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REVIEW

# TARGETED THERAPY IN CHOLANGIOCARCINOMA: CURRENT LANDSCAPE AND FUTURE HORIZON

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**ABSTRACT**: Cholangiocarcinoma (CCA) is a malignant disease of the biliary tree which accounts for 15% of primary liver cancers and 3% of gastrointestinal malignancies. For almost a decade, palliative chemotherapy remained the standard of care for advanced CCA with only modest disease control and survival rates, both in first- and second-line settings. In recent years, thanks to the advances of next-generation sequencing (NGS), an extensive range of targetable genetic alterations has been identified. In this review, we highlight different driver genomic alterations and their relative frequencies in advanced CCA, and we provide an updated overview of the current targeted treatment landscape.

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**Impact statement:** This review highlights different driver molecular alterations and their relative frequencies in advanced CCA and provides an updated overview of the current targeted treatment landscape.

**Key words**: *target therapy; advanced cancer; cholangiocarcinoma; biomarkers; resistance.*

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## INTRODUCTION

Cholangiocarcinoma (CCA) accounts for <3% (1) of all gastrointestinal malignancies and includes a cluster of heterogeneous tumours arising from the intrahepatic (iCCA), perihilar (pCCA) and distal (dCCA) biliary tree. CCA is a rare cancer, but its incidence (0.3–6 per 100,000 inhabitants per year (2)) and mortality (1–6 per 100,000 inhabitants per year, globally (3)) have been increasing in the past few decades worldwide, representing a global health problem.

In early stages, CCA is generally asymptomatic or presents with nonspecific symptoms, thus most patients are diagnosed when their disease is already inoperable (4). Unfortunately, prognosis for patients diagnosed with locally advanced or metastatic CCA is dismal, with a median overall survival (mOS) of only 19 and 9 months for patients with AJCC stage III and IV CCA, respectively, compared with 69 and 33 months for patients with stage I or II CCA, respectively (5).

Until recently, treatments for patients with advanced disease have relied on the use of palliative chemotherapy with only modest disease control and survival rates, both in first- and second-line settings. In recent years, the development of next generation sequencing (NGS) techniques, which can be performed both on tumour tissue and circulating blood tumour DNA (ctDNA), have identified an extensive range of molecular alterations in CCA. Genomic data suggest that up to 40-70% (6, 7) of patients with CCA harbor potentially actionable alterations (8) (**figure 1**) with different levels of evidence for clinical actionability of molecular targets according to the ESMO Scale for Clinical Actionability of molecular Targets (ESCAT) (9). Given the accumulating evidence that advanced CCAs could benefit from treatment personalization, the European Society of Medical Oncology (ESMO) recently recommended comprehensive NGS profiling of every patient with advanced CCA, to better define the treatment strategy (9).

In this review, we highlight different driver molecular alterations and their relative frequencies in advanced CCA, and we provide an updated overview of the current targeted treatment landscape.

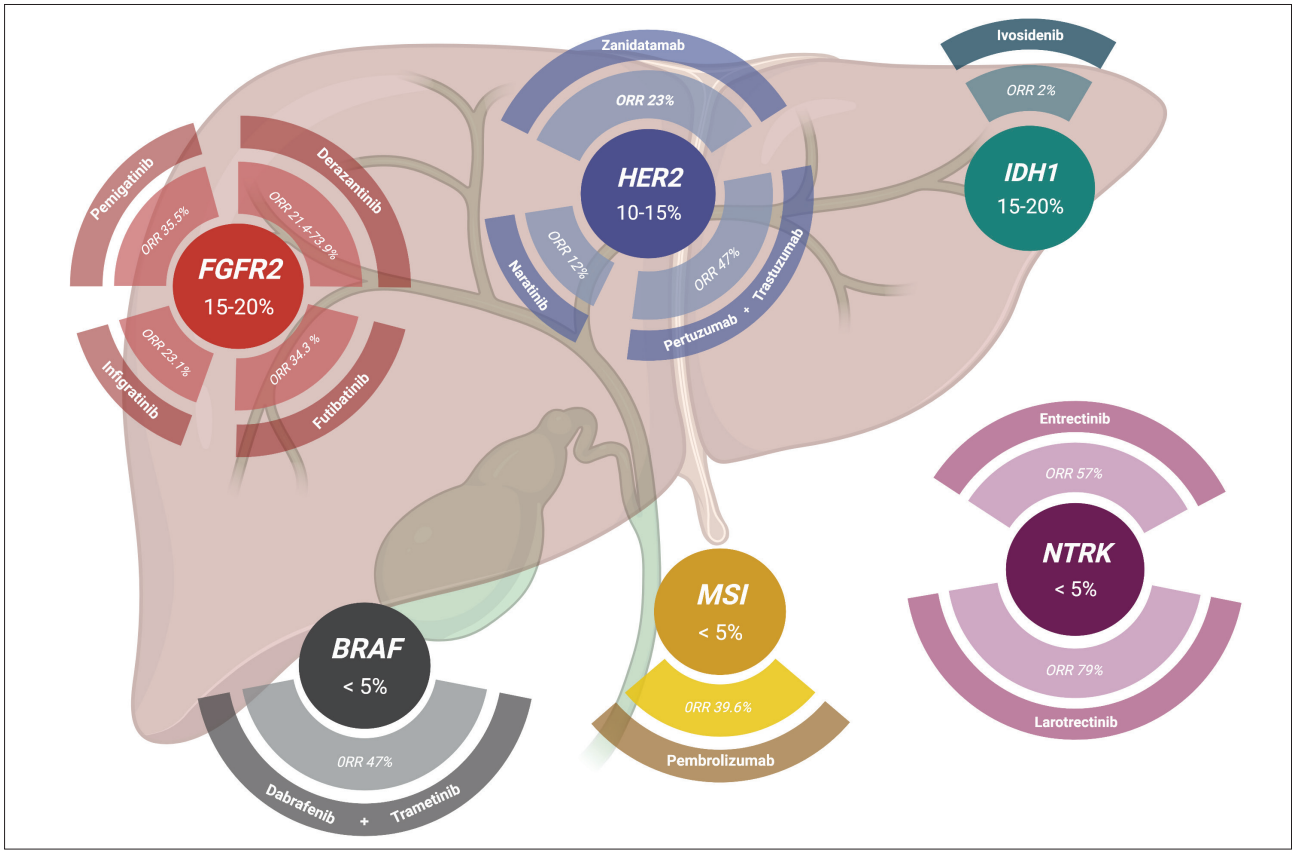


**Figure 1.** *Summary of relevant pathways responsible for development and progression of CCA. Red bubbles indicate molecular alterations of the gene which lead to hyperactivation or deregulation of signalling pathways, ultimately responsible for tumour growth, apoptosis evasion, proliferation, migration and invasion of cancer cells.*

## GENETIC LANDSCAPE AND ACTIONABLE ABERRATIONS

Data from several studies indicate TP53, KRAS, CDKN2A/B, ERBB2, BRAF, BAP1, PI3KCA, ARID1A, IDH1/2, FGFR1–3, PBRM1, SMAD4, BRCA 1/2 and MCL1 as the most frequently altered genes in tumour tissues from patients with CCA (7, 8, 10). ARID1A, CDKN2A/B, TP53, IDH1, BAP1 and FGFR2 gene aberrations are more commonly found in iCCA compared with eCCA, while HER2 amplification and TP53 mutations are most frequent in eCCA; in detail, alterations in IDH1, BAP1 and FGFR2 are almost limited to iCCA (7, 8, 10, 11), with a trend toward mutual exclusivity between FGFR2 fusions and KRAS, IDH1 or BRAF mutations (8, 12).

