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Practical guidelines for molecular testing of cholangiocarcinoma in clinical practice: Italian experts' position paper

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ABSTRACT

Biliary tract cancers (BTCs) represent a spectrum of malignancies associated with a dismal prognosis. Recent genomic profiling studies have provided a deeper understanding of the complex and heterogenous molecular landscape of BTCs, identifying several actionable genetic alterations, and expanding treatment options. Due to the high number and complexity of genetic alterations which require testing, next-generation sequencing (NGS) is currently the preferred approach over conventional methods (i.e., immunohistochemistry, fluorescence in-situ hybridization and PCR) for molecular profiling of BTCs and should be performed upfront in all BTC patients. However, BTC sampling often yields low tumor cellularity tissue, hampering NGS analysis. Future perspectives to overcome this obstacle include liquid biopsy and optimization of biopsy protocols. In this position paper, the authors discuss the current histopathologic, molecular, and therapeutic landscape of BTCs, provide a critical overview of the available testing methods for molecular diagnostics, and propose a practical diagnostic algorithm for molecular testing of BTC samples.

1. Introduction

Biliary tract cancers (BTCs) represent a spectrum of malignancies arising from epithelial cells of the bile ducts, including cholangiocarcinoma (CCA) arising in the intrahepatic, perihilar or distal biliary tree, and gallbladder carcinoma (Valle et al., 2021).

BTCs are the second most common primary hepato-biliary malignancies, comprising 3% of all gastrointestinal cancers (Rizvi et al., 2018). BTCs are relatively rare in high-income countries, but their incidence is currently increasing (especially intrahepatic CCA [iCCA]). The incidence of BTCs varies greatly depending on geographical regions, reaching the highest frequency in endemic regions of Thailand and China for CCA and in Bolivia, Chile and Bangladesh for gallbladder cancer (Sung et al., 2021).

The varying regional incidence of BTCs reflects different underlying etiological factors. Risk factors for CCA include primary sclerosing

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cholangitis, Caroli's disease, hepatolithiasis and hepatobiliary fluke infections (*Opisthorchis viverrini* and *Clonorchis sinensis*), as well as comorbid hepatic disorders including cirrhosis, hepatitis B and hepatitis C infection, metabolic-associated fatty liver disease (MAFLD) (Massarweh and El-Serag, 2017). In high-income countries, CCA is primarily associated with inflammatory diseases of the liver and the biliary tree, while in endemic areas of Thailand and China, liver fluke infection is the driving risk factor (Sithithaworn et al., 2014).

Gallbladder carcinoma has a different etiology than CCA. Predisposing conditions include cholelithiasis, primary sclerosing cholangitis and structural biliary tree abnormalities (i.e., congenital biliary dilatation and anomalous pancreaticobiliary ductal junction), chronic *Salmonella typhi* or *Helicobacter bilis* infections, and obesity (Rawla et al., 2019).

2. Current therapeutic approaches, ongoing clinical trials, and future perspectives

Surgery with curative intent is the current gold standard for patients diagnosed with resectable disease, followed by 6-month adjuvant chemotherapy with fluoropyrimidines for resected CCA and gallbladder carcinoma (Vogel et al., 2023). However, the relapse rate remains high, with a 3-year recurrence rate of up to 80% after curative-intent resection (Tsilimigras et al., 2020; Mavros et al., 2014).

Chemotherapy represents the current standard of care for first-line treatment of advanced and metastatic BTC (Vogel et al., 2023). The UK ABC-02 study (Valle et al., 2010) and the Japanese BT22 (Okusaka et al., 2010) study reported a longer overall survival (OS) for cisplatin/gemcitabine doublet over gemcitabine monotherapy. The TOPAZ-1 phase III study showed that the addition of the programmed death-ligand 1 (PD-L1) durvalumab to cisplatin/gemcitabine doublet resulted in a longer OS (HR 0.76, 95% CI 0.64–0.91), response rate and progression-free survival (PFS) (Oh et al., 2022a; Oh et al., 2022b). Thus, cisplatin-gemcitabine-durvalumab should be recommended for the first-line treatment of advanced BTC.

The recent KEYNOTE-966 phase III trials demonstrated a clinically meaningful improvement in OS for pembrolizumab plus gemcitabine and cisplatin compared with gemcitabine and cisplatin without any new safety signals, making this schedule a new potential option for patients with previously untreated metastatic or unresectable BTC (Kelley et al., 2023).

The UK ABC-06 study has reported a modest OS advantage for 5-fluorouracil-leucovorin-oxaliplatin (FOLFOX) compared with active symptom control (HR 0.69) (Lamarca et al., 2021). Thus, FOLFOX is currently recommended as second-line treatment, after cisplatin-gemcitabine.

Approximately 40% of BTCs harbor potential druggable genetic alterations (Nakamura et al., 2015; Normanno et al., 2022). For this reason, molecular analysis should be carried out before or during first-line treatment to timely establish the best therapeutic option for second or subsequent lines. The current "ESMO Clinical Practice Guideline for diagnosis, treatment and follow-up" (Vogel et al., 2023) recently supported the profiling of *IDH1* and *BRAF* mutations, *FGFR2* fusions, HER2 overexpression and MMRd/MSI-H status in all advanced/metastatic BTCs or locally advanced BTCs in patients who have progressed on or are intolerant to prior chemotherapeutic treatments (Table 1). In 2022, the Italian Government allocated 600,000 euros (2023–2025) to guarantee access to early NGS testing to all CCA patients (Ministero della Salute, 2023).

Ivosidenib is an IDH1 inhibitor approved by the United States Food and Drug Administration (FDA) in August 2021 and by the European Medicines Agency (EMA) in May 2023 for the treatment of adult patients with previously treated, advanced, or metastatic CCA harboring an *IDH1* mutation (Casak et al., 2022). Ivosidenib has shown activity in the ClarIDHy phase III clinical trial when compared with placebo, demonstrating a significant improvement of PFS (HR 0.49, 95% CI 0.34–0.70), and a superior OS (Abou-Alfa et al., 2020a; Zhu et al., 2021).

Table 1
List of genetic alterations to test for in biliary tract cancers (Vogel et al., 2023; Kendre et al., 2023).

