Criteria for preclinical models of cholangiocarcinoma: scientific and medical relevance

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

[1] Institute of Pathology, University of Regensburg, Regensburg, Germany;
[2] MRC-Human Genetics Unit, Institute of Genetics and Molecular Medicine, University of Edinburgh, Edinburgh, UK;
[3] TGF-β and Cancer Group, Oncobell Program, Bellvitge Biomedical Research Institute (IDIBELL), Barcelona, Spain;
[4] National Biomedical Research Institute on Liver and Gastrointestinal Diseases (CIBEREHD), Instituto de Salud Carlos III, Madrid, Spain;
[5] Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Hannover, Germany;
[6] Department of Molecular Medicine, University of Padua School of Medicine, Padua, Italy;
[7] Digestive Disease Section, Yale University School of Medicine, New Haven, CT, USA;
[8] Department of Liver and Gastrointestinal Diseases, Biodonostia Health Research Institute - Donostia University Hospital, University of the Basque Country (UPV/EHU), San Sebastian, Spain;
[9] Ikerbasque, Basque Foundation for Science, Bilbao, Spain;
[10] Inserm, Univ Rennes 1, OSS (Oncogenesis Stress Signaling), UMR_S 1242, Centre de Lutte contre le Cancer Eugène Marquis, F-35042, Rennes, France;
[11] Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, Lisbon, Portugal;
[12] Unit of Oncogenomics and Epigenetics, IRCCS Regina Elena National Cancer Institute, Rome, Italy;
[13] Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy;
[14] Department of Surgery, Erasmus MC Transplantation Institute, University Medical Center Rotterdam, The Netherlands;
[15] Department of Movement, Human and Health Sciences, Division of Health Sciences, University of Rome "Foro Italico", Rome, Italy;
[16] Institute of Pathology and Neuropathology, Ludwigsburg, Germany;
[17] Institute of Pathology, Heidelberg, Germany;
[18] Centre for Inflammation Research, University of Edinburgh, Edinburgh, UK;
[19] Department of Physiological Sciences, Faculty of Medicine and Health Sciences, University of Barcelona, Spain
[20] Department of Medical Oncology, The Christie NHS Foundation Trust, Manchester, UK;
[21] Division of Cancer Sciences, University of Manchester, Manchester, UK;
[22] Department of Medical Oncology, UCL Cancer Institute, London, UK;
[23] Mayo Clinic Cancer Center, Mayo Clinic, Phoenix, AZ, USA;
[24] Division of Gastroenterology and Hepatology, Mayo Clinic College of Medicine and Science, Rochester, MN, USA;

[25] Experimental Hepatology and Drug Targeting (HEVEPHARM), IBSAL, University of Salamanca, Salamanca, Spain;
[26] Biotech Research and Innovation Centre (BRIC), Department of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark;
[27] Department of Precision and Translational Medicine, Sapienza University of Rome, Rome, Italy;
[28] Liver Unit, Barcelona Clinic Liver Cancer (BCLC) Group, Hospital Clinic Barcelona, IDIBAPS, University of Barcelona, Barcelona, Spain;
[29] Department of Biochemistry and Genetics, School of Sciences, University of Navarra, Pamplona.
[30] Department of Medico-Surgical Sciences and Biotechnologies, Sapienza University of Rome, Rome, Italy;
[31] University of Navarra, Centre for Applied Medical Research, Program in Solid Tumours, Pamplona, Spain;
[32] IdiSNA, Navarra Institute for Health Research, Pamplona, Spain;
[33] Centro de Investigación Biomédica en Red de Cáncer (CIBERONC, Instituto de Salud Carlos III), Madrid, Spain;
[34] Department of Bioengineering and Therapeutic Sciences and Liver Center, University of California, San Francisco, USA;
[35] Institute of Cancer Sciences, University of Glasgow, Glasgow, UK;
[36] Sorbonne Université, Inserm, Centre de Recherche Saint-Antoine (CRSA), Paris, France

Corresponding author

Laura Fouassier, Ph.D.

Sorbonne Université, INSERM Centre de Recherche Saint-Antoine

27 rue Chaligny

75571 Paris cedex 12

France

E-mail:mailto: laura.fouassier@inserm.fr

Phone: +33-698774001

Abstract

Cholangiocarcinoma (CCA) is a rare malignancy developing at any point along the biliary tree. CCA has a poor prognosis, its clinical management remains challenging, and effective treatments are lacking. Preclinical research, therefore, 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 preclinical model is guided by the question(s) to be addressed, and ideally, results should be recapitulated in independent approaches. Here, a task force of 45 experts in CCA molecular and cellular biology, clinicians, including pathologists, from 10 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 37 (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 is to transfer (basic) laboratory research to the clinics through increased disease understanding and develop clinical biomarkers and innovative therapies for patients with CCA.

During the last decade, we witnessed considerable advances in understanding the molecular pathogenesis of cholangiocarcinoma (CCA). However, early diagnosis and effective treatments for this aggressive cancer lag behind 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 (i.e., intrahepatic, perihilar, or distal CCA), the cell(s) of origin (e.g., preneoplastic lesions), and the histomorphological tumour features (e.g., large vs. small bile duct type) [2].

Historically, 2D cell cultures have been widely used as *in vitro* model of CCA. In addition to experimentally-immortalized or primary cultures of normal cholangiocytes derived from normal bile ducts, over 50 CCA-derived cell lines have been established [3]. A limitation of these models is the lack of resemblance to the original tumours upon the continuous culturing, making it difficult to infer which therapeutics would have been efficient to treat the original neoplasm [4]. Moreover, 2D mono-cultures do not accurately mimic the characteristic features of biliary tumours, namely the three-dimensional architecture, cell-to-cell, and cell-to-matrix interactions, cellular heterogeneity, and the effect of the tumour microenvironment in cancer progression. To overcome these limitations, multicellular 3D models, such as spheroids and organoids, have been developed. Although they constitute valuable models to study CCA [5], spheroids usually do not precisely recapitulate the native tissue architecture and function of the tissue of origin [6]. In contrast, organoids maintain a higher and more predictable physical order in the 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. CCA induction through administering hepatocarcinogens or liver fluke infestation has the advantage of mimicking cancer pathogenesis. However, animal studies are time-consuming, expensive, ethically challenging, and sometimes, hepatocellular carcinoma (HCC) rather than CCA preferentially develops. To give *in vivo* context to 2D cell lines, CCA cells have been used to generate subcutaneous or orthotopic xenografts in mice. However, these approaches remain limited by poor rates of tumor engraftment. Technological advancements have made it possible to grow liver organoids, i.e., 3D cultures of bipotent liver precursors, and therefore develop mouse models based on transplantation of genetically modified liver organoids that undergo *in vivo* oncogenic transformation along the cholangiocellular lineage [10].

Alternatively, genetically-engineered mouse models (GEMMs) recapitulating the most frequent genetic alterations detected in CCA have been generated [11].

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

Methods

Panel of experts

A core group of 8 core 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 the initial Delphi questionnaire and are listed authors, and they proposed 10 additional experts to fill the second and final questionnaire. These 10 experts, not actively involved in writing the recommendations but providing their precious input by filling the second questionnaire, are listed as one collaborative author; CCA Model consortium. Thus, the final panel consisted of 45 individual experts from 10 countries located in Europe, Asia, and USA. **Supplementary Table S1** summarizes the expert panel's names, institutes, and demographics.

Building consensus

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

Questionnaires

The core team generated the questionnaires using an online Google Form (Alphabet Inc., CA) before sending them out to the experts. The first questionnaire consisted of 47 questions, divided over 4 parts:

Part 1.Defining minimal and advanced criteria for experimental models, Part 2.*In vivo* model for CCA, Part 3.*In vitro* models for CCA, and Part 4.Preclinical models for CCA. Based on questionnaire 1 (Supplemental data 1), a second questionnaire was designed, including 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 (**Figure 1**). From the second questionnaire, core recommendations were edited (new **Box 1**).

Clinical features to consider when using experimental models

Clinics

Experimental models of CCA must reflect the natural history of the known subtypes of CCA, their molecular heterogeneity, and the impact of clinical or therapeutic interventions. In ICD11, published in 2022, CCA is classified according to its origin as intrahepatic (iCCA) and extrahepatic (eCCA) (https://icd.who.int). iCCA arises from intrahepatic bile ducts, i.e., it grows in the liver. Consequently, it is more often surgically resectable than perihilar CCA (pCCA), the latter arising at the liver hilum where the likelihood of local vascular invasion is greater [12]. The impact 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 [13-16]. The reason why iCCAs are more molecularly heterogeneous than p/dCCAs is not fully understood and requires detailed examination. In addition, the influence of biology on the natural history of iCCA and its impact on surgical, local, and systemic treatment options necessitate further studies [17]. dCCA more closely resembles pCCA, but, again, the effect of both anatomy and biology on outcome has not been fully elucidated. However, many tools only seek to mimic iCCA, and there is a critical absence of pCCA and dCCA models.

