Unmet needs in basic and translational research in Cholangiocarcinoma

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Abstract

Background and Aims: Despite the impact of cutting-edge technologies in providing deep molecular phenotyping of many tumours, management of cholangiocarcinoma (CCA), a rare and insufficiently studied cancer with marked heterogeneity (including intrahepatic and extrahepatic variants), has remained limited and it has poor prognosis. Renewed interest in this enigmatic disease has been fostered in the last decade. Here, we will give an overview of the most important gaps in knowledge of the basic and translational research of CCA that must be prioritized to improve the CCA management in the future.

Methods: Exploration was initially conducted on the Consensus Statement on Cholangiocarcinoma 2020, where after careful discussion of priorities, basic/translational research were selected and approved. Then, systematic literature searches using PubMed were performed by authors from different disciplines to highlight the most relevant areas requiring further study.

Results: Genetic and molecular characterization studies have identified the presence of actionable mutations in both intrahepatic (50%-60%) and extrahepatic CCA (25%), but efficacy of targeted interventions is still hindered by several factors. They include a limited understanding of the intricate mechanisms of pharmacoresistance, the lack of accurate biomarkers enabling early detection of the disease, the complex role of the tumour microenvironment exerting both tumour-promoting and tumour-restraining functions, and the inadequacy of experimental models to recapitulate the wide heterogeneity of these tumours.

Conclusions: Promoting international collaborations among scientists with multidisciplinary skills, and creation of platforms to collect data and biological samples of
1 | INTRODUCTION

Cholangiocarcinoma (CCA) represents one of the biggest challenges that the scientific community, at multiple levels, beyond hepatologists and oncologists, is currently coping with. Indeed, because of its major aggressiveness and usual late diagnosis at an advanced stage, since the new millennium improvement in clinical management of CCA has been extremely limited, resulting in a 5-year survival that has remained dismal (7%-20%), reflecting a long and unfair neglected topic (Figure 1). Although in the last few years there has been some renewed interest in this enigmatic disease and its several controversial facets, CCA is still a sort of ‘child of a lesser God’ if compared with the ‘giants’ of the oncology research, breast and lung cancer above all, and even the major type of liver cancer, that is, hepatocellular carcinoma (HCC). Besides being epidemiologically rare, at least in Western countries, CCA is also an understudied cancer, with less than 10% of published works compared to breast and lung cancer, and about 17% as compared to the ‘closest relative’ HCC (Table 1). Moreover, the main issue making investigational approaches to CCA even more difficult and likely reluctant has been the high degree of heterogeneity of this cancer at both intertumoral and intratumoral levels. CCA encompasses different clinical entities, which reflect the variable anatomical site of origin within the biliary tree (intrahepatic, peri-hilar and distal CCA, the latter two considered as extrahepatic) and indeed, well-established classification criteria have been applied in the study design only recently. Heterogeneity is further compounded at the cellular level by a multifaceted microenvironment, containing a variety of stromal and immune cells, whose composition has only recently begun to be described. Against this background, in this review we will give an overview of the most important gaps in knowledge of the basic and translational research of CCA that must not be delayed further. The aim is to provide the reader with a series of research priorities that, from our point of view, should be raised to improve the CCA management in the next decade.

2 | METHODS

To perform this study, the initial search had been based on the Consensus Statement on Cholangiocarcinoma 2020 to which the authors herein involved contributed (RIRM, MS, JJGM, LF). In this consensus paper, priorities of either basic/translational or clinical research were proposed, selected and approved after careful discussion among experts in different fields. Starting from these outlines, a PubMed search was conducted by combining the term ‘cholangiocarcinoma’ with the following key words: ‘genetics’, ‘genome-wide association study (GWAS)’, ‘molecular classification’, ‘chemoresistance/pharmaco-resistance’, ‘biomarkers’, ‘microenvironment’, ‘cancer-associated fibroblasts’, ‘cancer stem cells’, ‘tumour-infiltrating lymphocytes’, ‘tumour-associated neutrophils’, ‘tumour-associated lymphangiogenesis’, ‘extracellular vesicles’, ‘miRNAs’, ‘circRNAs’, ‘ncRNAs’ and ‘in-vitro and in-vivo models’. No specific search dates were used. Sections were assigned according to the expertise of each author, and once pooled the whole document was thoroughly discussed and extensively revised by all co-authors.

