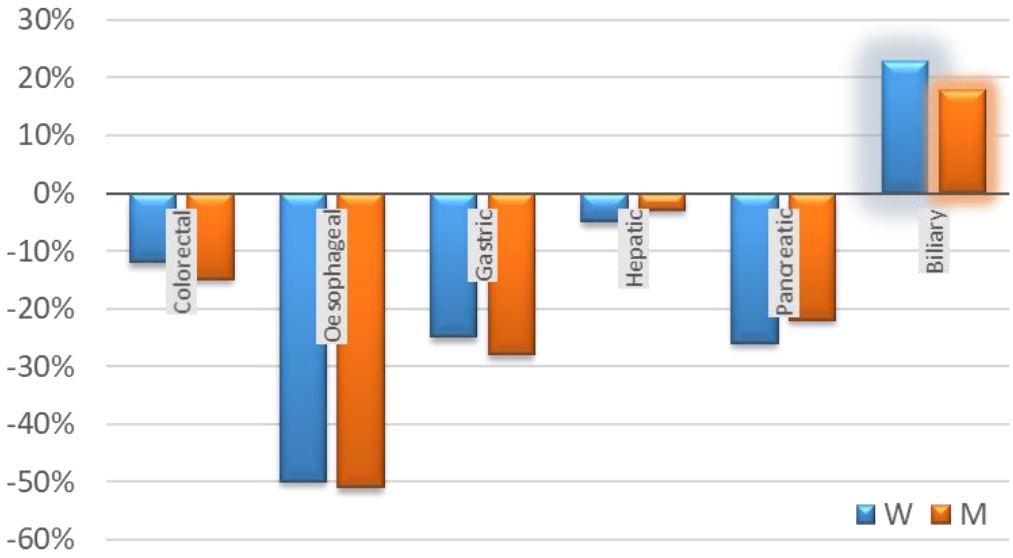

Figure 1. *Relative incidence in Southern vs Northern Italy (%). Re-elaboration from "I numeri del cancro in Italia 2016"*[7].

Nation-wide estimated cases were 4500 in 2012, as compared to 5400 in 2020[6,7], which represents an incremental trend of +18% over 8 years, or +2.25% yearly.

Italy emerges as an intermediate-incidence country also when considering data disaggregated for the sole cholangiocarcinoma (CC), with figures that fall under the definition of rare malignancy (less than 6 cases/100.000 inhabitants/year) but are increasing both for the intrahepatic and the extrahepatic sites [Figures 2, 3, and 4]. Among the most recent international epidemiology literature[1,8,9], the most comprehensive, granular and exhaustive analysis of CC incidence and temporal trends can be found in the International Association of Cancer Research (IARC)'s Cancer Incidence in Five Continents[9]. Mortality from CC is also on the rise[8] [Figure 5].

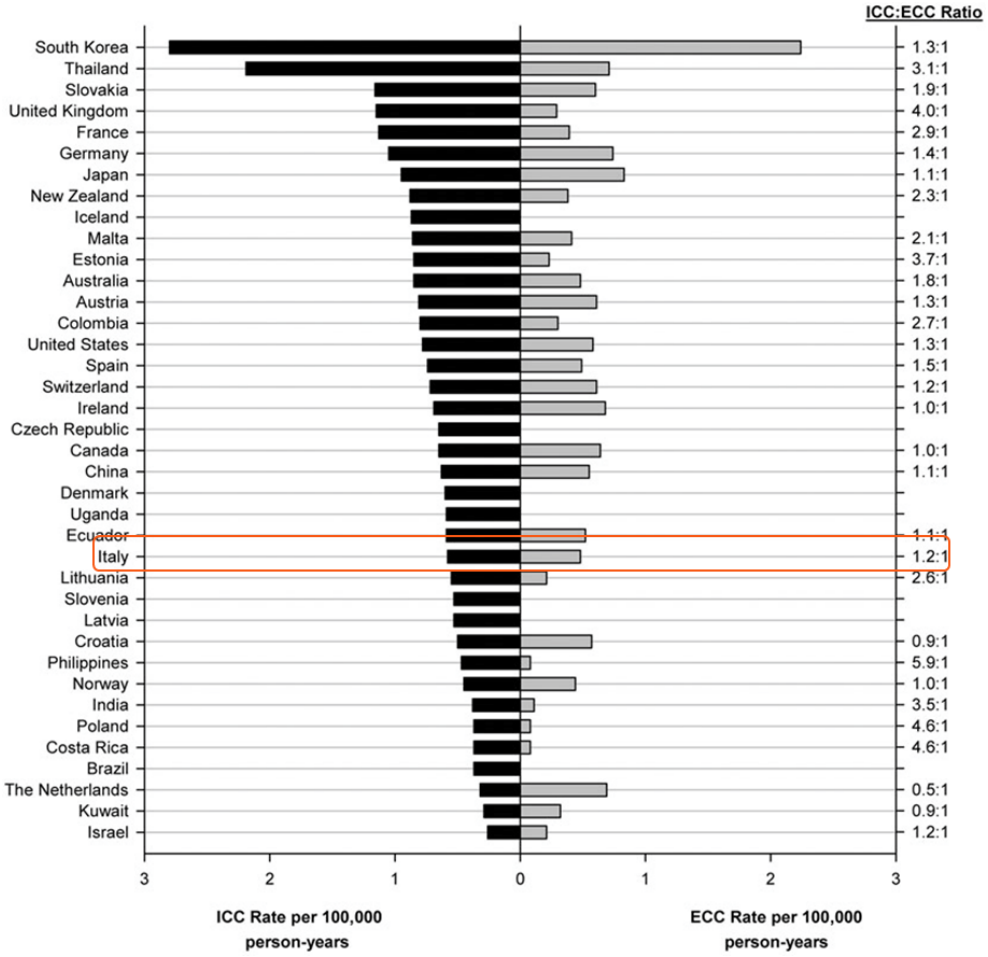

Figure 2. *CC incidence*[9].

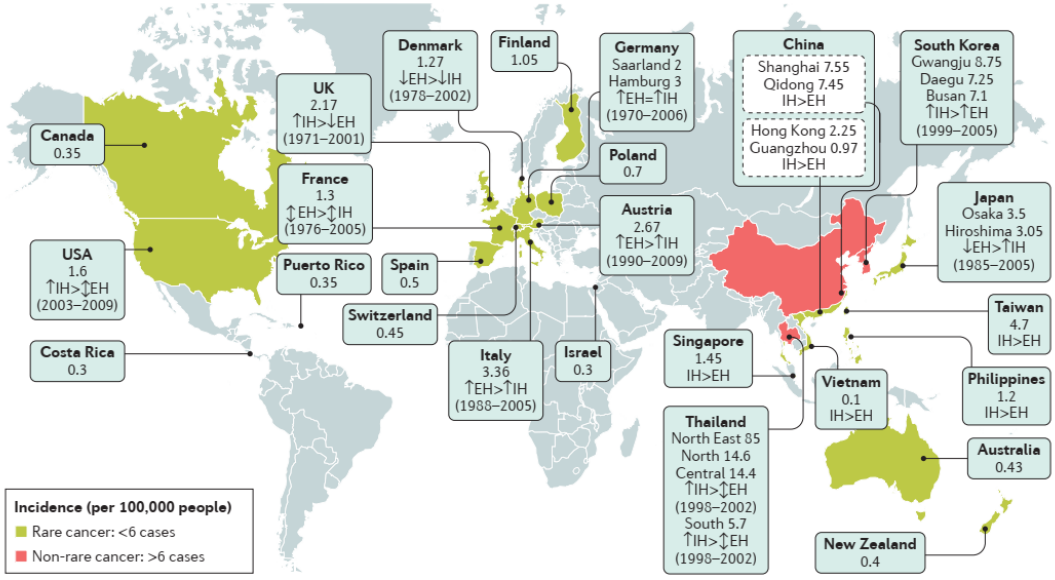

Figure 3. *CC incidence and trends*[1].

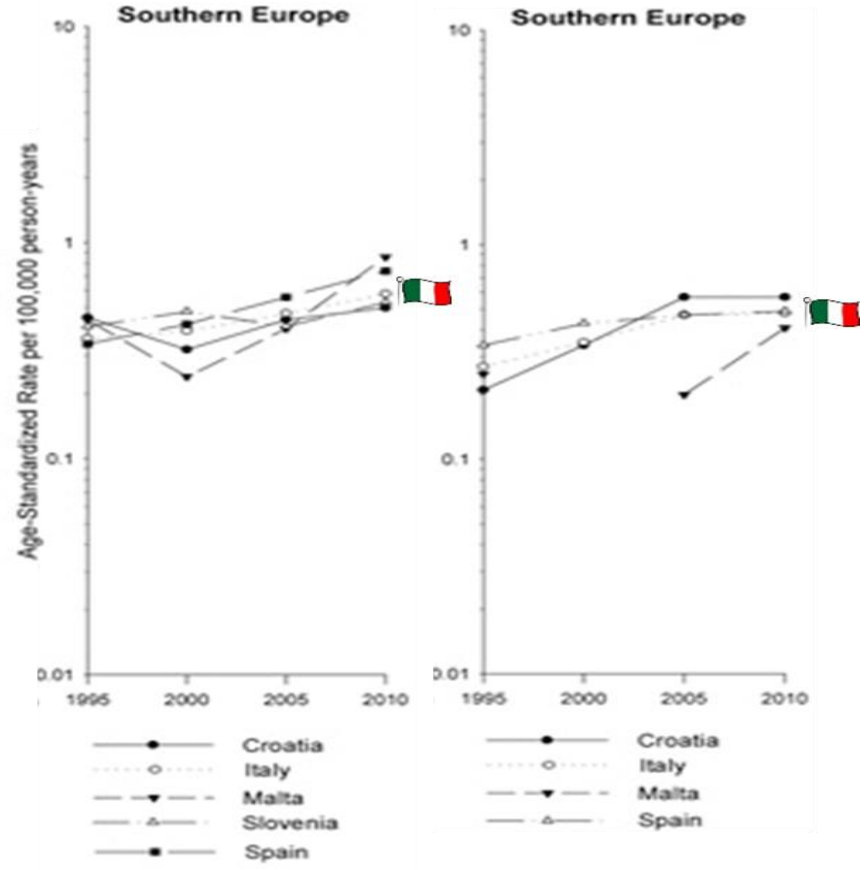

Figure 4. *Age-adjusted incidence trends of ICC (left) and ECC (right), re-elaborated from "Global trends in intrahepatic end extrahepatic cholangiocarcinoma incidence from 1993 to 2012"*[9].

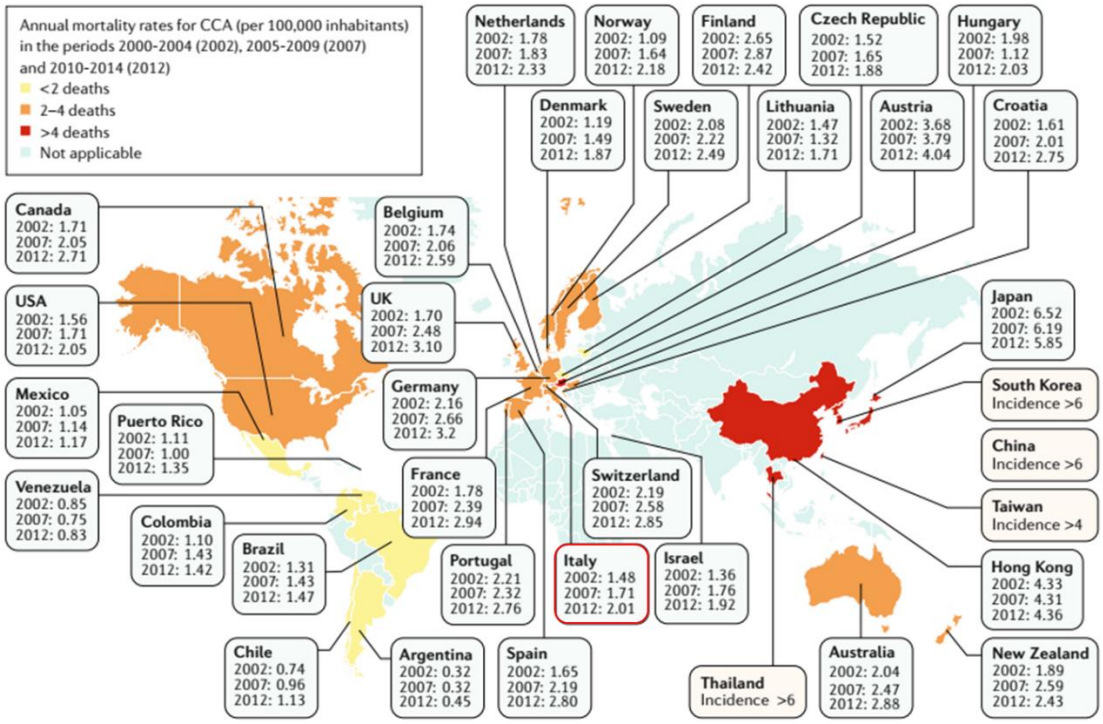

Figure 5. *Mortality of CC*[8].

Difficulties in interpretation of epidemiological data

The interpretation of CC epidemiology, including the rise in incidence, is not straightforward, as multiple confounders need to be taken into account.

1) Merging with other BTC sites. This is particularly relevant, as it prevents to obtain direct information from Italian tumor registries. They do not document CC cases *per se*: rather, they provide aggregated data with GC[6]. As seen, data that are granular for ICC and ECC ought to be derived from international studies, which instead apply this distinction.

2) Misclassification of ICC as HCC in clinical diagnoses or in the coding according to World Health Organization (WHO) International Codification of Diseases (ICD) classification system.

3) Misclassification of ICC as cancer of unknown primary (CUP). Both neoplasm can have similar clinical presentation (liver masses with or without lymphnode involvement), and histopathology; there is lack of ICC-specific diagnostic markers; the diagnosis of CUP (and, sometimes, of ICC) is frequently a diagnosis of exclusion. Indeed, one-fifth of CUPs are revealed of biliary origin upon molecular profiling[10]. A United States registry analysis over 18 years' time observed that the increase of new ICC diagnoses was paralleled by a decrease in new CUP cases. The authors argued that, given the higher steepness of the decreasing curve of CUP diagnoses, the rise of ICC could not be entirely attributed to the better diagnostics of CUP, i.e. that the phenomenon is "real" and not (only) a statistical artifact due to less misclassification as CUP[11].

4) The evolving updates of the ICD-O classification. Predominantly applied in Italy, WHO's ICD has unique codes for ECC, and ICC (as well as GC and AC). Conversely, IARC elaborated a separated codification system dedicated to oncologic disease, ICD-O, predominantly employed in the United States, that consists of two classification subsystems, which together describe the neoplasm: a topographic code (anatomical site) and a morphologic code (histology). The ICD-O-1 version (1979) did not even include a morphology or a histology code for pECC. In the ICD-O-2 version (1992) a specific histology code for pECC was elaborated but this was mapped as an ICC. Only in ICD-O-3 version (2001) the histology code for pECC could be mapped to either ICC or ECC[12]. Consequently, it is regarded that this could have resulted in an overestimation of ICC diagnoses at least until 2001, and unfortunately the present classification can still induce

the clinician or epidemiologist to miscode pECC as ICC. While these timepoints seem far away in time, we should remember that epidemiological trends can often be identified only over long time spans, hence the need for studies that encompass several years. Indeed, the miscoding of pECC as ICC could be a conspicuous confounder: a multicentric retrospective review of 625 cases from hepatobiliary tertiary care centers in United Kingdom revealed that 34% of ICC cases were actually pECC, and that 92% of all pECC were incorrectly coded as ICC[13]. As an additional consequence comes the need for a refined dissection of ICD-O codes in registry studies: for instance, in the mentioned United States registry study, the combined analysis of topography and histology codes was adopted to identify ICC and ECC cases. Interestingly, application of a sensitivity analysis with this new partition resulted in an observed higher age-adjusted incidence of ICC and lower for ECC, and did not affect the temporal trends of neither ICC or ECC, thus further corroborating the hypothesis of a "real", and not artefactual, increase[11]. Of this study it is also relevant the finding that, while the order of magnitude of the increase of ICC incidence was 150% over 18 years, ECC incidence remained stable[11]. If miscoding is a numerically relevant confounding factor, then "sifting out" the ICC data from pECC should reduce ICC incidence but magnify the ICC increasing trend. Indeed, a registry reclassification of all German Klatskin-Altemeier tumors to ECC reinforced the annual percentage change for ICC both in women (from +3.3% to +4.8%) and in men (from +3.8% to 4.8%)[14].

To summarize, for various reasons the exact, real incidence of CC primary sites is difficult to obtain; nevertheless, it is increasingly clear that, particularly for the intrahepatic primary site, it is increasing. Part of our work is the exploration of the causes that sustain this rising incidence of CC.

1.2. Etiology

Etiology of CC has long been matter of debate: long-time recognized, even historical (such as, Thorium-based contrast enhancers), risk contributors characterized by high strength of association but very low prevalence in Western countries (e.g. trematodes infestations, and primary biliary cirrhosis) can explain only a limited number of cases. On the other end of the spectrum, some conditions that are largely prevalent in the population (e.g. metabolic syndrome) seem to exhibit a weak correlation with CC

development, and their global etiologic contribution is unclear. In the middle, a plurality of patients develop CC in the absence of any apparent predisposing factor; these cases are therefore termed sporadic or cryptogenic.

Unfortunately, studies on CC etiology must overcome intrinsic difficulties: while observational studies are tainted by patient selection stemming from the retrospective nature, and by the small numbers of this rare disease, registry studies retain an unavoidable degree of inaccuracy (more on these limitations in par 2.1). Methodological issues render generally difficult a neat interpretation of the results. Nevertheless, this promising research line is accumulating a growing evidence corpus, that for some risk factors reached the meta-analytic level. In turn, meta-analyses too suffer from limitations. First, they are not conducted at a patient level. Second, the included studies are heterogeneous for design, and for exposure definitions: for instance, the presence of type-II diabetes mellitus can be variably derived from patients medications, medical records, from blood glucose blood work according to WHO criteria, or from the occurrence in patients-associated ICD codes. Third, in a context of small sample sizes, a numerical imbalance can be generated with the inclusion of just one large, registry study. Fourth, the over-representation of certain geographical areas (the Far East), and clinical settings limits the generalizability of the conclusions.

Metabolic disorders

Metabolic syndrome refers to the clustering of insulin resistance, visceral adiposity, atherogenic dyslipidemia, and arterial hypertension. These conditions, of which the first two appear to be crucial to the syndrome, are interrelated, and share underlying mediators, mechanisms, and pathways. Other phenomena often co-occur but are inessential to the syndrome: systemic inflammation, blood hypercoagulability, microalbuminuria[15], hepatic steatosis, hyperuricemia. Different scientific societies proposed sets of diagnostic criteria, but an attempt toward a shared definition dates back only to 2009[16]. Nowadays, falling into the definition requires three of five of the following :

-elevated waist circumference: population- and country-specific cutpoints (>102 cm in males, >88 cm in females for Mediterranean and Middle Easterners; >94 cm in males, >80 cm in females for European and North Americans[17];

-elevated triglycerides (TG): ≥150 mg/dl, or drug treatment for hypertriglyceridemia;

-reduced high-density lipoprotein (HDL)-cholesterol: <40 mg/dl in males, <50 mg/dl in females, or drug treatment for reduced HDL-cholesterol;
-elevated blood pressure: systolic ≥130 mmHg, diastolic ≥85 mmHg, or antihypertensive drug treatment in a patient with a history of arterial hypertension;
-elevated fasting glucose: ≥100 mg/dl, or drug treatment of elevate glucose. Initially investigated as a predisposing factor to cardiovascular disease and type-2 diabetes mellitus, metabolic syndrome is now considered a renowned risk factor for a variety of cancers, including pancreatic and "liver" cancers[18].
Currently, a correlative study between metabolic syndrome and CC onset is not available. However, associations were identified at a meta-analytic level for type-2 diabetes mellitus (odds ratio [OR] 1.73, 95%-confidence interval [$CI_{95\%}$] 1.47-2.04, for ICC, and 1.50, $CI_{95\%}$ 1.31-1.71 for ECC); arterial hypertension and obesity (again, an example of high inter-study heterogeneity of definitions) showed no correlation[19]. It is estimated (2020-'21) that 4.7% ($CI_{95\%}$ 4.4-4.9) of the Italian population suffers from type-2 diabetes mellitus[20], whereas, from 2018-'19 national data, 19.5% and 45.4% of men, and 22.7 and 27.8% of women suffer from obesity or excess weight according to the WHO definitions[21].

Liver disease

Strongly associated with obesity and metabolic syndrome, and a proven independent risk factor for type-2 diabetes mellitus and hepatic cirrhosis is non-alcoholic fatty liver disease (NAFLD). This umbrella term encompasses a spectrum of liver disease, that go from the simple hepatic steatosis to non-alcoholic steatohepatitis (NASH), which may progress to fibrosis and cirrhosis[22]. Up to 30% of the general population could be affected by NAFLD[23], and up to 5% of patients with NAFLD developed liver cirrhosis during a 8-year follow-up[24]. In multiple meta-analyses, NAFLD showed an association with ICC (ORs 2.19-2.22) and, with liminal significance, with ECC (ORs 1.48-1.55)[19,25]. It is increasingly recognized that in the NAFLD spectrum the real epidemiological role is played by the subgroup of NASH cases: although the mechanism is not well studied, tumor promotion in NASH occurs by direct means of the vicious cycle of inflammation, or indirectly through the induction of liver cirrhosis with its incessant cycles of tissue destruction and repair. NASH was highly prevalent in a multicentric ICC series (22.5%), and NASH-related ICC entailed shorter OS, regardless of liver fibrosis[26]. If some

molecular mediators of carcinogenesis appear aberrantly expressed in NAFLD[27,28], the future specific etiologic research needs to dissect NASH from NAFLD[26].

Liver cirrhosis represents the final stage of the natural history of a variety of liver conditions characterized by chronic inflammation, from NAFLD to chronic viral hepatitis infection, from cholestatic hepatopathies to alcoholic chronic injury. Liver cirrhosis shows one of the highest strength of association with ICC (OR 15.3), and to a lesser extent with ECC (OR 3.8)[19].

Lithiasis

Lithiasic disease is frequently reported during the diagnostic phases of CC, and it is associated at all anatomical levels of development (choledocholithiasis, cholecystolithiasis, hepatolithiasis, cholelithiasis) both with ICC (ORs 1.7-10.1) and ECC (ORs 2.9-18.6). Gallstones, along with the consequent chronic organ inflammation (cholecystitis), also represents the main risk factor for GC[29].

Bile ducts malformations or malfunctions

Caroli disease, choledochal cysts, primary biliary cholangitis, primary sclerosing cholangitis are all congenital conditions that manifest themselves with a progressive malfunction and inflammation of the bile ducts. Given the duration and the importance of the inflammation due to cholestasis, these conditions are strongest predictors of CC development: in primary sclerosing cholangitis the ORs range from 34 for dECC to 93 for ICC and 453 for pECC[30]; ORs in choledochal cysts carriers are 26.7 for ICC and 34.9 for ECC[19].

Asbestos

Exposure to asbestos is a putative risk factor supported by some interesting preliminary evidence. Asbestos fibers were found in gallbladder and bile ducts of BTC patients[31]: via ingestion or inhalation, asbestos fibers are presumed to make their way beyond the pulmonary alveoli and the enterocytic intestinal mucosa. After penetrating in the circulatory system, they are carried by the bloodstream towards the first filter, which is represented by the liver. Here they reside and accumulate in the canals of Hering where they cause formation of oxidative species, as well as direct DNA and cell membrane damage on cholangiocytes, with chronic inflammation. Exposure to asbestos may induce a specific genotype in ICC (*IDH1* and *BAP1* mutations). Indeed, the scarce available

literature provides a coherent evidence of a correlation with ICC (ORs up to 4) but not with ECC, a dose-response effect, and a potential explanation for sporadic cases, as 40% of ICC patients without any known risk factors resulted possibly or probably exposed to asbestos[32].

