**Title:** "*Clinical relevance of biomarkers in cholangiocarcinoma: critical revision and future directions*"

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**ABSTRACT**

Cholangiocarcinoma (CCA) is a malignant tumour arising from the biliary system. In Europe, this tumour frequently presents as a sporadic cancer in patients without defined risk factors and is usually diagnosed at advanced stages with a consequent poor prognosis. Therefore, the identification of biomarkers represents an utmost need for patients with CCA. Numerous studies proposed a wide spectrum of biomarkers at tissue and molecular levels.

With the present paper, a multidisciplinary group of experts within the European Network for the Study of Cholangiocarcinoma (ENSCCA) discusses the clinical role of tissue biomarkers and provide a selection based on their current relevance and potential applications in the framework of CCA. Recent advances are proposed by dividing biomarkers based on their potential role in diagnosis, prognosis, and therapy response. Limitations of current biomarkers are also identified, together with specific promising areas (i.e., artificial intelligence, patient-derived organoids, targeted therapy) where research should be focused to develop future biomarkers.

**Key words:** tissue, prognosis, diagnosis, biliary tract cancer

## KEY MESSAGES

- Routine histology is sufficient for a correct diagnosis and, when needed, a specific immunohistochemical panel leads to a definite diagnosis in most cases.
- Routine histology and several tissue biomarkers were described as useful for patient prognosis and risk stratification.
- Tissue biomarkers for prognosis needs to be assessed and validated in large multicentre studies, or in long-term observational or interventional studies.
- Pharmacoresistance is associated to the expression of uptake transporters or export pumps, and to PD-L1 expression.
- The main targeted therapies are those focused on fibroblast growth factor receptor (*FGFR*) 2 fusions and isocitrate dehydrogenase (*IDH*)-1 and -2 mutations but limited to a patient sub-cohort.

## 1. INTRODUCTION

Cholangiocarcinoma (CCA) is a malignant tumour arising in the biliary tree. The incidence of CCA currently accounts for ~15% of primary hepatobiliary cancers and its mortality represents ~2% of all cancer-related deaths.[1] Its silent presentation, aggressive nature, the lack of knowledge of specific risk factors, and/or suboptimal surveillance programs in individuals at high risk, among others, lead to diagnose CCA in advanced stages; moreover, refractoriness to chemotherapy results in dismal prognosis.[1,2]

The identification of biomarkers represents an utmost need; however, the fact that CCA is one of the most heterogeneous solid cancers is a major challenge. Anatomically, CCA is divided into three subtypes: intrahepatic (iCCA), perihilar (pCCA) and distal (dCCA), and each anatomical subtype is an independent entity from a biological and clinical point of view.[1]

The present multidisciplinary review is based on a collaboration among members of the European Network for the Study of Cholangiocarcinoma (ENSCCA), aimed to evaluate tissue biomarkers with clinical relevance for diagnosis, prognosis, and prediction of therapy response, thus providing clearer and updated guidance for clinicians. After acceptance of the manuscript outline proposal by the coordinators (RIRM, VC, GC), each section was distributed among 3-6 experts who worked together on the specific topics. A consensus was reached to select biomarkers based on the quality of evidence from the studies. The "*Research Need and Perspective*" sections have the scope of identifying specific promising areas on which research could be prospectively focused to go beyond current knowledge. Participants contributed with ideas in relation to all the topics during the revisions of the manuscript. Relevant articles were found by searching PubMed with the term "cholangiocarcinoma" or "bile duct cancer" in combination with the following terms: "biomarker", "histology", "classification", "cells of origin", "liquid biopsy", "diagnosis",

"progression", "survival", "chemoresistance", "radiology", "organoids", "artificial intelligence". Although no specific search dates were used, the most recent articles were preferred.

## 2. TISSUE BIOMARKERS FOR DIAGNOSIS

### Routine histology: diagnostic criteria and pitfalls

The vast majority of ***pCCA*** and ***dCCA*** are mucin-secreting adenocarcinomas characterized by widely spaced, well-formed irregular glands and small cell clusters, often rich in desmoplastic, sclerotic stroma (**Figure 1A**).[3,4] The main diagnostic issue is the need to distinguish well-differentiated p/dCCA from reactive ductal (peribiliary) glands, which is not always straightforward on morphology alone; in this case, the clinical history and radiological imaging must always be considered. Of note, differentiation between a diagnosis of dCCA and pancreatic ductal adenocarcinoma involving extrahepatic bile ducts may be impossible by histology and immunohistochemistry (IHC).

Two main histological subtypes of ***iCCA*** are recognized (**Figure 1B**): the large duct type, arising near the hepatic hilus, and the small duct type, which mainly occurs in the liver periphery.[4,5] *Large duct type iCCA* histologically resembles pCCA or dCCA and shows extensive portal infiltration, perineural invasion (PNI), mucin production, papillary structures, and features of intraductal dysplasia (**Figure 1C**). In contrast, *small duct type iCCA* is composed of cuboidal cells with uniform round nuclei, arranged in small-sized tubular or acinar structures, with no mucin production; less differentiated areas may show a small, solid cord-like or cribriform pattern.[6,7] Cholangiolo-carcinoma (CLC) and iCCA with ductal plate malformation pattern represent peculiar histologic subtypes, and could be considered as variants of the small duct type iCCA.[4]

### Molecular tissue biomarkers for diagnosis

Cytokeratin (CK) 7 and 19 are used in routine practice to establish CCA diagnosis by IHC, but both are non-specific markers that can be expressed in some hepatocarcinomas (HCC) and other adenocarcinomas[8-10]. Differential diagnosis with HCC is usually easy, unless the tumour is poorly differentiated. In this case, a wider IHC panel is recommended,[11] which should include markers of hepatocyte differentiation, such as hepatocyte paraffin 1 (HepPar-1), arginase-1, alpha-fetoprotein, CD10 and polyclonal carcinoembryonic antigen, or markers of malignant hepatocytes as glutamine synthetase, glypican 3, or heat shock protein 70.[3]

Epithelial cell adhesion molecule is a surface glycoprotein proposed as a marker for distinguishing between iCCA and HCC,[12] but it is also expressed in carcinomas of different origins and in poorly differentiated HCC. The high expression of tight junction proteins, such as claudins, in biliary tract cancers (BTC) suggests that they can be useful in differentiating these tumours from HCC.[13] The expression pattern of claudins varies in normal and in different parts of the neoplastic biliary tract, thus, when diagnosed in an advanced clinical stage, CCA of extrahepatic and intrahepatic origin and gallbladder cancer can be differentiated based on the claudin expression.[14] Unfortunately, claudin expression is similar in CCA and pancreatic ductal carcinomas, so it is not useful for this differential diagnosis.

The most important issue on biopsy is the differential diagnosis between iCCA and metastatic tumours. Indeed, secondary liver cancers are much more frequent than iCCA. The analysis of C-reactive protein, especially in combination with N-cadherin in whole tissue sections, could be useful to distinguish iCCA from intrahepatic metastases of various origins.[15] A panel of immuno-stains that may help in leading to a definite diagnosis in most cases is furnished in **Table 1**.

**Table 1. Immunohistochemical stains for differential diagnosis of iCCA and intrahepatic metastases and HCC.**

| | K7 | K19 | K20 | CDX2 | SATB2 | TTF1 | NAPSIN | ARGINASE | HepPar-1 | pCEA |
|---|---|---|---|---|---|---|---|---|---|---|
| **iCCA** | + | + | -/+ | -/+ | - | - | - | - | -/+ | + |
| **Colo-rectal cancer** | -* | - | + | + | + | - | - | - | - | + |
| **Gastric cancer** | + | + | +/- | +/- | - | - | - | - | - | + |
| **Lung cancer**** | + | - | - | - | - | + | + | - | - | + |
| **HCC** | -# | -^ | - | - | - | - | - | + | + | +§ |

* A subset of colo-rectal cancers, namely those originating in the rectum, may be CK7 positive
** Intestinal subtype of lung cancer displays the same immunophenotype of colo-rectal cancer
# K7 can be rarely seen in HCC, particularly poorly differentiated
^ Aberrant expression of K19 may occur in HCC and it is thought to be an adverse prognostic factor
§ In HCC a canalicular pattern is seen which is considered pathognomic, while in adenocarcinomas pCEA shows cytoplasmic/membranous stain.
*Abbreviations*: CDX2: Caudal Type Homeobox 2; HCC: hepatocellular carcinoma; HepPar-1: hepatocyte paraffin 1; K: cytokeratin; iCCA: intrahepatic cholangiocarcinoma; pCEA: polyclonal carcinoembryonic antigen; SATB2: Special AT-rich sequence-binding protein 2; TTF1: Thyroid transcription factor 1.

Unfortunately, metastatic adenocarcinoma from pancreas, gallbladder, and extrahepatic bile ducts may be undistinguishable from iCCA, both at histology and on IHC. The differential expression of a panel of 38 markers showed characteristic profiles for iCCA that distinguished them from metastatic and pancreatobiliary adenocarcinomas.[16] As such, it is likely that up to 10% of patients with liver metastasis from a pancreatic mass presenting with jaundice currently managed as metastatic pancreatic adenocarcinoma are likely to be dCCA. Ultimately, only routine multi-omic analysis is likely to be able to differentiate between these two histo-pathologically similar but prognostically very different cancers.

Over the last decade, non-coding RNAs (ncRNAs) have emerged as possible new clinically relevant biomarkers to assist in the diagnosis and also in the prognosis of multiple

cancers.[17,18] Among ncRNAs, microRNAs (miRNAs), long ncRNAs (lncRNAs) and circular RNAs (circRNAs) are the most studied in CCA[19,20]. Importantly, ncRNAs have been demonstrated to contribute to CCA onset and progression by regulating key signalling pathways. In addition, ncRNA deregulation has been shown to reflect CCA pathogenesis. Thus, numerous ncRNAs have been proposed as biomarkers for CCA diagnosis and prognosis.[21,22] The biochemical nature of ncRNAs constitutes an advantage for biomarkers: as nucleic acids, even low copies of ncRNAs can be easily and specifically detected, notably by quantitative RT-PCR, in situ hybridization (ISH) or Fluorescent ISH (FISH). Further studies would be needed to establish the validity of ncRNAs in clinical practice.

**Research Need and Perspective I. Liquid biopsy: from tissue analysis to serum biomarkers**

The only blood-based biomarker for CCA diagnosis widely used for clinical use is carbohydrate antigen 19-9 (CA19-9).[2] However, when used by itself, the sensitivity and specificity for diagnosing CCA are variable and, among other issues, depend on the used cut-off values.[23-25] In a large European cohort, CA19-9 showed low sensitivity in early stages, but increased sensitivity in advanced disease.[26] This finding could be of translational relevance for patient stratification and design of clinical trials. However, the determination of circulating levels of CA19-9 may be useful for patients' surveillance and follow-up, comprising primary sclerosing cholangitis (PSC), but its considerable limitations (e.g. non-specific elevation in other malignancies and even in benign disease associated to cholangitis and/or cholestasis) and complete absence in patients with Lewis antigen-negative blood type have triggered an active search for alternative circulating biomarkers (**Figure 2**). Changes in gut microbiota, bile acid metabolism and cytokines, as well as in other metabolites and proteins have been described in patients with CCA and some of them

proposed as biomarkers for diagnosis and prognosis.[27-31] Several studies have showed in biopsy proven CCA that the sensitivity and specificity of panels of serum metabolites or of proteins isolated from serum extracellular vesicles were better than CA19-9 for the early diagnosis of iCCA and dCCA, and for a differential diagnosis with HCC or pancreatic ductal adenocarcinoma, respectively.[27,30,32] Combination of CA19-9 with these innovative biomarkers improved their diagnostic capacity. Despite great efforts, no biomarker for CCA based on proteins, gut bacteria or metabolites have reached clinical practice yet,[24,30-32] but this could change if positive results are confirmed in ongoing validation studies.

Recent advances have allowed the identification of genomic alterations characteristic of each CCA subtype[1]. If translated into the field of diagnosis this knowledge would enable the development of a liquid biopsy-based on the analysis of cell-free circulating tumour DNA (ctDNA). Tumour DNA released from cancer cells into blood captures the tumour-specific and often heterogeneous genetic and epigenetic alterations, including point mutations, copy number alterations, chromosomal rearrangements, and DNA methylation patterns. Its analysis would avoid the limitations of invasive and often anatomically difficult conventional biopsies.[33] Although a ctDNA-based liquid biopsy may be limited by a low sensitivity in patients with early-stage disease this may be overcome in part by using digital PCR or capture-based next-generation sequencing, allowing for the simultaneous analysis of multiple disease-related mutations.[33-35] Additional potential advantages of a ctDNA-based liquid biopsy would be the identification of druggable targets, thus allowing the selection of the most effective therapies, and the detection of resistance mechanisms emerging during therapy through longitudinal sampling; also, it could allow the identification of mutations not found in the tissue biopsy or present in not-biopsied tumours. ctDNA genotyping in blood is currently being tested in CCA patients with promising results.[36,37] Although the number of published studies is still limited, a high concordance between alterations found in ctDNA and tumour tissue DNA has been generally observed.[38,39]

Bile is also emerging as a promising liquid biopsy matrix for CCA diagnosis; a recent study showed that detection of mutations in bile cell free DNA has a strong sensitivity for early malignancy detection within the biliary tract.[40] Identification of differentially methylated markers in bile cell free DNA has also been recently demonstrated to provide high sensitivity for CCA detection.[41,42] If confirmed, these approaches may significantly improve the management of patients with biliary strictures in which intraductal biopsy results inconclusive, and further support the biliary fluid as a relevant matrix for relatively low invasive liquid biopsy.

