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Consensus statement

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Criteria for preclinical models of cholangiocarcinoma: scientific and medical relevance

A list of authors and their affiliations appears at the end of the paper

Abstract

Cholangiocarcinoma (CCA) is a rare malignancy that develops at any point along the biliary tree. CCA has a poor prognosis, its clinical management remains challenging, and effective treatments are lacking. Therefore, preclinical research is of pivotal importance and necessary to acquire a deeper understanding of CCA and improve therapeutic outcomes. Preclinical research involves developing and managing complementary experimental models, from in vitro assays using primary cells or cell lines cultured in 2D or 3D to in vivo models with engrafted material, chemically induced CCA or genetically engineered models. All are valuable tools with well-defined advantages and limitations. The choice of a preclinical model is guided by the question(s) to be addressed; ideally, results should be recapitulated in independent approaches. In this Consensus Statement, a task force of 45 experts in CCA molecular and cellular biology and clinicians, including pathologists, from ten countries provides recommendations on the minimal criteria for preclinical models to provide a uniform approach. These recommendations are based on two rounds of questionnaires completed by 35 (first round) and 45 (second round) experts to reach a consensus with 13 statements. An agreement was defined when at least 90% of the participants voting anonymously agreed with a statement. The ultimate goal was to transfer basic laboratory research to the clinics through increased disease understanding and to develop clinical biomarkers and innovative therapies for patients with CCA.

Sections

e-mail: laura.fouassier@inserm.fr

Introduction

During the past decade, we have witnessed considerable advances in understanding the molecular pathogenesis of cholangiocarcinoma (CCA). However, early diagnosis and effective treatments for this aggressive cancer lag behind those for other fields. To accelerate the development of novel clinical strategies, preclinical models of CCA are essential[1]. Critical points to consider when using or developing these tools are the tumour anatomical origin (that is, intrahepatic CCA (iCCA), perihilar CCA (pCCA) or distal CCA (dCCA)), the cell or cells of origin (for example, preneoplastic lesions) and the histomorphological tumour features (for example, large versus small bile duct type)[2].

Historically, 2D cell cultures have been widely used as in vitro models of CCA. In addition to experimentally immortalized or primary cultures of normal cholangiocytes derived from normal bile ducts, over 50 CCA-derived human cell lines have been established[3]. A limitation of these models is the lack of resemblance to the original tumours as a result of continuous culturing, making it difficult to infer which therapeutics would have been effective to treat the original neoplasm[4]. Moreover, 2D monocultures do not accurately mimic the characteristic features of biliary tumours, namely the 3D architecture, cell-to-cell and cell-to-matrix interactions, cellular heterogeneity and the effect of the tumour microenvironment on cancer progression. To overcome these limitations, multicellular 3D models, such as spheroids and organoids, have been developed. Although they are valuable models to study CCA[5], spheroids usually do not precisely recapitulate the native tissue architecture and function of the tissue of origin[6]. By contrast, organoids maintain a higher and more predictable physical order in their cellular self-assembly and display a marked interaction with the extracellular matrix, thereby retaining most of the histological and malignant characteristics of the original neoplasm[6–9]. In addition to cell culture-based models, different in vivo CCA models have been developed. Inducing CCA through administering hepatocarcinogens or liver fluke infestation has the advantage of mimicking cancer pathogenesis. However, animal studies are time-consuming, expensive and ethically challenging, and sometimes hepatocellular carcinoma (HCC) rather than CCA preferentially develops[10]. To give in vivo context to 2D cell lines, CCA cells have been used to generate subcutaneous or orthotopic xenografts in mice[10,11]. However, these approaches remain limited by poor rates of tumour engraftment. Technological advances have made it possible to grow liver organoids (that is, 3D cultures of bipotent liver precursors) and therefore to develop mouse models based on transplantation of genetically modified liver organoids that undergo in vivo oncogenic transformation along the cholangiocellular lineage[12]. Alternatively, genetically engineered mouse models (GEMMs) that recapitulate the most frequent genetic alterations detected in CCA have been generated[10,12,13].

International collaborations to study CCA, spearheaded by the European Network for the Study of Cholangiocarcinoma and the European H2020 COST Action CA18122, have been crucial to fostering advances in this field. To improve the accuracy in obtaining and exchanging information among research groups, it is now essential to establish consensus criteria regarding the minimal standardized characteristics required from preclinical CCA models or when describing a new model. In this Consensus Statement, we detail these criteria for the available and forthcoming in vitro and in vivo models and document the international, interdisciplinary process used for their development.

Methods

Panel of experts

A core group of eight group members, all active researchers with significant contributions to the CCA field, initiated and led a Delphi study to define recommendations on the minimal criteria for experimental CCA models to provide a uniform approach for future studies. Furthermore, core group members identified 27 additional experts to be invited to join the steering committee and to be actively involved in implementing the Delphi process. These core and steering team members filled in the initial Delphi questionnaire and are listed authors, and they proposed ten additional experts to fill in the second and final questionnaire. These ten experts, who were not actively involved in writing the recommendations but provided their important input by filling in the second questionnaire, are listed as one collaborative author: the CCA Model Consortium. Thus, the final panel consisted of 45 experts from ten countries in Europe, Asia and the USA. The names, affiliations, locations and roles of the members of the expert panel are provided in Supplementary Table 1.

Building consensus

We used a modified Delphi method for two rounds of questionnaires. A statement consensus was reached when the agreement was ≥90%. Statements or questions that were agreed upon using this criterion in the first round were omitted from the second round.

Questionnaires

The core team generated the questionnaires using an online form (Google Forms, Alphabet, CA) before sending them out to the experts. The first questionnaire consisted of 47 questions, divided into four parts: defining minimal and advanced criteria for experimental models (part 1); in vivo model for CCA (part 2); in vitro models for CCA (part 3); and preclinical models for CCA (part 4). Based on questionnaire 1 (Supplementary Data 1), a second questionnaire was designed consisting of 13 statements, of which 12 could be solely answered with 'yes' or 'no' (Table 1). All experts could comment on every question. Both questionnaires and summaries of the outcome are shared in Supplementary Data 1. Through the consensus of experts in the field, we propose overarching criteria to be used when establishing or using preclinical models of CCA and linking this to the clinic (Fig. 1). From the second questionnaire, core recommendations were formulated (Box 1).

Clinical features to consider

Experimental models of CCA must reflect the natural history of the known subtypes of CCA, their molecular heterogeneity and the effect of clinical or therapeutic interventions. In the International Classification of Diseases 11th revision (ICD-11), published in 2022, CCA is classified according to its origin as iCCA or extrahepatic CCA (eCCA). iCCA arises from intrahepatic bile ducts, that is, it grows in the liver. Consequently, it is more often surgically resectable than pCCA, the latter of which arises at the liver hilum, where the likelihood of local vascular invasion is greater[14]. The effect of tumour biology on local invasion is poorly understood and requires further examination.

The biology of CCA subtypes also differs significantly. Approximately 50% of iCCAs have actionable molecular alterations, and targeted therapies against *FGFR2* fusions and *IDH1* mutation-driven cancers are already approved[15–18]. It is not fully understood why iCCAs are more molecularly heterogeneous than pCCAs or dCCAs, and this requires detailed examination. In addition, the influence of biology on the natural history of iCCA and its effect on surgical, local and systemic

Table 1 | Consensus statements

Number	Question	Statement	Response yes/ total responders	Grade[a]
Histological assessment				
1	Which of the following are malignant features of biliary tumours?	Invasion of the basement membrane	31/32	A
		Increased nucleus to cytoplasm ratio	18/31	C
		Distant metastasis	27/32	B
		Tumorigenic capacity of isolated cells after subcutaneous injection in immunodeficient mice	29/32	A
2	What type of histological investigation(s) should always be done to characterize an early-stage tumour in a preclinical CCA model?	Morphological examination of H&E	32/32	U
		Immunohistochemistry	27/30	A
		Immunohistochemistry for at least one biliary cytokeratin (for example, CK19, CK7, pan-CK, etc.)	16/25	C
		Markers for inflammatory cells and CAFs	12/26	D
		PAS reaction for highlighting mucin	13/26	C
		A broad panel of markers for hepatobiliary malignancies and metastasis	12/24	C
3	To allow correlation with the anatomical classification of human tumours, a preclinical model of CCA should specifically classify tumours induced as:	Intrahepatic CCA, perihilar CCA and distal CCA	25/30	B
		Intrahepatic CCA and extrahepatic CCA	12/25	D
		No need for such classification	1/23	D
4	Which of the following morphological and/or immunophenotypic features must be present to classify a lesion as CCA in a preclinical model?	Location within the liver or extrahepatic biliary tree	24/28	B
		Absence of an extrahepatic bile duct primary lesion	14/28	C
		Epithelial cytological features (cohesive groups or structures and/or pan-CK immunopositivity)	25/28	B
		At least focal gland formation	9/25	D
		Absence of hepatocellular differentiation (bile production and canalicular CD10 or BSEP)	14/24	D
		Immunopositivity for CK7 or CK19	31/31	U
		Focal desmoplastic stroma	22/30	B
		Presence of precursor lesions	4/24	D
		Primary origin within the intrahepatic or extrahepatic biliary tree	19/28	D
		Absence of primary hepatobiliary lesions	0/28	U
5	What histopathological features of human CCA must be verified in a preclinical model of CCA?	Intratumoural heterogeneity (high stroma, inflammatory response, epithelial phenotype)	27/30	A
		Intertumoural heterogeneity (large versus small bile duct tumour in intrahepatic CCA)	20/26	B
		Growth pattern (mass-forming, periductal infiltration, intraductal growth)	25/28	A
		Proportion of tumour showing gland formation	17/25	C
		Immunopositivity for CK7 or CK19	32/32	U
		Focal desmoplastic stroma	26/30	B
		Presence of precursor lesions	16/24	C
6	It has been proposed that intrahepatic CCA may originate from several cells of origin. Which of the following cell types may be the cells-of-origin for intrahepatic CCA?	Mature hepatocytes	27/32	B
		Mature cholangiocytes	23/32	B
		Hepatic progenitor/oval cells	32/33	A
		Peribiliary glands	29/30	A

