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Emerging molecular target antagonists for the treatment of biliary tract cancer

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Emerging molecular target antagonists for the treatment of biliary tract cancer

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Title

Emerging molecular target antagonists for the treatment of biliary tract cancer

Abstract

Introduction: Biliary tract cancers (BTCs) are a heterogeneous group of cancers, characterized by low incidence but poor prognosis. Even after complete surgical resection for early stage, relapse is frequent and the lack of effective treatments contributes to the dismal prognosis. To date, the only standard treatment in first-line is cisplatin/gemcitabine combination, whereas no standard in 2nd-line has been defined. Hence, the current goal is to better understand the biology of BTCs, discovering new treatment methods and improving clinical outcomes.

Areas covered: The development of next-generation-sequencing has unveiled the picture of the molecular signatures characterizing BTCs, leading to the identification of actionable mutations in biomarker-driven clinical trials. In this review we will cover the genetic landscape of BTC, focusing on the efficacy of existing treatments. Furthermore, we will discuss emerging molecular targets and evaluate the findings of pre-clinical studies. Finally, the encouraging results of clinical trials involving targeted therapies or immunotherapy will be reviewed.

Expert opinion: FGFR fusion rearrangements and IDH1 or IDH2 mutations are the most promising targeted treatments under evaluation. In addition, innovative trial design will allow to offer a chance for tailored medicine to infrequent subgroups of BTCs patients based on their molecular features rather than their histology.

Manuscript

1-Background

Biliary tract cancer (BTC) is a heterogeneous group of tumors arising from the epithelial lining of the biliary tree. The classification is based according to the anatomical location; BTC includes gallbladder cancer (GBC) and cholangiocarcinoma (CCA); the latter is further divided into intrahepatic (IH-CCA), and extrahepatic (EH-CCA), which includes EH-CCA perihilar (PCCA) and distal CCA (DCCA). IH-CCAs arise above the second order bile ducts, whereas PCCAs are located between the insertion of the cystic duct and the second order bile ducts, and DCCAs are located below the insertion of the cystic duct [1, 2].

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3 Even though GBC, intrahepatic, perihilar and distal extrahepatic CCA are grouped as BTCs, their clinical
4 presentation, pathobiology and management are different, and they should be viewed as separate entities.
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6 Despite being considered an infrequent cancer in the Western world, with an incidence of 1-2 cases every
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8 100,000 inhabitants, BTCs are extremely common in South America and in some areas of Asia, with up to 96
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10 cases/100,000 [3].

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12 Histologically, more than 95% of BTCs are adenocarcinomas, often poorly differentiated. About 10% of EH-CCA
13 are well differentiated papillary cancers, but mucinous (5%) and squamous cancers (2%) are also described [4].
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15 Several classifications have been proposed for IH-CCA owing to its highly heterogeneity. The two main
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17 histological subtypes are bile ductular type (mixed), arising from small intrahepatic bile ducts, and bile duct
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19 type (mucinous), arising from large intrahepatic bile ducts. These differences are also reflected in different
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21 molecular characteristics [5]. Notably, bile ductular type IH-CCAs share clinicopathological similarities with
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23 cytokeratin (CK) 19-positive hepatocellular carcinoma (HCC), and the bile duct type IH-CCAs share phenotypic
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25 traits with PCCA and pancreatic cancers [6].

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27 Beyond the differences, BTC have common features, such as a highly desmoplastic reaction, rich tumor
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29 microenvironment, and profound genetic heterogeneity, all contributing to the development of drug resistance
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31 and almost complete absence of curative therapies for metastatic disease.

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33 The vast majority of BTC (70%) occurs sporadically. Nevertheless, several pathologic conditions have been
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35 determined as possible risk factors such as Primary Sclerosing Cholangitis (PSC), choledochal cysts, parasitic
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37 infestation, and viral hepatitis B and C. Potential risk factors with less evidence are diabetes, alcohol, smoking,
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39 obesity and specific genetic polymorphisms [7]. Therefore, although a single trigger cannot be identified,
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41 different environmental, genetic and social factors may be rather involved and justify heterogeneous
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43 geographic distribution.

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45 Most patients with early stage disease are asymptomatic and diagnosing BTC at an early stage remains a
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47 challenge owing to its 'silent' clinical presentation, difficult to access anatomical location, and highly
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49 desmoplastic, paucicellular nature, which limits the sensitivity of cytological and pathological diagnostic
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51 approaches.

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53 Surgery is the preferred treatment option for localized BTC, but only a minority of patients (approximately
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55 35%) has early stage disease that is amenable to surgical resection with a curative intent. Survival after radical
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57 surgery generally depends on margin status (negative-R0 or positive-R1-R2 status), vascular invasion and lymph
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59 node involvement. Even after R0 resection 3-years survival rate is approximately 40-60%, and it can be lower in
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3 case of lymph node positivity [8].
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6 The unfavorable prognosis of recurrent disease drove efforts to lower the rate of relapse through
7 postoperative adjuvant therapy. So far, the great part of the available literature was made of retrospective,
8 non-randomized studies and meta-analyses: the majority took in consideration heterogeneous populations of
9 patients, treated with different systemic drugs with or without radiotherapy. Recently, following the
10 presentation of the preliminary results of the BILCAP study, a standard of care for adjuvant treatment is
11 available. In this randomized phase III trial, postoperative capecitabine was compared to surveillance: the
12 primary endpoint of the study was formally not met, despite a difference of 15 months in the median overall
13 survival (mOS) estimated values (51 versus 36 months, $p = 0.097$). However, mOS was significantly higher in the
14 chemotherapy arm both after sensitivity analyses and in the per protocol analysis [9].
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21 For patients with advanced-stage or unresectable disease, the available systemic therapies are of limited
22 effectiveness: the mOS with the current standard-of-care regimen (gemcitabine and cisplatin) is <1 year [10]
23 and a probability to outlive 5 years is about 5% [11].
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30 **2-Medical need**

31 Randomized trials have shown that systemic chemotherapy increases survival and improves quality of life (QoL)
32 in patients with metastatic BTC as compared with best supportive care (BSC). In the study by Glimelius, 90
33 patients with metastatic or locally advanced pancreatic carcinoma or BTC have been randomly assigned to 5-
34 fluorouracil (5-FU) -based chemotherapy or BSC. A clear benefit of chemotherapy on both mOS (6 vs 2.5
35 months in BSC group $p < 0.01$) and QoL was demonstrated [12]. More recently, modified gemcitabine and
36 oxaliplatin (mGEMOX) proved a significant improvement in mOS not only over BSC, but also over 5-FU/folinic
37 acid in unresectable GBC (9.5, 4.6 and 4.5 months respectively)[13].
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44 The majority of trials in the metastatic setting were performed with fluoropyrimidine or gemcitabine
45 monotherapy or in combination with other cytotoxic agents. 5-FU as single agent yields a variable response
46 rates (RR) from 10% to 40%. The combination of 5FU with other drugs (etoposide, interferon, cisplatin and
47 oxaliplatin) has proved moderate efficacy, limited overall survival (OS) benefit, and a significantly greater
48 toxicity profile. Since the late 90s, the adoption of gemcitabine as the standard of care for patients with
49 pancreatic cancer led to interest in its use for hepatobiliary tumors. As a single agent, gemcitabine has shown
50 RRs ranging from 0% to 30%, whereas its association with other agents has determined advantages in survival
51 and RRs (up to 41%) [14]. In 2007 a pooled analysis of 104 trials in advanced BTC, demonstrated the superiority
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3 of combination therapy compared to monotherapy in RR and tumor-control-rate (TCR). Subgroup analysis also
4 underlined that gemcitabine and platinum association had significantly higher response and TCRs compared to
5 fluoropyrimidine/gemcitabine monotherapy or fluoropyrimidine plus platinum regimens [15].
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9 Suggestions from this meta-analysis were turned into a standard of care by Valle's Phase III ABC- 02 trial. Four
10 hundred and ten patients with locally advanced or metastatic CCA, GBC and ampullary carcinoma were
11 randomly assigned to receive gemcitabine alone (1000 mg/m² days 1, 8, 15 q 28) vs gemcitabine and cisplatin
12 (1000 mg/m² + 25 mg/m² days 1, 8 q 21) for up to 24 weeks of treatment. After a median follow-up of 8.2
13 months, mOS, that was the primary endpoint of the study, was statistically improved in the combination arm
14 (11.7 vs. 8.1 months; $p < 0.001$). The benefit was preserved across the subgroups according to primary tumor
15 site, median progression-free-survival (mPFS) was improved (8.0 months vs. 5.0 months; $p < 0.001$), without
16 significant increase in toxicity. [10]. Following the results of this trial, a definite standard regimen for a disease
17 that has been "orphaned" for too long, was finally provided. Equivalent results have been replicated in the
18 japanese population [16]. Other combination schemes have also been evaluated as first line treatment in phase
19 II trials [17-19]. Among these, gemcitabine and oxaliplatin (GEMOX) is widely used in clinical practice for its
20 good response rate and favorable toxicity profile, although a direct comparison with the standard of care is
21 lacking [13, 20].
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30 Following a meta-analysis of the English and Japanese randomized trials, single agent gemcitabine is a
31 recommended option only for patients with Performance Status (PS) 2 according to Eastern Cooperative
32 Oncology Group (ECOG) scale [21].
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36 After the failure of the 1st line therapy, approximately half of the patients still have a good PS and satisfactory
37 organ function [22], but the advantages of 2nd line therapy are still unclear. In a large retrospective analysis, 196
38 patients who received 2nd line treatment after gemcitabine and cisplatin/oxaliplatin, were analyzed. The most
39 common regimens used were 5-FU/folinic acid, FOLFIRI, XELIRI, FOLFOX, XELOX, 5-FU and cisplatin: globally,
40 the outcome was poor, the mPFS and OS being 3.2 and 6.7 months, respectively, and no chemotherapy
41 regimen proved superiority over the others [23]. In another Italian retrospective analysis, PS emerged as the
42 most important prognostic factor to select the patients that may benefit from 2nd line treatment [24].
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51 **3-Existing treatment**

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54 In the past decade we have entered the era of targeted therapies: this strategy has modified the therapeutic
55 approach of many cancers but the first attempts of using targeted treatments in BTCs have been so far
56 unsatisfactory. Here, we revise the first studies that have explored targeted therapies in BTC.
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• 3.1 EGFR family pathway

Epidermal growth factor receptor (EGFR) is mutated and overexpressed in cancer human samples. EGFR is extensively represented in BTC, being expressed in 100% of IH-CCAs, 52.6% of EH-CCAs, and 38.5% of GBCs. [25]. Mutations have been found in up to 15% of BTCs [26, 27]. EGFR activation triggers the Mitogen Activated Proteine Kinases (MAPK)–ERK pathway, an oncogenic signalling pathway in cancer: it donates a proliferative advantage to cancer cell, contributes to progression through epithelian-mesenchymal transition (EMT) and leads to a poor clinical outcome [28, 29]. In preclinical studies, EGFR inhibitors tested in combination with chemotherapy have shown promising activity, providing a strong preclinical rationale for anti-EGFR therapy in BTC. Different strategies targeting EGFR have been studied such as tyrosine kinase inhibitors (TKIs) or monoclonal antibodies (mAbs) alone or in association with chemotherapy.

Erlotinib, a selective, reversible, orally-active EGFR inhibitor, after promising results in several phase II studies, failed to confirm its superiority in OS in a large phase III study. Compared to the combination of GEMOX, the addition of erlotinib in patients with newly diagnosed metastatic BTC significantly increased RR (40% vs 21%; $p=0.005$) but mPFS and OS were not increased (4.2 vs 5.8 months $p=0.087$ and 9.5 months in both arms, $p=0.611$ respectively). Subgroup analyses showed that in patients with CCA the addition of erlotinib prolonged mPFS (5.9 months vs 3.0 months; HR 0.73, 95% CI 0.53–1.00; $p=0.049$) [30].

Cetuximab is a mAb targeting EGFR, evaluated in combination with chemotherapy in BTC in several phase II trials. In a phase II study of cetuximab in combination with GEMOX, the treatment achieved a mPFS of 8.3 months and OS of 12.7 months, with high RR [31]. Following randomized trials failed to demonstrate a significant benefit of the GEMOX and cetuximab combination. In the BINGO study, cetuximab was tested in the 1st line setting in addition to GEMOX vs GEMOX alone. Despite being well tolerated, cetuximab did not significantly improve outcomes: mPFS was 6.1 months with cetuximab and 5.5 months without cetuximab, while mOS was 11.0 months with cetuximab and 12.4 months without cetuximab [32]. In a similar study in the Asiatic population, mPFS was 6.7 months with cetuximab and 4.1 months without cetuximab ($p = 0.05$), while mOS was 10.6 months vs 9.8 months respectively ($p = 0.91$). KRAS mutations, that are a well-known negative predictive factor of response to anti-EGFR mAbs, were identified in 36% of tumors, and did not affect the overall response rate (ORR) or mPFS [33].