Although more technically challenging, the application of serum cell-free DNA methylation assays of genes known to be hypermethylated in tumour tissues can be an alternative approach for early cancer detection.[33] Incipient studies suggest that such methylation assays may aid in the differential diagnosis of CCA and other biliary diseases.[43] Moreover, the possibility of analysing other nucleic acids, such as ncRNAs, in serum as biomarkers for CCA diagnosis and prognosis is also being actively investigated, indicating a promising diagnostic value of the circulating signatures of these molecules as diagnostic tools for CCA.[44-46]

It is foreseeable that the information emerging from these liquid biopsy assays will be complex to integrate into the clinical workflow. To leverage all its potential and combine these tests with clinical information and other “omics” data, the implementation of artificial intelligence tools and machine learning approaches will be essential as demonstrated in other solid tumours.[47-50]

## 3. TISSUE BIOMARKERS FOR PROGNOSIS

### Routine histology

Routine histology contains information that may be correlated with a patient’s prognosis and may be useful in risk stratification. Large duct type iCCA showed a worse overall survival

(OS) compared to small duct type, and is associated with perineural infiltration, and higher pathological tumour stage and CA19-9 levels compared to the other histological subtypes.[51-53] CLC areas can be found in both large bile duct and small bile duct iCCA;[54] interestingly, iCCA with CLC areas and pure CLC showed a better prognosis compared to the other biliary histo-types.[55-57] However, the lack of multicentric studies and the need of international consensus on histologic criteria and nomenclature are key limitations in these settings.

The presence of a desmoplastic and highly cellular stroma is a typical feature in iCCA (**Figures 1D-E**). The original identification of an association between stroma and outcome found that patients with resected tumours with more abundant desmoplastic stroma, categorised as 'scirrhous-type', had a poorer prognosis,[58,59] a finding supported by another study describing stromal immaturity as an independent predictor of poor outcome in iCCA.[60] However, more recent quantitative assessment of stromal content using extracellular matrix staining or stain-free evaluation of collagen fibres suggested that high stromal content conferred a survival benefit, although tumours with high collagen cross-linking were associated with a worse outcome.[61]

In cancers of the gastro-intestinal tract, mucins are considered prognostic markers.[62] Mucin production is observed in all anatomical subtypes of CCA (**Figure 1C**), but not in HCC; mucin-rich iCCA presents at an advanced stage upon diagnosis[63] and has a shorter survival time compared to conventional counterparts.[64] In parallel, the presence of mucin component in pCCA identifies tumours with greater parenchymal invasion, higher CA19-9 levels, and worse prognosis.[65]

Perineural invasion (**Figure 1E**) is defined as the invasion of tumour cells through the perineurium.[66] PNI has prognostic value in resected dCCA and was defined as a risk factor for poor survival by meta-analysis studies.[67] Furthermore, both in pCCA[68,69] and

iCCA,[66] PNI was indicated as an independent risk factor associated with worse recurrence and survival outcomes after resection.

**Research Need and Perspective II. Artificial intelligence tools: hidden information in H&E stain**

Haematoxylin and eosin (H&E)-stained histopathological slides are available for most cancer patients. In recent years, advances in artificial intelligence (AI) have made it possible to systematically extract subtle features from digitised H&E-stained slides. Currently, AI in histopathology relies almost exclusively on deep convolutional neural networks, a well-established technology. In principle, these tools can be used to automate tedious tasks in routine histopathology, easing the ever-increasing workload of pathologists. In addition, AI-based methods can extract hidden information from cellular or tissue morphology.[70] This hidden information can be three-fold: *i)* prognostic, helping to define the risk of cancer progression or death; *ii)* predictive, directly helping to forecast response to a given treatment; and *iii)* AI can infer genetic alterations in tumour tissue from histomorphology alone.[71] This could help in the future to pre-select patients for genetic testing or, ultimately, could replace genetic tests in certain circumstances.

From a clinical standpoint, the establishment of these AI-based biomarkers is very attractive because they do not require any tumour material additional to routine pathology slides. Therefore, AI-based image analysis could, in principle, be an inexpensive addition to routine workflows, even running predictive or prognostic models in the background while pathologists review the slides.[72]

In liver tumours, AI methods have been applied to predict prognosis of patients directly from routine histopathology images.[73] For example, an image segmentation pipeline developed by Liao *et al*. was used to calculate a risk score associated with OS after resection in HCC, allowing the stratification of patients into long- or short-term survivors.[74]

Similarly, a handcrafted feature from nuclei segmentation in HCC was used to predict early recurrence after resection.[75] Several studies have shown that deep learning algorithms were able to predict the survival of HCC patients from H&E-stained whole slide images.[73] Unfortunately, CCA is not yet a common application of these technologies, compared to HCC;[73] the reason could be ascribed to the fact that AI-based methods are known to be data-hungry, which means that they require a lot of data to yield reliable results. Indeed, in gastrointestinal cancer pathology it has been shown that the performance of AI systems increases if they are trained on more patients. Many studies use a few hundred patients to train AI biomarkers, but the best performing biomarkers have been reported from studies that trained on more than 5000 patients.[76] Therefore, the main problem is logistical, especially in a rare tumour type such as CCA. It is essential that, in addition to high-quality scans of pathological slides, the associated clinical and/or genetic data are in a format with a clean data structure. In practice, such impediments can slow down the development of clinical AI biomarkers, but multicentre academic consortia are a viable solution to these issues.

**Molecular tissue biomarkers for prognosis**

Molecular tissue biomarkers potentially embody prognostic value, allowing prediction of both the survival of patients undergoing tumour resection, the response to adjuvant therapies and the chance of tumour relapse, thus allowing patient stratification, and guiding therapeutic decisions (**Figure 3**). Patients with iCCA exhibiting genetic alterations on tumour protein P53 (*TP53*) and Kirsten ras oncogene homolog (*KRAS*) genes from two large international cohorts of resected patients were shown to display worse prognosis mainly related to shorter OS and higher tumour recurrence, when compared with patients with isocitrate dehydrogenase (*IDH*)-1 and -2 mutations or with the control group.[77,78] Recently, the presence of G12 KRAS variants, but not non-G12 KRAS variants, in iCCA was associated

with worse survival and increased risk of recurrence.[79] Transcriptomic analysis of resected iCCA tumours also revealed two distinct subtypes, the “inflammation” and the “proliferation” ones, with the latest being linked to the activation of oncogenes and worse outcome,[80] while a panel comprised of 36 genes, identified in a mRNA microarray conducted with tumour samples from patients with resectable iCCA, was directly associated with poor survival.[81] Interestingly, patients with either *KRAS/BRAF* mutations or increased Erb-B2 receptor tyrosine kinase 2 (*HER2*) levels showed worse prognosis. A meta-analysis including IHC-based studies (73, counting 4126 patients with CCA) allowed the identification of 77 tissue prognostic biomarkers for CCA.[82] In this setting, fascin, epidermal growth factor receptor (EGFR), Mucin (MUC) 1 , MUC4 and p27 were reported to be independent prognostic factors associated with worse OS in resected patients. In parallel, 39 transcriptomic prognostic biomarkers were reported in a cohort of patients with BTC, with all of them related to T-cell activation and immune response.[83] Moreover, the retained expression of BRCA1 Associated Protein 1 (BAP-1) and Polybromo 1 (PBRM-1) and overexpression of S100 Calcium Binding Protein P (S100P) has been related to a poor prognosis in iCCA.[84] More recently, a panel based on high expression levels of EGFR, HER4 and ephrin receptor A3 (EphA3) was shown to be an independent prognostic predictor for post-operative CCA recurrence.[85]

Finally, tumour tissue miRNAs might also help to predict prognosis; in this regard, increased levels of miR-21 in iCCA tumour specimens were positively linked to the clinical stage at diagnosis, tumour differentiation and with poor OS and progression-free survival (PFS)[86,87].

Although liver transplantation is already considered a potentially feasible option for highly selected patients with pCCA and iCCA,[88,89] it is to be hoped that ongoing trials will help determine if tissue biomarkers are also associated with prognosis after transplantation.

**“Host” response to malignancy: tissue biomarkers for prognosis prediction**

Non-epithelial histological features, partly reflecting a ‘host’ response to malignancy, but also of potential importance in carcinogenesis, associated with CCA may offer prognostic value.

*Tumour stroma and cancer-associated fibroblasts*

Prominent desmoplastic stroma produced by cancer-associated fibroblasts (CAFs) is a characteristic histological feature of CCA. Specific protein constituents of the stroma and CAFs have also been examined[90,91]. High levels of periostin assessed semi-quantitatively were associated with lower OS,[92] although this finding was not supported by more recent work in which periostin was quantified using an automated method.[61] The data linking osteopontin expression to outcome is also conflicting. Two studies found that decreased osteopontin expression in resected tumour was associated with poor outcomes,[93,94] although more recent studies described the opposite relationship.[95,96] There is also data supporting stromal tenascin expression predicting poor prognosis[93].

There is some evidence that the relative composition of CAFs is independently predictive of prognosis.[97,98]

*Immune cell populations*

The tumour immune microenvironment plays a role in carcinogenesis and immunotherapies modulating this environment are in clinical use. The composition of this ‘host’ immune infiltrate has been shown to provide additional prognostic information (**Figure 3**).

Patients whose tumours contain a larger number or higher density of infiltrating monocytes/macrophages have been shown to have a poorer prognosis;[99-101] however, there is conflicting evidence about whether the location of macrophages within the tumour

carries prognostic significance.[102,103]. Further, more recent evidence indicates that the macrophage phenotype may carry prognostic value.[104]

Intra- and peritumoral T-lymphocytes are commonly observed in CCA[105] and increased cluster of differentiation (CD)8-positive intra-tumoral T-cells counts are associated with a better prognosis.[100,106] Intra-epithelial CD4-positive T-cells are also predictive of a better outcome.[107] Mature CD83-positive intra-tumoral dendritic cells are positively correlated with intra-tumoral T-cells, and higher numbers are independently associated with better outcomes.[108] Patients with resected tumours that contained larger numbers of neutrophils[109] or higher neutrophil:lymphocyte ratio had a poorer prognosis.[110]

Multiplex immunofluorescence allows the investigation of the spatial arrangement of immune cells together with co-expression patterns.[111] The assay optimization has yield highly sensitive and reproducible characterization in several tumours, including lung and breast cancers.[112] Multiplex immunofluorescence has been also used for immune-profiling iCCA,[113] but its role as actual biomarker should be further assessed.

*Microvasculature and neoangiogenesis*

Neo-angiogenesis plays an important role in tumour progression,[114] although the evidence for the relationship between microvessel density and prognosis in CCA is conflicting. Some studies on resected tumours have shown that decreased microvessel density is associated with a poorer prognosis,[115,116] while other studies have suggested that increased microvessel density is predictive of poorer outcome.[117,118]

## 4. TISSUE BIOMARKERS, THERAPY RESPONSE, AND TARGETED THERAPY

### Tissue biomarkers and therapy response

Unfavourable responses to chemotherapy have been observed in the large duct compared with the small duct type iCCA and in advanced tumours with an increased DNA repair

capacity or with alterations in Transforming Growth Factor β pathway.[119] Reduced levels of uptake transporters and increased levels of export pumps in the plasma membrane by IHC have been associated with lower response to gemcitabine/cisplatin.[120,121] Increased levels of enzymes involved in the inactivation of anticancer agents or reduced expression of enzymes needed for the activation of prodrugs in tumours can predict a lower response to gemcitabine/cisplatin and, probably, to other drugs.[122] The heterogeneity in expression levels of molecular targets can predict the lack of effect of some drugs in CCA and this heterogeneity may potentially be used to select the best treatment. Resistance is a dynamic process and tumours, in response to the exposure to drugs, can change the levels of the proteins involved in resistance;[123,124] therefore, the occurrence of mutations that affect the activity of these proteins can also induce cross-resistance to different drugs.[125] New mutations in tyrosine kinase receptors and other targets are considered a mechanism of acquired resistance to targeted agents and are responsible for disease progression after an initial response.[123] Reduced expression of programmed cell death 1 ligand 1 (PD-L1) has been associated with worse outcome in CCA patients treated with monoclonal antibodies targeting programmed cell death protein 1 (PD-1), nivolumab or pembrolizumab.[126,127]

**Tissue biomarkers and targeted therapy**

The identification of targetable alterations and associated therapies have made precision medicine a reality in the management of CCA.[128] The main targeted therapies being developed in CCA are those focused on fibroblast growth factor receptor (*FGFR*) 2 fusions and IDH-1 and -2 mutations.[129]

*IDH as a biomarker and treatment target*

Around 15% of iCCA are expected to harbour a mutation in *IDH1*.[128] These mutations are predictive markers of benefit from IDH1 inhibitors. Ivosidenib is the most developed IDH

inhibitor in CCA.[130] Further assessment in the randomized ClarIDHy phase III clinical trial in patients with *IDH1* mutant (R132C/L/G/H/S mutation variants) CCA who had progressed to prior chemotherapy[131] confirmed that ivosidenib achieved a longer PFS (median 2.7 months) over placebo (median 1.4 months); HR 0.37 (95% CI 0.25-0.54; p-value <0.001).