Table 1 (continued) | Consensus statements

Number	Question	Statement	Response yes/ total responders	Grade[a]
In vivo models: xenograft models, genetically engineered mouse models (GEMM)				
7	Concerning newly developed patient-derived xenograft models	Should the model(s) be validated by an expert pathologist, and the histology of the tumour shown in publications?	37/37	U
		Should immune profiling also be reported?	20/31	C
		Should the model(s) be validated in more than one mouse strain?	8/34	D
		Should the expert pathologist specify what type of CCA is found in the model?	33/36	A
		Do orthotopic xenograft models represent the most disease-relevant tumour environment in which to test a drug, compared to ectopic xenograft models?	27/35	B
		Should a drug be tested in more than one model?	35/37	A
In vitro models: 2D culture models				
8	Which cell culture procedures should be standardized in experiments with cell lines or primary 2D cultures and be reported in publications?	Choice of plastic support (for example, TPP, Falcon, Corning, +/− ECM layer, etc.)	30/34	B
		Choice of cell culture medium	29/34	B
		Level of confluence when performing the experiments	27/33	B
		Isolation protocol for culture of primary cells	31/35	B
		Passaging and subculturing methods (for example, enzymatic versus mechanical dissociation, etc.)	29/34	B
9	The origin of any cell line (previously established or new) should be stated for publication according to the new CCA classification (that is, intrahepatic, perihilar, distal)	NA	37/38	A
In vitro models: 3D cultures				
10	Contaminating non-tumour organoids often grow in CCA organoid cultures. How should selection for tumour organoids be performed?	Specific tumour 'enrichment' medium (that is, tumour initiating medium, as described by Broutier et al. (2017)[7])	29/31	A
		Hand-picking of organoids with a different phenotype/removing the 'normal-looking' organoids	21/30	B
		Xenotransplantation in mice to select for tumour clones	22/30	B
11	Which analyses should be done to confirm the malignant origin of established organoid lines and be reported in publications?	Full genomic profiling	8/28	D
		Mutation analysis (targeted genomic profiling using a diagnostic panel)	28/31	A
		Phenotypic analysis	28/30	A
		Histological analysis (immunohistochemistry of EpCAM, CK7)	28/32	B
		Xenotransplantation in mice	26/32	B
12	Should every organoid culture be characterized (as proposed in question 11) before clinical applications such as drug screening?	NA	33/36	A
13	Personalized medicine applications, such as drug screenings to find the best treatment for the patient, will cost time. How much time is acceptable to initiate, grow and expand the organoids for these analyses? In other words, what is the maximum time acceptable to be relevant to the clinics?	<1 month	9/35	D
		<3 months	20/35	C
		<6 months	4/35	D
		Other; as short as possible/<1 month first-line treatment and <3 months second-line treatment	2/35	D

BSEP, bile salt export pump; CCA, cholangiocarcinoma; CK, cytokeratin; CAF, cancer-associated fibroblast; ECM, extracellular matrix; H&E, haematoxylin and eosin; NA, not applicable; PAS, periodic acid–Schiff stain. [a]Grading system: U, unanimous (100%) agreement; A, 90–99% agreement; B, 70–89% agreement; C, 50–69% agreement; D, <50% agreement.

treatment options require further study[19]. dCCA more closely resembles pCCA[19,20] but, again, the effects of both anatomy and biology on outcome have not been fully elucidated. However, many tools only seek to mimic iCCA, and there is a critical absence of models of pCCA and dCCA.

A second essential requirement of an experimental model is to reflect the interventional outcome. Although chemotherapy remains the standard of care, the increasing use of targeted therapies requires a deeper examination of molecular mechanisms and critical mechanisms of resistance[21–24]. As such, any model must reflect molecular changes in the patient that can be measured to provide hypotheses to overcome this commonly occurring resistance. Furthermore, such resistance mechanisms should be unravelled to develop and assess novel interventions to overcome resistance before clinical testing.

Pathology

Separate classifications (Union for International Cancer Control[25], American Joint Committee on Cancer[26] and WHO[20]) exist for iCCA, pCCA and dCCA. Macroscopic features divide iCCA into two subtypes: large duct and small duct[20]. Large duct iCCAs typically arise near large central ducts and grow along the ductal wall. Small duct iCCAs are usually peripheral mass-forming tumours in the hepatic parenchyma. Four patterns of growth are described for CCA: mass-forming, periductal infiltrating, intraductal and mixed types[27].

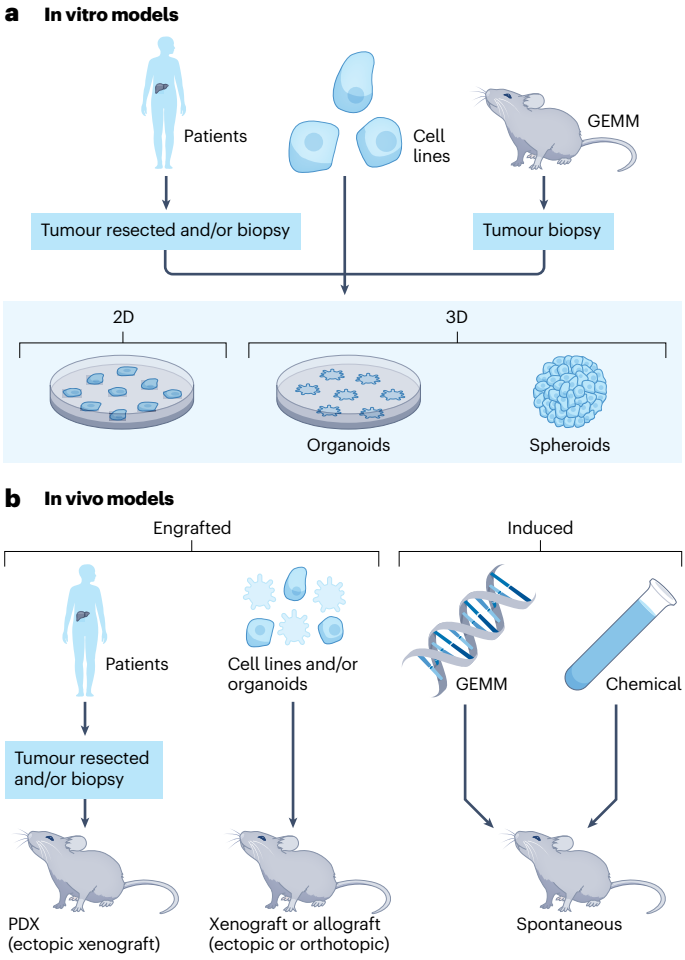

Fig. 1 | Panel of experimental models provided for cholangiocarcinoma preclinical studies. a, In vitro models. **b**, In vivo models. GEMM, genetically engineered mouse model; PDX, patient-derived xenograft.

Histopathology. Small duct iCCAs are typically non-mucin-secreting adenocarcinomas with a ductular or tubular pattern. Large duct iCCAs are generally mucin-secreting tubular adenocarcinomas resembling pCCA and dCCA[28]. Most pCCAs and dCCAs are adenocarcinomas with pancreaticobiliary morphology, comprising glandular structures and/or small groups of cells within the desmoplastic stroma[28].

Immunohistochemistry. No specific immunohistochemical pattern for CCA lesions exists. However, they typically show an upper gastrointestinal or pancreaticobiliary pattern of cytokeratin (CK) expression ($CK7^+$, $CK19^+$, $CK20^-$) when they still exhibit some degree of differentiation. In addition, large duct iCCAs sometimes express intestinal markers (for example, CK20 and CDX2)[29]. CCA is usually immunonegative for HepPar1, arginase 1 and glypican 3, distinguishing it from HCC and combined HCC–CCA[30,31]. Transcription factors that mark cell-specific lineages, such as thyroid transcription factor 1 (TTF1) (lung and thyroid cancers)[32], PAX8 (renal, thyroid, ovarian and endometrial cancers)[33] and GATA3 (breast and urothelial cancers)[34], are usually not expressed in CCA.

Biliary precursor lesions. CCA can develop from precursor lesions. Most large duct iCCA as well as pCCA and dCCA presumably originate from biliary intraepithelial neoplasia[35]. Intraductal papillary neoplasm of the bile duct (IPNB) is an intraductal papillary proliferation; 70% of IPNBs develop in intrahepatic ducts and 30% develop in perihilar ducts[36,37]. Invasive malignancy is evident in >50% of IPNBs at presentation[38,39]. Mucinous cystic neoplasm is a cystic epithelial tumour occurring almost exclusively in female patients, and it is debatable whether it represents a true biliary precursor lesion but approximately 5% of these tumours are associated with CCA[40,41].

Molecular profiling

Efforts to understand the heterogeneity of CCA have provided insights into the molecular pathogenesis and anatomical complexity of this disease[15,42–49]. The genetic landscapes are comparable to those of other carcinoma types of the gastrointestinal tract, show substantial similarities to genetic alterations of ductal adenocarcinoma of the pancreas, and exhibit an intermediate degree of alteration counts in the mutational spectrum of cancers[50], with shared genetic alterations between iCCA, pCCA and dCCA[47]. Although we have gained comprehensive insights into the underlying pathobiological processes of resectable invasive tumours, the precise genetic and epigenetic mechanisms involved in the onset of CCA are still unclear.

Integrated genomics approaches have been used to classify patients with CCA based on prognosis[39,51–53], emphasizing dysregulated oncogenic signalling pathways, including WNT–CTNNB1, MYC, PI3K–AKT–mTOR, ERBB, fibroblast growth factor receptor 2 (FGFR2), RAS–RAF–ERK, tumour necrosis factor (TNF), polo-like kinase 1 (PLK1), transforming growth factor-β (TGFβ), NOTCH, insulin-like growth factor receptor 1 (IGFR1), vascular endothelial growth factor (VEGF) and the Hippo cascade. This predominant molecular classification highlights distinct tumour phenotypes that are either inflammatory or proliferative in nature[52]. Moreover, iCCA can be classified on the basis of driver gene mutations, which elucidate unique mutational signatures, structural variants and epigenomic alterations[46]. This approach emphasized specific oncogenetic mechanisms in distinct

Box 1

Recommendations for cholangiocarcinoma experimental models

Histological assessment (all in vivo models)

- Invasion of the basement membrane and tumorigenic capacity of isolated cells engrafted subcutaneously in immunodeficient mice are the most important malignant features of cholangiocarcinoma (CCA) (97% and 91%, A).
- Immunohistochemistry of at least one biliary cytokeratin should always be performed to characterize an early-stage tumour in a preclinical CCA model (90%, A).
- A classification of preclinical CCA models as intrahepatic, perihilar and distal CCA is recommended (93%, A).
- Focal desmoplastic stroma is a morphological feature required to classify a lesion as CCA in a preclinical model (100%, U).
- Three histopathological features of human CCA must be assessed in a preclinical model: intratumoural heterogeneity (high stroma, inflammatory response, epithelial phenotype) (90%, A), the pattern of growth (mass-forming, periductal infiltration, intraductal growth) (90%, A), and immunopositivity for CK7 or CK19 (100%, U).