Additionally, programmed Death-Ligand 1 (PD-L1) expression has also been reported to be present in 10-70% of iCCA tumor specimens (13-15) while microsatellite unstable tumors (MSI-H) are uncommon and occur only in a minority of patients (ranges from 1 to 10%) (13, 16).

Among these alterations, there are at least 4 that are considered ESCAT level I, as there is enough data to drive treatment decisions in daily practice (IDH1 mutations, FGFR2 fusions, NTRK fusions and MSI-H) (9). Furthermore, there is growing evidence for treatments targeting ESCAT level II and III alterations such as BRAF and BRCA 1/2 mutations or ERBB2 amplification. **Table I** includes targeted agents with corresponding pivotal clinical trials, efficacy, and common adverse events whereas **table II** shows promising molecularly targeted therapies currently under investigation. The most relevant pathways (**figure 2**) and drugs are discussed below.

## FIBROBLAST GROWTH FACTOR RECEPTORS (FGFRS)

FGFRs consist of four transmembrane receptors (FGFR 1–4) with intracellular tyrosine kinase domains that carry out essential physiologic functions involving cell proliferation, differentiation, migration, and apoptosis via binding to their ligands (fibroblast

**Table I**. *Molecularly targeted therapies approved by regulatory agency or with concluded clinical trials showing relevant efficacy for CCA.*

| ACTIONABLE ALTERATION | DRUG (COMPARATOR) | TRIAL (PHASE) | SETTING | PATIENT NUMBER | ORR | MPFS (MO) | MOS (MO) | MAIN AES (FREQUENCY) | APPROVAL STATUS |
|---|---|---|---|---|---|---|---|---|---|
| IDH1 mutation | Ivosidenib (placebo) | ClarIDHy (III) | ≥2nd line, stage IV CCA | 187 | 2% | 2.7 | 10.3 | nausea (41%), diarrhea (35%), fatigue (31%) | Approved by FDA (2021) |
| FGFR2 fusion or rearrangement | Pemigatinib (none) | FIGHT-202 (II) | ≥2nd line, stage IV CCA | 108 | 37% | 7.0 | 17.5 | hyperphosphatemia (60%), alopecia (49%), diarrhea (47%) | Approved by FDA (2020), EMA (2021) |
| | Infigratinib (none) | NCT0215096 (II) | ≥2nd line, stage IV CCA | 108 | 23% | 7.3 | 12.2 | hyperphosphatemia (77%), eye disorders (68%), stomatitis (55%) | Approved by FDA (2021) |
| | Futibatinib (none) | FOENIX-CCA2 (II) | ≥2nd line, stage IV CCA | 103 | 42% | 9.0 | 21.7 | hyperphosphatemia (85%), alopecia (33%), dry mouth (30%) | Priority review by FDA |
| FGFR alteration | Erdafitinib (none) | NCT02699606 (II) | ≥2nd line, stage IV CCA | 22 | 41% | 5.6 | 40.2 | dry mouth (68%), stomatitis (64%) | Not approved |
| NTRK fusion | Larotrectinib (none) | LOXO (I), NAVIGATE (II) [pooled analysis] | stage IV solid tumor, no standard treatment available | 153 * | 79% * | 28.3 * | 44.4 * | increased AST/ALT (45%), anemia (45%), fatigue (37%) * | FDA (2018), EMA (2019) |
| | Entrectinib (none) | ALKA (I), STARTRK-1 (I), STARTRK-2 (II) [pooled analysis] | stage IV solid tumor, no standard treatment available | 54 * | 57% * | 11.0 * | 21.0 * | increased creatinine (73%), anemia (67%), hyperuricemia (52%) * | FDA (2019), EMA (2020) |
| BRAF V600E mutation | Dabrafenib + Trametinib (none) | ROAR (II) | stage IV BTC, no standard treatment available | 43 | 51% | 9.0 | 14.0 | pyrexia (60%), nausea (42%), fatigue (33%) | Not approved |
| dMMR/MSI | Pembrolizumab (none) | KEYNOTE-158 (II) | ≥2nd line, stage IV noncolorectal tumor | 233 * | 34% * | 4.1 * | 23.5 * | fatigue (14%), pruritus (13%), diarrhea (12%) | FDA (2017), EMA (2022) |
| TMB-H (≥10 mut/Mb) | Pembrolizumab (none) | KEYNOTE-158 (II) | ≥2nd line, stage IV noncolorectal tumor | 102 * | 29% * | 2.1 * | 11.7 * | fatigue (16%), hypothyroidism (12%), pruritus (10%) | FDA (2020) |
| ERBB2/HER2 amplification or overexpression | Trastuzumab + Pertuzumab | MyPathway (II) | ≥2nd line, stage IV BTC | 39 | 23% | 4.0 | 10.9 | diarrhea (33%), increased AST/ALT (31%), anemia (20%) | Not approved |
| EBB2/HER2 activating mutation | Neratinib | SUMMIT (II) | ≥2nd line, stage IV BTC | 25 | 16% | 2.8 | 5.4 | diarrhea (56%), vomiting (48%), fatigue (40%) | Not approved |
| ERBB2/HER2 amplification or overexpression | Zanidatamab | NCT02892123 (I) | ≥2nd line, stage IV BTC | 20 | 47% | - | - | diarrhea (43%), infusion-related reactions (33%) | Not approved |
| HER2-positive | Trastuzumab deruxtecan | JMA-IIA00423 | ≥2nd line, stage IV BTC | 22 | 36% | 5.1 | 7.1 | anemia (53.1%), neutropenia (31.3%), and leukopenia (31.3%), interstitial lung disease (25%) | Not approved |
| HER2-low-expressing | | | | 8 | 12% | 3.5 | 8.9 | | |