*FGFR fusions*

FGFR2 fusions are of special relevance in CCA, with around 15% of iCCA showing these aberrations.[128] Multiple selective FGFR tyrosine kinase inhibitors have been developed, all these with an adequate safety profile and promising efficacy in phase II studies in patients with refractory CCA.[128] These compounds have reported consistently high partial response rates (varying between 20.7% and 35.5%) in the presence of FGFR2 fusions, with median PFS of around 6 months.[128] Some of these agents are now moving into randomized phase III clinical trials in the first-line setting, in which their activity is being compared with the current standard of care [pemigatinib-INCB054828 (FIGHT-302; NCT03656536), infigratinib-BGJ398 (PROOF; NCT03773302), futibatinib-TAS-120 (FOENIX-CCA3; NCT04093362)].

Further second generation IDH inhibitors are being developed, which would address IDH2 mutations that occur less frequently but have an identical pathogenicity (https://www.cancer.gov/publications/dictionaries/cancer-drug/def/idh1-idh2-dual-inhibitor-hmpl-306).

*Other predictive markers for selection of targeted therapies*

Other molecular alterations seem to be linked to activity of specific targeted therapies, but their presence is rare (<5%) in CCA.

The human epidermal growth factor receptor (HER) family includes four members: EGFR or HER1, HER2, HER3, and HER4.[132] Relevance of involvement of this pathway

in cancer progression has been shown previously.[133] Some trials in CCA with HER overexpression have shown disappointing activity,[134-136] with some ongoing clinical trials aiming to clarify their role (NCT02892123). A recent study indicates that pertuzumab plus trastuzumab has promising, durable activity in patients with HER2-positive metastatic BTC, with good tolerability relative to traditional cytotoxic treatments.[137]

Ring finger protein 43 (*RNF43*) mutations are rarely present in CCA, but they may allow targeted approaches with porcupine inhibitors,[129,138] with some ongoing trials in this setting (NCT03447470, NCT04907851).

CCA rarely presents with fusions of the neurotrophic tyrosine receptor kinase (*NTRK*) gene,[139] but when these are present, tropomyosin receptor kinase inhibitors are known to achieve high rates of objective response (57%-75%) in previously treated advanced solid tumours, as a tumour-agnostic approach.[140,141]

*BRAF* mutations have been described in a small proportion of CCA.[142] For patients harbouring *BRAF* V600E mutations, dual inhibition of BRAF and MEK with dabrafenib and trametinib have achieved high partial response rates (42%-36%) and a median PFS of 9.2 months.[143]

Microsatellite instability (MSI) is also rare in CCA.[1] Pembrolizumab has become available for MSI-high advanced cancers;[144,145] although preliminary data were encouraging, the efficacy of pembrolizumab in patients with MSI-high CCA remains to be defined.[1,146]

*Targeted therapies without predictive biomarkers*

Unfortunately, not all targeted therapies have an identified predictive biomarker to allow patient selection and, despite this, seem to be still effective for “all-comers”. The phase II REACHIN study[147] recruited 66 patients diagnosed with BTC who were randomized to the multi-tyrosine kinase inhibitor, regorafenib, or placebo after standard chemotherapy.

This study showed a benefit in terms of PFS (HR 0.49 (95% CI 0.29-0.81; p-value 0.005); despite an absence of objective responses or OS benefit.

**Research Need and Perspective III. Organoids: "mini-tumours" for drug selection?**

Organoids are defined as three-dimensional structures derived from primary cells that self-organize through cell-to-cell and cell-to-matrix interactions and recapitulate aspects of the native tissue architecture and function *in vitro*.[148,149] Increasing evidence supported the feasibility of deriving cancer organoids from fresh tumour tissue to establish the so-called "mini-tumours" that can be grown in plastic and contribute to the advancement in tumour biology and precision medicine[150]. There is growing evidence demonstrating the feasibility of CCA organoids in either resectable[151-153] or advanced disease.[154] Organoids recapitulate the morphology and genomics of the source tissue; these features are maintained over culture[154,155] and thus enable the use of organoids as disease models that better represent the multicellular interaction occurring within the tumour (**Figure 4**).[156] More interestingly, patient-derived organoids established as part of patients' based co-clinical trials mimic drug response in gastrointestinal cancer with a positive predictive value of 70% and a negative predictive value of 100%.[155] As organoids are constituted mainly by epithelial cells, their predictive value as a pure culturing line is enriched in the testing of epithelial-directed drugs such as conventional chemotherapy compounds or targeted drugs.[151,154] These interesting results have encouraged the use of organoids as "real life" predictive tools to aid drug selection for patient management and have prompted the initiation of clinical trials. One example is the SCORE trial (NCT04279509) where the choice of chemotherapy drugs for patients with refractory solid cancers is based on the response score assessed on an organoid-based semi-high throughput platform. However, the evolution of therapeutic approaches and the introduction of immuno-oncology require an evolution of these ex-vivo predictive models towards the incorporation of tumour

microenvironment components. Co-culture between organoids from mismatch repair (MMR)-deficient human cancers and autologous peripheral blood lymphocytes could be used to enrich tumour-reactive T cells, which could then recognize and kill MMR deficient organoids.[157] The role of myeloid-derived suppressive cells (MDSC) in modulating response to PD-1 inhibitors was assessed in co-cultures amongst murine pancreatic cancer organoids, cytotoxic T cells and MDSC, showing the usefulness of these models in investigating mechanisms of resistance to immunotherapy.[158] However, unanswered questions remain before these models can be used in clinical practice to implement precision medicine. To date, there are still scarce reports of human CCA development and, in contrast with other tumour types, the efficiency rate of organoid establishment in sequential series of patients are still lacking in CCA. The impact of CCA subtyping on the success rate of organoids has not been explored. In addition, feasibility data are missing regarding the possibility of establishing co-cultures between CCA organoids and autologous immune cells based on their life span.

## 5. RADIOLOGY

### Radiology in diagnosis and staging

Although the gold standard of diagnosis and grading for CCA is still pathological examination, radiology (**Figure 5**) has a pivotal role in the management of CCA in terms of diagnosis, staging, follow-up, and response to therapy.[159,160] Some radiological findings at computed tomography (CT) and magnetic resonance imaging (MRI), such as capsular retraction and the presence of a homogeneous mass with rim-like enhancement and progressive contrast uptake, are highly indicative of a CCA diagnosis. In addition, CCA does not exhibit, in general, the radiological hallmarks of HCC by MRI/CT, allowing the differential diagnosis with HCC, particularly in the setting of chronic liver diseases.[161,162] Also, radiology is key for evaluating resectability.[159,163]

In the last decade, major efforts have been implemented in correlating some specific imaging findings with pathology traits. Differences in CT enhancement pattern among the morphologic subtypes of CCA were noted, since mass-forming tumours are usually hyperenhanced at the periphery, with central hypoenhancing, while periductal-infiltrating tumours appear hyperenhanced, and intraductal tumours, hypoenhanced at arterial phase.[164] When correlated with MRI findings, large duct type iCCA generally showed concentric filling at venous phase, whereas small duct type iCCA and CLC showed washout in various patterns.[63] Finally, there is scarce information regarding the radiological appearance of combined HCC-CCA; its radiological pattern most commonly overlaps with those of iCCA,[165] and the imaging features that are preponderantly HCC or iCCA appear to correspond to the predominant histopathology components.[166] **Figures 6A-B** show the workflow diagrams combining imaging modalities and tissue biomarkers to reach a final diagnosis of iCCA or p/dCCA when an intrahepatic mass or biliary stricture is detected, respectively.

**Radiology in prognosis assessment**

Radiology may also offer some relevant information regarding the biological tumour behaviour, closely associated with outcome. Moreover, some imaging features may correlate with some specific molecular profile. For instance, the degree of diffusion restriction on diffusion-weighted imaging (DWI) has been independently associated with OS in mass-forming iCCA,[167] and the tumour apparent diffusion coefficient (ADC) quantification has reasonable accuracy for predicting iCCA grade.[168] Furthermore, Ki-67 expression in extrahepatic CCA was predicted through intravoxel incoherent motion (IVIM) combined with DWI, which could reflect the proliferative activity of CCA.[169] Also, the pattern of arterial contrast uptake has been correlated with clinical outcome. In that regard, resected mass-forming iCCA with diffuse arterial hyperenhancement showed better

prognosis in terms of tumour recurrence and OS than did those with peripheral rim enhancement or diffuse hypoenhancement.[170] Finally, the presence of necrosis, satellite nodules and vascular encasement were associated with increased risk of recurrence/death.[171]

### Research Need and Perspective IV. Radiomics and radiogenomics

The advent of radiomic, AI and machine learning, together with the increasing awareness of CCA heterogeneity at morphology and molecular levels, are revitalising the study of radiological correlates. Radiomics extracts quantitative radiologic data from medical images and explores the correlation with clinical outcomes. Radiogenomics aims to identify relationships between quantitative image data with genome and molecular measurements in order to construct association maps to be correlated with outcome.[172] Radiomic and radiogenomic studies in CCA are scarce and most of them include a relatively small number of patients and lack external validation. **Table S1** summarizes the most relevant studies conducted on CCA. Examples of the applicability of AI on imaging are the capability of identifying EGFR and vascular endothelial growth factor expression levels through the identification of certain texture parameters by CT.[173] Also, the MRI texture signature, including three wavelets and one 3D feature, has a favourable ability to discriminate inflamed from non-inflamed immunophenotyping based on the density of CD8+ T cells and in predicting OS.[174] Similarly, a machine learning approach by MRI could serve as a non-invasive biomarker in predicting PD-1/ PD-L1 expression and prognosis of iCCA patients, which may guide clinical decision-making in selecting iCCA patients who may potentially benefit from PD-1/PD-L1 blockage.[175] On the same line, reasonable accuracy has been demonstrated in predicting tumour grade and higher AJCC (American Joint Committee on Cancer) stage in iCCA using certain qualitative and quantitative imaging traits.[176]

These promising data from radiomics and radiogenomics are still preliminary and future studies based on large and multicentre prospective studies are needed.

## 6. CONCLUSIONS AND CLINICAL REMARKS

CCA is characterised by heterogeneity at many levels. In the last ten years different clinical-pathological entities comprised within the CCA spectrum have been identified. To be used in clinical practice, surrogate biomarkers should reflect disease pathobiology and be associated with important outcomes. In a heterogeneous malignancy such as CCA, biomarker discovery is complex. For this reason, as multidisciplinary international investigators, we have focused the attention at the tissue level, the closest observation possible into the complex pathobiology of CCA. Beyond classical serological biomarkers, clinicians may find clinically relevant information for diagnosis, prognosis and therapy response by using tissue biomarkers (**Table 2**).

Remarkably, the performance of tests as relevant surrogate biomarkers for diagnosis or for prediction of solid outcomes in CCA needs to be assessed and validated in large multicentre studies, or in long-term observational or interventional studies, respectively. A coordinated multicentre and multidisciplinary effort seems the sole strategy for the discovery of clinically relevant biomarkers in CCA.