Xenograft models, genetically engineered mouse models

- The type of CCA should be specified for patient-derived xenograft models (92%, A).
- Drugs should be tested in more than one model (95%, A).

2D cultures

- Cell culture procedures should be standardized in experiments with cell lines or primary 2D cultures and be reported in publications. Procedures include the choice of plastic support and cell culture medium, and the level of confluence when performing the experiments should be mentioned (88%, 85%, 82%, B).
- The isolation protocol for primary cells, including passaging and subculturing methods, should be reported in publications (for example, enzymatic versus mechanical dissociation, etc.) (9% and 85%, B).
- The origin of any cell line (previously established or new) should be stated for publication according to the new CCA classification (that is, intrahepatic, perihilar, distal) (90–99%, A).
- The origin of any cell line (previously established or new) should be presented in a publication according to the new CCA classification (that is, intrahepatic, perihilar, distal) (97%, A).

3D cultures

- A specific tumour 'enrichment' medium (that is, tumour initiating medium, as described by Broutier et al.[7]) is recommended to minimize contamination in non-tumour organoids (94%, A).
- Mutation analysis (targeted genomic profiling using a diagnostic panel) (90%, A), and phenotypic analysis should be done to confirm the malignant origin of established organoid lines and reported in publications (93%, A).
- Every organoid culture should be characterized before clinical applications such as drug screening (92%, A).
- The shorter period for patient-organoid initiation, expansion and analysis has to be less than 3 months (57%, C).

Grading system: U, unanimous (100%) agreement; A, 90–99% agreement; B, 70–89% agreement; C, 50–69% agreement; and D, <50% agreement.

patient subsets each associated with potential unique drugs such as RNA synthesis inhibitors in *IDH*-mutant tumours, microtubule modulators in *KRAS*-mutant tumours, topoisomerase inhibitors in *TP53*-mutant tumours and mTOR inhibitors in wild-type tumours enriched in *FGFR2* fusions[15].

As the three anatomical CCA subtypes differ in their molecular alterations[47] and potentially in their cell of origin[54–57], the CCA subtypes should be studied in separate experimental models[2]. However, the stepwise progression of human CCA and, thus, the accumulation of a wide variety of molecular alterations might not be reflected in the mouse models in which tumours develop most rapidly. Furthermore, the available experimental models represent specific subsets of patients with CCA, and it is essential to consider the molecular heterogeneity of patients with CCA when using these models. With this in mind, integrative transcriptomics might represent a relevant strategy to define the best-fit models, as previously demonstrated for HCC[58,59].

In vivo CCA models

Engrafted models

Xenografts. Xenografts are grafts of tissue or cells transplanted from a different species into an immunodeficient host[60]. Xenograft CCA models are generated by either implanting human neoplastic CCA cells subcutaneously into the flanks of immunodeficient or athymic mice (ectopic grafts) or directly into the liver (orthotopic grafts). These experimental animal models help to evaluate the therapeutic efficacy and safety of novel candidate drugs or physical-based therapies for treating CCA in vivo. They are highly reproducible, cost-efficient, technically easy and feasible, with limited adverse effects related to the procedure, and they only require short periods for evaluation[60–63]. Furthermore, when engrafted subcutaneously, the generated tumours are easily accessible throughout the duration of the in vivo model, which enables the real-time measurement of tumour volume growth with a caliper. Several studies have investigated the therapeutic efficacy and safety of different compounds such as sorafenib or an epigenetic inhibitor[61,64–67]. Additionally, the role of various proteins[68–73] and microRNAs[74–78] were evaluated in ectopic xenograft models by implanting genetically manipulated CCA cells. Nevertheless, ectopic xenografts also have intrinsic limitations. Xenografts usually reflect advanced tumour stages, growing rapidly and making the study of early CCA challenging. At the same time, distinct CCA cell lines display different tumorigenic activity, with some being unable to generate tumours after injection. Furthermore, these tumours are implanted in a non-physiological site, seldom metastasize, and might lose the molecular heterogeneity characteristic of human CCA. Most importantly, they

do not enable study of the crosstalk between tumour cells, the multicellular microenvironment milieu and the immune system[10,60,62,63].

The use of orthotopic xenograft models might overcome some of these limitations by developing tumours directly in the organ of origin. Orthotopic grafts are more likely to trigger tumour dissemination, with the development of distant metastases[79]. Intrahepatic implantation of CCA cells can be achieved either by injecting cells directly into the liver parenchyma using ultrasound-guided injection[80] or through the portal or splenic vein[60]. Small fragments of CCA tumours previously generated in subcutaneous xenografts or cancer stem cell-derived spheroids can also be orthotopically implanted[81,82]. Although intrasplenic injection is technically easier than intraportal administration and carries fewer postoperative complications, the implantation of CCA cells by intrasplenic injection resulted in successful engraftment in the liver and the spleen[83]. Notably, intrasplenic injection of EGI-1 CCA cells also induced the development of lung metastases[84]. Still, generating orthotopic models is more time-consuming, and some postoperative complications can arise. Furthermore, the assessment of tumour development and growth and metastases requires imaging techniques or is performed only when the animal is killed[60,63]. In this sense, using luciferase-expressing CCA cells is an excellent choice to monitor tumour growth over time[83]. However, this tool might not be accessible to all.

Engrafting cells or tissues obtained directly from patients can result in the development of patient-derived xenografts (PDXs). The original genetic and epigenetic features and surrounding stroma as observed in the initial mass are usually maintained in subcutaneous or orthotopic tumours, which are thus the ideal model to predict therapeutic responses and are excellent tools in personalized medicine. Indeed, several studies have already used PDXs to examine CCA that harbour specific mutational patterns and to test the use of specific targeted therapies[85–91]. Nevertheless, the success of PDX engraftment is relatively low, depending on the primary tumour itself and the experimental design for tumour engraftment. Thus, they constitute a time-intensive and resource-intensive model and might require several months for successful implantation[60]. Based on the available data and unanimous agreement, the expert panel strongly suggests that the type of CCA should be defined by a pathologist for PDX models, with the histology of the tumour shown in publications (Box 1).

Allografts (syngeneic). Syngeneic models enable the implantation of murine CCA cells into an immunocompetent host, and thereby display a fully functional immune system. The first syngeneic model was developed when two rat CCA cell lines (BDEneu and BDEsp) were directly implanted into the biliary tract of Fischer 344 rats. While BDEsp engraftment induces the development of non-metastatic iCCA, BDEneu-derived tumours are more aggressive, with the rapid and consistent formation of CCA lesions and metastases[92,93]. This model was used to elucidate the mechanisms that underlie tumour progression and to evaluate the efficacy of novel drug candidates[93–97]. A development of this approach using a novel syngeneic murine model was reported in which the malignant mouse cell lines SB1-7, obtained from a bile duct ligation (BDL) and transposon-based CCA model, were engrafted into the mice[98,99]. The obtained cell lines were successfully implanted, leading to CCA lesions resembling human CCAs[99]. In addition, fetal liver cells obtained from genetically modified mouse embryos can be implanted into mouse liver, inducing CCA formation[100]. Furthermore, the CCA cells can be genetically manipulated before engraftment, revealing insights into the mechanisms that govern cholangiocarcinogenesis and enabling implantation of the cells into already established knockout mouse strains, thereby permitting the study of alterations in specific genes in the tumour stroma[101]. In this line, unpublished observations from the SB1 orthotopic model indicate that extending the frequently used end point (4 weeks) by 2 additional weeks enables the formation of extrahepatic metastases in the lung (J.V. and E.G.-S., unpublished work). Therefore, further characterization of this timeline in a genetically treatable immunocompetent host, coupled with the isolation of tumour cells from the original site of injection and the metastatic sites, could provide an excellent model to understand, and perhaps even prevent, a rather under-studied process such as CCA metastatic spreading. Overall, these models can overcome the limitations of xenografts, such as the absence of the immune system, are ideal for studying tumour–stroma interactions, and are an excellent alternative for testing immunotherapy-based strategies. Still, they require microsurgical procedures, increasing the probability of procedure-related complications.

Chemically induced models

High levels of inflammation, fibroblast activation and rich extracellular matrix deposition in the tumour typify CCA in patients[102]. In some cases, these tumours develop in patients with chronic diseases such as chronic liver fluke infection or in patients with primary sclerosing cholangitis[2], and the cells associated with these pre-cancerous conditions contribute to cancer formation. Several chemical models that generate chronic and iterative injury, leading to tumour formation, have been developed to recapitulate this complex microenvironment in CCA.

Early work demonstrated that administering thiourea or thioacetamide (TAA) to rats triggers liver cancer formation over 2 years[103]. TAA is a potent hepatotoxin that induces hepatic fibrosis and cirrhosis in rodents owing to progressive damage of hepatocytes and biliary epithelium. TAA-induced biliary damage reproduces the typical dysplasia–carcinoma sequence, ultimately evolving to invasive iCCA[104]. Consequently, the use of TAA to induce tumour-initiating injury in rodents has become a cornerstone of CCA research. However, as detailed in this early work, CCA formation in TAA-treated rats is very variable, with only ~50% of animals developing frank carcinomas. Results are even more variable in wild-type mice. TAA is not mutagenic per se; instead, the initiation of chronic sclerosing inflammation and continuous regeneration drives the spontaneous accumulation of mutations in biliary cells, which then become cancerous, as is observed in patients with chronic cholangiopathies[105,106]. Therefore, combined with BDL, a classic model of obstructive cholestasis and subsequent bile duct proliferation, TAA accelerates the formation of biliary tumours in rats[106,107]. In addition to TAA, several other mutagenic models have also been developed to induce CCA in rodents. For instance, diethylnitrosamine and dimethylnitrosamine generate DNA adducts in the liver and are sufficient for liver carcinogenesis[108] and, in combination with inflammatory injury (BDL or *Opisthorchis viverrini* infection), drive CCA development in mice and hamsters[109–111]. Furan is a potent mutagen capable of initiating CCA in rats[112]. Long-term furan treatment is currently the only chemically induced model of CCA with a tumour incidence of nearly 100%, which results in multi-organ metastases and closely recapitulates the primary and secondary pathologies of human CCA. Available models are summarized in Table 2 and Fig. 2.