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 [18-21]. 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 unraveled to develop and assess novel interventions to overcome resistance before clinical testing.

Pathology

Separate classifications (UICC, AJCC, WHO) exist for iCCA, pCCA, and dCCA. Macroscopic features divide iCCA into two subtypes: large duct and small duct [22]. 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 [23].

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 perihilar and distal CCAs [24]. Most p/dCCAs are adenocarcinomas with pancreaticobiliary morphology, comprising glandular structures and/or small groups of cells within the desmoplastic stroma [24].

Immunohistochemistry. No specific immunohistochemical pattern for CCA lesions exists. However, they typically show an upper gastrointestinal/pancreaticobiliary pattern of cytokeratin (CK) expression (CK7+, CK19+, CK20-negative) when they still exhibit some degree of differentiation. In addition, large duct iCCAs sometimes express intestinal markers (e.g., CK20 and CDX2) [25]. CCA is usually immunonegative for HepPar-1, arginase-1, and glypican-3, distinguishing it from HCC and combined HCC/CCA. Transcription factors marking cell-specific lineages such as TTF-1 (lung and thyroid cancers), PAX8 (renal, thyroid, ovarian, and endometrial cancers), and GATA-3 (breast and urothelial cancers) are not usually expressed in CCA.

Biliary precursor lesions. CCA could develop from precursor lesions. Most cases of large duct iCCA and p/dCCA presumably originate from biliary intraepithelial neoplasia [26]. Intraductal papillary neoplasm of the bile duct (IPNB) is an intraductal papillary proliferation that develops in intrahepatic (70%) or perihilar ducts (30%) [27,28]. Invasive malignancy is evident in > 50% of IPNBs at presentation. Furthermore, the mucinous cystic neoplasm is a cystic epithelial tumour occurring almost exclusively in females, associated with CCA in 5% of cases [29,30].

Molecular profiling

Efforts to understand the heterogeneity of CCA have provided insights into the molecular pathogenesis and anatomical complexity of this disease [13,31-38]. The genetic landscapes fall midway in the mutational spectrum of cancers [39], with shared genetic alterations between iCCA, pCCA, and dCCA [36]. Although

the gained comprehensive insight into the underlying pathobiological processes of resectable invasive tumours, the precise involvement of genetic and epigenetic mechanisms in the onset of CCA is still insufficient.

Integrated genomics approaches have been used to classify CCA patients based on prognosis [40-43], emphasizing dysregulated oncogenic signalling pathways, including WNT-CTNNB1, MYC, PI3K-AKT-mTOR, ERBB, RAS-RAF-ERK, TNF, PLK1, TGFβ, NOTCH, IGFR1, VEGF, and the Hippo cascade. This predominant molecular classification highlights distinct tumour phenotypes of either inflammatory or proliferative in nature [41]. Moreover, iCCA can be classified based on driver-gene mutations elucidating unique mutational signatures, structural variants, and epigenomic alterations [35]. Of note, specific oncogenic mechanisms in distinct patient subsets with potential unique drug responses like RNA synthesis inhibition in *IDH*-mutant, microtubule modulator in *KRAS*-mutant, topoisomerase inhibition in *TP53*-mutant, and mTOR inhibitors in wild-type tumours enriched in FGFR2 fusions [13].

As the three anatomical CCA subtypes differ in their molecular alterations [36] and potentially in the cell-of-origin [44-47], the CCA subtypes should be studied in separate experimental models [2]. However, the step-wise progression of human CCA and thus the accumulation of a wide variety of molecular alterations may not be reflected in the most rapid mouse models. 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 may represent a relevant strategy to define the best-fit models as previously demonstrated for HCC [48,49].

In vivo CCA models

Engrafted models

Xenograft. Xenografts consist of transplanting tissues or cells from a different species into an immunodeficient host [50]. 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 in the liver (orthotopic grafts). These experimental animal models help 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 [50-53]. 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 [54 55-58]. Additionally, the role of various proteins [59-64] and miRNAs [65-69] 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 implantation rates, with some not generating tumours after injection. Furthermore, these tumours are implanted in a non-physiological site, seldom metastasize, and may lose the molecular heterogeneity characteristic of human CCA. Most importantly, they do not allow the study of the crosstalk between tumour cells, the multicellular microenvironment milieu, and the immune system [50-53].

Using orthotopic xenograft models may overcome some of these limitations by developing tumours directly in the organs of origin. Orthotopic grafts are more likely to trigger tumour dissemination, with the development of distant metastases. Intrahepatic implantation of CCA cells can be achieved either by injecting cells directly into the liver parenchyma using ultrasound-guided injection [70] or through the portal or splenic vein [50]. Small fragments of CCA tumours previously generated in subcutaneous xenografts or cancer stem cell-derived spheroids can also be orthotopically implanted [71,72]. Although intrasplenic injection is technically easier than intraportal administration and carries fewer post-operative complications, the implantation of CCA cells by intrasplenic injection resulted in successful engraftment not only in the liver, but also in the spleen [73]. Of note, intrasplenic injection of EGI-1 CCA cells also induced the development of lung metastases [74]. Still, generating orthotopic models is more time-consuming, and some post-operative complications may arise. Furthermore, the tumour development, growth, and metastases assessment requires imaging techniques or is only determined at sacrifice [50,53]. In this sense, using luciferase-expressing CCA cells is an excellent choice to monitor tumour growth over time [73]. However, this tool might not be accessible to all.

Engrafting cells or tissues directly obtained from patients may result in the development of patient-derived xenografts (PDXs). Subcutaneous or orthotopic tumours usually maintain the original genetic and epigenetic features and surrounding stroma observed in the initial mass, thus constituting the ideal model to predict therapeutic responses and being excellent tools in personalized medicine. Indeed, several studies have already used PDXs to examine tumours harbouring specific mutational patterns and test the use of specific targeted therapies [75-79]. Nevertheless, the success of PDX engraftment is

relatively low, depending on the primary tumour itself and the experimental design for tumor engraftment. Thus, they constitute a time and resource-intense model and may require several months for successful implantation [50]. 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 tumor shown in the publication (Box1).

Allograft (syngeneic). Syngeneic models have the advantage of implanting murine CCA cells into an immunocompetent host, displaying a fully-functional immune system. The first syngeneic model was developed when 2 rat CCA cell lines (BDEneu and BDEsp) were directly implanted in the biliary tract of Fisher 344 rats. While BDEsp engraftment induces the development of non-metastatic iCCA, BDEneu-derived tumours were more aggressive, with the rapid and consistent formation of CCA lesions and metastases [80,81]. This model was used to elucidate the mechanisms underlying tumour progression and evaluate the efficacy of novel drug candidates [81-85]. More recently, a novel syngeneic murine model was reported by engrafting the malignant mouse cell lines SB1-7, obtained from a bile-duct ligation and transposon-based CCA model into mice [86,87]. The obtained cell lines were successfully implanted, leading to CCA lesions resembling human CCAs [87]. In addition, foetal liver cells obtained from genetically-modified mouse embryos may also be implanted in the mouse liver, inducing CCA formation [88]. Furthermore, the cells mentioned above can be genetically manipulated before engraftment, revealing insights into the mechanisms governing cholangiocarcinogenesis and allowing the implantation of the cells in already established knockout mice strains, thus permitting the study of alterations in specific genes in the tumour stroma [89]. In this line, unpublished observations from the SB1 orthotopic model indicate that extending 2 weeks the frequently used endpoint (4 weeks) allows the formation of extrahepatic metastases in the lung. Therefore, further characterization of this timeline in a genetically malleable immunocompetent host, coupled with the isolation of tumor cells from the original site of injection and the metastatic sites, could provide an excellent model to understand, and perhaps even prevent, a rather understudied process such as CCA metastatic spreading. Overall, these models may overcome xenograft limitations, such as the absence of the immune system, are ideal for studying tumour-stroma interactions, and are an excellent alternative to test 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 [90]. In some cases, these tumours develop in the context of chronic diseases, 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 two years [91]. 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 [92]. 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, akin to what is observed in patients with chronic cholangiopathies. Therefore, combined with bile duct ligation (BDL), a classical model of obstructive cholestasis and subsequent bile duct proliferation, TAA accelerates the formation of biliary tumours [93]. Different from TAA, several mutagenic models have also been developed to induce CCA in rodents. For instance, diethylnitrosamine (DEN) and dimethylnitrosamine (DMN) generate DNA adducts in the liver and suffice for liver carcinogenesis [94], and in combination with inflammatory injury (BDL or *O. viverrini* infection), drive CCA development in mice and hamsters [95-97]. Furan is a potent mutagen capable of initiating CCA in rats [98]. Long-term furan treatment is currently the only chemically-induced model of CCA with nearly 100% of tumour incidence, which results in multi-organ metastases and closely recapitulates the primary and secondary pathologies of human CCA. Available models are summarised in **Table 2** and **Figure 2**.