**Table 2. Usefulness of biomarkers in diagnosis, prognosis and therapy response in CCA**

| | | ***Clinical relevance*** | ***Current limitations*** |
|---|---|---|---|
| **Diagnosis** | *Routine Histology* | ***Routine histology*** is key but needs IHC support | ***Differential diagnosis:***<br>• iCCA vs metastasis from PDAC & BTC<br>• dCCA vs PDAC |
| | *Molecular tissue biomarkers* | An **IHC panel** (**Table 1**) **leads** to a definite diagnosis in most cases | |
| | *Radiology* | ***CT/MRI relevant features***:<br>• capsular retraction<br>• homogeneous mass with:<br>  ○ rim-like enhancement<br>  ○ progressive contrast uptake<br>• no hallmarks of HCC<br>***Resectability evaluation*** | ***Differential diagnosis:***<br>• iCCA vs combined HCC-CCA |
| **Prognosis** | | ***Clinical relevance*** | ***Current limitations*** |
| | *Routine Histology* | **i/p/dCCA**<br>• mucin presence<br>• perineural invasion<br>**iCCA**<br>• histologic subtyping (small/large/CLC)<br>• stroma maturity | Histologic criteria and nomenclature are not fully standardized |
| | *Molecular tissue biomarkers* | ***Most relevant biomarkers****:*<br>• TP53/KRAS/BRAF mutation<br>• HER2 expression levels<br>• mucins (MUC1, MUC4)<br>• immune cell populations<br>*See Figure 3 for complete list.* | No international consensus on molecular tissue biomarkers for patients' stratification |
| | *Radiology* | • arterial contrast uptake<br>• *others* | Monocentric studies & no definite correlation with tissue biomarkers |
| **Therapy response & targeted therapy** | | ***Clinical relevance*** | ***Current limitations*** |
| | *Therapy response* | Chemoresistance is associated to:<br>• large-duct type iCCA<br>• ↓ expression of uptake transporters<br>• ↑ expression of export pumps<br>• ↓ PD-L1 expression | No marker is currently recommended in clinical practice |
| | *Targeted Therapy* | • ***FGFR2* fusions**<br>• ***IDH1/2* mutations**<br>• others in <5% CCA | Restricted to a small percentage of patients |

*Abbreviations*: BTC, biliary tract cancer; CCA, cholangiocarcinoma; CLC, cholangiolo-carcinoma; CT, computed tomography; dCCA, distal cholangiocarcinoma; FGFR2, fibroblast growth factor

receptor; HCC, hepatocellular carcinoma; HCC-CCA, hepatocellular cholangiocarcinoma; HER2, Erb-B2 receptor tyrosine kinase 2; iCCA, intrahepatic cholangiocarcinoma; IDH1/2, isocitrate dehydrogenase 1/2; IHC, immunohistochemistry; MRI magnetic resonance imaging; pCCA, perihilar cholangiocarcinoma; PDAC, pancreatic ductal adenocarcinoma; PD-L1, programmed cell death protein ligand 1; TP53/KRAS/BRAF, tumour protein P53/Kirsten ras oncogene homolog/B-type Raf proto-oncogene.

**Patient and Public Involvement**

Patients or members of the public were not directly involved in the design, writing, or revising this manuscript. However, the present manuscript is based upon work from COST Action European Cholangiocarcinoma Network (Euro-Cholangio-Net: CA18122) and represents a deliverable of this action. AMMF - The Cholangiocarcinoma Charity (UK) is directly involved in Euro-Cholangio-Net and participates in defining the mission and the deliverables of the Action. AMMF endorsed this manuscript and will participate in its dissemination.

**Competing interests**

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**REFERENCES**

1 Banales JM, Marin JJG, Lamarca A, et al. Cholangiocarcinoma 2020: the next horizon in mechanisms and management. *Nat Rev Gastroenterol Hepatol* 2020;17(9):557-88. doi: 10.1038/s41575-020-0310-z

2 Valle JW, Kelley RK, Nervi B, et al. Biliary tract cancer. *Lancet* 2021;397(10272):428-44. doi: 10.1016/S0140-6736(21)00153-7

3 Cholangiocarcinoma Working G. Italian Clinical Practice Guidelines on Cholangiocarcinoma - Part I: Classification, diagnosis and staging. *Dig Liver Dis* 2020;52(11):1282-93. doi: 10.1016/j.dld.2020.06.045

4 Board WCoTE. Digestive system tumours. Fifth Edition ed. Lyone (France): International Agency for Research on Cancer 2019.

5 Akita M, Fujikura K, Ajiki T, et al. Dichotomy in intrahepatic cholangiocarcinomas based on histologic similarities to hilar cholangiocarcinomas. *Mod Pathol* 2017;30(7):986-97. doi: 10.1038/modpathol.2017.22

6 Nakanuma Y, Kakuda Y. Pathologic classification of cholangiocarcinoma: New concepts. *Best Pract Res Clin Gastroenterol* 2015;29(2):277-93. doi: 10.1016/j.bpg.2015.02.006

7 Komuta M. Intrahepatic cholangiocarcinoma: tumour heterogeneity and its clinical relevance. *Clin Mol Hepatol* 2022 doi: 10.3350/cmh.2021.0287

8 Ma H, Xiao W, Wang M, et al. CDH17+/CDX2+ Can be Helpful in Providing Support for Small Intestinal Origin Versus Pancreatic or Biliary Origin. *Appl Immunohistochem Mol Morphol* 2021;29(7):541-45. doi: 10.1097/PAI.0000000000000913

9 Yang F, Wan Y, Xu L, et al. MRI-Radiomics Prediction for Cytokeratin 19-Positive Hepatocellular Carcinoma: A Multicenter Study. *Front Oncol* 2021;11:672126. doi: 10.3389/fonc.2021.672126

10 Zhuo JY, Lu D, Tan WY, et al. CK19-positive Hepatocellular Carcinoma is a Characteristic Subtype. *J Cancer* 2020;11(17):5069-77. doi: 10.7150/jca.44697

11 Takahashi Y, Dungubat E, Kusano H, et al. Application of Immunohistochemistry in the Pathological Diagnosis of Liver Tumors. *Int J Mol Sci* 2021;22(11) doi: 10.3390/ijms22115780

12 Proca DM, Niemann TH, Porcell AI, et al. MOC31 immunoreactivity in primary and metastatic carcinoma of the liver. Report of findings and review of other utilized markers. *Appl Immunohistochem Mol Morphol* 2000;8(2):120-5. doi: 10.1097/00129039-200006000-00006

13 Lodi C, Szabo E, Holczbauer A, et al. Claudin-4 differentiates biliary tract cancers from hepatocellular carcinomas. *Mod Pathol* 2006;19(3):460-9. doi: 10.1038/modpathol.3800549

14 Nemeth Z, Szasz AM, Tatrai P, et al. Claudin-1, -2, -3, -4, -7, -8, and -10 protein expression in biliary tract cancers. *J Histochem Cytochem* 2009;57(2):113-21. doi: 10.1369/jhc.2008.952291

15 Yeh YC, Lei HJ, Chen MH, et al. C-Reactive Protein (CRP) is a Promising Diagnostic Immunohistochemical Marker for Intrahepatic Cholangiocarcinoma and is Associated With Better Prognosis. *Am J Surg Pathol* 2017;41(12):1630-41. doi: 10.1097/PAS.0000000000000957

16 Fernandez Moro C, Fernandez-Woodbridge A, Alistair D'souza M, et al. Immunohistochemical Typing of Adenocarcinomas of the Pancreatobiliary System Improves Diagnosis and Prognostic Stratification. *PLoS One* 2016;11(11):e0166067. doi: 10.1371/journal.pone.0166067

17 Merdrignac A, Papoutsoglou P, Coulouarn C. Long Noncoding RNAs in Cholangiocarcinoma. *Hepatology* 2021;73(3):1213-26. doi: 10.1002/hep.31534

18 Salati M, Braconi C. Noncoding RNA in Cholangiocarcinoma. *Semin Liver Dis* 2019;39(1):13-25. doi: 10.1055/s-0038-1676097

19 Louis C, Leclerc D, Coulouarn C. Emerging roles of circular RNAs in liver cancer. *JHEP Rep* 2022;4(2):100413. doi: 10.1016/j.jhepr.2021.100413

20 Shi T, Morishita A, Kobara H, et al. The Role of microRNAs in Cholangiocarcinoma. *Int J Mol Sci* 2021;22(14) doi: 10.3390/ijms22147627

21 Louis C, Desoteux M, Coulouarn C. Exosomal circRNAs: new players in the field of cholangiocarcinoma. *Clin Sci (Lond)* 2019;133(21):2239-44. doi: 10.1042/CS20190940

22 Wada Y, Shimada M, Morine Y, et al. A blood-based noninvasive miRNA signature for predicting survival outcomes in patients with intrahepatic cholangiocarcinoma. *Br J Cancer* 2022 doi: 10.1038/s41416-022-01710-z

23 Bowlus CL, Lim JK, Lindor KD. AGA Clinical Practice Update on Surveillance for Hepatobiliary Cancers in Patients With Primary Sclerosing Cholangitis: Expert Review. *Clin Gastroenterol Hepatol* 2019;17(12):2416-22. doi: 10.1016/j.cgh.2019.07.011

24 Fung BM, Tabibian JH. Cholangiocarcinoma in patients with primary sclerosing cholangitis. *Curr Opin Gastroenterol* 2020;36(2):77-84. doi: 10.1097/MOG.0000000000000616

25 Rizzo A, Ricci AD, Tavolari S, et al. Circulating Tumor DNA in Biliary Tract Cancer: Current Evidence and Future Perspectives. *Cancer Genomics Proteomics* 2020;17(5):441-52. doi: 10.21873/cgp.20203

26 Izquierdo-Sanchez L, Lamarca A, La Casta A, et al. Cholangiocarcinoma landscape in Europe: diagnostic, prognostic and therapeutic insights from the ENSCCA Registry. *J Hepatol* 2021 doi: 10.1016/j.jhep.2021.12.010

27 Herraez E, Romero MR, Macias RIR, et al. Clinical relevance of the relationship between changes in gut microbiota and bile acid metabolism in patients with intrahepatic cholangiocarcinoma. *Hepatobiliary Surg Nutr* 2020;9(2):211-14. doi: 10.21037/hbsn.2019.10.11

28 Jia X, Lu S, Zeng Z, et al. Characterization of Gut Microbiota, Bile Acid Metabolism, and Cytokines in Intrahepatic Cholangiocarcinoma. *Hepatology* 2020;71(3):893-906. doi: 10.1002/hep.30852

29 Arbelaiz A, Azkargorta M, Krawczyk M, et al. Serum extracellular vesicles contain protein biomarkers for primary sclerosing cholangitis and cholangiocarcinoma. *Hepatology* 2017;66(4):1125-43. doi: 10.1002/hep.29291

30 Banales JM, Inarrairaegui M, Arbelaiz A, et al. Serum Metabolites as Diagnostic Biomarkers for Cholangiocarcinoma, Hepatocellular Carcinoma, and Primary Sclerosing Cholangitis. *Hepatology* 2019;70(2):547-62. doi: 10.1002/hep.30319

31 Macias RIR, Munoz-Bellvis L, Sanchez-Martin A, et al. A Novel Serum Metabolomic Profile for the Differential Diagnosis of Distal Cholangiocarcinoma and Pancreatic Ductal Adenocarcinoma. *Cancers (Basel)* 2020;12(6) doi: 10.3390/cancers12061433

32 Intuyod K, Armartmuntree N, Jusakul A, et al. Current omics-based biomarkers for cholangiocarcinoma. *Expert Rev Mol Diagn* 2019;19(11):997-1005. doi: 10.1080/14737159.2019.1673162

33 Ignatiadis M, Sledge GW, Jeffrey SS. Liquid biopsy enters the clinic - implementation issues and future challenges. *Nat Rev Clin Oncol* 2021;18(5):297-312. doi: 10.1038/s41571-020-00457-x

34 Newman AM, Bratman SV, To J, et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. *Nat Med* 2014;20(5):548-54. doi: 10.1038/nm.3519

35 Lamarca A, Kapacee Z, Breeze M, et al. Molecular Profiling in Daily Clinical Practice: Practicalities in Advanced Cholangiocarcinoma and Other Biliary Tract Cancers. *J Clin Med* 2020;9(9) doi: 10.3390/jcm9092854

36 Andersen RF, Jakobsen A. Screening for circulating RAS/RAF mutations by multiplex digital PCR. *Clin Chim Acta* 2016;458:138-43. doi: 10.1016/j.cca.2016.05.007

37 Zill OA, Greene C, Sebisanovic D, et al. Cell-Free DNA Next-Generation Sequencing in Pancreatobiliary Carcinomas. *Cancer Discov* 2015;5(10):1040-8. doi: 10.1158/2159-8290.CD-15-0274

38 Ettrich TJ, Schwerdel D, Dolnik A, et al. Genotyping of circulating tumor DNA in cholangiocarcinoma reveals diagnostic and prognostic information. *Sci Rep* 2019;9(1):13261. doi: 10.1038/s41598-019-49860-0

39 Okamura R, Kurzrock R, Mallory RJ, et al. Comprehensive genomic landscape and precision therapeutic approach in biliary tract cancers. *Int J Cancer* 2021;148(3):702-12. doi: 10.1002/ijc.33230

40 Arechederra M, Rullan M, Amat I, et al. Next-generation sequencing of bile cell-free DNA for the early detection of patients with malignant biliary strictures. *Gut* 2021 doi: 10.1136/gutjnl-2021-325178

41 Loi E, Zavattari C, Tommasi A, et al. HOXD8 hypermethylation as a fully sensitive and specific biomarker for biliary tract cancer detectable in tissue and bile samples. *Br J Cancer* 2022 doi: 10.1038/s41416-022-01738-1

42 Vedeld HM, Grimsrud MM, Andresen K, et al. Early and accurate detection of cholangiocarcinoma in patients with primary sclerosing cholangitis by methylation markers in bile. *Hepatology* 2022;75(1):59-73. doi: 10.1002/hep.32125