3 | GENETICS, MOLECULAR PROFILING AND CLASSIFICATION

In recent years, the application of innovative technologies, such as next-generation sequencing (NGS) or single cell analysis, to the study of CCA has allowed an extensive profiling of the genetic and molecular alterations of this neoplasm. Thanks to these approaches, it has become clear that CCA is an extremely diverse cancer that besides different histopathological variants, includes a wide number of mutations with related signalling perturbations that underpin its pathophysiology. This in-depth analysis has led to the classification...
of the different forms of CCA based on specific genetic and transcriptomic features.

The first attempt to unravel the genetic landscape of CCA was performed with the intrahepatic form (iCCA). A seminal study of Sia et al. analyzed the genetic alterations of a large series of 149 ICCAs identifying two main subclasses, inflammatory and proliferative. The inflammatory type was characterized by the overexpression of cytokines and chemokines, such as interleukin (IL)-3, -4, -6, -10, -17a and C-C Motif Chemokine Ligand (CCL)-19, while the proliferative type was characterized by a higher prevalence of Epidermal Growth Factor Receptor (EGFR), Insulin-like Growth Factor Receptor 1 (IGFRI), MET and Kirsten rat sarcoma virus (KRAS) mutations, several copy number variations and deletion of Salvador Family WW Domain Containing Protein 1 (SAV1). Clinically, the proliferative class showed a poorer survival rate and greater tendency to recurrence compared with the inflammatory class. Other studies performed in different iCCA cohorts gave substantially confirmatory results.10,11 Some years later, the molecular landscape of the extrahepatic CCA (eCCA) was similarly dissected,12 appearing much more complex than iCCA, with the description of four subclasses: proliferation, mesenchymal, metabolic and immune, each harbouring specific genetic and signaling alterations (Figure 2). Among these subclasses, the mesenchymal type was the most aggressive. According to OncoKB targets, only 25% of eCCAs were characterized by the presence of actionable targets and less than half with respect to iCCA (50%-60%), highlighting the concept that eCCA is an even less attractive disease for targetable genetic alterations (Figure 2). Among these subclasses, the mesenchymal type was the most aggressive. According to OncoKB targets,13 only 25% of eCCAs were characterized by the presence of actionable targets and less than half with respect to iCCA (50%-60%), highlighting the concept that eCCA is an even less attractive disease for targeted therapies.12,14 In particular, potentially targetable genetic alterations, such as Fibroblast Growth Factor Receptor (FGFR) aberrations (fusions/mutations/amplifications) and Isocitrate dehydrogenase 1 (IDH) mutant-enriched subtypes, while quite common in iCCA (20% for FGFR, 15% for IDH),15 are instead rare in eCCA (1% and 4.7% respectively).12 Based on these observations, only iCCAs are currently included in clinical trials assessing efficacy of FGFR and IDH inhibitors.16 Among genetic mutations, KRAS shows a quite relevant proportion in both iCCA ([20%-54%]17 and eCCA (36.7%))12, but unfortunately targeting this pathway is troublesome, since no approved drugs capable of targeting mutated KRAS proteins directly are available. Additionally targeting KRAS indirectly at the level of its downstream effectors is unsuccessful as shown in pancreatic ductal adenocarcinoma (PDAC), a similarly aggressive malignancy frequently demonstrating activation of KRAS.18,19

The analysis of a cohort of mixed HCC-CCA20 revealed the presence of a peculiar subtype, named cholangiolocellular carcinoma (CLC), deriving from cholangiocytes rather than hepatocyte transdifferentiation, with genetic signatures which differed from the classic mixed HCC-CCA. Furthermore, in mixed HCC-CCA, the most aggressive forms expressed stemness traits consistent with a tumour initiating cell phenotype. Unfortunately, to date, information on mixed HCC-CCA are rather limited because of the rarity of this tumour. Thus, ad hoc studies of this tumour conducted by international consortia through comprehensive transcriptomic analysis performed by targeted DNA-sequencing and whole-genome profiling would be strongly encouraged. They could lead to the identification of specific single nucleotide polymorphisms (SNPs) possibly linked to the early development of CCA that may reveal patients at greater risk of CLC.21

Altogether, studies devoted to genetic and molecular characterization are technically sound and highly refined, but the deliverables are still preliminary since they lack a consensus and leave open several discrepancies between the histopathological and the molecular classifications, which need to be analysed and standardized. Moreover, given the low prevalence of actionable driving pathways, in particular in eCCA, further efforts aimed at dissecting the intimate mechanisms of tumour invasiveness, from epigenetic modifications, to 3D chromatin conformation, post-translational modifications (SUMOylation, NEDDylation), and secretome and transcriptomic alterations, will be necessary to improve the success of therapies. Another aspect of molecular profiling with therapeutic potential is the assessment of mismatch repair (MMR) deficiency and microsatellite instability (MSI) as well as programmed death-ligand 1 (PD-L1) expression, which support the use of immunotherapy. Unfortunately, these features can be found in only 8% of iCCA22 and 2% of eCCA,12 thereby making this treatment option unlikely to be useful for CCA.