Although many rat and mouse models driven by chemical insults reflect both the pre-cancerous disease history and 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). Recent work has focused on combining the disease-inducing aspects of these models, such as inflammation and fibrosis, with GEMMs, discussed in more detail in the following section. A critical point to consider is the control tissue that should be compared with malignant biliary

cells. Indeed, as the whole liver is inappropriate since hepatocytes are the prevalent cell population, isolated bile ducts should be considered the best control.

Genetically Engineered Mouse Models (GEMMs)

GEMMs are advanced animal models of human cancer (**Table 3**). They are rationally designed to mimic human CCA's genetic and epigenetic alterations, aberrant activation of signalling pathways, and the sequence of preneoplastic and early and late tumour stages, including metastasis. In addition, GEMMs can be coupled to *in vivo* transfection (HTVI and/or electroporation) or injection (adeno-associated-viruses, AAV) approaches to activate/express transgenes in adult hepatocytes to further expand the mouse model toolbox [99].

General concerns precluding the use of GEMMs are their high cost, tumour latency, and embryonic Cre expression in non-inducible models that may 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. A summary of selected GEMMs is provided herein.

Most CCA GEMMs incorporate common oncogenic alterations found in humans, including inactivation of tumour suppressor genes (*PTEN*, *SMAD4*, *P53*) or induction of oncogenes (*KRAS*, *IDH1/2*, *AKT1*, *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 [100]. *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 the concomitant *Trp53* abrogation and *KrasG12D* expression in the Alb-Cre mouse background [101]. This model features premalignant biliary lesions (intraductal papillary neoplasms and Von Meyenburg complexes), leading to invasive carcinoma and distal metastases. To directly probe the cell of origin in this model, *Kras*$^{LSL-G12D/+}$; *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) [102]. *KrasG12D* activation and *Trp53* loss in adult hepatocytes required co-administration of DDC-diet to form tumours (iCCA and HCC with a similar incidence, in addition to combined HCC/CCA), highlighting the role of inflammation on liver cancer

formation. By contrast, activation of the transgenes in the adult ductal compartment in the *Sox9-Cre*$^{ERT2+}$ accelerated the development of hepatic tumours, mainly iCCA, from preneoplastic lesions (not found in AAV8-injected mice) without the need for inflammatory cues.

Targeting *KrasG12D* activation and *Pten* deletion triggered the fastest GEMM in Alb-Cre mice [103]. In Kras$^{LSL-G12D/+}$; 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 *KrasG12D* activation developed tumours after longer latency, showing hepatocyte and cholangiocyte differentiation features. By using Alb-Cre^{ERT2+} or K19Cre$^{ERT/+}$ mouse strains to activate the oncogenic alterations in adult hepatocytes or cholangiocytes, respectively, the authors reported the development of HCC and HCC-precursor lesions, but not iCCA, in 8-week old Alb-Cre^{ERT2+}; *Kras*$^{LSL-G12D}$; *Pten*$^{flox/flox}$ mice, while tamoxifen injection on day 10 elicited iCCA. The formation of iCCA in Alb-Cre^{ERT2+}; *Kras*$^{LSL-G12D}$; *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 [104].

IDH1/2 oncogene modelling in mice was employed [105,106]. Breeding of *Idh2*$^{LSL-R172K}$ and *Kras*$^{LSL-G12D}$ 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 intra-epithelial neoplasia-like lesions, suggestive of preneoplastic stages, occurred. In more recent work, the same group generated *Idh1*^{LSLR132C} mice that developed iCCA upon crossing with *Kras*$^{LSL-G12D}$ mice in the *Alb-Cre* background [107]. Another oncogene investigated in *Alb-Cre* mice was *Notch1*, via a mouse strain expressing the Notch 1 intracellular domain (NICD) from the *Rosa26* locus [108]. By 8 months post-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 [109]. Mice developed hepatocyte and cholangiocyte regenerative foci, the latter resembling human biliary intra-epithelial 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, *KrasG12D* expression and deletion of both *Tgfβr2* and *Cdh1* (E-cadherin) were achieved in adult CK19^{+} biliary cells, leading to early-onset metastatic tumours in the extrahepatic and hilar bile duct [110]. Dying

cholangiocytes in response to E-cadherin ablation released IL-33 to foster a proliferative 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 [109] or derivation of tumour organoids from mice [110] allowed follow-up experiments otherwise limited by the mice's short life span.

Additional carcinogen-exposed GEMMs modeling the consequences of an inflammatory environment, a frequent risk factor in human CCA, have also been reported. However, both the low penetrance and the high latency jeopardized their use [111,112]. Nonetheless, co-exposure with carcinogens might be a strategy in GEMMs to accelerate cholangiocarcinogenesis by providing a pro-inflammatory and pro-fibrogenic environment recapitulating the human context [113].

Orthotopic or subcutaneous allografts 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 [10,64,88,107]. These cellular models are amenable to gene editing, and their orthotopic transplantation in syngeneic mice enables tumour growth in an immune-competent microenvironment. Additionally, the plasticity of LPCs and liver organoids to originate CCA- or HCC-like tumours, depending on the genetic context, is preserved.

GEMMs showed that LPCs, cholangiocytes (intra- and extrahepatic), and mature hepatocytes can be the cell of origin of CCA in mice [47,114]. However, the relevance of these findings for human CCA remains under evaluation. Indeed, various elements, including the targeted cell population (differentiated vs. stem cells; additional cell types only present in humans), the tissue location (intra- vs. -extrahepatic), the increased complexity of oncogenic alterations, the type, degree, and duration of the pro-oncogenic and pro-inflammatory stimuli, the liver status, etc., might ultimately affect CCA development.

For all preclinical *in vivo* models, based on statements on histological assessment and a unanimous agreement (Table 1 & Box 1), the expert panel strongly suggests that :

- The invasion of the basement membrane and tumorigenic capacity of isolated cells engrafted subcutaneously in immune-deficient 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: (a.) intra-tumoral heterogeneity (high stroma, inflammatory response, epithelial phenotype), (b) pattern of growth (mass-forming, periductal infiltration, intraductal growth), and (c) immunopositivity for CK7 or CK19.
- The expert panel recommends classifying preclinical CCA models as intrahepatic, perihilar, and distal CCA, and suggests that focal desmoplastic stroma is a morphological feature required to classify a lesion as CCA in a preclinical model.
- A drug should be tested in more than one model.

Lastly, to adopt a shared tool for defining the CCA experimental models homogeneously, an "experimental model sheet" was generated based on an initial expert discussion done in a physical ad hoc meeting (Malta meeting 20189; WG1 meeting) (**Table 4**) 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 characterized [115]. Later, an assortment of CCA cell lines of intrahepatic and extrahepatic origin was generated from primary tumours, ascites, metastases, and patient-derived xenografts (**Supplementary Table 2**). Besides human CCA cells, several lines derived from mouse, rat, and hamster models have been described (**Supplementary Table 2**). As proper control cells, primary cultures of normal cholangiocytes should be used.

Molecular studies performed in human CCA tissues have uncovered recurring genomic alterations in specific genes such as mutations in *TP53*, *IDH1*, *KRAS*, and *SMAD4* genes, *FGFR2* receptor fusions, or *ERBB* family gene amplifications [116], which, in part, 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 recently, with the development of high-throughput sequencing techniques, three studies have used exome sequencing or RNA-seq analyses to perform deep molecular phenotyping of some of the most widely used CCA cell lines (**Supplementary Table 2**) [117-119]. This has allowed 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 [120], and potential misclassification of the origin of some cell lines may impact the clinical translation of some molecular and functional studies. For instance, Mz-ChA-1 cells have been traditionally used as a CCA cell line [121,122], but they are classified as a gallbladder carcinoma cell line. Thus, results extrapolated from this cell line should be considered for patients with this specific type of tumour.