Genetic alteration	Prevalence	Testing methods	ESCAT scale
IDH1 mutations	1%– 18% (10-20% iCCA)	DNA-based NGS (PCR if sample inadequate for NGS)	I-A
FGFR2 fusions	5-7% (10-15% iCCA)	RNA-based NGS (FISH if sample inadequate for NGS)	I-B
ERBB2 amplifications	5%– 10%	IHC and/or FISH or DNA-based NGS*	I-B
$BRAF^{V600E}$ mutations	1-7%	DNA-based NGS (PCR if sample inadequate for NGS)	I-B
NTRK fusions	< 1%	IHC and/or FISH and validation by RNA-based NGS	I-C
dMMR/MSI	< 1%	IHC, PCR or DNA-based NGS* *	I-C

Abbreviations: iCCA: intrahepatic cholangiocarcinoma; NGS: Next Generation Sequencing; FISH: Fluorescent in situ hybridization; IHC: immunohistochemistry

* NGS copy number gain/amplification should be orthogonally confirmed by FISH with validated assays; * * not validated yet

Pemigatinib is an ATP-competitive FGFR kinase inhibitor (Merz et al., 2021). Pemigatinib received accelerated approval as second-line treatment in April 2020 by the FDA (Hoy, 2020) and in March 2021 by the EMA (Anon) in CCA patients harboring *FGFR2* gene fusions or other rearrangements, following the results of the seminal FIGHT-202 study (Patel et al., 2023). In May 2022, pemigatinib received also AIFA (Italian Medicines Agency) approval with the label indication for "treatment of adults with locally advanced or metastatic cholangiocarcinoma with a fibroblast growth factor receptor 2 (FGFR2) fusion or rearrangement that have progressed after at least one prior line of systemic therapy" (Regime di rimborsabilita).

In the FIGHT-202 study, in the cohort with FGFR2 fusions/rearrangements the ORR (95% CI) was 37.0% (27.9–46.9%), the median (95% CI) PFS was 7.0 (6.1–10.5) months and the median OS (95% CI) was 17.5 (14.4–22.9) months. Pemigatinib showed a manageable safety profile in the cohort with FGFR2 fusions/rearrangements (Vogel et al., 2022). The ongoing phase III FIGHT-302 study is evaluating first-line pemigatinib vs gemcitabine plus cisplatin in patients with unresectable or metastatic CCA and FGFR2 fusions/rearrangements (Abou-Alfa et al., 2020b).

Futibatinib is an oral agent pan-inhibitor of FGFR1–4. The phase II FOENIX-CCA2 trial showed an overall response rate (ORR) of 41.7% and a 12-month survival rate of 73% and a favorable safety profile, which led to accelerated FDA approval in September 2022 and EMA approval in July 2023 (Goyal et al., 2023).

Other candidate drugs are currently under development and clinical trials for patients with CCA harboring *FGFR* genetic alterations are underway.

RLY-4008 is a highly selective inhibitor of FGFR2 designed to limit off-target toxicity and to overcome resistance mechanisms (Subbiah et al., 2023). Preliminary data from the phase 1/2 ReFocus trial demonstrated potent efficacy, with an ORR of 88% (95% CI 63.6–98.5) in the first 17 patients who had not received prior anti-FGFR therapy (Subbiah et al., 2023; Park et al., 2022).

In the phase II MyPathway basket trial, the anti-HER2 doublet pertuzumab-trastuzumab achieved an ORR of 23% (95% CI 11–39) in patients with HER2 overexpression/amplification (Meric-Bernstam et al., 2019). The phase II KCSG-HB19–14 evaluated the clinical activity of FOLFOX plus trastuzumab and reported an ORR of 29.4% (95% CI 16.7–46.3) (Lee et al., 2023). In the phase II HERIZON-BTC-01 trial, zanidatamab, a bispecific antibody targeting two distinct HER2 epitopes, demonstrated confirmed objective responses in 41.3% of patients with a manageable safety profile in patients with treatment-refractory,

HER2-positive BTC (Harding et al., 2023). According to the preliminary results of DESTINY-PanTumor02 trial, the antibody drug conjugate targeting HER2 trastuzumab deruxtecan (T-DXd) showed encouraging ORR, particularly in patients with immunohistochemistry (IHC) 3 + expression, and durable clinical benefit (Meric-Bernstam et al., 2023).

These results support the use of anti-HER2 therapies in BTC patients with HER2 amplification who have no other therapeutic options, although no HER2-directed therapies are approved for this indication.

The ROAR basket trial has investigated the combination of the BRAF inhibitor dabrafenib and the MEK inhibitor trametinib, reporting an ORR of 51% with a median OS of 14 months in pretreated patients with $BRAF^{V600E}$ (Subbiah et al., 2020). This combination has been approved only by the FDA, but not by the EMA.

Following the results of the phase II KEYNOTE-158 trial (Marabelle et al., 2019), the anti-PD-1 pembrolizumab is recommended in patients with MSI/dMMR tumors, including BTCs, who have progressed on or are intolerant to prior treatment.

3. Histopathology of biliary tract cancers

BTCs comprise two distinct entities: CCA and gallbladder cancer. CCA can be further classified based on the anatomic location into iCCA, perihilar CCA (pCCA) and distal CCA (dCCA). iCCA accounts for 10–20% of CCAs and arises from ductules or segmental ducts of the intrahepatic biliary tree; pCCA is the most common CCA, comprising 50–60% of cases, and develops in the right and/or left hepatic duct and/or at their junction; dCCA occurs in the common bile duct. pCCA and dCCA are classified as extrahepatic cholangiocarcinoma (eCCA) (Krasinskas, 2018; Kendall et al., 2019).

Macroscopically, iCCA can present three main patterns of growth: mass-forming (MF), periductal infiltrating (PI) and intraductal growing (IG). MF type iCCA is the most common and appears as a nodular lesion in the liver parenchyma; PI type iCCA grows as periductal nodular and sclerosing lesions; IG type iCCA displays polypoid or papillary tumor growths within the duct lumina (Nakanuma et al., 2010). eCCAs have a nodular sclerosing appearance with an infiltrating growth pattern.