Panitumumab, a fully human antibody against EGFR, has been tested in combination with chemotherapy in non-randomized and randomized, phase II trials. In particular, in the Vecti-BIL trial advanced BTC patients were selected upfront for KRAS mutational status. The study revealed that the addition of panitumumab to GEMOX compared to GEMOX alone did not significantly improve mPFS, (5.3 months in experimental arm vs 4.4 months

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3 in control arm), neither OS (10.2 months in experimental arm vs 9.9 months in control arm) in KRAS wild type
4 (WT) BTC patients [34].

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7 Despite the results of a recent meta-analysis suggesting a potential role of anti-EGFR therapy in prolonging PFS
8 and RR, the current evidence does not support its use in BTC [35].

11 • 3.2 MEK pathway

12 Targeting the RAS/RAF/MEK/ERK pathway, a major player in the cellular processes, including proliferation and
13 apoptosis, is thought to be a winning strategy in many cancers including BTC.

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17 The second-generation, uncompetitive inhibitor of MEK 1/2 - **selumetinib** - was investigated in a phase II study
18 of 28 advanced BTC patients. Median PFS was 3.7 months and the mOS was 9.8 months, with a 12% of ORR
19 [52]. Recently a Phase Ib trial has tested the pharmacokinetics and toxicity profile of selumetinib in
20 combination to cisplatin and gemcitabine. Other trials are needed to demonstrate its applicability in clinical
21 practice [53].

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26 Another uncompetitive inhibitor of MEK 1/2, **binimetinib**, after encouraging results in safety and activity during
27 phase I studies [54], is currently under evaluation in phase II studies (NCT02151084, NCT01828034).

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31 Recently, the open-label, multicentre, single-arm trial, evaluating pazopanib - an orally available multikinase
32 inhibitor of VEGF (vascular endothelial growth factor) receptor, PDGF (platelet-derived growth factor) receptor,
33 c-KIT (stem-cell growth factor receptor), fibroblast growth factor receptor (FGFR) and RAF - in addition to
34 **trametinib** - an orally available highly specific inhibitor of MEK 1 and MEK 2 - showed discouraging results.
35 Despite the trend towards increased 4-month PFS, the difference did not reach statistical significance. The mOS
36 was 6.4 months (95% CI: 4.3–10.2) and the ORR was 5% (95% CI: 0.13–24.9%) [55].

41 • 3.3 VEGF

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44 The vascular endothelial growth factor (VEGF) pathway promotes tumor growth - by angiogenesis and vascular
45 permeability - in several tumors including BTC [36]. In CCA, VEGF expression is reported in about 30-40% of
46 cases and is correlated with lymphnode metastasis and poor prognosis [37, 38]. VEGF expression is reported in
47 50% of GBC and also in this sub-group a poor prognosis is observed [39]. Based on preclinical and clinical data
48 supporting a VEGF-targeted approach, several studies have evaluated the effectiveness and applicability of
49 targeted therapy (bevacizumab, sorafenib, cediranib, vandetanib) in metastatic BTC, providing only limited
50 information, principally because of their phase II design. Therefore, the role of antiangiogenic agents in the
51 treatment of BTC is not well defined.

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3 **Bevacizumab** is a recombinant humanized mAb whose efficacy has been tested in patients affected by
4 advanced BTC. In two studies it was tested in combination with erlotinib and in sequence with gemcitabine and
5 oxaliplatin, without favorable results [40, 41]. More recently, a small phase II study evaluated the combination
6 of gemcitabine, capecitabine, and bevacizumab in a first-line setting, showing a mPFS and mOS similar to those
7 observed with standard chemotherapy (8.1 months and 11.3 months, respectively) [42]. Larsen et al. recently
8 presented the results of a phase II trial of capecitabine, irinotecan, gemcitabine, and bevacizumab as second-
9 line setting in 50 BTC patients. Median PFS was 3.6 months and mOS was 6.4 months [43]. To date, the role of
10 bevacizumab on BTC has been evaluated only in single-arm studies, with no randomized study performed.

11
12 **Cediranib** is a potent inhibitor of the VEGF receptor tyrosine kinases -also directed against PDGF receptors and
13 c-KIT being investigated in a recent phase II study [44]. Patients were randomized to receive cisplatin and
14 gemcitabine plus either cediranib or placebo. Cediranib did not significantly improved mPFS (7.7 months in the
15 cediranib arm vs. 7.4 months in the placebo arm). There was a trend towards longer OS in the experimental
16 arm compared to placebo (14.1 vs 11.9 months, respectively) [45].

17 For definitive conclusions, we will have to wait for the results of the ongoing phase II trial, investigating
18 cediranib in combination with modified FOLFOX6 in advanced BTC (NCT01229111).

19
20 **Sorafenib** is an oral multikinase inhibitor directed against VEGF receptors and PDGF receptor- β , and blocking
21 downstream intracellular serine/threonine kinases, like Raf-1, WT and mutant B-Raf.

22 After the defeats in monotherapy in several studies, the more recent conducted by Luo et al [46], Sorafenib
23 was evaluated in combination with standard chemotherapy.

24 First, Moehler et al. investigated Sorafenib in the first line setting, treating patients with gemcitabine with
25 either sorafenib or placebo. In this study, longer mPFS and OS were found in the gemcitabine plus placebo arm
26 [47]. Then, a randomized, double-blinded, multicenter phase II trial investigating a combination of gemcitabine,
27 cisplatin with either sorafenib or placebo demonstrated no significant difference in mPFS and mOS between
28 the two arms [48]. Finally, a small trial conducted by Lee et al. showed that the addition of sorafenib to
29 gemcitabine and cisplatin did not improve efficacy over historical data, but there was an increase of toxicity
30 [49].

31
32 **Sunitinib** is another oral small molecular-targeted drug inhibiting several intracellular and receptor protein
33 kinases, including VEGF and PDGF receptors, c-KIT, and rearranged during transfection (RET). In the only
34 available phase II study, the role of sunitinib was investigated as a second-line treatment in advanced BTC
35 demonstrating only marginal efficacy; ORR was 8.9 % and mPFS was 1.7 months [50].

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3 **Vandetanib** is an orally active, multikinase inhibitor targeting VEGF receptor 2, EGFR and RET kinase. In the
4 Italian "VanGogh" study, 165 BTC patients chemotherapy-naïve were randomized into three groups:
5 vandetanib monotherapy, vandetanib plus gemcitabine and gemcitabine plus placebo. Median PFS was 105
6 days (95 % CI 72–155), 114 days (95 % CI 91–193), and 148 days (95 % CI 71–225) respectively, while mOS was
7 228 days (95 % CI 190–364), 284 days (95 % CI 213–359), and 307 days (95 % CI 254–523) for the three arms
8 respectively. Thus, vandetanib alone or in association with gemcitabine did not demonstrate any superiority
9 when compared with gemcitabine alone [51].

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16 In conclusion, the role of VEGF inhibition in addition to chemotherapy for patients with advanced BTCs remains
17 still investigational, but increasingly burdened by the absence of a biomarker of efficacy for VEGF inhibitors.
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20 21 22 **4-Market review**

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25 Throughout the therapeutic course of BTC patients, complications are a constant. Infections, sepsis and
26 cholangitis often undermine patients' health. Furthermore, chemotherapy is associated with high rate of side
27 effects that requires spending of many economic resources.
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31 We have considered two cost-effectiveness analysis conducted in USA and Japan. They evaluate cost-
32 effectiveness of combination treatment with gemcitabine and cisplatin compared to treatment with
33 gemcitabine alone. Despite being two comparable analyses in different populations, their conclusions are
34 opposite; Roth et al. demonstrated that cisplatin and gemcitabine combination was a cost-effective alternative
35 to gemcitabine monotherapy. Tsukiyama et al., on the contrary, suggested that gemcitabine monotherapy was
36 a better treatment strategy for advanced BTC. These different conclusions may be due to varying health care
37 conditions and different long-term palliative care cost between the countries.
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44 Surprisingly, in both studies the majority of the resources were employed for supportive and palliative care
45 rather than for drugs [56, 57].
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48 For these reasons, systemic treatments are required not only to prolong survival, but also to limit palliative
49 interventions. Recently, immunotherapy and target therapies have demonstrated to increase QoL and to be
50 associated to lower rates of side effects in several cancer types as compared to chemotherapy [58-60]. For
51 these reasons, alternative treatment strategies are also strongly encouraged in BTC patients.
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5-Current research goals

Carcinogenesis is a multistep process encountering imbalance between proliferative and inhibitory stimuli, dysregulation of apoptotic signals and abnormal response to angiogenetic factors which are responsible for invasion and metastatization events [61]. It involves specific cell genome derangements that contribute to the selective growth advantage of cancer. Specific pathways govern cell fate through proliferation, cell survival, differentiation, epigenetic changes and maintenance of genome integrity [62].

The genomic heterogeneity of BTC is well-known. As previously discussed, it's not only related to the diverse anatomical location of the tumor but also to etiology, the various risk factors and associated pathologies. Interestingly, mutations in TP53 responsible for genome integrity are very common in all subgroup of BTC but are more often observed in liver-fluke related CCA [63]. These differences will need to be considered when assessing outcome and in devising therapeutic strategies for CCA and GBC [2, 61, 64-67]. Advances in genome-wide technologies have made feasible the discovery of other possible targetable or actionable molecular alterations in BTC. Precision therapy for this group of tumors is subordinated to an enhanced understanding of genetic and molecular alteration for each subtype.

6-Scientific rationale and molecular pathogenesis

- **6.1 Proliferation and cell survival.**

Several growth pathways are implicated in arising and progression of BTCs. Here we revise the most promising preclinical evidences that could, in the near future, have a clinical counterpart.

ErbB family, that includes four different receptors (ErbB1 or EGFR, ErbB2 or HER-2/neu, ErbB3 and ErbB4), have a main role in carcinogenesis processes and represents the most extensively mutated pathway of the GBC samples [68]. We have already introduced the role of EGFR and its clinical implications.

Human epidermal growth factor receptor 2 (HER2) amplification has been observed in BTCs patients with a prevalence in EH-CCA (about 20%), GBC (9.8%) and a rare incidence in IH-CCA. The prognostic meaning of HER2 has not yet been completely clarified but could become a relevant predictive factor [68, 69].

KRAS mutations occur in BTC and the reported incidence is 7-47%. It is usually associated with the alteration of other factors, such as EGFR, HER2 or MET [68, 70]. A recent work showed poor prognosis for KRAS mutated CCA that is more pronounced in the subgroup with transcriptional enrichment of genes that regulate proteasome activity [71].

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3 Some studies reported BRAF mutations in a percentage between 0% and 22%. In a large cohort, BRAF V600E
4 mutation was observed in 5.9% of GBC, 3% of IH-CCA and 0% of EH-CCA and no clear correlation with prognosis
5 was detected [72, 73].
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8 Recently, ARAF mutation (ARAFm), a member of the RAS family, has been reported in 11% of IH-CCA iCCA.
9 ARAFm leads to an increased basal and inducible activity when compared to WT protein. Further studies need
10 to investigate the oncogenic potential of this new target. [74].
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13 ROS1 fusion proteins have been recently implicated in CCA carcinogenesis. The fused-in-glioblastoma-c-ros-
14 oncogene 1 (FIG-ROS) was found in 14-16% of EH-CCA [75] and it mediated cancerogenesis in KRAS dependent
15 mode with a high aggressiveness [76].
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18 The expression level of hepatocyte growth factor (HGF) receptor (also known as c-Met) is amplified either in
19 CCA and GBC. Miyamoto analyzed 247 patients and noted MET high expression in 11.7% of IH-CCA and 16.2%
20 in EH-CCA. MET seems to play a central role in carcinogenesis through the protection from apoptosis and
21 promoting angiogenesis and tumor invasion. In CCA it is associated with EGFR expression and represents a poor
22 prognostic factor. Moreover, it could be linked to acquired resistance to EGFR or HER2 inhibitors [77, 78].
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27 FGFR is a tyrosine kinase receptor involved in numerous cellular process including proliferation, angiogenesis
28 and tissue repair. Disruption of this pathway has been implicated as driver event in biliary cancer formation.
29 Fusions of FGFR gene have been reported in some cancer type; in BTC several gene fusions have been
30 described, the most frequent being FGFR2-BICC1 fusion. This new protein seems to have a higher incidence in
31 female and a link with hepatitis infection. It is constitutively active and leading to the activation of MAPK and
32 phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA)/ mammalian target of
33 rapamycin (mTOR) pathways. Moreover, FGFR translocation may confer a survival benefit. Indeed, in a western
34 dataset a superior cancer specific survival was observed in patients with FGFR2 translocation compared to non
35 translocated patients (123 months vs 37 months) [65, 74, 79].
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43 Aberration in tumor suppressive gene phosphatase and tensin homolog (PTEN) has been correlated with
44 clinical outcome. Indeed, PTEN loss has been associated with poor outcome in all BTC, particularly in
45 combination with either activated Protein kinase B (PKB), also known as Akt or mTOR. However, genetic
46 alteration in AKT genes, with normal level of PTEN, were associated with cancerogenesis but also with
47 favorable prognosis. These findings suggest isolated AKT alteration could have an important role in the
48 initiation of IH-CCA but not in progression of this subgroup [80-82].
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54 Different pathways have been implicated in carcinogenesis and proliferative advantage in CCA. However, these
55 pathways overlap in several points, providing a molecular reason for resistance to target therapies.
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Besides, not every cancer has a proliferative signature. Sia et al., using an integrative molecular analysis technique, have identified gene signatures in IH-CCA and have correlated them with pathological features and clinical patients' outcome. The authors describe two categories of IH-CCA: a proliferative class and an inflammatory class. The proliferative class, that accounts for 62% of cases, was typified by alterations in several oncogenes included in RAS-RAF-MEK-ERK or PIK3CA-AKT-mTOR signaling pathway, implicated in cell proliferation and cell survival, respectively.