In general, the well-established cell lines represent an easy model to explore mechanisms of tumourigenesis and gain 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 favouring the accumulation of new genomic alterations [123-126]. Furthermore, *in vitro* maintenance often supports the selection of cell clones that are not representative of the genetic heterogeneity of the original tumor. In addition, cell cultures grown as a monolayer may lack polarization and realistic cell-cell contacts within the tumour bulk. Finally, the absence of cancer stromal cells and cell-matrix interactions do not recreate the fundamental interaction with the tumour microenvironment [3,123].

In addition to immortalized 2D cell lines, primary cultures of CCA tissue were established [127-130]. The overall success rate for CCA cell line isolation and establishment is relatively low (around 10%), partly due to insufficient numbers of tumour cells in resected tissues. Notably, contaminating non-tumour cells (*i.e.,* 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, retaining more of the morphological and functional characteristics of their tissue of origin [131]. Primary cultures constrain cell differentiation and partially preserve the stem-like component, thus reflecting tumour heterogeneity. However, the short time window to reach senescence hampers long-term experiments and their reproducibility.

A major limitation, independently of whether cell lines or primary CCA cultures are used, is the absence of components of the tumour microenvironment. To address this problem [132,133], different strategies have emerged in 2D cell culture, including conditioned media experiments, indirect co-culture through porous membrane cell culture inserts [134], and direct co-culture [135]. In some cases, these experiments are performed with primary cultures of tumour and stromal cells (i.e., cancer-associated fibroblasts, CAFs; monocytes/macrophages) [5,136]. In other cases, CCA cell lines are made to interact with immortalized stromal cell lines (**Table 3**) [132,134,137]. Although these systems do not fully recapitulate the complex tumour microenvironment, they enable the 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 [132,133,136].

Based on statements on histological assessment (Table 1) and a unanimous agreement, the expert panel (Box 1) strongly suggests to state in publication the origin of any cell line (previously established or new) according to the new CCA classification (intrahepatic, perihilar, distal). In addition, information regarding cell culture conditions should be provided in the publication to standardize the procedures (choice of plastic support and cell culture medium, level of confluence, isolation procedure for primary culture, passaging and sub-culturing methods, etc.).

3D-culture recapitulating tumour organization

To facilitate personalized/precision medicine, patient material is used to study treatment responses. While 2D CCA models are a step closer to the *in vivo* situation in the patient compared to 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 [5,138], while organoids self-organize in a matrix-rich 3D environment with which they interact [139,140] [6,141]. While 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 (e.g., fibroblasts and/or immune cells), and extracellular matrix components to enable the analysis of cell-cell and cell-matrix interactions.

Spheroids

Tumour spheroids, mostly 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 [5,138]. They can be grown in natural and/or synthetic hydrogels [141,142], and the increased

complexity of the model 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.

Organoids

Robust protocols for deriving biliary organoids from both mouse and human primary tissue explants or biopsies have been established [6,140], and complemented by methods that allow for the derivation and propagation of organoids from iPS cells [143], or cells collected from bile [144,145]. Apart from organoids derived from healthy donors, the successful establishment of organoid cultures from tumour tissues [6,7,9,146,147] 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 (CCAOs) 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 CCAOs inevitably has limitations, including the overgrowth by non-malignant cholangiocyte organoids. Using specific tumour enrichment medium [148], resort to hand picking non-malignant or tumour organoids to clean up the culture, and xenotransplantations are ways to address this challenge. It is agreed upon that tumourigenicity needs to be confirmed for all CCAO lines, preferably done by mutation analysis (stand-alone or as part of whole genomic profiling). Proof of organoid tumourigenicity in immunocompromised mice and histopathological analysis are additional tests that can be performed. A shortcoming of CCAOs 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 CCAOs, 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, as well as FGFR2 fusions and MYC overexpression) were engineered *in vitro* in either human [149,150] or mouse [151]. Collectively, these studies provided convincing evidence that liver organoids, in which few genetic hits were introduced to recapitulate recurrent patterns of putative iCCA driver mutations, gave rise to CCA upon sub-cutaneous or orthotopic transplantation in mice. This approach is therefore suitable for modelling genetically-defined cholangiocarcinogenesis in bipotent liver precursors and generating models for precision oncology research [10].

Based on the available data and a unanimous agreement, the expert panel strongly suggests:

- The use of a specific tumour « enrichment » medium (i.e., tumour initiating medium as described by Broutier et al., 2017, DOI: 10.1038/nm.4438) to minimize contamination in non-tumour organoids.
- To perform mutation and phenotypic analyses to confirm the malignant origin of established organoid lines and to report them in publication.
- To characterize every organoid culture before clinical applications such as drug screening.

Complex 3D culture systems

Although a hydrogel-based extracellular matrix (ECM) 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) not fully comprising human or tumour ECM. Moreover, additional stromal cells such as fibroblasts and immune cells are generally lacking in these cultures. The tumour microenvironment plays a crucial role in the initiation, progression, and invasion of CCA through a complex interaction between tumour cells, stromal cells, and the extracellular matrix [152]. Targeting this desmoplastic, stroma-rich tumour microenvironment might be essential to overcome chemoresistance [153-155]. Thus, including the CCA extracellular environment *in vitro* seems vital to mimic tumour composition, cell-cell and cell-matrix interaction [156], morphology, and tumour architecture more closely.

Current efforts is focussed on the generation of future complex models (assembloids) that integrate the epithelial CCA component with 3D bio-printed scaffolds that recapitulate the anatomy of the biliary system; immune cells that shape tumour growth and drug sensitivity through direct- or paracrine-interaction; 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 peculiar growth dynamic and timeline. The use of cryopreservation protocols and human iPSC-derived generation of cell types from the same background cell may overcome these issues.

How can clinical needs be addressed using currently available experimental models

The experimental models described here will facilitate the translation from experimental and preclinical work to the clinical setting. While some models provide relevant insights into the basic mechanisms of cancer progression, unraveling pathway and cell signaling analysis, cell-cell, or tumour-microenvironment interactions, others provide results that can be cautiously translated into the design of more effective treatments for CCA or the development of new human clinical trials. A few recent studies indicate that genetically defined cellular and animal models can advance the discovery of actionable vulnerabilities associated with druggable iCCA oncogenic drivers. Specifically, three independent studies reported that a) RAS-ERK signalling is necessary and sufficient to support the oncogenic activity of FGFR2 fusions in PDX [157], GEMMs [158], and organoid-based iCCA models [151]; b) combination therapies capable of providing for more robust and durable suppression of RAS-ERK, improved the therapeutic efficacy of clinically approved FGFR tyrosine kinase inhibitors [151,157,158]. Likewise, Idh1/Kras-driven models revealed that pharmacological targeting of mutated Idh1 sensitized iCCA to host-mediated immune responses, which could be enhanced by concomitant administration of immune checkpoint inhibitors [107].

The increasing availability of novel circulating biomarkers beyond the conventional serum tumour markers warrants validation for specific uses. Additional prognostic biomarkers may allow for a more accurate patient risk assessment and stratification in clinical trials. Predictive biomarkers for selecting the optimal therapy, such as ctDNA-based assays for FGFR2 fusions and IDH-1 mutations [159,160], are already in clinical use and will push the field forward. Finally, additional pharmacodynamic biomarkers able to track disease evolution more accurately than the carbohydrate antigen (CA) 19-9 and that can reveal the emergence of drug resistance are warranted [161], as shown for FGFR2 resistance [162].

CCA organoids have proven helpful for understanding fundamental mechanisms of cancer progression and biomarker discovery [7]. Though successful derivation of CCA organoids has lagged behind some other tumour types, organoids hold high potential as tools for improving CCA research and therapy [163]. With further improvement of clinical applicability, through continued advances in stem cell biology, organoid culture, 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 ECM dynamics constantly remodeled by tumour cells, CAFs, and tumor-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 essential in assessing drug resistance and tumour progression. Due to the lack of the tumour microenvironment, drug screenings performed *in vitro* do not fully reflect the *in vivo* efficacy, resulting in newly developed drugs failing in phase I-III clinical trials [164]. Finally, common risk factors and co-existing diseases characterizing human CCA (primary sclerosing cholangitis, liver flukes, chronic viral hepatitis, liver cirrhosis, etc.) 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 above might recapitulate human CCA more accurately.