Chemical cholangiocarcinogenesis

As the liver exerts both the so-called first-passage effect for ingested substances, and the biological functions of metabolism and detoxification of xenobiotics, certain chemicals, accumulating in the liver, can induce tumorigenesis locally.

Among them, 1-2-dichloropropane is worth a mention as its tumorigenic potential is a recent acquisition, made possible by the observation of a cluster of CC cases among Japanese workers in printing industry, where 1,2-dichloropropane was employed as organic oil solvent. The relationship between exposure and risk of CC is now proven, and today 1,2-dichloropropane is listed by IARC among class-1 carcinogens[33–36].

Ethanol consumption is another known risk factor for both ICC (OR 3.1), and ECC (OR 1.7), however the definition of exposure is heterogeneous: >80 g/day, any history of exposure, 1 day/week for >6 months, >5 g (135 ml)/day for >10 years, presence of alcohol-related liver disease or ICD9 coding[19]. Alcoholic liver disease is a potent risk factor for ICC (OR 4.5)[37], as it ultimately has a cirrhotic evolution. Ethanol could also exert a direct local carcinogenic effect, being metabolized by cytochrome P450 isoform 2E1 to acetaldehyde. On the one hand, extensive metabolization facilitated by the enzymatic induction of the microsomal cytochrome-based enzymes can increase the intracellular levels of reactive oxygen species, with DNA damage and lipid peroxidation. On the other hand, acetaldehyde exerts direct mutagenic and tumorigenic effects[38].

Tobacco carcinogens are metabolized in the liver[39], and several constituents of tobacco smoke (e.g. 2-acetylaminofluorene, and 4-aminobiphenil) were identified as hepatocarcinogens[40], supposedly acting as tumorigenesis initiators. One proposed mechanism is the formation of DNA adducts, as levels of polycyclic aromatic hydrocarbon-DNA adducts and 4-aminobiphenil-DNA adducts were found at higher concentrations in HCC tissue than in non-neoplastic liver tissue[41,42]. A smoking habit is associated with both ICC and ECC risk (ORs 1.25, and 1.69, respectively)[19]. In 2010-2013, 28% of Italians aged 18-69 years were active smokers (one out of four smoked at

least 1 pack per day), with a lower prevalence among older people (≥65 years old), only 10% of whom had a current smoking habit. During the years 2010, a long-term decreasing trend in consumption was in place, and we find smoke prevalence slightly reduced to 25.3% in the 2016-2019 period (21.9% in the age range 50-69 y.o.). The proportion of past smokers was stable at 17-18%, as it was the average number of cigarettes smoked by active consumers (around 12 per day). In more recent times (biennium 2020-2021), current smokers went down to 24.2% ($CI_{95\%}$ 23.7-24.7) of the same reference population, and former smokers to 16.7% ($CI_{95\%}$ 16.3-17.2)[43], still posing a major and acknowledged public health threat.

Infections and infestations

Chronic bile ducts inflammation can also arise from infection and infestation. Indeed, the highest incidence of CC localizes in the lower Mekong region, where it ranges from 90 to 320 cases/100.000 inhabitants/year[44,45]. The liver fluke *Opistorchis Viverrini*, endemic in this area, is a foodborne helminth spread by ingestion of infected cyprinid fish, a frequent ingredient in the traditional local cuisine, and is associated with CC development, as it resides in the bile ducts of the infested host[46,47]. *O. Viverrini*-associated tumorigenesis relies on multiple pathways: direct damage to the biliary epithelium causes a perpetual activation of the wounds repair mechanisms; inflammation determines a direct paracrine release of reactive species of oxygen and of nitric oxide; in addition, fluke-secreted proteins exert direct induction of cell proliferation and inhibition of DNA repair and apoptosis. These pathways converge in creating multiple genetic lesions, which are fixed along cell replications, eventually leading to malignant transformation of cholangiocytes and tumor promotion[47,48].

O. Viverrini is not the sole cholangiocarcinogenic parasite: infestations by *Clonorchis sinensis* and *Schistosoma haematobium* increase the risk of CC development, and both share with the former the IARC classification as group-1 carcinogens[49,50]. Although their respective habitats include Europe, *Opistorchis felineus*[51] and *Salmonella typhi*[52] tumorigenic potential and relevance as etiologic factors for CC and GC, respectively, are less studied. Among viral infections, hepatotropic Epstein-Barr virus infection is another example of an understudied putative risk factor, although its genome could be found in 6% of ICC DNA samples in a large Chinese case series[53]. In the following paragraph, we review in detail the role played by viral hepatitis (HBV and HCV) in CC development.

1.2.1. Role of viral hepatitis

Epidemiology

Viral hepatitis is an infection that causes inflammation of the liver. Being caused by different viruses, the focus is traditionally directed to HBV and HCV viruses. Both can cause acute and chronic infections, which in turn are leading causes of liver cirrhosis, hepatocellular carcinoma, and CC. In spite of a reduction in incidence, HBV and HCV infections are still responsible of a global burden of 1.1 million deaths per year[54]. Based on registry data from 2015, it is estimated that HBV and HCV infections are responsible of 55% of all hepatocellular carcinoma and of 45% of all chronic liver disease/liver cirrhosis deaths in European Union (EU) and United Kingdom. Of note, this registry study did not evaluate CC as a cause of mortality from liver disease[55].

The EU's European Centre for Disease prevention and Control (ECDC) agency produces periodic reports on the incidence of hepatitis C and B, solely based on virologic and serologic laboratory findings. In these reports, data on hepatitis coming from Italy are not partitioned into acute and chronic infections, although a definition for both conditions is long-time accepted by ECDC[56], with limited variations over time[57] [Table 1].

Hepatitis B	
Acute	Detection of IgM core antigen-specific antibody (anti-HBc IgM) or Detection of hepatitis surface antigen (HBsAg) and prior negative HBV markers in the previous six months or Detection of hepatitis B nucleic acid (HBV-DNA) and prior negative HBV markers in the previous six months
Chronic	Detection of HBsAg or HBeAg or HBV-DNA and No detection of anti-HBc IgM (negative result) or Detection of HBsAg or HBeAg or HBV-DNA on two occasions that are six months apart (in the event the case was not previously reported)
Unknown	Any newly diagnosed case which cannot be classified according the above description of acute or chronic infection
Hepatitis C	

Acute	Recent HCV seroconversion (prior negative test for hepatitis C in last 12 months) or Detection of hepatitis C virus nucleic acid (HCV RNA) or hepatitis C virus core antigen (HCV-core) in serum/plasma and no detection of hepatitis C virus antibody (negative result)
Chronic	Detection of hepatitis C virus nucleic acid (HCV RNA) or hepatitis C core antigen (HCV-core) in serum/plasma in two samples taken at least 12 months apart (in the event the case was not previously reported)
Unknown	Any newly diagnosed case which cannot be classified according the above description of acute or chronic infection

Table 1. *ECDC definitions of viral hepatitis conditions[57].*

Newly reported cases of hepatitis C infection were 0.5/100.000 inhabitants/year (crude incidence, not age-standardized) in 2006[56], and then gradually declined to 0.3 cases/100.000 inhabitants per year (2017-2019), to sharply decrease to 0.1-0.05 cases/100.00 inhabitants per year during the coronavirus disease (COVID-19) pandemic (years 2020-2021)[58]. Prevalence among randomly selected individuals in metropolitan areas in 2017 was estimated at 2.3% (2.8% males, 1.9% females), with a neat cohort effect, ranging from 0.2% and 1.2% for people born after 1984 and between 1975-1984, respectively, to 7.0% and 4.2% for people born in 1935-1944 and before 1935, respectively[59]. These figures appear lower if compared to an overall HCV Ab prevalence of 5.9% ($CI_{95\%}$ 5.2-6.6), derived by a pooled analysis of 15 Italian studies (n=4826) with sampling periods ranging from 1996 to 2014, which ranked Italy at the first place in EU as for HCV prevalence[60]. Prevalence is lower in Northern Italy than the South of the country[61].

Similarly, a decreasing trend in reported new transmissions can be demonstrated for HBV infection. When restricting the analysis to the 20 EU countries that reported consistently from 2011–2020 (which exclude Italy), the rate for acute cases showed a steady decline from 0.8 cases/100.000 inhabitants in 2011 to 0.2 cases/100.000 inhabitants in 2020[57]. This long-term decrease is attributed for the most part to national hepatitis B vaccination programmes[62]. Crude incidence of hepatitis B in Italy was 2.0 cases/100.000 inhabitants per year in 2006, 0.7 cases/100.000 inhabitants per year in 2011[56], 0.7 cases/100.000 inhabitants per year in 2017, and 0.3/0.2 in the first COVID-19 years (2020-2021)[57]. In those countries that reported the disease status according to EU criteria, 7% of new cases

of year 2021 were reported as acute, 43% as chronic, 43% as "unknown" and 7% could not be classified[58]. Prevalence of HBsAg in the general population in the aforementioned pooled analysis (10 studies, with sampling periods ranging from 2001 to 2009, for a total sample size of n=3982) was 0.7% ($CI_{95\%}$ 0.4-1.0)[60].

Etiopathogenesis

A mounting corpus of evidence highlighted the link between viral hepatitis and CC. Nowadays, this accumulating literature, essentially composed of cohort studies, case-control studies, and registry studies, which were mainly performed in Eastern countries, reached the meta-analytic level [Table 2]. While the single studies may yield to conflicting, inconclusive results, meta-analyses consistently show a positive correlation between HBV and HCV infection, and the risk of CC development.

Meta-analysis	Design of included studies	Risk estimator	HCV on ICC	HCV on ICC	HBV on ECC	HCV on ECC
Zhou Y et al.[63]	Case-control, cohort	OR	3.17 (1.88-5.34)	3.42 (1.96-5.99)	not assessed	not assessed
Li M et al.[64]	Case-control, cohort	RR, OR	3.42 (2.46-4.74)	not assessed	1.68 (1.14-2.47)	not assessed
Li H et al.[65]	Case-control	OR	not assessed	3.38 (2.72-4.21)	not assessed	1.75 (1.00-3.05)
Tian T, et al.[66]	Case-control, cohort	OR	4.05 (2.78-5.90)	not assessed	1.73 (1.30-2.30)	not assessed
Zhang H et al.[67]	Case-control	OR	3.18 (2.36-4.30)	not assessed	1.41 (0.93-2.14)	not assessed
Wang Y et al.[68]	Case-control, cohort	OR	3.96 (3.05-5.15)	2.90 (2.07-4.08)	1.55 (1.25-1.92)	1.60 (1.14-2.23)
Clements J et al.[19]	Case-control	OR	4.57 (3.43-6.09)	4.28 (2.98-6.16)	2.11 (1.64-2.73)	1.98 (1.33-2.94)

Table 2. *Meta-analyses on HBV/HCV epidemiology in CC.*

The underlying mechanisms of HBV and HCV infections in the development of ICC have not yet been fully elucidated.

It is likely that the chronic inflammation of biliary epithelia play a primary role in cholangiocarcinogenesis through the mechanisms of reactive oxygen species-induced DNA damage, and of repeated cycles of cell death and regeneration with increased cell turnover[69–71].

Integration of HBV DNA into human genome may also contribute to cholangiocarcinogenesis, as several HBV DNA integration sites can be detected in ICC

and in the mixed cholangiocellular-hepatocellular carcinoma samples, with integration events preferentially recurring in specific regions which may affect gene expression and regulation in cells, such as the human telomerase reverse transcriptase (*TERT*) gene.

The HBx protein, encoded by the HBV *X* gene, is widely recognized as a primary causal factor in hepatocarcinogenesis[72–75] by a number of mechanisms: proto-oncogene upregulation (*c-Jun*, *c-myc*)[73,76] and of transcription factors (AP-1, NF-κB)[77,78], activation of MAPK/ERK, stress-activated protein kinase/Jun N-terminal kinase, and PKC signaling pathways[78], and binding of p53[79]. This ultimately results in cell cycle disruption, DNA repair impairment, inhibition of apoptosis. HBx is frequently expressed in ICC and the surrounding liver tissue[75,80]. HBx expression correlates to a significantly higher prevalence of elevated serum alpha-fetoprotein (AFP)[80]. One proposed additional mechanism of action for HBx in ICC concerns the transcriptional expression of *TERT*[81].

Similarly to HBx, the HCV core protein may be involved in the tumorigenic process, promoting the cellular proliferation of hilar cholangiocarcinoma cells, inhibiting apoptosis[82] and inducing epithelial-mesenchymal transition in ICC cell lines[83].

1.3. Biological and molecular pathogenesis

Pathogenesis models

The liver is an organ with an important regenerative capacity, and contains precursors cells with a double potential, in that they can originate hepatocytes or cholangiocytes, which in turn are subject to malignant transformation into HCC and CC. In support of the hypothesis of the cancer stem cell[84], histopathology and genetic profiling allow to identify two entities with mixed characteristic, the hepatocellular-cholangiocarcinoma, supposed to derive from the bipotent precursor cell[85], and the cholangiocarcinoma-like hepatocellular cholangiocarcinoma[86]. In fact, depending on the differentiation status reached before the maturation arrest, a heterogeneous phenotype spectrum can be observed.

An alternative hypothesis for biliary cancerogenesis is the clonal evolution model, a multistep process of tumor development from precancerous lesions to the invasive carcinoma, driven by the progressive accumulation of genetic and epigenetic alterations, in the context of a chronic inflammation[87]. Indeed, the finding of the biliary intraepithelial

neoplasia (BilIN), a known precursor of the invasive form, is frequent in ECC surgical specimens. BilIN-associated neoplasms show less invasiveness and dedifferentiation, but possibily faster proliferation kinetics[88]. In the traditional model of cholangiocarcinogenesis, tumor promotion occurs against a backdrop of cholestasis and/or chronic biliary inflammation: the increased cell turnover and the abundant cytokine release induce the accumulation of mutations and the proliferation of mutated cells. For instance, the activation-induced histidine deaminase, an enzyme that is involved in nucleic acids synthesis and exerts a mutagenic effect, inflammatory cytokines and is found at high concentrations in cholangiocarcinoma cells, where it catalyzes mutations to *MYC*, *TP53* and $p16^{INK4a}$ genes[89].

On the other hand, the oncogenic process can occur also in the absence of inflammation. Two molecular classes can be identified in ICC[90], characterized by distinct genomic profiles and clinical behavior. The inflammatory class (38% of the cases) is characterized by the activation of the inflammatory pathways, the abundant production of cytokines (IL-6, IL-10, IL-17), and the hyperactivation of STAT3. In the proliferation class (62%), the mitogen pathways (such as, RAS, MAPK, Met) are overactive, and outcomes are poorer.

Gallbladder carcinogenesis, depending upon similar mechanism to those sustaining cholangiocarcinogenesis, involves cytokines, TGFbeta, mitogen pathways, and loss of tumor suppressor genes (such as, TP53, and RaSSF1A) and cell cycle regulators (such as, $p21^{Waf1}$ and $p27^{Kip1}$). The multi-step clonal evolution is proposed as the predominant histogenetic model: dysplasia - carcinoma in situ – invasive adenocarcinoma[91]. This hypothesis is supported both by the frequent finding of dysplastic areas in proximity of tumor tissue[92] and by the co-occurrence of the same or similar mutations in the dysplastic and cancerous epithelia[93]. An alternative histogenetic sequence, based on the transition adenoma-carcinoma, seems plausible only for a minority of cases[94].

Actionable genomic alterations

The molecular heterogeneity of BTC constitutes a big obstacle to the development of efficient target therapy. The application of the most recent sequencing techniques to BTC brought to light an intricate genomic and transcriptomic landscape, unveiling a considerable molecular complexity. Against this backdrop, biological differences

emerged among primary sites[95,96] and within each single primary site[90,97], and eventually oncogenic pathways of pharmacological interest could be identified[98,99], mostly for ICC.

Isocitrate dehydrogenase (IDH, isoforms 1 and 2) is physiologically involved in the cell energetic metabolism. Point mutations at hotspot codons 132 and 172 are gain-of-function: mutated IDH1/2 aberrantly synthesizes 2-hydroxyglutarate, an oncometabolite that promotes epigenetic changes[100]. Disrupting this mechanism via targeted IDH1 inhibition decreases 2-hydroxyglutarate blood levels and produces morphological and molecular changes in CC tissue, increasing the expression of liver-related genes[101].

Fibroblast-derived growth factor receptor 2 (FGFR2) is a receptor tyrosine kinase with downstream pathways that include cell proliferation and angiogenesis. FGFR2 gene rearrangements code for constitutively activated fusion products, that result in aberrant mitogenic signalling. FGFR-altered disease exhibits a more indolent clinical course[102]. More rarely, activating gene fusions can involve other receptor tyrosine kinases, of note NTRK, ROS1, ALK[103–105].

An aberrant epidermal growth factor receptor (EGFR) signalling, which can occur via protein overexpression or mutation, induces cell proliferation and tumor progression[106,107]. The interference with EGFR signalling in aBTC, alone or in combination with chemotherapy (CT), repeatedly proved an unsuccessful strategy in single randomized trials[108–110], but pooled data from multiple studies suggest a certain degree of biological activity of conventional EGFR interfering agents[111]. More promising agents have a broader or different spectrum within the family of EGFR. In particular, HER-2 appears to be an interesting candidate target for molecularly-directed treatments, particularly in GC[112].

CC may also harbour targetable BRAF mutations[113] and BRCA1/2 alterations[114]. Prevalence of actionable genomic alterations are summarized in Table 3.

Molecular alteration	**Incidence**
IDH1/2 mutation	8-36% ICC, 0-7% ECC
FGFR2 fusion	6% BTC, 5.5-16% ICC
EGFR overexpression; EGFR mutation	4.5-8% BTC; 13.5-15% BTC
HER2 overexpression	11.5-28% AC, 15.5-19% GC, 5-17.5% ECC, 5% ICC

BRAF point mutation	up to 22% CC
PIK3CA mutation	7-12.5% GC, 4-8% ICC, 4% ECC, 5% BTC
NTRK fusion	3.5% ICC
ROS1 rearrangement; FIG-ROS1 fusion	1.4% ICC; 14% GBC, 16% ECC
BRCA1/2 alteration	11% GC, 5% ECC, 5-8% ICC

Table 3. *Actionable genomic alterations in BTC. Re-elaboration from Filippi et al[115]. Specific references in the publication.*

1.4. Principles of treatment of the advanced disease

For the purpose of the present work it is useful to review in brief how aBTC is currently treated, what are the expected outcomes, and the main avenues of evolution in sight[115].

Chemotherapy

The standard treatment of the aBTC, which consists of locally advanced unresectable cases, initially metastatic disease, locoregional and distant recurrences after surgery, is systemic CT, namely the doublet with a platinoid (oxaliplatin, cisplatin) and gemcitabine. After preliminary encouraging results in the ABC-01 trial[116], the combination of gemcitabine and cisplatin (GemCis) became the regimen of choice based on the benefit showed in the Western phase-III trial ABC-02, in which 410 patients with aBTC were randomized to receive GemCis (gemcitabine 1000 mg/m^2 + cisplatin 25 mg/m^2 days 1,8/21) or gemcitabine monotherapy. The combination CT yielded a significant benefit in terms of disease control rate (DCR) (81.4% vs 71.8% in the control arm; p 0.049), progression-free survival (PFS) (8 months vs 5, p<0.001), and overall survival (OS) (11.7 months vs 8.1, p<0.001; HR 0.64, $CI_{95\%}$ 0.52-0.80), with no increase in toxicity, save for neutropenia[4]. Similar gains in favor of GemCis were independently observed in the Japanese randomized phase-II trial BT-22[117], that showed a PFS of 5.8 months vs 3.7 with gemcitabine monotherapy, and an OS of 11.2 months versus 7.7. In patients with tolerability concerns and/or contraindications to cisplatin, oxaliplatin can serve as an accepted alternative agent (GemOx regimen), based on single-arm trials achieving numerically similar results (i.e., OS ranging from 8.3 to 12.4 months)[108–110,118]. In Eastern countries only, the fluoropyrimidine mix S-1 (tegafur, gimeracil, oteracil) is utilized to avoid platinoid-related toxicity, as the gemcitabine-S-1 combination proved non-inferior

to the standard gemcitabine-platinum doublet[119]. Similarly, the XelOx regimen (capecitabine, oxaliplatin) showed non-inferiority to gemcitabine-oxaliplatin in a Korean trial[120]. Gemcitabine monotherapy maintains a role for those patients unfit for a doublet regimen, frail and/or with Eastern Cooperative Oncology Group (ECOG) performance status (PS) ≥2[121].

On the other edge of the spectrum, strategies for treatment intensification in highly fit patients are a matter of scientific debate. The triplet gemcitabine-cisplatin-S-1 demonstrated a moderate benefit in an Eastern population (OS 13.5 months vs 12.6 with GemCis, HR 0.79), with no significant differences in toxicity[122]. Conversely, three recent randomized trials yielded disappointing results, with a global lack of benefit observed from the addition of nab-paclitaxel to GemCis[123], and from the introduction of first-line modified FOLFIRINOX (oxaliplatin, irinotecan, and 5-fluorouracyl)[124].

Options for second-line treatment after progression to first-line chemotherapy include the FOLFOX and FOLFIRI regimens[125–127].

Target-therapies

Progress in the identification of the driver molecular alterations in BTC (see par 1.4) ushered in new therapeutic scenarios, envisaging a larger therapeutic armamentarium. R132-mutated IDH1 inhibitor Ivosidenib achieved an OS benefit in CT-refractory, R132-mutated IDH1 positive aBTC patients in the ClarIDHy trial (n=187, phase-III trial, randomized against placebo, cross-over at progressive disease [PD] permitted): 7.5 months vs 5.1, p 0.0001[128]. This molecule received the European Medicine Agency (EMA) regulatory approval for the treatment of pre-treated R132-mutated IDH1 positive CC in February 2023, but it is not yet approved or reimbursed in Italy. Resistance mechanisms to ivosidenib that are developed under the selective pressure operated by the drug are being elucidated[129]. A second-generation IDH1-inhibitor, LY3410738, able to interact with different enzimatic sites than ivosidenib, showed preliminary efficacy[130].

FGFR gene fusions are the most frequent actionable targets in CC. Specific inhibitors (pemigatinib, infigratinib, erdafitinib) are under phase-III evaluation[131] after promising phase-II testing. For instance, in the FIGHT-202 trial, conducted on 107 pretreated

patients whose CC carried FGFR gene fusions, permigatinib achieved a RR of 36%, 1-year PFS rate of 29%, and 1-year OS rate of 68%[132].

The phase-II basket ROAR trial[133] analyzed activity and tolerability of the combination of dabrafenib and trametinib (a BRAF and a MEK inhibitor, respectively) in a number of neoplasms characterized by the BRAF V600E point mutations. This trial enrolled 43 patients whose primary site was biliary: these patients experienced an OS of 13.5 months ($CI_{95\%}$ 10.4-17.6) and a response rate (RR) of 51% ($CI_{95\%}$ 36-67).

While trastuzumab never entered the clinical routine for HER2-amplified aBTC (mostly, GC), last-generations molecules will likely represent the future of this clinical niche. In the phase-II basket DESTINY-Pantumor02 trial the antibody-drug conjugate trastuzumab-deruxtecan obtained a 22% RR among the 42 patients with pretreated biliary primary (56% in immunohistochemistry 3+/3+ cases)[134]. Zanidatamab, a bi-specific monoclonal antibody targeting two distinct HER2 epitopes, yielded a RR of 41% in a similar population in the HERIZON-BTC-01 trial[135], and similar figures (RR 46.7%) were obtained by the combination of tucatinib and trastuzumab in a smaller Japanese trial[136].