The inflammatory class showed activation of inflammatory pathways, overexpression of cytokines (i.e. IL-6) and signal transducer and activator of transcription 3 (STAT3). STAT3 is a mediator that modulates cell growth and survival while IL-6 is an inflammatory cytokine produced either by CCAs and cholangiocytes stimulated by an inflammatory *noxa*. IL-6 is involved in cell survival through upregulation of Mcl-1 via AKT-dependent mechanism. Mcl-1 mediates tumor necrosis factor-related apoptosis inducing ligand (TRAIL) resistance and cell survival. IL-6 is also implicated in upregulation of Mcl-1 through a STAT3 dependent mechanism [83, 84].

- **6.2 Cell differentiation**

The Notch signaling pathway has a main role in cell differentiation, inflammation and carcinogenesis. Notch activation was implicated in de-differentiation of adult hepatocytes into precursors of IH-CCA. These studies not only show the high plasticity of liver cell but also change the traditional model according to which CCA cells derived from cholangiocytes or hepatic common progenitor cells [85]. These observations could be combined to Sia et al. data. In this work, the authors describe how gene signatures, especially the proliferation class one's, overlap with those identified in HCC. These data provides us a second model of CCA carcinogenesis; not only based on alteration of epithelial cells of biliary tree but also on de-differentiation of adult hepatocytes.[84].

- **6.3 Epigenetic changes.**

Tumor cells may also acquire an advantage in survival and proliferation through epigenetic changes that lead to silencing of onco-suppressor genes. Mutations of isocitrate dehydrogenase 1 (IDH1) and 2 (IDH2) are among the most common genetic alteration in IH-CCA (14-36%) [70, 86], with unclear prognostic significance [87]. No mutations in these genes were observed in EH-CCA and GBC. Mutant IDH proteins lead to an abnormal enzymatic activity inducing to production of 2-hydroxyglutarate (2-HG) from α -ketoglutarate (α KG), which is considered an oncometabolite and causes epigenetic changes. Furthermore, deficiency in α KG inhibits activity of α KG-dependent dioxygenase and results in altered cell differentiation, survival and extracellular matrix maturation [88, 89]. Mutant IDH blocks liver progenitor cells that, as a result of altered hepatocyte response to hepatic injury, could lead to the development of malignant lesions. Indeed, a recent work has shown that mutant IDH blocks hepatocyte differentiation through the production of 2-HG and the suppression of HNF-4 α ,

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3 the main regulator of hepatocytes identity and quiescence [90]. Farshidfar et al. have associated mutant IDH
4 with a specific gene signature characterized by high mitochondrial and low chromatin modifier gene
5 expression. Speculating about the significance of this signature is complex for the wide number of genes and
6 processes involved. Potentially, the combination of anti-IDH targeted therapies with anti-mitochondrial activity
7 drugs could be exploited in the future as a therapeutic strategy [91].
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11 12 • **6.4 Angiogenesis and tumor environment.** 13

14 VEGF has a central role in carcinogenesis and it has been targeted in different malignancies. In CCA, VEGF
15 expression is reported in about 30-40% of cases and is correlated with lymph node metastasis and poor
16 prognosis [38]. VEGF expression is reported in 50% of GBC and also in this sub-group a poor prognosis is
17 observed [39].
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21 BTC have a characteristic hypovascular, desmoplastic stroma that plays an important role in tumor
22 pathogenesis and is consisting of cancer associated fibroblasts (CAF) expressing α -smooth muscle actin (α -
23 SMA), activated macrophages and a fibrotic collagen rich extracellular matrix [92].
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27 α -SMA positive CAFs are involved in cancer progression through production of matricellular proteins, growth
28 factors, chemokines, and matrix metalloproteinases. Patients expressing high levels of α -SMA have poorer
29 survival [93]. The desmoplastic matrix also allows the development of a niche fostering tumor spread rather
30 than as a response to the anticancer treatments [94]. Transforming growth factor β (TGF- β) seems to be
31 implicated in the generation of the niche. Indeed, preclinical models have demonstrated a reduction in fibrosis
32 and tumor spread with TGF- β antagonist [95]. CAFs produce numerous factors involved in autocrine and
33 paracrine signalling that promote oncogenic processes like periostin, tenascin-c, thrombospondin 1, stromal
34 cell derived factor 1 (SDF-1), HGF and Wnt inducible signalling protein-1v (WISP1) [93]. These interact with cell
35 signalling pathways. For example, periostin interacts with tenascin-C, HGF and SDF-1, which bind to their
36 respective receptors, integrin, MET and CXCR4 on CCA cells, leading to activation of the PIK3CA/AKT signalling
37 pathway.
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47 Finally, desmoplastic stroma may be influenced also by sonic Hedgehog (Hh) signalling pathway. Preclinical
48 models have disclosed the interplay between Hh and CAF through platelet-derived growth factor BB [96].
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51 Cancer associated macrophages (CaM) are implicated in formation and maintaining of the stromal
52 microenvironment and appear to have prognostic significance. A high number of CD163+ macrophages in the
53 stroma of resected IH-CCA correlates with poor disease-free survival [97].
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3 Desmoplastic stroma formation is also associated with increased level of IL-6 that promotes tumor growth via
4 autocrine and paracrine mechanisms [93].
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7 For all these reasons, targeting stromal factors involved in cholangiocarcinogenesis or improving drug delivery
8 through the desmoplastic stroma are attractive targets for novel therapeutics.
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11 • **6.5 Immune system.**

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13 In BTC, a number of clinical and epidemiological factors might predict the efficacy of immunotherapies. Several
14 chronic infections, such as viral hepatitis and bacterial cholangitis, are established risk factors for
15 cholangiocarcinoma. Notably, immunotherapies have shown promising efficacy in cancer associated with
16 infections, probably thanks to the presentation of non-self or neoantigens related with infections [98].
17 Furthermore, in a patient subgroup of BTC with poor prognosis has been revealed had a high mutational load,
18 resulting in abundant tumor-specific neoantigens, and enrichment for expression of immune-related genes,
19 including genes encoding inhibitory immune-checkpoint proteins [65]. In these patients, immune-checkpoint
20 inhibition could permit to overcome cancer related mechanism of immune-silencing.
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24 A central role in immune response is played by CaM that, through the production of soluble factor such as
25 interleukins or cytokines, modulates anticancer immune response and maintain stromal environment [97].
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29 In some small studies, programmed death-ligand 1 (PD-L1) expression has been evaluated on cancer specimens
30 and on immune cells within the tumor microenvironment [99, 100]. PD-L1 is one of the most studied biomarker
31 and levels of tumor PD-L1 expression have been associated with sensitivity to immune checkpoint inhibitor
32 monotherapy in several tumor and they could predict response to the immunotherapies.
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42 **7- Competitive environment and potential development issues**

43 • **7.1 HER2**

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45 HER2 also belongs to the ErbB family of tyrosine kinases receptor and it is seems to be overexpressed in
46 approximately 10% of GBC and 26.3% EH-CCA [25]. Given the efficacy of agents targeting HER2 in other cancers
47 types, they were tested in BTC patients, nevertheless none improved outcomes so far.
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51 **Lapatinib** is a dual tyrosine kinase EGFR and HER2 inhibitor; it was tested in two phase II studies in patients
52 with advanced BTC. The first one was conducted on hepatobiliary cancer patients including 19 BTC, reaching
53 poor results (mPFS was 1.8 months and mOS was 5.2 months) and no objective responses [101]. Later, similar
54 results have emerged from a phase II trial involving only BTC patients: the response was extremely poor (0%),
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3 leading to premature discontinuation of the trial [102]. The role of **Trastuzumab**, a mAb that targets the HER2
4 receptor, is so far not well defined in treatment of BTC. The only data available comes from a retrospective
5 analysis in which Javle et al. studied BTC patients with HER2 genetic alterations or protein overexpression
6 treated with HER2-directed therapy in combination with concurrent therapy of physician's choice. Among the
7 eight GBC patients who received trastuzumab, one patient experienced complete response (CR), four patients a
8 partial response (PR), and three had stable disease (SD). The median duration of response in these patients was
9 40 weeks. In contrast, among five CCA patients no response was observed and disease progression occurred
10 during treatment with trastuzumab [103]. Likewise, in 2012 Law reported a case of a patient affected by HER2
11 positive GBC, showing a dramatic response after nine weeks of treatment with trastuzumab and paclitaxel
12 [104].

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14
15 **Neratinib**, a more recent tyrosine kinase inhibitor HER2-directed is actually under evaluation in SUMMIT, an
16 ongoing basket trial involving a variety of tumor types harboring HER2 mutations, including BTC
17 (NCT01953926). Preliminary data, recently presented during the American Association for Cancer Research's
18 Annual Meeting 2017 showed an ORR of 22% in BTC (9 patients) [105].

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21 Similarly, in a phase II trial evaluating the efficacy of **Afatinib** - a potent, orally bioavailable ErbB Family Blocker
22 - was tested in different malignancies including 5 BTCs presenting HER2 amplification [106]. As shown in table
23 1, afatinib is currently under evaluation, in association with capecitabine, in a phase I/Ib trial addressed to
24 patients with advanced refractory solid tumors, comprising pancreatic cancer and BTCs (NCT02451553) (Table
25 1, New therapies under evaluation in biliary tract cancers).

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28 Given these contradictory outcomes, HER2-directed therapy remains an open chance of treatment for BTC
29 patients with gene amplification, especially in GBC. A phase II trial of trastuzumab-emtansine is currently
30 ongoing in HER2-positive BTC patients (NCT02999672) (Table 1.)

31 32 33 34 35 36 37 38 39 40 41 42 43 44 • **7.2 PIK3CA/PTEN/AKT/mTOR pathway**

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46 The PIK3CA/PTEN/AKT/mTOR pathway has drawn attention in last years as a target for new drugs
47 development. About 12.5% of GBC patients have activating mutations of PIK3CA [107].

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50 In a phase II Italian study, 39 patients with advanced and pre-treated BTC received **everolimus** - an mTOR
51 inhibitor; mPFS was 3.2 months, and mOS was 7.7 months [108]. Since only patients who had received no more
52 than one previous systemic chemotherapy regimen were enrolled, these results are at least in line with
53 conventional 2nd-line chemotherapy results in advanced BTC, reporting a mPFS and mOS of 2.8 and 7.5 months
54 respectively.
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3 At the same time, in the Australian phase II study, everolimus was investigated in the first line setting in
4 advanced BTC. Final results are not yet available, but preliminary findings are encouraging: ORR of 12 % and
5 mPFS of 6.0 months [109].
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9 More extensive studies are needed to clarify the efficacy of mTOR inhibitors in the treatment of BTC.
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11 As shown in table 1, a phase II trial using a PIK3CA inhibitor, **copanlisib** in CCA, in first line setting in
12 combination with cisplatin and gemcitabine is ongoing (NCT02631590) .
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- 15 • **7.3 BRAF**

16 BRAF mutations occur in a small portion of BTC patients; the most common mutation is V600E, whose
17 frequency range from 5 to 8% [110].
18

19 In the study of Hyman the efficacy of **vemurafenib** was evaluated in non-melanoma cancers. In the cohort of 8
20 CCA patients harboring BRAF V600E mutation, it was described one PR, which have persisted for more than 12
21 months, four SD and three progression disease (PD) [111].
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24 Furthermore, given the impressive activity noted in metastatic melanoma with BRAF V600 mutations using a
25 combination of **dabrafenib** (BRAF inhibitor) and **trametinib** (MEK1 and MEK2 inhibitor), dual blockade merits
26 evaluation also in this subset. A case report of a notable response in a IH-CCA patient who received this
27 combination supports this hypothesis [112].
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- 32 • **7.4 ROS, ALK, NTRK**

33 The presence of NTRK fusions in patients diagnosed with IH-CCA have been stated around 3.5% [113]. Sporadic
34 fusions of ROS1 (up to 8.7%) and ALK (2.6%) are also described [114].
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38 Compounds targeting an NTRK1/2/3, ROS1, or ALK gene rearrangement have demonstrated impressive ORR
39 (57-86%) in a selected population of solid tumors [115].
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41 Based on this data and the well-known clinical activity of ALK inhibitors in non-small cell lung cancer (NSCLC)
42 with ALK or ROS1 translocation, a phase II trial is ongoing. This trial evaluates the efficacy and safety of ceritinib
43 in patients with IH-CCA over-expressing ROS1 or ALK (NCT02374489). The recently identified FIG-ROS fusion
44 suggests that crizotinib therapy merits evaluation: NCT02034981 is a phase II trial of crizotinib in patients
45 harbouring an ALK, MET, or ROS1 alteration (Table 1).
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50 During the most recent ASCO annual meeting, the presentation of preliminary data on larotrectinib showed a
51 consistent and durable antitumor activity of this drug in NTRK fusion cancers, across a wide range of tumor
52 types. Among 55 NTRK fusion patients enrolled, 2 were CCA. The ORR for the 46 patients evaluated was 78%,
53 with responses in 12 tumor types; no data on CCA were presented [116].
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• 7.5 FGFR

FGFR pathway is involved in cellular migration, proliferation, survival, and differentiation. FGFR mutations and fusions predominate in IH-CCA in about 16% of cases [110]. In particular, genome-wide structural analyses showed numerous translocation events concerning the FGFR2 locus, ranging between 11 and 45% in IH-CCA patients [117]. The discovery of recurrent FGFR aberrations has opened a promising therapeutic avenue.