Consensus 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, testing anti-tumour therapies, and deciphering therapeutic resistance mechanisms. The panoply of CCA experimental models is wide, from simple, practical, and inexpensive to more complex models resembling human cancer biology, with a more challenging implementation and higher costs. The choice of the 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 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 reviewed above. GEMMs appear to mimic pathobiological features of human tumourigenesis more closely, 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 not only the relatively high costs to set up the culture but also the availability of starting material (human CCA tissue).

In addition to providing an inventory, including evaluating (dis)advantages, 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, to provide a comprehensive tumour mimic, results gathered using different models are highly recommended. This fosters the consolidation of scientific data with well-defined minimal criteria before validating them on humans by manipulating *ex vivo* samples or clinical trials.

Conclusions **[Au: please provide a short concluding paragraph]**

Biomedical research relies entirely on *in vitro* and *in vivo* experimental models, a prerequisite for research in basic and applied sciences. In this Consensus Statement, an international group of experts developed and endorsed a set of consensus statements and recommendations on CCA experimental models, and provided guidance on the models proposed to the scientific community and the information that should be specified in publications on these models. As a complement, the experts provided he scientific community with a brief overview of currently available models to the scientific community, highlighting the advantages and disadvantages that scientists should be aware of. Importantly, This Consensus Statement has been prepared based on the expertise of both researchers and clinicians from different specialties (cell biologists, molecular biologists, oncologists, hepatologists, pathologists), thus ensuring the relevance of these statements and recommendations for a broad range of scientific public, from medical healthcare to scientists who are directly investigating this fatal cancer.

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Author contributions

LF coordinated the workgroups and the process of generating the manuscript, the review, MV and RC coordinated the Delphi questionnaire, and all authors contributed equally to the redaction and final revision of the manuscript.

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Consortium Authorship

Diego F Calvisi, Institute of Pathology, University of Regensburg, Regensburg, Germany (author), Luke Boulter, MRC-Human Genetics Unit, Institute of Genetics and Molecular Medicine, University of Edinburgh, Edinburgh, UK (author), Javier Vaquero, TGF-β and Cancer Group, Oncobell Program, Bellvitge Biomedical Research Institute (IDIBELL), Barcelona, Spain & National Biomedical Research Institute on Liver and Gastrointestinal Diseases (CIBEREHD), Instituto de Salud Carlos III, Madrid, Spain (author), Anna Saborowski, Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Hannover, Germany (author), Luca Fabris, Department of Molecular Medicine, University of Padua School of Medicine, Padua, Italy & Digestive Disease Section, Yale University School of Medicine, New Haven, CT, USA (author), Pedro M Rodrigues, National Biomedical Research Institute on Liver and Gastrointestinal Diseases (CIBEREHD), Instituto de Salud Carlos III, Madrid, Spain & Department of Liver and Gastrointestinal Diseases, Biodonostia Health Research Institute - Donostia University Hospital, University of the Basque Country (UPV/EHU), San Sebastian, Spain & Ikerbasque, Basque Foundation for Science, Bilbao, Spain (author), Cedric Coulouarn, Inserm, Univ Rennes 1, OSS (Oncogenesis Stress Signaling), UMR_S 1242, Centre de Lutte contre le Cancer Eugène Marquis, Rennes, France (author), Rui E Castro, Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, Lisbon, Portugal (author), Oreste Segatto, Unit of Oncogenomics and Epigenetics, IRCCS Regina Elena National Cancer Institute, Rome, Italy (author), Chiara Raggi, Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy (author), Luc JW van der Laan, Department of Surgery, Erasmus MC Transplantation Institute, University Medical Center Rotterdam, The Netherlands (author), Guido Carpino, Department of Movement, Human and Health Sciences, Division of Health Sciences, University of Rome "Foro Italico", Rome, Italy (author), Benjamin Goeppert, Institute of Pathology and Neuropathology, Ludwigsburg, Germany (author), Stephanie Roessler, Institute of Pathology, Heidelberg, Germany (author), Timothy Kendall, Centre for Inflammation Research, University of Edinburgh, Edinburgh, UK (author), Matthias Evert, Institute of Pathology, University of Regensburg, Regensburg, Germany (author), Ester Gonzalez-Sanchez, TGF-β and Cancer Group, Oncobell Program, Bellvitge Biomedical Research Institute (IDIBELL), Barcelona, Spain & National Biomedical Research Institute on Liver and Gastrointestinal Diseases (CIBEREHD), Instituto de Salud Carlos III, Madrid, Spain & Department of Physiological Sciences, Faculty of Medicine and Health Sciences, University of Barcelona, Spain (author), Juan W Valle, Department of Medical Oncology, The Christie NHS Foundation Trust,

Manchester, UK & Division of Cancer Sciences, University of Manchester, Manchester, UK (author), Arndt Vogel, Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Hannover, Germany (author), John Bridgewater, Department of Medical Oncology, UCL Cancer Institute, London, UK (author), Mitesh J Borad, Mayo Clinic Cancer Center, Mayo Clinic, Phoenix, AZ, USA (autor), Gregory J Gores, Division of Gastroenterology and Hepatology, Mayo Clinic College of Medicine and Science, Rochester, MN, USA (author), Lewis Roberts, Division of Gastroenterology and Hepatology, Mayo Clinic College of Medicine and Science, Rochester, MN, USA (author), Jose JG Marin, National Biomedical Research Institute on Liver and Gastrointestinal Diseases (CIBEREHD), Instituto de Salud Carlos III, Madrid, Spain & Experimental Hepatology and Drug Targeting (HEVEPHARM), IBSAL, University of Salamanca, Salamanca, Spain (author), Jesper B Andersen, Biotech Research and Innovation Centre (BRIC), Department of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark (author), Domenico Alvaro, Department of Precision and Translational Medicine, Sapienza University of Rome, Rome, Italy (author), Alejandro Forner, National Biomedical Research Institute on Liver and Gastrointestinal Diseases (CIBEREHD), Instituto de Salud Carlos III, Madrid, Spain & Liver Unit, Barcelona Clinic Liver Cancer (BCLC) Group, Hospital Clinic Barcelona, IDIBAPS, University of Barcelona, Barcelona, Spain (author), Jesus M Banales, National Biomedical Research Institute on Liver and Gastrointestinal Diseases (CIBEREHD), Instituto de Salud Carlos III, Madrid, Spain & Department of Liver and Gastrointestinal Diseases, Biodonostia Health Research Institute - Donostia University Hospital, University of the Basque Country (UPV/EHU), San Sebastian, Spain & Ikerbasque, Basque Foundation for Science, Bilbao, Spain & Department of Biochemistry and Genetics, School of Sciences, University of Navarra, Pamplona (author), Vincenzo Cardinale,Department of Medico-Surgical Sciences and Biotechnologies, Sapienza University of Rome, Rome, Italy (author), Rocio IR Macias, National Biomedical Research Institute on Liver and Gastrointestinal Diseases (CIBEREHD), Instituto de Salud Carlos III, Madrid, Spain & Experimental Hepatology and Drug Targeting (HEVEPHARM), IBSAL, University of Salamanca, Salamanca, Spain (author), Silve Vicent, University of Navarra, Centre for Applied Medical Research, Program in Solid Tumours, Pamplona, Spain & IdiSNA, Navarra Institute for Health Research, Pamplona, Spain & Centro de Investigación Biomédica en Red de Cáncer (CIBERONC, Instituto de Salud Carlos III), Madrid, Spain (author), Xin Chen, Department of Bioengineering and Therapeutic Sciences and Liver Center, University of California, San Francisco, USA (author), Chiara Braconi, Institute of Cancer Sciences, University of

Glasgow, Glasgow, UK (author), Alexander Scheiter, Department of Pathology, University Regensburg, Regensburg, Germany (consortium member), Florin M Selaru, Division of Gastroenterology and Hepatology and Sidney Kimmel Cancer Center, Johns Hopkins University, Baltimore, USA (consortium member), Katja Evert, Institute for Pathology, University Regensburg, Regensburg, Germany (consortium member), Kirsten Utpatel, Institute of Pathology, University of Regensburg, Regensburg, Germany (consortium member), Laura Broutier, Childhood Cancer & Cell Death, Centre de Recherche en Cancérologie de Lyon (CRCL), Lyon, France (consortium member), Massimiliano Cadamuro, Department of Molecular Medicine - DMM, University of Padova, Padova, Italy (consortium member), Meritxell Huch, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany (consortium member), Rob Goldin, Centre of Pathology, Imperial College London, London, UK (consortium member), Sergio A Gradilone, The Hormel Institute, University of Minnesota, Austin, USA, Masonic Cancer Center & University of Minnesota, Minneapolis, USA (consortium member), Yoshimata Saito, Division of Pharmacotherapeutics, Keio University Faculty of Pharmacy, Tokyo, Japan & Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan (consortium member), Monique MA Verstegen, Department of Surgery, Erasmus MC Transplantation Institute, University Medical Center Rotterdam, The Netherlands (author), Laura Fouassier, Sorbonne Université, Inserm, Centre de Recherche Saint-Antoine (CRSA), Paris, France (author).