Conversely, the relatively old research line on the pharmacological inhibition of EGFR[108,109,118] and VEGF/VEGFR[137–139] pathways achieved only marginal results in clinics, so that EGFR inhibition and antineoangiogenic treatment never made into the therapeutic armamentarium.

Immunotherapy

After the notable, sometimes revolutionary, results in other neoplasms, only recently immunocheckpoint inhibitors (ICIs) showed evidence of efficacy in unselected aBTC patients. The addition of durvalumab (TOPAZ-1 trial)[140] or pembrolizumab (KEYNOTE-966 trial)[141] to the standard GemCis prolonged OS (HRs of 0.76, and 0.83, respectively). In the small subset of aBTC characterized by high microsatellite instability (MSI-H)/mismatch repair deficit, ICI showed a noteworthy efficacy, in line with what observed for other MSI-H primaries[142–144]. Pembrolizumab received the EMA regulatory approval for the treatment of a variety of MSI-H tumors, agnostic to the primary site.

1.4.1. Locoregional treatments for post-surgical relapse

The therapeutic decision making process of some recurrences of BTC may benefit of a discussion in a multidisciplinary team (MDT). Among them, low-burden recurrent disease in fit patients can theoretically be subject to locoregional treatments (LRTs). A second resection may be forgone due to fears of the physical and psychological burden deriving from a surgical intervention. Most of the available evidence regards ICC, whereas other primaries are less studied. While retrospectively identified differences in OS between cases of relapsing ICC that were surgically treated vs those who underwent CT (such as, 29.2 vs 11.8 months, p 0.003 in Ohira et al)[145,146] only carry little practical value, given the intrinsic selection of these patients, the absolute figures of OS reveal this strategy as a promising research field: these patients bear a *per se* good prognosis. In this context, we previously demonstrated an interesting PFS for LRTs (mainly surgery)[2].

The largest meta-analysis available collected 366 clinical histories of recurrent ICC treated with surgery, and reported 1-y, 3-y, and 5-y OS rates of 87% ($CI_{95\%}$ 81%-91%), 58% ($CI_{95\%}$ 48%-68%) and 39% ($CI_{95\%}$ 29%-50%), respectively[147]. Patient selection should rely on practical criteria, such as patient's comorbidities and global fitness, the availability of residual liver parenchyma in locoregional relapses, the disease-free survival (DFS) from the first resection, as well as the radicality obtained with the first resection and the carbohydrate antigen 19.9 (Ca19.9) blood levels. Indeed, DFS from first resection appears to influence the success of resection of the recurrent disease in ICC: in a conspicuous retrospective series of ICC resected after postoperative recurrence (n=72), patients with DFS from the first resection <1 year could expect an OS substantially lower than cases recurring after 1 year[148]. The prognostic role of Ca19.9 levels and resection margin status is highlighted in a larger collection of 113 ICC clinical histories[149]. However, these studies are quite an exception: owing to the small clinical niche explored, literature in the field is mostly represented by small-size retrospective studies.

Information on relapsed ECC are scant, as their recurrences are less frequently amenable to surgery. Given the smaller numbers, ECC cases are frequently found within all-primary recurrent BTC cohorts. In a retrospective analysis of 52 recurring BTC cases (20 GC, 11 pECC, 14 dECC, 7 ICC), surgery of the recurrence was associated with a 3-y cancer-specific survival rate of 53%[150].

A variety of non-surgical LRTs may be applied to relapsed BTC: trans-arterial chemioembolization (TACE), trans-arterial radioembolization (TARE), hepatic artery infusion (HAI), percutaneous hepatic perfusion (PHP), stereotactic beam radiotherapy (SBRT), concurrent chemioradiation (CRT), and ablation with radiofrequencies (RFA) or microwaves (MWA). However, the therapeutic room for non-surgical LRTs in recurrent BTC is still matter of debate, and, apart from the obvious case of patients who are unfit for surgery, it is not clear when to offer these techniques in lieu of surgery[151].

RFA is a safe, feasible and efficacious technique for the treatment of ICC as a whole[152]. In the specific setting of recurring ICC a recent retrospective analysis described the outcomes of 64 hepatic lesions of 40 patients that were treated with RFA, experiencing an OS of 26.6 months; larger (>20 mm) lesions correlated with worse prognosis (HR: 2.77, p 0.005)[153]. A direct comparison between surgery and ablation in relapsed ICC was performed in two Chinese retrospective studies: OS was similar in the two treatment groups: 21.3-31.3 months for surgery, and 20.3-29.4 months for ablative techniques[154,155]. Major complications were less frequent with MWA than with surgery (5.3% vs 13.8%, $p<0.001$)[155].

Survival outcomes of TACE compared favorably with MWA (OS 26.9 months vs 12.0, $p<0.05$), but, again, this is too a retrospective comparison with potential for selection bias[156]. TARE and radiation therapy are even more understudied, available data being limited to the setting of locally-advanced ICC, aggregating untreated and pretreated cases. A recent Italian study reported the outcomes of 29 patients whose ICC was subject to TARE: OS among patients who were pretreated with surgery or liver-directed therapy was 14 months; low rates of post-procedural and late complications were recorded[157].

Because of the heterogeneity of study populations and methodologies, it is not possible to draw definitive conclusions on the best type of LRT for relapsed BTC.

1.5. Prognostication in the advanced disease

Against a backdrop of a severe prognosis, a certain degree of variability can be observed in the natural history of aBTC patients undergoing systemic CT. In around 10% of patients OS exceeds two years[2]. Several prognostic factors were proposed, that could be immediately available to the clinician. The pertaining literature is abundant, and for some

of them it was possible to perform meta-analyses, that more solidly confirmed their roles. This is the case, for instance, of the derived parameters platelet-to-lymphocyte ratio (PLR)[158,159] and neutrophil-to-lymphocyte ratio (NLR)[160,161], both these indicators representing a consolidated proxy of the systemic inflammation that accompanies several neoplasms, including BTC. The inflammation status can also be measured with C-reactive protein (CRP)[162], the modified Glasgow Prognostic Score (mGPS)[163] and the Systemic Inflammation Index (SII)[164].

As a plurality of BTC patients develops liver metastases and ultimately deteriorates due to liver impairment (cholestasis, cholangitis, hepatic insufficiency), residual organ function, measured with albumin or other derived indicators, appears a promising prognostic parameter[165–167] which was incorporated in at least one prognostic model[168].

Estimation of PS, generally performed according to the ECOG scale or the Karnofski Index, is still one of the strongest, immediate tool available to the clinician to rapidly assess the patient condition, which heavily affects his resilience towards CT side effects and disease symptoms. Large studies confirmed its strong prognostic role in aBTC undergoing medical treatment[169–172].

Circulating carcinoembryonic antigen (CEA) and Ca19.9 are routinely used as tumor markers in clinical practice to monitor the disease response, along with seriated imaging. Being expression of the quantity of tumor cells, these two molecules can serve the function of indicator of the tumor burden, and their prognostic role is recognized[169,170,173]. Less studied in BTC is a lactate dehydrogenase (LDH)[174].

A number of prognostic models were devised to predict prognosis in aBTC, in form of scores[168,170,172,175] or indexes[162,164,176], that classify the population in risk groups (usually, three: good, intermediate, poor prognosis), or nomograms[3,175], that specifically estimate the individual residual survival.

2. Rationale and objectives

As presented in Chapter 1, CC represents the most interesting BTC subsite for an etiology investigation, but BTC as a whole is a rare and understudied disease. Gaps in basic knowledge of the disease are frequent, numerous questions await answers, and preliminary hypotheses need confirmation. In particular, if different causes underlie a different biology, then it is reasonable to expect signals of distinct clinical courses across etiologies. Hence, the need for a close interconnection for the study of etiology and prognosis in BTC.

Objective n°1: a prospective, systematic assessment of CC etiology landscape.

The available literature concerning CC etiology is mostly composed of studies that are variably flawed by one or more of the following limitations. Retrospective nature comes with the issues of not *ad hoc* data collection, missing data, and lack of pre-planned statistical design; small sample size can entail difficulties in finding meaningful and statistically significant differences; monocentricity can lead to a lack of generalizability of the conclusions. Patient selection, either explicit (e.g. a surgical cohort) or intrinsic, can lead to confounding ramifications difficult to identify. Furthermore, most studies are being directed towards a single condition, which can fail to capture the impact of other, perhaps co-segregating, comorbidities. In addition to these methodological limitations, a diverse range of confounding biases (e.g. lead-time bias, [un]healthy-user bias), inherent with retrospective collections, may further complicate the interpretation and the validity of the results. Upon these considerations, as our contribution to this research line we proposed the BI-CAUSE study, a prospective, multi-center, observational trial, directed to a wide range of conditions, which appeared the most efficient and methodologically sound way of addressing the mentioned issues.

Objective n°2: a comprehensive evaluation of viral hepatitis causative, prognostic, and predictive roles.

For its shifting epidemiology, the well-established biological alterations, and its public health implications, viral hepatitis emerges as a topic of increasingly recognized relevance in CC prevention. We sought to replicate the specific results from the BI-CAUSE trial, utilizing the extensive retrospective cohort of the BICC study. We hypothesized that, relying on the strengths of multicentricity and large numerosity, this approach, while not being the ideal design, could still generate sufficiently solid

conclusions for our purpose: investigating on a more extensive sample the role and the impact on CC of viral hepatitis in Western countries. In fact, while the biological role of hepatotropic viruses infection has been elucidated in preclinical studies, clinical-epidemiological literature is mainly based on series from Eastern countries, characterized by higher prevalence of both viral hepatitis and BTC, particularly ICC, while European cohorts are typically under-represented. Indeed, in the recent pooled analysis by Clements et al[19], which only included case-control studies, only one European study[177] was included (five from the US), contributing with only 26 out of over 14.000 cases. Similarly, the same small case-control trial represented the only European study in Zhou's, Li M's, Li H's and Wang's meta-analyses[63–65,68]. Another limitation is represented by the heterogeneity of the definition of HBV infection. Most of the case-control studies found in literature only evaluated HBsAg, thus failing to discriminate between chronic and recent, acute hepatitis, as well as missing both acute, resolved hepatitis and chronic cases with reduced HBsAg levels below threshold of detection (the so-called "HBc-alone" infections). Indeed, at least two case-control studies report a significant association for HBcAb in the absence of a HBsAg positivity[178,179]. Scant and conflicting literature is currently available as for the prognostic and predictive roles of viral hepatitis.

Objective n°3: development of a useful and reasoned prognostic model for aBTC patients. However, prognostication, i.e. the practice of estimating *ex ante* the survival trajectory of a patient or a group of patients with similar demographic and disease characteristics, is a broader exercise, that combines a variety of parameter to elaborate a prognostic models. As mentioned in par. 1.4, a few prognostic models for aBTC already exist. However, from a methodological standpoint, some papers lack a measurement of validity (c-statistics, AUC)[162,176], and some would not provide an external validation of their findings[3,173,176]. More importantly, clinical applicability to aBTC is questionable when the training populations contain patients candidate to best supportive care[170,173] or resected (and never recurred) cases[3]; likewise, monocentricity[172] could reflect the clinical practice of a single center and limit the generalizability of the model. Some models were derived from specific primary sites[173,175]. Other models incorporate only few aspects of the tumor-host interaction (for instance, Du et al elaborated a prognostic model from only CEA and dNLR)[173]. But, most of all, some models lack clinical usefulness: there is no point, for instance, for a three-tier model to partition patients in prognostic groups that are almost equal in size, such as Park et al did in one of the seminal attempts to modelling in aBTC[176]:

some groups need to be maximized and others to be kept as low as possible according to the clinical needs (more on these considerations in par. 4.3). In the presented work we sought to devise a practical tool, that took into account multiple different spheres of the tumor-host interaction, and resulted useful to accurately discriminate patients in a clinically reasoned way.

Objective n°4: exploring treatment intensification for BTC post-surgical relapse: outcomes and safety.

Even if prognostication has a direct application to the clinics, as a medical oncologist I aim to figure out new solutions for BTC patients, which means 1) to identify and characterize an unmet clinical need, and 2) to find an answer. As explained in subpar. 1.4.1, BTC post-operative recurrence amenable to LRTs represents a small fraction of relapsed cases, endowed with a favorable prognosis. As a consequence, literature in the field mostly consists of small-size retrospective studies. For instance, in the meta-analysis by Ramouz et al[147], only six of the 28 included studies had a sample size ≥10. The rarity of the condition is also the main reason why a large prospective trial with comparative intent is unlikely to be conducted in the future, therefore the evidence able to inform therapeutic decisions will still originate from retrospective studies. Unfortunately, as of today, heterogeneity in study populations and methodologies renders impossible to draw definitive conclusions on the best LRT for relapsed BTC. We therefore aimed to contribute to the existing literature with further retrospective evidence from an extensive Western cohort.

3. Prospective, systematic assessment of CC etiology landscape

3.1. Patients and methods

The BI-CAUSE trial (Biliary cancer in Italy: a study on Cholangiocarcinoma cAUSEs and risk factors) is an observational cohort study, aimed at investigating a comprehensive range of established and putative risk factors of CC. This study is based on a multi-institutional, prospective design. The study has been conducted and coordinated at Istituto di Ricovero e Cura a Carattere Scientifico di Candiolo (leading center), and recruited patients from a total of 7 participating centres in Italy. The study conformed to the principles set forth in "Helsinki Declaration" (64th WMA General Assembly, Fortaleza, Brazil, October 2013) and to the Good Clinical Practice. Ethical approval was received from the leading center and from the local ethics committees of the participating centers. Enrolment in the BI-CAUSE study required the voluntary signing of a written, dated informed consent form by the patients. Data were collected on CRFs and stored in a database in anonymized fashion.

Eligible patients were considered all patients who received a histologic diagnosis of CC (ICC or ECC), and received systemic CT. Patients who had already undergone a systemic treatment at the moment of enrolment in BI-CAUSE trial would not be included in the survival analysis, but only in the characterization of the patient's risk landscape.

The "Anamnesi fisiologica" CRF contained fields about: date and number of local enrolment; previous systemic treatment; birthdate, sex, ethnic group; height and body weight; blood group; smoking habit: past vs present, quantity, duration; drinking habit: quality (wine, beer or hard liquors) and quantity; trips and sojourns to Asian countries.
The "Anamnesi patologica" CRF contained fields about:
- established or putative CC risk factors, namely: viral hepatitis B and C, type I and type II diabetes mellitus, dyslipidemia, cholecystectomy, cholelithiasis, choledocolithiasis, hepatolithiasis, cholangitis, chronic cholecistitis, chronic pancreatitis, ulcerative colitis, Crohn's disease, primary sclerosis cholangitis, primary biliary cirrhosis, thyroideal abnormalities, primary hemochromatosis, Wilson's disease, *porphyria cutanea tarda*, *porphyria cutanea intermittens*, hepatobiliary malformations, familiar history of bile duct cancer, personal history of neoplasia and past CT treatments;

- the source of the information: orally reported by the patient vs documental evidence (e.g.: medical reports displayed by the patient, written records of radiological/laboratory findings, etc.) or disease under treatment (e.g. use of oral hypoglycemic drugs for type II diabetes mellitus).

The "Caratteristiche di malattia" CRF contained fields about: date of histological diagnosis; ECOG PS; clinical presentation: abdominal pain, subjective sensation of abdominal weight, objective abdominal mass/hepatomegaly, jaundice/rise of laboratory markers of cholestasis, cholangitis, ascites, weight loss; primary site (ICC, dECC, pECC); histological type as per the WHO classification; non-neoplastic liver appearance; tumor grading; tumor staging according to Union for International Cancer Control (UICC)/ American Joint Committee on Cancer (AJCC) TNM staging classification, 7th edition; for advanced disease only: metastasis pattern.

The "Analisi di laboratorio" CRF contained fields about: white blood cells count (WBC), neutrophils, lymphocytes, hemoglobin (Hb), platelets count; humoral inflammation: CRP; liver damage, cholestasis and residual hepatic function: aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (GGT), prothrombin time international normalized ratio (PT INR), total proteins, albumin, total and fractionated bilirubin; residual renal function: creatinine; metabolic conditions: total cholesterol, low-density lipoprotein (LDL), HDL, TG, serum glucose, thyroid-stimulating hormone, glycated hemoglobin (HbA1c); iron metabolism: iron, transferrin; Anti-Mitochondrial Antibodies (AMA), positive in 90% of primary biliary cirrhosis; tumor markers: Ca19.9, CEA; blood type.

The "Epatite virale" CRF contained fields about:

- first-line laboratory screening in patients with either a negative anamnesis for hepatic viral infections or a positive one for past acute hepatitis, namely HBsAg, IgG anti-HBc and HCV Ab.
- anamnestic or serological positivity to viral hepatitis infection would lead to different paths of laboratory exams (precisely defined in the form) in order to differentiate and ascertain conditions like inactive chronic carrier, chronic hepatitis, "HBc-alone" chronic hepatitis, pre-core mutant, hepatitis delta concurrent infection.

The "Storia naturale" CRF contained fields about: surgery and adjuvant therapy; subsequent treatments: number of lines, drugs, number of cycles for each line; date of death or loss to follow-up.

Data analysis consisted of two separate tasks (Task-1, Task-2).

Task-1 was the characterization of the etiologic landscape. Its core focused on identifying and weighing risk factors in the study population. The present study not being a case-control study, a comparison vs a control CC-free population was not envisaged. However, the prevalence data still retain a descriptive value, useful in indirect confrontations with prevalence data in general population taken from literature and statistic databases.

Task-2 analysis was a further evaluation of the correlation of the above described, taken-at-diagnosis data with outcomes (OS, PFS, response).

Statistical analyses were performed with advanced biostatistics softwares, namely IBM SPSS. The significance level of comparisons performed (type-1 error, α) was set at values 5%, hence 95%-confidence intervals were calculated, and p values < 0.05 (or lower) were considered as statistically significant. Bonferroni correction was applied when appropriate.

Data analysis employed derivative values and standardization procedures including:

- Body Mass Index (BMI) in Kg/m^2. Stratification relied on WHO "International Classification of adult underweight, overweight and obesity according to BMI" definitions of overweight (BMI ≥25.00) and obesity (BMI ≥30.00);
- alcohol consumption was standardized in alcohol units (UA, 1 UA = 8 grams of ethanol) per day. Definitions of above-moderate drinkers (>2 UA/day for females, >4 UA/day for males)[180], and heavy drinkers (>5 UA/day for females, >10 UA/day for males[181,182]; and/or history of alcoholism) were derived from commonly accepted thresholds.
- smoking habit intensity and duration was annotated as number of cigarettes/day; cumulative exposure was expressed in pack-years, and on its basis patients were categorized as light, moderate, and heavy smokers (1-19, 19-39, ≥40 pack-years, respectively)[183].
- a patient was deemed positive for dyslipidemia in case of occurrence of the condition in his/her medical history (as reported in the study interview) and/or if under the specific medical treatment (as reported in the study interview) and/or in case of laboratory alterations of the bloodwork required for the study, namely, HDL <40 md/dl (males) / 50 mg/dl (females), and/or TG >150 mg/dl, and/or total cholesterol 200 mg/dl;
- a patient was deemed positive for type-2 diabetes mellitus in case of occurrence of the condition in his/her medical history (as reported in the study interview) and/or if under the specific medical treatment (as reported in the study interview) and/or in case of

laboratory alterations of the bloodwork required for the study, namely HbA1c >48 mmol/mol.

OS and PFS were defined as the time from the first cycle of systemic treatment of the advanced disease to death, and to death or evidence of progressive disease, respectively. In case of loss to follow-up or survival event not occurred at the time of the data analysis, the case was censored at the last follow-up date in the survival curves. RR and DCR were employed as derivative parameters from response descriptors as defined in the RECIST v.1.1 criteria[184].

3.2. Results

Study population

A total of 151 cases of CC were collected. Median age was 66.3 years; males represented 54.3% of the population. The most represented primary was ICC (58.3%), followed by dECC (24.5%) and pECC (14.6%) [Table 4].

Age (y), median [IQR]	66.3 [58.5-73.6]
Sex, n (%)	
Male	82 (54.3)
Female	69 (45.7)
Primary site, n (%)	
ICC	88 (58.3)
ECC	59 (39.1)
- pECC	22 (14.6)
- dECC	37 (24.5)
Not attributable	4 (2.6)

Table 4. *General characteristics of the accrued population (n=151). IQR, interquartile range.*

Risk factors

Lifestyle habits were investigated first. Smoking and alcohol drinking were largely prevalent in the study population, with no statistical differences observed between primary sites, save for a lower proportion of past smokers (31.0% vs 54.2%, p 0.006) but

a more frequent active habit (23.0% vs 8.5%, p 0.026) among ICC patients vs ECC patients. Data are presented in detail in Table 5.

Smoking habit				
	Total sample (n=150)	**ICC (n=87)**	**ECC (n=59)**	***p***
Never smoker	64 (42.7)	40 (46.0)	22 (37.3)	0.312
Ever smoker	86 (57.3)	47 (54.0)	37 (62.7)	
- past smoker	59 (39.3)	27 (31.0)	32 (54.2)	0.006
- current smoker	25 (16.7)	20 (23.0)	5 (8.5)	0.026
Not attributable	1 (0.7)	1 (1.1)	0 (0.0)	-
Cumulative exposition among ever smokers				
	Total sample (n=86)	**ICC (n=47)**	**ECC (n=37)**	***p***
Light smoker (1-19 py)	36 (41.9)	20 (42.6)	15 (40.5)	0.481
Moderate smoker (20-39 py)	16 (18.6)	7 (14.9)	8 (21.6)	
Heavy (≥40 py)	27 (31.4)	16 (34.0)	11 (29.7)	
Not attributable	7 (8.1)	4 (8.5)	3 (8.1)	-
Alcohol intake				
	Total sample (n=151)	**ICC (n=87)**	**ECC (n=59)**	***p***
Astemious	43 (28.5)	28 (32.2)	14 (23.7)	0.449
Drinkers	100 (66.2)	56 (64.4)	40 (67.8)	
- above-moderate drinkers	35 (23.2)	21 (24.1)	14 (23.7)	0.999
- heavy drinkers	35 (6.0)	6 (6.9)	3 (5.1)	0.740
Not attributable	9 (5.3)	3 (3.4)	5 (8.5)	-

Table 5. *Smoking and drinking habits. Values expressed in n (%). ICC and ECC subgroups do not add up perfectly to the total sample owing to few cases non categorized for primary site. Comparisons between primary sites (Fisher's exact test) were calculated among cases with an attributable smoking/drinking habit.*

The median BMI in good health was 24.8 Kg/m^2; 47.2% had excess weight, and 15.5% were obese. Globally, 16.9% of patients had an anamnestic or laboratory finding of type-II diabetes mellitus. While 14.8% of patients had a history of dyslipidemia or was under treatment for the condition, a further 29.6% had at least one parameter out of range (low HDL, elevated TG, elevated total cholesterol). Two thirds of the population had at least one of these three features of metabolic syndrome (excess weight, type-II diabetes mellitus, and dyslipidemia), and one third had at least two [Table 6].