** Data related to overall trial population of patients with an advanced solid tumor, not only BTC.*

*BTC: biliary tract cancer; CCA: cholangiocarcinoma; EMA: European Medicines Agency; FDA: Food and Drug Administration; dMMR: deficient mismatch repair; mOS: median overall survival; mPFS: median progression-free survival; MSI: microsatellite instability; ORR: objective response rate; TMB-H: high tumor mutational burden.*

***Table II.*** *Main molecularly targeted therapies currently under investigation for CCA.*

| ACTIONABLE ALTERATION | DRUG (COMPARATOR 1) [COMPARATOR 2] | TRIAL (PHASE) | SETTING | ORR (%) * | MPFS (MO) * | MOS (MO) * |
|---|---|---|---|---|---|---|
| FGFR2 fusion or rearrangement | Pemigatinib (GemCis) | FIGHT-302 (III) | 1st line, stage IV CCA | - | - | - |
| | Infigratinib (GemCis) | PROOF-301 (III) | 1st line, stage IV CCA | - | - | - |
| | Futibatinib (GemCis) | FOENIX-CCA3 (III) | 1st line, stage IV CCA | - | - | - |
| | Derazantinib + Atezolizumab (none) | NCT05174650 (II) | 2nd line, stage IV iCCA | - | - | - |
| FGFR2 fusion, mutation or amplification | Derazantinib (none) | FIDES-01 (III) | ≥2nd line, stage IV iCCA | 8.7% | 7.3 | - |
| IDH1 or IDH2 mutations | LY3410738 (none) | NCT04521686 (I) | 1st line, stage IV CCA | - | - | - |
| | Vorasidenib (none) | NCT02481154 (I) | ≥1st line, stage IV CCA | - | - | - |
| | Ceralasertib + Olaparib (none) | NCT03878095 (II) | ≥2nd line, stage IV CCA | - | - | - |
| | Olaparib + Durvalumab (none) | NCT03991832 (II) | 1st–3rd line stage IV BTC | - | - | - |
| KRAS G12C mutation | Adagrasib (none) | KRYSTAL-1 (I/II) | ≥2nd line, stage IV solid tumor | 41% | 6.6 | - |
| HRD | Olaparib | NCT04042831 (II) | ≥2nd line stage IV BTC | - | - | - |
| [I-O] | Pembrolizumab + Lenvatinib (none) | LEAP-005 (II) | ≥2nd line stage IV BTC | 10% | 6.1 | 8.6 |
| | Pembrolizumab + GemCis (placebo + GemCis) | KEYNOTE-966 (III) | 1st line stage IV BTC | - | - | - |
| | Durvalumab + GemCis (placebo + GemCis) | TOPAZ-1 (III) | 1st line stage IV BTC | 27% | 7.2 | 12.8 |
| | Nivolumab + Ipilimumab (none) | CA209-538 (II) | ≥1st line stage IV BTC | 24% | 3.1 | 6.1 |
| | Nivolumab (none) | NCT02829918 (II) | ≥2nd line stage IV BTC | 22% | 3.7 | 14.2 |
| | Nivolumab + Gem Cis (Nivolumab + Ipilimumab) | NCT03101566 (II) | 1st line stage IV BTC | - | - | - |
| | Tremelimumab + GemCis (Durvalumab + GemCis) [Tremelimumab + Durvalumab + GemCis] | NCT03046862 (II) | 1st line stage IV BTC | 50% (73%) [73%] | 13 (11) [12] | 15 (18) [21] |

** Ongoing studies, preliminary results reported when available.*

*HRD: homologous recombination deficiency; I-O: immune-oncology; mOS: median overall survival; mPFS: median progression-free survival; ORR: objective response rate.*