Alexander Scheiter, [37] Florin M Selaru, [38] Katja Evert, [39] Kirsten Utpatel, [39] Laura Broutier, [40,41] Massimiliano Cadamuro, [42] Meritxell Huch, [43] Rob Goldin, [44] Sergio Gradilone, [45,46] Yoshimata Saito, [47]

[37] Institute of Pathology, University of Regensburg, Regensburg, Germany
[38] Division of Gastroenterology, Department of Medicine and Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, Baltimore, MD
[39] Institute of Pathology, University of Regensburg, Regensburg, Germany
[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, Dept 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

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Box 1. Benefits and limitations of cholangiocarcinoma experimental models and recommendations.

	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
Allograft	- Full immune system - Ideal to study 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 of 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
RECOMMENDATIONS **Histological assessment (all *in vivo* models)** 1. Invasion of the basement membrane and tumorigenic capacity of isolated cells engrafted subcutaneously in immune-deficient mice are the most important malignant features of CCA (97% and 91%, A). 2. Immunohistochemistry of at least one biliary cytokeratin should always be performed to characterize an early-stage tumour in a preclinical CCA model (90%, A). 3. A classification of preclinical CCA models as intrahepatic, perihilar, and distal CCA is recommended. (93%, A). 4. Focal desmoplastic stroma is a morphological feature required to classify a lesion as CCA in a preclinical model (100%, U). 5. Three histopathological features of human CCA must be assessed in a preclinical model: intra-tumoral 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 (GEMM)** 6. The type of CCA should be specified for patient-derived xenograft models (92%, A). 7. Drugs should be tested in more than one model (95%, A).		
***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	- Absence of stromal cells - Cultures grown as a monolayer

	- Cells available with genetic alteration(s)	
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 - Increased complexity	- Limited use for high-throughput analysis - Often made from cell lines - Do not fully reflect the polyclonal nature of a CCA tumour
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 in culture initiation - Absence of stromal cells

RECOMMENDATIONS

2D cultures

8. Cell culture procedures should be standardised in experiments with cell lines or primary 2D cultures and be reported in publications. Procedures include the choice of plastic support, 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 sub-culturing methods, should be reported in publications (i.e., enzymatic vs. mechanical dissociation, etc.) (89% and 85%, B).
   The origin of any cell line (previously established or new) should be stated for publication according to the new CCA classification (i.e., intrahepatic, perihilar, distal) (90-99, A)
9. The origin of any cell line (previously established or new) should be presented in a publication according to the new CCA classification (i.e., intrahepatic, perihilar, distal) (97%, A).

3D cultures

10. A specific tumour "enrichment" medium (i.e., tumour initiating medium as described by Broutier et al., 2017, DOI: 10.1038/nm.4438) is recommended to minimize contamination in non-tumour organoids (94%, A).
11. 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).
12. Every organoid culture should be characterized before clinical applications such as drug screening (92%, A).
13. The shorter period for patient-organoids initiation, expansion, and analysis has to be less than 3 months (57%, C).

Grading system: U, denotes unanimous (100%) agreement; A, 90–99% agreement; B, 70–89% agreement; C, 50-69% agreement; and D, <50% agreement.
ECM, extracellular matrix; HCC/CCA, hepatocholangiocarcinoma; TAA, thioacetamide.

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[Au: please add details of the grading system used as a footnote as each display item must be understood as a standalone item]

Table 1. Consensus statements.

#	Statement	Response yes / total responders	Grade
Histological assessment			
1	Which of the following ones are malignant features of biliary tumours?		
	1. Invasion of the basement membrane	31/32	A
	2. Increased nucleus/cytoplasm ratio	18/31	C
	3. Distant metastasis	27/32	B
	4. Tumorigenic capacity of isolated cells after subcutaneous injection in immune-deficient 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?		
	1. Morphological examination of H&E	32/32	U
	2. Immunohistochemistry	27/30	A
	3. Immunohistochemistry for at least one biliary cytokeratin (e.g., CK19, CK7, pan CK, etc.)	16/25	C
	4. Markers for inflammatory cells and CAFs	12/26	D
	5. PAS reaction for highlighting mucin	13/26	C
	6. 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:		
	1. Intrahepatic CCA, perihilar CCA, and distal CCA	25/30	B
	2. Intrahepatic CCA and extrahepatic CCA	12/25	D
	3. 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?		
	1. Location within the liver or extrahepatic biliary tree	24/28	B
	2. Absence of an extrahepatic bile duct primary lesion	14/28	C
	3. Epithelial cytological features (cohesive groups or structures and/or pan-cytokeratin immunopositivity)	25/28	B
	4. At least focal gland formation	9/25	D
	5. Absence of hepatocellular differentiation (bile production and canalicular CD10 or BSEP)	14/24	D
	6. Immunopositivity for CK7 or CK19	31/31	U
	7. Focal desmoplastic stroma	22/30	B
	8. Presence of precursor lesions	4/24	D
	9. Primary origin within the intra- or extra-hepatic biliary tree	19/28	D
	10. Absence of primary hepatobiliary lesions	0/28	U
5	What histopathological features of human CCA must be verified in a preclinical model of CCA?		
	1. Intra-tumoral heterogeneity (high stroma, inflammatory response, epithelial phenotype)	27/30	A

	2. Inter-tumoral heterogeneity (large versus small bile duct tumour in iCCA)	20/26	B
	3. Growth pattern (mass-forming, periductal infiltration, intraductal growth)	25/28	A
	4. Proportion of tumour showing gland formation	17/25	C
	5. Immunopositivity for CK7 or CK19	32/32	U
	6. Focal desmoplastic stroma	26/30	B
	7. Presence of precursor lesions	16/24	C
6	It has been proposed that iCCA may originate from several cells of origin. Which of the following cell types may be the cells-of-origin for iCCA?		
	1. Mature hepatocytes	27/32	B
	2. Mature cholangiocytes	23/32	B
	3. Hepatic progenitor/oval cells	32/33	A
	4. Peribiliary glands	29/30	A
In vivo and in vitro models			
Xenograft models, Genetically Engineered Mouse Models (GEMM)			
7	Concerning newly developed patient-derived xenograft models		
	1. Should the model(s) be validated by an expert pathologist and the histology of the tumour shown in publications?	37/37	U
	2. Should immune profiling also be reported?	20/31	C
	3. Should the model(s) be validated in more than one mouse strain?	8/34	D
	4. Should the expert pathologist specify what type of CCA is found in the model?	33/36	A
	5. 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
	6. Should a drug be tested in more than one model?	35/37	A
2D culture models			
8	Which cell culture procedures should be standardised in experiments with cell lines or primary 2D cultures and be reported in publications?		
	1. Choice of plastic support (i.e., TPP, Falcon, Corning, +/- ECM layer, etc.)	30/34	B
	2. Choice of cell culture medium	29/34	B
	3. Level of confluence when performing the experiments	27/33	B
	4. Isolation protocol for culture of primary cells	31/35	B
	5. Passaging and sub-culturing methods (i.e., enzymatic vs. 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 (i.e., intrahepatic, perihilar, distal)	37/38	A
3D cultures			

10	Contaminating non-tumour organoids often grow in CCA organoid cultures. How should selection for tumour organoids be performed?		
	1. Specific tumour "enrichment" medium (i.e., tumour initiating medium (as described by Broutier *et al.,* 2017, DOI: 10.1038/nm.4438)	29/31	A
	2. Hand-picking of organoids with a different phenotype / removing the 'normal-looking' organoids	21/30	B
	3. 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?		
	1. Full genomic profiling	8/28	D
	2. Mutation analysis (targeted genomic profiling using a diagnostic panel)	28/31	A
	3. Phenotypic analysis	28/30	A
	4. Histological analysis (immunohistochemistry of EpCAM, CK7)	28/32	B
	6. Xenotransplantation in mice	26/32	B
12	Should every organoid culture be characterized (as proposed in Q 11) before clinical applications such as drug screening?	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; the less as possible / <1 mo 1st line treatment and <3 mo 2nd line treatment	2/35	D

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

Table 2. Carcinogen-based rodent models of cholangiocarcinoma.