The majority of pCCA and dCCA are mucin-producing adenocarcinomas characterized by irregular glands and small cell clusters, surrounded by desmoplastic stroma. iCCAs are heterogeneous in terms of microscopic appearance. Conventional iCCA can be classified into two main histologic subtypes: small bile duct type iCCAs present as small-sized tubular or acinar adenocarcinoma invading the hepatic parenchyma with no or minimal mucin production and may derive from hepatic stem or progenitor cells and cuboidal cholangiocytes; large duct type iCCAs are composed of large, irregular, dilated glands, with abundant stroma, and seem to derive from columnar mucous cholangiocytes or peribiliary glands (Liau et al., 2014; Hayashi et al., 2016; Komuta et al., 2012). Rare variants of iCCA include squamous or adenosquamous carcinoma, lymphoepithelioma-like carcinoma related to *Epstein-Barr virus* infection and sarcomatous carcinoma (Nagtegaal et al).

Besides conventional and rare variants of iCCA, cholangiolocarcinoma and iCCA with ductal plate malformation pattern are further histological variants of small duct iCCA (Moeini et al., 2017).

Cancer of unknown primary (CUP) is a malignant widespread metastatic disease without an identifiable primary site after extensive clinical, radiological and histopathological investigation (Varadhachary and Raber, 2014). Due to the lack of specific biomarkers, CCAs are difficult to differentiate from other tumor types (i.e., pancreatic and upper gastrointestinal adenocarcinomas) and they tend to be overrepresented in CUP cohorts, especially in liver-involved CUPs (Conway et al., 2022). Due to this reason, in 2023, ESMO published clinical practice guideline for diagnosis, treatment and follow-up related to cancer of unknown primary, including a dedicated algorithm for the differential diagnosis of iCCA versus CUP (Krämer et al., 2023).

Access to molecular profiling may facilitate a confident diagnosis of CCA in CUP patients and could enable prompt access to targeted therapies.

The majority of gallbladder carcinomas arise in the fundus of the gallbladder and appear as poorly defined lesions with infiltrating growth. Biliary type-adenocarcinoma is the most common histotype of gallbladder carcinoma, followed by intestinal-type adenocarcinomas. However, numerous variants have been described, including mucinous adenocarcinoma, clear cell carcinoma, poorly cohesive carcinoma, adenosquamous carcinoma and sarcomatoid carcinoma (Nagtegaal et al).

Adequate reporting of macroscopic and microscopic findings is critical for cancer staging and prediction outcomes of BTC patients. However, due to the rarity of these neoplasms, pathological expertise remains scarce and essential information is often missing from reports (de Bitter et al., 2021). The adoption of standardized models for BTC reporting, as for any other cancer type, has led to improvements in the reporting of key prognostic factors by pathologists (Burt et al., 2018).

4. How to choose the best sample for molecular diagnostics

Large genomic studies (Silverman et al., 2021; Lowery et al., 2018) suggest that 40–50% of CCAs harbor clinically actionable genomic alterations. A genomic alteration is to be considered clinically actionable if a targeted therapy is approved for any indication or under investigation in a pivotal phase II or III trial in patients with BTC (i.e., level 1 of ESMO Scale for Clinical Actionability of molecular Targets [ESCAT]). Of note is that in the aforementioned studies (Silverman et al., 2021; Lowery et al., 2018) > 80% of the patients had iCCA because of its greater accessibility to adequate biopsy sampling; moreover, iCCA shows the higher rates of druggable alterations. Few quantitative data are available for actionable genetic alterations specifically in eCCA patients, but a recent study of 189 eCCA patients suggests that approximately 25% of the cases harbor actionable mutations. Based on the current evidence, ESMO guidelines have recommended routine use of NGS multigene panels covering level I alterations on all BTCs (Mosele et al., 2020).

A study by Lowery and colleagues (Lowery et al., 2018) did not find any significant difference in the frequencies of genetic alterations in primary tumor biopsies in comparison with relapses. Of note, *FGFR2* fusions are thought to be early events and drivers of carcinogenesis and would be expected to be found in the primary tumor and metastatic site and not to be modulated by adjuvant chemotherapy (Borad et al., 2015; Lin et al., 2022).

Three types of tissue samples can be used for molecular analysis in BTC patients: surgical resection specimens, biopsy samples and biliary brush cytology/microbiopsies (Fig. 1). Surgical resection specimens are the gold standard for molecular diagnostics, because of the higher tumor content and because of the possibility of selecting the best tissue block and a tumor area enriched in neoplastic cells. Unfortunately, since patients with PI iCCA and dCCA are often not eligible for curative surgery, BTC surgical specimens are often not available. For this reason, a great number of patients rely on biopsy specimens for the identification of actionable targets (Vogel et al., 2023). Finally, biopsy sample is to be preferred to biliary brush cytology, due to the higher tumor content (Vogel et al., 2023).

Collecting adequate biopsy samples for molecular profiling from the biliary tree is technically challenging, especially in dCCA and pCCA, and often results in failure of molecular analysis due to the small size of the samples and low tumor content.

Lamarca and colleagues (Lamarca et al., 2020a) reported a failure rate of 26.8% in molecular profiling of BTC biopsies, mainly due to small biopsy samples with insufficient tumor cell content. This may be attributed to tumor location or desmoplastic fibrosis. The collaboration of a pathologist, responsible for choosing the most representative tissue block when multiple blocks are available and for selecting a tumor area enriched in neoplastic cells, can minimize the failure rate of molecular