Although many rat and mouse CCA models that are driven by chemical insults reflect both the pre-cancerous disease history and the molecular and histopathological features of human CCA, their use is becoming less popular, primarily due to their long latency, cost and

variability (both in terms of tumour penetrance and high molecular heterogeneity). Advances in CCA modelling have focused on combining the disease-inducing aspects of these models, such as inflammation and fibrosis, with GEMMs, which are discussed in more detail in the next section. A critical point is the choice of the control tissue to compare with malignant biliary cells. Indeed, as the whole liver is inappropriate because hepatocytes are the prevalent cell population, isolated bile ducts should be considered the best control.

Genetically engineered mouse models

GEMMs are advanced animal models of human cancer (Table 3). They are rationally designed to mimic the genetic and epigenetic alterations, the aberrant activation of signalling pathways and the sequence of preneoplastic and early and late tumour stages, including metastasis, in human CCAs. In addition, GEMMs can be coupled to in vivo transfection (hydrodynamic tail vein injection and/or electroporation) or injection (adeno-associated viruses (AAV)) approaches to activate or express transgenes in adult hepatocytes to further expand the mouse model toolbox[113].

General concerns precluding the use of GEMMs are their high cost, tumour latency and embryonic Cre expression in non-inducible models that might compromise translation to human disease. However, adopting CRISPR–Cas9 strategies to generate new GEMM strains, and the development of tamoxifen-inducible, organ-specific Cre recombinase strains, circumvented some of these limitations.

Most CCA GEMMs incorporate common oncogenic alterations found in humans, including inactivation of tumour suppressor genes (*PTEN*, *SMAD4* and *P53*) or induction of oncogenes (*KRAS*, *IDH1/2*, *AKT1* and *NOTCH1*) to investigate the consequences of cell-autonomous effects on cholangiocarcinogenesis. In the first reported CCA GEMM, ablation of *Pten* and *Smad4* in fetal bipotential hepatic progenitors (liver progenitor cells (LPCs)) was achieved during embryogenesis using an albumin–Cre (*Alb*–Cre) strain[114]. *Alb*–Cre,*Smad4*$^{flox/flox}$,*Pten*$^{flox/flox}$ mice displayed the histopathological stages detected in human disease, from bile duct hyperplasia and dysplasia to carcinoma in situ and invasive CCA.

Another model closely recapitulating human cholangiocarcinogenesis consists of concomitant *Trp53* abrogation and *Kras*^{G12D} expression in the *Alb*–Cre mouse background[115]. This model features premalignant biliary lesions (IPNB and von Meyenburg complexes), leading to invasive carcinoma and distal metastases. To directly probe the cell of origin in this model, *Kras*$^{LSL\text{-}G12D/+}$,*Tp53*$^{flox/flox}$ mice were bred to the tamoxifen-inducible *Sox9*–Cre^{ERT2+} strain (targeting cholangiocytes) or intravenously administered the AAV8 vector expressing Cre under the thyroxine-binding protein (targeting adult hepatocytes)[116]. *Kras*^{G12D} activation and *Trp53* loss in adult hepatocytes required co-administration of the 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) pro-cholestasis diet to form tumours (iCCA and HCC with a similar incidence, in addition to mixed HCC–CCA), highlighting the role of inflammation in liver cancer formation. By contrast, *Kras*^{G12D} activation and *Trp53* loss in the adult ductal compartment in the *Sox9*–Cre^{ERT2+} mouse strain accelerated the development of hepatic tumours, mainly iCCA, from preneoplastic lesions (not found in AAV8-injected mice) without the need for inflammatory cues[116].

Targeted *Kras*^{G12D} activation and *Pten* deletion triggered the fastest GEMM of CCA in *Alb*–Cre mice[117]. In *Kras*$^{LSL\text{-}G12D/+}$,*Pten*$^{flox/flox}$,*Alb*–Cre mice, early hyperplastic biliary foci were detected by 4 weeks of age, and mice died by 7 weeks. Tumours were multifocal, stroma-rich localized iCCA. Interestingly, mice with heterozygous *Pten* deletion and *Kras*^{G12D} activation developed tumours after a longer latency, showing hepatocyte and cholangiocyte differentiation features. The use of *Alb*–Cre^{ERT2+} or *K19*–Cre$^{ERT/+}$ mouse strains to activate the oncogenic alterations in adult hepatocytes or cholangiocytes, respectively, led to the development of HCC and HCC precursor lesions, but not iCCA precursor lesions, in 8-week-old *Alb*–Cre^{ERT2+},*Kras*$^{LSL\text{-}G12D}$,*Pten*$^{flox/flox}$ mice, whereas tamoxifen injection on day 10 elicited iCCA. The formation of iCCA in *Alb*–Cre^{ERT2+},*Kras*$^{LSL\text{-}G12D}$,*Pten*$^{flox/flox}$ mice might be because *Alb*–Cre is still active in biliary cells at 10 days of age, and indicates that cholangiocytes are the cell of origin of CCA in these models, which was later independently confirmed using similar approaches[118].

IDH1 and IDH2 oncogene modelling in mice was employed[119,120]. Breeding of *Idh2*$^{LSL\text{-}R172K}$ and *Kras*$^{LSL\text{-}G12D}$ mice in the *Alb*–Cre background yielded multifocal iCCA-like liver masses with invasive growth and metastatic capacity. Furthermore, adjacent to the tumours, oval cell expansion and biliary intraepithelial neoplasia-like lesions, suggestive of preneoplastic stages, occurred. Subsequently, the same group generated *Idh1*$^{LSL\text{-}R132C}$ mice that developed iCCA upon crossing with *Kras*$^{LSL\text{-}G12D}$ mice in the *Alb*–Cre background[121]. Another oncogene investigated in *Alb*–Cre mice was *Notch1*, via a mouse strain expressing the NOTCH1 intracellular domain from the *Rosa26* locus[122]. By 8 months after birth, malignant foci were detected, leading to CCA formation in transplanted immunodeficient mice.

Two GEMMs highlighted the importance of a pro-inflammatory environment in cholangiocarcinogenesis. In the first model, severe liver damage by inflammatory cues originating from mitochondrial dysfunction characterized *Hspd1*$^{flox/flox}$ mice bred to the *Alb*–Cre strain[123]. Mice developed hepatocyte and cholangiocyte regenerative foci, the latter resembling human biliary intraepithelial neoplasia. The lesions arose in the context of an injured microenvironment and not through cell-autonomous mechanisms, as most regenerative liver foci exhibited HSPD1 expression. In the second model, *Kras*^{G12D} expression and deletion of both *Tgfbr2* and *Cdh1* (encoding E-cadherin) were achieved in adult CK19^{+} biliary cells, leading to early-onset metastatic tumours in the extrahepatic and hilar bile duct[124]. In response to E-cadherin ablation, dying cholangiocytes released IL-33 to foster a proliferative

Table 2 | Carcinogen-based rodent models of cholangiocarcinoma

Carcinogenic agent	Animal	Mechanism of action	Biliary lesions	Refs.
TAA	Rat and mouse	Membrane protein and phospholipid modifications	Intense fibrosis with dysplasia	103,104
Furan	Rat	DNA adduct generation	Chronic inflammation, proliferation of bile duct cells	112
DEN, DMN (even combined with BDL)	Hamster and mouse	DNA adduct generation	Desmoplasia, cystic hyperplasia of bile ducts	108–110,182
Opisthorchis viverrini	Hamster	DNA oxidative damage	Alterations of oxidative metabolism and proliferation of bile ducts	111

BDL, bile duct ligation; DEN, diethylnitrosamine; DMN, dimethylnitrosamine; TAA, thioacetamide.

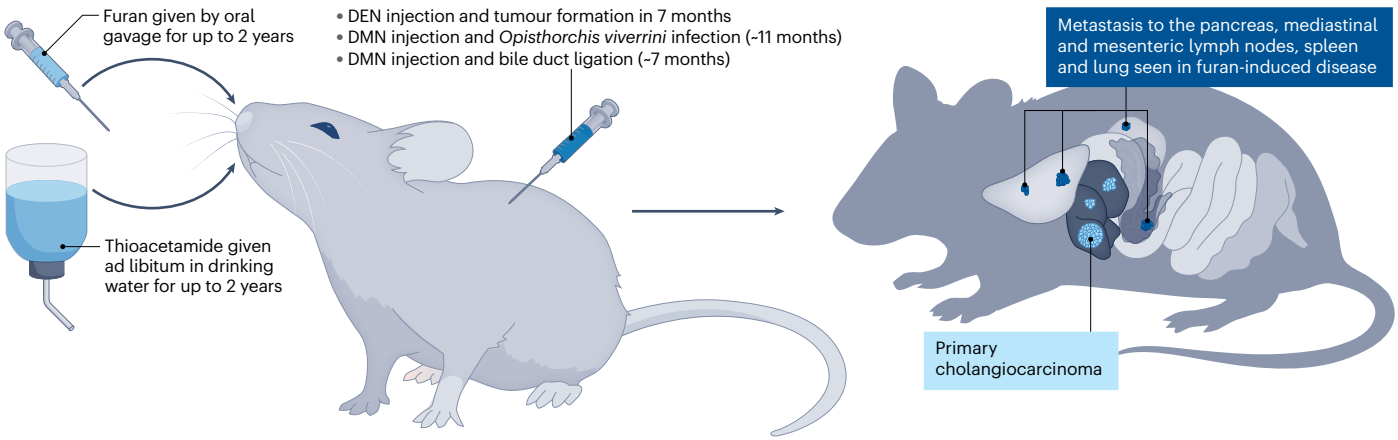

Fig. 2 | Chemical models. Schematic summary of available chemical models to initiate cholangiocarcinoma in rodents and induce metastatic dissemination. DEN, diethylnitrosamine; DMN, dimethylnitrosamine.

phenotype in biliary epithelial cells that contributed to neoplastic transformation. However, after 4 weeks of tamoxifen administration, mice succumbed to liver and/or respiratory failure. In these models, transplantation of liver tissues in immunodeficient mice[123] or derivation of tumour organoids from mice[124] enabled follow-up experiments otherwise limited by the short lifespan of the mice.