Carcinogenic agent	Animal	Mechanism of action	Biliary lesions	Ref.
TAA	Rat and mouse	Membrane protein and phospholipid modifications	Intense fibrosis with dysplasia	91,92
Furan	Rat	DNA adduct generation	Chronic inflammation, proliferation of bile duct cells	98
DEN, DMN (even combined with BDL)	Hamster and mouse	DNA adduct generation	Desmoplasia, cystic hyperplasia of bile ducts	94-96,165
Opisthorchis viverrini	Hamster	DNA oxidative damage	Alterations of oxidative metabolism and proliferation of bile ducts	97

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

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

Genetic strategy	Key features	Advantages and Disadvantages	Ref.
Alfp-Cre, *Trp53*$^{f/f}$	Advanced HCC/CCA (from LPCs)	A: *Trp53* mutation found in human CCA D: Long latency (14- to 20-month-old mice), tumours of bilinear origin (mixed HCC/CCA)	166
Alb-Cre, *Smad4*$^{f/f}$, *Pten*$^{f/f}$	Multistep progression involving hyperplasia, dysplasia, carcinoma *in situ*, and well-established iCCA (from LPCs)	A: 100% tumour penetrance D: Cre activation during embryogenesis, long tumour latency (4-5 months) and lack of metastasis	100
Alb-Cre, *Kras*$^{LSLG12D/+}$, *Pten*$^{f/f}$	Invasive iCCA with an abundant desmoplasia, primarily showing glandular morphology resembling well-differentiated human CCA (from LPCs)	A: 100% penetrance, rapid development (7 weeks of age), abundant desmoplastic stroma, iCCA exclusive D: Cre activation during embryogenesis, no apparent metastases or invasion to other organs	103 104
Alb-Cre, *Idh2*$^{LSL-R172}$, *Kras*$^{LSL-G12D}$	Multifocal liver masses of iCCA (from LPCs)	A: 100% penetrance, splenic invasion and peritoneal metastases D: Cre activation during embryogenesis, long tumour latency (33-58 weeks)	105
Alb-Cre, *NotchICD*	Development of transplantable CCA, likely progenitor cell-derived (transplantation of cells from 8 months-old mice in immunodeficient animals gives rise to CCA) (from LPCs)	A: Notch expression is characteristic of human disease D: Cre activation during embryogenesis, no obvious cancer development after 8 months in transgenic mice, requires additional transplantation model	167
Alb-Cre, *Trp53*$^{f/f}$; *NotchICD*	Development of iCCA abortive glandular pattern (moderate to high pleomorphic nuclei with some atypic mitoses) and dense fibrous tissue with inflammatory cells (from LPCs)	A: 100% penetrance, development of fibrous/inflammatory microenvironment D: Long tumour latency (>8-9 months), no metastases	168
Alb-Cre, *Kras*$^{LSL-G12D/+}$; *Fbxw7*$^{LSL-R468C/LSL-R468C}$	Dysplastic dust-like structures surrounded by fibrosis in all mice (only bile duct dilation and hyperplasia in some heterozygous Fbxw7$^{LSL-R468C}$ mice at the age of 8 months) (from LPCs)	A: Low latency (2 months of age) D: Cre activation during embryogenesis, homozygous Fbxw7 mutations not occurring in human disease	169
Alb-Cre, *Hspd1*$^{f/f}$	Cholangiocellular lesions, characterized by irregular glands, loss of polarity, multilayering of cells, and frequent mitosis resembling human BIN	A: Low latency, possibility of transplanting cholangiocellular lesions, activation of human CCA pathways D: Not related to known oncogenic drivers of human disease, no metastases, not established iCCA	109
Alb-Cre, *Jnk1*$^{f/f}$, *Jnk2*$^{-/-}$	JNK deletion causes changes in cholesterol and bile acid metabolism that foster cholestasis, bile duct proliferation, and iCCA	A: iCCA exclusive D: ~95% penetrance, long tumour latency (14 months)	170
Alb-Cre, *NEMO*$^{f/f}$, *Jnk1*$^{f/f}$, *Jnk2*$^{-/-}$	Hyperproliferative ductular lesions with atypia compatible with CCA	A: Elevated ROS associated with cholangiocellular proliferation D: Not full penetrance, long latency (50 weeks)	171
Alb-Cre, *Kras*$^{LSL-G12D/+}$, *Trp53*$^{f/f}$	Multistage progression including stroma-rich tumours and premalignant biliary lesions (IPBN and) (from LPCs)	A: 100% penetrance, average latency 16 weeks, metastatic lesions D: Cre activation during embryogenesis, wide latency range, CCA in ~80% of mice	101

Kras$^{LSLG12D/+}$, *Trp53*$^{f/f}$ infected with AAV8-TBG-Cre	Development of ICC (40%), HCC (40%), mixed HCC/CCA (20%) (from hepatocytes)	A: Recombination event in adult mice, higher CCA frequency in combination with DCC diet (all tumours ICC or mixed HCC/CCA) D: Cre-recombinase administration via adeno-associated virus (AAV), large tumour latency range (12-66 weeks post-AAV infection)	102
AhCre^{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)	A: 100% penetrance, low latency (43 days), tumour development starts in adult mice D: Not specific to liver tissue, lack of invasive tumour or metastasis	172
Sox9-Cre^{ERT2}; *Kras*$^{LSL-G12D/+}$, *Trp53*$^{f/f}$	iCCA tumours accompanied by adjacent extensive ductular reactions and desmoplasia, with areas resembling BIN (from cholangiocytes)	A: 100% penetrance, iCCA exclusive, recombination in mature cholangiocytes D: 30 weeks average latency	102
Ck19-Cre^{ER}, *Kras*$^{LSL-G12D}$, *Tgfbr2*$^{flox/flox}$; *Cdh1*$^{flox/flox}$;	Markedly thickened EHBD wall with a swollen gallbladder involving invasive periductal infiltrating-type eCCA and lymphatic metastasis (from biliary cells)	A: Low latency (4 weeks), eCCA exclusive B: Concurrent development of lung adenocarcinomas leads to mice asphyxiation	110
Pdx1-Cre, *Pik3ca*$^{LSLH1047R/+}$	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)	A: eCCA exclusive, only one genetic hit driving CCA B: ~40 weeks average latency, 90% penetrance, wide tumour latency range	173
GEM-based implantation models			
LPCs from *Alb-Cre*, *Kras*$^{LSL-G12D}$, *Trp53*$^{LSL-R172H/lox}$ +/- FIG-ROS fusion	Allografted tumours resemble advanced CCA	A: Quick model, orthotopic implantation in the liver, iCCA exclusive, stroma presence D: Requires technical training to isolate LPC	88
LPCs or cholangiocytic progenitor cells or hepatocytes from *Trp53*$^{-/-}$ mice	Tumours exhibit a high stromal content and a mixed hepatocellular and cholangiocellular differentiation	A: Quick model D: Not CCA exclusive	166
Adult liver organoids from *Kras*$^{LSL-G12D}$, *Trp53*$^{f/f}$ mice	Kras driven organoids lead to CCA while c-Myc expression in wild-type organoids induces HCC formation	A: Tumours latency of 6-8 weeks for Kras mut and Trp53 ko organoids D: Requires training in organoid isolation, growth and manipulation	10
Cholangiocytes from *Kras*$^{LSL-G12D}$, *Trp53*$^{f/f}$ mice	Tumours with a high stromal component expressing CCA markers	A: Quick and reproducible model, orthotopic implantation in the liver, iCCA exclusive, stroma presence D: Requires technical training to isolate mouse cholangiocytes	64
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	A: 100% penetrance, iCCA exclusive D: Long latency (30 weeks)	174
Ck19-Cre$^{ERT/eYFP}$; *Trp53*$^{f/f}$ mice treated with TAA	Treatment with TAA generates oncogenic stress yielding multifocal invasive iCCA	A: iCCA exclusive D: 80% penetrance, long latency (>6 months)	111
Trp53$^{-/-}$ mice treated with CCl_4	Bile duct injury/necrosis, proliferation and fibrosis development triggered by CCl_4	A: Exclusive iCCA D: 50% mice develop tumours, metastatic lesions rarely observed	112

GSTA3$^{-/-}$ mice treated with aflatoxin B1	Macro- and microscopic liver cysts, hepatocellular nodules, cholangiomas, iCCA and oval cell proliferation	D: Long latency (12 and 24 weekly AFB1 injections followed by a rest period of 12 and 6 months)	[175]
Alb-Cre, *Jnk1*$^{f/f}$, *Jnk2*$^{-/-}$ treated with DEN	Cystogenesis and cholangioma-like structures in liver parenchyma with strong infiltration of immune cells	A: Participation of inflammatory insult D: No established CCA, long latency	[171]

A: Advantages; BIN: biliary intraepithelial neoplasia; CCl_4: carbon tetrachloride; D: disadvantages; DEN: diethylnitrosamine; GSTA3: glutathione-S-transferase A3; IPBN: intraductal papillary biliary neoplasms; LPCs: bipotent liver progenitor cells; ROS: reactive oxygen species; TTA: tetradecylthioacetic acid; VMC Von Meyenburg complexes.