BMI (Kg/m^2), median [IQR]	24.8 [22.8-28.4]
Excess weight (BMI >25 Kg/m^2)	67 (47.2)
Obesity (BMI >30 Kg/m^2)	22 (15.5)
Type-II diabetes mellitus	24 (16.9)
- history and/or medication	20 (14.1)
- laboratory*	4 (2.8)
Dislipidemia	63 (44.4)
- history and/or medication	21 (14.8)
- laboratory**	42 (29.6)
1 feature	49 (34.5)
2 features	39 (27.5)
3 features	9 (6.3)
At least 1 feature	97 (68.3)
At least 2 features	48 (33.8)

Table 6. *Metabolic syndrome features (n=150, save for BMI distribution analysis, where n=142). Values expressed in n (%), unless otherwise specified. Considered features: excess weight, type-II diabetes mellitus, dyslipidemia. *HbA1c >48 mmol/mol, in the absence of history or specific drug treatment.**HDL <40 md/dl (males) / 50 mg/dl (females) and/or TG >150 mg/dl and/or total cholesterol 200 mg/d, in the absence of history or specific drug treatment.*

Bile ducts lithiasis (cholelithiasis, choledocolithiasis, prior cholecistectomy) and chronic inflammation (cholecystitis, cholangitis) was reported in a substantial minority

of patients, with no appreciable differences between primary sites [Table 7]. No cases of hepatolithiasis, prior pancreatitic episodes, or chronic pancreatitis were reported.

	Total sample	**ICC**	**ECC**	***p***
Lithiasis	28 (18.7)	14 (16.1)	12 (20.3)	0.512
Inflammation	10 (6.7)	5 (5.7)	5 (8.5)	0.526
Lithiasis and/or inflammation	33 (22.0)	17 (19.5)	14 (23.7)	0.544

Table 7. *Bile ducts lithiasic and inflammatory pathology. Values expressed in n (%). ICC and ECC subgroups do not add up perfectly to the total sample owing to few cases non categorized for primary site.*

The results of the screening for viral hepatitis (HBsAg, HBcAb, HCV Ab) identified a low prevalence of HBsAg; a trend towards a more frequent HBcAb positivity among ICC patients (26.7% vs 13.1 among ECC patients, p 0.061); and a statistically higher prevalence of HCV Ab in ICC (7.0 vs 0.0 in ECC subgroup, p 0.036) [Table 8]. When the screening serology was integrated with medical history from the study interview and the second-level laboratory exams (HBV DNA, HCV RNA, HBsAg, HBeAb, HDV Ab), categories were derived for past acute hepatitis B, chronic hepatitis B, and hepatitis C. Higher prevalence in ICC patients vs ECC patients could be demonstrated for both hepatitis C (six cases, all among ICC patients, of which only one confirmed to have been pharmacologically eradicated) (p 0.036), and resolved acute hepatitis B (p 0.015), which peaked at 26.7% in ICC patients. Chronic hepatitis B remained an infrequent finding in both groups [Table 9]. Four patients, including the one with eradicated infection, had both hepatitis C and past, acute hepatitis B; in one patient chronic hepatitis B and hepatitis C infections concurred.

	Total sample (n=149)	**ICC (n=86)**	**ECC (n=59)**	***p***
HBsAg				
Positive	4 (2.7)	1 (1.2)	3 (5.1)	0.305
Negative	133 (89.3)	78 (90.7)	52 (88.1)	
na	12 (8.1)	7 (8.1)	4 (6.8)	-

HBcAb				
Positive	31 (20.8)	23 (26.7)	8 (13.6)	0.061
Negative	100 (67.1)	53 (61.6)	44 (74.6)	
na	18 (12.1)	10 (11.6)	7 (11.9)	-
HCV Ab				
Positive	6 (4.0)	6 (7.0)	0 (0.0)	0.036
Negative	119 (79.9)	64 (74.4)	53 (89.8)	
na	24 (16.1)	16 (18.6)	6 (10.2)	-

Table 8. *Viral hepatitis serology. Values expressed in n (%). ICC and ECC subgroups do not add up perfectly to the total sample owing to few cases non categorized for primary site. Comparisons between primary sites (Fisher's exact test) were calculated among cases with an available serology. Na, not available.*

	Evaluable patients (total sample/ICC/ECC)	**Cases**			
		Total sample	**ICC**	**ECC**	***p***
Past acute hepatitis B	132 / 77 / 52	26 (19.7)	21 (27.3)	5 (9.6)	0.015
Chronic hepatitis B	137 / 79 / 55	5 (3.6)	2 (2.5)	3 (5.5)	0.400
Hepatitis C	127 / 71 / 54	6 (4.7)	6 (8.5)	0 (0.0)	0.036

Table 9. *Viral hepatitis conditions. Values expressed in n (%). ICC and ECC subgroups do not add up perfectly to the total sample owing to few cases non categorized for primary site. Comparisons between primary sites (Fisher's exact test) were calculated among cases with an attributable positive or negative status.*

Among rare causes of CC, no cases of primary biliary cholangitis were observed, although one patient, without any evident risk factor for ICC, tested positive for AMA. One ICC had a hepatic background of secondary hemochromatosis, whereas no cases of primary sclerosing cholangitis, Wilson disease, or porphyria were detected. Two ICC patients had a concurrent inflammatory bowel disease (ulcerative colitis, unconfirmed Crohn's disease, respectively). One pECC arised in an asymptomatic incomplete *pancreas divisum*; one ICC arised in a Caroli disease, already subject to left hepatectomy and bilio-digestive anastomosis on the right hepatic duct six years before the cancer diagnosis; one ICC was a malignant transformation of a hepatic cyst, known for eleven years before the cancer diagnosis.

Prognostic factors

After excluding those patients who already were receiving oncologic treatment at the moment of the inclusion in the study, and those who would never reach oncologic care, ultimately receiving sole best supportive care, 87 clinical histories were evaluable for prognostic analysis. Median PFS to first-line CT (CT1) was 7.4 months ($CI_{95\%}$ 4.6-10.2); median OS 10.4 months ($CI_{95\%}$ 5.1-15.7), not significantly different between primary sites. The only variable that showed a prognostic value was hypoalbuminemia for OS, and, with marginal significance, PFS [Table 10].

	PFS HR ($CI_{95\%}$)	**p**	**OS HR ($CI_{95\%}$)**	**p**
Age: <65 y.o vs ≥65 y.o	0.71 (0.22-2.26)	0.564	0.49 (0.15-1.60)	0.239
ECOG PS: 0-1 vs 2	-	-	0.12 (0.01-1.38)	0.090
Primary site: ICC vs ECC	1.40 (0.42-4.62)	0.584	1.61 (0.53-4.91)	0.400
BMI: <30 Kg/m^2 vs ≥30 Kg/m^2	0.33 (0.04-2.66)	0.297	1.00 (0.22-4.59)	0.998
Hypoalbuminemia: yes vs no	4.27 (0.93-19.52)	0.061	3.66 (1.06-12.6)	0.040
Anemia: yes vs no	1.70 (0.51-5.65)	0.387	1.95 (0.60-6.30)	0.266
NLR: <3.0 vs ≥3.0	-	-	0.27 (0.03-2.41)	0.268
PLR: <median value vs ≥median value	0.58 (0.16-2.11)	0.409	0.40 (0.12-1.30)	0.128
Viral hepatitis: yes vs no	2.59 (0.71-9.47)	0.150	0.80 (0.17-3.74)	0.780

Table 10. *Univariate analysis.*

3.3. Discussion

The BI-CAUSE trial is the first prospective etiologic study on CC, covering a broad range of putative contributors. This represents an innovative contribution to the relevant literature, chiefly composed of retrospective observations. A variegated landscape emerged, based on intertwined features of dysmetabolism, lithiasis, chronic inflammation, and viral hepatitis, whereas, even when considered together, rare causes did not represent a meaningful proportion of cases. Globally considered, this study further circumscribed the proportion of truly idiopathic cases. In particular, the large proportion of HBcAb-positive cases in a low-incidence country like Italy, underlines the role of resolved hepatitis B, in accordance with the hypothesis of mutagenesis and HBx

expression from viral DNA integration, as described in subpar 1.2.1. On the contrary, the etiologic weight of chronic, often cirrhosis-related, hepatitis B in the present population was marginal. Smoking habit was frequent, as compared to the figures (reported in par 1.2) of the Italian population, even more so considering an age range similar to our pool of cases. Our data suggest smoke as a major, under-recognized, etiologic contributor, particularly to ICC.

Restricting the cases eligible to prognostic analysis to the actual new diagnoses, which was intended to minimize selection and lead-time bias, unfortunately led to decreased sample size. Missing data further reduced the available pool for analysis, so that only one variable (hypoalbuminemia) emerged as a significant prognostic determinant, for OS, and marginally for PFS. Analysis on larger samples is clearly the way toward meaningful findings.

In particular, among the other, non-statistically significant factors (each having already shown a prognostic value in at least one of the existing studies), one was striking, even if not reaching the threshold for statistical significance. The presence of hepatitis was correlated to a pejorative trend for PFS (p 0.15), and a null-effect on OS with a largely non-significant HR of 0.80. Unique among the variables tested on univariate analysis, this discordance may suggest an ambivalent role for hepatitis-related CC: in other words, on larger sample sizes hepatitis could be a demonstrated a favorable or null prognostic factor, while resulting an unfavorable predictive factor of response/efficacy from CT1. Intriguingly, could viral hepatitis result in slowly growing, chemo-resistant CC cases? Considering that the literature -again, predominantly Eastern-based- comes to all the three logically possible conclusions (positive[185], negative[186,187], and null[188] impact of hepatitis on advanced CC survival), and that hepatitis-related CC is sometimes considered a distinct entity[189], the hypothesis is worth a deeper look. Chapter 3 is dedicated to explore the impact of viral hepatitis.

4. Comprehensive evaluation of viral hepatitis causative, prognostic, and predictive roles in intrahepatic cholangiocarcinoma

4.1. Patients and methods

The BICC (Biliary tract cancer in Italy - a Comprehensive Characterization) study is a multipurpose collaborative effort consisting in an extensive retrospective dataset of aBTC cases which underwent CT1 in various centers adhering to the Gruppo Italiano Colangiocarcinoma Onlus (GICO). On behalf of GICO, I wrote down the study protocol, produced the documentation for ethical approval, and performed the data entry from the peripheral centers, the database polishing, the control of data consistency and integrity, the generation of the queries, the database correction and maintenance.

The focus was directed on ICC cases of the BICC database, although comparisons were also drawn versus the other primary sites. Patients with insufficient treatment information were excluded.

In the BICC database hepatitis status was assessed through anamnestic collection of clinical history, and available serology (HBsAg, HBcAb, HCV Ab) and molecular biology (HBV DNA, HCV RNA) laboratory exams. Five different conditions, three for hepatitis B, and two for hepatitis C, were defined [Table 11], for which each patient could be categorized as positive or negative. Positivity for a specified hepatitis condition was defined as at least one available positive value among the considered hepatitis variables; negativity, as negative values for all the available variables. Exclusion of a case for insufficient data occurred if no variable could be assigned a value.

Condition	Variables considered	Clinical meaning
cB-L	HBsAg, HBV DNA	Laboratory findings of chronic hepatitis B
B-L	HBsAg, HBV DNA, HBcAb	Laboratory findings of hepatitis B*
B-LH	HBsAg, HBV DNA, HBcAb, clinical history of hepatitis B	Laboratory and/or anamnestic findings of hepatitis B*
C-L	HCV Ab, HCV RNA	Laboratory findings of hepatitis C#
C-LH	HCV Ab, HCV RNA, clinical history of hepatitis C	Laboratory and/or anamnestic findings of hepatitis C#

Table 11. *Hepatitis conditions. *includes chronic hepatitis B, acute resolved hepatitis B, and "core-only" chronic hepatitis B. #includes chronic hepatitis C, and acute resolved hepatitis C.*

Values recorded at the beginning of CT1 (up to two weeks before the treatment start) were collected for an extensive panel of variables, whose relation with hepatitis status was assessed. Clinical and pathology parameters consisted of age, sex, primary site, tumor grading, prior R0-R1 surgery, disease extension (locoregional, which grouped locally advanced disease and locoregional relapse; distant spread, which grouped initially metastatic disease and recurrence with metastases), metastatic sites (liver, lung, peritoneum, bone), ECOG PS. Laboratory variables included: CEA, Ca19.9, Hb, red blood cells distribution width (RDW), platelets, lymphocytes, neutrophyles, monocytes, total bilirubin, GGT, ALT, albumine, CRP. Derived indicators were also calculated: NLR (neutrophils/lymphocytes), LMR (lymphocytes/monocytes), PLR (platelets/lymphocytes), systemic inflammation index (SII, neutrophil×platelets/lymphocytes/1000), HRR (Hb/RDW), prognostic nutritional index (PNI, 10 x albumine + 0.005 x lymphocytes).

Statistical analysis

Prevalence of the mentioned hepatitis conditions was measured on the whole cohort. The differential prevalence among primary sites was also examined, and represented by means of ORs between the prevalence in ICC and prevalence in all the other sites combined.

The panel of variables was screened for differences in distribution between hepatitis-positive and hepatitis-negative cases. Continuous variables were subject to dichotomization according to cutpoints, chosen in advance as clinically meaningful (such as commonly recognized thresholds for anemia or neutrophilia) or as the median values on the entire cohort.

Time-to-progression (TTP), defined as the primary endpoint, and OS were calculated from the first cycle of CT1 to disease progression and death, respectively. Data censoring in case of lack of a disease progression event was applied: at the last follow-up in case of follow-up loss, at the last CT cycle in case of deaths deemed related to clinical progression, at the day of death occurred during treatment, at the start of second-line CT in case of switch to a different CT regimen before disease progression (e.g. for toxicities). The switch between two platinum salts in combination with gemcitabine, and the de-escalation to monotherapy, were considered as a single CT line. Dates of death were retrieved from administrative files or electronic medical records; histories lacking a date of death were censored at the last available follow-up. Both for TTP and OS, subgroups

analyses were carried out with respect to CT1 regimens and primary sites (ICC vs all other sites combined). Potential differences in prognostic factors between hepatitis-positive and hepatitis-negative cases were explored. Response assessment to CT1 employed the radiology categories of complete response, partial response, stable disease, progressive disease, and adverse event in the absence of a restaging. If imaging evidence of PD lacked, an event of clinical progression (e.g. worsening of disease-related symptoms, or general deterioration) was used as proxy of progression. RR and DCR were defined as sum of complete and partial responses, and of RR and stable disease, respectively.

The Kaplan-Meier estimator and the Cox regression model were used in the survival analysis. Comparisons between frequencies employed Chi square test. Statistical significance was considered for $p < 0.05$, or lower values in multiple comparisons according to Bonferroni correction. Median values and distributions were compared through median test and Mann-Whitney's test U, respectively. SPSS Statistic v. 20 (IBM Analytics®, Armonk, NY) was used as main statistical program.

4.2 Results

Study population

Overall, 940 clinical histories from 14 Italian medical institutions, spanning from April, 2001 to August, 2019, fulfilled the eligibility criteria. The 472 patients with ICC constituted 50.2% of the cohort. Only a minority of patients, ranging from 292 to 395 in the whole cohort, and from 139 to 194 among ICC cases, depending on the specific hepatitis condition considered, could be categorized according to the presence of the mentioned conditions.

However, the ICC populations with and without available data on hepatitis status did not significantly differ as for distribution of clinical and demographic characteristics, commonly recognized prognostic factors, CT1 regimen [Table 12], or prognosis [Table 13]. While some differences could be observed in ECOG PS, when cases with this specific variable missing where removed no significant heterogeneity emerged. Differences in distribution of CEA values, and in median levels of ALT, did not reach the statistical threshold after Bonferroni correction (p 0.01) [Table 12].

	cB-L			B-L			B-LH			C-L			C-LH		
	A	**NA**	**p**	**A**	**NA**	**p**	**A**	**NA**	**p**	**A**	**NA**	**p**	**A**	**NA**	**p**
n	183	289		185	287		194	278		139	333		149	323	
Age															
≥ 70 years	27.9	27.9	0.95	28.1	27.5	0.97	27.3	28.1	0.86	28.8	27.3	0.75	28.2	27.6	0.89
Sex															
male	57.4	53.0	0.31	57.8	52.3	0.24	58.8	51.4	0.12	58.3	52.9	0.28	59.1	52.3	0.17
ECOG PS															
0-1	82.0	85.4		82.2	84.7		82.5	84.5		77.7	86.2		78.5	86.1	
≥2	7.1	10.1	0.046 - 0.37	7.0	10.1	**0.049** - 0.33	7.2	10.1	0.09 - 0.37	8.6	9.0	**0.004** - 0.87	8.7	9.0	0.011 - 0.86
na	10.9	5.2		10.8	5.2		10.3	5.4		13.7	4.8		12.8	5.0	
Disease extension															
Locoregional	20.2	20.9		20.0	20.9		20.6	20.5		18.0	21.6		18.1	21.7	
Distant spread	78.1	79.4	0.32	78.4	78.7	0.33	77.8	79.1	0.38	79.9	78.1	0.10	79.9	78.0	0.12
na	1.6	0.3		1.6	0.3		1.5	0.4		2.2	0.3		2.0	0.3	
Prior R0-R1 surgery															
yes	25.7	18.5		25.4	18.5		25.3	18.3		21.6	21.0		23.5	20.1	
no	72.7	79.8	0.15	73.0	79.1	0.18	73.2	79.1	0.16	77.7	76.3	0.39	75.2	77.4	0.62
na	1.6	2.4		1.6	2.4		1.5	2.5		0.7	2.7		1.3	2.5	
Tumor grade*															
Low (G1-G2)	23.5	28.6		23.8	28.2		23.2	28.8		23.7	27.6		23.5	27.9	
High (G3-G4)	32.8	34.5	0.33	33.0	34.1	0.41	33.5	33.8	0.31	35.3	33.0	0.68	35.6	32.8	0.60
na or Gx	43.7	37.6		43.2	37.6		43.3	37.4		41.0	39.3		40.9	39.3	
CT1															
Gemcitabine monotherapy	26.2	19.2		25.9	19.2		25.8	19.1		26.6	19.8		26.2	19.8	
Gemcitabine-Platinum salt	61.2	63.1	0.08	61.6	62.4	0.09	61.3	62.6	0.11	62.6	61.9	0.06	61.1	62.5	0.18
Other regimens	12.6	18.5		12.4	18.5		12.9	18.3		10.8	18.3		12.8	17.6	
Overall survival															
median (months)	10.9	11.4	0.83	10.9	11.4	0.90	10.3	11.5	0.94	11.0	11.5	0.41	11.0	11.4	0.82
Laboratory [median, (IQR)]**															
CEA (ng/ml)	2.2 (1.4-6.3)	3.4 (1.6-11.5)	0.10, 0.03	2.2 (1.4-6.3)	3.6 (1.6-11.9)	0.06, 0.03	2.3 (1.5-6.4)	3.5 81.5-12.7)	0.063, 0.047	2.2 (1.4-5.9)	3.3 (15-9.6)	0.06, 0.08	2.4 (1.4-5.9)	3.3 (1.5-3.3)	0.08, 0.07
Ca19.9 (U/ml)	72.5 (12.2-313.4)	109.3 (24.4-787)	0.39, 0.11	72.5 (12.4-308.9)	109.3 (24.0-791.1)	0.40, 0.11	12.8 (69.0-290.0)	111.1 (24.0-833.4)	0.21, 0.08	67.9 (11.9-282.9)	24.2 (106.7-705.0)	0.14, 0.10	67.4 (12.4-282.9)	109.7 (23.6-789.0)	0.10, 0.08
Hb (g/dl)	12.9 (11.4 - 14.2)	12.6 (11.5-14.0)	0.67, 0.26	12.9 (11.4-14.2)	12.6 (11.5-14.0)	0.67, 0.26	12.8 (11.4-14.2)	12.8 (11.5-14.0)	0.97, 0.32	13.1 (11.7-14.3)	12.6 (11.4-14.0)	0.22, 0.08	13.1 (11.7-14.3)	12.6 (11.4-14.0	0.22, 0.06
NLR	3.2 (2.2-4.8)	3.4 (2.3-5.3)	0.72, 0.38	3.3 (2.3-4.5)	3.4 (2.3-5.4)	0.80, 0.42	3.2 (2.2-4.5)	3.4 (2.4-5.4)	0.58, 0.35	3.4 (2.4-4.5)	3.4 (2.3-5.3)	0.97, 0.98	3.3 (2.2-4.2)	3.4 (2.4-5.4)	0.70, 0.47
Albumine (g/dl)	3.7 (3.2 – 4.2)	3.8 (3.3-4.2)	0.28, 0.89	3.8 (3.2-4.2)	3.8 (3.3-4.2)	0.23, 0.88	3.8 (3.2-4.2)	3.8 (3.3-4.2)	0.28, 0.75	3.7 (3.2-4.1)	3.8 (3.3-4.2)	0.12, 0.49	3.7 (3.2-4.2)	3.8 (3.3-4.2)	0.26, 0.85
ALT (U/L)	29 (19-45)	34 (20-51)	0.04, 0.11	29 (18-45)	34 (20-52)	0.03, 0.07	29 (19-46)	34 (20-51)	0.04, 0.15	29 (18-45)	34 (20-52)	0.03, 0.06	29 (19-45)	33.5 (20.0-51)	0.09, 0.15

Table 12. *Characteristics of patients with and without sufficient information to be assigned a hepatitis status, according to each condition. In bold, p values below Bonferroni-corrected threshold of statistical significance for 5 comparisons (p<0.01). Categories of not available data representing less than 5% of the sample were excluded from the respective χ2 test. *p values for χ2 test excluding and including patients with not available data, respectively. ** p values for median test and Mann-Whitney test, respectively. p values for ECOG PS were calculated including (first value) and excluding (second value) cases without an available value for this variable. (N)A, information on hepatitis status (not) available; na, data not available.*

Comparison groups	cB-L	B-L	B-LH	C-L	C-LH
	Prevalence of hepatitis conditions between selected primary sites*				
pECC vs dECC	0.77	0.66	0.64	0.57	0.33
ECC vs GC and AC	0.83	0.90	0.94	0.95	0.68
	Overall survival among ICC patients**				
Hepatitis status available vs data unavailable	0.70	0.74	0.80	0.34	0.63

Table 13*. Chi square (*) and log rank (**) p values for selected comparisons.*

The absence of relevant biological and clinical heterogeneity between cases with available and cases without available information of hepatitis status being demonstrated for each and every hepatitis condition, we proceeded with the analysis of prevalence, predictive and prognostic role of hepatitis infection in ICC.

Hepatitis prevalence among ICC patients ranged from 9.3% (cB-L) to 25.3% (B-LH), and was compared with all other primary sites combined: a larger prevalence was demonstrated for cB-L, B-LH, C-L (all p values <0.05, yet not <0.01), and C-LH (p <0.0001), but statistical significance was not reached for B-L (p 0.226). Interestingly, prevalence figures of all the derived ORs were consistently in favor of ICC, peaking at 5.42 for C-LH [Table 14].