Additional carcinogen-exposed GEMMs that model the consequences of an inflammatory environment, which is a frequent risk factor in human CCA, have also been reported. However, both the low penetrance and the high latency limited their use[125,126]. Nonetheless, co-exposure with carcinogens might be a strategy in GEMMs to accelerate cholangiocarcinogenesis by providing a pro-inflammatory and pro-fibrogenic environment that recapitulates the human context[127].

Orthotopic or subcutaneous allograft models of premalignant liver cells (LPCs or adult liver organoids) or GEMM-derived CCA cell lines provide an alternative experimental strategy to time-consuming GEMMs[12,73,100,121]. These cellular models are amenable to gene editing, and their orthotopic transplantation into syngeneic mice enables tumour growth in an immune-competent microenvironment. Additionally, the plasticity of LPCs and liver organoids to originate CCA-like or HCC-like tumours, depending on the genetic context, is preserved.

GEMMs have shown that LPCs, cholangiocytes (intrahepatic and extrahepatic) and mature hepatocytes can be the cell of origin of CCA in mice[57,128]. However, the relevance of these findings for human CCA remains under evaluation. Indeed, various elements, including the targeted cell population (differentiated versus stem cells and additional cell types only present in humans), the tissue location (intrahepatic versus extrahepatic), the increased complexity of oncogenic alterations, the type, degree and duration of the pro-oncogenic and pro-inflammatory stimuli, the liver status, and other factors, might ultimately affect CCA development.

For all preclinical in vivo models, based on statements on histological assessment and unanimous agreement (Box 1 and Table 1), the expert panel strongly suggests that: the invasion of the basement membrane and tumorigenic capacity of isolated cells engrafted subcutaneously in immunodeficient mice are the most critical malignant features of CCA; morphological examination by H&E and immunohistochemistry should be conducted to characterize an early-stage tumour in the preclinical CCA model; immunohistochemistry of at least one biliary cytokeratin (CK7 or CK19) should always be performed to characterize a lesion as CCA in the absence of hepatobiliary primary lesions in a preclinical model; three histopathological features of human CCA must be assessed in a preclinical model: 1) intra-tumoural heterogeneity (high stroma, inflammatory response, epithelial phenotype); 2) pattern of growth (mass-forming, periductal infiltration, intraductal growth); and 3) immunopositivity for CK7 or CK19; the expert panel recommends classifying preclinical CCA models as iCCA, pCCA and dCCA, and suggests that focal desmoplastic stroma is a morphological feature required to classify a lesion as CCA in a preclinical model; and finally, a drug should be tested in more than one model.

Finally, to adopt a shared tool for homogeneously defining experimental models of CCA, an 'experimental model sheet' was generated, based on an initial expert discussion by all members of the CCA Model Consortium in a physical ad hoc meeting (Malta meeting 20189, WG1 meeting, European H2020 COST Action CA18122) (Box 2 and Supplementary Table 3) to provide complete information on animal experimentations to the scientific community through publications.

In vitro CCA models

2D culture with cell lines or primary cells

The urgent need to understand the biological processes of CCA progression and drug resistance has led to the widespread use of in vitro models represented by human and animal primary cultures and established cell lines. In 1985, the first CCA cell line – HChol-Y1 – was established from a patient with iCCA and then characterized[129]. Later, an assortment of CCA cell lines of intrahepatic and extrahepatic origin were generated from primary tumours, ascites, metastases and PDXs (Supplementary Table 2). In addition to human CCA cells, several lines derived from mouse, rat and hamster models have been described (Supplementary Table 2). Primary cultures of normal cholangiocytes should be used as control cells.

Molecular studies performed in human CCA tissues have uncovered recurring genomic alterations in specific genes such as mutations in *TP53*, *IDH1/2*, *FGFR2*, *KRAS*, *BRAF* and *SMAD4*, *FGFR2* receptor fusions and *ERBB* family gene amplifications[130], which qualify as targets for molecular approaches. Although most described CCA cell lines have been studied in terms of phenotypic and functional characterization of some parameters, only in the past few years, with the development

Table 3 | Summary of the most representative in vivo CCA models based on genetically engineered mice

Genetic strategy	Key features	Advantages	Disadvantages	Refs.
Alfp–Cre, *Trp53*$^{f/f}$	Advanced HCC–CCA (from LPCs)	*Trp53* mutation found in human CCA	Long latency (mice 14–20 months of age), tumours of bilinear origin (combined HCC–CCA)	183
Alb–Cre, *Smad4*$^{f/f}$, *Pten*$^{f/f}$	Multistep progression involving hyperplasia, dysplasia, carcinoma in situ, and well-established iCCA (from LPCs)	100% tumour penetrance	Cre activation during embryogenesis, long tumour latency (4–5 months) and lack of metastasis	114
Alb–Cre, *Kras*$^{LSL\text{-}G12D/+}$, *Pten*$^{f/f}$	Invasive iCCA with an abundant desmoplasia, primarily showing glandular morphology resembling well-differentiated human CCA (from LPCs)	100% penetrance, rapid development (7 weeks of age), abundant desmoplastic stroma, iCCA exclusive	Cre activation during embryogenesis, no apparent metastases or invasion to other organs	117,118
Alb–Cre, *Idh2*$^{LSL\text{-}R172}$, *Kras*$^{LSL\text{-}G12D}$	Multifocal liver masses of iCCA (from LPCs)	100% penetrance, splenic invasion and peritoneal metastases	Cre activation during embryogenesis, long tumour latency (33–58 weeks)	119
Alb–Cre, *NotchICD*	Development of transplantable CCA, probably progenitor cell-derived (transplantation of cells from 8-month-old mice in immunodeficient animals gives rise to CCA) (from LPCs)	Notch expression is characteristic of human disease	Cre activation during embryogenesis, no obvious cancer development after 8 months in transgenic mice, requires additional transplantation model	184
Alb–Cre, *Trp53*$^{f/f}$, *NotchICD*	Development of iCCA abortive glandular pattern (moderate to high pleomorphic nuclei with some atypical mitoses) and dense fibrous tissue with inflammatory cells (from LPCs)	100% penetrance, development of fibrous or inflammatory microenvironment	Long tumour latency (>8–9 months), no metastases	185
Alb–Cre, *Kras*$^{LSL\text{-}G12D/+}$, *Fbxw7*$^{LSL\text{-}R468C/LSL\text{-}R468C}$	Dysplastic dust-like structures surrounded by fibrosis in all mice (only bile duct dilation and hyperplasia in some heterozygous *Fbxw7*$^{LSL\text{-}R468C}$ mice at the age of 8 months) (from LPCs)	Low latency (2 months of age)	Cre activation during embryogenesis, homozygous *Fbxw7* mutations not occurring in human disease	186
Alb–Cre, *Hspd1*$^{f/f}$	Cholangiocellular lesions, characterized by irregular glands, loss of polarity, multilayering of cells and frequent mitosis resembling human BIN	Low latency, possibility of transplanting cholangiocellular lesions, activation of human CCA pathways	Not related to known oncogenic drivers of human disease, no metastases, not established iCCA	123
Alb–Cre, *Jnk1*$^{f/f}$, *Jnk2*$^{-/-}$	JNK deletion causes changes in cholesterol and bile acid metabolism that foster cholestasis, bile duct proliferation and iCCA	iCCA exclusive	~95% penetrance, long tumour latency (14 months)	187
Alb–Cre, *NEMO*$^{f/f}$, *Jnk1*$^{f/f}$, *Jnk2*$^{-/-}$	Hyperproliferative ductular lesions with atypia compatible with CCA	Elevated ROS associated with cholangiocellular proliferation	Not full penetrance, long latency (50 weeks)	188
Alb–Cre, *Kras*$^{LSL\text{-}G12D/+}$, *Trp53*$^{f/f}$	Multistage progression including stroma-rich tumours and premalignant biliary lesions (IPBN) (from LPCs)	100% penetrance, average latency 16 weeks, metastatic lesions	Cre activation during embryogenesis, wide latency range, CCA in ~80% of mice	115
Kras$^{LSLG12D/+}$, *Trp53*$^{f/f}$ infected with AAV8-*TBG*–Cre	Development of iCCA (40%), HCC (40%), combined HCC–CCA (20%) (from hepatocytes)	Recombination event in adult mice, higher CCA frequency in combination with DDC diet (all tumours ICC or combined HCC–CCA)	Cre-recombinase administration via AAV, large tumour latency range (12–66 weeks after AAV infection)	116
Ah–Cre^{ERT}, *Kras*$^{G12V/+}$, *Pten*$^{f/f}$	Multifocal non-invasive papillary neoplasms in the intrahepatic biliary tract (from major interlobular bile ducts to small bile duct radicles in portal tracts)	100% penetrance, low latency (43 days), tumour development starts in adult mice	Not specific to liver tissue, lack of invasive tumour or metastasis	189
Sox9–Cre^{ERT2}, *Kras*$^{LSL\text{-}G12D/+}$, *Trp53*$^{f/f}$	iCCA tumours accompanied by adjacent extensive ductular reactions and desmoplasia, with areas resembling BIN (from cholangiocytes)	100% penetrance, iCCA exclusive, recombination in mature cholangiocytes	30 weeks average latency	116
Ck19–Cre^{ER}, *Kras*$^{LSL\text{-}G12D}$, *Tgfbr2*$^{flox/flox}$, *Cdh1*$^{flox/flox}$	Markedly thickened extrahepatic bile duct wall with a swollen gallbladder involving invasive periductal infiltrating-type eCCA and lymphatic metastasis (from biliary cells)	Low latency (4 weeks), eCCA exclusive	Concurrent development of lung adenocarcinomas leads to asphyxiation of mice	124
Pdx1–Cre, *Pik3ca*$^{LSL\text{-}H1047R/+}$	Adult mice develop enlarged extrahepatic bile duct and BIN with complete penetrance leading to eCCA (from well-differentiated, stroma-rich ductal adenocarcinomas to more undifferentiated)	eCCA exclusive, only one genetic hit driving CCA	~40 weeks average latency, 90% penetrance, wide tumour latency range	190