BGJ 398, an oral FGFR inhibitor, is under evaluation in a phase II trial in advanced CCA with FGFR gene fusion/translocation after first-line chemotherapy (NCT02150967). The recent interim report of this trial was the following: 50 patients with BTC having FGFR genetic alterations were enrolled, the majority with IH-CCA. The ORR was 22% (all 8 patients with a partial response had an FGFR2 fusion) and the disease control rate (DCR) was 95% with PFS of 6 months [103].

A number of other clinical trials involving selective FGFR small molecule inhibitors - including INCB54828 (NCT02924376), BAY1163877 (NCT01976741), and the irreversible FGFR inhibitor TAS-120 (NCT02052778) - are currently in progress in early-phase trials in patients with advanced solid tumors, including BTC (Table 1).

Moreover, nonselective multi-TKIs targeting also FGFR, including **ponatinib** and **pazopanib**, have showed some activity in patients with highly pretreated IH-CCA [117].

Finally, a nonselective TKI, ARQ 087 (NCT01752920), which inhibits RET, PDGF receptor, KIT, SRC, and FGFR1–3, is currently under evaluation in a phase II trial of patients with FGFR-aberrant tumors, including FGFR2 fusion-positive advanced IH-CCA. Preliminary data from the phase I/II basket trial show that 3 of the 12 IH-CCA patients with FGFR2 fusion had a PR (DCR of 75%).

In conclusion, the preliminary data for FGFR inhibitors in advanced IH-CCA are encouraging.

• 7.6 IDH1-2

IDH 1 and **2** mutations are frequent in IH-CCA (9 of 40, 23%), while lacking in EH-CCA and GBC patients. Additionally, results from several researches have shown that IDH1 mutation is more common than mutation of IDH2.

Recently the findings of a dose escalation study of AG-120, an IDH1 inhibitor, in patients with advanced solid tumors having these mutations was presented by Burris et al. Of the 20 IH-CCA patients enrolled, 1 patient (5%) reached a PR and 11 patients (55%) had SD, with disease stability beyond 6 months [118].

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3 As shown in table 1, the potential efficacy of IDH1 and IDH2 inhibitors is currently being evaluated in clinical
4 trials involving solid tumors such as BTC that harbor these mutations (NCT02481154, NCT02073994,
5 NCT02381886).
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8 9 • **7.7 Immunotherapy**

10 Mechanisms involved in DNA repair are indispensable for the maintenance of genomic stability. Acquired or
11 genetic mutations leading to defective DNA mismatch repair (MMR) are common in several tumors such as
12 colorectal, gastric and endometrial cancer [110].
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16 In the study of Jaivle et al regarding mutational profiling of BTC, mutations in genes involved in DNA repair -
17 MSH6, BRCA1, BRCA2, ATM, MLH1 or MSH2 - was highly represented: 13% in IH-CCA, 26% in EH-CCA and 6% of
18 GBC cases [103].
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22 Available data comes from KEYNOTE-028, an ongoing, phase 1b trial of **pembrolizumab** monotherapy for
23 advanced solid tumors PD-L1-positive. Patients with PD-L1-positive BTC were included, with the exception of
24 cancer of the ampulla of Vater. Of 89 BTC patients screened for PD-L1 expression, 42% were PD-L1-positive
25 tumors. 24 pretreated patients - including 38% who received ≥ 3 prior therapies - were enrolled, showing an
26 ORR (confirmed and unconfirmed) of 17%, with 4 PR, 4 SD, and 12 PD [119].
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31 Given the demonstrated sensibility to programmed cell death protein 1 (PD-1) blockade with checkpoint
32 inhibitor agents (e.g pembrolizumab or nivolumab), BTC patients presenting these mutations can represent a
33 subset where immunotherapy may be effective. A number of immunotherapy studies are currently recruiting
34 (Table 1).
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42 **8-Conclusion**

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44 Treatment for advanced BTC is challenging; although cisplatin and gemcitabine are considered the standard 1st
45 line on the basis of a consolidated phase III study, survival outcomes remain dismal; TCR is achieved in
46 approximately 80% of cases, but mOS is generally less than one year. Second-line treatment should be offered
47 to patients who maintain a good PS, but no schedule should be preferred above the others. To date, there is no
48 strong evidence to support the use of combination instead of single-agent therapy neither. Data from
49 retrospective series show that 2nd line treatment yields mPFS of 3 months and mOS of 7 months approximately.
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51 Moreover, treatment of BTC patients is encumbered with a high rate of complications, such as infections, need
52 of biliary stenting, gastrointestinal toxicity, that have a high burden not only on patient's care but also on social
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3 and economical aspects. It goes without saying that every patient should be carefully evaluated for potential
4 risks and benefit of active therapy and that every treatment, in addition to efficacy, should provide a
5 manageable safety profile.
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9 In the past years, despite initial encouraging reports, targeted agents have failed to provide significant changes
10 in the history of BTC patients. In particular, anti-EGFR and anti-VEGF treatment used in addition to cisplatin and
11 gemcitabine as 1st line treatment have provided no benefit in randomized control trials. Other targeted agents
12 used, such as anti-MEK or anti-HER2, and data from some small non-randomized trials have shown little value
13 and conflicting results. More recently, some other druggable targets are raising renewed interest in tailored
14 medicine for BTC care.
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19 20 **9-Expert opinion** 21

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23 Despite a great effort to improve patients' outcomes, in the past decades only few studies have provided
24 practice-changing findings. The milestones of BTC care include the superiority of chemotherapy over BSC [12]
25 and the identification of gemcitabine-cisplatin as standard treatment for advanced disease [10]. More recently,
26 the role of adjuvant chemotherapy after resection of localized disease is being supported by randomized
27 controlled trials [9]. These results have been achieved over a period of about 20 years and appear inadequate
28 considering the progresses that have been made in other diseases, including gastrointestinal malignancies, in
29 the same time span.
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35 Undoubtedly, dealing with a rare disease is the main disadvantage researchers have to face when designing
36 clinical trials; in the 90ies BTCs were often grouped with ampullary and pancreatic cancers. The bulk of
37 literature available includes small trials that, in spite of being multicentric, generally have a small sample size of
38 40-100 patients. In addition, some of the first studies with targeted therapies were not supported by a strong
39 rationale or an accurate patient selection; in some cases, researchers have tried to apply the same paradigms
40 that showed success in other cancer types to BTC, an orphan disease with very few options.
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45 We have already pointed out that the different entities that are commonly included in BTC, such as IH-CCA, EH-
46 CCA e GBC, have also different clinical and molecular features. Sometimes they are used as stratification factors
47 for randomization or to conduct subgroup analyses. In our opinion, this issue is a great limitation especially for
48 studies with targeted agents because the different pathways activated or inhibited are strongly influenced by
49 the molecular features of the tumor, which vary strongly among the entities. For example, in the first studies
50 with anti-EGFR therapies, slightly better results were observed in the IH-CCA groups, but their real impact was
51 never significant because of the small sample size or the retrospective character of the analyses.
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57 With the advent of new, advanced technologies like next-generation sequencing the underpinnings of genomic
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3 landscape of BTCs are being disclosed. To date, no distinct molecular mutation characterizes BTCs, but the
4 scenario is scattered into several alterations that have a relatively low rate, especially if we consider the
5 different anatomical locations. Among the druggable alterations, IDH1-2 mutations and FGFR translocations are
6 those that show the most potential because of higher frequency and the investigation of different compounds
7 in clinics.
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12 In parallel with the progress of technologies, drug development in the past few years is substantially changing;
13 trial design is moving from the standard approach from phase I to phase III trials and approval, to newest
14 methods. More often we are observing proof-of-concept studies that lead to an accelerated approval by
15 regulatory agencies, thanks to striking results on a limited number of patients in non-randomized phase I or II
16 trials.
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21 Moreover, revolutionary trials designs permit to handle multiple related questions with fewer patients. In
22 basket trials the effect of one drug is tested at the same time but in a variety of tumor types that share the
23 same single mutation. Umbrella trials indeed have many different treatment arms within one trial; people are
24 assigned to a particular treatment arm of the trial based on their type of cancer and the specific molecular
25 makeup of their disease.
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29 Nivolumab and pembrolizumab have recently been granted FDA approval for microsatellite instability-high
30 (MSI-H) tumors; this is the first time a cancer treatment to be approved based on a common biomarker rather
31 than tumor type. A percentage of BTCs patients are potentially involved by this progress: similarly, some
32 ongoing basket trials that we have introduced in the previous paragraphs, such as those directed against ROS,
33 ALK, NTRK1/2/3 are deemed to provide interesting results for patients harboring low-frequency mutations.
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37 Thanks to these new approaches and to the better knowledge of potentially actionable genomic alterations in
38 BTCs it will be possible to offer a chance for tailored medicine to BTCs patients ant to and to meet an unmet
39 clinical need.
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50 **10-References**

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38 etiology
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47 This work refers to the integrated analysis of somatic mutations, RNA expression, copy number, and DNA
48 methylation by The Cancer Genome Atlas of a set of predominantly intrahepatic CCA
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Table 1 New therapies under evaluation in biliary tract cancers

Therapeutic regimen	Target	Phase	ClinicalTrials.gov identifier	Status
VEGF and multitarget				
Ramucirumab	VEGFR2 2	2	NCT02520141	Recruiting
GEMCIS and ramucirumab or merestinib	VEGFR2 c-MET	2	NCT02711553	Active (not recruiting)
FOLFOX and cediranib	VEGFR	2	NCT01229111	Not recruiting (results expected)
Regorafenib	VEGFR1-3, c-KIT, TIE-2, PDGFR- β , C-Raf, B-Raf, p38 MAPK, FGFR1-2, Ret 2	2 2	NCT02115542 NCT02053376	Active (not recruiting) Active (not recruiting)
Apatinib	VEGFR-2	2	NCT03144856	Recruiting
Lenvatinib	VEGFR-2, VGFR-3	2	NCT02579616	Active (not recruiting)
Varlitinib + GEMCIS	EGFR	2	NCT02992340	Recruiting
MAPK				
Trametinib versus CAP/5FU	MEK 1/2	2	NCT02042443	Not recruiting (results expected)
Binimetinib (MEK162) and GEMCIS	MEK 1/2	2	NCT01828034	Active (not recruiting)
Selumetinib (at different doses) and GEMCIS	MEK 1/2	2	NCT02151084	Recruiting
HER 2				
Afatinib	HER2	1/1b	NCT02451553	Recruiting
Trastuzumab Emtansine	HER2	2	NCT02999672	Recruiting
Neratinib	HER2	2	NCT01953926	Recruiting
Other				
Copanlisib + GEMCIS	PIK3CA	2	NCT02631590	Recruiting
Entrectinib	NTRK1/2/3, ROS1, ALK	2	NCT02568267	Recruiting
Larotrectinib	NTRK1/2/3	2	NCT02576431	Recruiting
LDK378	ALK, ROS1	2	NCT02374489	Recruiting
Crizotinib	ALK, MET, ROS1	2	NCT02034981	Recruiting
Immunotherapy				
Pembrolizumab	PD1	2	NCT03260712	Recruiting

Cisplatin Gemcitabine				
Nivolumab	PD1	2	NCT02829918	Recruiting
Pembrolizumab and GM-CSF	PD1	2	NCT02703714	Recruiting
Ipilimumab and nivolumab	CTLA4 and PD1	2	NCT02923934	Recruiting
Pembrolizumab + CAPOX	PD1	2	NCT03111732	Recruiting
Infigratinib	FGFR2	2	NCT02150967	Recruiting
INCB054828	FGFR2	2	NCT02924376	Recruiting
BAY1163877	Pan-FGFR	1	NCT01976741	Recruiting
TAS-120	FGFR		NCT02052778	Recruiting
ARQ 087	FGFR1-3, RET, PDGFR, KIT	1/2	NCT01752920	Not recruiting (results expected)
AG881	IDH1-2	1	NCT02481154	Active (not recruiting)
AG-120	IDH1	1	NCT02073994	Active (not recruiting)
IDH305	IDH1	1	NCT02381886	Suspended
Multiagent				
Multiple arms based on molecular profiling (cetuximab, trastuzumab, gefitinib, lapatinib, everolimus, sorafenib, crizotinib)	Multiple (EGFR, HER2, mTOR, VEGF/EGFR/ PDGFR, ALK/ROS1)	2	NCT02836847	Recruiting
Sulfatinib	VEGFR 1,2,3, FGFR 1, CSF1R	2	NCT02966821	Recruiting
Regorafenib + modified GEMOX	VEGFR1-3, c-KIT, TIE-2, PDGFR-β, C-Raf, B-Raf, p38 MAPK, FGFR1-2, Ret 2	2	NCT02386397	Recruiting