Although CCA is classically recognized as a rare cancer, there are considerable geographical variations in age-standardized incidence. Indeed, CCA is not rare in the South-East Asia (China, South Korea and Thailand), where the incidence is greater than six cases per 100,000 people.21 Moreover, CCA mortality is higher in men than in women worldwide.5 Thus, it is tempting to speculate on the one hand, that beyond local environmental factors, genetic predisposition is different across ethnic groups, and on the other, that gender-related differences may sustain diverse aggressive tumour phenotypes. Today, data on the inherited factors involved in CCA pathogenesis are very limited. 'Omic' studies aimed at addressing these differences should help to bridge this gap.

Overall, these refined molecular classifications, although methodologically sound and relevant as role models for experimental protocols applied to the study of heterogeneous cancers,
still have limited translational value, in terms of generation of new diagnostic tools to enable early tumour detection, and better allocation of patients to appropriate tailored therapies. Although several clinical trials adopting criteria of personalized therapy in CCA are ongoing, a real impact on its poor prognosis remains a long way off.34,15,23,24

4 | MECHANISMS OF PHARMACORESISTENCE

Although pharmacotherapy is crucial in the management of advanced CCA, the success of this palliative treatment is hampered by the development of drug resistance in cancer cells. Moreover, even a complete clinical and radiological response after surgery does not mean that all tumour cells have been eradicated. Accordingly, administration of adjuvant chemotherapy is a frequent practice after curative treatments, above all when the null-resection margin cannot be obtained.

Multiple mechanisms of pharmacoresistance (MPRs) permit CCA cells to overcome the challenge of antitumour agents. Pharmacoresistance can be primary (intrinsic) or secondary (acquired) triggered by the exposure to drugs. Unfortunately, MPRs usually induce resistance to multiple drugs (for a complete recent review, see25). MPRs are based on the existence of a dynamic resistome,26,27 consisting of more than one hundred genes classified into seven groups (MRP-1/7). Those included in MRP-1 are involved in lowering the intracellular concentrations of active drugs by hindering their uptake or increasing their release, which in CCA markedly determines the efficacy of gemcitabine, 5′-FU, and platinum-derived drugs. Their uptake through equilibrative nucleoside transporter 1 (ENT1) and copper transporter 1 (CTR1) is usually hampered in chemoresistant CAs.8,28 Changes in the expression and function of other plasma membrane transport proteins, such as organic cation transporters (OCTs), affect the response of CCA to cationic drugs, such as several tyrosine kinase inhibitors (TKIs).30 On the other hand, the high expression of ABC proteins, mainly MRP1,31 and MRP3,32 also plays an important role in the sensitivity of CCA to pharmacotherapy. Besides, the proportion of active drugs can be lowered in resistant CCA (MPR-2).33

Other genes of the resistome are related to altered interaction with molecular targets (MPR-3). For instance, the response of CCA to pemigatinib has been associated with the presence of mutations in FGFR2.34 Moreover, IDH inhibitors can lose their efficacy in fighting CCA if mutations altering the IDH1 or IDH2 sequence are found in these tumours.35 To overcome this problem, several drugs, such as ivosidenib, are being developed.36 The efficacy of DNA-damaging drugs, such as cisplatin and oxaliplatin, depends on DNA repair machinery (MPR-4), which can be enhanced by up-regulation of the genes involved, such as P53R2 and KPNA2.37 A considerable part of the resistome is associated with genes included in MPR-5, which alter death-related signalling pathways favouring survival over apoptosis. An example is the up-regulation of the anti-apoptotic protein Bcl-2 and down-regulation of the pro-apoptotic Bax, which occurs in gemcitabine-resistant CCA cells.38

Recently, extracellular mechanisms that involve crosstalk between tumour cells and environmental factors affecting drug response (MPR-6), and those that favour phenotypic transitions, such as epithelial-to-mesenchymal or development of stemness characteristics, have been recognized as critical elements of CCA resistome (MPR-7). These are promoted by HMGA1, highly expressed in resistant CCA.39 Regarding modern immunotherapy, the appearance of epigenetic alterations in CCA constitutes one important mechanism contributing to drug resistance by helping tumour cells to escape host immune surveillance, which has prompted the development of novel drugs that target DNA methylation or histone modifications.40

Further research efforts should be made into treatments that are currently showing promising results in clinical trials, especially on targeted therapies and immunotherapies.41,42 Targeted therapies aimed at MPRs can be ‘on-target’ when the resistance occurs because of mutations in the primary molecular target, which results in poor or no response to the specific drug or it may be ‘off-target’ when the resistance occurs through activation of signalling pathways parallel to that in which the target of interest is involved. Furthermore, since most molecular targets are localized inside tumour cells, insufficient intracellular drugs levels because of MPR-1 and MPR-2 markedly affect the response to these drugs.