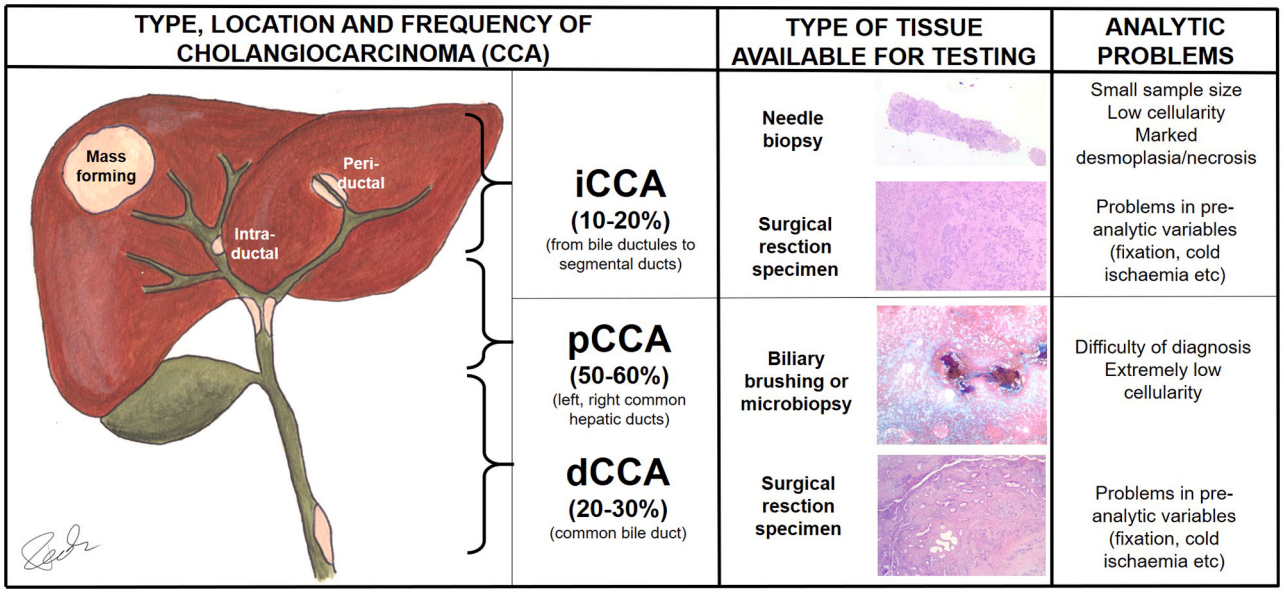

Fig. 1. Different types of cholangiocarcinomas, tissue characteristics and analytical problems.

profiling (Cappello et al., 2022). However, the main hurdle remains the high rates of "scant" tumor biopsy and cytology samples. If the biopsy sample is inadequate for NGS analysis due to low tumor content, a re-biopsy should be considered. However, this may not be practicable in locally advanced and metastatic patients, who are often unfit to undergo this procedure (Levit et al., 2019).

To maximize the diagnostic yield, multiple biopsy sampling of the lesions should be performed (Penault-Llorca et al., 2022). Complementary biliary brushing is also recommended (EJCA et al., 2021). Biopsy samples are preferred over cytology alone, due to the higher tumor content. In order to maximize tissue availability for molecular testing, the tissue fragments obtained from a patient should be subdivided into two paraffin blocks and should be exploited as little as possible for histopathological classification. The threshold number of tumor cells required for successful nucleic acid extraction for molecular analysis has not been defined in BTCs, but for other tumors (i.e., lung cancer), the desirable number is at least 200–400 cells (Thunnissen et al., 2012). Furthermore, the pathologist should always ensure that the tumor cellularity of a given specimen is adequate for the limit of detection and sensitivity of the downstream molecular test to be performed (Nibid et al., 2023). Moreover, the quality (i.e., fragmentation) and quantity of DNA/RNA extracted from a given sample should be evaluated before molecular testing. While DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissue remains relatively well-preserved, RNA can be heavily degraded and fragmented so that only short sequences (approximately 100–200 nucleotides) can be recognized (Cappello et al., 2022).

The authors recommend performing NGS as a reflex test, regardless of the stage of presentation. In other cancer types, NGS is usually not recommended in early-stage tumors undergoing potentially curative treatment (Remon and Dienstmann, 2018; Schwartzberg et al., 2017). However, because only a small subset of BTCs are candidates for curative surgery, the rate of relapse following surgical resection is high, and the number of actionable genetic alterations is large, patients with early-stage CCA benefit from NGS profiling at the time of diagnosis (Javle et al., 2016; Ross et al., 2014). Moreover, early molecular profiling could help match patients to basket trials recruiting for a specific genetic alteration (Mosele et al., 2020).

5. Molecular classification of biliary tract cancers and therapeutic targets

In recent years, numerous efforts have been made to unveil the genomic profile of BTCs and to improve the understating of their complex molecular landscape. The prevalence of genetic alterations varies across different studies, due to the heterogeneous nature and rarity of this cancer type and the different sequencing methods used.

In the largest genomic study reported in the literature, Javle and colleagues (Javle et al., 2019) profiled 3634 samples of CCA and found the most commonly altered genes to be *TP53*, *CDKN2A/B*, *KRAS*, *ARID1A*, *IDH1*, *BAP1*, *PBRM1* and *FGFR2* (mostly fusions).

Comprehensive genomic profiling was performed in over 1000 patients during screening for enrollment in the phase II FIGHT-202 trial. Similarly to the previously mentioned study, the most commonly altered genes were *TP53*, *CDKN2A/B*, *KRAS*, *ARID1A*, *SMAD4*, *IDH1*, *BAP1* and *PBRM1*; *FGFR2* alterations were found in 7% of patients (Silverman et al., 2021).

Fewer data are available for the genomic profile of eCCAs. iCCA and eCCA have been shown to harbor a different genomic profile, with *FGFR* fusions, mutations, or amplifications and *IDH* mutations being much more common in iCCA than in eCCA, while *KRAS* mutations and *ERBB2* amplification and overexpression are more frequent in eCCA (Nakamura et al., 2015; Weinberg et al., 2019; Jusakul et al., 2017; Simbolo et al., 2014). Differences also exist between small duct iCCAs, which often harbor *IDH1* and *IDH2* mutations and FGFR2 alterations, and large duct iCCAs, which are frequently mutated in *KRAS* and/or *TP53,* making them more similar to pCCA and eCCA than small duct iCCA ((Lowery et al., 2018); (Kipp et al., 2012); (Graham et al., 2014).

A recent study by de Bitter and colleagues (de Bitter et al., 2022) has investigated the genomic profile of gallbladder carcinoma, reporting that half of the tumors harbor potentially actionable alterations, such as mutations in *CDKN2A* (11%), *PIK3CA* (10%), and *KRAS* (*KRAS* (8%, including *KRAS* p.G12C variants which are now considered targetable) and *ERBB2* amplifications (6%).