1
2
3 **2-HG** 2-Hydroxyglutarate

4
5 **5-FU** 5-Fluorouracil

6
7 **αKG** α-Ketoglutarate

8
9 **ARAFm** ARAF Mutation

10
11 **α-SMA** α-Smooth Muscle Actin

12
13 **BSC** Best Supportive Care

14
15 **BTC** Biliary Tract Cancer

16
17 **CaM** Cancer Associated Macrophages

18
19 **CAF** Cancer Associated Fibroblasts

20
21 **CCA** Cholangiocarcinoma

22
23 **CK** Cytokeratin

24
25 **c-KIT** Stem Cell Growth Factor Receptor

26
27 **CR** Complete Response

28
29 **DCCA** Distal Cholangiocarcinoma

30
31 **DCR** Disease Control Rate

32
33 **ECOG** Eastern Cooperative Oncology Group

34
35 **EGFR** Epidermal Growth Factor Receptor

36
37 **EH-CCA** Extrahepatic Cholangiocarcinoma

38
39 **EMT** Epithelial- Mesenchymal Transition

40
41 **FGFR** Fibroblast Growth Factor Receptor

42
43 **FIG-ROS** Fused In Glioblastoma c-ROS Oncogene 1

44
45 **FOLFIRI** 5- Fluorouracil/Leuovorina- Irinotecan

46
47 **FOLFOX** 5- Fluorouracil/Leuovorina- Oxaliplatin

48
49 **GBC** Gallbladder Cancer

1
2
3 **GEMOX** Gemcitabine- Oxaliplatin

4
5 **HCC** Hepatocellular Carcinoma

6
7 **HER-2** Human Epidermal Growth Factor Receptor 2

8
9
10 **HGF** Hepatocyte Growth Factor

11
12 **Hh** Hedgehog

13
14 **IDH1** Isocitrate Dehydrogenase 1

15
16 **IDH2** Isocitrate Dehydrogenase 2

17
18 **IH-CCA** Intrahepatic Cholangiocarcinoma

19
20 **mAbs** Monoclonal Antibodies

21
22 **MAPK** Mitogen Activated Proteine Kinases

23
24 **mGEMOX** Modified Gemcitabine- Oxaliplatin

25
26 **MMR** Mismatch Repair

27
28 **mOS** Median Overall Survival

29
30 **mPFS** Median Progression Free Survival

31
32 **MSI-H** Microsatellite Instability-High

33
34 **mTOR** Mammalian Target of Rapamycin

35
36 **NSCLC** Non- Small Cell Lung Cancer

37
38 **ORR** Overall Response Rate

39
40 **OS** Overall Survival

41
42 **PCCA** Perihilar Cholangiocarcinoma

43
44 **PD** Progression Disease

45
46 **PD-1** Programmed Cell Death Protein 1

47
48 **PDGF** Platelet-Derived Growth Factor

49
50 **PD-L1** Programmed Cell Death- Ligand 1

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2
3 **PFS** Progression Free Survival
4

5 **PIK3CA** Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
6

7 **PKB** Protein Kinase B
8

9 **PR** Partial Response
10

11 **PS** Performance Status
12

13
14 **PSC** Primary Sclerosing Cholangitis
15

16 **PTEN** Phosphatase and Tensin Homolog
17

18 **QoL** Quality of Life
19

20 **RET** Rearranged During Transfection
21

22 **RR** Response Rates
23

24 **SD** Stable Disease
25

26 **SDF-1** Stromal Cell Derived Factor 1
27

28 **STAT3** Signal Transducer and Activator of Transcription 3
29

30 **TCR** Tumor Control Rate
31

32 **TGF- β** Transforming Growth Factor β
33

34 **TKIs** Tyrosine Kinase Inhibitors
35

36 **TRAIL** Tumor Necrosis Factor Related Apoptosis Inducing Ligand
37

38 **VEGF** Vascular Endothelial Growth Factor
39

40 **WISP1** Wnt Inducible Signaling Protein-1v
41

42 **WT** Wild Type
43

44 **XELIRI** Capecitabine- Irinotecan
45

46 **XELOX** Capecitabine- Oxaliplatin
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Title

Emerging molecular target antagonists for the treatment of biliary tract cancer

Abstract

Introduction: Biliary tract cancers (BTCs) are a heterogeneous group of cancers, characterized by low incidence but poor prognosis. Even after complete surgical resection for early stage, relapse is frequent and the lack of effective treatments contributes to the dismal prognosis. To date, the only standard treatment in first-line is cisplatin/gemcitabine combination, whereas no standard in 2nd-line has been defined. Hence, the current goal is to better understand the biology of BTCs, discovering new treatment methods and improving clinical outcomes.

Areas covered: The development of next-generation-sequencing has unveiled the picture of the molecular signatures characterizing BTCs, leading to the identification of actionable mutations in biomarker-driven clinical trials. In this review we will cover the genetic landscape of BTC, focusing on the efficacy of existing treatments. Furthermore, we will discuss emerging molecular targets and evaluate the findings of pre-clinical studies. Finally, the encouraging results of clinical trials involving targeted therapies or immunotherapy will be reviewed.

Expert opinion: FGFR fusion rearrangements and IDH1 or IDH2 mutations are the most promising targeted treatments under evaluation. In addition, revolutionary-innovative trial design will allow to offer a chance for tailored medicine to infrequent subgroups of BTCs patients based on their molecular features rather than their histology.

Manuscript**1-Background**

Biliary tract cancer (BTC) is a heterogeneous group of tumors arising from the epithelial lining of the biliary tree. The classification is based according to the anatomical location; BTC includes gallbladder cancer (GBC) and cholangiocarcinoma (CCA); the latter is further divided into intrahepatic (IH-CCA), and extrahepatic (EH-CCA), which includes EH-CCA perihilar (PCCA) and distal CCA (DCCA). IH-CCAs arise above the second order bile ducts, whereas PCCAs are located between the insertion of the cystic duct and the second order bile ducts, and DCCAs are located below the insertion of the cystic duct [1, 2].

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9 Even though GBC, intrahepatic, perihilar and distal extrahepatic CCA are grouped as BTCs, their clinical
10 presentation, pathobiology and management are different, and they should be viewed as separate entities.
11 Despite being considered an infrequent cancer in the Western world, with an incidence of 1-2 cases every
12 100,000 inhabitants, BTCs are extremely common in South America and in some areas of Asia, with up to 96
13 cases/100,000 [3].
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16 Histologically, more than 95% of BTCs are adenocarcinomas, often poorly differentiated. About 10% of EH-CCA
17 are well differentiated papillary cancers, but mucinous (5%) and squamous cancers (2%) are also described [4].
18 Several classifications have been proposed for IH-CCA owing to its highly heterogeneity. The two main
19 histological subtypes are bile ductular type (mixed), arising from small intrahepatic bile ducts, and bile duct
20 type (mucinous), arising from large intrahepatic bile ducts. These differences are also reflected in different
21 molecular characteristics [5]. Notably, bile ductular type IH-CCAs share clinicopathological similarities with
22 cytokeratin (CK) 19-positive hepatocellular carcinoma (HCC), and the bile duct type IH-CCAs share phenotypic
23 traits with PCCA and pancreatic cancers [6].
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27 Beyond the differences, BTC have common features, such as a highly desmoplastic reaction, rich tumor
28 microenvironment, and profound genetic heterogeneity, all contributing to the development of drug resistance
29 and almost complete absence of curative therapies for metastatic disease.
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31 The vast majority of BTC (70%) occurs sporadically. Nevertheless, several pathologic conditions have been
32 determined as possible risk factors such as Primary Sclerosing Cholangitis (PSC), choledochal cysts, parasitic
33 infestation, and viral hepatitis B and C. Potential risk factors with less evidence are diabetes, alcohol, smoking,
34 obesity and specific genetic polymorphisms [7]. Therefore, although a single trigger cannot be identified,
35 different environmental, genetic and social factors may be rather involved and justify heterogeneous
36 geographic distribution.
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40 Most patients with early stage disease are asymptomatic and diagnosing BTC at an early stage remains a
41 challenge owing to its 'silent' clinical presentation, difficult to access anatomical location, and highly
42 desmoplastic, paucicellular nature, which limits the sensitivity of cytological and pathological diagnostic
43 approaches.
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46 Surgery is the preferred treatment option for localized BTC, but only a minority of patients (approximately
47 35%) has early stage disease that is amenable to surgical resection with a curative intent. Survival after radical
48 surgery generally depends on margin status (negative-R0 or positive-R1-R2 status), vascular invasion and lymph
49 node involvement. Even after R0 resection 3-years survival rate is approximately 40-60%, and it can be lower in
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9 case of lymph node positivity [8].

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11 The unfavorable prognosis of recurrent disease drove efforts to lower the rate of relapse through
12 postoperative adjuvant therapy. So far, the great part of the available literature was made of retrospective,
13 non-randomized studies and meta-analyses: the majority took in consideration heterogeneous populations of
14 patients, treated with different systemic drugs with or without radiotherapy. Recently, following the
15 presentation of the preliminary results of the BILCAP study, a standard of care for adjuvant treatment is
16 available. In this randomized phase III trial, postoperative capecitabine was compared to surveillance: the
17 primary endpoint of the study was formally not met, despite a difference of 15 months in the median overall
18 survival (mOS) estimated values (51 versus 36 months, $p = 0.097$). However, mOS was significantly higher in the
19 chemotherapy arm both after sensitivity analyses and in the per protocol analysis [9].
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23 For patients with advanced-stage or unresectable disease, the available systemic therapies are of limited
24 effectiveness: the mOS with the current standard-of-care regimen (gemcitabine and cisplatin) is <1 year [10]
25 and a probability to outlive 5 years is about 5% [11].
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30 **2-Medical need**

31 Randomized trials have shown that systemic chemotherapy increases survival and improves quality of life (QoL)
32 in patients with metastatic BTC as compared with best supportive care (BSC). In the study by Glimelius, 90
33 patients with metastatic or locally advanced pancreatic carcinoma or BTC have been randomly assigned to 5-
34 fluorouracil (5-FU) -based chemotherapy or BSC. A clear benefit of chemotherapy on both mOS (6 vs 2.5
35 months in BSC group $p < 0.01$) and QoL was demonstrated [12]. More recently, modified gemcitabine and
36 oxaliplatin (mGEMOX) proved a significant improvement in mOS not only over BSC, but also over 5-FU/folinic
37 acid in unresectable GBC (9.5, 4.6 and 4.5 months respectively)[13].
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41 The majority of trials in the metastatic setting were performed with fluoropyrimidine or gemcitabine
42 monotherapy or in combination with other cytotoxic agents. 5-FU as single agent yields a variable response
43 rates (RR) from 10% to 40%. The combination of 5FU with other drugs (etoposide, interferon, cisplatin and
44 oxaliplatin) has proved moderate efficacy, limited overall survival (OS) benefit, and a significantly greater
45 toxicity profile. Since the late 90s, the adoption of gemcitabine as the standard of care for patients with
46 pancreatic cancer led to interest in its use for hepatobiliary tumors. As a single agent, gemcitabine has shown
47 RRs ranging from 0% to 30%, whereas its association with other agents has determined advantages in survival
48 and RRs (up to 41%) [14]. In 2007 a pooled analysis of 104 trials in advanced BTC, demonstrated the superiority
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of combination therapy compared to monotherapy in RR and tumor-control-rate (TCR). Subgroup analysis also underlined that gemcitabine and platinum association had significantly higher response and TCRs compared to fluoropyrimidine/gemcitabine monotherapy or fluoropyrimidine plus platinum regimens [15].

Suggestions from this meta-analysis were turned into a standard of care by Valle's Phase III ABC- 02 trial. Four hundred and ten patients with locally advanced or metastatic CCA, GBC and ampullary carcinoma were randomly assigned to receive gemcitabine alone (1000 mg/m² days 1, 8, 15 q 28) vs gemcitabine and cisplatin (1000 mg/m² + 25 mg/m² days 1, 8 q 21) for up to 24 weeks of treatment. After a median follow-up of 8.2 months, mOS, that was the primary endpoint of the study, was statistically improved in the combination arm (11.7 vs. 8.1 months; $p < 0.001$). The benefit was preserved across the subgroups according to primary tumor site, median progression-free-survival (mPFS) was improved (8.0 months vs. 5.0 months; $p < 0.001$), without significant increase in toxicity. [10]. Following the results of this trial, a definite standard regimen for a disease that has been "orphaned" for too long, was finally provided. Equivalent results have been replicated in the Japanese population [16]. Other combination schemes have also been evaluated as first line treatment in phase II trials [17-19]. Among these, gemcitabine and oxaliplatin (GEMOX) is widely used in clinical practice for its good response rate and favorable toxicity profile, although a direct comparison with the standard of care is lacking [13, 20].