Table 3 (continued) | Summary of the most representative in vivo CCA models based on genetically engineered mice

Genetic strategy	Key features	Advantages	Disadvantages	Refs.
GEM-based implantation models				
LPCs from *Alb*–Cre, *Kras*$^{LSL\text{-}G12D}$, *Trp53*$^{LSL\text{-}R172H/lox\ +/-}$, FIG–ROS fusion	Allografted tumours resemble advanced CCA	Quick model, orthotopic implantation in the liver, iCCA exclusive, stroma presence	Requires technical training to isolate LPCs	100
LPCs or cholangiocytic progenitor cells or hepatocytes from *Trp53*$^{-/-}$ mice	Tumours exhibit a high stromal content and a mixed hepatocellular and cholangiocellular differentiation	Quick model	Not CCA exclusive	183
Adult liver organoids from *Kras*$^{LSL\text{-}G12D}$, *Trp53*$^{f/f}$ mice	*Kras*-driven organoids lead to CCA, while *Myc* expression in wild-type organoids induces HCC formation	Tumour latency of 6–8 weeks for *Kras*-mutated and *Trp53*-knockout organoids	Requires training in organoid isolation, growth and manipulation	12
Cholangiocytes from *Kras*$^{LSL\text{-}G12D}$, *Trp53*$^{f/f}$ mice	Tumours with a high stromal component expressing CCA markers	Quick and reproducible model, orthotopic implantation in the liver, iCCA exclusive, stroma presence	Requires technical training to isolate mouse cholangiocytes	73
GEM-based carcinogenic models				
Alb–Cre^{ERT2}, *R26*$^{RlacZ/+}$ or *Ck19*–Cre^{ERT2}, *R26*$^{RlacZ/+}$ mice treated with TAA	Macronodular liver cirrhosis containing cells the typical histology of CCA	100% penetrance, iCCA exclusive	Long latency (30 weeks)	191
Ck19–Cre$^{ERT/eYFP}$, *Trp53*$^{f/f}$ mice treated with TAA	Treatment with TAA generates oncogenic stress yielding multifocal invasive iCCA	iCCA exclusive	80% penetrance, long latency (>6 months)	125
Trp53$^{-/-}$ mice treated with CCl_4	Bile duct injury or necrosis, proliferation and fibrosis development triggered by CCl_4	Exclusive iCCA	50% of mice develop tumours, metastatic lesions rarely observed	126
Gsta3$^{-/-}$ mice treated with aflatoxin B1	Macroscopic and microscopic liver cysts, hepatocellular nodules, cholangiomas, iCCA, and oval cell proliferation	Participation of oval cells in tumorigenesis	Long latency (12 and 24 weekly aflatoxin B1 injections followed by a rest period of 12 and 6 months)	192
Alb–Cre, *Jnk1*$^{f/f}$, *Jnk2*$^{-/-}$ mice treated with DEN	Cystogenesis and cholangioma-like structures in liver parenchyma with strong infiltration of immune cells	Participation of inflammatory insult	No established CCA, long latency	188

AAV, adeno-associated virus; BIN, biliary intraepithelial neoplasia; CCA, cholangiocarcinoma; CCl_4, carbon tetrachloride; DDC, 3,5-diethyoxycarbonyl-1,4-dihydrocollidine; DEN, diethylnitrosamine; eCCA, extrahepatic CCA; GEM, genetically engineered mouse; HCC, hepatocellular carcinoma; iCCA, intrahepatic CCA; IPBN, intraductal papillary biliary neoplasm; LPC, bipotent liver progenitor cell; ROS, reactive oxygen species; TAA, thioacetamide; TTA, tetradecylthioacetic acid.

of high-throughput sequencing techniques, have three studies used exome sequencing or RNA sequencing analyses to perform deep molecular phenotyping of some of the most widely used CCA cell lines[131–133] (Supplementary Table 2). This has enabled the selection of cell lines with specific genetic alterations representing valuable drug screening tools, particularly for targeted therapy.

Most cell lines were established before the release of the latest WHO guidelines on the classification of tumours of the digestive system[134], and potential misclassification of the origin of some cell lines might affect the clinical translation of some molecular and functional studies. For instance, Mz-ChA-1 cells have traditionally been used as a CCA cell line[135,136], but they are classified as a gallbladder carcinoma cell line. Thus, results acquired using this cell line should be helpful for patients with this specific type of tumour.

In general, the well-established cell lines described in Supplementary Table 2 are easy models to explore tumorigenesis mechanisms and achieve high experimental reproducibility, mainly due to their long-term growth ability, short replication doubling time and low maintenance costs. However, several significant weaknesses have been described, such as long-term serum-based culture conditions that favour the accumulation of new genomic alterations as seen in many other long-term cultured cell lines[137–140]. New mutations obviously are not wanted in studying the effects of mutations leading to malignant outgrowth. Furthermore, in vitro maintenance often supports the selection of cell clones that are not representative of the genetic heterogeneity of the original tumour[123,137,141]. In addition, cell cultures grown as a monolayer might lack polarization and realistic cell–cell contacts within the tumour bulk. Finally, in the absence of cancer stromal cells and cell–matrix interactions, the fundamental tissue architecture provided by cellular and molecular components of the tumour microenvironment is not recapitulated[3,137].

In addition to immortalized 2D cell lines, primary cultures of human CCA tissue have been established[142–145]. The overall success rate for CCA cell line isolation and establishment is relatively low (approximately 10%)[146], partly due to insufficient numbers of tumour cells in resected tissues. Notably, contaminating non-tumour cells, such as fibroblasts, must be removed. Primary cultures are grown under serum-free and growth factor-enhanced conditions, which better resemble the in vivo tumour condition. Also, primary CCA cultures can be used shortly after derivation, so that more of the morphological and functional characteristics of their tissue of origin are retained[147]. In primary cultures, cell differentiation is constrained and the stem-like component is partially preserved, and thereby these cultures reflect tumour heterogeneity. However, the short time window to reach senescence in primary cultures hampers long-term experiments and their reproducibility.

A major limitation, independent of whether cell lines or primary CCA cultures are used, is the absence of acellular and cellular components of the tumour microenvironment, the presence of which would benefit the model. To address this problem[148,149], different strategies have emerged in 2D cell cultures, including conditioned medium experiments, indirect co-culture through porous membrane cell culture inserts[150] and direct co-culture[151]. In some cases, these experiments are performed with primary cultures of tumour and stromal cells (that are cancer-associated fibroblasts (CAFs) and monocytes/macrophages)[5,152]. In other cases, CCA cell lines are made to interact with immortalized stromal cell lines[148,150,153] (Supplementary Table 2). Although these systems do not fully recapitulate the complex tumour microenvironment, they enable study of the crosstalk between CCA cells and other cell types, deepening our understanding of the role of different stromal cell types in tumour progression and drug response mechanisms[148,149,152].

Based on statements on histological assessment (Table 1) and unanimous agreement, the expert panel (Box 1) strongly suggests to state in publications the origin of any cell line (previously established or new) according to the new CCA classification (iCCA, pCCA or dCCA). In addition, information regarding cell culture conditions should be provided in publications to standardize the procedures (such as choice of plastic support and cell culture medium, level of confluence, isolation procedure for primary culture, and passaging and subculturing methods).

3D culture recapitulating tumour organization

To facilitate personalized or precision medicine, patient material is used to study treatment responses. Although 2D CCA models are a step closer to the in vivo conditions in the patient compared with the established CCA cell lines, 3D culture models, including spheroids and organoids, resemble physiological conditions even more thoroughly. Spheroids are 3D aggregates of cells grown without a predefined culture substrate to adhere to[5,154], whereas organoids self-organize in a matrix-rich 3D environment with which they interact[155–157]. Although traditional organoids represent an epithelial cell culture, there is a consensus that 3D models should ideally be upgraded to include epithelial stem cells, cells from the tumour microenvironment (for example, fibroblasts and/or immune cells) and extracellular matrix components to enable the analysis of cell–cell and cell–matrix interactions.

Spheroids. Tumour spheroids, typically generated as 3D multicellular aggregates from 2D-grown adherent cells, sometimes including stromal cells such as fibroblasts and endothelial cells, are used to model tumour biology[5,154]. They can be grown in natural and/or synthetic hydrogels[157,158], and the increased complexity of these models enhances the understanding of tumour pathobiology, including tumour homeostasis and organization. In contrast to 2D cultures, tumour spheroids inherently recapitulate the gradient of oxygen supply and drug diffusion occurring within the tumour. However, their use as high-throughput, robust platforms is still limited due to the complexity of the culture conditions.

Organoids. Robust protocols for deriving biliary organoids from both mouse and human primary tissue explants or biopsy samples have been established[6,156], and are complemented by methods that enable the derivation and propagation of organoids from induced pluripotent stem cells[159] or cells collected from bile[160,161]. In addition to organoids derived from healthy donors, the successful establishment of organoid cultures from tumour tissues[6,7,9,162,163] can substantially add to the toolbox of preclinical and translational CCA research. The overall consensus in the field is that the efficiency of establishing these CCA organoids from different patient tumours should be at least 25%. Efficiency should reach over 50% to guarantee the applicability of organoids to personalized medicine. Working with CCA organoids inevitably has limitations, including the overgrowth of non-malignant cholangiocyte organoids. Using specific tumour enrichment medium[164], resorting to hand-picking non-malignant or tumour organoids to clean up the culture and xenotransplantation are ways to address this challenge. It is agreed that tumorigenicity needs to be confirmed for all CCA

Box 2

Experimental model sheet criteria

List of complete information on animal experimentation that should be provided for publication (the full table is available in Supplementary Table 3).