Based on the current evidence, ESMO (Mosele et al., 2020) and the United States National Comprehensive Cancer Network (NCCN) (Benson et al., 2023) guidelines recommend routine use of NGS multigene panels on advanced BTCs to identify clinically actionable genetic alterations. ESMO recommends NGS testing for level I (i.e., improved outcomes in clinical trials) genetic alterations according to ESCAT, including *IDH1*

mutations, *FGFR2* fusions, and *NTRK* fusions, microsatellite instability (MSI)/mismatch repair deficiency (dMMR), *BRAF* mutations, *ERBB2* amplifications. The following level II and III actionable genetic alterations have available targeted therapies that do not have any approved therapeutic indication for BTCs: *IDH2* mutations, *FGFR2* mutations, *ERBB2* mutations, *BRCA1* and *BRCA2* mutations and *PALB2* mutations (Vogel et al., 2023).

IDH1/2 mutations occur in approximately 15% of iCCAs and in 7% of eCCAs. In a retrospective analysis of 6130 iCCA patients by Kendre and colleagues (Kendre et al., 2023), *IDH1* (14.3%) and *IDH2* (4.0%) mutations were mutually exclusive. *IDH1* mutations frequently involve the arginine 132 residue, withIDH1-R132C and IDH1-R132G representing the commonest *IDH* variants (Rizzo et al., 2021). Interestingly, *IDH1* mutations rarely coexist with *FGFR2* fusions, are mutually exclusive with *KRAS/NRAS* mutations, and are frequently associated with *ARID1A* mutations and with a hypermethylated phenotype (Ntanasis--Stathopoulos et al., 2020; Boscoe et al., 2019). Numerous studies have investigated the prognostic significance of *IDH1* mutations in iCCA patients, although none reported a statistically significant association with clinical outcome (Javle et al., 2016).

The fibroblast growth factor receptors (FGFRs) are a family of tyrosine kinase receptors that include FGFR1, FGFR2, FGFR3, and FGFR4. Amplification, single-nucleotide variants, or gene fusions of the *FGFR* genes are seen in a wide range of solid tumors (Katoh, 2019). Various studies aimed at profiling CCAs estimated the frequency of *FGFR2* fusions to be approximately 5–7% in patients with any CCA and in 10% in patients with iCCA (Kendre et al., 2023; Lamarca et al., 2020b). Genomic alterations in *FGFR1* or *FGFR3* are observed in ~1% of CCA patients (Silverman et al., 2021). Over 150 different *FGFR2* fusion partners have been observed in CCA, the most common partner being *BICC1 ((*Arai et al., 2014); (Wu et al., 2013). FGFR2 fusions are mutually exclusive with *FGFR2* mutations and commonly co-occur with *BAP1* alterations (Silverman et al., 2021). *FGFR2* fusions have been associated with a better clinical outcome in iCCA (Goyal et al., 2021) and *FGFR* alterations have also been associated with a poor response to gemcitabine-platinum chemotherapy (Boileve et al., 2019).

BRAF is a proto-oncogene that encodes for a cytoplasmic serine/threonine kinase, which activates the MAPK pathway. Mutations in the *BRAF* gene occur in 1–7% of CCAs, mainly in iCCAs (Weinberg et al., 2019; Simbolo et al., 2014). According to Kendre and colleagues, $BRAF^{V600E}$ (2.3%) and $BRAF^{non\text{-}V600E}$ (2.3%) mutations occur at similar frequencies in iCCAs (Kendre et al., 2023).

HER2 is an oncogenic driver and therapeutic target in many solid tumors. HER2 amplification and/or overexpression are present in about 13% of eCCAs and 5% of iCCAs (Normanno et al., 2022). In the setting of iCCA, Kendre and colleagues reported a frequency of *ERBB2* amplifications of 3.8% and of *ERBB2* mutations of 1.6% (Kendre et al., 2023). ERRB2 amplifications have also been described in gallbladder carcinoma. A recent work by de Bitter and colleagues reported a frequency of *ERBB2* amplifications of 6.4% in gallbladder carcinoma (de Bitter et al., 2022).

NTRK genes encode a range of tropomyosin receptor kinase (TRK) proteins. *NTRK* fusions can be found in a plethora of solid tumors; however, they are a rare finding in CCA (approximately 4%) and limited data are available (Boilève et al., 2021).

MMRd is defined as the loss of at least one MMR protein. MSI consists of the accumulation of mutations in the microsatellite regions, which are repetitive sequences distributed throughout the human genome. MSI is the molecular fingerprint of MMRd. BTCs can be MSI/MMRd, but to a lower extent in comparison with other gastrointestinal neoplasms (i.e., colorectal, or gastroesophageal cancer). Previous studies have reported rates of MSI/MMRd in BTCs ranging from 1% to 3% (Kendre et al., 2023; Goeppert et al., 2013; Rizzato et al., 2022).

Tumor mutational burden (TMB) is defined as the number of mutations per megabase of coding DNA. High TMB (TMB-h), defined as TMB $\geq$ 10 mutations per megabase, is associated with MSI in some cancer types (Galuppini et al., 2019). In BTCs the association between MSI and TMB-h is still debated (ZHAO et al., 2020). Javle and colleagues (Javle et al., 2019) found that 1% of the CCAs profiled had a TMB > 20 mutations per megabase and 3% had a TMB > 10 mutations per megabase.

6. Methods for molecular testing

Common conventional tests are based on IHC, fluorescent in situ hybridization (FISH), or PCR-based DNA or RNA sequencing strategies (Table 2). While these conventional tests are distributed in most diagnostic laboratories, they do not screen for multiple genetic alterations and generally require knowledge of the targeted alterations (Normanno et al., 2022; Bekaii-Saab et al., 2021).

Validated IHC tests are available to determine the presence of *HER2* amplification, MMRd, $BRAF^{V600E}$ mutation, and *NTRK* fusions. However, these tests have limited utility in BTCs, and are discouraged by ESMO guidelines, except for MMR IHC, which should be performed according to tissue availability (Vogel et al., 2023).

FISH uses fluorescence-labeled DNA probes to target specific chromosomal locations within the nucleus to detect and quantify gene amplifications and known rearrangements, including gene fusions (Chrzanowska et al., 2020). Break-apart FISH is a common approach to detecting gene fusions. It requires the use of two differently labeled DNA probes (red and green fluorescence) encompassing the fusion breakpoint

Table 2
Pros and cons of available methods for molecular testing in BTCs.