	Overall population	Population with available data on hepatitis status				
		All primaries	ICC	Other primaries	p value	OR ($CI_{95\%}$)
cB-L	2.6% (24)	6.3% (24)	9.3% (17)	3.6% (7)	0.022	2.77 (1.12-6.83)
B-L	6.9% (65)	17.0% (65)	19.5% (36)	14.8% (29)	0.226	1.39 (0.81-2.38)
B-LH	8.6% (80)	20.5% (81)	25.3% (49)	16.1% (32)	0.024	1.76 (1.07-2.90)
C-L	2.1% (20)	6.8% (20)	10.8% (15)	3.3% (5)	0.011	3.56 (1.22-1.26)
C-LH	3.5% (33)	10.9% (33)	18.1% (27)	3.9% (6)	<0.0001	5.42 (2.17-13.56)

Table 14*. Prevalence of hepatitis conditions [%, (n)] among all patients (n=935) and among patients with available hepatitis status. Differences in prevalence between intrahepatic cholangiocarcinoma and all other sites combined.*

Interestingly, very few cases of differences in distribution of the collected variables were observed between hepatitis-positive and hepatitis-negative patients. In particular, highly significant differences in immune-inflammatory activation parameters emerged only

across the partitions according to C-L and/or C-LH status: positive patients exhibited lower proportions of cases with above-median WBC, monocytes, platelets and SII. Conversely, these populations were homogeneous for the majority of other parameters, and no significant differences were observed in conditions regarding hepatitis B [Table 15].

	cB-L			B-L			B-LH			C-L			C-LH		
	-	+	p	-	+	p	-	+	p	-	+	p	-	+	p
n	166	17		149	36		145	49		124	15		122	27	
Age															
≥ 70 years	27.7 (46)	29.4 (5)		28.9 (43)	25.0 (9)		29.0 (42)	22.4 (11)		25.8 (32)	53.3 (8)		26.2 (32)	37 .0 (10)	0.26
Sex															
male	56.0 (93)	70.6 (12)	0.25	57.0 (85)	61.1 (22)	0.66	57.2 (83)	63.3 (31)	0.46	58.9 (73)	53.3 (8)	0.68	59.0 (72)	59.3 (16)	0.98
ECOG PS															
0-1	83.1 (138)	70.6 (12)		82.6 (123)	80.6 (29)		82.1 (119)	83.7 (41)		78.2 (97)	73.3 (11)		77.9 (95)	81.5 (22)	
≥2	7.8 (13)	0 (0)	0.29	8.1 (12)	2.8 (1)	0.31	8.3 (12)	4.1 (2)	0.35	8.9 (11)	6.7 (1)	0.84	9.0 (11)	7.4 (2)	0.76
Disease extension															
Locoregional	21.1 (35)	11.8 (2)	0.35	21.5 (32)	13.9 (5)	0.32	20.7 (30)	20.4 (10)	0.98	17.7 (22)	20.0 (3)	0.87	17.2 (21)	22.2 (6)	0.58
Distant spread	77.1 (128)	88.2 (15)		77.2 (115)	83.3 (30)		77.9 (113)	77.6 (38)		79.8 (99)	80.0 (12)		80.3 (98)	77.8 (21)	
Prior R0-R1 surgery															
no	72.9 (121)	70.6 (12)	0.92	71.1 (106)	80.6 (29)	0.19	71.7 (104)	77.6 (38)	0.38	79.0 (98)	66.7 (10)	0.25	78.7 (96)	59.3 (16)	0.053
yes	25.9 (43)	23.5 (4)		27.5 (41)	16.7 (6)		26.9 (39)	20.4 (10)		20.2 (25)	33.3 (5)		20.5 (25)	37 (10)	
Tumor grade*															
Low (G1-G2)	24.1 (40)	17.6 (3)	0.43	24.8 (37)	19.4 (7)	0.78	24.8 (36)	18.4 (9)	0.45	23.4 (29)	26.7 (4)	0.99	23.8 (29)	22.2 (6)	0.84
High (G3-G4)	31.9 (53)	41.2 (7)		33.6 (50)	30.6 (11)		33.1 (48)	34.7 (17)		34.7 (43)	40.0 (6)		35.2 (43)	37.0 (10)	
Metastatic sites															
Liver	47.6 (79)	58.8 (10)	0.39	46.3 (69)	55.6 (20)	0.34	46.2 (67)	53.1 (26)	0.43	52.4 (65)	46.7 (7)	0.65	52.5 (64)	48.1 (13)	0.66
Lung	14.5 (24)	11.8 (2)	0.75	14.8 (22)	16.7 (6)	0.79	15.2 (22)	14.3 (7)	0.87	16.9 (21)	6.7 (1)	0.30[a]	17.2 (21)	7.4 (2)	0.20[a]
Peritoneum	11.4 (19)	5.9 (1)	0.48	10.7 (16)	11.1 (4)	0.96[a]	11 (16)	12.2 (6)	0.83[a]	12.1 (15)	6.7 (1)	0.53[a]	12.3 (15)	7.4 (2)	0.46[a]
Bone	8.4 (14)	5.9 (1)	0.71	9.4 (14)	2.8 (1)	0.19[a]	9.7 (14)	2.0 (1)	0.08[a]	8.9 (11)	6.7 (1)	0.77[a]	8.2 (10)	11.1 (3)	0.64[a]
CT1															
Gemcitabine monotherapy	25.9 (43)	29.4 (5)	0.63	26.2 (39)	25.0 (9)	0.94	26.2 (38)	24.5 (12)	0.87	25.8 (32)	33.3 (5)	0.75[a]	26.2 (32)	25.9 (7)	0.96
Gemcitabine-Platinum salt	62.0 (103)	52.9 (9)		61.7 (92)	61.1 (22)		61.4 (89)	61.2 (30)		62.1 (77)	66.7 (10)		61.5 (75)	59.3 (16)	
Laboratory															

Neutrophilia	23.5 (39)	23.5 (4)	0.91	24.2 (36)	22.2 (8)	0.81	24.1 (35)	22.4 (11)	0.82	28.2 (35)	6.7 (1)	0.08 a	27.9 (34)	7.4 (2)	0.02 2
Lymphocytosis	1.2 (2)	0 (0)	0.65	1.3 (2)	0 (0)	0.48	1.4 (2)	0 (0)	0.41[a,]	0.8 (1)	6.7 (1)	0.05 1[a,]	0.8 (1)	3.7 (1)	0.23[a,]
Monocytosis	5.4 (9)	5.9 (1)	0.96	5.4 (8)	5.6 (2)	0.99	5.5 (8)	6.1 (3)	0.95[a]	7.3 (9)	0 (0)	0.38[a]	7.4 (9)	0 (0)	0.19[a]
Anemia	41,6 (69)	23,5 (4)	0.15	38,9 (58)	44,4 (16)	0.60	39,3 (57)	49.0 (24)	0.23	36,3 (45)	33,3 (5)	0.88	36,9 (45)	37.0 (10)	0.99
Thrombocytosis	4,2 (7)	0 (0)	0.39[a,]	4,7 (7)	0 (0)	0.19[a]	4,8 (7)	0 (0)	0.12[a]	5,6 (7)	0 (0)	0.35[a,]	5,7 (7)	0 (0)	0.20[a]
Laboratory [median, (IQR)]**															
NLR	3.2 (2.6-4.4)	3.4 (2.1-5.5)	0.57, 0.77	3.2 (2.2-4.4)	3.4 (2.4-5.1)	0.70, 0.73	3.2 (2.2-4.4)	3.2 (2.4-5.1)	0.97, 0.71	2.6 (3.5-4.7)	2.1 (1.3-2.3)	0.036, **0.001**	3.5 (2.6-4.6)	2.2 (1.5-3.5)	0.047, **0.001**
PLR	148.0 (97.6-205.3)	128.2 (77.5-164.0)	0.61, 0.21	142.0 (97.3-204.5)	132.5 (88.8-178.9)	0.99, 0.42	141.8 (96.7-204.3)	132.5 (92.1-193.6)	0.97, 0.51	150.3 (102.0-210.2)	96.1 (82.6-126.8)	0.036, 0.011	150.3 (101.4-214.5)	105.4 (90.6-146.6)	0.015, 0.011
LMR	2.4 (1.8-3.4)	2.4 (1.5-2.9)	0.78, 0.53	2.4 (1.7-3.4)	2.5 (1.9-3.1)	0.75, 0.78	2.4 (1.7-3.4)	2.6 (1.9-4.2)	0.39, 0.26	2.4 (1.7-3.3)	4.6 (3.5-5.9)	0.030, **0.002**	2.4 (1.7-3.3)	3.8 (3.2-5.4)	0.025, **0.001**
SII	734 (392-1171)	547 (352-1063)	0.29, 0.36	733 (392-1171)	678 (392-1116)	0.70, 0.70	732 (389-1170)	678 (402-1096)	0.76, 0.70	789 (484-1225)	354 (248-652)	**0.007, 0.001**	789 (483-1220)	375 (248-708)	**0.001, <0.001**
HRR	0.95 (0.78-1.10)	1.00 (0.62-1.12)	0.81, 0.90	0.96 (0.79-1.10)	0.94 (0.77-1.07)	0.76, 0.55	0.95 (0.78-1.10)	0.95 (0.78-1.09)	0.99, 0.78	0.99 (0.83-1.11)	0.82 (0.60-1.03)	0.25, 0.13	0.99 (0.83-1.11)	0.91 (0.74-1.09)	0.69, 0.33
PNI	45.0 (39.4-50.5)	45.6 (42.3-50.6)	0.97, 0.39	45.5 (39.4-51.1)	44.5 (40.4-49.5)	0.50, 0.58	45.1 (39.4-51.0)	45.3 (41.3-49.2)	0.88, 0.81	44.4 (39.6-50.3)	49.7 (44.2-55.2)	0.46, 0.10	44.6 (39.7-50.4)	48.2 (45.4-52.0)	0.025, 0.06
CEA (ng/ml)	2.4 (1.4-6.3)	1.5 (0.9-5.5)	0.58, 0.16	2.4 (1.4-6.2)	1.9 (1.4-7.0)	0.86, 0.68	2.2 (1.4-6.1)	2.4 (1.6-8.8)	0.97, 0.61	2.5 (1.5-8.0)	1.0 (1.8-3.1)	0.08, 0.04	2.5 (1.5-7.8)	2.0 (1.0-4.3)	0.26, 0.20
Ca19.9 (U/ml)	69.0 (12.2-392.5)	80.6 (11.2-127.9)	0.75, 0.60	68.5 (11.4-311.2)	86.0 (23.2-303.5)	0.63, 0.80	67.7 (11.4-290.0)	85.3 (31.0-301.4)	0.54 0.54	67.7 (11.5-279.8)	84.0 (16.4-1105.4)	0.99, 0.67	67.0 (11.4-280.5)	84.0 (24.0-350.0)	0.99, 0.51
Albumine (g/dl)	3.8 (3.2-4.2)	4.0 (3.5-4.4)	0.94, 0.19	3.8 (3.2-4.3)	36 (3.2-4.1)	0.24, 0.39	3.8 (3.2-4.3)	3.65 (3.2-4.1)	0.49, 0.57	3.6 (3.2-4.1)	3.9 (3.5-4.4)	0.42, 0.24	3.6 (3.2-4.1)	3.9 (3.6-4.3)	0.24, 0.13
ALT (U/L)	30 (19-45)	21 (18-53)	0.22, 0.32	29 (19-45)	26 (18-45)	0.52, 0.81	29 (20-46)	28 (18-55)	0.89, 0.68	29 (18-45)	29 (15-45)	0.88, 0.62	29 (18-44)	36 (19-63)	0.36, 0.23
GGT (U/L)	148 (57-313)	110 (31-196)	0.80, 0.14	139 (60-310)	147 (45-228)	0.81, 0.38	138 (59-309)	147 (50-212)	0.86, 0.44	148 (65-315)	53 (38-134)	0.08, 0.013	147 (65-305)	62 (43-185)	0.024, 0.032
CRP	5.5 (2.0-13.5)	2.0 (0.1-2.0)	0.97, 0.18	4.5 (1.9-19.6)	5.5 (2.0-7.2)	0.99, 0.73	4.7 (1.9-25.3)	5.5 (2.1-10.3)	0.99, 0.82	6.4 (1.8-25.3)	4.1 (2.4-6.2)	0.64, 0.67	6.4 (1.8-25.3)	4.1 (2.5-6.2)	0.64, 0.67

Table 15. *Characteristics of patients according to hepatitis status (negative vs positive), according to each condition. In bold, p values below Bonferroni-corrected threshold of statistical significance for 5 comparisons (p<0.01). Categories of not available data representing less than 5% of the sample were excluded from the respective χ2 test. *p values for χ2 test excluding and including patients with not available data, respectively. ** p values for median test and Mann-Whitney test, respectively. (N)A, information on hepatitis status (not) available; na, data not available.*

Prognostic relevance of hepatitis status

Median OS in the whole aBTC series was 10.3 months ($CI_{95\%}$ 9.5-11.2), and among ICC patients was 11.3 months ($CI_{95\%}$ 10.0-12.6). No heterogeneity of prognosis was observed among primary sites other than ICC (data not shown, will appear in the publication). When these cases were considered as a whole, OS appeared positively influenced by B-

L and B-LH positive status. In contrast, none of the hepatitis conditions significantly impacted OS of ICC patients [Table 16].

	aBTC			non-ICC sites			ICC		
	Median OS (pos vs neg)	**HR**	**p**	**Median OS (pos vs neg)**	**HR**	**p**	**Median OS (pos vs neg)**	**HR**	**p**
cB-L	11.8 vs 10.0	0.83	0.41	21.6 vs 9.5	0.63	0.23	11.8 vs 10.9	1.04	0.88
B-L	11.3 vs 10.0	0.82	0.18	14.3 vs 9.1	**0.49**	**0.001**	8.9 vs 11.3	1.39	0.12
B-LH	11.2 vs 10.0	0.83	0.18	14.3 vs 9.1	**0.51**	**0.009**	8.9 vs 11.3	1.28	0.17
C-L	9.5 vs 10.1	1.16	0.55	6.3 vs 9.7	1.80	0.26	9.5 vs 11.2	1.12	0.71
C-LH	11.0 vs 10.1	0.86	0.47	6.3 vs 9.7	1.70	0.30	11.0 vs 11.2	0.83	0.41
Any condition	10.2 vs 10.1	0.82	0.12	8.3 vs 14.3	**0.55**	**0.005**	9.5 vs 12.7	1.13	0.47

Table 16. *Prognostic impact of each hepatitis condition.* In **bold**, p values <0.01.

Impact on treatment efficacy

No significant differences were observed in terms of RR achieved by CT1 in ICC across the partitions for hepatitis conditions. Conversely, hepatitis-positive patients experienced a higher DCR (47.3% vs 25.0%, p 0.041), largely secondary to a significantly high DCR in C-L positive patients (87.5% vs 41.5% in negative patients, p 0.014) and C-LH patients (84.6% vs 30.0% in negative patients, p 0.001) [Table 17].

	Response			Disease control		
	-	**+**	**p**	**-**	**+**	**p**
Any condition	*3 (10.7)*	*7 (12.7)*	*0.464*	*7 (25.0)*	*26 (47.3)*	***0.041***
cB-L	15 (17.2)	2 (28.6)	0.454	39 (44.8)	3 (42.9)	0.920
B-L	9 (16.7)	2 (14.3)	0.829	22 (40.7)	6 (42.9)	0.886
B-LH	2 (6.3)	5 (21.7)	0.089	10 (31.3)	11 (47.8)	0.212
C-L	14 (21.5)	1 (12.5)	0.550	27 (41.5)	7 (87.5)	**0.014**
C-LH	3 (10.0)	3 (23.1)	0.256	9 (30.0)	11 (84.6)	**0.001**

Table 17*. Antitumor activity of CT1 in ICC according to hepatitis status. In* ***bold*** *p values <0.05.*

TTP in the whole cohort of ICC was 5.1 months with Gemcitabine-Platinum vs 2.9 with Gemcitabine (p 0.01), resulting in a HR of 0.74 ($CI_{95\%}$ 0.59-0.94). This benefit retained its statistical significance in the small subgroup of patients negative for all of the hepatitis

categories (p 0.024, n=26), as expected, but it did not among hepatitis-positive patients (p 0.41, n=50) [Figure 6].

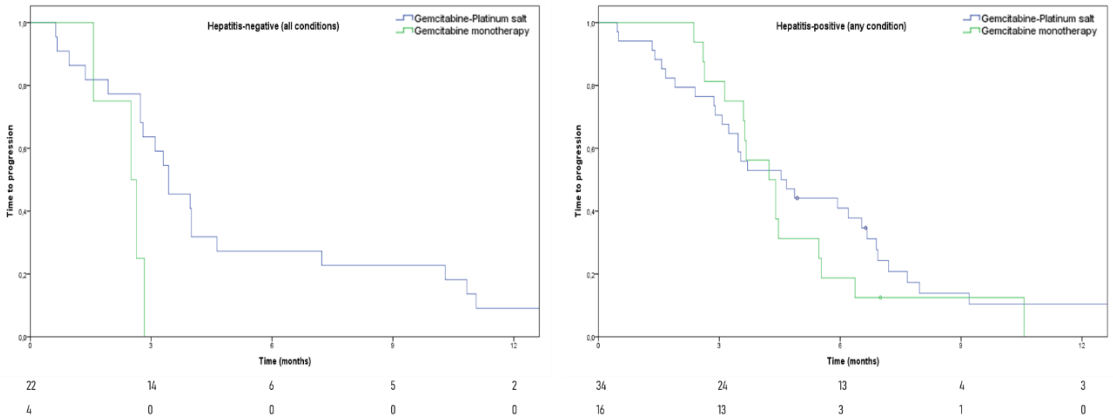

***Figure 6**. Differential impact on TTP of gemcitabine-platinoid doublets vs gemcitabine monotherapy according to hepatitis status.*

Subgroups according to hepatitis condition were tainted by small sample size; none of the performed comparisons reached the Bonferroni-corrected threshold of p<0.01 [Table 18].

	-			+		
	n (Gem-Pt; Gem)	**median TTP in months (Gem-Pt vs Gem); p**	**HR [$CI_{95\%}$]**	**n (Gem-Pt; Gem)**	**median TTP in months (Gem-Pt vs Gem); p**	**HR [$CI_{95\%}$]**
Any condition	22; 4	3.4 vs 2.5; **0.024**	0.27 [0.08-0.92]	34; 16	4.5 vs 4.2; 0.409	0.77 [0.41-1.45]
cB-L	89; 40	4.5 vs 2.7; 0.207	0.78 [0.54-1.15]	7; 5	3.5 vs 4.2; 0.989	1.01 [0.27-3.8]
B-L	55; 17	4.0 vs 2.6; 0.217	0.61 [0.26-1.40]	15; 7	3.7 vs 4.2; 0.755	0.85 [0.31-2.31]
B-LH	33; 17	4.0 vs 2.6; 0.233	0.71 [0.41-1.23]	24; 11	3.7 vs 4.2; 0.582	0.81 [0.37-1.74]
C-L	65; 29	5.6 vs 4.7; 0.387	0.53 [0.83-1.29]	8; 5	6.9 vs 4.4; **0.039**	0.20 [0.04-1.07]
C-LH	30; 8	3.4 vs 2.6; **0.044**	0.43 [0.19-1.007]	13; 7	6.9 vs 4.5; 0.537	0.74 [0.28-1.96]

***Table 18.** Differential efficacy of Gemcitabine-Platinum salt doublets (Gem-Pt) over Gemcitabine monotherapy (Gem) in ICC according to hepatitis status. In **bold** p values <0.05.*

4.3. Discussion

While gallbladder cancer and extrahepatic cholangiocarcinoma are traditionally linked to biliary lithiasis, viral hepatitis has an established biologic role in promoting carcinogenesis in ICC.

This biologic rationale justified an analysis of hepatitis significance restricted to ICC cases, which was supported by the finding, in our series, of potentially higher rates of hepatitis comorbidity in ICC cases compared to other primaries. Indeed, these data suggested that the prognostic role of HBV infection differs in ICC vs the other primary sites of aBTC. The observed favorable impact in the latter ones was an unexpected finding, which was not further explored, not being part of the study objectives. Conversely, hepatitis comorbidity did not seem to influence OS in ICC cases. However, those hepatitis conditions that correlated with longer OS in other primary sites (B-L, B-LH), potentially revealed themselves as negative prognostic impactors of ICC, exerting a negative influence to a moderate extent (HRs 1.28-1.39). However, given the lack of statistical significance (p values 0.12-0.17), this should could be very well an incidental finding, and should be regarded as hypothesis-generating at best. As such, while speculations on biologic explanations could include the negative impact of the underlying cirrhosis or an aggressive nature of hepatitis-related ICC, a confirmation is still warranted.

While providing an extensive Western case series to this corpus, which is mainly built on Eastern studies, our work also aimed to evaluate the predictive value of efficacy of CT1. In particular, to the best of my knowledge, this is the first assessment of the influence of hepatitis status specifically on the addition of platinoids to gemcitabine. With this regard, a non-significant beneficial trend could be observed consistently in the hepatitis-negative subgroups across all five of the hepatitis categories [Table 18]. Both the narrowness of observed deltas and the small sample sizes likely concurred to prevent the demonstration of statistical differences. Conversely, the benefit of the addition of platinoids to gemcitabine seemed to outright vanish among patients positive for any hepatitis condition. This could have resulted from the sum of opposite effects: a trend towards a higher efficacy in HCV patients, and a null or slightly detrimental effect in HBV cases [Table 18]. But again, although corroborated by high DCRs among HCV cases, these considerations remain at a speculation level: the statistical volatility secondary to the small subgroups sizes prevents definite conclusions.

From a statistical point of view, while being explicitly focused on observing a difference in OS and TTP, a prespecified statistical hypothesis was not considered given the retrospective nature; missing values and the mere dichotomization of continuous variables entail a loss of information, and potentially lead to misleading interpretations[190].

Other limitations of the present study need to be discussed. First, the retrospective nature of the study -common to most of the field literature- involves not-on-purpose data reporting and missing information. For instance, presence and severity of the associated liver disease, or weight loss, could not be assessed. The proportion of ICC cases that could be assigned a positive or negative value ranged from 29.4% to 41.1% according to the hepatitis category. While the composition of the assigned vs not assigned cases with respect to relevant variables did not show meaningful differences, we cannot entirely rule out an intrinsic patient selection. Even the mere data of hepatitis prevalence, albeit in line with the available literature, should be confirmed prospectively, ideally employing reasoned definitions of hepatitis that go beyond the mere finding of serologic positivity. To this regard, this is the first study in the field that used, in an exploratory fashion, five different categories for hepatitis (three for HBV and two for HCV), to better catch the variegated panorama of the hepatotropic virus infections. Indeed, in accordance with the presented preclinical observations (see subpar. 1.2.1), the present study suggested a role in colangiocarcinogenesis for past, resolved infections. This is very much in line with what observed in the BI-CAUSE trial.

In conclusion, with the presented analysis from the BICC cohort, we took one step further from the BI-CAUSE study (Chapter 2) with regard to viral hepatitis, as we observed a prognostic and predictive role that is nuanced by the specific type of hepatitis condition. The treatment of this recalcitrant disease still poses a challenge. As a medical oncologist, I complemented this research with the exploration of niches with potential for marginal gains in oncologic care efficacy. The first task, a natural evolution of the research on prognostic factors, was to put them to practical use, by combining them into a useful and reasoned prognostic tool. This research is presented in Chapter 4.

5. Development of a prognostic model for advanced biliary tract cancer patients

5.1. Patients and methods

This project was based on a multi-center retrospective analysis of the BICC database of aBTC cases, treated with at least one cycle of CT1. Patients with insufficient treatment information were excluded. The primary objective of the study was to find any variable associated with prognosis (OS) from the vast array of variables contained in the BICC database. Secondary objective was to build a reasoned prognostic model, capable to overcome the conceptual and methodologic limitations of the available models.