An in-depth understanding of the molecular basis of MPRs to each drug is required for the advance of pharmacological treatment of CCA (Figure 3) by: (i) predicting the response to available drugs and identifying tumour weaknesses to select the best options for each CCA patient as the disease evolves25; (ii) developing more effective drugs; (iii) designing optimized combined regimens of chemotherapeutic and targeted drugs; and (iv) developing sensitizing strategies. Several approaches designed to enhance the response of CCA to pharmacotherapy with the aim of increasing drug intracellular concentration and enhancing efficiency have been developed recently in HCC45 and CCA (unpublished). These include the encapsulation of anticancer agents into nanoparticles to improve the entry into CCA cells by endocytosis,43 the binding of drugs to bile acids to promote their entry by transport proteins present in CCA cells44 and the coadministration of molecules able to inhibit drug export pumps.

Gene therapy has also been proposed as a strategy to overcome drug resistance: for instance, using vectors to induce the expression of a drug transporter or a tumour suppressor protein under the control of cancer-specific promoters upregulated in CCA cells, such as Baculoviral IAP Repeat Containing 5 (BIRC5), Telomerase Reverse Transcriptase (TERT), Cytokeratin 19 (CK19) or Cyclooxygenase-2 (COX-2).46,47

5 | BIOMARKERS

Given the difficulty in obtaining a quality biopsy to confirm the diagnosis of CCA, in many cases, the availability of minimally invasive
markers is an important unmet need in the management of these patients. Currently, the only serum biomarker recommended for routine clinical practice in CCA is carbohydrate antigen 19-9 (CA19-9). Although for the diagnosis of CCA its sensitivity and specificity are far from ideal, especially in the early stages, the determination of this glycoprotein in serum is helpful in the follow-up after surgery to detect tumour recurrence and evaluate the response during pharmacological and radiological treatments. During the last few years, alternative serum biomarkers have been actively sought, especially using ‘omics’ technologies, combinations of proteins and metabolites, and RNA profiles determined in extracellular vesicles (EVs). Despite the fact that some combinations have shown potential usefulness in CCA diagnosis, at present, none of these biomarkers has reached the clinical setting although, some validation studies are underway.

Recent advances have identified genomic alterations characteristic of CCA associated with the anatomical origin of the tumour. Liquid biopsy to evaluate circulating tumour cells (CTCs) and circulating cell-free tumour DNA (ctDNA) released by primary or metastatic sites is an alternative diagnostic approach to overcome the limitation in obtaining biopsies to evaluate tumour progression. Although monitoring CTCs is showing promising results for other types of cancers, such as HCC, and breast and colorectal cancer, the findings in CCA are still preliminary. It has been suggested that the presence of viable CTCs in portal blood after resection and their interaction with immune cells and myeloid fibroblasts (cell elements actively engaged in the tumour microenvironment), may be responsible for the metastatic spread of CCA. In addition, the presence of CTCs has been shown to correlate with tumour extent and reduced overall survival in CCA patients. Despite the marked inter-tumoural and intra-tumoural genetic heterogeneity, several studies have described a correlation between genetic and epigenetic alterations in tissue-based tumour DNA and ctDNA, supporting the usefulness of the latter in the diagnosis of CCA. It has been demonstrated that using NGS, ctDNA analysis is feasible and accurate, although its sensitivity may be low in early-stage CCA tumours. Interestingly, more significant amounts of ctDNA have been associated with advanced disease and worse prognosis. The ability to repeat blood analysis more frequently than other traditional techniques (biopsy, imaging) can make it easier to follow the evolution of this malignancy. In addition, this approach

### Table 1: Incidence and recent published literature of cholangiocarcinoma with respect to the main epithelial cancers

| Cancer       | Incidence (x 100,000) | Articles in 2020 |
|--------------|-----------------------|------------------|
| Breast       | 30-100                | 20,504           |
| Lung         | 30-50                 | 25,942           |
| Colorectal   | 14-40                 | 17,315           |
| HCC          | 4-20                  | 10,128           |
| PDAC         | 8-15                  | 9,250            |
| CCA          | 0.5-10                | 1,701            |

Note: CCA, cholangiocarcinoma; PDAC, pancreatic ductal adenocarcinoma; HCC, hepatocellular carcinoma.