Method	
IHC	✓ Good agreement with molecular testing for MMR
	✗ Can only be used to detect HER2 overexpression and MMRd and as a screening tool for NTRK fusions
Break-apart FISH	✓ Can detect *FGFR2* fusions with unknown partners
	✗ Low sensitivity for *FGFR2* intrachromosomal rearrangements
	✗ Low sensitivity for *FGFR2* C-terminal truncation
PCR	✓ Widespread technique, usually present in low-volume and peripheral centers
	✗ Can identify only a predetermined set of mutations
DNA-based NGS	✓ Can detect multiple genetic alterations (i.e., suited for tumors with a large number of actionable genetic alterations, such as BTCs)
	✓ Possibility to perform Comprehensive Genomic Profiling
	✗ Lower sensitivity for gene fusions
RNA-based NGS	✓ Identification of fusion transcripts, including novel fusions
	✗ The quality of FFPE samples is critical for a successful analysis due to RNA fragmentation

Abbreviations: MMRd: Mismatch repair deficiency; FFPE: Formalin-fixed paraffin-embedded

to create a combined fluorescent signal that is specific to the unaltered gene. Rearrangements at the fusion breakpoint increase the distance between the probes, resulting in the separation of the red and green fluorescent signals. Break-apart FISH can be used to detect *FGFR2* fusions because it is able to identify rearrangements in a partner-agnostic manner. However, it provides no data on fusion gene partners nor on the expression of the fusion protein and intrachromosomal rearrangements, which account for ~50% of all *FGFR2* rearrangements in CCA, as these may not be identified if the distance between the probes after rearrangement remains too short (Neumann et al., 2022).

DNA-based PCR can be used to screen for mutations or copy number variations, while RNA-based real-time RT-PCR is a fast and sensitive method to detect transcribed gene fusions. However, the latter is not suitable for *FGFR2* fusions, where both fusion partners and the location of the breakpoint are often unknown (Angerilli et al., 2023).

In the era of precision oncology, selective single-gene testing has been outdated by NGS and other multiplexed platforms. At present, targeted NGS panels find application in molecular diagnostics because they target genes of clinical significance, with high sensitivity, fast turnaround time and relatively low costs (Cappello et al., 2022; Angerilli et al., 2021).

NGS is the gold standard and the preferred technique for molecular testing in BTCs, because it allows the detection of multiple actionable genetic alterations through massive parallel sequencing of several genes. NGS is well suited for FFPE samples and biopsy specimens. However, high failure rates of NGS testing are reported in BTCs, due to insufficient neoplastic cell content in the diagnostic biopsies.

NGS testing can rely on DNA, RNA, or circulating cell-free DNA (cfDNA) as a source of genetic material. However, the use of DNA or RNA can lead to the detection of different types of genetic alterations.

DNA-based NGS can detect any type of genomic alteration, including single-nucleotide variants (SNVs), copy number variations, rearrangements, tumor mutational burden (TMB), and MSI. However, different DNA-based NGS panels can have variable performance, according to the type of sequences targeted and the size of the panel. On the other hand, RNA-based NGS can identify alternative splicing events and gene fusions, which can go undetected by DNA-based NGS, and can also quantify gene expression levels, but often miss SNVs with low variant allele frequency. The greatest challenge associated with RNA-based NGS is that RNA extracted from FFPE is more unstable and prone to degradation, resulting in higher rates of failure of NGS analysis (van Maldegem et al., 2008; Heyer et al., 2019; Reeser et al., 2017).

NGS technology is becoming increasingly complex and producing progressively larger amounts of data. NGS testing can be time-consuming and expensive, requiring specialized equipment, skilled personnel and extensive bioinformatics analysis. For this reason, low-volume laboratories in small and mid-tiered hospitals may not be equipped to perform in-house NGS testing. If complete testing cannot be performed, the samples should be outsourced to a laboratory with adequate technology, experienced staff, short turnaround time, and successful participation in external quality assessment (Brcic and Kern, 2020).

According to ESMO guidelines, *FGFR2* and *NTRK* gene fusions should preferably be interrogated at the transcriptomic level using an RNA-based NGS panel that can detect known and unknown fusion partners. For this reason, hybrid capture or anchored multiplex PCR technologies are recommended while amplicon-based assays can only detect a predefined set of fusions using gene-specific primer pairs. However, amplicon-based technology has the advantage of requiring a lower input of nucleic acids and, therefore, can provide relevant information also for small biopsies or cytology specimens. The best approach is to combine RNA testing with a DNA-based approach to identify breakpoints (Vogel et al., 2023). While RNA-based NGS directly interrogates the fusion transcript, DNA-based assays need computational inference to identify fusion partners. In the latter case, if the breakpoint in the partner gene is not in-frame or on-strand or is in the intergenic space, the fusion will be reported as rearrangement with no partner gene (Neumann et al., 2022). However, if RNA-based assays are not available, gene fusions can also be investigated using exclusively a DNA-based panel, with an optimized design and bioinformatic pipeline.

The molecular pathology report should be clear and concise, and guide clinicians in the therapeutic decision-making process (Schmid et al., 2022). It should contain the following information:

- Identification of laboratory, patient (name, surname, date of birth, sex), ordering physician, sample (ID, date of specimen collection).
- Features of tumor tissue specimen: microscopic diagnosis, tumor cell content, whether microdissection was performed, and the identification of the pathologist who performed it.
- Methodology: assay, limit of detection, target genes with exons/codons analyzed (if applicable).
- Test results: list of genetic alterations detected using standard nomenclature, variant allele frequency, additional analytic and clinical interpretative comments.

7. Liquid biopsy

The term liquid biopsy is referred to the minimally invasive sampling and analysis of prognostic and predictive biomarkers isolated from biological fluids (usually plasma). Liquid biopsy may be a valid alternative testing method to overcome issues caused by low-quality tissue samples of BTCs. Liquid biopsy approaches are also promising tools to capture tumor heterogeneity and monitor treatment response and the onset of resistance to targeted therapies.