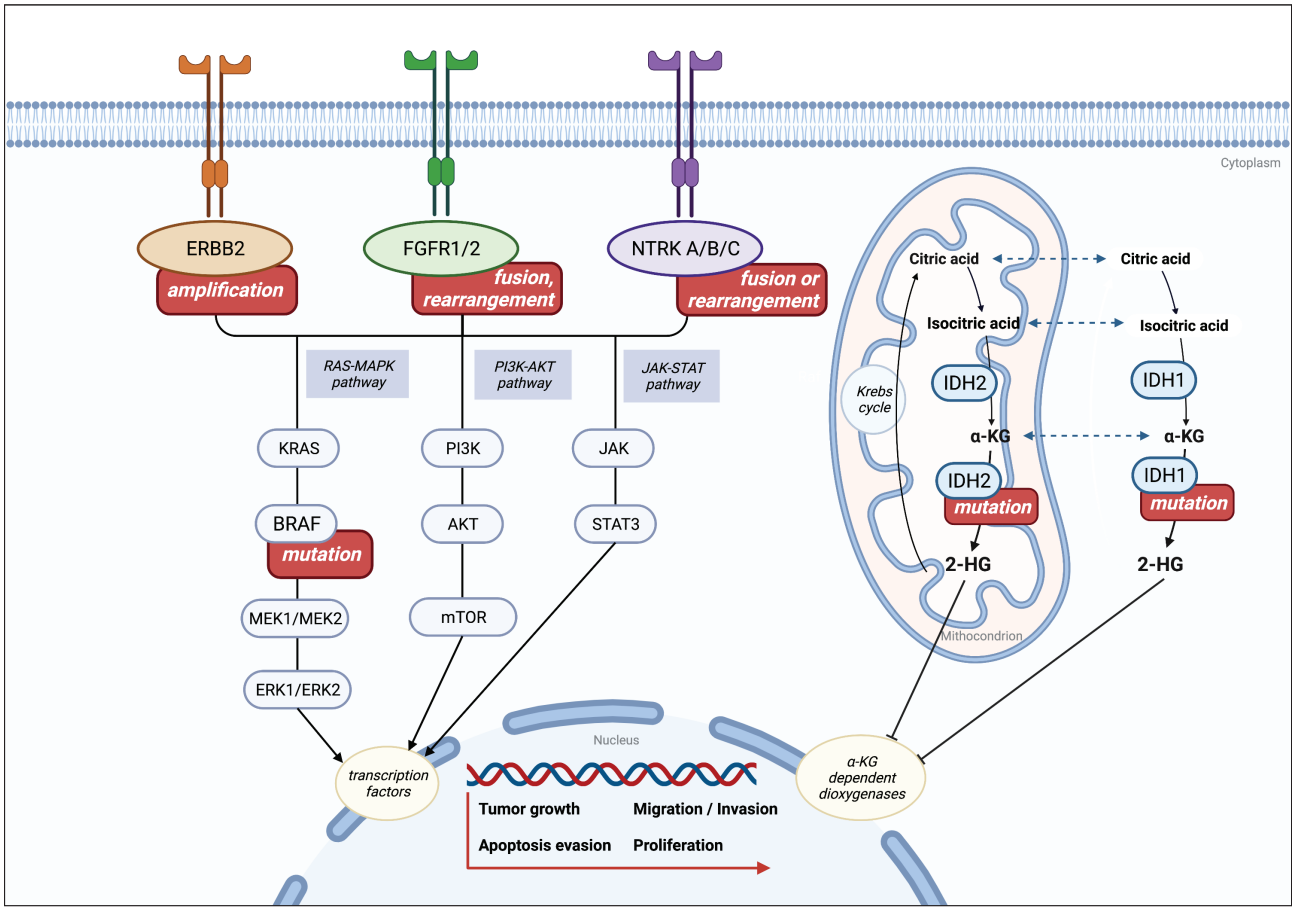


**Figure 2.** *Main targets of molecular therapies in CCA. Each sphere shows the relative frequency of targetable alterations. In semi-circles are shown therapies with relative objective response rate (ORR). MSI, BRAF and NTRK alterations represent agnostic target which are relatively rare in BTC, while FGFR2 fusion/rearrangements and IDH1 mutations are peculiar of iCCA and ERBB2/HER2 amplification/overexpression is frequent in every type of CCA. ORRs of Larotrectinib and Entrectinib refers to overall population of patients with advanced solid tumor harboring a NTRK fusion or rearrangement.*

growth factors, FGFs) (17). FGFRs aberrations are mostly located in the gene coding for FGFR2, with a minority of FGFR1 alterations (0.9% [FGFR1] *vs*. 6.1% [FGFR2] *vs*. 0% for the other family members), and there is a predominance of rearrangements or fusions (3.5%) over amplifications (2.6%), while mutation events are rare (0.9%) (18). FGFR2 fusion proteins are activated by the spontaneous dimerization of their respective partners, thus inducing the activation of downstream oncogenic signalling pathways including RAS-RAF-MEK-ERK/MAPK, PI3K/AKT/mTOR and JAK/STAT pathways.

Interestingly, FGFR2 fusion-positive tumours are almost exclusively found in iCCAs with an incidence of 10-20%, mainly in non Opistorchis Viverrini (11, 19) related iCCA and in females (20) and seems to be associated with indolent disease, even if the impact on prognosis is yet to be confirmed (21, 22).

Several FGFR inhibitors are currently being developed, many of which have already shown adequate safety and early efficacy in phase I-II studies for heavily pre-treated patients with iCCA harbouring an FGFR2 fusion; recent data show efficacy of FGFR inhibitors also across a broader spectrum of FGFR aberrations like FGFR2 mutations or amplification or uncharacterized FGFR1-3 aberrations (23, 24).

Currently, three of these agents are moving into phase III clinical trials, exploring their potential role in the first-line setting compared to standard Cisplatin and Gemcitabine (CisGem) chemotherapy, but the trials experience some difficulties of accrual.

## Pemigatinib

Pemigatinib (INCB054828) is a selective, reversible, oral inhibitor of FGFR 1-3. Its efficacy and safety were assessed in a single-arm phase 2 trial (FIGHT- 202) of patients with previously treated locally advanced/metastatic CCA with documented FGFR status (25, 26).

One hundred and seven of the enrolled 146 patients harboured FGFR2 gene fusions or rearrange-

ments. Of these, 35.5% achieved objective disease response (3 had complete responses and 35 had partial responses), and the disease control rate (DCR) was 82%. Median Progression Free Survival (mPFS) was 6.9 months and mOS was 21.1 months. Instead, no responses occurred in the groups of patients with other FGFR aberration or without FGFR alterations (26).

Based on these positive results, pemigatinib received approval by the FDA (27) and EMA for the treatment of adults with previously treated, unresectable locally advanced or metastatic CCA with an FGFR2 fusion or other rearrangement. Moreover, the antitumor activity and manageable toxicities associated with pemigatinib prompted the ongoing phase 3 clinical trial (FIGHT-302; NCT03656536) which compares pemigatinib with CisGem chemotherapy for advanced CCA patients with FGFR2 rearrangements in the first-line setting.

## Infigratinib

Infigratinib (BGJ398) is an oral bioavailable, selective, ATP-competitive pan-FGFR kinase inhibitor. Its therapeutic activity was assessed in a single-arm phase II clinical trial in patients with advanced CCA containing FGFR1-3 alterations, including FGFR fusions, mutations and amplifications who have progressed on or were intolerant to cytotoxic therapy (NCT02150967). The preliminary results of 61 patients with FGFR2 alteration reported that all responsive tumour contain FGFR2 fusions; the overall response rate (ORR) was 14.8%, the DCR was 75.4% and the mPFS was 5.8 months, which is comparable to first-line chemotherapy (20, 28). Subsequently, Javle *et al.* reported an ORR of 23.1% (with one confirmed complete response) in the 108 patients with FGFR2 fusions or other rearrangements (29). Recently, based on these encouraging data, infigratinib received approval by the FDA for the treatment of adults with previously treated, unresectable locally advanced or metastatic CCA with an FGFR2 fusion or other rearrangements. Recently, a phase III random controlled trial has started recruiting subjects with CCA harboring FGFR2 gene alterations to evaluate the efficacy and safety of infigratinib *vs.* chemotherapy (NCT03773302) in the first-line setting.

## Derazantinib

Derazantinib is an oral bioavailable, selective, ATP-competitive pan-FGFR kinase inhibitor which demonstrated good tolerability and single-agent activity in heavily pre-treated patients with unselected advanced tumours in a dose-escalation phase I study (30). In a *post hoc* analysis, the anti-tumour activity of derazantinib as measured by DCR and PFS seems to be similar for patients with FGFR2 fusions and FGFR2 mutations/amplifications while patients without any detectable FGFR gene aberration appear to derive no benefit (31). These data were confirmed in a subsequent phase I/II study of derazantinib in 29 FGFR2 gene fusion-positive, unresectable iCCA patients showing a mPFS of 5.7 months, an ORR of 20.7%, and a DCR of 82.8% (32).

A larger pivotal trial of Derazantinib targeting FGFR2 gene fusion-, mutation- or amplification-positive iCCA is ongoing (NCT03230318). The interim analysis suggest that Derazantinib provided clinical benefit not only in the subgroup of 103 patients with advanced iCCA harbouring FGFR2 fusion (ORR 21.4%, including 22 partial responses, DCR 74.8%, mPFS 7.8 months and mOS 15.5 months (33)) but also in the subgroup of 23 patients harbouring FGFR2 mutations/amplifications (ORR 73.9%, including 2 partial responses, mPFS 7.3 months) (24).