Source: Globocan and PubMed databases.

### Figure 2: Graph representation of the molecular subclasses in intrahepatic (iCCA) and extrahepatic cholangiocarcinoma (eCCA) as defined by comprehensive multi-platform molecular profiling studies. In iCCA, two main subclasses, proliferation and inflammation, have been identified, whereas in eCCA, the proliferation class is accompanied by metabolic, mensenchymal and immune classes. For each subclass, the most relevant molecular signatures along with the relative proportion are indicated. Data for iCCA and eCCA are reported according to and  respectively.
and lymphatic spreading. However, this observation stands at odds with enhanced epithelial- to-mesenchymal transition (EMT), in PDAC, whereby CAF depletion yielded undifferentiated tumours, in stark conflict to that reported in other tumour contexts, that is, increased tumour cell proliferation and more invasive phenotype.

10 may also influence tumour cell behaviour. Since TME is crucial in populating the TME are sorely needed to better appreciate their role.

FIGURE 3 Schematic representation of the three essential points in the current pharmacological research of cholangiocarcinoma pharmacoresistance, that is, (i) understanding mechanisms of pharmacoresistance (MPR), which have been classified into seven groups (MPR-1/7); (ii) characterization, if possible, by non-invasive techniques, of the resistome as the set of genes whose expression is dynamically changed during cancer development and therapy; and (iii) development of new drugs and combinations, as well as novel strategies to sensitize tumour cells and improve the response of cholangiocarcinoma to available pharmacotherapy.

6 | TUMOUR MICROENVIRONMENT (TME)

As already mentioned, regardless of the anatomical subtype, a defining feature of CCA is the development of an exuberant desmoplastic reaction. Besides stromal cells (fibroblasts, lymphatic and vascular endothelial cells), this includes a vast assortment of immune cells (macrophages, neutrophils, lymphocytes, NK cells, myeloid-derived suppressor cells), whose phenotyping and functional characterization are still largely incomplete. Stromal and immune cells lie embedded in a stiff and dense extracellular matrix (ECM) in close contact with the neoplastic bile ducts, which as with the cellular counterpart may also influence tumour cell behaviour. Since TME is crucial in regulating the invasive functions of the tumour ducts, in both a promoting and restraining fashion, a more detailed understanding of the complex interplay between tumour and stroma/immune milieu is a prerequisite to uncover novel and effective therapeutic targets.

Targeting cancer-associated fibroblasts (CAF) by pro-apoptotic agents (le navitoclax) is paradigmatic, and in rodent models of CCA it has significant anti-tumour effects, with impaired growth, invasiveness and lymphatic spreading. However, this observation stands in stark conflict to that reported in other tumour contexts, that is, PDAC, whereby CAF depletion yielded undifferentiated tumours, with enhanced epithelial-to-mesenchymal transition (EMT), increased tumour cell proliferation and more invasive phenotype. Dual effects of targeting CAF may depend upon a pronounced CAF heterogeneity, which in iCCA has been recently analysed at single cell resolution, with identification of five CAF subtypes with distinctive functions. These include vascular, matrix, inflammatory, antigen-presenting, and EMT-like, each variably affecting the tumour phenotype. In CCA, mechanisms underlying CAF heterogeneity are uncharted, and their elucidation may clarify the therapeutic significance of CAF targeting. Among them, the cellular origin of CAFs is controversial. It may involve several different cell types, and this implies a range of activation states that variably affect cancer development. In turn, paracrine signals released by cancer cells may induce phenotypic differences in CAF subsets, possibly by re-programming their epigenome. In particular, CAFs may interact with a population of cancer stem cells (CSCs), that is, tumour cells displaying stem cell-like features, which sustain tumour growth, invasion, metastasis, pharmacoresistance, and recurrence. From this point of view, characterization of a subset of CAF localized in the tumour niche with more specific functions supporting tumour initiation and progression, by regulating CSC-like properties, is an issue of utmost translational value in iCCA. In addition to soluble factors, including cytokines, chemokines, and growth factors, CAFs and tumour cells are metabolically coupled by exchanging non-coding RNAs (specifically miRNAs, circular RNAs, and long non-coding RNAs), reactive oxygen species (ROS) and energy-rich metabolites, which are mainly mediated by EVs. This is a field of research awaiting discovery with interesting perspectives and therapeutic implications. Indeed, selective cargo delivery via EVs provides an attractive tool to potentially induce more effective responses in the target cells with fewer side effects or harmful reactions in other cells. However, the underlying methodology needs to be refined before its applicability and efficacy to be confirmed. Furthermore, given the multicellular composition of the TME, other ways of communicating are likely to operate and in particular, those that engage immune cells have been largely ignored and may provide hints for therapeutic intervention. In this scenario, tumour-associated macrophages (TAM) displaying M2 features are classically recognized as tumour-supportive players, but like CAF, they encompass a highly heterogeneous population with a range of phenotypes endowed with a wide tumour-related functionality (including angiogenesis, ECM remodelling and T cell inhibition) that cannot be reduced to a simple M1 and M2 polarization.