The sensitivity of liquid biopsy assays is challenged by the low fraction of circulating tumor DNA (ctDNA) retrieved from blood samples. However, a study by Lamarca and colleagues reported a lower sample failure rate for ctDNA in comparison with tissue, pinpointing that liquid biopsy may be a valid alternative in case of scarce tissue samples (Lamarca et al., 2020a). Another study by Ettrich and colleagues reported a blood/tissue concordance of 74% for CCAs and 92% for iCCAs only (Ettrich et al., 2019). According to ESMO guidelines (Pascual et al., 2022), ctDNA testing is recommended when tissue testing is not feasible. The increasing clinical relevance of liquid biopsy testing is demonstrated by the approval of two ctDNA-based assays, FoundationOne Liquid CDx (Foundation Medicine, Cambridge, MA, USA) (No Title Internet. cited) and Guardant360 CDx (Guardant Health, Redwood city, CA, USA) (No Title Internet. cited) for various solid tumors, including BTCs.

In a recent study by Berchuck and colleagues, Guardant360® CDx was used to profile 1671 patients with advanced BTCs. *IDH1* mutations and $BRAF^{V600E}$ were detected at similar rates to tissue biopsies, but the concordance rate for *FGFR2* fusions detection was only 18%, due to the diversity of fusion partners. In fact, the sensitivity for *FGFR2-BICC1* fusions was 58%, but only 2% for non-*BICC1* fusions (Berchuck et al., 2022). The post-hoc analysis of the phase II FOENIX-CCA2 trial of futibatinib in advanced/metastatic CCA harboring *FGFR2* fusion/rearrangement reported a concordance rate of 87% between ctDNA and tissue sample, by using a custom version of the Illumina® TruSight Oncology 500 ctDNA sequencing assay (Goyal et al., 2022).

8. Real-world data on molecular diagnostics of CCA: the Italian perspective

While most clinical trials have well-resourced and organized molecular testing strategies, what truly happens in the real-world setting? With this in mind, a collaborative effort from the Italian group of gastrointestinal pathologists (GIPAD) and the Italian group for molecular pathology and predictive medicine (PMMP) of the Italian society of Pathology (SIAPeC-IAP) gave a better insight of the Italian scenario. A questionnaire to evaluate a series of parameters regarding histopathological and molecular diagnostics of CCA was sent to all Italian surgical

pathology units in March 2023 and 22 centers responded.

- *Where is the molecular analysis performed?* The majority of centers (16; 73%) profile CCA in the surgical pathology departments, while only a small subgroup (3; 14%) performs the molecular analysis in another hospital or in another department of the same hospital. Three centers do not profile CCA for molecular alterations.
- *Which NGS panel is used?* 84% of centers use DNA-based NGS panels (two centers perform Comprehensive Genomic Profiling) and 68% of centers use an adjunctive RNA-based NGS panels for testing *FGFR2* alterations.
- Which samples are tested? In half of the participating centers, molecular testing is performed upon clinical request, in 5 centers (23%) all CCAs are profiled, and in 3 (14%) only iCCAs are tested.
- *MMR/MSI.* All participating centers but one performs MMR/MSI testing. The majority (14; 64%) of centers perform both IHC and molecular testing, while 6 (27%) use only IHC in the characterization of MMR/MSI status and only one (5%) use MSI molecular testing without performing IHC.
- *IDH1.* All participating centers but three perform *IDH1* testing. More than half of the centers (13; 69%) use only DNA-based NGS, while the rest use either only Real-Time PCR (3; 14%) or both technologies (3; 14%). Overall, the participating centers report a 15% rate of failure of *IDH1* testing. In the majority of cases the failure was caused by low tumor cellularity, followed by low quality of nucleic acids and sequencing issues.
- *FGFR2.* All participating centers but three perform *FGFR2* testing. More than half of the centers use only RNA-based NGS (12; 55%), 5 (23%) use either RNA-based NGS or FISH, one center use only FISH, and another one uses DNA-based NGS (Comprehensive Genomic Profiling). Overall, the participating centers report 29% rate of failure of *FGFR2* testing. In the majority of cases, the failure was caused by low tumor cellularity, followed by low quality of nucleic acids and sequencing issues.

Table 3
Practical guide for molecular testing in BTCs.

Sample	Testing method
Biopsy/cytology sample Surgical resection specimen	RNA-based NGS and/or DNA-based NGS§ (gene panel should include *IDH1* mutations, *FGFR2* fusions, *ERBB2* amplifications, *BRAF*^{V600E} mutations, *NTRK* fusions, MSI) IHC for MMR status/*ERBB2* amplifications
Biopsy or surgical resection specimen inadequate for NGS	DNA-based PCR for *IDH1* mutations FISH for *FGFR2* fusions IHC for MMR status§ IHC and/or FISH for *ERBB2* amplifications§ IHC or FISH for *NTRK* fusions§

§according to tumor tissue availability
Abbreviations: NGS: Next Generation Sequencing; FISH: Fluorescent In Situ Hybridization; IHC: immunohistochemistry

9. Diagnostic algorithm for BTCs

Three types of samples can be used for molecular analysis in BTCs (Fig. 2; Table 3): surgical resection specimens, biopsy samples, and (biliary brush) cytology. Targeted RNA and DNA-based NGS is the preferred testing method in the BTC setting. Comprehensive Genomic Profiling (CGP) can be also considered if the NGS panel is covering *FGFR2* fusions/rearrangements. According to current clinical practice, in case of scanty tissue, the RNA-based NGS approach should be preferred. Immunohistochemistry for MMR proteins and HER2 should also be performed if enough tissue is available. Surgical resection specimens are the gold standard for molecular diagnostics, because of higher tumor content. A hematoxylin and eosin (HE) stained slide of the designated block for NGS testing should be evaluated by the pathologist to select a tumor area enriched in neoplastic cells. If tumor cellularity is not adequate for NGS testing (<5–10%), another block should be chosen. Tumor content and cellularity should be assessed in biopsy samples and biliary brush cytology to decide whether they are suitable for NGS analysis. In case of scant samples (<100 cells) inadequate for NGS,