## Futibatinib

Futibatinib (TAS-120) is an irreversible and highly selective inhibitor which targets all four FGFR subtypes (34). It is under evaluation in a phase II study (NCT02052778) of 103 biliary tract cancer (BTC) patients with FGFR2 fusion or rearrangement, of which 67 were evaluable for efficacy in a recently presented interim analysis. In this group of patients (82% harbouring FGFR2 fusions), futibatinib gained an ORR of 34.3% and a DCR of 76.1% (35). In a preliminary phase I study, although patients with iCCA harbouring FGFR2 fusion/rearrangements experienced the greater benefit, objective responses were seen also in 2 patients with FGFR mutated CCA (23). In addition, activity of futibatinib seems significant even following progression on previous FGFR inhibitors, with a response rate of 17.9% in this setting, suggesting that it may be able to overcome mechanisms of resistance (23).

Currently, futibatinib is moving into phase III clinical trial exploring the potential role in the first-line setting compared to CisGem chemotherapy [FOENIX-CCA3; NCT04093362]) for patients with FGFR2 rearrangements.

## Erdafitinib

Erdafitinib is an orally bioavailable, selective pan-FGFR kinase inhibitor (36), already approved for the treatment of patients with unresectable urothelial

carcinoma harbouring FGFR2 or 3 genomic alterations (37).

Its efficacy has been assessed in adults with advanced CCA containing FGFR alterations who had failed at least one prior systemic treatment in an open-label phase IIa study conducted in China, Korea and Taiwan (NCT02699606). At interim results from this ongoing study, 15 of the 17 treated Asian patients with advanced CCA and FGFR alterations (10 FGFR2 fusion, 4 FGFR2 mutation, 1 FGFR3 fusion, and 2 FGFR3 mutation) had an evaluable response with an objective response rate (ORR) of 7/15 (47%) and a DCR of 12/15 (80%) (38).

## METABOLIC PATHWAY LINKED TO IDH1/2 MUTATIONS

Isocitrate dehydrogenase (IDH) is an essential enzyme for the citric acid cycle and converts isocitrate to α-ketoglutarate (α-KG) by oxidative decarboxylation, thus providing ATP and precursors for cellular metabolism. There are 3 human isoforms of IDH (IDH1, IDH2, and IDH3), which can be found in the cytoplasm and mitochondria (39, 40). Several studies indicate that IDH1/2 genes can be interested by gain-of-function point mutations, which means that mutant IDH1 (mIDH1) and mutant IDH2 (mIDH2) become able to catalyze the conversion of α-KG to 2-hydroxy-glutarate (2-HG), a substrate which competitively inhibits over 60 dioxygenases requiring α-KG as a cofactor, such as enzymes involved in the demethylation of DNA (41, 42). In this way, the accumulation of 2-HG, which can be detected in tumor tissue and blood (43, 44) in patients with IDH1/2 mutant tumors, impairs cellular differentiation through effects on chromatin structure and DNA methylation, finally leading to tumor initiation and progression.

Mutations in IDH genes are present in up to 10-15% (IDH1) and approximately 3% (IDH2) of CCAs; mutations are particularly frequent (up to 20%) in intrahepatic cases (7, 45, 46), especially if not related to infections (43). Prognostic implications of an IDH mutation in CCA is still under debate (47-50).

Several selective inhibitors targeting tumor harboring IDH-mutant alleles have been developed. These block the function of mutant IDH1 or IDH2 at nanomolar concentrations, leading to reduced 2-HG levels. In particular, ivosidenib has been the first mIDH1-inhibitor approved by the US Food and Drug Administration (FDA), in August 2021, for treatment of adults with previously treated, locally advanced or metastatic CCA harboring IDH1 mutation. Ivosidenib (AG-120) is a potent oral mIDH1-inhibitor and initially showed good activity in a combined phase I/II study (51), where 4 partial responses (5%), a 3.8 mPFS and 13.8 mOS were observed in 73 patients with pretreated advanced IDH1-mutated CCA. Benefit for ivosidenib in this population was further addressed in the placebo-controlled randomized phase III ClarIDHy trial (52, 53), which enrolled 187 patients with previously treated, advanced (93% metastatic) mIDH1-CCA (R132C/L/G/H/S mutation variants). Patients were randomized 2:1 to ivosidenib or placebo with cross-over permitted from placebo to ivosidenib following disease progression. The ORR with ivosidenib was 2.4%; however, DCR was 50.8% and mPFS was longer with ivosidenib (2.7 months) *vs.* placebo (1.4 months). With 70% of patients who crossed over from placebo to ivosidenib, the mOS (accounting for crossover) in the placebo group was 5.1 months *vs*. 10.3 months in the ivosidenib group. Based on these results, ClarIDHy was the first phase III trial reporting positive results with targeted therapies in CCA patients, and ivosidenib is now recommended by the NCCN guidelines for second line therapy in IDH1-mutant cholangiocarcinoma. This landmark study resulted in FDA approval of ivosidenib in this population of patients and mandates the provision of molecular profiling in CCA (53).

Several other mIDH1/2-inhibitors that might be effective in CCA are still undergoing testing in clinical trials (see **table II**).

The combination of mIDH-inhibitors with first-line chemotherapy may represent another step in improving the efficacy of this drugs if safety profile is acceptable. For instance, a phase I/II trial is studying the combination of an oral selective mIDH1-inhibitor, Olutasidenib (FT-2102), with standard first-line chemotherapy (CisGem) in patients with advanced IDH1-mutated iCCAs (NCT03684811).

Furthermore, in preclinical studies, IDH1/2-mutated tumor cells show an impaired mechanism of homologous recombination, which makes them vulnerable to poly ADP ribose polymerase (PARP) inhibitors (54). Indeed, several phase I/II trials are actually investing the efficacy of PARP-inhibitors in the treatment of advanced pretreated solid tumors with IDH1/2 mutations, including CCA, alone (NCT03212274; NCT03207347) or in combination with other agents affecting related pathways, such as ataxia telangiectasia and rad3 related (ATR) inhibitors (NCT02576444;

NCT03878095) or immune checkpoint inhibitors, such as durvalumab (NCT03991832).

Finally, there is currently focus on the biological rational of combining IDH inhibition with immunotherapy, in particular with anti-CTLA4 antibodies, in relation to immunosuppressive effects mediated by 2-HG and to the preclinical evidence that treatment with mIDH inhibitors results in the simultaneous recruitment of effector and immunoregulatory cells: thus, additional CTLA4 blockade may favor the immune effector response, resulting in synergistic antitumor effect (55, 56).

## EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR)/HER2

The HER family receptors consist of four distinct receptors: epidermal growth factor receptor (EGFR) or HER1, HER-2, HER-3, and HER-4, corresponding to the EGFR, ERBB1/2/3/4 genes. In normal cells, binding of the ligands to the extracellular domain of these receptors leads to the dimerization with eventual phosphorylation of the intracellular tyrosine kinase domain and activation of the downstream pathways, which include MAPK, PI3K/AKT/mTOR, and STAT pathways that control and regulate cell proliferation, differentiation, and metabolism (57). In CCA as well as in various human cancers, EGFR/ERBB2 alterations commonly lead to EGFR and HER2 overexpression which is highly related to tumorigenesis, tumour cell invasion and metastasis. Notably, 5-15% of CCA patients could harbour EGFR mutations (58), whereas ERBB2 amplification, overexpression or both are observed in approximately 17% of extrahepatic and 5% of intrahepatic CCA (45).