Data collection

Anamnestic and pathology data were retrieved, including sex, primary site, tumor grading, prior biliary stenting, and prior surgery. Inter-center heterogeneity in tumor grading was standardized as follows: well-differentiated tumors were termed as G1, moderately differentiated as G2, poorly differentiated as G3, and undifferentiated or anaplastic as G4; in the case of intratumor heterogeneity, the maximum grade was recorded; Gx defined a lack of information in the pathology report, such as in the case of certain cytology reports. Values at CT1 initiation were collected for age, disease extension (locoregional, which grouped locally advanced, unresectable disease, and locoregional relapse; distant spread, which grouped initially metastatic disease and recurrence with metastases), number of metastatic sites (none, single, or multiple metastases), ECOG PS. Laboratory analyses recorded at the medical visit for CT1 initiation or up until two weeks prior to CT1 initiation were also analyzed in the present work, and included: CEA, Ca19.9, Hb, red blood cells distribution width (RDW), platelets, leucocytes, neutrophils, lymphocytes, monocytes, prothrombin time international normalized ratio, total bilirubin, GGT, ALP, AST, albumin, and LDH. Several derived indicators, already evaluated either in BTC or in malignancies of the same district (HCC, pancreatic adenocarcinoma), were also calculated: NLR, PLR, LMR, ALT/lymphocytes, albumin/GGT, GGT/ALT, albumin/ALP, GGT/platelets, and HRR, in addition to SII and PNI. Dates of death were retrieved from electronic medical records or administrative files. Data were collected until December, 2019, and subsequently analyzed.

Statistical analysis

Median OS, defined as the time interval from the first CT1 cycle to death from any cause, was the primary endpoint, and was calculated using the Kaplan-Meyer estimator. Cases still under treatment or lost to follow-up were censored at the last follow-up. Confidence intervals were set at 95%, and two-sided p values < 0.05 were considered statistically significant, unless Bonferroni correction was applied for multiple comparisons. Given its retrospective nature, the study did not consider a pre-planned sample size.

After dichotomization of continuous variables, a multivariate analysis by backward stepwise elimination was performed on a limited panel of variables. These variables were selected *post hoc*, from those that had displayed a significant correlation with OS on univariate analysis, and were chosen without prespecified criteria as the most meaningful and clinically relevant. Continuous variables were dichotomized using relevant cut points (e.g. definition of anemia). A prognostic model was devised in the form of a score, and calculated as follows: the value of 1 was assigned to each of the four highly-significant independent predictors of OS whenever the case fell in the favorable category (e.g. albumin levels ≥3.50 mg/dl), or the respective HR, rounded to the first decimal place, identified by univariate analysis if patients were identified as being in the unfavorable category (e.g. albumin levels <3.50). Individual prognostic index (PI) values were derived from the sum of these figures. Patients were then categorized into three groups according to their PI. A prognostic model was elaborated from the significant variables: the form of the model, a three-tier prognostic score (favorable, intermediate, and unfavorable prognosis), had been established in advance, whereas optimal cut-off values were empirically researched to achieve an optimized stratification. In particular, the model aimed to maximize the favorable-prognosis group, and to minimize the unfavorable-prognosis one, while maintaining very distinct group-specific survivals. Patients with unavailable data were excluded, according to complete case analysis.

Survival analysis employed the log-rank test and Cox regression model. Harrell's c-index, calculated as average from time 0 to 12 months, as well as AUC for OS events at 6 (AUC_6) and 12 (AUC_{12}) months, were employed as estimators of the discriminatory

power of the prognostic index. OS curves for selected subgroups of interest were also calculated.

An external validation was conducted on a cohort from Modena Cancer Centre (Italy) selected with identical criteria (validation cohort). Differences in characteristics between the cohorts were assessed by Chi Square and Mann-Whitney U tests.

IBM SPSS Statistics for Windows, version 20 (IBM Corp., Armonk, NY, USA) was used as the main statistical program.

The manuscript was checked for adherence to STROBE (STrengthening the Reporting of OBservational studies in Epidemiology) and TRIPOD (Transparent Reporting of a multivariable prediction model for Individual Prognosis Or Diagnosis) statements[191,192]; revisions and interactions with the editor.

5.2 Results

Study population

Clinical histories of 935 patients (training cohort) from 14 Italian medical institutions fulfilled the eligibility criteria. The diagnosis period ranged from April, 2001 to August, 2019. Demographic and baseline clinical, pathological and laboratory characteristics of the study population are listed in Table 19. In particular, median age was 65.9 years; 51.4% were male; the main primary site was ICC in 50.3% of patients, followed by GC in 21.1%, dECC in 14.2%, pECC in 8.2%, AC in 5.9%, and unknown site in 0.3%. Regarding the treatment, 562 patients (60.1%) received per intention-to-treat a gemcitabine-platinum salt doublet as CT1, 224 (24.0%) received gemcitabine monotherapy, and 149 (15.9%) received other regimens. Second-line treatment was received by 45.3% of patients. A total of 814 OS events had already occurred at the data cut-off, and the median OS of the entire cohort was 10.3 months ($CI_{95\%}$ 9.5-11.1). Patients treated with gemcitabine monotherapies (7.5 months, $CI_{95\%}$ 6.6-8.4), but not those treated with other regimens (12.4 months, $CI_{95\%}$ 10.4-14.4), experienced shorter OS ($p<0.001$, and p 0.12, respectively) than patients treated with gemcitabine-platinum salts (11.7 months, $CI_{95\%}$ 10.8-12.6).

Variable	Training cohort (n=935)	Validation cohort (n=159)	p value
Age in years			
median (IQR)	65.9 (58.5-72.1)	67.0 (59.0-75.0)	0.038
≥ 70 years	312 (33.3)	66 (41.5)	0.046
Sex			
female	454 (48.6)	85 (53.5)	0.253
male	481 (51.4)	74 (46.5)	
ECOG PS			
0-1	789 (84.4)	112 (70.4)	<0.001
≥2	84 (9.0)	26 (16.4)	
na	62 (6.6)	21 (13.2)	
Disease extension			
Locoregional	210 (22.5)	19 (11.9)	0.01
Distant spread	720 (77.0)	139 (87.4)	
na	5 (0.5)	1 (0.6)	
Prior R0 surgery			
yes	236 (25.2)	40 (25.2)	0.945
no	674 (72.1)	114 (71.7)	
na	25 (2.7)	5 (3.1)	
Tumor grade			
Low (G1-G2)	265 (28.3)	23 (14.4)	<0.001
High (G3-G4)	330 (35.3)	18 (11.3)	
Gx or na	340 (36.3)	118 (74.2)	

Variable	Training cohort (n=935)	Validation cohort (n=159)	p value
Laboratory [median, (IQR)]			
CEA (ng/ml)	3.4 (1.6-10.6)	2.6 (1.5-8.7)	0.047
Ca19.9 (U/ml)	119.5 (24.35-822.75)	119.4 (26.7-1234.0)	0.082
Hb (g/dl)	12.6 (11.4-13.9)	12.5 (11.2-13.3)	0.297
Neutrophils (c/µl)	4970 (3507-7330)	5620 (3997-8173)	0.011
NLR	3.05 (2.09-4.55)	3.54 (2.47-5.26)	0.236
Platelets(/µl)	239 (184-319)	251 (200-321)	0.092
Albumin (g/dl)	3.66 (3.20-4.03)	3.70 (3.40-4.10)	0.290
ALT (U/L)	31 (19-54)	33.5 (19-69)	0.017
Primary site			
ICC	470 (50.3)	76 (47.8)	<0.001
pECC	77 (8.2)	15 (9.4)	
dECC	133 (14.2)	10 (6.3)	
GC	197 (21.1)	58 (36.5)	
AC	55 (5.9)	0 (0.0)	
na	3 (0.3)	0 (0.0)	
CT1			
Gem-Pt	562 (60.1)	75 (47.2)	0.003
Gem	224 (24.0)	44 (27.7)	
Other regimens	149 (15.9)	40 (25.1)	
Overall survival in months			
median ($CI_{95\%}$)	10.3 (9.5-11.1)	8.0 (6.7-9.3)	0.052

Table 19*. Characteristics of the training and validation cohorts. Values in n (%), unless otherwise specified. nr, not reported.*

Prognostic factors

Among the several variables that had a prognostic association with OS on univariate regression analysis [data not shown, in Supplementary Table 1 in the publication], eight of the most relevant ones were dichotomized and subject to multivariate analysis. ECOG PS ≥2, Ca19.9 >120 U/l, albumin <3.50 mg/dl, and NLR ≥3.1 retained a high statistical significance (p<0.01), whereas Hb <12.5 g/dl, prior R0 surgery, disease status, and platelet count >400.000 /µl did not [Table 20].

Variable	Discrete categories	Univariate analysis		Multivariate analysis	
		HR ($CI_{95\%}$)	**p value**	**HR ($CI_{95\%}$)**	**p value**

ECOG PS	2-3 *vs* 0-1	2.95 (2.33-3.72)	<0.001	2.05 (1.46-2.89)	<0.001
Ca19.9	≥120 U/l *vs* <120 U/l	1.52 (1.30-1.78)	<0.001	1.37 (1.11-1.69)	0.004
Albumin	<3.50 mg/dl *vs* ≥3.50 mg/dl	1.97 (1.64-2.36)	<0.001	1.41 (1.13-1.77)	0.002
NLR	<3.1 *vs* ≥3.1	1.73 (1.49-2.01)	<0.001	1.51 (1.23-1.87)	<0.001
Hemoglobin	<12.5 g/dl *vs* ≥12.5 g/dl	1.52 (1.32-1.75)	<0.001	1.28 (1.03-1.59)	0.023
Prior surgery	yes *vs* no	0.66 (0.56-0.78)	<0.001	not significant	
Disease status	distant spread *vs* locoregional disease	1.30 (1.10-1.54)	0.002	not significant	
Platelet count	≤400.000/µl *vs* >400.000/µl	1.27 (1.60-2.01)	<0.001	not significant	

Table 20. *Univariate and multivariate analysis performed on dichotomized variables.*

Prognostic model

All of necessary data for a PI to be calculated were available for 421 patients (training set). The distribution of relevant variables in the training and validation sets was not significantly different from their respective original cohorts [Table 21].

Variable	**Training cohort (n=935)**	**Training set (n=421)**	**p value**	**Validation cohort (n=159)**	**Validation set (n=129)**	**p value**	**Data completeness**			
							Training cohort	**Training set**	**Validation cohort**	**Validation set**
Age ≥70 y	33.4	32.8	0.82	40.9	41.1	0.97	99.9	100	100	100
Gem-Pt CT1	60.1	64.6	0.12	47.2	49.6	0.68	100	100	100	100
CT2 receipt	45.3	44.0	0.69	46.5	51.9	0.36	98.9	99.3	100	100
Gallbladder primary	21.1	21.7	0.81	36.5	33.3	0.58	99.7	99.5	100	100
ECOG PS ≥2	9.6[#]	10.2[#]	0.74	18.8	17.8	0.83	93.4	100*	86.8	100*
Distant spread	77.4	79.7	0.35	86.7	88.3	0.69	99.5	99.5	99.4	99.2
Prior R0 surgery	25.9	23.6	0.37	25.2	24.0	0.82	97.3	97.6	100	100
Platelet count >400.000/µl	10.3	10.2	0.97	9.6	9.3	0.93	92.7	100	84.9	100
Hb <12.5 g/dl	47.3	49.2	0.53	49.6	48.8	0.90	92.7	99.5	84.9	100
NLR ≥3.1	48.6	48.2	0.90	57.3	58.1	0.88	87.8	100*	82.4	100*
Ca19.9 ≥120 U/l	50.1	51.3	0.70	50.0	49.6	0.95	77.2	100*	84.3	100*
Albumin <3.50 mg/dl	40.5	40.4	0.97	68.4	68.2	0.97	58.4	100*	83.6	100*

Table 21*. Distribution and data completeness of selected variables in the different populations considered in the study. Distribution is expressed as % of the population, after exclusion of cases with data not available; comparisons*

were performed through Chi-square test. Data completeness, expressed in %, is defined as the share of cases with availability of a specified variable value. Availability of ECOG PS, NLR, Ca19.9, and albumin was mandatory for the inclusion in the training and validation sets. # Three patients in the training cohort and in the training set had an ECOG PS 3.*

The resulting equation of the index was: PI = [1 or 3.0 (ECOG PS)] + [1 or 1.5 (Ca19.9)] + [1 or 2.0 (albumin)] + [1 or 1.7 (NLR)]. Values ranged from 4.0 to 8.2, with a median of 5.0. Patients were categorized into three risk groups, with prognoses categorized as favorable (PI ≤5.0, n=217, 51.5% of the training set), intermediate (5.0< PI ≤6.5, n=165, 39.2%), and unfavorable (PI >6.5, n=39, 9.3%). These groups showed a clear OS gradient, with median values of 12.7 months ($CI_{95\%}$ 11.0-14.4), 7.1 months ($CI_{95\%}$ 5.8-8.4), and 3.2 months ($CI_{95\%}$ 1.7-4.7), respectively, and 1-y OS rates of 55%, 27%, and 13%, respectively. The early and persistent separation of the corresponding survival curves translated into marked and statistically significant differences (all between-groups p values ≤0.001) [Table 22, Figure 7].

Prognostic group	**n**	**% of the sample**	**median OS in months ($CI_{95\%}$) [IQR]**	**OS HR (comparison with subsequent group)**	**p value**	**1-y OS**	**2- OS**
training set (n=421)							
favorable	217	51.5%	12.7 (11.0-14.4) [7.0-18.4]	0.53 (0.43-0.66)	<0.001	55%	18%
intermediate	165	39.2%	7.1 (5.8-8.4) [3.7-11.9]	0.54 (0.37-0.77)	0.001	27%	9%
unfavorable	39	9.3%	3.2 (1.7-4.7) [2.1-8.0]	-	-	13%	0%
validation set (n=129)							
favorable	66	51.2%	12.7 (11.0-14.3) [6.8-17.9]	0.66 (0.44-1.00)	0.050	57%	17%
intermediate	43	33.3%	7.5 (6.1-8.9) [4.1-13.6]	0.30 (0.17-0.53)	<0.001	33%	14%
unfavorable	20	15.5%	1.4 (0.1-2.7) [0.8-4.4]	-	-	5%	0%

Table 22. *Survival according to prognostic group in the training and validation sets.*

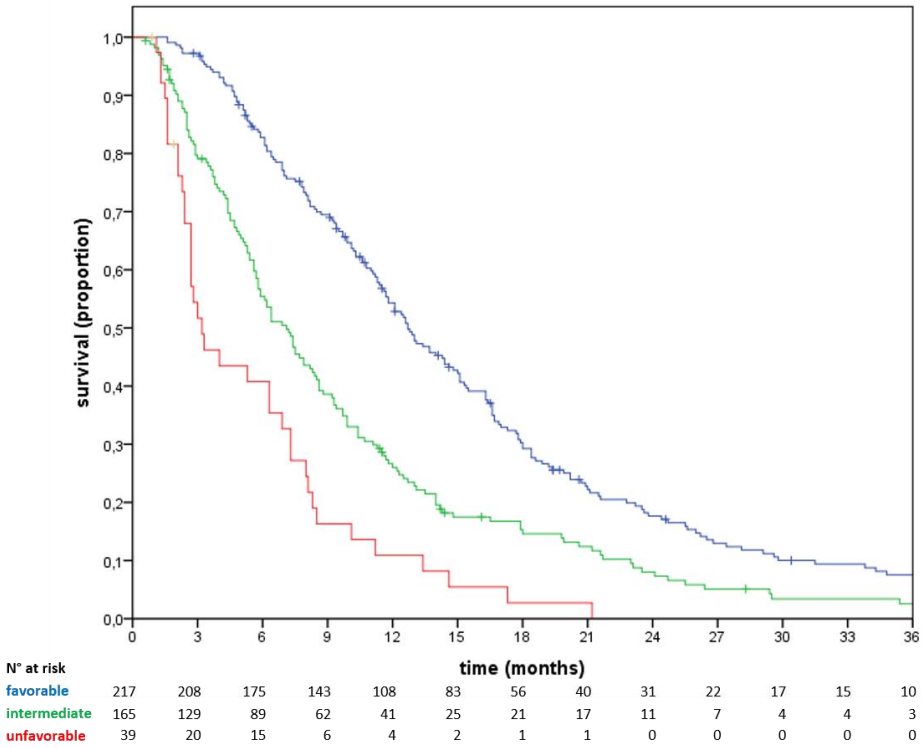

Figure 7. *Overall survival in the training set. Blue line, favorable prognosis; green line, intermediate prognosis; red line, unfavorable prognosis.*

The c-index, AUC_6, and AUC_{12} of the prognostic model in the training set were 0.69, 0.68 ($CI_{95\%}$ 0.62-0.73), and 0.66 ($CI_{95\%}$ 0.61-0.72), respectively [Figure 8].

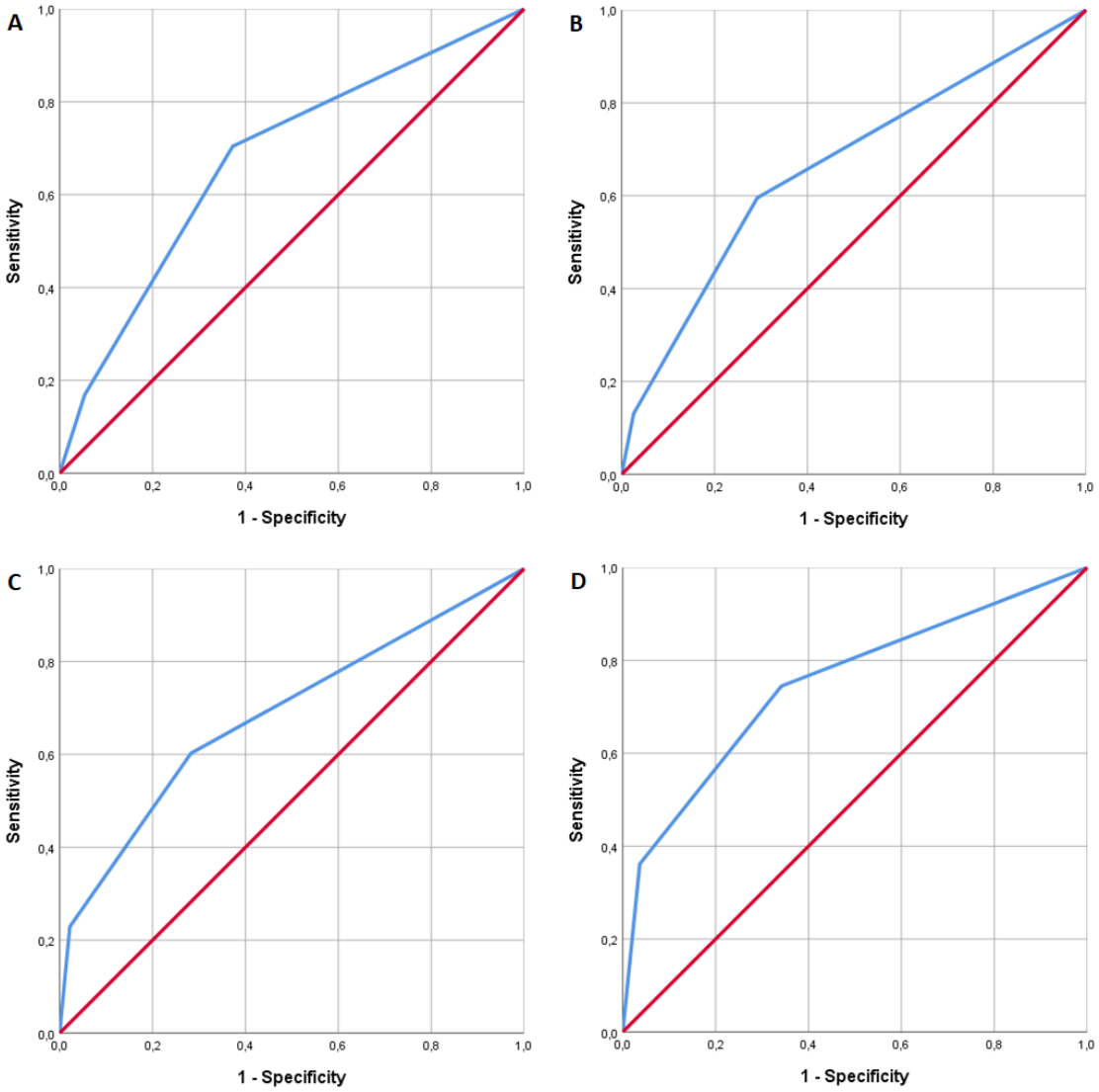

Figure 8. *Receiver operating characteristics curves of the prognostic model, for prediction of overall survival status at 6 and 12 months (A and B, respectively) in the training set, and in the validation set (C and D, respectively).*

The model retained its prognostic performance in most of the subgroups explored (primary sites, CT1 regimen, advanced age) [Figure 9; crude numbers not shown, retrievable in the Supplementary Figure 3 in the publication].

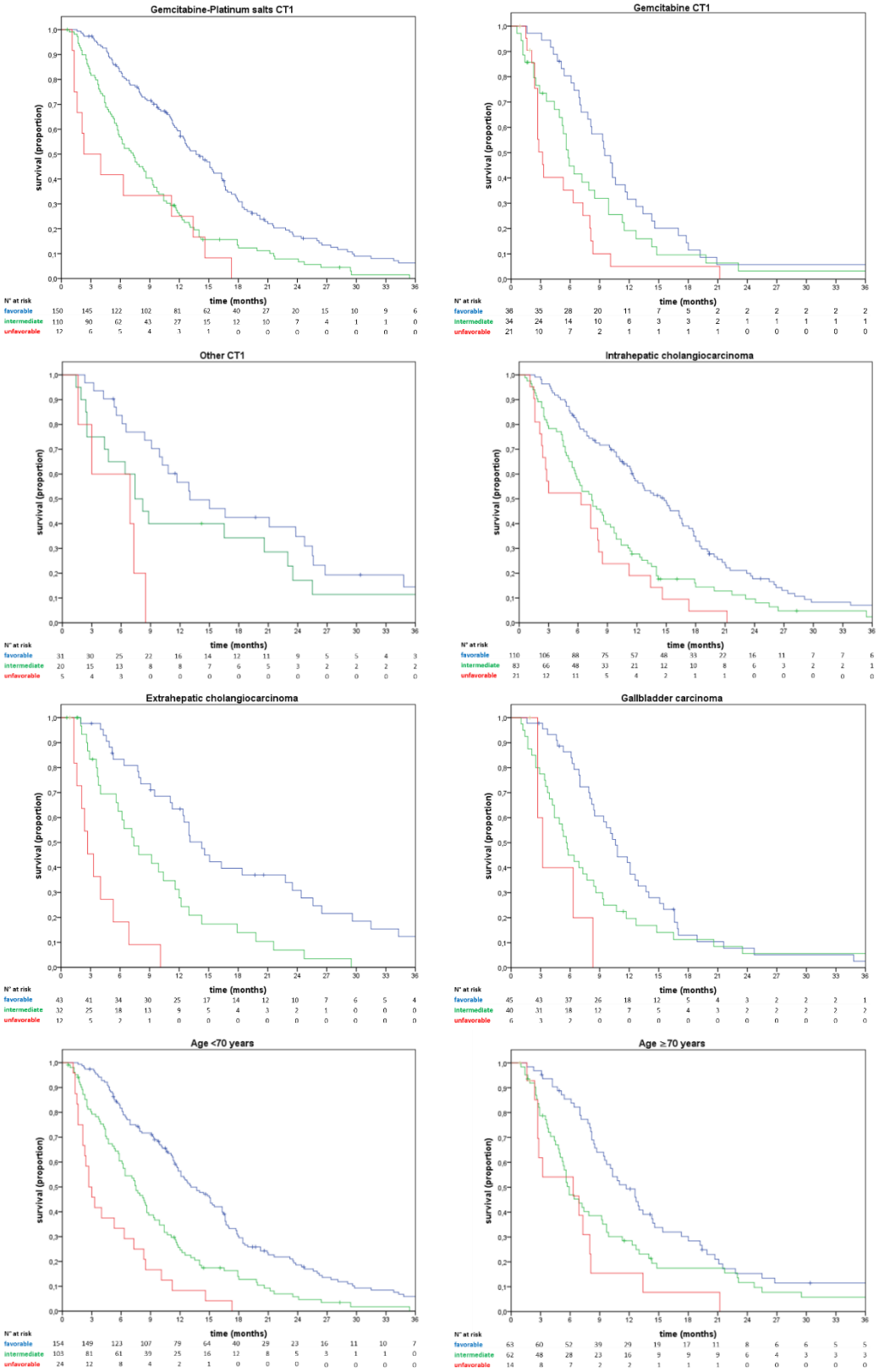

Figure 9. *Prognostic stratification in selected subgroups of the training set. Blue line, favorable prognosis; green line, intermediate prognosis; red line, unfavorable prognosis. ECC includes distal and proximal sites.*

The mere PI, not categorized in prognostic groups, achieved an AUC_6 of 0.72 ($CI_{95\%}$ 0.67-0.77) and an AUC_{12} of 0.69 ($CI_{95\%}$ 0.63-0.74). Patients with ECOG PS of 0, 1, and 2 achieved median OS of 13.1 ($CI_{95\%}$ 11.1-15.1), 7.7 ($CI_{95\%}$ 6.1-9.3), and 4.0 ($CI_{95\%}$ 0.4-7.6) months, respectively. Stratification according to ECOG PS achieved AUC_6 0.65 ($CI_{95\%}$ 0.60-0.71) and AUC_{12} 0.67 ($CI_{95\%}$ 0.62-0.72), similar to those obtained with the risk score (p=ns).