43 Wasenang W, Chaiyarit P, Proungvitaya S, et al. Serum cell-free DNA methylation of OPCML and HOXD9 as a biomarker that may aid in differential diagnosis between cholangiocarcinoma and other biliary diseases. *Clin Epigenetics* 2019;11(1):39. doi: 10.1186/s13148-019-0634-0

44 Lapitz A, Arbelaiz A, O'Rourke CJ, et al. Patients with Cholangiocarcinoma Present Specific RNA Profiles in Serum and Urine Extracellular Vesicles Mirroring the Tumor Expression: Novel Liquid Biopsy Biomarkers for Disease Diagnosis. *Cells* 2020;9(3) doi: 10.3390/cells9030721

45 Laschos K, Lampropoulou DI, Aravantinos G, et al. Exosomal noncoding RNAs in cholangiocarcinoma: Laboratory noise or hope? *World J Gastrointest Surg* 2020;12(10):407-24. doi: 10.4240/wjgs.v12.i10.407

46 Limb C, Liu DSK, Veno MT, et al. The Role of Circular RNAs in Pancreatic Ductal Adenocarcinoma and Biliary-Tract Cancers. *Cancers (Basel)* 2020;12(11) doi: 10.3390/cancers12113250

47 Chabon JJ, Hamilton EG, Kurtz DM, et al. Integrating genomic features for non-invasive early lung cancer detection. *Nature* 2020;580(7802):245-51. doi: 10.1038/s41586-020-2140-0

48 Cristiano S, Leal A, Phallen J, et al. Genome-wide cell-free DNA fragmentation in patients with cancer. *Nature* 2019;570(7761):385-89. doi: 10.1038/s41586-019-1272-6

49 Nuzzo PV, Berchuck JE, Korthauer K, et al. Detection of renal cell carcinoma using plasma and urine cell-free DNA methylomes. *Nat Med* 2020;26(7):1041-43. doi: 10.1038/s41591-020-0933-1

50 Wan N, Weinberg D, Liu TY, et al. Machine learning enables detection of early-stage colorectal cancer by whole-genome sequencing of plasma cell-free DNA. *BMC Cancer* 2019;19(1):832. doi: 10.1186/s12885-019-6003-8

51 Akita M, Sofue K, Fujikura K, et al. Histological and molecular characterization of intrahepatic bile duct cancers suggests an expanded definition of perihilar cholangiocarcinoma. *HPB (Oxford)* 2019;21(2):226-34. doi: 10.1016/j.hpb.2018.07.021

52 Ma B, Meng H, Tian Y, et al. Distinct clinical and prognostic implication of IDH1/2 mutation and other most frequent mutations in large duct and small duct subtypes of intrahepatic cholangiocarcinoma. *BMC Cancer* 2020;20(1):318. doi: 10.1186/s12885-020-06804-6

53 Sigel CS, Drill E, Zhou Y, et al. Intrahepatic Cholangiocarcinomas Have Histologically and Immunophenotypically Distinct Small and Large Duct Patterns. *Am J Surg Pathol* 2018;42(10):1334-45. doi: 10.1097/PAS.0000000000001118

54 Kendall T, Verheij J, Gaudio E, et al. Anatomical, histomorphological and molecular classification of cholangiocarcinoma. *Liver Int* 2019;39 Suppl 1:7-18. doi: 10.1111/liv.14093

55 Rhee H, Ko JE, Chung T, et al. Transcriptomic and histopathological analysis of cholangiolocellular differentiation trait in intrahepatic cholangiocarcinoma. *Liver Int* 2018;38(1):113-24. doi: 10.1111/liv.13492

56 Takamura H, Gabata R, Obatake Y, et al. Clinical features and diagnostic imaging of cholangiolocellular carcinoma compared with other primary liver cancers: a surgical perspective. *Technol Cancer Res Treat* 2020;19:1533033820948141. doi: 10.1177/1533033820948141

57 Kusano H, Naito Y, Mihara Y, et al. Distinctive clinicopathological features and KRAS and IDH1/2 mutation status of cholangiolocellular carcinoma. *Hepatol Res* 2020;50(1):84-91. doi: 10.1111/hepr.13428

58 Kojima S, Hisaka T, Midorikawa R, et al. Prognostic Impact of Desmoplastic Reaction Evaluation for Intrahepatic Cholangiocarcinoma. *Anticancer Res* 2020;40(8):4749-54. doi: 10.21873/anticanres.14476

59 Kajiyama K, Maeda T, Takenaka K, et al. The significance of stromal desmoplasia in intrahepatic cholangiocarcinoma: a special reference of 'scirrhous-type' and 'nonscirrhous-type' growth. *Am J Surg Pathol* 1999;23(8):892-902. doi: 10.1097/00000478-199908000-00006

60 Jing CY, Fu YP, Huang JL, et al. Prognostic Nomogram Based on Histological Characteristics of Fibrotic Tumor Stroma in Patients Who Underwent Curative Resection for Intrahepatic Cholangiocarcinoma. *Oncologist* 2018;23(12):1482-93. doi: 10.1634/theoncologist.2017-0439

61 Guedj N, Blaise L, Cauchy F, et al. Prognostic value of desmoplastic stroma in intrahepatic cholangiocarcinoma. *Mod Pathol* 2021;34(2):408-16. doi: 10.1038/s41379-020-00656-y

62 Kasprzak A, Adamek A. Mucins: the Old, the New and the Promising Factors in Hepatobiliary Carcinogenesis. *Int J Mol Sci* 2019;20(6) doi: 10.3390/ijms20061288

63 Komuta M, Govaere O, Vandecaveye V, et al. Histological diversity in cholangiocellular carcinoma reflects the different cholangiocyte phenotypes. *Hepatology* 2012;55(6):1876-88. doi: 10.1002/hep.25595

64 Chi Z, Bhalla A, Saeed O, et al. Mucinous intrahepatic cholangiocarcinoma: a distinct variant. *Hum Pathol* 2018;78:131-37. doi: 10.1016/j.humpath.2018.04.010

65 Lu J, Li B, Li FY, et al. Prognostic significance of mucinous component in hilar cholangiocarcinoma after curative-intent resection. *J Surg Oncol* 2019;120(8):1341-49. doi: 10.1002/jso.25722

66 Zhang Z, Zhou Y, Hu K, et al. Perineural invasion as a prognostic factor for intrahepatic cholangiocarcinoma after curative resection and a potential indication for postoperative chemotherapy: a retrospective cohort study. *BMC Cancer* 2020;20(1):270. doi: 10.1186/s12885-020-06781-w

67 Wellner UF, Shen Y, Keck T, et al. The survival outcome and prognostic factors for distal cholangiocarcinoma following surgical resection: a meta-analysis for the 5-year survival. *Surg Today* 2017;47(3):271-79. doi: 10.1007/s00595-016-1362-0

68 Hu HJ, Jin YW, Shrestha A, et al. Predictive factors of early recurrence after R0 resection of hilar cholangiocarcinoma: A single institution experience in China. *Cancer Med* 2019;8(4):1567-75. doi: 10.1002/cam4.2052

69 Ruzzenente A, Bagante F, Olthof PB, et al. Surgery for Bismuth-Corlette Type 4 Perihilar Cholangiocarcinoma: Results from a Western Multicenter Collaborative Group. *Ann Surg Oncol* 2021;28(12):7719-29. doi: 10.1245/s10434-021-09905-z

70 Echle A, Rindtorff NT, Brinker TJ, et al. Deep learning in cancer pathology: a new generation of clinical biomarkers. *Br J Cancer* 2021;124(4):686-96. doi: 10.1038/s41416-020-01122-x

71 Kather JN, Heij LR, Grabsch HI, et al. Pan-cancer image-based detection of clinically actionable genetic alterations. *Nat Cancer* 2020;1(8):789-99. doi: 10.1038/s43018-020-0087-6

72 Kather JN, Calderaro J. Development of AI-based pathology biomarkers in gastrointestinal and liver cancer. *Nat Rev Gastroenterol Hepatol* 2020;17(10):591-92. doi: 10.1038/s41575-020-0343-3

73 Nam D, Chapiro J, Paradis V, et al. Artificial intelligence in liver diseases: Improving diagnostics, prognostics and response prediction. *JHEP Rep* 2022;4(4):100443. doi: 10.1016/j.jhepr.2022.100443

74 Liao H, Xiong T, Peng J, et al. Classification and Prognosis Prediction from Histopathological Images of Hepatocellular Carcinoma by a Fully Automated Pipeline Based on Machine Learning. *Ann Surg Oncol* 2020;27(7):2359-69. doi: 10.1245/s10434-019-08190-1

75 Saito A, Toyoda H, Kobayashi M, et al. Prediction of early recurrence of hepatocellular carcinoma after resection using digital pathology images assessed by machine learning. *Mod Pathol* 2021;34(2):417-25. doi: 10.1038/s41379-020-00671-z

76 Echle A, Grabsch HI, Quirke P, et al. Clinical-Grade Detection of Microsatellite Instability in Colorectal Tumors by Deep Learning. *Gastroenterology* 2020;159(4):1406-16 e11. doi: 10.1053/j.gastro.2020.06.021

77 Nakamura H, Arai Y, Totoki Y, et al. Genomic spectra of biliary tract cancer. *Nat Genet* 2015;47(9):1003-10. doi: 10.1038/ng.3375

78 Nepal C, O'Rourke CJ, Oliveira D, et al. Genomic perturbations reveal distinct regulatory networks in intrahepatic cholangiocarcinoma. *Hepatology* 2018;68(3):949-63. doi: 10.1002/hep.29764

79 Zhou SL, Xin HY, Sun RQ, et al. Association of KRAS Variant Subtypes With Survival and Recurrence in Patients With Surgically Treated Intrahepatic Cholangiocarcinoma. *JAMA Surg* 2022;157(1):59-65. doi: 10.1001/jamasurg.2021.5679

80 Sia D, Hoshida Y, Villanueva A, et al. Integrative molecular analysis of intrahepatic cholangiocarcinoma reveals 2 classes that have different outcomes. *Gastroenterology* 2013;144(4):829-40. doi: 10.1053/j.gastro.2013.01.001

81 Andersen JB, Spee B, Blechacz BR, et al. Genomic and genetic characterization of cholangiocarcinoma identifies therapeutic targets for tyrosine kinase inhibitors. *Gastroenterology* 2012;142(4):1021-31 e15. doi: 10.1053/j.gastro.2011.12.005

82 Ruys AT, Groot Koerkamp B, Wiggers JK, et al. Prognostic biomarkers in patients with resected cholangiocarcinoma: a systematic review and meta-analysis. *Ann Surg Oncol* 2014;21(2):487-500. doi: 10.1245/s10434-013-3286-x

83 Ghidini M, Cascione L, Carotenuto P, et al. Characterisation of the immune-related transcriptome in resected biliary tract cancers. *Eur J Cancer* 2017;86:158-65. doi: 10.1016/j.ejca.2017.09.005

84 Sarcognato S, Gringeri E, Fassan M, et al. Prognostic role of BAP-1 and PBRM-1 expression in intrahepatic cholangiocarcinoma. *Virchows Arch* 2019;474(1):29-37. doi: 10.1007/s00428-018-2478-y

85 Padthaisong S, Thanee M, Namwat N, et al. A panel of protein kinase high expression is associated with postoperative recurrence in cholangiocarcinoma. *BMC Cancer* 2020;20(1):154. doi: 10.1186/s12885-020-6655-4

86 Chusorn P, Namwat N, Loilome W, et al. Overexpression of microRNA-21 regulating PDCD4 during tumorigenesis of liver fluke-associated cholangiocarcinoma contributes to tumor growth and metastasis. *Tumour Biol* 2013;34(3):1579-88. doi: 10.1007/s13277-013-0688-0

87 Wang LJ, He CC, Sui X, et al. MiR-21 promotes intrahepatic cholangiocarcinoma proliferation and growth in vitro and in vivo by targeting PTPN14 and PTEN. *Oncotarget* 2015;6(8):5932-46. doi: 10.18632/oncotarget.3465

88 Sapisochin G, Ivanics T, Heimbach J. Liver Transplantation for Intrahepatic Cholangiocarcinoma: Ready for Prime Time? *Hepatology* 2022;75(2):455-72. doi: 10.1002/hep.32258

89 Twohig P, Peeraphatdit TB, Mukherjee S. Current status of liver transplantation for cholangiocarcinoma. *World J Gastrointest Surg* 2022;14(1):1-11. doi: 10.4240/wjgs.v14.i1.1

90 Byrling J, Kristl T, Hu D, et al. Mass spectrometry-based analysis of formalin-fixed, paraffin-embedded distal cholangiocarcinoma identifies stromal thrombospondin-2 as a potential prognostic marker. *J Transl Med* 2020;18(1):343. doi: 10.1186/s12967-020-02498-3

91 Lan C, Kitano Y, Yamashita YI, et al. Cancer-associated fibroblast senescence and its relation with tumour-infiltrating lymphocytes and PD-L1 expressions in intrahepatic cholangiocarcinoma. *Br J Cancer* 2022;126(2):219-27. doi: 10.1038/s41416-021-01569-6