### EGFR Inhibitors

Several clinical trials have recently evaluated the role of EGFR-targeted drugs, usually divided into EGFR tyrosine kinase inhibitors (EGFR-TKIs) and monoclonal antibodies targeting EGFR (EGFR-mAbs), but in mostly in unselected populations. After the modest benefit showed in phase II studies, a phase III study compared the efficacy and safety of erlotinib plus gemcitabine and oxaliplatin (GEMOX) regimen with GEMOX regimen alone in therapy-naive patients with advanced BTC. Patients treated with chemotherapy plus erlotinib achieved a higher ORR (29.6% *vs*. 16.5%) but mOS (9.5 months in both groups) and mPFS (5.8 *vs*. 4.2 months) did not improve significantly; however, in a subgroup analysis, patients with CCA had an improved mPFS with the combination therapy (5.9 months *vs*. 3.0 months; p = 0.049) (59). In addition, two trials evaluated the efficacy of anti-EGFR antibodies cetuximab (in patients not selected for molecular alterations with sensitivity) and panitumumab (in patients with KRAS wild type tumours) plus gemcitabine-platinum doublets with disappointing results (60, 61).

Thus, based on this previous evidence, anti EGFR inhibitors do not seem to be a promising treatment option for patients with BTC.

### HER2 Inhibitors

MyPathway, a phase IIa multiple basket study, evaluated the combination of HER2-directed monoclonal antibodies pertuzumab and trastuzumab in 39 patients with previously treated BTC harbouring ERBB2 gene amplification or protein overexpression. After a median follow-up of 8.2 months, 9 patients had an objective response (all partial response), yielding an ORR of 23% (62).

Studies with other dual EFGR and HER2 tyrosine kinase inhibitors, including Afatinib and Lapatinib, failed to demonstrate the therapeutic effects in CCA patients (63-65). Preliminary results of the phase II SUMMIT basket trial testing Neratinib, an oral tyrosine kinase inhibitor of EGFR, HER2, and HER4, in pre-treated patients affected by solid tumours with activating somatic ERBB2 mutations showed, among the 25 evaluable patients, an ORR of 12%, a clinical benefit rate of 20%, a mPFS of 2.8 months and an OS of 5.4 months (66).

Furthermore, a phase I study of Zanidatamab, a bispecific HER2-targeted antibody, demonstrated among 17 evaluable patients an ORR of 47% and a DCR of 65% (67). Zanidatamab is now under further investigation as treatment option following standard-of-care for patients affected by ERBB2 amplified BTC in a phase II multicentric trial (NCT04466891).

Finally, the HERB trial evaluated the safety and efficacy of Trastuzumab deruxtecan, an antibody-drug conjugate composed of a humanized monoclonal anti-HER2 antibody and a topoisomerase I inhibitor, in patients with HER2-expressing (HER2-positive: IHC3+ or IHC2+/ISH+, and HER2-low-expressing: IHC/ISH status of 0/+, 1+/-, 1+/+, or 2+/-) BTC. Among 22 treated cases, ORR was 36% and DCR 82%, with mPFS and OS of 5.1 months and 7.1 months, respectively. In addition, encouraging effi-

cacy data were seen even in the 8 patients with low HER2 expression. Concerning safety, 8 patients (25%) had interstitial pulmonary disease, two of which were life-threatening events (68).

## RAS/RAF/MEK/ERK SIGNALING PATHWAY INHIBITORS

The RAS/RAF/MEK/ERK signaling cascade is an oncogenic pathway that is activated in several cancer types, including CCA, and promotes proliferation of cancer cells, migration, and metastasis. The frequency of detected KRAS mutations for intrahepatic and extrahepatic CCA ranges from 9% to 45% and from 15% to 67%, respectively, and 4% and 3% for NRAS (69-71). Although RAS mutations serve as oncogenic drivers in many different types of malignancies, targeting RAS has mostly been unsuccessful because of its intricate interactions with other signalling pathways. Consequently, inhibiting targets downstream of RAS, such as BRAF and MEK, has been attempted in various diseases.

BRAF mutations have been described in less than 5% of patients with CCA, primarily in the cohort with intrahepatic disease (7, 72). Dual targeting with dabrafenib (BRAF inhibitor) and trametinib (MEK inhibitor) within the ROAR trial, a phase II basket trial of 178 patients harbouring the BRAF V600E mutations, provided promising result. Among 43 patients with BRAF V600E-mutated BTCs (of which 91% were cholangiocarcinoma), this regimen achieved an ORR of 51%, a mPFS of 7.2 months and a mOS of 11.3 months (73).

Selumetinib, a MEK inhibitor, has been studied in combination with CisGem in a phase Ib study (ABC-04) in therapy-naive patients with advanced BTC, unselected for molecular alterations (74). Among eight evaluable patients, three had a partial response, and five had stable disease with a median PFS of 6.4 months; the most frequent adverse event were G1-2 oedema and rash. A phase II study with selumetinib, which did not select patients for molecular alterations, included 39% of chemotherapy-refractory patients, showing an ORR of 12%, a median PFS of 3.7 months, and a median OS of 9.8 months (75).

Recently, Adagrasib, a KRAS G12C-selective covalent inhibitor, demonstrated clinical activity and a manageable safety profile in pancreatic ductal adenocarcinoma and other GI cancer in a phase II study; in particular, it showed an ORR of 50% (4/8) in patients with BTC (76).

Based on these promising results, further studies are ongoing to assess the efficacy of drugs inhibiting this pathway.

## NEUROTROPHIC TROPOMYOSIN RECEPTOR KINASE (NTRK)

The neurotrophic receptor tyrosine kinase (NTRK) genes, NTRK1, NTRK2, and NTRK3, which are reported as altered in less than 5% of patients with iCCA (6, 77), encode the tropomyosin receptor kinases (TRK), a receptor family composed of three transmembrane proteins: TRKA, TRKB and TRKC (78). In a normal state, TRK receptor signalling has a role in neuronal development, function, survival, and proliferation whereas intrachromosomal rearrangements of NTRK1–3 resulting in gene fusions may activate signal transduction leading to oncogenesis (79). Point mutations and amplifications of NTRK genes have also been reported in human tumours (80).