Studies of phenotype-function correlation, as well as deciphering TAM interplay with CAF, vascular cells and other immune cells populating the TME are sorely needed to better appreciate their
suitability to and therapeutic effects of therapeutic manipulation. Compared with other epithelial cancers, immunotherapy appears to be less effective in CCA,\textsuperscript{99,100} consistent with a still limited understanding of the role and contribution of tumour-infiltrating lymphocytes (TIL). Using laser capture microdissection, four immune subtypes have been identified in the TME of CCA.\textsuperscript{91} Interestingly, TIL subtypes harboured different gene expression profiles in inflammatory and immune checkpoint pathways, thereby supporting the notion that effects of immunotherapy such as PD-1 antibodies may depend on the quantity and quality of T-lymphocyte infiltration in CCA.\textsuperscript{91} On the other hand, an increase in NK cell numbers or functions has been proposed as a promising approach for the treatment of CCA.\textsuperscript{92} A protective role has been attributed to CD20\textsuperscript{+} B cells, based on the observation that elevated population of B cells have been found in the lymphoepithelioma-like CCA, a rare type of iCCA associated with Epstein-Barr virus infection characterized by a more favourable prognosis.\textsuperscript{93} Myeloid-derived suppressor cells (MDSC) are a heterogeneous group of immune cells from the myeloid lineage with remarkable ability to suppress T-cell responses,\textsuperscript{94} which in CCA are recruited and induced by CAF to promote stenosis features in the tumour niche and thus pharmacoresistance.\textsuperscript{95,96} Thus, strategies to deplete MDSC may open interesting perspectives of combined therapy in iCCA to improve responses to chemotherapy.\textsuperscript{94} A morphologic hallmark of CCA TME is the rich lymphatic bed, which escorts growth and expansion of tumour bile ducts upon fine regulation by CAF.\textsuperscript{72} Of note, lymphatic rather than blood vessels represent the preferential route of early dissemination of CCA, in keeping with the observation that anti-angiogenic therapies have been so far largely unsuccessful in the treatment of CCA.\textsuperscript{97} Surprisingly, mechanisms underlying tumour-associated lymphangiogenesis have been overlooked in CCA research, but this subject is of paramount importance in the development of new strategies of therapeutic interference with lymphatic metastasisization.\textsuperscript{98} Finally, another theme so far scarcely investigated but with translational significance is the role of ECM proteins, not only fibronectin and laminin (normal constituents), but also osteopontin, tenascin and periostin (pathological components), in regulating the behaviour of cells present in the TME, beyond tumoural cholangiocytes.\textsuperscript{98-100} In fact, changes in quantity or quality of ECM proteins behaving as mechanoreceptors or influencing tissue stiffness can modulate tumour aggressiveness and metastasization acting on protein complexes and transcription factors.\textsuperscript{70}

Thus, dissecting further the composite TME landscape by accurate spatial analysis and phenotypic signature discovery is a promising avenue to explore for CCA with relevant implications. On the one hand, the potential of emerging and conventional treatments, such as immunotherapy, anti-angiogenesis and chemotherapy, would be better harnessed, and on the other, novel strategies possibly relevant to other deadly desmoplastic cancers (ie PDAC) might be devised using CCA as a model.\textsuperscript{101} For this purpose, tools integrating imaging based single-cell spatial phenotyping data with complementary transcriptomic and genomic datasets could represent on outstanding asset.