92 Utispan K, Thuwajit P, Abiko Y, et al. Gene expression profiling of cholangiocarcinoma-derived fibroblast reveals alterations related to tumor progression and indicates periostin as a poor prognostic marker. *Mol Cancer* 2010;9:13. doi: 10.1186/1476-4598-9-13

93 Iguchi T, Yamashita N, Aishima S, et al. A comprehensive analysis of immunohistochemical studies in intrahepatic cholangiocarcinoma using the survival tree model. *Oncology* 2009;76(4):293-300. doi: 10.1159/000207506

94 Terashi T, Aishima S, Taguchi K, et al. Decreased expression of osteopontin is related to tumor aggressiveness and clinical outcome of intrahepatic cholangiocarcinoma. *Liver Int* 2004;24(1):38-45. doi: 10.1111/j.1478-3231.2004.00886.x

95 Sulpice L, Rayar M, Desille M, et al. Molecular profiling of stroma identifies osteopontin as an independent predictor of poor prognosis in intrahepatic cholangiocarcinoma. *Hepatology* 2013;58(6):1992-2000. doi: 10.1002/hep.26577

96 Zheng Y, Zhou C, Yu XX, et al. Osteopontin promotes metastasis of intrahepatic cholangiocarcinoma through recruiting MAPK1 and mediating Ser675 phosphorylation of beta-Catenin. *Cell Death Dis* 2018;9(2):179. doi: 10.1038/s41419-017-0226-x

97 Chuaysri C, Thuwajit P, Paupairoj A, et al. Alpha-smooth muscle actin-positive fibroblasts promote biliary cell proliferation and correlate with poor survival in cholangiocarcinoma. *Oncol Rep* 2009;21(4):957-69. doi: 10.3892/or_00000309

98 Zhang XF, Dong M, Pan YH, et al. Expression pattern of cancer-associated fibroblast and its clinical relevance in intrahepatic cholangiocarcinoma. *Hum Pathol* 2017;65:92-100. doi: 10.1016/j.humpath.2017.04.014

99 Hasita H, Komohara Y, Okabe H, et al. Significance of alternatively activated macrophages in patients with intrahepatic cholangiocarcinoma. *Cancer Sci* 2010;101(8):1913-9. doi: 10.1111/j.1349-7006.2010.01614.x

100 Miura T, Yoshizawa T, Hirai H, et al. Prognostic Impact of CD163+ Macrophages in Tumor Stroma and CD8+ T-Cells in Cancer Cell Nests in Invasive Extrahepatic Bile Duct Cancer. *Anticancer Res* 2017;37(1):183-90. doi: 10.21873/anticanres.11304

101 Subimerb C, Pinlaor S, Khuntikeo N, et al. Tissue invasive macrophage density is correlated with prognosis in cholangiocarcinoma. *Mol Med Rep* 2010;3(4):597-605. doi: 10.3892/mmr_00000303

102 Atanasov G, Dietel C, Feldbrugge L, et al. Tumor necrosis and infiltrating macrophages predict survival after curative resection for cholangiocarcinoma. *Oncoimmunology* 2017;6(8):e1331806. doi: 10.1080/2162402X.2017.1331806

103 Atanasov G, Hau HM, Dietel C, et al. Prognostic significance of macrophage invasion in hilar cholangiocarcinoma. *BMC Cancer* 2015;15:790. doi: 10.1186/s12885-015-1795-7

104 Sun D, Luo T, Dong P, et al. CD86(+)/CD206(+) tumor-associated macrophages predict prognosis of patients with intrahepatic cholangiocarcinoma. *PeerJ* 2020;8:e8458. doi: 10.7717/peerj.8458

105 Kasper HU, Drebber U, Stippel DL, et al. Liver tumor infiltrating lymphocytes: comparison of hepatocellular and cholangiolar carcinoma. *World J Gastroenterol* 2009;15(40):5053-7. doi: 10.3748/wjg.15.5053

106 Kitano Y, Okabe H, Yamashita YI, et al. Tumour-infiltrating inflammatory and immune cells in patients with extrahepatic cholangiocarcinoma. *Br J Cancer* 2018;118(2):171-80. doi: 10.1038/bjc.2017.401

107 Goeppert B, Frauenschuh L, Zucknick M, et al. Prognostic impact of tumour-infiltrating immune cells on biliary tract cancer. *Br J Cancer* 2013;109(10):2665-74. doi: 10.1038/bjc.2013.610

108 Takagi S, Miyagawa S, Ichikawa E, et al. Dendritic cells, T-cell infiltration, and Grp94 expression in cholangiocellular carcinoma. *Hum Pathol* 2004;35(7):881-6. doi: 10.1016/j.humpath.2004.03.016

109 Mao ZY, Zhu GQ, Xiong M, et al. Prognostic value of neutrophil distribution in cholangiocarcinoma. *World J Gastroenterol* 2015;21(16):4961-8. doi: 10.3748/wjg.v21.i16.4961

110 Lin G, Liu Y, Li S, et al. Elevated neutrophil-to-lymphocyte ratio is an independent poor prognostic factor in patients with intrahepatic cholangiocarcinoma. *Oncotarget* 2016;7(32):50963-71. doi: 10.18632/oncotarget.7680

111 Parra ER, Ferrufino-Schmidt MC, Tamegnon A, et al. Immuno-profiling and cellular spatial analysis using five immune oncology multiplex immunofluorescence panels for paraffin tumor tissue. *Sci Rep* 2021;11(1):8511. doi: 10.1038/s41598-021-88156-0

112 Taube JM, Roman K, Engle EL, et al. Multi-institutional TSA-amplified Multiplexed Immunofluorescence Reproducibility Evaluation (MITRE) Study. *J Immunother Cancer* 2021;9(7) doi: 10.1136/jitc-2020-002197

113 Huang YH, Zhang CZ, Huang QS, et al. Clinicopathologic features, tumor immune microenvironment and genomic landscape of Epstein-Barr virus-associated intrahepatic cholangiocarcinoma. *J Hepatol* 2021;74(4):838-49. doi: 10.1016/j.jhep.2020.10.037

114 Carpino G, Cardinale V, Di Giamberardino A, et al. Thrombospondin 1 and 2 along with PEDF inhibit angiogenesis and promote lymphangiogenesis in intrahepatic cholangiocarcinoma. *J Hepatol* 2021;75(6):1377-86. doi: 10.1016/j.jhep.2021.07.016

115 Nanashima A, Shibata K, Nakayama T, et al. Relationship between microvessel count and postoperative survival in patients with intrahepatic cholangiocarcinoma. *Ann Surg Oncol* 2009;16(8):2123-9. doi: 10.1245/s10434-009-0494-5

116 Yugawa K, Itoh S, Yoshizumi T, et al. Prognostic impact of tumor microvessels in intrahepatic cholangiocarcinoma: association with tumor-infiltrating lymphocytes. *Mod Pathol* 2021;34(4):798-807. doi: 10.1038/s41379-020-00702-9

117 Thelen A, Scholz A, Benckert C, et al. Microvessel density correlates with lymph node metastases and prognosis in hilar cholangiocarcinoma. *J Gastroenterol* 2008;43(12):959-66. doi: 10.1007/s00535-008-2255-9

118 Thelen A, Scholz A, Weichert W, et al. Tumor-associated angiogenesis and lymphangiogenesis correlate with progression of intrahepatic cholangiocarcinoma. *Am J Gastroenterol* 2010;105(5):1123-32. doi: 10.1038/ajg.2009.674

119 Yoon JG, Kim MH, Jang M, et al. Molecular Characterization of Biliary Tract Cancer Predicts Chemotherapy and Programmed Death 1/Programmed Death-Ligand 1 Blockade Responses. *Hepatology* 2021;74(4):1914-31. doi: 10.1002/hep.31862

120 Brandi G, Deserti M, Vasuri F, et al. Membrane Localization of Human Equilibrative Nucleoside Transporter 1 in Tumor Cells May Predict Response to Adjuvant Gemcitabine in Resected Cholangiocarcinoma Patients. *Oncologist* 2016;21(5):600-7. doi: 10.1634/theoncologist.2015-0356

121 Marin JJG, Lozano E, Briz O, et al. Molecular Bases of Chemoresistance in Cholangiocarcinoma. *Curr Drug Targets* 2017;18(8):889-900. doi: 10.2174/1389450116666150223121508

122 Marin JJG, Lozano E, Herraez E, et al. Chemoresistance and chemosensitization in cholangiocarcinoma. *Biochim Biophys Acta Mol Basis Dis* 2018;1864(4 Pt B):1444-53. doi: 10.1016/j.bbadis.2017.06.005

123 Marin JJG, Sanchon-Sanchez P, Cives-Losada C, et al. Novel Pharmacological Options in the Treatment of Cholangiocarcinoma: Mechanisms of Resistance. *Cancers (Basel)* 2021;13(10) doi: 10.3390/cancers13102358

124 Marin JJ, Macias RI. Understanding drug resistance mechanisms in cholangiocarcinoma: assisting the clinical development of investigational drugs. *Expert Opin Investig Drugs* 2021;30(7):675-79. doi: 10.1080/13543784.2021.1916912

125 Marin JJ, Briz O, Monte MJ, et al. Genetic variants in genes involved in mechanisms of chemoresistance to anticancer drugs. *Curr Cancer Drug Targets* 2012;12(4):402-38. doi: 10.2174/156800912800190875

126 Kim RD, Chung V, Alese OB, et al. A Phase 2 Multi-institutional Study of Nivolumab for Patients With Advanced Refractory Biliary Tract Cancer. *JAMA Oncol* 2020;6(6):888-94. doi: 10.1001/jamaoncol.2020.0930

127 Piha-Paul SA, Oh DY, Ueno M, et al. Efficacy and safety of pembrolizumab for the treatment of advanced biliary cancer: Results from the KEYNOTE-158 and KEYNOTE-028 studies. *Int J Cancer* 2020;147(8):2190-98. doi: 10.1002/ijc.33013

128 Lamarca A, Barriuso J, McNamara MG, et al. Molecular targeted therapies: Ready for "prime time" in biliary tract cancer. *J Hepatol* 2020;73(1):170-85. doi: 10.1016/j.jhep.2020.03.007

129 Valle JW, Lamarca A, Goyal L, et al. New Horizons for Precision Medicine in Biliary Tract Cancers. *Cancer Discov* 2017;7(9):943-62. doi: 10.1158/2159-8290.CD-17-0245

130 Lowery MA, Burris HA, 3rd, Janku F, et al. Safety and activity of ivosidenib in patients with IDH1-mutant advanced cholangiocarcinoma: a phase 1 study. *Lancet Gastroenterol Hepatol* 2019;4(9):711-20. doi: 10.1016/S2468-1253(19)30189-X

131 Abou-Alfa GK, Macarulla T, Javle MM, et al. Ivosidenib in IDH1-mutant, chemotherapy-refractory cholangiocarcinoma (ClarIDHy): a multicentre, randomised, double-blind, placebo-controlled, phase 3 study. *Lancet Oncol* 2020;21(6):796-807. doi: 10.1016/S1470-2045(20)30157-1

132 Friedlaender A, Subbiah V, Russo A, et al. EGFR and HER2 exon 20 insertions in solid tumours: from biology to treatment. *Nat Rev Clin Oncol* 2022;19(1):51-69. doi: 10.1038/s41571-021-00558-1

133 Mitri Z, Constantine T, O'Regan R. The HER2 Receptor in Breast Cancer: Pathophysiology, Clinical Use, and New Advances in Therapy. *Chemother Res Pract* 2012;2012:743193. doi: 10.1155/2012/743193

134 El-Khoueiry AB, Rankin C, Siegel AB, et al. S0941: a phase 2 SWOG study of sorafenib and erlotinib in patients with advanced gallbladder carcinoma or cholangiocarcinoma. *Br J Cancer* 2014;110(4):882-7. doi: 10.1038/bjc.2013.801

135 Peck J, Wei L, Zalupski M, et al. HER2/neu may not be an interesting target in biliary cancers: results of an early phase II study with lapatinib. *Oncology* 2012;82(3):175-9. doi: 10.1159/000336488

136 Ramanathan RK, Belani CP, Singh DA, et al. A phase II study of lapatinib in patients with advanced biliary tree and hepatocellular cancer. *Cancer Chemother Pharmacol* 2009;64(4):777-83. doi: 10.1007/s00280-009-0927-7

137 Javle M, Borad MJ, Azad NS, et al. Pertuzumab and trastuzumab for HER2-positive, metastatic biliary tract cancer (MyPathway): a multicentre, open-label, phase 2a, multiple basket study. *Lancet Oncol* 2021;22(9):1290-300. doi: 10.1016/S1470-2045(21)00336-3

138 Koo BK, van Es JH, van den Born M, et al. Porcupine inhibitor suppresses paracrine Wnt-driven growth of Rnf43;Znrf3-mutant neoplasia. *Proc Natl Acad Sci U S A* 2015;112(24):7548-50. doi: 10.1073/pnas.1508113112