Several small-molecule TRK inhibitors are currently in clinical development. In particular, two agents have recently received FDA and EMA approval for the tumour agnostic treatment of patients with solid tumours harbouring an NTRK gene fusion; these are the selective TRK inhibitor Larotrectinib and the TRK/ROS1/ALK multikinase inhibitor entrectinib, which have yielded an impressive ORR of 57% to 75% in advanced solid tumours harbouring NTRK fusions (the percentage of patients with cholangiocarcinoma enrolled in the two pivotal trials were 4% and 2%, respectively) (81, 82). Of note, TRK inhibitors have activity also against ROS1 and ALK fusions, which are reported in 0-8.7% and 2.7% of patients with CCA, respectively (6, 83).

### PI3K/AKT/mTOR PATHWAY

PI3K/AKT/mTOR is an intracellular signalling pathway in which the tumour suppressor gene PTEN plays an essential regulatory role (84). It encodes the PTEN protein, a phosphatase which reverses the actions of PI3K to control the level of PIP3, thus modulating the activation of the PI3K/Akt/mTOR pathway (85). Various types of tumours are interested by hyperactivation of PI3K or inactivation of PTEN, which results in deregulation of this signalling pathway, conferring cells oncogenic potential (86).

According to several studies, PI3K mutations were detected in 4.4% of iCCA patients and 6.5% of eCCA patients. PTEN mutations were observed in 4.4% of iCCAs and 3.9% of eCCAs (87). Blocking this sig-

nalling pathway might inhibit tumour growth. As a result, many inhibitors targeting the effector proteins in the the PI3K/Akt/mTOR pathway are under development and investigation.

Copanlisib (BAY 80–6946) is a selective pan-class I PI3K inhibitor which has been evaluated in a phase I study with 50 patients with advanced solid tumours not selected for molecular alterations, including 23 BTC patients (NCT01460537). Response rate was 6.3% in the copanlisib with gemcitabine and 12% in the copanlisib with CisGem arm. Among BTC patients, response rate was 17% (88). Currently, the therapeutic effects of the copanlisib plus CisGem regimen is being assessed in a phase II trial with CCA patients (NCT02631590).

The mTOR inhibitor everolimus has been evaluated in several clinical studies. A phase I study assessing the safety and antitumor activity of everolimus in combination with gemcitabine enrolled in 10 BTC patients, with 6 patients achieving stable disease (60%) (89). Subsequently, a phase II clinical trial was conducted to evaluate the therapeutic efficacy and safety of everolimus in 39 advanced BTC patients who were previously treated with chemotherapy. It reported a DCR of 44.7%, ORR of 5.1%, mPFS of 3.2 months and a mOS of 7.7 months (90). Another relevant phase II clinical trial of everolimus in cancer patients with PI3K mutation or PTEN loss did not show any general clinical benefit, though the only CCA patient achieved stable disease (91). In recent years, the data of the RADiChol study, a phase II clinical trial, was published (NCT00973713). Twenty-seven patients with advanced BTC were enrolled in this study. The primary endpoint DCR at 12 weeks was 48%, with a mPFS of 5.5 months and mOS of 9.5 months (92). Currently, the therapeutic efficacy of everolimus in BTC treatment has not been confirmed, so more clinical data are expected to be released.

## DDR AND BRCA1/2 MUTATIONS

Poly adenosine diphosphate-ribose polymerase inhibitors (PARPi) represent a novel therapeutic option for cancer patients harbouring germline and somatic aberrations in DNA damage repair (DDR) genes. Across BTC patients, BRCA1/2 mutations are rare (1-7%) but a broader spectrum of DDR gene alterations is reported in 28.9-63.5% (93, 94). A retrospective analysis by Golan *et al.* included 18 patients, 5 with germline BRCA1/2 mutations and 13 with somatic mutations; interestingly four stage III-IV patients were treated with PARP inhibitors with an OS from 11 to 65 months (95). To date there is no evidence regarding the efficacy of PARP inhibitor in BTC patients harboring DDR gene alteration, but phase II clinical trials evaluating Olaparib and Niraparib in this setting are now ongoing (96) (NCT04042831, NCT03207347).

Furthermore, there is preclinical evidence that IDH1 and IDH2 mutations could induce a homologous recombination defect similar to a "BRCAness" phenotype (97), due to the capacity of 2HG to inhibit DNA and protein demethylation leading to a hypermethylation profile (98). This hypothesis is currently being tested in several clinical trials (NCT03212274, NCT03991832, NCT03878095). Similarly, Niger *et al.* recently suggested that even MGMT may be involved in platinum-induced DNA damage response (DDR) by playing a role in the homologous recombination signaling in cancer cells, providing the rational to explore the combinations of TMZ with DDR inhibitors such as PARP and ATR inhibitors (100).

## BIOMARKERS FOR CHECKPOINT INHIBITORS SENSITIVITY

The only FDA-approved immunotherapy in BTCs is pembrolizumab, an anti-PD-1 antibody, which received tissue-agnostic approval for the treatment of advanced solid tumours with DNA mismatch repair deficiency (dMMR), microsatellite instability (MSI) and high tumours mutational burden, including those of the biliary tract (<3% cases) (100-102). However, MSI-H BTC are uncommon (ranges from 1 to 10%) and other clinical trials evaluating checkpoint inhibitors as monotherapy for advanced BTC have shown controversial and overall disappointing results. Clinical benefit seems limited only to a small percentage of BTC and, with the exception of MSI, no predictive biomarker has been identified yet (13, 103). However, various trials focused on evaluating the impact of programmed Death-Ligand 1 (PD-L1) expression, which is reported to be present in 10-70% of iCCA (13-15), on responses to checkpoint inhibitors.

In the phase Ib KEYNOTE 028, which enrolled exclusively patients with positive PD-L1 tumor expression (PD-L1 ≥1%), the ORR, PFS and OS of 23 patients with previously treated BTC were 13%, 1.8 and 5.7 months respectively (104, 105). Similarly in the KEYNOTE-158, assessing the efficacy of pembrolizumab in an unselected cancer patient population, the

ORR was 5.8% (6/104), mOS 7.4 months and mPFS 2 months. In a subgroup analysis, ORR was 6.6% (4/61) and 2.9% (1/34) among PD-L1-expressers and PD-L1-non-expressers, respectively (102). Furthermore, regarding 22 dMMR/MSI patients, response rate was 40.9% (including complete response in 2 patients), mPFS 4.2 months, and mOS 24.3 months (106).

Nivolumab has also been evaluated as second-line therapy for unselected refractory BTC patients in a multicenter phase II study which demonstrated an ORR of 22% (10 of 46) with a disease control rate of 59% (27 of 46) (107); PDL-1 expression in tumors was associated with prolonged progression-free survival (HR 0.23, P < .001 with PD-L1 cutoff of 1%; HR 0.37, P = .02 with cutoff of 10%).