### Animal and In-vitro Models

As mentioned before, massive intratumoural and intertumoural heterogeneity are hallmarks of CCAs.\textsuperscript{1,3} This makes the study of these neoplasms and correlation with clinical data extremely difficult, a limit emphasized by the lack of standardized classification criteria and staging methods. Beside issues related to the anatomical classification, strictly related to the ‘geolocalization’ where the CCA arises, the big picture is further complicated by additional factors, which cannot be disregarded. They include the wide variety of histological features, such as mucin production, presence of ductular reaction or CLC elements, and the putative cell of origin, which beyond the small duct or large duct cholangiocytes, encompass even the hepatocytes and the stem cell niche of the canals of Hering (iCCA) or the peribiliary glands (eCCA).\textsuperscript{102-105} In this complex scenario, genetic mutations and molecular aberrations occur variably across the different subtypes without clustering in clear distinctive patterns.\textsuperscript{9,112,20} This calls into question whether the generation of experimental models is able indeed to recapitulate this protein disease condition and to reproduce the consistency of management of CCA. In fact, despite the availability of several animal models with different approaches, including chemically induced, implantation, genetically-engineered mouse models (GEMM) or hydrodynamic gene delivery,\textsuperscript{106-110} it is still uncertain whether and to what extent they may reproduce the various features of CCA, as essentially they are all models of iCCA, which is the less frequent type among clinical CCAs.\textsuperscript{108,109} A further gap in our knowledge of these models is the lack of characterization of the pathophysiological sequence that leads from biliary inflammation and fibrosis to dysplasia and cancer. This would be of paramount importance for the identification of biomarkers which were predictive of cancer development, in particular in premalignant conditions, such as primary sclerosing cholangitis, and congenital hepatic fibrosis/Caroli’s disease, which are not reliant on effective surveillance programs. While implantation approaches are useful for studying tumours at an advanced stage, and thus provide insights into the mechanisms of cancer invasiveness and progression, they fail to model tumourigenesis.\textsuperscript{108,111} On the other hand, GEMM although able to capture some traits of tumour development, do not reproduce the background of chronic biliary inflammation from which CCAs often arise. As with GEMM, in-vivo models based on the use of transposons or ‘sleeping beauty’ technology coupled with the tail vein hydrodynamic gene delivery are characterized by the co-expression of genes that have been found to be relevant in the molecular pathogenesis of human CCAs.\textsuperscript{108,111,112} However, in these models, mature hepatocytes represent the cell of origin of CCA, and while they are suitable for recapitulating the steps of malignant transformation, they seem to be specific only in a minority of CCAs.\textsuperscript{109,110} The chemically induced (or toxicant) rodent models, based on the thioacetamide or furan administration, are the most widely used to reproduce the different phases of tumour development by taking advantage of their ability to initiate inflammation and activate fibrosis, in line with the known risk factors of CCA.\textsuperscript{1,106} However, they are flawed because of several limitations. Firstly, they often give rise to mixed neoplasia, where areas of iCCA develop in
conjunction with areas of HCC. Second, they require a long time span to develop neoplasia and as mortality rates are quite significant, it may occur before the tumour onset. Third, tumour susceptibility is quite different across various strains of mice and rats, and therefore, molecular aberrations associated with tumour development are scarcely reproducible, resulting in a wide heterogeneity of tumour lesions in animals during the course of the experiments.106,111

In this evolving scenario, a major drawback remains the lack of animal models of eCCA. To date, eCCA models are obtained by xenografting immunocompromised mice with human tumour cells (either stabilized cell lines or primary cells) derived from eCCA, implanted in the hepatic parenchyma (directly or through intrasplenic/intraportal injection) or subcutaneously. Although tumours are successfully generated with high rates of engraftment, they fail to reproduce the interactions of tumour cholangiocytes with the extrahepatic biliary microenvironment because of the site of implantation.106,108,111 Moreover, given the immunodeficiency of the recipient animals, the relationship with the immune compartment, featuring a distinct molecular subclass in eCCA,112 cannot be investigated.