Following a meta-analysis of the English and Japanese randomized trials, single agent gemcitabine is a recommended option only for patients with Performance Status (PS) 2 according to Eastern Cooperative Oncology Group (ECOG) scale [21].

After the failure of the 1st line therapy, approximately half of the patients still have a good PS and satisfactory organ function [22], but the advantages of 2nd line therapy are still unclear. In a large retrospective analysis, 196 patients who received 2nd line treatment after gemcitabine and cisplatin/oxaliplatin, were analyzed. The most common regimens used were 5-FU/folinic acid, FOLFIRI, XELIRI, FOLFOX, XELOX, 5-FU and cisplatin: globally, the outcome was poor, the mPFS and OS being 3.2 and 6.7 months, respectively, and no chemotherapy regimen proved superiority over the others [23]. In another Italian retrospective analysis, PS emerged as the most important prognostic factor to select the patients that may benefit from 2nd line treatment [24].

3-Existing treatment

In the past decade we have entered the era of targeted therapies: this strategy has modified the therapeutic approach of many cancers but the first attempts of using targeted treatments in BTCs have been so far unsatisfactory. Here, we revise the first studies that have explored targeted therapies in BTC.

- **3.1 EGFR family pathway**

Epidermal growth factor receptor (EGFR) is mutated and overexpressed in cancer human samples. EGFR is extensively represented in BTC, being expressed in 100% of IH-CCAs, 52.6% of EH-CCAs, and 38.5% of GBCs. [25]. Mutations have been found in up to 15% of BTCs [26, 27]. EGFR activation triggers the Mitogen Activated Proteine Kinases (MAPK)–ERK pathway, an oncogenic signalling pathway in cancer: it donates a proliferative advantage to cancer cell, contributes to progression through epithelian-mesenchymal transition (EMT) and leads to a poor clinical outcome [28, 29]. In preclinical studies, EGFR inhibitors tested in combination with chemotherapy have shown promising activity, providing a strong preclinical rationale for anti-EGFR therapy in BTC. Different strategies targeting EGFR have been studied such as tyrosine kinase inhibitors (TKIs) or monoclonal antibodies (mAbs) alone or in association with chemotherapy.

Erlotinib, a selective, reversible, orally-active EGFR inhibitor, after promising results in several phase II studies, failed to confirm its superiority in OS in a large phase III study. Compared to the combination of GEMOX, the addition of erlotinib in patients with newly diagnosed metastatic BTC significantly increased RR (40% vs 21%; $p=0.005$) but mPFS and OS were not increased (4.2 vs 5.8 months $p=0.087$ and 9.5 months in both arms, $p=0.611$, respectively). Subgroup analyses showed that in patients with CCA the addition of erlotinib prolonged mPFS (5.9 months vs 3.0 months; HR 0.73, 95% CI 0.53–1.00; $p=0.049$) [30].

Cetuximab is a mAb targeting EGFR, evaluated in combination with chemotherapy in BTC in several phase II trials. In a phase II study of cetuximab in combination with GEMOX, the treatment achieved a mPFS of 8.3 months and OS of 12.7 months, with high RR [31]. Following randomized trials failed to demonstrate a significant benefit of the GEMOX and cetuximab combination. In the BINGO study, cetuximab was tested in the 1st line setting in addition to GEMOX vs GEMOX alone. Despite being well tolerated, cetuximab did not significantly improve outcomes: mPFS was 6.1 months with cetuximab and 5.5 months without cetuximab, while mOS was 11.0 months with cetuximab and 12.4 months without cetuximab [32]. In a similar study in the Asiatic population, mPFS was 6.7 months with cetuximab and 4.1 months without cetuximab ($p = 0.05$), while mOS was 10.6 months vs 9.8 months respectively ($p = 0.91$). KRAS mutations, that are a well-known negative predictive factor of response to anti-EGFR mAbs, were identified in 36% of tumors, and did not affect the overall response rate (ORR) or mPFS [33].

Panitumumab, a fully human antibody against EGFR, has been tested in combination with chemotherapy in non-randomized and randomized, phase II trials. In particular, in the Vecti-BIL trial advanced BTC patients were selected upfront for KRAS mutational status. The study revealed that the addition of panitumumab to GEMOX compared to GEMOX alone did not significantly improve mPFS, (5.3 months in experimental arm vs 4.4 months

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in control arm), neither OS (10.2 months in experimental arm vs 9.9 months in control arm) in KRAS wild type (WT) BTC patients [34].

Despite the results of a recent meta-analysis suggesting a potential role of anti-EGFR therapy in prolonging PFS and RR, the current evidence does not support its use in BTC [35].

- **3.2 MEK pathway**

Targeting the RAS/RAF/MEK/ERK pathway, a major player in the cellular processes, including proliferation and apoptosis, is thought to be a winning strategy in many cancers including BTC.

The second-generation, uncompetitive inhibitor of MEK 1/2 - **selumetinib** - was investigated in a phase II study of 28 advanced BTC patients. Median PFS was 3.7 months and the mOS was 9.8 months, with a 12% of ORR [52]. Recently a Phase Ib trial has tested the pharmacokinetics and toxicity profile of selumetinib in combination to cisplatin and gemcitabine. Other trials are needed to demonstrate its applicability in clinical practice [53].

Another uncompetitive inhibitor of MEK 1/2, **binimetinib**, after encouraging results in safety and activity during phase I studies [54], is currently under evaluation in phase II studies (NCT02151084, NCT01828034).

Recently, the open-label, multicentre, single-arm trial, evaluating pazopanib - an orally available multikinase inhibitor of VEGF (vascular endothelial growth factor) receptor, PDGF (platelet-derived growth factor) receptor, c-KIT (stem-cell growth factor receptor), fibroblast growth factor receptor (FGFR) and RAF - in addition to **trametinib** - an orally available highly specific inhibitor of MEK 1 and MEK 2 - showed discouraging results. Despite the trend towards increased 4-month PFS, the difference did not reach statistical significance. The mOS was 6.4 months (95% CI: 4.3–10.2) and the ORR was 5% (95% CI: 0.13–24.9%) [55].

- **3.3 VEGF**

The **v**ascular endothelial growth factor (VEGF) pathway promotes tumor growth - by angiogenesis and vascular permeability - in several tumors including BTC [36]. In CCA, VEGF expression is reported in about 30-40% of cases and is correlated with lymphnode metastasis and poor prognosis [37, 38]. VEGF expression is reported in 50% of GBC and also in this sub-group a poor prognosis is observed [39]. Based on preclinical and clinical data supporting a VEGF-targeted approach, several studies have evaluated the effectiveness and applicability of targeted therapy (bevacizumab, sorafenib, cediranib, vand~~t~~etanib) in metastatic BTC, providing only limited information, principally because of their phase II design. Therefore, the role of antiangiogenic agents in the treatment of BTC is not well defined.

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9 **Bevacizumab** is a recombinant humanized mAb whose efficacy has been tested in patients affected by
10 advanced BTC. In two studies it was tested in combination with erlotinib and in sequence with gemcitabine and
11 oxaliplatin, without favorable results [40, 41]. More recently, a small phase II study evaluated the combination
12 of gemcitabine, capecitabine, and bevacizumab in a first-line setting, showing a mPFS and mOS similar to those
13 observed with standard chemotherapy (8.1 months and 11.3 months, respectively) [42]. Larsen et al. recently
14 presented the results of a phase II trial of capecitabine, irinotecan, gemcitabine, and bevacizumab as second-
15 line setting in 50 BTC patients. Median PFS was 3.6 months and mOS was 6.4 months [43]. To date, the role of
16 bevacizumab on BTC has been evaluated only in single-arm studies, with no randomized study performed.
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20 **Cediranib** is a potent inhibitor of the VEGF receptor tyrosine kinases -also directed against PDGF receptors and
21 c-KIT being investigated in a recent phase II study [44]. Patients were randomized to receive cisplatin and
22 gemcitabine plus either cediranib or placebo. Cediranib did not significantly improved mPFS (7.7 months in the
23 cediranib arm vs. 7.4 months in the placebo arm). There was a trend towards longer OS in the experimental
24 arm compared to placebo (14.1 vs 11.9 months, respectively) [45].
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26 For definitive conclusions, we will have to wait for the results of the ongoing phase II trial, investigating
27 cediranib in combination with modified FOLFOX6 in advanced BTC (NCT01229111).
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30 **Sorafenib** is an oral multikinase inhibitor directed against VEGF receptors and PDGF receptor- β , and blocking
31 downstream intracellular serine/threonine kinases, like Raf-1, WT and mutant B-Raf.

32 After the defeats in monotherapy in several studies, the more recent conducted by Luo et al [46], Sorafenib
33 was evaluated in combination with standard chemotherapy.
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35 First, Moehler et al. investigated Sorafenib in the first line setting, treating patients with gemcitabine with
36 either sorafenib or placebo. In this study, longer mPFS and OS were found in the gemcitabine plus placebo arm
37 [47]. Then, a randomized, double-blinded, multicenter phase II trial investigating a combination of gemcitabine,
38 cisplatin with either sorafenib or placebo demonstrated no significant difference in mPFS and mOS between
39 the two arms [48]. Finally, a small trial conducted by Lee et al. showed that the addition of sorafenib to
40 gemcitabine and cisplatin did not improve efficacy over historical data, but there was an increase of toxicity
41 [49].
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45 **Sunitinib** is another oral small molecular-targeted drug inhibiting several intracellular and receptor protein
46 kinases, including VEGF and PDGF receptors, c-KIT, and rearranged during transfection (RET). In the only
47 available phase II study, the role of sunitinib was investigated as a second-line treatment in advanced BTC
48 demonstrating only marginal efficacy; ORR was 8.9 % and mPFS was 1.7 months [50].
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9 **Vandetanib** is an orally active, multikinase inhibitor targeting VEGF receptor 2, EGFR and RET kinase. In the
10 Italian "VanGogh" study, 165 BTC patients chemotherapy-naive were randomized into three groups:
11 vandetanib monotherapy, vandetanib plus gemcitabine and gemcitabine plus placebo. Median PFS was 105
12 days (95 % CI 72–155), 114 days (95 % CI 91–193), and 148 days (95 % CI 71–225) respectively, while mOS was
13 228 days (95 % CI 190–364), 284 days (95 % CI 213–359), and 307 days (95 % CI 254–523) for the three arms
14 respectively. Thus, vandetanib alone or in association with gemcitabine did not demonstrate any superiority
15 when compared with gemcitabine alone [51].

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18 In conclusion, the role of VEGF inhibition in addition to chemotherapy for patients with advanced BTCs remains
19 still investigational, but increasingly burdened by the absence of a biomarker of efficacy for VEGF inhibitors.
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24 **4-Market review**

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26 Throughout the therapeutic course of BTC patients, complications are a constant. Infections, sepsis and
27 cholangitis often undermine patients' health. Furthermore, chemotherapy is associated with high rate of side
28 effects that requires spending of many economic resources.
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30 We have considered two cost-effectiveness analysis conducted in USA and Japan. They evaluate cost-
31 effectiveness of combination treatment with gemcitabine and cisplatin compared to treatment with
32 gemcitabine alone. ~~These studies get to different conclusion, probably for different conditions in each~~
33 ~~nation~~ ~~Despite being two comparable analyses in different populations, their conclusions are opposite; indeed,~~
34 Roth et al. demonstrated that cisplatin and gemcitabine combination was a cost-effective alternative to
35 gemcitabine monotherapy. ~~However,~~ Tsukiyama et al., on the contrary, suggested that gemcitabine
36 monotherapy was a better treatment strategy for advanced BTC. These different conclusions may be due to
37 varying health care conditions and probably because of different long-term palliative care cost ~~in Japan than in~~
38 ~~the USA~~ between the countries.
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43 Surprisingly, in both studies the majority of the resources were employed for supportive and palliative care
44 rather than for drugs [56, 57].
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46 For these reasons, systemic treatments are required not only to prolong survival, but also to limit palliative
47 interventions. Recently, immunotherapy and target therapies have demonstrated to increase QoL and to be
48 associated to lower rates of side effects in several cancer types as compared to chemotherapy [58-60]. For
49 these reasons, alternative treatment strategies are also strongly encouraged in BTC patients.
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5-Current research goals

Carcinogenesis is a multistep process encountering imbalance between proliferative and inhibitory stimuli, dysregulation of apoptotic signals and abnormal response to angiogenic factors which are responsible for invasion and metastatization events [61]. It involves specific cell genome derangements that contribute to the selective growth advantage of cancer. Specific pathways govern cell fate through proliferation, cell survival, differentiation, epigenetic changes and maintenance of genome integrity [62].

The genomic heterogeneity of BTC is well-known. As previously discussed, it's not only related to the diverse anatomical location of the tumor but also to etiology, the various risk factors and associated pathologies. Interestingly, mutations in TP53 responsible for genome integrity are very common in all subgroup of BTC but are more often observed in liver-fluke related CCA [63]. These differences will need to be considered when assessing outcome and in devising therapeutic strategies for CCA and GBC [2, 61, 64-67]. Advances in genome-wide technologies have made feasible the discovery of other possible targetable or actionable molecular alterations in BTC. Precision therapy for this group of tumors is subordinated to an enhanced understanding of genetic and molecular alteration for each subtype.