139 Ross JS, Wang K, Gay L, et al. New routes to targeted therapy of intrahepatic cholangiocarcinomas revealed by next-generation sequencing. *Oncologist* 2014;19(3):235-42. doi: 10.1634/theoncologist.2013-0352

140 Doebele RC, Drilon A, Paz-Ares L, et al. Entrectinib in patients with advanced or metastatic NTRK fusion-positive solid tumours: integrated analysis of three phase 1-2 trials. *Lancet Oncol* 2020;21(2):271-82. doi: 10.1016/S1470-2045(19)30691-6

141 Drilon A, Laetsch TW, Kummar S, et al. Efficacy of Larotrectinib in TRK Fusion-Positive Cancers in Adults and Children. *N Engl J Med* 2018;378(8):731-39. doi: 10.1056/NEJMoa1714448

142 Javle M, Bekaii-Saab T, Jain A, et al. Biliary cancer: Utility of next-generation sequencing for clinical management. *Cancer* 2016;122(24):3838-47. doi: 10.1002/cncr.30254

143 Subbiah V, Lassen U, Elez E, et al. Dabrafenib plus trametinib in patients with BRAF(V600E)-mutated biliary tract cancer (ROAR): a phase 2, open-label, single-arm, multicentre basket trial. *Lancet Oncol* 2020;21(9):1234-43. doi: 10.1016/S1470-2045(20)30321-1

144 Kam AE, Masood A, Shroff RT. Current and emerging therapies for advanced biliary tract cancers. *Lancet Gastroenterol Hepatol* 2021;6(11):956-69. doi: 10.1016/S2468-1253(21)00171-0

145 Naing A, Meric-Bernstam F, Stephen B, et al. Phase 2 study of pembrolizumab in patients with advanced rare cancers. *J Immunother Cancer* 2020;8(1) doi: 10.1136/jitc-2019-000347

146 Le DT, Durham JN, Smith KN, et al. Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. *Science* 2017;357(6349):409-13. doi: 10.1126/science.aan6733

147 Demols A, Borbath I, Van den Eynde M, et al. Regorafenib after failure of gemcitabine and platinum-based chemotherapy for locally advanced/metastatic biliary tumors: REACHIN, a randomized, double-blind, phase II trial. *Ann Oncol* 2020;31(9):1169-77. doi: 10.1016/j.annonc.2020.05.018

148 Amato F, Rae C, Prete MG, et al. Cholangiocarcinoma Disease Modelling Through Patients Derived Organoids. *Cells* 2020;9(4) doi: 10.3390/cells9040832

149 Marsee A, Roos FJM, Verstegen MMA, et al. Building consensus on definition and nomenclature of hepatic, pancreatic, and biliary organoids. *Cell Stem Cell* 2021;28(5):816-32. doi: 10.1016/j.stem.2021.04.005

150 Yuan B, Zhao X, Wang X, et al. Patient-derived organoids for personalized gallbladder cancer modelling and drug screening. *Clin Transl Med* 2022;12(1):e678. doi: 10.1002/ctm2.678

151 Broutier L, Mastrogiovanni G, Verstegen MM, et al. Human primary liver cancer-derived organoid cultures for disease modeling and drug screening. *Nat Med* 2017;23(12):1424-35. doi: 10.1038/nm.4438

152 Saito Y, Muramatsu T, Kanai Y, et al. Establishment of Patient-Derived Organoids and Drug Screening for Biliary Tract Carcinoma. *Cell Rep* 2019;27(4):1265-76 e4. doi: 10.1016/j.celrep.2019.03.088

153 Li L, Knutsdottir H, Hui K, et al. Human primary liver cancer organoids reveal intratumor and interpatient drug response heterogeneity. *JCI Insight* 2019;4(2) doi: 10.1172/jci.insight.121490

154 Lampis A, Carotenuto P, Vlachogiannis G, et al. MIR21 Drives Resistance to Heat Shock Protein 90 Inhibition in Cholangiocarcinoma. *Gastroenterology* 2018;154(4):1066-79 e5. doi: 10.1053/j.gastro.2017.10.043

155 Vlachogiannis G, Hedayat S, Vatsiou A, et al. Patient-derived organoids model treatment response of metastatic gastrointestinal cancers. *Science* 2018;359(6378):920-26. doi: 10.1126/science.aao2774

156 van Tienderen GS, Li L, Broutier L, et al. Hepatobiliary tumor organoids for personalized medicine: a multicenter view on establishment, limitations, and future directions. *Cancer Cell* 2022 doi: 10.1016/j.ccell.2022.02.001

157 Dijkstra KK, Cattaneo CM, Weeber F, et al. Generation of Tumor-Reactive T Cells by Co-culture of Peripheral Blood Lymphocytes and Tumor Organoids. *Cell* 2018;174(6):1586-98 e12. doi: 10.1016/j.cell.2018.07.009

158 Holokai L, Chakrabarti J, Lundy J, et al. Murine- and Human-Derived Autologous Organoid/Immune Cell Co-Cultures as Pre-Clinical Models of Pancreatic Ductal Adenocarcinoma. *Cancers (Basel)* 2020;12(12) doi: 10.3390/cancers12123816

159 Forner A, Vidili G, Rengo M, et al. Clinical presentation, diagnosis and staging of cholangiocarcinoma. *Liver Int* 2019;39 Suppl 1:98-107. doi: 10.1111/liv.14086

160 Yang CM, Shu J. Cholangiocarcinoma Evaluation via Imaging and Artificial Intelligence. *Oncology* 2021;99(2):72-83. doi: 10.1159/000507449

161 Iavarone M, Piscaglia F, Vavassori S, et al. Contrast enhanced CT-scan to diagnose intrahepatic cholangiocarcinoma in patients with cirrhosis. *J Hepatol* 2013;58(6):1188-93. doi: 10.1016/j.jhep.2013.02.013

162 Rimola J, Forner A, Reig M, et al. Cholangiocarcinoma in cirrhosis: absence of contrast washout in delayed phases by magnetic resonance imaging avoids misdiagnosis of hepatocellular carcinoma. *Hepatology* 2009;50(3):791-8. doi: 10.1002/hep.23071

163 Saleh M, Virarkar M, Bura V, et al. Intrahepatic cholangiocarcinoma: pathogenesis, current staging, and radiological findings. *Abdom Radiol (NY)* 2020;45(11):3662-80. doi: 10.1007/s00261-020-02559-7

164 Kim NR, Lee JM, Kim SH, et al. Enhancement characteristics of cholangiocarcinomas on mutiphasic helical CT: emphasis on morphologic subtypes. *Clin Imaging* 2008;32(2):114-20. doi: 10.1016/j.clinimag.2007.08.022

165 Potretzke TA, Tan BR, Doyle MB, et al. Imaging Features of Biphenotypic Primary Liver Carcinoma (Hepatocholangiocarcinoma) and the Potential to Mimic Hepatocellular Carcinoma: LI-RADS Analysis of CT and MRI Features in 61 Cases. *AJR Am J Roentgenol* 2016;207(1):25-31. doi: 10.2214/AJR.15.14997

166 Brunt E, Aishima S, Clavien PA, et al. cHCC-CCA: Consensus terminology for primary liver carcinomas with both hepatocytic and cholangiocytic differentation. *Hepatology* 2018;68(1):113-26. doi: 10.1002/hep.29789

167 Lee J, Kim SH, Kang TW, et al. Mass-forming Intrahepatic Cholangiocarcinoma: Diffusion-weighted Imaging as a Preoperative Prognostic Marker. *Radiology* 2016;281(1):119-28. doi: 10.1148/radiol.2016151781

168 Lewis S, Besa C, Wagner M, et al. Prediction of the histopathologic findings of intrahepatic cholangiocarcinoma: qualitative and quantitative assessment of diffusion-weighted imaging. *Eur Radiol* 2018;28(5):2047-57. doi: 10.1007/s00330-017-5156-6

169 Cui X, Chen H, Cai S, et al. Correlation of apparent diffusion coefficient and intravoxel incoherent motion imaging parameters with Ki-67 expression in extrahepatic cholangiocarcinoma. *Magn Reson Imaging* 2019;63:80-84. doi: 10.1016/j.mri.2019.08.018

170 Min JH, Kim YK, Choi SY, et al. Intrahepatic Mass-forming Cholangiocarcinoma: Arterial Enhancement Patterns at MRI and Prognosis. *Radiology* 2019;290(3):691-99. doi: 10.1148/radiol.2018181485

171 Aherne EA, Pak LM, Goldman DA, et al. Intrahepatic cholangiocarcinoma: can imaging phenotypes predict survival and tumor genetics? *Abdom Radiol (NY)* 2018;43(10):2665-72. doi: 10.1007/s00261-018-1505-4

172 Jeong WK, Jamshidi N, Felker ER, et al. Radiomics and radiogenomics of primary liver cancers. *Clin Mol Hepatol* 2019;25(1):21-29. doi: 10.3350/cmh.2018.1007

173 Sadot E, Simpson AL, Do RK, et al. Cholangiocarcinoma: Correlation between Molecular Profiling and Imaging Phenotypes. *PLoS One* 2015;10(7):e0132953. doi: 10.1371/journal.pone.0132953

174 Zhang J, Wu Z, Zhao J, et al. Intrahepatic cholangiocarcinoma: MRI texture signature as predictive biomarkers of immunophenotyping and survival. *Eur Radiol* 2021;31(6):3661-72. doi: 10.1007/s00330-020-07524-y

175 Zhang J, Wu Z, Zhang X, et al. Machine learning: an approach to preoperatively predict PD-1/PD-L1 expression and outcome in intrahepatic cholangiocarcinoma using MRI biomarkers. *ESMO Open* 2020;5(6):e000910. doi: 10.1136/esmoopen-2020-000910

176 King MJ, Hectors S, Lee KM, et al. Outcomes assessment in intrahepatic cholangiocarcinoma using qualitative and quantitative imaging features. *Cancer Imaging* 2020;20(1):43. doi: 10.1186/s40644-020-00323-0

177 Yoshikawa D, Ojima H, Iwasaki M, et al. Clinicopathological and prognostic significance of EGFR, VEGF, and HER2 expression in cholangiocarcinoma. *Br J Cancer* 2008;98(2):418-25. doi: 10.1038/sj.bjc.6604129

178 Liu YF, Zhao R, Guo S, et al. Expression and clinical significance of hepatoma-derived growth factor as a prognostic factor in human hilar cholangiocarcinoma. *Ann Surg Oncol* 2011;18(3):872-9. doi: 10.1245/s10434-010-1303-x

179 Hui AM, Cui X, Makuuchi M, et al. Decreased p27(Kip1) expression and cyclin D1 overexpression, alone and in combination, influence recurrence and survival of patients with resectable extrahepatic bile duct carcinoma. *Hepatology* 1999;30(5):1167-73. doi: 10.1002/hep.510300506

180 Park BK, Paik YH, Park JY, et al. The clinicopathologic significance of the expression of vascular endothelial growth factor-C in intrahepatic cholangiocarcinoma. *Am J Clin Oncol* 2006;29(2):138-42. doi: 10.1097/01.coc.0000204402.29830.08

181 Wu WR, Shi XD, Zhang R, et al. Clinicopathological significance of aberrant Notch receptors in intrahepatic cholangiocarcinoma. *Int J Clin Exp Pathol* 2014;7(6):3272-9.

