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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 11

(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.

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 yielded 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 21

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

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

Abbreviations: BTC, biliary tract cancer; CCA, cholangiocarcinoma; CLC, cholangiolocarcinoma; 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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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. Cholangiolocarcinoma (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), cholangiopancreatographic 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.