6-Scientific rationale and molecular pathogenesis

• 6.1 Proliferation and cell survival.

Several growth pathways are implicated in arising and progression of BTCs. Here we revise the most promising preclinical evidences that could, in the near future, have a clinical counterpart.

ErbB family, that includes four different receptors (ErbB1 or EGFR, ErbB2 or HER-2/neu, ErbB3 and ErbB4), have a main role in carcinogenesis processes and represents the most extensively mutated pathway of the GBC samples [68]. We have already introduced the role of EGFR and its clinical implications.

Human epidermal growth factor receptor 2 (HER2) amplification has been observed in BTCs patients with a prevalence in EH-CCA (about 20%), GBC (9.8%) and a rare incidence in IH-CCA. The prognostic meaning of HER2 has not yet been completely clarified but could become a relevant predictive factor [68, 69].

KRAS mutations occur in BTC and the reported incidence is 7-47%. It is usually associated with the alteration of other factors, such as EGFR, HER2 or MET [68, 70]. A recent work showed poor prognosis for KRAS mutated CCA that is more pronounced in the subgroup with transcriptional enrichment of genes that regulate proteasome activity [71].

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9 Some studies reported BRAF mutations in a percentage between 0% and 22%. In a large cohort, BRAF V600E
10 mutation was observed in 5.9% of GBC, 3% of IH-CCA and 0% of EH-CCA and no clear correlation with prognosis
11 was detected [72, 73].

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13 Recently, ARAF mutation (ARAFm), a member of the RAS family, has been reported in 11% of IH-CCA iCCA.
14 ARAFm leads to an increased basal and inducible activity when compared to WT protein. Further studies need
15 to investigate the oncogenic potential of this new target. [74].

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17 ROS1 fusion proteins have been recently implicated in CCA carcinogenesis. The fused-in-glioblastoma-c-ros-
18 oncogene 1 (FIG-ROS) was found in 14-16% of EH-CCA [75] and it mediated cancerogenesis in KRAS dependent
19 mode with a high aggressiveness [76].

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21 The expression level of hepatocyte growth factor (HGF) receptor (also known as c-Met) is amplified either in
22 CCA and GBC. Miyamoto analyzed 247 patients and noted MET high expression in 11.7% of IH-CCA and 16.2%
23 in EH-CCA. MET seems to play a central role in carcinogenesis through the protection from apoptosis and
24 promoting angiogenesis and tumor invasion. In CCA it is associated with EGFR expression and represents a poor
25 prognostic factor. Moreover, it could be linked to acquired resistance to EGFR or HER2 inhibitors [77, 78].

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28 FGFR is a tyrosine kinase receptor involved in numerous cellular process including proliferation, angiogenesis
29 and tissue repair. Disruption of this pathway has been implicated as driver event in biliary cancer formation.
30 Fusions of FGFR gene have been reported in some cancer type; in BTC several gene fusions have been
31 described, the most frequent being FGFR2-BICC1 fusion. This new protein seems to have a higher incidence in
32 female and a link with hepatitis infection. It is constitutively active and leading to the activation of MAPK and
33 phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA)/ mammalian target of
34 rapamycin (mTOR) pathways. Moreover, FGFR translocation may confer a survival benefit. Indeed, in a western
35 dataset a superior cancer specific survival was observed in patients with FGFR2 translocation compared to non
36 translocated patients (123 months vs 37 months) [65, 74, 79].

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40 Aberration in tumor suppressive gene phosphatase and tensin homolog (PTEN) has been correlated with
41 clinical outcome. Indeed, PTEN loss has been associated with poor outcome in all BTC, particularly in
42 combination with either activated Protein kinase B (PKB), also known as Akt or mTOR. However, genetic
43 alteration in AKT genes, with normal level of PTEN, were associated with cancerogenesis but also with
44 favorable prognosis. These findings suggest isolated AKT alteration could have an important role in the
45 initiation of IH-CCA but not in progression of this subgroup [80-82].

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49 Different pathways have been implicated in carcinogenesis and proliferative advantage in CCA. However, these
50 pathways overlap in several points, providing a molecular reason for resistance to target therapies.

Besides, not every cancer has a proliferative signature. Sia et al., using an integrative molecular analysis technique, have identified gene signatures in IH-CCA and have correlated them with pathological features and clinical patients' outcome. The authors describe two categories of IH-CCA: a proliferative class and an inflammatory class. The proliferative class, that accounts for 62% of cases, was typified by alterations in several oncogenes included in RAS-RAF-MEK-ERK or PIK3CA-AKT-mTOR signaling pathway, implicated in cell proliferation and cell survival, respectively.

The inflammatory class showed activation of inflammatory pathways, overexpression of cytokines (i.e. IL-6) and signal transducer and activator of transcription 3 (STAT3). STAT3 is a mediator that modulates cell growth and survival while IL-6 is an inflammatory cytokine produced either by CCAs and cholangiocytes stimulated by an inflammatory *nox*a. IL-6 is involved in cell survival through upregulation of Mcl-1 via AKT-dependent mechanism. Mcl-1 mediates tumor necrosis factor-related apoptosis inducing ligand (TRAIL) resistance and cell survival. IL-6 is also implicated in upregulation of Mcl-1 through a STAT3 dependent mechanism [83, 84].

• **6.2 Cell differentiation.**

The Notch signaling pathway has a main role in cell differentiation, inflammation and carcinogenesis. Notch activation was implicated in de-differentiation of adult hepatocytes into precursors of IH-CCA. These studies not only show the high plasticity of liver cell but also change the traditional model according to which CCA cells derived from cholangiocytes or hepatic common progenitor cells [85]. These observations could be combined to Sia et al. data. In this work, the authors describe how gene signatures, especially the proliferation class one's, overlap with those identified in HCC. These data provides us a second model of CCA carcinogenesis; not only based on alteration of epithelial cells of biliary tree but also on de-differentiation of adult hepatocytes.[84].

• **6.3 Epigenetic changes.**

Tumor cells may also acquire an advantage in survival and proliferation through epigenetic changes that lead to silencing of onco-suppressor genes. Mutations of isocitrate dehydrogenase 1 (IDH1) and 2 (IDH2) are among the most common genetic alteration in IH-CCA (14-36%) [70, 86], with unclear prognostic significance [87]. No mutations in these genes were observed in EH-CCA and GBC. Mutant IDH proteins lead to an abnormal enzymatic activity inducing to production of 2-hydroxyglutarate (2-HG) from α -ketoglutarate (α KG), which is considered an oncometabolite and causes epigenetic changes. Furthermore, deficiency in α KG inhibits activity of α KG-dependent dioxygenase and results in altered cell differentiation, survival and extracellular matrix maturation [88, 89]. Mutant IDH blocks liver progenitor cells that, as a result of altered hepatocyte response to hepatic injury, could lead to the development of malignant lesions. Indeed, a recent work has shown that mutant IDH blocks hepatocyte differentiation through the production of 2-HG and the suppression of HNF-4 α ,

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the main regulator of hepatocytes identity and quiescence [90]. Farshidfar et al. have associated mutant IDH with a specific gene signature characterized by high mitochondrial and low chromatin modifier gene expression. Speculating about the significance of this signature is complex for the wide number of genes and processes involved. Potentially, the combination of anti-IDH targeted therapies with anti-mitochondrial activity drugs could be exploited in the future as a therapeutic strategy [91].

• **6.4 Angiogenesis and tumor environment.**

VEGF has a central role in carcinogenesis and it has been targeted in different malignancies. In CCA, VEGF expression is reported in about 30-40% of cases and is correlated with lymph node metastasis and poor prognosis [38]. VEGF expression is reported in 50% of GBC and also in this sub-group a poor prognosis is observed [39].

BTC have a characteristic hypovascular, desmoplastic stroma that plays an important role in tumor pathogenesis and is consisting of cancer associated fibroblasts (CAF) expressing α -smooth muscle actin (α -SMA), activated macrophages and a fibrotic collagen rich extracellular matrix [92].

α -SMA positive CAFs are involved in cancer progression through production of matricellular proteins, growth factors, chemokines, and matrix metalloproteinases. Patients expressing high levels of α -SMA have poorer survival [93]. The desmoplastic matrix also allows the development of a niche fostering tumor spread rather than as a response to the anticancer treatments [94]. Transforming growth factor β (TGF- β) seems to be implicated in the generation of the niche. Indeed, preclinical models have demonstrated a reduction in fibrosis and tumor spread with TGF- β antagonist [95].

CAFs produce numerous factors involved in autocrine and paracrine signalling that promote oncogenic processes like periostin, tenascin-c, thrombospondin 1, stromal cell derived factor 1 (SDF-1), HGF and Wnt inducible signalling protein-1v (WISP1) [93]. These interact with cell signalling pathways. For example, periostin interacts with tenascin-C, HGF and SDF-1, which bind to their respective receptors, integrin, MET and CXCR4 on CCA cells, leading to activation of the PIK3CA/AKT signalling pathway.

Finally, desmoplastic stroma may be influenced also by sonic Hedgehog (Hh) signalling pathway. Preclinical models have disclosed the interplay between Hh and CAF through platelet-derived growth factor BB [96].

Cancer associated macrophages (CaM) are implicated in formation and maintaining of the stromal microenvironment and appear to have prognostic significance. A high number of CD163+ macrophages in the stroma of resected IH-CCA correlates with poor disease-free survival [97].

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Desmoplastic stroma formation is also associated with increased level of IL-6 that promotes tumor growth via autocrine and paracrine mechanisms [93].

For all these reasons, targeting stromal factors involved in cholangiocarcinogenesis or improving drug delivery through the desmoplastic stroma are attractive targets for novel therapeutics.

• **6.5 Immune system.**

In BTC, a number of clinical and epidemiological factors might predict the efficacy of immunotherapies ~~coexist~~.

Several chronic infections, such as viral hepatitis and bacterial cholangitis, are established risk factors for cholangiocarcinoma. Notably, immunotherapies have shown promising efficacy in cancer associated with infections, probably thanks to the presentation of non-self or neoantigens related with infections [98].

Furthermore, in a patient subgroup of BTC with poor prognosis has been revealed had a high mutational load, resulting in abundant tumor-specific neoantigens, and enrichment for expression of immune-related genes, including genes encoding inhibitory immune-checkpoint proteins [65]. In these patients, immune-checkpoint inhibition could permit to overcome cancer related mechanism of immune-silencing.

A central role in immune response is played by CaM that, through the production of soluble factor such as interleukins or cytokines, modulates anticancer immune response and maintain stromal environment [97].

In some small studies, programmed death-ligand 1 (PD-L1) expression has been evaluated on cancer specimens and on immune cells within the tumor microenvironment [99, 100]. PD-L1 is one of the most studied biomarker and levels of tumor PD-L1 expression have been associated with sensitivity to immune checkpoint inhibitor monotherapy in several tumor and they could predict response to the immunotherapies.

7- Competitive environment and pPotential development issues

• **7.1 HER2**

HER2 also belongs to the ErbB family of tyrosine kinases receptor and it is seems to be overexpressed in approximately 10% of GBC and 26.3% EH-CCA [25]. Given the efficacy of agents targeting HER2 in other cancers types, they were tested in BTC patients, nevertheless none improved outcomes so far.

Lapatinib is a dual tyrosine kinase EGFR and HER2 inhibitor; it was tested in two phase II studies in patients with advanced BTC. The first one was conducted on hepatobiliary cancer patients including 19 BTC, reaching poor results (mPFS was 1.8 months and mOS was 5.2 months) and no objective responses [101]. Later, similar results have emerged from a phase II trial involving only BTC patients: the response was extremely poor (0%),

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9 leading to premature discontinuation of the trial [102]. The role of **Trastuzumab**, a mAb that targets the HER2
10 receptor, is so far not well defined in treatment of BTC. The only data available comes from a retrospective
11 analysis in which Javle et al. studied BTC patients with HER2 genetic alterations or protein overexpression
12 treated with HER2-directed therapy in combination with concurrent therapy of physician's choice. Among the
13 eight GBC patients who received trastuzumab, one patient experienced complete response (CR), four patients a
14 partial response (PR), and three had stable disease (SD). The median duration of response in these patients was
15 40 weeks. In contrast, among five CCA patients no response was observed and disease progression occurred
16 during treatment with trastuzumab [103]. Likewise, in 2012 Law reported a case of a patient affected by HER2
17 positive GBC, showing a dramatic response after nine weeks of treatment with trastuzumab and paclitaxel
18 [104].

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22 **Neratinib**, a more recent tyrosine kinase inhibitor HER2-directed is actually under evaluation in SUMMIT, an
23 ongoing basket trial involving a variety of tumor types harboring HER2 mutations, including BTC
24 (NCT01953926). Preliminary data, recently presented during the American Association for Cancer Research's
25 Annual Meeting 2017 showed an ORR of 22% in BTC (9 patients) [105].