182 Sugiura K, Mishima T, Takano S, et al. The Expression of Yes-Associated Protein (YAP) Maintains Putative Cancer Stemness and Is Associated with Poor Prognosis in Intrahepatic Cholangiocarcinoma. *Am J Pathol* 2019;189(9):1863-77. doi: 10.1016/j.ajpath.2019.05.014

183 Lee D, Do IG, Choi K, et al. The expression of phospho-AKT1 and phospho-MTOR is associated with a favorable prognosis independent of PTEN expression in intrahepatic cholangiocarcinomas. *Mod Pathol* 2012;25(1):131-9. doi: 10.1038/modpathol.2011.133

184 Huang Q, Liu L, Liu CH, et al. Expression of Smad7 in cholangiocarcinoma: prognostic significance and implications for tumor metastasis. *Asian Pac J Cancer Prev* 2012;13(10):5161-5. doi: 10.7314/apjcp.2012.13.10.5161

185 Gu MJ, Jang BI. Clinicopathologic significance of Sox2, CD44 and CD44v6 expression in intrahepatic cholangiocarcinoma. *Pathol Oncol Res* 2014;20(3):655-60. doi: 10.1007/s12253-014-9745-2

186 Matsushima H, Kuroki T, Kitasato A, et al. Sox9 expression in carcinogenesis and its clinical significance in intrahepatic cholangiocarcinoma. *Dig Liver Dis* 2015;47(12):1067-75. doi: 10.1016/j.dld.2015.08.003

187 Huang XY, Ke AW, Shi GM, et al. Overexpression of CD151 as an adverse marker for intrahepatic cholangiocarcinoma patients. *Cancer* 2010;116(23):5440-51. doi: 10.1002/cncr.25485

188 Morine Y, Shimada M, Iwahashi S, et al. Role of histone deacetylase expression in intrahepatic cholangiocarcinoma. *Surgery* 2012;151(3):412-9. doi: 10.1016/j.surg.2011.07.038

189 Kim JY, Kim HJ, Park JH, et al. Epidermal growth factor upregulates Skp2/Cks1 and p27(kip1) in human extrahepatic cholangiocarcinoma cells. *World J Gastroenterol* 2014;20(3):755-73. doi: 10.3748/wjg.v20.i3.755

190 Liu R, Yang Z, Huang S, et al. The expressions of HMGA2 and Thy1 in extrahepatic cholangiocarcinoma and their clinicopathological significances. *Surg Oncol* 2019;29:41-47. doi: 10.1016/j.suronc.2019.01.013

191 Lee CT, Wu TT, Lohse CM, et al. High-mobility group AT-hook 2: an independent marker of poor prognosis in intrahepatic cholangiocarcinoma. *Hum Pathol* 2014;45(11):2334-40. doi: 10.1016/j.humpath.2014.04.026

192 Yang XW, Li L, Hou GJ, et al. STAT3 overexpression promotes metastasis in intrahepatic cholangiocarcinoma and correlates negatively with surgical outcome. *Oncotarget* 2017;8(5):7710-21. doi: 10.18632/oncotarget.13846

193 Zhang C, Bai DS, Huang XY, et al. Prognostic significance of Capn4 overexpression in intrahepatic cholangiocarcinoma. *PLoS One* 2013;8(1):e54619. doi: 10.1371/journal.pone.0054619

194 Chung JY, Hong SM, Choi BY, et al. The expression of phospho-AKT, phospho-mTOR, and PTEN in extrahepatic cholangiocarcinoma. *Clin Cancer Res* 2009;15(2):660-7. doi: 10.1158/1078-0432.CCR-08-1084

195 Ishii N, Araki K, Yokobori T, et al. Poor prognosis in cholangiocarcinoma patients with low FBXW7 expression is improved by chemotherapy. *Oncol Lett* 2017;13(5):3653-61. doi: 10.3892/ol.2017.5946

196 Dong LW, Hou YJ, Tan YX, et al. Prognostic significance of Beclin 1 in intrahepatic cholangiocellular carcinoma. *Autophagy* 2011;7(10):1222-9. doi: 10.4161/auto.7.10.16610

197 Wang TT, Cao QH, Chen MY, et al. Beclin 1 deficiency correlated with lymph node metastasis, predicts a distinct outcome in intrahepatic and extrahepatic cholangiocarcinoma. *PLoS One* 2013;8(11):e80317. doi: 10.1371/journal.pone.0080317

198 Merino-Azpitarte M, Lozano E, Perugorria MJ, et al. SOX17 regulates cholangiocyte differentiation and acts as a tumor suppressor in cholangiocarcinoma. *J Hepatol* 2017;67(1):72-83. doi: 10.1016/j.jhep.2017.02.017

199 Fan L, He F, Liu H, et al. CD133: a potential indicator for differentiation and prognosis of human cholangiocarcinoma. *BMC Cancer* 2011;11:320. doi: 10.1186/1471-2407-11-320

200 Morine Y, Imura S, Ikemoto T, et al. CD44 Expression Is a Prognostic Factor in Patients with Intrahepatic Cholangiocarcinoma After Surgical Resection. *Anticancer Res* 2017;37(10):5701-05. doi: 10.21873/anticanres.12007

201 Gardini A, Corti B, Fiorentino M, et al. Expression of connective tissue growth factor is a prognostic marker for patients with intrahepatic cholangiocarcinoma. *Dig Liver Dis* 2005;37(4):269-74. doi: 10.1016/j.dld.2004.11.001

202 Boonla C, Sripa B, Thuwajit P, et al. MUC1 and MUC5AC mucin expression in liver fluke-associated intrahepatic cholangiocarcinoma. *World J Gastroenterol* 2005;11(32):4939-46. doi: 10.3748/wjg.v11.i32.4939

203 Shibahara H, Tamada S, Higashi M, et al. MUC4 is a novel prognostic factor of intrahepatic cholangiocarcinoma-mass forming type. *Hepatology* 2004;39(1):220-9. doi: 10.1002/hep.20031

204 Higashi M, Yamada N, Yokoyama S, et al. Pathobiological implications of MUC16/CA125 expression in intrahepatic cholangiocarcinoma-mass forming type. *Pathobiology* 2012;79(2):101-6. doi: 10.1159/000335164

205 Mao X, Chen D, Wu J, et al. Differential expression of fascin, E-cadherin and vimentin: Proteins associated with survival of cholangiocarcinoma patients. *Am J Med Sci* 2013;346(4):261-8. doi: 10.1097/MAJ.0b013e3182707108

206 Aishima S, Asayama Y, Taguchi K, et al. The utility of keratin 903 as a new prognostic marker in mass-forming-type intrahepatic cholangiocarcinoma. *Mod Pathol* 2002;15(11):1181-90. doi: 10.1097/01.MP.0000032537.82380.69

207 Jiao X, Yu W, Qian J, et al. ADAM-17 is a poor prognostic indicator for patients with hilar cholangiocarcinoma and is regulated by FoxM1. *BMC Cancer* 2018;18(1):570. doi: 10.1186/s12885-018-4294-9

208 Ieta K, Tanaka F, Utsunomiya T, et al. CEACAM6 gene expression in intrahepatic cholangiocarcinoma. *Br J Cancer* 2006;95(4):532-40. doi: 10.1038/sj.bjc.6603276

209 Xu Y, Zhu M, Zhang S, et al. Expression and prognostic value of PRL-3 in human intrahepatic cholangiocarcinoma. *Pathol Oncol Res* 2010;16(2):169-75. doi: 10.1007/s12253-009-9200-y

210 Watanabe A, Suzuki H, Yokobori T, et al. Forkhead box protein C2 contributes to invasion and metastasis of extrahepatic cholangiocarcinoma, resulting in a poor prognosis. *Cancer Sci* 2013;104(11):1427-32. doi: 10.1111/cas.12249

211 Chen Y, Jiang P, Tian F, et al. Effects of Y Box Binding Protein-1 in Progression and Prognosis of Cholangiocarcinoma. *J Invest Surg* 2021;34(1):55-63. doi: 10.1080/08941939.2019.1604916

212 Shi RY, Yang XR, Shen QJ, et al. High expression of Dickkopf-related protein 1 is related to lymphatic metastasis and indicates poor prognosis in intrahepatic cholangiocarcinoma patients after surgery. *Cancer* 2013;119(5):993-1003. doi: 10.1002/cncr.27788

213 Wan P, Chi X, Du Q, et al. miR-383 promotes cholangiocarcinoma cell proliferation, migration, and invasion through targeting IRF1. *J Cell Biochem* 2018;119(12):9720-29. doi: 10.1002/jcb.27286

214 Lu X, Zhou C, Li R, et al. Long Noncoding RNA AFAP1-AS1 Promoted Tumor Growth and Invasion in Cholangiocarcinoma. *Cell Physiol Biochem* 2017;42(1):222-30. doi: 10.1159/000477319

215 Xu Y, Jiang X, Cui Y. Upregulated long noncoding RNA PANDAR predicts an unfavorable prognosis and promotes tumorigenesis in cholangiocarcinoma. *Onco Targets Ther* 2017;10:2873-83. doi: 10.2147/OTT.S137044

216 Oshikiri T, Miyamoto M, Shichinohe T, et al. Prognostic value of intratumoral CD8+ T lymphocyte in extrahepatic bile duct carcinoma as essential immune response. *J Surg Oncol* 2003;84(4):224-8. doi: 10.1002/jso.10321

**FIGURE LEGENDS.**

**Figure 1. Anatomical classification and histological features of cholangiocarcinoma.**

**A.** According to its anatomical location, cholangiocarcinoma (CCA) can be classified into intrahepatic (iCCA), perihilar (pCCA) and distal (dCCA). Haematoxylin and eosin (H&E) stain; scale bar: 200 μm. **B.** Different iCCA histological subtypes can be identified, including large bile duct type (which histologically resembles pCCA and dCCA) and small bile duct type. Cholangiolo-carcinoma (CLC) represents a peculiar variant of the small bile duct type iCCA. This classification is based on the anatomical organization of the intrahepatic biliary tree and recapitulates the level or size of the displayed bile duct. Immunohistochemistry for cytokeratin 7; scale bar: 100 μm. **C-D.** CCA histological heterogeneity comprises variable expression of mucins (periodic acid-Schiff – PAS stain, panel C) and fibrous stroma component (Sirius red – SR stain, panel D). **E.** Specific histological features have been associated with a dismal prognosis in CCA, such as large bile duct type and immature stroma in iCCA, and mucin production and perineural infiltration in all CCA subtypes. H&E and PAS stain (mucin).

**Figure 2. Liquid biopsy and tissue biomarkers.**

Liquid biopsy and conventional tissue biopsy both allow the study of specific biomarkers correlated with CCA diagnosis, prognosis and response to therapy. Abbreviations: ctDNA: circulating tumour DNA; dPCR: digital polymerase chain reaction; ERCP, endoscopic retrograde cholangiopancreatography; (F)ISH: (fluorescence) in situ hybridization; IF: immunofluorescence; IHC: immunohistochemistry; ncRNA: non-coding RNA; NGS: next-generation sequencing.

**Figure 3. Tissue and molecular biomarkers in cholangiocarcinoma.**

List of molecular biomarkers associated with cholangiocarcinoma (CCA) and their role in influencing overall survival (OS) and recurrence-free survival (RFS), categorized according to their functions. * long non-coding RNAs. Abbreviations: ADAM-17; A-disintegrin and metalloproteinase 17; AKT, protein kinase B; BRAF, B-type Raf proto-oncogene; CAPN4, calpain small subunit 1; CD, cluster of differentiation; CEACAM6, carcinoembryonic antigen-related cell adhesion molecule 6; c-MET, tyrosine-protein kinase Met or hepatocyte growth factor receptor; CTGF, connective tissue growth factor; CTLA4, cytotoxic T-lymphocyte antigen-4; DKK1, dickkopf-related protein 1; EGFR, epidermal growth factor receptor; FBXW7, F-box and WD repeat domain-containing 7; FOXC2, forkhead box protein C2; HDAC1, histone deacetylase 1; HDGF, hepatoma-derived growth factor; HMGA2, high-mobility group AT-hook 2; IHC, immunohistochemistry; KRAS, Kirsten ras oncogene homolog; KRT903; keratin 903; mTOR, mammalian target of rapamycin; MUC, mucin; NLR, neutrophil-to-lymphocyte ratio; PCR: polymerase chain reaction; PTEN, tensin homolog deleted on chromosome 10; PTP4A3, protein tyrosine phosphatase 4A3; PTPN14, protein tyrosine phosphatase non-receptor type 14; SKP2, S-phase kinase-associated protein 2; Sox, SRY-related HMG-box; STAT3; signal transducer and activator of transcription 3; TP53, tumour protein P53; VEGF-C, vascular endothelial growth factor C; WES, whole-exome sequencing; YAP, yes-associated protein; YBOX-1, Y Box Binding Protein 1.

**Figure 4. Patient-derived organoids for personalized approaches.**

The development of patient-derived organoids from tumour samples allows the building of a platform for disease modelling studies, and for drug screening analyses in a personalized medicine approach to patient care.

**Figure 5. Radiological features of cholangiocarcinoma.**

**A.** Mass forming intrahepatic cholangiocarcinoma (iCCA) arising from small bile ducts. The mass forming tumour can be assessed in T2 weighted images (i), diffusion-weighted images (ii), portal venous phase (iii), and hepatospecific phase (iv). The mass is limited to the left lobe and a typical capsular retraction can be observed (arrow) in all sequences. **B.** Periductal infiltrating perihilar cholangiocarcinoma (pCCA). The tumour (*) can be assessed in diffusion-weighted images (i), T2 weighted images (ii), cholangiopancreato magnetic resonance (CPMR) (iii) and portal venous phase computed tomography (CT) (iv). Dilated distal bile ducts (arrow) can be observed on both CT and magnetic resonance imaging (MRI).

**Figure 6. Clinical workflow diagrams for the diagnosis of cholangiocarcinoma.**

**A.** Workflow diagram for the final diagnosis of intrahepatic cholangiocarcinoma (iCCA) combining imaging modalities and tissue biomarkers when an intrahepatic mass is detected. **B**. Workflow diagram for the diagnosis of perihilar/distal cholangiocarcinoma (p/dCCA) combining imaging modalities and tissue biomarkers when a biliary stricture is detected. ERCP, endoscopic retrograde cholangiopancreatography; EUS, endoscopic ultrasound guided fine needle aspiration (FNA)/biopsy (FNB), FISH: fluorescence in situ hybridization; IHC: immunohistochemistry; MDCT, multidetector computed tomography; MRCP, magnetic resonance cholangiopancreatography; MRI, magnetic resonance imaging; US, ultrasonography.