Of note the recent phase III TOPAZ-1 trial (108) reported, for the first time, positive results of immunotherapy plus chemotherapy as first-line treatment for advanced BTC; durvalumab plus CisGem demonstrated statistically significant prolonged OS (12.8 mo *vs*. 11.5 mo) and PFS (7.2 mo *vs*. 5.7 mo) compared with placebo plus CisGem, regardless of the level of PD-L1 expression; in detail, the HR for OS with durvalumab *vs* placebo was 0.79 and 0.86 in patients with a PD-L1 tumour positivity area (TAP) ≥1% (>1% of tumour area occupied by tumour cells with PD-L1 staining) and <1%, respectively, with only a trend towards major efficacy for PD-L1 positive cases. Moreover, overall response rate was 26.7% with chemo-immunotherapy compared with 18.7% with chemotherapy alone (P = .011). Durvalumab did not add additional toxicity to that observed with chemotherapy alone, with similar grade 3 and 4 adverse events rates (109).

Based on these data, FDA has recently approved Durvalumab plus chemotherapy for the treatment of locally advanced or metastatic bile tract cancer (110).

## RESISTANCE TO TARGET THERAPY

As previously discussed, several target therapies and immunotherapies are being assessed in clinical trials for the treatment of advanced or metastatic CCA patient; however, despite evidence of initial responses and disease control, disease progression inevitably occurs in most patients and PFS were frequently dismal up to date little is known about mechanisms of resistance to these compounds and potential ways to overcome them.

Regarding primary FGFR inhibitor resistance, Silverman and colleagues demonstrated that patients with co-occurring tumor suppressor gene alterations including BAP1, CDKN2A/B, PBRM1 and TP53 had shorter median PFS on pemigatinib than those without alterations in these genes, although the numbers of patients do not allow any significant conclusions (111).

As far as secondary resistance to FGFR2 inhibition is concerned, Goyal *et al.* first reported a small case series of three patients with FGFR2 fusion-positive iCCA developing FGFR2 V565F gate-keeper mutation (and in two cases also polyclonal secondary mutations in the FGFR2 kinase domain) as a mechanism of acquired resistance to infigratinib (112). Later, Goyal *et al.* showed that sequential treatment with futibatinib after progression on infigratinib or Debio 1347 (an ATP-competitive FGFR1–3 inhibitor) led to prolonged clinical benefit from FGFR inhibition in four patients with acquired FGFR2 kinase domain mutations (113).

In addition, Wu *et al.* recently demonstrate that EGFR-dependent signaling limits the effectiveness of FGFR inhibitor therapy determining adaptive resistance in patient-derived models with FGFR2 positive cholangiocarcinoma. This supports the potential of combination treatment with FGFR and EGFR inhibitors which would suppress MEK/ERK and mTOR signaling, increasing apoptosis, and causing marked tumor regressions (114).

Primary resistance mechanisms to IDH1 inhibitors are not yet fully elucidated. The quantity of 2-HG accumulated, however, seems to play a role in the response to mIDH-inhibitors: in the ClarIDHy trial, in fact, treatment duration appeared to be longer for patients with plasma 2-HG levels below 100 ng/mL after 1 cycle of ivosidenib (53).

With respect to acquired resistance, new mutations in other genes like TP53, ARID1A, POLE, PIK3R and TXB3 or a second site mutation in IDH1 or isoform switching from mutant IDH1 to mutant IDH2 or vice versa (115) avoid the binding of ivosidenib and increase 2-HG production (116-118).

As far as immunotherapy is concerned, even if there are no definitive data, there are some reports of acquired resistance to checkpoint inhibitors due to a deregulated Wnt/β-catenin pathway, also in MSI-H cholangiocarcinoma (119).

## PITFALLS OF TISSUE NGS AND POTENTIAL OF LIQUID BIOPSY

Until recently, tissue biopsies have been represented the standard not only for pathological diagnosis and molecular characterization but also, at progression, to identify mechanism of resistance. However,

the complexity of tumor heterogeneity, which may be underrepresented in a single site biopsy, the ethical considerations, and the procedural difficulties in obtaining longitudinal tumor samples, which is often inadequate for molecular profiling, represent important issues. In a recent report by Lamarca *et al.* of real-life profiling of BTC, the rate of tissue sample failure was ~25%, mainly due to insufficient tumor representation or low DNA extracted for analysis (120). Limited access to tissue can result in a restraint of personalized treatment approaches and option for patients. Developing new strategies to identify BTC and to obtain an adequate molecular profiling is still an unmet need. In this scenario, there is mounting evidence on the use of liquid biopsy as a non-invasive and repeatable method that allows for tumor molecular profiling and treatment response (121-127).

In a recently published large cohort of 1671 patients with advanced BTC profiled with Guardant360, genetic alterations were detected in cell free DNA (cfDNA) in 84% of patients. Targetable alterations detected in 44% of patients and high pre-treatment cfDNA variant allele fraction (VAF) were associated with poor prognosis and shorter response to chemotherapy and targeted therapy. Of note, cfDNA analysis was quite reliable in detecting IDH1 mutations and BRAF V600E at similar rates to tissue profiling, but it was able to identify only 18% of FGFR2 fusions (128).

This study the utility of cfDNA analysis in current management of BTC and adds up to already mentioned literature showing the role of liquid biopsy in uncovering putative mechanisms of resistance to targeted therapies (112, 119).

## CONCLUSIONS

Advanced cholangiocarcinoma is a malignancy associated with poor prognosis and rising incidence worldwide. Treatment options have been historically based on platinum combination chemotherapy however, during the past decade, immuno-oncology and mutation-targeting drugs are revolutionizing the treatment of advanced CCA.

Consequently, current clinical guidelines, including ESMO, advocate for molecular characterization, which can be performed on the tumor tissue and/or on circulating tumor DNA (ctDNA), to define the best therapeutic strategy for patients affected by CCA.

However, despite these drugs are revolutionizing our clinical practice, all patients on target therapies eventually develop treatment resistance. Understanding mechanisms involved in targeted treatment resistance and how they evolve after exposure to each drug represents a compelling and urgent need to develop new rational combination strategies. In addition, there is a lack of reliable biomarkers to identify the BTC patients most likely to benefit from immunotherapy.

In conclusion, despite major improvements in CCA characterization and treatment, further research is needed to elucidate resistance mechanisms to immune checkpoint inhibitors and targeted therapies in these patients. Moreover, the widespread of novel treatment options is still limited, and new accessible strategies are still to be defined in this difficult to treat tumor entity.

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### Availability of data and materials

The data underlying this article can be shared just before a reasonable request to the Corresponding Author.

### Authors' contributions

CP: conceptualization; CP, CS: methodology; MN, EJ: validation; CP, CS, FN: writing - original draft preparation; CP, CS, FN, FC, AR: writing, review and editing; FN, MdB, MN, EJ: supervision. All Authors have read and agreed to the published version of the manuscript.

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