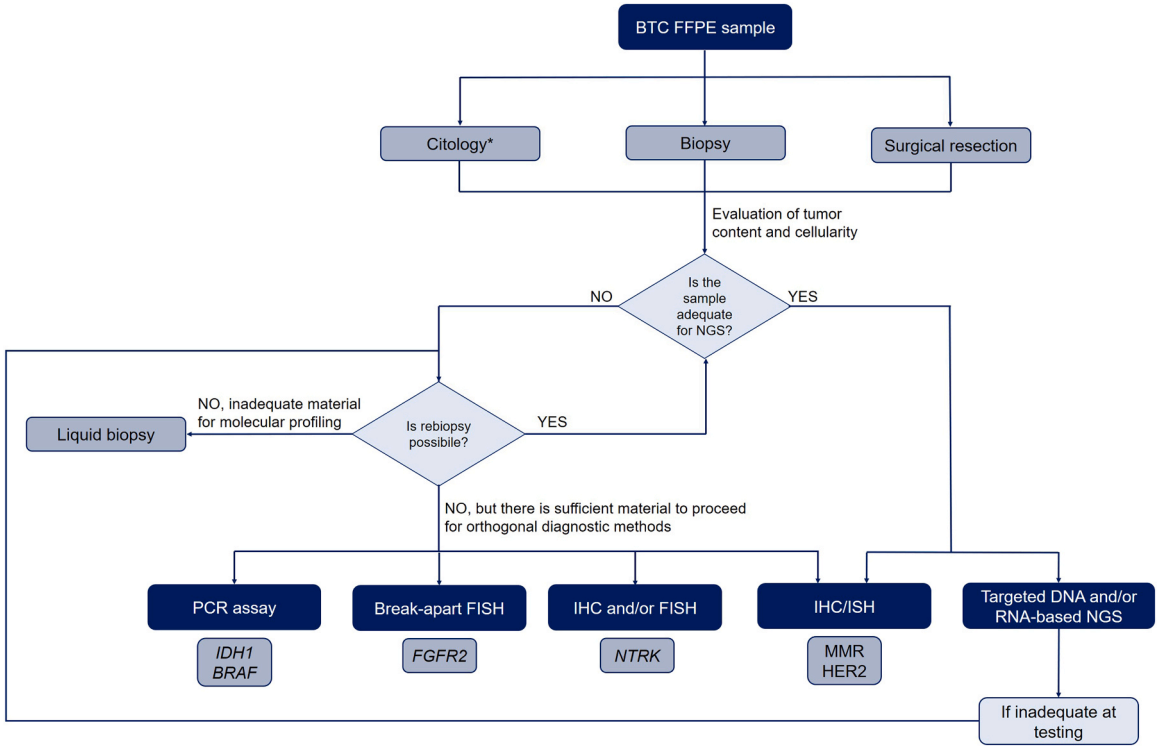

Fig. 2. Proposed diagnostic algorithm for BTCs molecular testing. *most cytology samples are limited to brushing-derived smears and thus are compatible with only PCR-based assays.

break-apart FISH for *FGFR2* fusions, DNA-based PCR for *IDH1*, IHC for MMR proteins, and IHC or FISH for *NTRK* fusions should be performed. FISH analysis can be performed also on cytology samples if the material is processed as cell block. Of note, IHC and FISH for *NTRK* fusion are screening methods and, alone, are not reliable for the detection of *NTRK* fusion due to the high rates of false negatives. NGS sequencing is needed to confirm the presence of *NTRK* fusions. However, positive FISH or IHC for *NTRK* fusions provides a strong indication for a re-biopsy, in order to perform NGS sequencing (Hechtman, 2022).

10. Conclusions

While the incidence of BTC is rising, the prognosis of advanced BTC patients remains dismal, due to the aggressive biology of these neoplasms and the low efficacy of conventional treatments.

Recent genomic profiling studies have provided a greater understanding of the complex and heterogenous molecular landscape of BTCs, identifying several druggable genetic alterations, including alterations not commonly found in other solid tumors, such as *IDH1* mutations and a large variety of *FGFR2* rearrangements. Notably, level I actionable genetic alterations, which offer the possibility to treat patients with already approved therapies or in advanced phase of clinical development, can be found in up to 40% of BTCs.

Due to the high number of alterations which require testing, their complexity (i.e., *FGFR2* fusions), and the low amount of tissue available for many patients with advanced disease, NGS is the preferred approach for BTC molecular diagnostics. As only a small subset of BTCs are candidates for curative surgery and the rate of relapse following surgical resection is high, reflex testing with targeted NGS should be implemented. One major challenge that still needs to be addressed is the high rate of failure in molecular profiling that is often caused by small biopsy samples with insufficient tumor content. To overcome this obstacle, we need to focus on optimizing biopsy protocols to increase the amount of tumor tissue obtained. Obtaining an adequate sample in size and tumor content is crucial to perform extensive molecular profiling. Additionally, the use of liquid biopsies can also be implemented to improve success rates.

The continuing efforts and advances in the development of targeted therapies for patients with BTC suggest that genomic profiling will progressively become more important to guide treatment decisions. In this scenario, the pathologist must be responsible for the delivery of Personalized diagnostics and should be in charge of the selection of the most suitable sample and testing method.

10.1. Critical view

Recent genomic profiling studies have identified several actionable genetic alterations, and expanded treatment options. Due to the high number and complexity of genetic alterations which require testing, next-generation (NGS) is preferred over conventional methods (i.e., immunohistochemistry, fluorescence in-situ hybridization and PCR) for molecular profiling of BTCs and should be performed upfront in all BTC patients. One major challenge that still needs to be addressed is the high rate of failure in molecular profiling caused by low tumor content in biopsy samples.

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CRediT authorship contribution statement

Matteo Fassan: Funding acquisition, Supervision. **Aldo Scarpa:** Supervision. **Lorenza Rimassa:** Conceptualization, Supervision. **Valentina Angerilli:** Data curation, Writing – original draft. **Federica Grillo:** Data curation, Writing – original draft. **Nicola Normanno:** Writing – review & editing. **Giancarlo Pruneri:** Writing – review & editing. **Antonio Marchetti:** Writing – review & editing. **Giuseppe Tonini:** Writing – review & editing, Su

Declaration of Competing Interest

All other authors declare that they have no conflict of interest related to the present work.

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Conflict of interest statement

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