- Type of model (in vitro, ex vivo, in vivo)
- Species (mouse, rat, hamster, human, etc.)
- Sex (male, female, both)
- Strain
- Condition of the surrounding liver (apparently healthy, cirrhosis, fibrosis, etc.)
- Method of generation (spontaneous, carcinogenic, chronic injury, infectious, transgenic, knockout, transposon-mediated, patient-derived xenograft, organoids, isolated from animal tumours, isolated from human tumours, etc.)
- Tumour development (fast, slow)
- Metastasis (yes, no, locations, etc)
- Anatomical location of the lesions (when applicable) (intrahepatic, extrahepatic, both)
- Cell of origin (if available) (cholangiocyte, stem/progenitor cell, hepatocyte)
- Types of samples and storage conditions for future analyses
- Presence of preneoplastic lesions (yes/no)
- Type of preneoplastic lesions (IPNB, IPMN, BilIN, etc.)
- Type of cholangiocarcinoma (iCCA, pCCA, dCCA, combined HCC-CCA)
- Histology of tumours (large duct type, small duct type, CCA, lymphoepithelioma-like CCA, etc.)
- Microenvironment features (presence of stroma/desmoplastic reaction, absence of stroma, immune infiltration (yes/no))
- Phenotype of the lesions (CK7, CK19, MUC1, MUC2, MUC5AC, MUC6, HNF4A, AFP, markers of stemness, markers of EMT, etc.)
- Control samples used if applicable (bile duct freshly isolated from liver or cell line)

AFP, α-fetoprotein; BilIN, biliary intraepithelial neoplasm; CCA, cholangiocarcinoma; CK, cytokeratin; dCCA, distal CCA; EMT, epithelial–mesenchymal transition; HCC, hepatocellular carcinoma; HNF4A, hepatocyte nuclear factor 4α; iCCA, intrahepatic CCA; IPNB, intraductal papillary neoplasm of the bile duct; IPMN, intraductal papillary mucinous neoplasm; MUC, mucin; pCCA, perihilar CCA.

organoid lines, preferably via mutation analysis (standalone or as part of whole-genomic profiling). Proof of organoid tumorigenicity in immunocompromised mice and histopathological analysis are additional tests that can be performed. A shortcoming of CCA organoids is that an established line does not fully reflect the polyclonal nature of the original tumour. This might hamper insights into drug sensitivity or clonal regrowth of treated CCA tumours.

In addition to fully transformed CCA organoids, non-malignant cholangiocyte organoids can be a genetically flexible platform to functionally annotate the influence of specific genetic alterations on CCA pathobiology. Thus, recurrent iCCA genetic alterations (such as *BAP1*, *NF1*, *SMAD4*, *PTEN*, *KRAS*, *AKT* and *IDH1/2* mutations, and *FGFR2* fusions and MYC overexpression) were engineered in vitro in both human[165,166] and mouse liver organoids[167]. Collectively, these studies provide convincing evidence that liver organoids, in which few genetic hits were introduced to recapitulate recurrent patterns of putative iCCA driver mutations, give rise to CCA upon subcutaneous or orthotopic transplantation into mice. Therefore, this approach is suitable for modelling genetically-defined cholangiocarcinogenesis in bipotent liver precursors and generating models for precision oncology research[12].

Based on the available data and unanimous agreement, the expert panel strongly suggests (Box 1): the use of a specific tumour 'enrichment' medium (that is, tumour initiating medium as described by Broutier et al.[7]) to minimize contamination in non-tumour organoids; mutation and phenotypic analyses should be done to confirm the malignant origin of established organoid lines and reported in publications; and every organoid culture should be characterized before clinical applications such as drug screening.

Complex 3D culture systems

Although a hydrogel-based extracellular matrix is used to support the 3D growth of cells for both spheroids and organoids, this is typically a mouse tumour-derived basement membrane extract (Matrigel or BME) that does not represent the full human or tumour extracellular matrix[155]. Moreover, additional stromal cells, such as fibroblasts and immune cells are generally lacking in these cultures. The tumour microenvironment is crucial in the initiation, progression and invasion of CCA through a complex interaction between tumour cells, stromal cells and the extracellular matrix[168]. Targeting this desmoplastic, stroma-rich tumour microenvironment might be essential to overcome chemoresistance[169–171]. Thus, it would seem vital to include the CCA extracellular environment in vitro to mimic tumour composition, cell–cell and cell–matrix interactions[172], morphology and tumour architecture more closely.

Current efforts are focused on the generation of future complex models (assembloids) that integrate the epithelial CCA component with 3D bioprinted scaffolds that recapitulate the anatomy of the biliary system[173,174]. This includes immune cells that shape tumour growth and drug sensitivity through direct or paracrine interaction, and stromal cells that create a physical barrier for drug delivery in addition to a pro-tumorigenic microenvironment. The challenges reside in the co-culture of autologous cell types derived from the same patient, as each cell type will have a unique growth dynamic and timeline. The use of cryopreservation protocols and human-induced pluripotent stem cell-derived generation of cell types from the same background cell might overcome these issues.

Addressing clinical needs

The experimental models described previously will facilitate the translation from experimental and preclinical work to the clinical setting. Whereas some models have provided relevant insights into the basic mechanisms of cancer progression, unravelling and allowing the analysis of cell signalling pathways, and cell–cell or tumour–microenvironment interactions, others have provided results that can be cautiously translated into the design of more effective treatments for CCA or the development of new clinical trials in humans. A few studies have indicated that the use of genetically defined cellular and animal models can advance the discovery of actionable vulnerabilities associated with druggable iCCA oncogenic drivers. Specifically, three independent studies found that RAS–ERK signalling is necessary and sufficient to support the oncogenic activity of *FGFR2* fusions in PDXs[91], GEMMs[175] and organoid-based iCCA models[167], and that combination therapies capable of more robust and durable suppression of RAS–ERK improve the therapeutic efficacy of clinically approved FGFR tyrosine kinase inhibitors[91,167,175]. Likewise, *IDH1*/*KRAS*-driven models have revealed that pharmacological targeting of mutated IDH1 sensitizes iCCA to host-mediated immune responses, which can be enhanced by concomitant administration of immune checkpoint inhibitors[121].

The increasing availability of novel circulating biomarkers beyond the conventional serum tumour markers warrants validation for specific uses. Additional prognostic biomarkers might enable more accurate patient risk assessment and stratification in clinical trials. Predictive biomarkers for selecting the optimal therapy, such as circulating tumour DNA-based assays for *FGFR2* fusions and *IDH1* mutations[176,177], are already in clinical use and will push the field forward. Finally, additional pharmacodynamic biomarkers capable of tracking disease evolution more accurately than the carbohydrate antigen 19-9 (CA 19-9), which is a tumour marker used in the management of biliary and pancreatic cancer, and that can reveal the emergence of drug resistance are warranted[178], as shown for resistance to FGFR2 inhibitors[179]. Exposing FGFR2-mutated cells to an irreversible FGFR inhibitor (TAS-120) was found to provide a clinical benefit in patients who had developed resistance to reversible FGFR inhibitors via on-target mutations, the only exception being mutations targeting the gatekeeper residue.

CCA organoids have proven helpful for elucidating fundamental mechanisms of cancer progression and biomarker discovery[7]. Although the successful derivation of CCA organoids has lagged behind some other tumour types, organoids have high potential as tools for improving CCA research and therapy[180]. With further improvement in clinical applicability through continued advances in stem cell biology, organoid cultures and single-cell sequencing, a possible golden era for CCA organoids in personalized medicine is within reach.

A common limitation of experimental models is their inability to fully mimic all aspects of the tumour biology and personalized cancer features of individual patients. For example, the tumour microenvironment is a complex mix of cancerous and non-cancerous cells. The extracellular matrix dynamics, that is constantly remodelled by tumour cells, CAFs and tumour-associated macrophages, create a desmoplastic environment. In addition, there is considerable heterogeneity within and between tumours. It is challenging to capture this in experimental models, but it is essential in assessing drug resistance and tumour progression. Owing to the lack of the tumour microenvironment, drug screenings performed in vitro do not fully reflect in vivo efficacy, resulting in newly developed drugs failing in phase I to phase III clinical trials[181]. Finally, common risk factors and co-existing diseases that characterize human CCA (primary sclerosing cholangitis, liver flukes, chronic viral hepatitis, liver cirrhosis and others) are generally absent in the existing models. Thus, generating new models that combine

established risk factors and concomitant morbidities for the human tumour with specific genetic alterations such as those reported earlier might recapitulate human CCA more accurately.

Study strengths and limitations

The Delphi method was applied to reach a consensus on the criteria required to establish valid preclinical models for the study of CCA. For this purpose, we built a task force of 45 renowned experts. Although we recognized that a more extensive panel could be preferred, we believe that the number of experts, their relevance in the CCA field, and the variety of backgrounds represented, including basic scientists, pathologists and clinicians, strengthened the validity of the consensus. During the process, the experts raised numerous comments, suggestions and questions, which were openly and rigorously discussed and incorporated into the study. This interactive and dynamic approach and the absence of dominant voices, which often inhibit the expression of minority viewpoints, resulted in fair and balanced contributions and the achievement of the final consensus statements and recommendations.

Experimental models are essential for a better understanding of carcinogenesis and tumour progression, for testing antitumour therapies and for deciphering therapeutic resistance mechanisms. A wide range of experimental models of CCA are available from simple, practical and inexpensive to more complex models resembling human cancer biology, albeit with a more challenging implementation process and higher costs (Table 4). The choice of model depends on what is requested of it, its accessibility, and, most importantly, its ability to answer a well-defined scientific question. 2D cultures and engrafted subcutaneous murine models are the most-used models to dissect signalling pathways, identify therapeutic targets and investigate drug resistance mechanisms. Depending on the type of research, in vivo orthotopic implantation models are preferred over ectopic CCA models. Both have advantages and limitations, as previously discussed. GEMMs seem to mimic pathobiological features of human tumorigenesis more closely than other models, despite being complex and expensive. Regarding in vitro models, tremendous progress has been made in better recapitulating the tumour 3D structure. The difficulty in employing these models includes the relatively high

Table 4 | Benefits and limitations of CCA experimental models

Model	Benefits	Limitations
In vivo models		
Engrafted models: xenograft	Engraftment of human cells or tissue Ectopic engraftment inexpensive and easy to implement Easy-to-measure ectopic tumours Commonly used for drug testing	Defective immune system Ectopic allograft poorly relevant Rate of human CCA tissue ectopic engraftment (PDX) very low Orthotopic engraftment difficult to perform
Engrafted models: allograft	Full immune system Ideal for studying tumour–stroma interplay Fully compatible for testing immunotherapy-based therapies	Ectopic allograft poorly relevant Orthotopic engraftment difficult to perform
Chemically induced	Recapitulate development of CCA (TAA) with pre-cancerous disease history Long-term furan treatment induces 100% of tumour incidence	Highly variable Control tissue: isolated bile duct and not whole liver
GEMM	Design to mimic genetic alterations in human CCA Model of advanced CCA Valuable tool for testing targeted therapies	Fast tumour development Origin of CCA multiple Appearance of mixed HCC–CCA tumour Costly
In vitro models		
2D culture with cell lines or primary cells	Easy and low maintenance costs High experimental reproducibility Large panels of cell lines commercially available Cells available with genetic alteration(s)	Absence of stromal cells Cultures grown as a monolayer
3D culture recapitulating a tumour organization: spheroids	Can be patient-derived Increased complexity through 3D multicellular aggregates of epithelial cells and stromal cells Recapitulate the gradient of oxygen supply and drug diffusion	Limited use for high-throughput analysis Often made from cell lines Do not fully reflect the polyclonal nature of a CCA tumour
3D culture recapitulating a tumour organization: organoids	Increased complexity by 3D tumour cell growth in ECM Well-established protocol Specific mutations can be introduced in non-tumour organoids to analyse CCA driver mutations	Low initiation efficiency from human tumours An established line does not fully reflect the polyclonal nature of the original tumour Overgrowth of non-tumour cells on culture initiation Absence of stromal cells

CCA, cholangiocarcinoma; ECM, extracellular matrix; GEMM, genetically engineered mouse model; HCC, hepatocellular carcinoma; PDX, patient-derived xenograft; TAA, thioacetamide.

costs of setting up the culture and the availability of starting material (human CCA tissue).