26
27
28 Similarly, in a phase II trial evaluating the efficacy of **Afatinib** - a potent, orally bioavailable ErbB Family Blocker
29 - was tested in different malignancies including 5 BTCs presenting HER2 amplification [106]. [As shown in table](#)
30 [1, afatinib](#) is currently under evaluation, in association with capecitabine, in a phase I/Ib trial addressed to
31 patients with advanced refractory solid tumors, comprising pancreatic cancer and BTCs (NCT02451553) (Table
32 [1, New therapies under evaluation in biliary tract cancers](#)).

33
34
35 Given these contradictory outcomes, HER2-directed therapy remains an open chance of treatment for BTC
36 patients with gene amplification, especially in GBC. A phase II trial of trastuzumab-emtansine is currently
37 ongoing in HER2-positive BTC patients (NCT02999672) (Table [1, New therapies under evaluation in biliary](#)
38 [tract cancers](#))

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42 • **[7.2 PIK3CA/PTEN/AKT/mTOR pathway](#)**

43 The PIK3CA/PTEN/AKT/mTOR pathway has drawn attention in last years as a target for new drugs
44 development. About 12.5% of GBC patients have activating mutations of PIK3CA [107].

45
46
47 In a phase II Italian study, 39 patients with advanced and pre-treated BTC received **everolimus** - an mTOR
48 inhibitor; mPFS was 3.2 months, and mOS was 7.7 months [108]. Since only patients who had received no more
49 than one previous systemic chemotherapy regimen were enrolled, these results are at least in line with
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9 conventional 2nd-line chemotherapy results in advanced BTC, reporting a mPFS and mOS of 2.8 and 7.5 months
10 respectively.

11
12 At the same time, in the Australian phase II study, everolimus was investigated in the first line setting in
13 advanced BTC. Final results are not yet available, but preliminary findings are encouraging: ORR of 12 % and
14 mPFS of 6.0 months [109].

15
16 More extensive studies are needed to clarify the efficacy of mTOR inhibitors in the treatment of BTC.

17
18 As shown in table 1, a phase II trial using a PIK3CA inhibitor, **copanlisib** in CCA, in first line setting in
19 combination with cisplatin and gemcitabine is ongoing (NCT02631590) [Table 1](#).

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21
22 • **7.3 BRAF**

23 BRAF mutations occur in a small portion of BTC patients; the most common mutation is V600E, whose
24 frequency range from 5 to 8% [110].

25
26 In the study of Hyman the efficacy of **vemurafenib** was evaluated in non-melanoma cancers. In the cohort of 8
27 CCA patients harboring BRAF V600E mutation, it was described one PR, which have persisted for more than 12
28 months, four SD and three progression disease (PD) [111].

29
30 Furthermore, given the impressive activity noted in metastatic melanoma with BRAF V600 mutations using a
31 combination of **dabrafenib** (BRAF inhibitor) and **trametinib** (MEK1 and MEK2 inhibitor), dual blockade merits
32 evaluation also in this subset. A case report of a notable response in a IH-CCA patient who received this
33 combination supports this hypothesis [112].

34
35 • **7.4 ROS, ALK, NTRK**

36 The presence of NTRK fusions in patients diagnosed with IH-CCA have been stated around 3.5% [113]. Sporadic
37 fusions of ROS1 (up to 8.7%) and ALK (2.6%) are also described [114].

38
39 Compounds targeting an NTRK1/2/3, ROS1, or ALK gene rearrangement have demonstrated impressive ORR
40 (57-86%) in a selected population of solid tumors [115].

41
42 Based on this data and the well-known clinical activity of ALK inhibitors in non-small cell lung cancer (NSCLC)
43 with ALK or ROS1 translocation, a phase II trial is ongoing. This trial evaluates the efficacy and safety of ceritinib
44 in patients with IH-CCA over-expressing ROS1 or ALK (NCT02374489). The recently identified FIG-ROS fusion
45 suggests that crizotinib therapy merits evaluation: NCT02034981 is a phase II trial of crizotinib in patients
46 harbouring an ALK, MET, or ROS1 alteration [Table 1](#).

47
48 During the most recent ASCO annual meeting, the presentation of preliminary data on larotrectinib showed a
49 consistent and durable antitumor activity of this drug in NTRK fusion cancers, across a wide range of tumor
50

types. Among 55 NTRK fusion patients enrolled, 2 were CCA. The ORR for the 46 patients evaluated was 78%, with responses in 12 tumor types; no data on CCA were presented [116].

- **7.5 FGFR**

FGFR pathway is involved in cellular migration, proliferation, survival, and differentiation. FGFR mutations and fusions predominate in IH-CCA in about 16% of cases [110]. In particular, genome-wide structural analyses showed numerous translocation events concerning the FGFR2 locus, ranging between 11 and 45% in IH-CCA patients [117]. The discovery of recurrent FGFR aberrations has opened a promising therapeutic avenue.

BGJ 398, an oral FGFR inhibitor, is under evaluation in a phase II trial in advanced CCA with FGFR gene fusion/translocation after first-line chemotherapy (NCT02150967). The recent interim report of this trial was the following: 50 patients with BTC having FGFR genetic alterations were enrolled, the majority with IH-CCA. The ORR was 22% (all 8 patients with a partial response had an FGFR2 fusion) and the disease control rate (DCR) was 95% with PFS of 6 months [103].

A number of other clinical trials involving selective FGFR small molecule inhibitors - including INCB54828 (NCT02924376), BAY1163877 (NCT01976741), and the irreversible FGFR inhibitor TAS-120 (NCT02052778) - are currently in progress in early-phase trials in patients with advanced solid tumors, including BTC (Table 1).

Moreover, nonselective multi-TKIs targeting also FGFR, including **ponatinib** and **pazopanib**, have showed some activity in patients with highly pretreated IH-CCA [117].

Finally, a nonselective TKI, ARQ 087 (NCT01752920), which inhibits RET, PDGF receptor, KIT, SRC, and FGFR1–3, is currently under evaluation in a phase II trial of patients with FGFR-aberrant tumors, including FGFR2 fusion-positive advanced IH-CCA. Preliminary data from the phase I/II basket trial show that 3 of the 12 IH-CCA patients with FGFR2 fusion had a PR (DCR of 75%).

In conclusion, the preliminary data for FGFR inhibitors in advanced IH-CCA are encouraging.

- **7.6 IDH1-2**

IDH 1 and 2 mutations are frequent in IH-CCA (9 of 40, 23%), while lacking in EH-CCA and GBC patients. Additionally, results from several researches have shown that IDH1 mutation is more common than mutation of IDH2.

Recently the findings of a dose escalation study of AG-120, an IDH1 inhibitor, in patients with advanced solid tumors having these mutations was presented by Burris et al. Of the 20 IH-CCA patients enrolled, 1 patient (5%) reached a PR and 11 patients (55%) had SD, with disease stability beyond 6 months [118].

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9 | As shown in table 1, the potential efficacy of IDH1 and IDH2 inhibitors is currently being evaluated in clinical
10 trials involving solid tumors such as BTC that harbor these mutations (NCT02481154, NCT02073994,
11 NCT02381886). Table 1

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14 • **7.7 Immunotherapy**

15 Mechanisms involved in DNA repair are indispensable for the maintenance of genomic stability. Acquired or
16 genetic mutations leading to defective DNA mismatch repair (MMR) are common in several tumors such as
17 colorectal, gastric and endometrial cancer [110].

18
19 In the study of Jaivle et al regarding mutational profiling of BTC, mutations in genes involved in DNA repair -
20 MSH6, BRCA1, BRCA2, ATM, MLH1 or MSH2 - was highly represented: 13% in IH-CCA, 26% in EH-CCA and 6% of
21 GBC cases [103].

22
23 Available data comes from KEYNOTE-028, an ongoing, phase 1b trial of **pembrolizumab** monotherapy for
24 advanced solid tumors PD-L1-positive. Patients with PD-L1-positive BTC were included, with the exception of
25 cancer of the ampulla of Vater. Of 89 BTC patients screened for PD-L1 expression, 42% were PD-L1-positive
26 tumors. 24 pretreated patients - including 38% who received ≥ 3 prior therapies - were enrolled, showing an
27 ORR (confirmed and unconfirmed) of 17%, with 4 PR, 4 SD, and 12 PD [119].

28
29 Given the demonstrated sensibility to programmed cell death protein 1 (PD-1) blockade with checkpoint
30 inhibitor agents (e.g pembrolizumab or nivolumab), BTC patients presenting these mutations can represent a
31 subset where immunotherapy may be effective. A number of immunotherapy studies are currently recruiting
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35 (Table 1).

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39 **8-Conclusion**

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41 Treatment for advanced BTC is challenging; although cisplatin and gemcitabine are considered the standard 1st
42 line on the basis of a consolidated phase III study, survival outcomes remain dismal; TCR is achieved in
43 approximately 80% of cases, but mOS is generally less than one year. Second-line treatment should be offered
44 to patients who maintain a good PS, but no schedule should be preferred above the others. To date, there is no
45 strong evidence to support the use of combination instead of single-agent therapy neither. Data from
46 retrospective series show that 2nd line treatment yields mPFS of 3 months and mOS of 7 months approximately.
47
48 Moreover, treatment of BTC patients is encumbered with a high rate of complications, such as infections, need
49 of biliary stenting, gastrointestinal toxicity, that have a high burden not only on patient's care but also on social
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and economical aspects. It goes without saying that every patient should be carefully evaluated for potential risks and benefit of active therapy and that every treatment, in addition to efficacy, should provide a manageable safety profile.

In the past years, despite initial encouraging reports, targeted agents have failed to provide significant changes in the history of BTC patients. In particular, anti-EGFR and anti-VEGF treatment used in addition to cisplatin and gemcitabine as 1st line treatment have provided no benefit in randomized control trials. Other targeted agents used, such as anti-MEK or anti-HER2, and data from some small non-randomized trials have shown little value and conflicting results. More recently, some other druggable targets are raising renewed interest in tailored medicine for BTC care.

9-Expert opinion

Despite a great effort to improve patients' outcomes, in the past decades only few studies have provided practice-changing findings. The milestones of BTC care include the superiority of chemotherapy over BSC [12] and the identification of gemcitabine-cisplatin as standard treatment for advanced disease [10]. More recently, the role of adjuvant chemotherapy after resection of localized disease is being supported by randomized controlled trials [9]. These results have been achieved over a period of about 20 years and appear inadequate considering the progresses that have been made in other diseases, including gastrointestinal malignancies, in the same time span.

Undoubtedly, dealing with a rare disease is the main disadvantage researchers have to face when designing clinical trials; in the 90ies BTCs were often grouped with ampullary and pancreatic cancers. The bulk of literature available includes small trials that, in spite of being multicentric, generally have a small sample size of 40-100 patients. In addition, some of the first studies with targeted therapies were not supported by a strong rationale or an accurate patient selection; in some cases, researchers have tried to apply the same paradigms that showed success in other cancer types to BTC, an orphan disease with very few options.

We have already pointed out that the different entities that are commonly included in BTC, such as IH-CCA, EH-CCA e GBC, have also different clinical and molecular features. Sometimes they are used as stratification factors for randomization or to conduct subgroup analyses. In our opinion, this issue is a great limitation especially for studies with targeted agents because the different pathways activated or inhibited are strongly influenced by the molecular features of the tumor, which vary strongly among the entities. For example, in the first studies with anti-EGFR therapies, slightly better results were observed in the IH-CCA groups, but their real impact was never significant because of the small sample size or the retrospective character of the analyses.

With the advent of new, advanced technologies like next-generation sequencing the underpinnings of genomic

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9 landscape of BTCs are being disclosed. To date, no distinct molecular mutation characterizes BTCs, but the
10 scenario is scattered into several alterations that have a relatively low rate, especially if we consider the
11 different anatomical locations. Among the druggable alterations, IDH1-2 mutations and FGFR translocations are
12 those that show the most potential because of higher frequency and the investigation of different compounds
13 in clinics.
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16 In parallel with the progress of technologies, drug development in the past few years is substantially changing;
17 trial design is moving from the standard approach from phase I to phase III trials and approval, to newest
18 methods. More often we are observing proof-of-concept studies that lead to an accelerated approval by
19 regulatory agencies, thanks to striking results on a limited number of patients in non-randomized phase I or II
20 trials.
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23 Moreover, revolutionary trials designs permit to handle multiple related questions with fewer patients. In
24 basket trials the effect of one drug is tested at the same time but in a variety of tumor types that share the
25 same single mutation. Umbrella trials indeed have many different treatment arms within one trial; people are
26 assigned to a particular treatment arm of the trial based on their type of cancer and the specific molecular
27 makeup of their disease.
28

29 Nivolumab and pembrolizumab have recently been granted FDA approval for microsatellite instability-high
30 (MSI-H) tumors; this is the first time a cancer treatment to be approved based on a common biomarker rather
31 than tumor type. A percentage of BTCs patients are potentially involved by this progress: similarly, some
32 ongoing basket trials that we have introduced in the previous paragraphs, such as those directed against ROS,
33 ALK, NTRK1/2/3 are deemed to provide interesting results for patients harboring low-frequency mutations.
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36 Thanks to these new approaches and to the better knowledge of potentially actionable genomic alterations in
37 BTCs it will be possible to offer a chance for tailored medicine to BTCs patients ant to and to meet an unmet
38 clinical need.
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45 **10-References**

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