Criteria for preclinical models of cholangiocarcinoma: scientific and medical relevance

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 J.W. 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 I.R. Macias,[4,25] Silve Vicent,[31,32,33] Xin Chen,[34] Chiara Braconi,[35] Monique M.A. Verstegen,[14] Laura Fouassier[36]; CCA model consortium **[Au: please check author list carefully]**

[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

Email : laura.fouassier@inserm.fr

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 preclinical model is guided by the question(s) to be addressed, and, 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 10 countries **[Au: addition of 'and' OK? Is this the intended meaning? The sentence didn't quite work previously, but feel free to modify to your intended meaning]** 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 to develop clinical biomarkers and innovative therapies for patients with CCA.

[H1] Introduction [Au: I have added [H] heading markers throughout to aid our production team during the layout – please do not delete.]

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 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, perihilar or distal CCA), 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 model of CCA. In addition to experimentally-immortalized or primary cultures of normal cholangiocytes derived from normal bile ducts, over 50 CCA-derived **[Au: human cell lines, specifically?]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**[Au: by 'upon the', do you mean 'as a result of'?]** continuous culturing, making it difficult to infer which therapeutics would have been effective **[Au: by 'efficient', do you mean 'effective'?]** to treat the original neoplasm [4]. Moreover, 2D mono-cultures 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 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. Inducing CCA 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 **[Au: please reference]** . To give in vivo context to 2D cell lines, CCA cells have been used to generate subcutaneous or orthotopic xenografts in mice **[Au: please reference]** . However, these approaches remain limited by poor rates of tumour engraftment. Technological

advancements have made it possible to grow liver organoids, that is, 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) that recapitulate 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 **[Au: is this the exact name of this initiative?]** , have been crucial to fostering advances in this field. To improve the accuracy in obtaining and exchanging information among research groups**[Au:OK?]** , it is now essential to establish consensus criteria regarding the minimal standardized characteristics required from preclinical CCA models or when describing a new model **[Au:OK?]**. In this Consensus Statement, 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.

[H1] Methods

[H2] Panel of experts

A core group of 8 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 10 additional experts to fill in the second and final questionnaire. These 10 experts, who were not actively involved in writing the recommendations but provided their important input by filling in the second questionnaire **[Au:OK?]** , are listed as one collaborative author: the CCA Model consortium. Thus, the final panel consisted of 45 experts from 10 countries located in Europe, Asia and USA. **Supplementary Table S1** summarizes the expert panel's names, institutes and demographics.

[H2] Building consensus

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

[H2] 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 into 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 (Supplementary 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 formulated **[Au: can you clarify what is meant by 'edited' here?]** (**Box 1**).

[H1] Clinical features to consider [Au:OK? This heading was too long previously]

[H2] Clinics [Au: Is 'Clinics' the appropriate heading for this subsection? Perhaps a little more detail could be provided.]

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 ICD11, published in 2022, CCA is classified according to its origin as intrahepatic CCA (iCCA) and extrahepatic CCA (eCCA) (https://icd.who.int). iCCA arises from intrahepatic bile ducts, that is, it grows in the liver. Consequently, it is more often surgically resectable than perihilar CCA (pCCA), the latter of which arises at the liver hilum where the likelihood of local vascular invasion is greater [12]. 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 [13-16]. It is not fully understood why iCCAs are more molecularly heterogeneous than pCCAs or dCCAs, **[Au: this term has not yet been introduced, please define – perihiliar or distal CCAs?]** 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 treatment options necessitate further

studies [17]. dCCA more closely resembles pCCA **[Au: please reference]** , 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 **[Au: in the reference list, please complete the reference details for ref 21]** [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 unravelled to develop and assess novel interventions to overcome resistance before clinical testing.

[H2] Pathology

Separate classifications (Union for International Cancer Control (UICC), American Joint Committee on Cancer (AJCC), and World Health Organization (WHO)) exist for iCCA, pCCA and dCCA **[Au: is it possible to cite these classifications here?]** . 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].

[H3] 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].