Validation

The validation cohort consisted of 159 clinical histories, diagnosed from November, 2000 to March, 2018. The median OS in the validation cohort was 8.0 months ($CI_{95\%}$ 6.7-9.3) [Table 19]. The necessary data were available for 129 patients (validation set). The stratification performed by the prognostic model retained its statistical significance and clinical validity. In particular, patients classified with favorable (51.2% of the validation set), intermediate (33.3%), and unfavorable (15.5%) groups experienced median OS of 12.7 months ($CI_{95\%}$ 11.1-14.3), 7.5 months ($CI_{95\%}$ 6.2-8.8), and 1.4 months ($CI_{95\%}$ 0.1-2.7), respectively. The 1-y OS was 57%, 33%, and 5%, respectively [Table 22, Figure 10]. C-index, AUC_6, and AUC_{12} values of the model in the validation set were 0.73, 0.75 ($CI_{95\%}$ 0.66-0.84), and 0.69 (0.59-0.78), respectively [Figure 8].

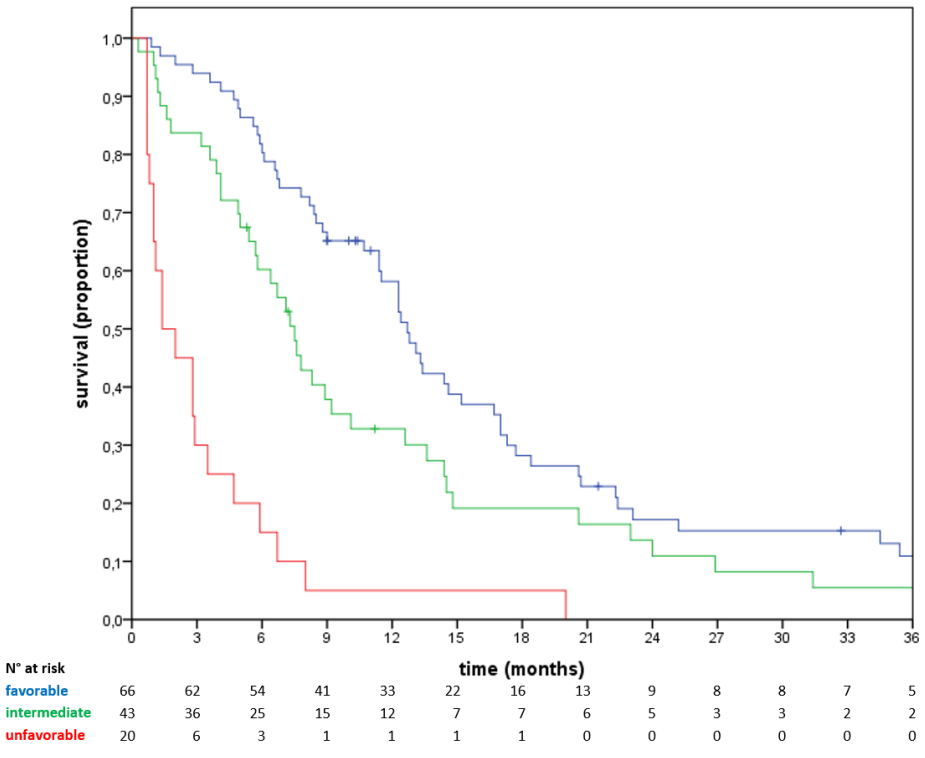

***Figure 10**. Overall survival in the validation set. Blue line, favorable prognosis; green line, intermediate prognosis; red line, unfavorable prognosis.*

5.3. Discussion

Against the backdrop of a poor global prognosis, a certain variability in aBTC clinical histories can be demonstrated. Separating clinical trajectories *ex ante* carries the ultimate goal of selecting patients for specific tailored treatments. Indeed, some particularly fragile patients do not benefit from chemotherapy, due to primarily progressive disease, treatment complications and/or clinical deterioration. Conversely, other fitter patients will reach second-line CT[193,194]. While modelling in pretreated aBTC provided interesting results[195,196], the first-line setting appears even more compelling, considering the high dropout rate between lines of treatment observed in real-world practice[2,197].

As described in par. 1.5, a pattern emerges from the heterogeneous available literature on OS predictors: variables most frequently associated with prognosis concern the areas of patient general condition (reflected, for example, by age[162], and ECOG PS[172,176,198]), nutritional status and residual organ synthetic function (hypoalbuminemia and anemia[171], PNI[199]), inflammatory status (most notably, NLR)[159,160,163,169,198], biological aggressiveness (tumor grade, prior surgery)[3,195,198,200], and tumor burden (metastatic disease, carcinoembryonic antigen, Ca19.9)[169–171,173].

Considering the risks of collinearity and interference related to a high initial number of variables, not all the significant prognosticators on univariate analysis were indiscriminately tested on a multivariate level. Instead, eight relevant prognosticators were chosen, so that all five of the mentioned areas of the disease-patient interaction were covered. Four variables emerged as strong independent predictors of OS, each reflecting a distinct domain of the tumor-host dyad: ECOG PS, NLR, albumin, and Ca19.9. We regard this as a conceptual amelioration, compared to other recent reports[168].

By combining these prognosticators, we devised a reliable prognostic estimator. Prognostic modelling in aBTC is heterogeneous, regarding examined population, study design, and final outcome. Firstly, a few studies are dedicated to specific primary sites[173,175]; conversely, following the consideration that the treatment is not differential according to the primary site, our analysis considered all disease sites together. In

particular, our model included AC cases (which only accounted for a small minority of the sample), similar to other relevant models[3,170,172]. While consistent with most of the existing literature in the field, this approach could be theoretically flawed by differences in prognosis and prognostic criteria between primary sites. Indeed, in accordance with other studies[3], our analysis suggested poorer outcomes for GC and a trend towards a favorable impact for AC. However, this issue has not yet been unequivocally demonstrated, with studies reporting a particularly unfavorable prognosis for other primary sites[176,201], and the majority of the available literature not observing a differential impact of disease site on OS. On the top of these considerations, our model retains discriminative ability across primary sites [Figure 9].

Following the paradigm of treatment homogeneity, unlike others[170,173], we included the locally advanced, unresectable disease. Different from other studies, clinical histories that would not consider CT1, such as surgically cured disease[3], or patients who would never reach active oncologic care[170,173], were excluded for homogeneity's sake. A minority of patients received non-standard CT regimens, which lack level-1 evidence of efficacy. However, given that the intention was to define subsets of very high and low benefits from CT *per se*, we included these patients in the analysis.

The oncology institutions contributing to the BICC database ranged from small peripheral facilities to high-volume, academic centers. This type of multi-center nature added to the real-life blueprint of the work, as not all patients are treated in large hospitals or enrolled in clinical trials.

We developed a prognostic model in the form of a three-tier prognostic score. In contrast to the mere addition of risk factors[172], assigning a coefficient to each addend allows accounting for the weight of each prognostic contributor: in previous comparable models[164,168,170,176], these coefficients were variably derived from rounded Chi-square values, regression coefficients, or HRs. Among the models with c-index or AUC provided, these parameters ranged from 0.68 to 0.83, and from 0.63 to 0.65, respectively[164,168,170]. Therefore, the discriminatory performance of our PI resulted as being in line with the comparable literature.

Our model, in which ECOG PS was assigned the highest weight, did not outperform this parameter in mere terms of AUC. However, our attempt was triggered by the specific

intent of convenience from the clinician's point of view. In particular, patients identified as having a favorable- prognosis would be good candidates for future, intensified regimens (NCT02591030, NCT03768414), or clinical trials. In modelling, we prioritized the maximization of this subpopulation over obtaining higher survivals in smaller groups[162,168]. Favorable-prognosis patients constituted more than half of the study population, and could expect a satisfactory median OS of 12.7 months, and a 75% chance to live longer than 7 months. At the other end of the spectrum, chances of benefitting from CT were very low for the unfavorable-prognosis group. Importantly, unlike other comparable models[164,168], our prognostic score circumscribed this population, most suited to best supportive care due to an expected median OS of 3.2 months, to less than 10% of patients. However, we could not compare these outcomes with those of patients undergoing palliative care[170], and only a prospective evaluation might ultimately confirm the lack of benefit from CT1. Therefore, we could not recommend referral of these patients to palliation on the sole basis of our work.

Our work has some limitations. Firstly, the retrospective nature of our study entails not-on-purpose data collection, missing information, numerical imbalance between cohorts, and intrinsic patient selection. Important variables such as CRP levels, cachexia, weight loss, and state and severity of liver disease were not collected. Only a minority of patients in the training cohort could be evaluated for all the necessary variables to the model. However, the sample size remained large (421 patients), and no significant differences were demonstrated regarding relevant variables, including those prognosticators tested on multivariate analysis. Dichotomization of continuous variables introduces a loss of informativity[190]. To reduce this problem, cut points were chosen to be clinically meaningful (thresholds for anemia, thrombocytosis, hypoalbuminemia), or to approximate consolidated ones (NLR)[169,170,198]; the median value was used for Ca19.9.

However reasoned, some passages of the model construction were made arbitrarily and *post hoc*. This called for further testing on an external validation dataset. This model showed good reproducibility in this population, which globally trended towards worse prognosis.

In brief, after exploring CC etiology, I complemented my research path with a more clinically-oriented line. As mentioned in par 3.4, I sought for niches of clinical needs that

needed further clarification. The first one was the lack of a reasoned prognostic model, hereby presented.

The second was the particular setting oligo-recurrent disease (background in subpar 1.4.1): I concluded my research in these doctoral years facing the issue of treatment intensification. In other words, is it worth to subject patients with an apparently brief residual survival expectancy to repeat (non)surgical procedures? I'll attempt to answer this question in the next chapter.

6. Treatment intensification for BTC post-surgical relapse: outcomes and safety

6.1. Patients and methods

ALT-rBTC (Ablative-intent Locoregional Treatments in Recurrent Biliary Tract Cancer undergoing chemotherapy: a retrospective analysis) is a multicentric retrospective study on BTC recurrence treated with ablative-intent LRTs (surgical or not surgical) in patients which also received systemic CT for the advanced disease. The primary objective was to describe the survival outcomes of LRT. Secondary objectives were: the description of the demographic and disease (clinical, histological, further treatments) characteristics of this niche of patients; the identification of prognostic factors, with the ultimate goal of constructing of a prognostic model specific for this subpopulation.

PFS was the primary endpoint, defined as the time from the date of the LRT (or the first intervention, in case of multiple sequential LRTs) and the date of PD or death. Secondary endpoints were: OS; prevalence of complications occurring in the 60 days following the LRT; time-to-chemotherapy (TTC), defined as the time from the date of LRT and the start of a CT treatment for systemic disease not amenable to LRTs; statistically significant prognostic factors. An additional exploratory endpoint was the creation of a prognostic model.

Patients were eligible to inclusion if they were affected by BTC with the following eligibility criteria:

-had been radically (R0 or R1) surgically resected, with or without a prior neoadjuvant therapy. Clinical histories including R2 or exploratory-intent surgical interventions were excluded.

-experienced a documented (TC, RM , PET) locoregional or distant relapse of BTC, to which one or multiple surgical or non-surgical LRT were applied, with an ablative (not palliative) goal. Non-surgical LRTs included: TACE, TARE, RFA/MWA, HAI, SBRT, CRT, PHP.

-received at least one systemic CT line for the advanced disease. As a consequence, the following cases did not meet the inclusion criteria: patient receiving adjuvant-intent post-surgical CT only, and patients that only received LRT for the relapsed disease.

Data collection started in September, 2022, and was concluded in May, 2023. The clinical histories were retrieved from clinical records, and a central integrated database was elaborated. Collected data concerned:

-patients' age and sex;

-diagnosis, surgical procedure on the primary, staging according to AJCC/UICC TNM (2018 edition), histopathology features (margins, tumor diameters, grading, vascular invasion, presence of necrosis);

-for patients undergoing surgical LRTs: histopathology features of relapsed disease;

-for patients undergoing non-surgical LRTs: number of treatment sessions, treatment characteristics (e.g., total radiation dose in Grays [Gy] for SBRT)

-peri-procedural complications, eventual subsequent LRTs;

-regimens and dates of start and end of CT lines;

-bloodwork up to 28 days prior to the (first) LRT: WBC, neutrophils, lymphocytes, Hb, platelets, AST, ALT, GGT, ALP, total bilirubin, albumin, Ca19.9, CEA. The following parameters were derived: NLR, PLR, PNI.

Statistical analysis

Population characteristics were reported as absolute numbers and proportion (%) of the sample. Their distribution between subgroups was analyzed with Fisher exact test, with a statistical significance threshold set at $p < 0.05$. Survival times were calculated with the Kaplan-Meier estimators; for numerical measures 95%-confidence intervals were calculated; comparisons between survival curves were drawn through the log rank test, for which the statistical significance threshold was set at $p < 0.05$.

The following variables were input to regression analysis (Cox model) to evaluate their prognostic significance: patient sex, primary site, nodal involvement at diagnosis, DFS (defined as time from date of surgery on the primary to date of relapse), neoadjuvant CT receipt, relapse site, treatment of relapse (surgical vs non surgical), number and maximum diameter of the treated lesions, along with ECOG PS and laboratory analytes collected before the (first) LRT. Continuous variables were dichotomized first according to clinically relevant cutpoints (e.g., the definition of anemia), then according to the median values. Analysis was performed only for dichotomizations that resulted in both subgroups having $n \geq 5$. The dataset was elaborated in MS Excel, whereas IBM SPSS Statistics for Windows, version 20 (IBM Corp., Armonk, NY, USA) was employed as main statistical program.

6.2. Results

Study population

Sixty-five clinical histories from fourteen Italian centers met the inclusion criteria, with diagnoses of BTC ranging from July, 2003 and March 2022. The accrued population was equally split by sex; importantly, ICC was the predominant primary site, and less than one-fifth of cases had a nodal involvement at the moment of diagnosis / surgery on the primary [Table 23].

Median age, y [range; IQR]	66 [36-83; 60-71]
Sex, n (%)	
Female	32 (49.2)
Male	33 (50.8)
Primary site, n (%)	
ICC	39 (60.0)
pECC	7 (10.8)
dECC	6 (9.2)
GC	6 (9.2)
AC	7 (10.8)
Locoregional lymphnodes involvement, n (%)	
N0	42 (64.6)
N1	12 (18.5)
na	5 (7.7)

***Table 23**. Population characteristics.*

Only five patients had a primary that was not upfront resectable, and had undergone neoadjuvant CT, performed with the first-line regimen GemCis, before surgery. Given the high representation of ICC in this cohort, the most frequent surgical treatment on the primary was hepatectomy or its variations (which also constitute the treatment of localized pECC); duodenocephalopancreasectomy (dECC, AC) was the second most frequent class of surgical interventions. Surgical treatment resulted in R0 resections in 84.6% of cases, and yielded a median DFS of 14.2 months. Histopathology information was available for 52 cases (80.0%), being almost equally split between moderately and poorly differentiated neoplasm; no well-differentiated BTC cases were collected.

Conversely, more detailed information was only seldom reported, so that neither perineural/vascular invasion, nor necrosis prevalences could be calculated. Following the treatment, only about half of patients underwent adjuvant CT. Two-thirds of the relapses in this population occurred in the liver, which comprehended both hematogenous metastases and locoregional relapse on the resection margins; the second most frequent site of relapse was lymphnodal. One-third of patients had the LRT performed after one or more lines of systemic CT (eventual adjuvant CT is not counted) [Table 24].

Primary	
Neoadjuvant CT, n (%)	
Yes	5 (7.7)
- of which, with GemCis	- 5 (100)
No	45 (69.2)
na	14 (21.5)
Surgical intervention, n (%)	
Duodenocephalopancreasectomy	14 (21.5)
Hepatectomy	24 (36.9)
Bile duct resection	1 (1.5)
Colecystectomy	4 (6.2)
na	22 (33.8)
Radicality, n (%)	
R0	55 (84.6)
R1	7 (10.8)
na	3 (4.6)
Adjuvant CT, n (%)	
Yes	31 (47.7)
- of which, with Gemcitabine	- 13 (41.9)
- of which, with Capecitabine	- 11 (35.5)
No	34 (52.3)
Tumor grading, n (%)	
G2	27 (41.5)
G3	25 (38.5)
na	13 (20.0)

Relapse	
Median DFS, mo [IQR]	14.2 [7.3-6.3]
Site of relapse, n (%)	
Liver	44 (67.7)
Peritoneum	3 (4.6)
Lung	2 (3.1)
Bone	1 (1.5)
Lymphnodes	10 (15.4)
na	1 (1.5)
Systemic CT prior to LRT, n (%)	
Yes	22 (33.8)
- of which, 1 line	- 15 (68.2)
- of which, 2 lines	- 6 (27.3)
- of which, >2 lines	- 1 (4.5)
No	37 (56.9)
na	6 (9.2)

Table 24. *Population characteristics, as of treatment of the primary tumor, and of type of relapse.*

The study population was then characterized for laboratory analytes at the moment of LRT. Values distribution and occurrence of pathological alterations are presented in Table 25.

Variable, *unit of measurement* (n with available data)	**Median [IQR]**	**Alteration (numerical definition)**	**n (%)**
WBC, *10^6/l* (33)	5.46 [4.31-7.08]	Leucocytosis (>8.00)	3 (9.1)
Neutrophils, *10^6/l* (20)	3.23 [2.22-3.87]	Neutrophilia (>6.00)	2 (10.0)
Limphocytes, *10^6/l* (20)	1.71 [1.12-1.81]	Lymphopenia (<1.00)	4 (20.0)
Hb, *g/dl* (32)	12.85 [11.62-14.08]	Anemia (<12.0)	14 (43.8)
Platelets, *10^6/l* (32)	212.50 [182.25-270.25]	Piastrinosis (>450.00)	3 (9.4)
AST, *UI/l* (27)	22 [20-36]	Elevated AST (>35)	7 (25.9)
AST, *UI/l* (27)	21.5 [15-43]	Elevated ALT (>35)	8 (26.7)
GGT, *UI/l* (11)	29 [16-48]	Elevated GGT (>35)	2 (18.2)
ALP, *UI/l* (17)	98 [80.5-150.5]	Elevated ALP (>35)	7 (41.2)
Total bilirubin, *g/dl* (24)	0.64 [0.49-1.05]	Hyperbilirubinemia (>1.20)	1 (4.2)
Albumina, *g/dl* (11)	4.2 [3.6-4.5]	Hypoalbuminemia (<3.5)	1 (9.1)
CEA, *ng/ml* (25)	1.7 [0.95-2.80]	Elevated CEA (>5.00)	5 (20.0)
Ca19.9, *U/ml* (32)	50.0 [14.35-117.23]	Elevated Ca19.9 (>37)	18 (56.3)
Derived variables			
NLR (20)	1.81 [1.44-3.44]		
PLR (20)	167950 [110357-253894]		
PNI (4)	48.1 [42.2-55.9]		

***Table 25**. Laboratory analysis at the moment of the (first) LRT.*

Locoregional treatments

Twenty-six patient (40%) were treated with surgery for their relapse, 39 (60%) with non-surgical LRT. In line with the prevalence of hepatic sites of relapse, liver resections (lobectomies, segmentectomies, atypical resections as the metastasectomies) were predominant among surgical patients. In addition, three patients underwent the hepatectomy intervention and then received thermoablation (2 patients) and hyperthermic intraperitoneal chemotherapy (1 patient). Globally, the R0 resection rate in this subgroup

was high (73.1%). The most employed LRTs were TACE (38.5%), SBRT (25.6%), and RFA/MWA (17.9%); no cases of PHP or HAI were identified. Excluding the cases for which this information could not be retrieved, single lesions were treated in 47.4% and 56.2% of patients in the surgical and non-surgical cohorts (p 0.57), respectively; similarly, non-significant differences in distribution were found for double lesions (26.3% vs 12.5%, p 0.27), and multiple lesions (26.3% vs 31.2%, p 0.14). Periprocedural complication rates were low in both groups (11.5% vs 15.4%, in the surgical and non-surgical cohort, respectively; p 0.99): surgical complications were represented by two cases of anemia (one associated with a biliary fistula), and one dehiscent surgical wound; in the non-surgical cohort, six cases of post-ablation/post-embolization syndromes (fever, abdominalgia, nausea), one of which complicated with an hepatic abscess. Almost one-quarter (23.1%) of the treated patients received a post-LRT CT treatment with a "pseudo-adjuvant" intent [Table 26].

Surgical LRT		**Non-surgical LRT**	
Treated patients	26 (40.0)	Treated patients	39 (60.0)
Surgical procedure		*Intervention*	
Liver resections	11 (42.3)	TACE	15 (38.5)
Lymphadenectomy	2 (7.7)	TARE	3 (7.7)
Abdominal/peritoneal resection, omentectomy	3 (11.5)	RFA/MWA	7 (17.9)
Lung resection	2 (7.7)	SBRT	10 (25.6)
Hepatic surgery followed by LRT	3 (11.5)	Radiosurgery	3 (7.7)
Radicality		CRT	1 (2.6)
R0	19 (73.1)	HAI	0 (0.0)
R1	5 (19.2)	PHP	0 (0.0)
na	2 (7.7)		
N° of treated lesions		*N° of treated lesions*	
1	9 (34.6)	1	18 (46.2)
2	5 (19.2)	2	4 (10.3)
>2	5 (19.2)	>2	10 (38.5)
na	9 (34.6)	na	6 (15.4)
Median diameter, mm [IQR]	15 [9.25-28]	Median diameter, mm [IQR]	21.5 [16.25-25.5]
Periprocedural complications		*Periprocedural complications*	

Yes	3 (11.5)	Yes	6 (15.4)
No	13 (50.0)	No	25 (64.1)
na	10 (38.5)	na	8 (20.5)
"Pseudo-adjuvant" CT			
Yes	15 (23.1)		
- of which, with capecitabine	- 4 (26.7)		
- of which, gemcitabine	- 3 (20.0)		
- of which, with other regimens	- 6 (40.0)		
- of which with regimen na	- 2 (13.8)		
No	26 (40.0)		
na	24 (36.9)		

***Table 26**. Surgical and non-surgical LRT for relapse. Values in n (%), unless specified otherwise. na, not available.*

Outcomes of locoregional treatments

Median PFS in the study population was 5.1 months ($CI_{95\%}$ 4.5-5.7), not significantly impacted by the type of LRT received: 6.7 months ($CI_{95\%}$ 4.6-8.8) for surgical patients, and 4.1 months ($CI_{95\%}$ 3.1-5.1) in the non-surgical cohort (p 0.27) [Figure 11].