In addition to in-vivo approaches, different types of in-vitro cell models have been increasingly used in CCA research. In the last decade, two-dimensional (2D) cultures obtained from stabilized or primary tumour epithelial cells, have been supplemented by the 3D culture systems, such as spheroids and organoids.108,113 Historically, the first in-vitro models used were the 2D cultures, because of the fast growth, the low maintenance costs and the high experimental reproducibility. However, these culture types tended to accumulate mutations, did not mimic interactions with TME and nor create polarized monolayers, factors that were detrimental for oncology research. In addition, because of the presence of serum in the culture media, they usually did not contain CSCs, which were unable to remain viable in these conditions. Furthermore, purification procedures provided a selection of tumour cell clones, thereby missing the intratumoural variability typically reported in CCAs.108 To circumvent some of these limitations, 3D culture systems, divided into spheroids and organoids, have been proposed.113 Spheroids are cellular aggregates of broad origin (tumoural, embryonic, etc) that grow from a single cell in suspension, while organoids are cultures that grow on matrix, such as hydrogel or matrigel, and derive from embryonic stem cells, induced pluripotent stem cells, progenitor cells or primary cells.113 The spheroids are easy to generate and handle, and their culture is relatively cheap. Spheroids are genetically stable and form polarized cell layers but do not reproduce the histological and morphological characteristics of the tissue, from which they derive. Organoids are genetically very stable and, given the absence of serum in the culture medium, they maintain the CSC phenotype. They form polarized cell structures with stable cell-to-cell junctions and preservation of the native tissue architecture, but without reproducing the interactions with the ECM, which is a prerequisite for TME studies. Of note, the organoid technique can be extended to in-vivo settings to generate primary tumours that, once excised, may generate secondary tumours in a second recipient animal.114 Unfortunately, the high costs of cell maintenance, and the duration of generating such cultures (taking several weeks), are major deterrents to their widespread use even in skilled labs.108,113 A recap of pros and cons of experimental models of CCA is given in Table 2.

Conceivably, future efforts will be required to develop in-vivo and in-vitro models that besides being compliant with the molecular categorization of CCAs, will mimic better the liver background whereby the malignant biliary transformation arises.

8 | ACTIONS TO TACKLE CHALLENGES OF CCA RESEARCH

As previously outlined, a major drawback of CCA research particularly that devoted to basic and translational studies has epidemiological grounds, since the low prevalence of the disease limits the availability of biological samples and tissues.5,21 This disadvantage is accentuated further by the difficulties related to the generation of animal models able to faithfully recapitulate a markedly heterogeneous disease.108,111 Given these issues, research proposals supported by an overt interest in a disease that at least from a clinical point of view, is poorly rewarding, have remained confined for a long time to a restricted group of investigators with limited funding opportunities. However, these limitations are turning into new opportunities that have encouraged cooperation between groups with the development of concerted actions. These have resulted in the creation of international collaborative networks involving scientists and clinicians with different background and multidisciplinary skills, such as the European Network for the Study of Cholangiocarcinoma (ENS-CCA). Among the various initiatives conducted by ENS-CCA are the creation of platforms where data and biological samples, including tumour tissue, bile, serum and urine of patients from different countries are collected in registries. By joining forces, this cooperation allows easy access to biological samples and clinical correlates, to encourage translational and cross-sectional proposals aimed at bridging the gaps in CCA investigations taking different approaches. Moreover, thanks to endorsement by the main international scientific societies for the study of the liver diseases (EASL, AASLD), dedicated single topic conferences bringing together basic, translational and clinical researchers, along with patients and stakeholders, have started to be considered creating the opportunities of heated debates/discussions that were unthinkable a few years ago. However, these actions cannot be achieved without the engagement of government and plurinational agencies to make available dedicated funding calls, in not only Europe and North America, but also Latin America and Asia, which only may stop a dangerous vicious circle ‘less money – fewer studies – lack of progress’. Indeed, CCA research is still overshadowed by associations devoted to fight cancer that prefer to finance proposals focused on the ‘big killers’ (lung, breast, colorectal but even HCC) more attractive for drug design and clinical trials, where identification of driver mutations has led to the development of increasingly effective molecularly targeted therapies. In this respect, initiatives such as the ESCALON project, within the Horizon2020 program (https://escalon.eu/project), which support international collaborative projects, are sorely needed.
Although there has been renewed interest in CCA recently and progress has been made in the last few years, there is still much more work that needs to be done to improve the understanding of a disease with yet many dark features. Thus, promoting basic and translational research in CCA is a moral imperative to pursue better management of these patients, often left to basket clinical trials designed for cancer types much better characterized at the molecular level. Thanks to comprehensive multi-platform molecular profiling studies, some promising results have emerged in CCA, leading to the identification of distinctive oncogenic fingerprints and possibly, paving the way to targeted interventions, at least in a subset of patients. However, CCA research still faces many obstacles and challenges. Deciphering the intricate mechanisms of pharmacoresistance, discovering accurate biomarkers for the early detection of...
the disease, unravelling the complexity of the tumour microenvironment and its dual interplay with the tumour counterpart additionally with the aid of more reliable experimental models, these are some examples of matters to put on the CCA research agenda. In this context, prioritization of the key objectives, with definitions of timescales and evaluation of the cost-benefit ratio, are the next steps for future combined efforts. We believe that this approach may result in considerable progress in the field, and turn the multilevel heterogeneity of CCA into an exciting opportunity for personalized medicine.

Data sharing is not applicable as no new data were created or analysed in this study.

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