[H3] Immunohistochemistry. No specific immunohistochemical pattern for CCA lesions exists. However, they typically show an upper gastrointestinal or pancreaticobiliary **[Au:OK?]** 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 (for example, CK20 and CDX2) [25]. CCA is usually immunonegative for HepPar1, arginase 1, and glypican 3, distinguishing it from HCC and combined HCC-CCA **[Au: please reference]** . Transcription factors that mark cell-specific lineages such as thyroid transcription factor 1 (TTF1) (lung and thyroid cancers), PAX8 (renal,

thyroid, ovarian and endometrial cancers), and GATA3 (breast and urothelial cancers) are not usually expressed in CCA **[Au: please reference]** .

***[H3] Biliary precursor lesions*.** CCA can **[Au: do you mean 'can'?]** 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%) **[Au: can you please clarify precisely what these percentages mean]** [27,28]. Invasive malignancy is evident in >50% of IPNBs at presentation **[Au: please reference]** . Furthermore, the mucinous cystic neoplasm is a cystic epithelial tumour occurring almost exclusively in female patients **[Au:OK?]** , associated with CCA in 5% of cases [29,30].

[H2] 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 **[Au: can you provide more detail regarding what is meant by 'the genetic landscapes fall midway'?]** in the mutational spectrum of cancers [39], with shared genetic alterations between iCCA, pCCA and dCCA [36]. Although we have 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 **[Au:OK?]** .

Integrated genomics approaches have been used to classify patients with CCA on the basis of prognosis [40-43], emphasizing dysregulated oncogenic signalling pathways, including WNT-CTNNB1, MYC, PI3K-AKT-mTOR, ERBB, 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 **[Au:OK?]** . This predominant molecular classification highlights distinct tumour phenotypes that are either inflammatory or proliferative in nature [41]. Moreover, iCCA can be classified on the basis of driver-gene mutations elucidating **[Au: is 'elucidating' the appropriate word here? I wonder whether this could perhaps be made a bit clearer]** unique mutational signatures, structural variants and epigenomic alterations [35]. Notably, **[Au: I think there is a word or two in this sentence missing. Should this be 'there are specific'? Please take another look at this sentence]** specific oncogenic mechanisms in distinct patient subsets with potential unique drug responses such as RNA synthesis inhibition in *IDH*-mutant tumours, microtubule

modulator in *KRAS*-mutant tumours, topoisomerase inhibition in *TP53*-mutant tumours, 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 their 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 might not be reflected in the most rapid mouse models **[Au: as in, 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 [48,49].

[H1] In vivo CCA models

[H2] Engrafted models

[H3] 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 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 [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 **[Au: such as? A couple of examples would be good]** [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 **[Au: might or can?]** 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 [50-53].

Using orthotopic xenograft models might 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 **[Au: please reference]** . 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]. Notably, 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 can arise **[Au:OK?]** . Furthermore, the tumour development, growth and metastases assessment either 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 **[Au: might or can?]** 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 **[Au: CCA, specifically?]** tumours that harbour specific mutational patterns and to 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 tumour engraftment. Thus, they constitute a time and resource-intensive model and may **[Au: might or can?]** require several months for successful implantation [50]. On the basis of 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).

[H3] Allograft (syngeneic). Syngeneic models have the advantage of implanting murine CCA cells into an immunocompetent host, thereby displaying 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 Fisher 344 rats. While BDEsp engraftment induces the development of non-metastatic iCCA, BDEneu-derived tumours were more aggressive, with rapid and consistent formation of CCA lesions and metastases [80,81]. This model was used to elucidate the mechanisms that underlie tumour progression and to evaluate the efficacy of novel drug candidates [81-85]. More recently **[Au: we tend to avoid use of the word 'recent' as it can be a bit vague – please either delete or replace with a more-specific timeframe]** , 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, fetal liver cells obtained from genetically-modified mouse embryos can also be implanted into the mouse liver, inducing CCA formation **[Au:OK?]** [88]. Furthermore, the cells mentioned above **[Au: which cells, specifically?]** can be genetically manipulated before engraftment, revealing insights into the mechanisms that govern cholangiocarcinogenesis and enabling implantation of the cells in already established knockout mice strains, thereby 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 the frequently used endpoint (4 weeks) by 2 additional weeks **[Au: is this the intended meaning? Please check]** enables formation of extrahepatic metastases in the lung **[Au: please provide more information regarding these unpublished observations – unpublished by who? Have these data been submitted to a journal? In general, personal communications or unpublished work involving a third party need to be accompanied by an e-mail from the third party giving permission for the inclusion of this information. If you could provide more detail about the source of these unpublished observations, I will be able to provide further guidance.]** . Therefore, further characterization of this timeline in a genetically malleable 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 understudied process such as CCA metastatic spreading. Overall, these models might **[Au: or can?]** 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.