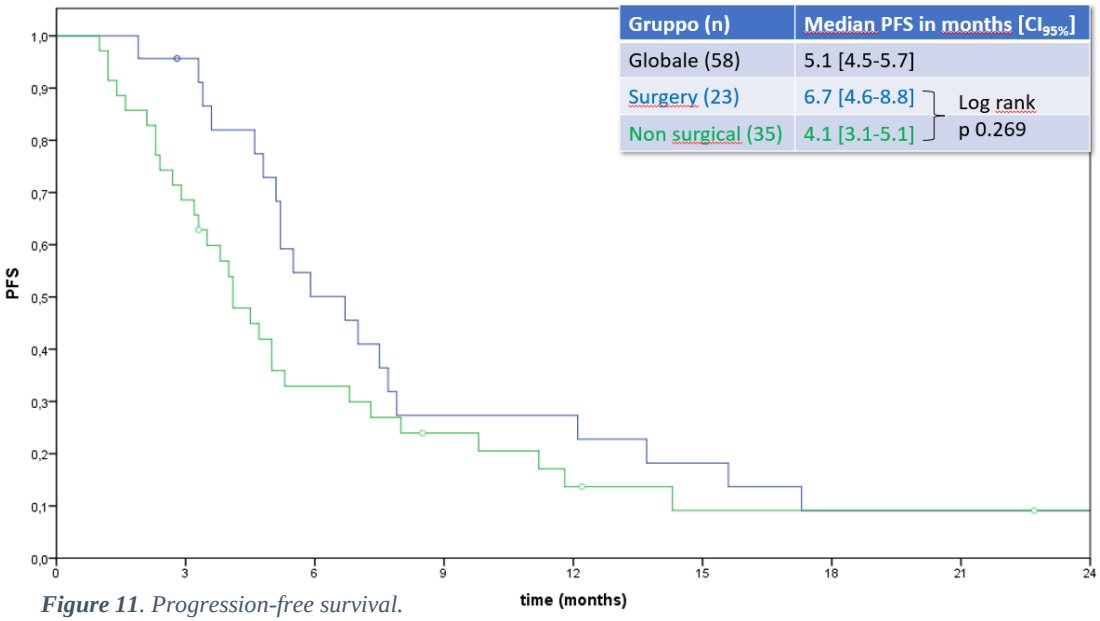

***Figure 11**. Progression-free survival.*

Median OS was 24.7 ($CI_{95\%}$ 13.3-36.1), 31.0 ($CI_{95\%}$ 13.8-48.2), and 20.7 months ($CI_{95\%}$ 11.6-29.8) in the overall population, in the surgical cohort, and in the non-surgical cohort, respectively. Albeit numerically wide, the gap between the treatment cohorts was not statistically significant (p 0.51) [Figure 12].

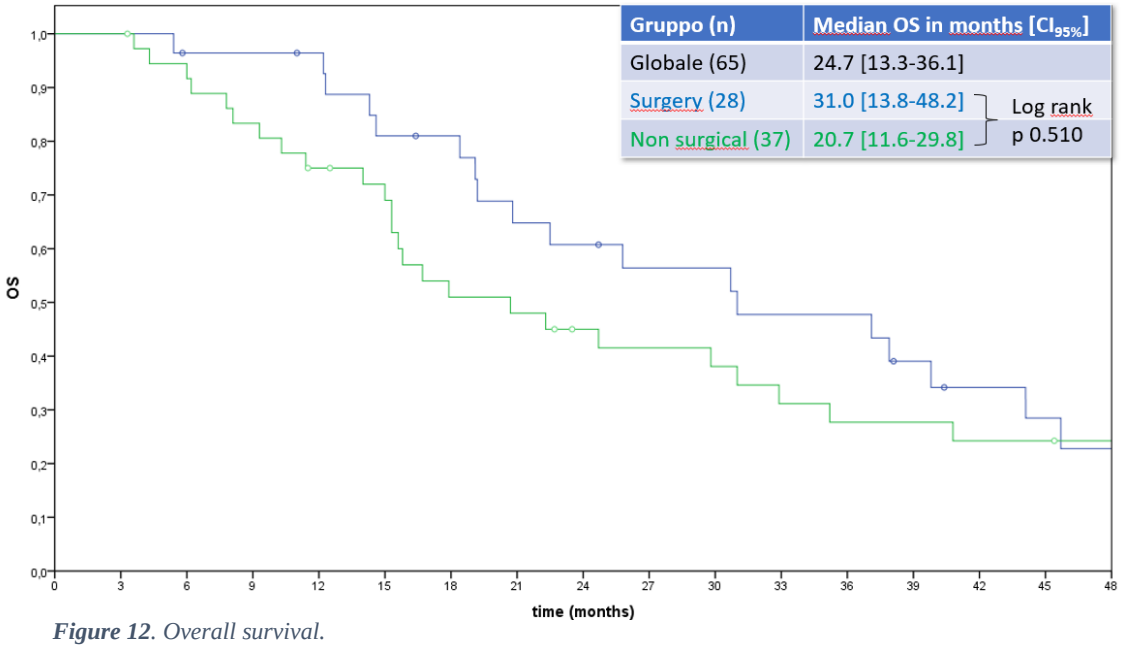

***Figure 12**. Overall survival.*

Subgroup analysis was performed for site of relapse. Of note, in the subpopulation of patients whose disease relapsed in the liver, the OS gap between the treatment groups was even wider, but again not statistically significant [Table 27]. Outcomes of surgical resections in other sites appeared lower to some extent. Given the very limited sample sizes, these figures are reported only for a descriptive purpose: pulmonary resections (n=2) achieved a PFS of 7.7 months and an OS of 19.2 months; abdominal/peritoneal resections (n=4) were associated with a PFS of 5.5 months and an OS of only 12.2 months.

	Surgery	**n**	**Non-surgical LRT**	**n**	**HR**	**p**
PFS	5.9 (3.2-8.6)	13	4.1 (2.9-5.3)	23	0.61 (0.30-1.24)	0.173
OS	37.1 (24.5-49.8)	14	16.7 (12.9-20.5)	22	0.58 (0.29-1.15)	0.118

***Table 27**. Outcomes in hepatic relapses. Overall survival and progression-free survival are expressed in months, HRs in absolute numbers; in brackets the 95%-confidence intervals.*

Subsequent treatments

One-third (22) of patients were subject to a second LRT, 6 of them to multiple treatments. The most frequent approach in these strategies of repeat LRT was TACE, employed as second LRT in 7 patients, followed by SBRT (5 patients), RFA/MWA (4 patients), and surgery or other treatments (3 patients each) [Table 28]. Of interest, PFS in this subgroup (n° patients with available information=5) was 4.0 months ($CI_{95\%}$ 3.3-4.7), and OS (n=22) 21.2 months ($CI_{95\%}$ 1.9-40.4).

Subsequent LRTs	
Yes	22 (33.8)
- of which, TACE	- 7 (31.8)
- of which, SBRT	- 5 (22.7)
- of which, RFA/MWA	- 4 (18.2)
- of which, surgery	- 3 (13.6)
- of which, other LRT	- 3 (13.6)
No	- 24 (36.9)
na	- 19 (29.2)

***Table 28**. Subsequent LRTs. Values expressed in n (%).*

Following the (first) LRT, most of the study population (51 patients, or 78.5%) underwent a systemic CT for disease not any longer amenable to a LRT [Table 29].

Subsequent systemic CT	
No	14 (21.5)
Yes	51 (78.5)
- 1	23 (45.1)
- 2	14 (27.5)
- >2	14 (27.5)
CT1	
GemCis	15 (29.4)
Gemcitabine	14 (27.5)
GemOx	6 (11.8)
Pemigatinib	4 (7.8)
Other	12 (23.5)
Second-line CT regimen	

FOLFIRI	6 (21.4)
Capecitabine	5 (17.9)
GemCis	4 (14.3)
Gemcitabine	3 (10.7)
Other	10 (35.7)

***Table 29**. Subsequent systemic CT. Values expressed in n (%).*

Median TTC among these patients was 8.0 months (IQR 5.2-13.4). CT1 regimens consisted chiefly in gemcitabine-based doublets and gemcitabine monotherapy; median PFS to CT1 was 7.7 months (IQR 2.9-12.7). Following this further disease progression, 55% of patients went on to receive a second-line CT (half of them received further lines): these patients were endowed with a particularly favorable prognosis, as they experienced a PFS (n° patients with available information=37) of 8.7 months ($CI_{95\%}$ 5.3-12.1) and an OS (n=41) of 17.4 months ($CI_{95\%}$ 9.3-25.4).

Regression analysis

The univariate analysis did not demonstrate statistically significant associations with OS or PFS for any of the tested clinical or laboratory variables [Tables 30, 31, and 32].

Variable (categories)	**n**	**PFS HR ($CI_{95\%}$)**	**OS HR ($CI_{95\%}$)**
Sex (F vs M)	65	1.00 (0.56-1.77)	0.81 (0.44-1.47)
Primary site (other sites vs ICC)	65	0.91 (0.50-1.67)	1.24 (0.70-2.18)
Nodal involvement at diagnosis (N+ vs N0)	51	0.97 (0.49-1.93)	0.85 (0.39-1.84)
DFS (>1 year vs <1 year)	57	0.64 (0.36-1.11)	0.86 (0.49-1.53)
Neoadjuvant CT (yes vs no)	45	0.62 (0.19-2.04)	0.63 (0.15-2.67)
ECOG PS (1 vs 0)	32	1.65 (0.75-3.63)	1.42 (0.63-3.19)
Relapse site (liver vs lymphnodes)	52	0.94 (0.78-1.12)	0.97 (0.81-1.17)
LRT (non-surgical vs surgical)	58	1.37 (0.78-2.38)	1.21 (0.69-2.11)
N° of treated lesion (single vs multiple)	46	0.76 (0.40-1.43)	0.99 (0.52-1.88)
Max diameter (>20 mm vs <20 mm)	25	0.85 (0.37-1.98)	1.06 (0.42-2.66)
Max diameter (>30 mm vs <30 mm)	25	1.76 (0.69-4.5)	1.97 (0.71-5.43)
Adjuvant CT (yes vs no)	38	0.76 (0.38-1.52)	0.89 (0.43-1.85)

***Table 30**. Univariate analysis of clinical variables. n = n° patients with available information.*

Variable	PFS HR ($CI_{95\%}$)	OS HR ($CI_{95\%}$)
WBC	1.15 (0.54-2.45)	1.14 (0.50-2.60)
Neutrophils	1.53 (0.52-4.48)	1.02 (0.28-3.67)
Lymphocytes	1.35 (0.46-3.92)	1.54 (0.43-5.47)
Hb	1.46 (0.65-3.27)	1.63 (0.67-3.95)
Platelets	1.59 (0.73-3.48)	1.14 (0.49-2.66)
NLR	0.78 (0.27-2.27)	0.46 (0.13-1.65)
PLR	0.96 (0.33-2.76)	0.34 (0.09-1.24)

***Table 31**. Univariate analysis of laboratory variables. Comparisons for values above median vs below median.*

Variable	PFS HR ($CI_{95\%}$)	OS HR ($CI_{95\%}$)
Anemia	1.46 (0.65-3.39)	1.50 (0.61-3.71)
Elevated AST	1.81 (0.68-4.82)	2.86 (0.97-8.44)
Elevated ALT	2.09 (0.84-5.18)	2.85 (0.90-7.49)
Elevated ALP	2.78 (0.82-9.36)	3.24 (0.89-11.76)
Elevated CEA	1.31 (0.47-3.63)	0.86 (0.28-2.64)
Elevated Ca19.9	2.09 (0.90-4.86)	1.03 (0.45-2.33)

***Table 32**. Univariate analysis of laboratory variables. Comparisons for condition present vs absent.*

Additionally, in the surgical cohort the prognostic role of margin status was assessed, by directly comparing R0 resections with R1. In presence of very limited sample sizes for R1-resected subgroups, no significant differences emerged: PFS was 5.9 months ($CI_{95\%}$ 3.5-8.3) in R0-resected patients (n° patients with available information=17), and 12.1 months ($CI_{95\%}$ 0.0-28.4) in R1-resected patients (n=3) (HR 2.26, $CI_{95\%}$ 0.51-10.2; p 0.286). OS was 31.0 months ($CI_{95\%}$ 16.3-45.7) and 22.5 months ($CI_{95\%}$ 14.3-47.7) in R0-resected (n=19) and R1-resected (n=5) patients (HR 0.84, $CI_{95\%}$ 0.30-2.37; p 0.740).

6.3. Discussion

Even in case of radical (R0, R1) resection, BTC bears a severe prognosis, owing to the high rates of locoregional and distant relapse[202,203]. There is still lack of guidelines recommendations regarding the best management of recurrent disease[121]. When clinically

and anatomically feasible, LRTs may add to the severe prognosis of BTC recurrences. Indeed, prior studies have hypothesized a therapeutic window of opportunity for repeat surgeries[147,150] or other LRTs, such as RFA[153] and TACE[156]. Even in the absence of a pre-specified statistical hypothesis, the ALT-rBTC study suggests that an integrated, multimodal strategy, based on the addition of either a surgical or non-surgical LRT to systemic CT is feasible, safe, and promising. The median PFS 5.1 months is comparable to a first-line pharmacological treatment in the general aBTC population[4,117].

The fact that interrogating 14 centers for diagnoses covering a timespan of up to 20 years resulted in only selecting 65 clinical histories points to the scarcity of suitable cases for this approach. As such, it is unlikely that a prospective trial will emerge to confirm prospectively the results; as a consequence it is important to enrich the existing retrospective literature. Indeed, the present study collected one of the largest samples available.

Caution should be adopted when interpreting the survival outcomes in a retrospective study, particularly OS that is most influenced by selection biases. Prognosis of all-comers aBTC lies around 10-14 months in clinical practice[2], as only little improvement is observed even in the most recent phase-III clinical trials[123,140]. We[2] and others[200] previously demonstrated that receiving prior surgery strongly associates with OS from the beginning of CT1; in other words, recurrent BTC carries a better prognosis than *de novo* metastatic disease. Presumptively, having undergone a surgical curative-intent selected both for fit, less comorbid individuals, and against aggressive tumor behaviors: in order to be caught at a localized stage, tumors need to display slower growth and favorable biology. This is suggested by the absence of ECOG PS 2 patients; by the over-representation in our study of G2 tumors (half of the sample), in a generally poorly differentiated disease[2]; and by the protracted median DFS (14 months) from the resection of the primary tumor. Another level of selection is represented by the tumor burden at relapse: LRTs are generally reserved to mono- or oligo-recurrent neoplasms, with small diameters. Indeed, residual OS of unselected recurrent disease varies from 4 to 15.6 months[145,200,204,205], but even those series that excluded patients not receiving further oncologic treatments did not achieve comparable results to the survival figures observed in the present study. A relevant patient selection is therefore very apparent. OS in our study (24.7 months; 31.0 months after a surgical LRT) appears instead similar to the

existing literature on repeat resection of BTC (38.0 months)[150] or ICC (26.7-36.8 months)[150,206].

Particularly the latter studies, maintaining a surgical perspective, tend not to characterize the subsequent path of the patient after the repeat resection, which instead was described in the present work. Another strength of the ALT-rBTC study was the inclusion of a relevant fraction of ECC, whose clinical behavior is less studied than ICC[175].

Although a wide gap was highlighted in the median OS values, no significant differences in terms of survival were observed between surgical and non-surgical LRTs. This finding is consistent with previous studies, that were focused on the treatment of liver recurrences of ICC, and specifically compared surgery to ablation[154,155]. Infrequent complications were observed both in surgical or non-surgical LRT; however, once again the retrospective and not *ad hoc* data collection calls for caution in interpreting these results, which compare favorably with other surgical series[149,206]. The issue of missing data could very well be more extensive than the 38.5% of patients with no available information, and could instead reflect the underreporting of less severe complications in discharge reports or complications occurred after the discharge and treated in other facilities.

Disappointingly, none of the laboratory and clinical variables tested showed a prognostic role even on univariate analysis. This prevented further analysis and the creation of a prognostic model. The most promising variables were elevated Ca19.9 (PFS only), elevated AST, elevated ALT, elevated ALP, and the maximum diameter of the treated lesion (cutpoint 30 mm). An inverse relation between the maximum diameter and differentiation was previously observed in resected ICC[207].

7. Final discussion and conclusions

In this doctoral thesis we reported an intense, coherent research that began with the etiology of CC, providing information for future prevention measures, to end with practical insights for the clinical setting.

In Chapter 3, we reported on the results of the first prospective, broad etiologic study on CC. Metabolic syndrome, lithiasic pathology, and viral hepatitis were the predominant risk factors. High prevalence of smoke and past, acute hepatitis B infections command more future consideration for these risk factors. On small numbers, prognostic analysis failed to catch statistically significance (with the exception of hypoalbuminemia) but suggested a prognosis-modifying role for hepatitis that was further dealt with in Chapter 3. Results of the BI-CAUSE trial await publication: while an abstract earned the right to a poster presentation at a recent congress of the Associazione Italiana di Oncologia Medica (AIOM), a paper *in extenso* is under elaboration.

In Chapter 4, we presented the analysis of the prognostic and predictive roles exerted on ICC by five categories of hepatitis, derived from the combination of anamnestic findings and laboratory data. A high prevalence of hepatitis was confirmed in our large Western retrospective cohort. With some due caveats, our findings suggested interesting hypotheses to be subject to further confirmation: a cholangiocarcinogenic role even from past hepatitis infection; the correlation of HCV infection with lower markers of inflammation; the lack of prognostic impact of the said hepatitis conditions, save for a moderate non-significant detrimental trend for HBV infection; interestingly, the lack of benefit of the standard doublet over gemcitabine monotherapy in hepatitis-related ICC, at least in HBV-related cases.

In Chapter 5, we proposed a set of easily retrievable prognostic variables, capable of predicting OS in a large, unselected, real-life population of aBTC patients undergoing CT1. Computation of selected variables into a prognostic score provided a tool to perform prognostic stratification with moderate accuracy. This tool could result in being useful to the clinician, in order to ascertain the potential benefit from CT1 during the clinician-patient discussion at the start of treatment. From the BICC study database a number of studies were published, two of which I first-authored: *A prognostic model in patients with advanced biliary tract cancer receiving first-line chemotherapy* (Filippi R et al; Acta

Oncol. 2021 Oct;60(10):1317-1324. doi: 10.1080/0284186X.2021.1953704); *Clinical insights and prognostic factors from an advanced biliary tract cancer case series: a real-world analysis* (Filippi R et al; J Chemother. 2022 Apr;34(2):123-132. doi: 10.1080/1120009X.2021.1953887), which is not presented in this doctoral thesis. The work on viral hepatitis in ICC is ready for submission.

In Chapter 6 we presented a strategy of intensification of the treatment of a niche of aBTC. The integration of LRTs into the therapeutic CT sequencing in suitable relapsed BTC (mostly ICC) patients proved safe and promising. With the limits of missing data, periprocedural complications were low, and the approach achieved a PFS comparable to a CT line alone, with no appreciable differences between surgical and non-surgical approaches. One-third of patients underwent repeat LRTs. In addition to the existing contributions in literature, ALT-rBTC study provides useful evidence to inform multidisciplinary team discussion of relapsing BTC.

BTC and CC remain understudied and poorly understood nosological entities. While evidence slowly begins amassing, a deeper comprehension of causative mechanisms and influence of the disease course, as well as new therapeutic solutions, is urgently needed. Indeed, incidence is on the rise, while surgery of the localized forms represents the only curative treatment, as survival of the advanced forms remains globally dismal. With this work we provided insights on the whole description of BTC, from etiology to prognostication, to treatment, with specific objectives chosen to fill the gaps of the existing knowledge. The present work shed new lights on particular aspects of the disease, from a Western point of view. As it is the case with research, answers were found, but new questions were raised. My future work will seek to further our comprehension of BTC along this directions.

8. List of abbreviations

Ab: antibody
aBTC: advanced biliary tract cancer
AC: ampullary cancer
AFP: alpha-fetoprotein
AIOM: Associazione Italiana di Oncologia Medica
AJCC: American Joint Committee on Cancer
ALP: alkaline phosphatase
ALT: alanine transaminase
AMA: anti-mitochondrial antibodies
AST: aspartate transaminase
AUC: area under the receiving operator curve
BilIN: biliary intraepithelial neoplasia
BMI: Body Mass Index
BTC: biliary tract cancer
Ca19.9: carbohydrate antigen 19.9
CC: cholangiocarcinoma
CEA: carcinoembryonic antigen
CRF: case report form
CRT: concurrent chemioradiation
CT: chemotherapy
CT1: first-line CT
CUP: cancer of unknown primary
DCR: disease control rate
dECC: distal extrahepatic cholangiocarcinoma
DFS: disease-free survival
ECC: extrahepatic cholangiocaricnoma
ECDC: European Centre for Disease prevention and Control
ECOG: Eastern Cooperative Oncology Group
EGFR: epidermal growth factor receptor
EMA: European Medicine Agency
EU: European Union
FGFR2: Fibroblast-derived growth factor receptor 2
FOLFIRI: irinotecan + 5-fluorouracyl regimen
FOLFIRINOX: oxaliplatin + irinotecan + 5-fluorouracyl regimen
FOLFOX: oxaliplatin + 5-fluorouracyl regimen
GC: gallbladder cancer
GemCis: gemcitabine + cisplatin regimen
GemOx: gemcitabine + oxaliplatin regimen
GGT: γ-glutamil transpeptidase
GICO: Gruppo Italiano Colangiocarcinoma Onlus
Glycated hemoglobin: HbA1c
HAI: hepatic artery infusion
Hb: hemoglobin
HBc Ab/IgM: hepatitis B core antigen antibodies/immunoglobulins M
HBeAg: hepatitis B “e” antigen
HBsAg: hepatitis B surface antigen
HBV: hepatitis B virus
HCC: hepatocellular carcinoma

HCV: hepatitis C virus
HDL: high-density lipoprotein
HR: hazard ratio
HRR: Hb/RDW
IARC: International Association of Cancer Research
ICC: intrahepatic cholangiocarcinoma
ICD(-O): International Codification of Diseases (for Oncology)
ICI: immunocheckpoint inhibitors
IDH: Isocitrate dehydrogenase
Ig: immunoglobulin
IQR: interquartile range
LDH: lactate dehydrogenase
LDL: low-density lipoprotein
LMR: lymphocyte-monocyte ratio
LRT: locoregional treatment
MDT: multidisciplinary team
MSI-H: high microsatellite instability
MWA: microwave ablation
NAFLD: non-alcoholic fatty liver disease
NASH: non-alcoholic steatohepatis
NLR: neutrophil-lymphocyte ratio
OR: odds ratio
OS: median overall survival
PD: progressive disease
pECC: proximal extrahepatic cholangiocarcinoma
PFS: median progression-free survival
PHP: percutaneous hepatic perfusion
PI: prognostic index
PLR: platelet-lymphocyte ratio
PNI: prognostic nutritional index
PS: performance status
PT INR: prothrombin time international normalized ratio (PT INR)
RDW, red blood cells distribution width
RFA: radiofrequency ablation
RR: response rate
SBRT: stereotactic beam radiotherapy
SII: systemic inflammation index
TACE: trans-arterial chemioembolization
TARE: trans-arterial radioembolization
TERT: human telomerase reverse transcriptase
TG: triglycerides
TTC: time-to-chemotherapy
TTP: time-to-progression
UA: unità alcolica, alcohol unit
UICC: Union for International Cancer Control
WBC: white blood cells count
WHO: World Health Organization
XelOx: capecitabine + oxaliplatin regimen
y: year(s)

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