Table 4. Experimental model sheet.

Experimental model sheet	
1. **Type of model**: (*in vitro, ex vivo, in vivo*)	
2. **Species**: (mouse, rat, hamster, human, etc.)	
3. **Gender**: (male, female, both)	
4. **Strain:**	
5. **Condition of the surrounding liver** (apparently healthy, cirrhosis, fibrosis, etc.) :	
6. **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.):	
7. **Tumour development:** (fast, slow)	
8. **Metastasis:** (yes, no, locations,...)	
9. **Anatomical location of the lesions (when applicable)**: (intrahepatic, extrahepatic, both)	
10. **Cell of origin (if available)**: (cholangiocyte, stem/progenitor cell, hepatocyte)	
11. **Types of samples and storage conditions for future analyses**	
12. **Presence of preneoplastic lesions**: (yes/no)	
13. **Type of preneoplastic lesions**: (IPNB, IPMN, BilIN, etc.)	
14. **Type of cholangiocarcinoma**: (iCCA, pCCA, dCCA, combined HCC/CCA)	
15. **Histology of tumours**: (large duct type, small duct type, CCA, lymphoepithelioma-like CCA, etc.)	
16. **Microenvironment features**: (presence of stroma/desmoplastic reaction, absence of stroma, immune infiltration yes/no)	
17. **Phenotype of the lesions**: (CK7, CK19, MUC1, MUC2, MUC5AC, MUC6, HNF4A, AFP, markers of stemness, markers of EMT, etc.)	
18. **Control samples used if applicable** (bile duct freshly isolated from liver or cell line)	

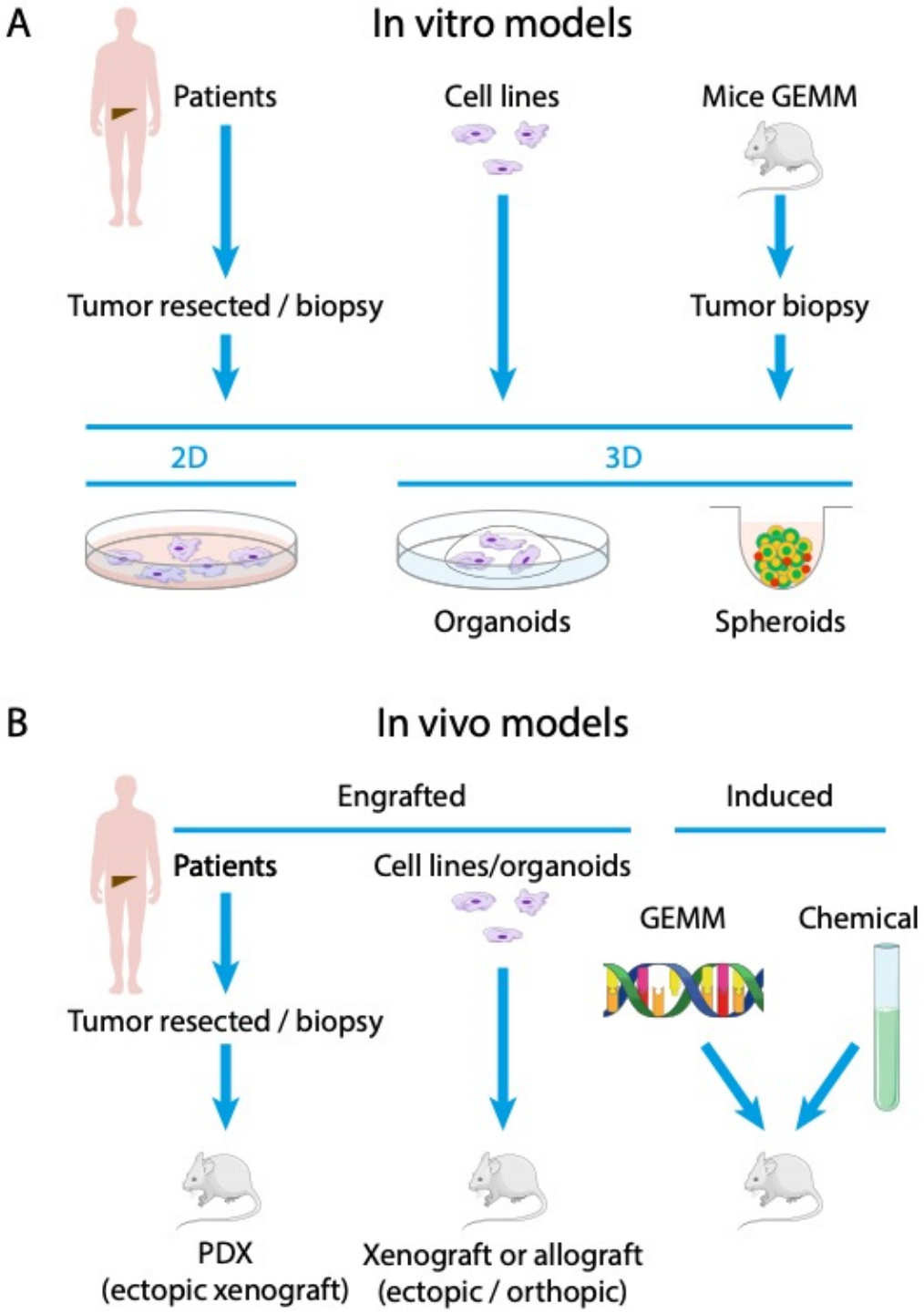

Figure 1. Panel of experimental *in vitro* (A) and *in vivo* (B) models provided for cholangiocarcinoma preclinical studies.

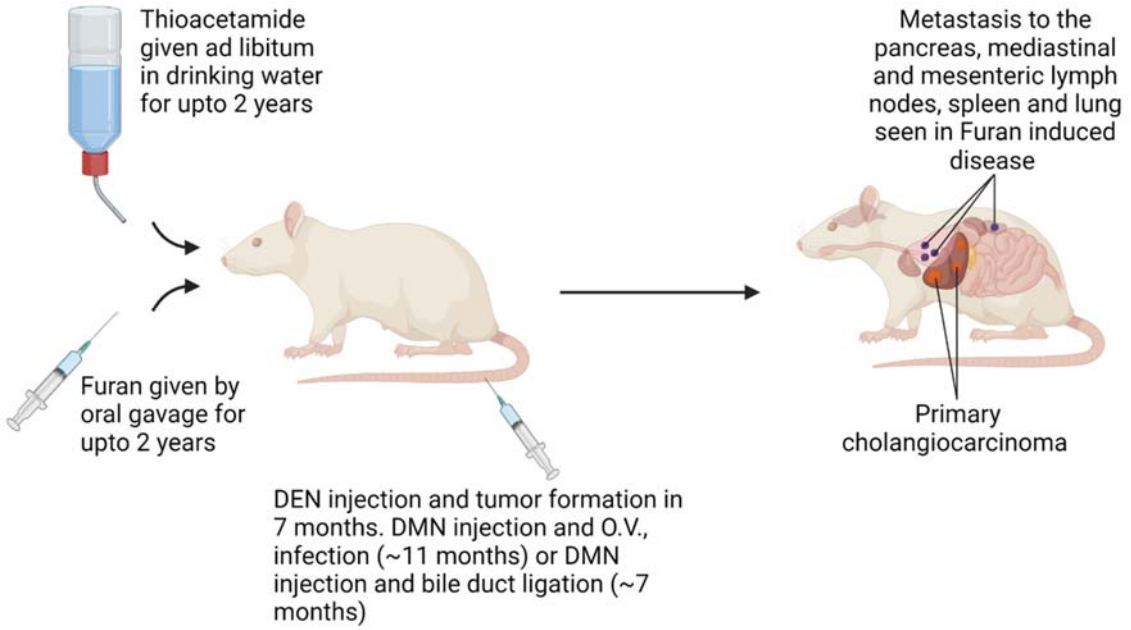

Figure 2. Schematic summary of available chemical models to initiate cholangiocarcinoma in rodents and induce metastatic dissemination.