In addition to providing an inventory, including evaluating advantages and disadvantages, of the most accurate experimental models currently available to the CCA scientific community, we present recommendations on minimal criteria for using these models. Using a Delphi-based process, a panel of experts in the field reached a consensus on these criteria as proposed herein. Obviously, disease models should ultimately lead to knowledge transfer from (basic) laboratory research to the clinic, to better understand the disease and offer innovative therapies. As the choice of model is highly dependent on the research question, it is highly recommended that results are gathered using different models to provide a comprehensive tumour mimic. This fosters the consolidation of scientific data with well-defined minimal criteria before validating them in humans by manipulating ex vivo samples or clinical trials.

Conclusions

Biomedical research relies entirely on in vitro and in vivo experimental models, a prerequisite for research in basic and applied sciences. This Consensus Statement is based on a set of recommendations on experimental models of CCA and information that should be specified in publications on these models developed and endorsed by an international group of experts to provide guidance to the scientific community. As a complement, the experts provided a brief overview of currently available models, highlighting the advantages and disadvantages that scientists should be aware of. Importantly, this Consensus Statement was prepared based on the expertise of both researchers and clinicians from different specialties (cell biologists, molecular biologists, oncologists, hepatologists and pathologists), and thus ensures the relevance of these statements and recommendations for a broad range of scientific communities, from health-care professionals to scientists directly investigating this fatal cancer.

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Correspondence should be addressed to Laura Fouassier.

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Diego F. Calvisi[1], Luke Boulter[2,3], Javier Vaquero[4,5], Anna Saborowski[6], Luca Fabris[7,8], Pedro M. Rodrigues[5,9,10], Cédric Coulouarn[11], Rui E. Castro[12], Oreste Segatto[13], Chiara Raggi[14], Luc J. W. van der Laan[15], Guido Carpino[16], Benjamin Goeppert[17,18], Stephanie Roessler[19], Timothy J. Kendall[20], Matthias Evert[1], Ester Gonzalez-Sanchez[4,5,21], Juan W. Valle[22,23], Arndt Vogel[6], John Bridgewater[24], Mitesh J. Borad[25], Gregory J. Gores[26], Lewis R. Roberts[26], Jose J. G. Marin[5,27], Jesper B. Andersen[28], Domenico Alvaro[29], Alejandro Forner[5,30], Jesus M. Banales[5,9,10,31], Vincenzo Cardinale[32], Rocio I. R. Macias[5,27], Silve Vicent[33,34,35], Xin Chen[36], Chiara Braconi[37], Monique M. A. Verstegen[15], Laura Fouassier[38] ✉ & CCA Model Consortium*

CCA Model Consortium

Diego F. Calvisi[1], Luke Boulter[2,3], Javier Vaquero[4,5], Anna Saborowski[6], Luca Fabris[7,8], Pedro M. Rodrigues[5,9,10], Cédric Coulouarn[11], Rui E. Castro[12], Oreste Segatto[13], Chiara Raggi[14], Luc J. W. van der Laan[15], Guido Carpino[16], Benjamin Goeppert[17,18], Stephanie Roessler[19], Timothy J. Kendall[20], Matthias Evert[1], Ester Gonzalez-Sanchez[4,5,21], Juan W. Valle[22,23], Arndt Vogel[6], John Bridgewater[24], Mitesh J. Borad[25], Gregory J. Gores[26], Lewis Roberts[26], Jose J. G. Marin[5,27], Jesper B. Andersen[28], Domenico Alvaro[29], Alejandro Forner[5,30], Jesus M. Banales[5,9,10,31], Vincenzo Cardinale[32], Rocio I. R. Macias[5,27], Silve Vicent[33,34,35], Xin Chen[36], Chiara Braconi[37], Alexander Scheiter[1], Florin M. Selaru[39], Katja Evert[1], Kirsten Utpatel[1], Laura Broutier[40,41], Massimiliano Cadamuro[42], Meritxell Huch[43], Robert Goldin[44], Sergio A. Gradilone[45,46], Yoshimasa Saito[47], Monique M. A. Verstegen[15] & Laura Fouassier[38]

[1]Institute of Pathology, University of Regensburg, Regensburg, Germany. [2]MRC Human Genetics Unit, Institute of Genetics and Cancer, University of Edinburgh, Edinburgh, UK. [3]Cancer Research UK Scottish Centre, Institute of Genetics and Cancer, Edinburgh, UK. [4]TGF-β and Cancer Group, Oncobell Program, Bellvitge Biomedical Research Institute (IDIBELL), Barcelona, Spain. [5]National Biomedical Research Institute on Liver and Gastrointestinal Diseases (CIBEREHD), Instituto de Salud Carlos III, Madrid, Spain. [6]Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical

School, Hannover, Germany. [7]Department of Molecular Medicine, University of Padua School of Medicine, Padua, Italy. [8]Digestive Disease Section, Yale University School of Medicine, New Haven, CT, USA. [9]Department of Liver and Gastrointestinal Diseases, Biodonostia Health Research Institute - Donostia University Hospital, University of the Basque Country (UPV/EHU), San Sebastian, Spain. [10]Ikerbasque, Basque Foundation for Science, Bilbao, Spain. [11]Inserm, Univ Rennes 1, OSS (Oncogenesis Stress Signalling), UMR_S 1242, Centre de Lutte contre le Cancer Eugène Marquis, Rennes, France. [12]Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, Lisbon, Portugal. [13]Translational Oncology Research Unit, IRCCS Regina Elena National Cancer Institute, Rome, Italy. [14]Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy. [15]Department of Surgery, Erasmus MC Transplantation Institute, University Medical Center Rotterdam, Rotterdam, Netherlands. [16]Department of Movement, Human and Health Sciences, Division of Health Sciences, University of Rome "Foro Italico", Rome, Italy. [17]Institute of Pathology and Neuropathology, Ludwigsburg, Germany. [18]Institute of Pathology, Kantonsspital Baselland, Liestal, Switzerland. [19]Institute of Pathology, Heidelberg University Hospital, Heidelberg, Germany. [20]Centre for Inflammation Research, University of Edinburgh, Edinburgh, UK. [21]Department of Physiological Sciences, Faculty of Medicine and Health Sciences, University of Barcelona, Barcelona, Spain. [22]Department of Medical Oncology, The Christie NHS Foundation Trust, Manchester, UK. [23]Division of Cancer Sciences, University of Manchester, Manchester, UK. [24]Department of Medical Oncology, UCL Cancer Institute, London, UK. [25]Mayo Clinic Cancer Center, Mayo Clinic, Phoenix, AZ, USA. [26]Division of Gastroenterology and Hepatology, Mayo Clinic College of Medicine and Science, Rochester, MN, USA. [27]Experimental Hepatology and Drug Targeting (HEVEPHARM), IBSAL, University of Salamanca, Salamanca, Spain. [28]Biotech Research and Innovation Centre (BRIC), Department of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark. [29]Department of Translational and Precision Medicine, Sapienza University of Rome, Rome, Italy. [30]Liver Unit, Barcelona Clinic Liver Cancer (BCLC) Group, Hospital Clinic Barcelona, IDIBAPS, University of Barcelona, Barcelona, Spain. [31]Department of Biochemistry and Genetics, School of Sciences, University of Navarra, Pamplona, Spain. [32]Department of Medico-Surgical Sciences and Biotechnologies, Sapienza University of Rome, Rome, Italy. [33]University of Navarra, Centre for Applied Medical Research, Program in Solid Tumours, Pamplona, Spain. [34]IdiSNA, Navarra Institute for Health Research, Pamplona, Spain. [35]Centro de Investigación Biomédica en Red de Cáncer (CIBERONC, Instituto de Salud Carlos III), Madrid, Spain. [36]Department of Bioengineering and Therapeutic Sciences and Liver Center, University of California, San Francisco, CA, USA. [37]School of Cancer Sciences, University of Glasgow, Glasgow, UK. [38]Sorbonne Université, Inserm, Centre de Recherche Saint-Antoine (CRSA), Paris, France. [39]Division of Gastroenterology, Department of Medicine and Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, Baltimore, MD, USA. [40]Childhood Cancer & Cell Death (C3), Université Claude Bernard Lyon 1, INSERM 1052, CNRS 5286, Centre Léon Bérard, Centre de Recherche en Cancérologie de Lyon (CRCL), Lyon, France. [41]The Wellcome Trust/CRUK Gurdon Institute, University of Cambridge, Cambridge, UK. [42]University of Padua, Department of Molecular Medicine (DMM), Padua, Italy. [43]Max Planck Institute of Molecular Cell Biology and Genetics (MPI-CBG), Dresden, Germany. [44]Division of Digestive Sciences, Imperial College, London, UK. [45]The Hormel Institute, University of Minnesota, Austin, MN, USA. [46]Masonic Cancer Center, University of Minnesota, Minneapolis, MN, USA. [47]Division of Pharmacotherapeutics, Keio University Faculty of Pharmacy, Tokyo, Japan.