[H1] Chemically-induced models [Au: should this be a 2nd tier heading? Underneath 'In vivo 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 **[Au: such as?]** , 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 [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 **[Au: please reference]** . 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 **[Au: in what model? Rats?]** [93]. In addition to TAA, several other mutagenic models have also been developed to induce CCA in rodents **[Au:OK?]** . For instance, diethylnitrosamine (DEN) and dimethylnitrosamine (DMN) generate DNA adducts in the liver and are sufficient for liver carcinogenesis [94], and, in combination with inflammatory injury (BDL or *Opisthorchis 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 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 **Figure 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). Recent work **[Au: please replace 'Recent' with a more-specific timeframe]** has 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 to consider is the control tissue that should be compared 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.

[H2] Genetically engineered mouse models [Au: should this be a 2nd tier heading? Underneath 'In vivo models'?]

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 (hydrodynamic tail vein injection (HTVI) 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 [99].

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. **[Au: I don't think this previous sentence was necessary – OK to delete?]**

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 [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 that closely recapitulates human cholangiocarcinogenesis consists of concomitant *Trp53* abrogation and *Kras*^{G12D} 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]. *Kras*^{G12D} activation and *Trp53* loss in adult hepatocytes required co-administration of DDC **[Au: please add the definition for DDC]** -diet to form tumours (iCCA and HCC with a similar incidence, in addition to combined HCC-CCA), highlighting the role of inflammation in liver cancer formation. By contrast, activation of the transgenes **[Au: which transgenes?]** in the adult ductal compartment in the *Sox9-Cre*$^{ERT2+}$ **[Au: strain?]** accelerated the development of hepatic tumours, mainly iCCA, from preneoplastic lesions (not found in AAV8-injected mice) without the need for inflammatory cues. **[Au: is this still ref 102?]**

Targeting **[Au: can 'Targeting' be deleted? Or changed to 'Targeted'?]** *Kras*^{G12D} activation and *Pten* deletion triggered the fastest GEMM of CCA **[Au:OK?]** in *Alb-Cre* mice [103]. In *Kras*$^{LSL-G12D/+}$;*Pten*$^{flox/flox}$;*Alb-Cre* mice **[Au:OK?]** , 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 longer latency, showing hepatocyte and cholangiocyte differentiation features. By using *Alb-Cre*$^{ERT2+}$ or *K19Cre*$^{ERT/+}$ **[Au:OK?]** mouse strains to activate the oncogenic alterations in adult hepatocytes or cholangiocytes, respectively, the researchers 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, whereas 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 **[Au: as in, both?]** oncogene modelling in mice was employed [105,106]. Breeding of *Idh2*$^{LSL-R172K}$ and *Kras*$^{LSL-G12D}$ mice **[Au:OK?]** 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 **[Au: please provide a more specific timeframe]** , 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 Notch1 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 **[Au: do you wish to refer to the gene or protein here?]** expression. In the second model, *Kras*^{G12D} 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] enabled follow-up experiments otherwise limited by the mice's short life span.

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 jeopardized their use **[Au: which carcinogens, specifically?]** [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 that recapitulates the human context [113]. **[Au: in the reference list, please complete the reference details for ref 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 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 [47,114]. 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 others, 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; and finally, a drug should be tested in more than one model. **[Au: We are unable to include bullet points in the main text, so I have separated these with semi-colons instead. OK?]**

Finally, to adopt a shared tool for defining the CCA experimental models homogeneously, an "experimental model sheet" was generated, based on an initial expert discussion in a physical ad hoc meeting (Malta meeting 20189; WG1 meeting) (**Box 2; Supplementary Table 3**), to provide complete information on animal experimentations to the scientific community through publications. **[Au: who attended this meeting? A subset of the 45 experts mentioned previously? Please clarify.]**

[H1] In vitro CCA models

[H2] 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 [115]. Later, an assortment of CCA cell lines of intrahepatic and extrahepatic origin was 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**). **[Au: is 'proper' the appropriate word here?]** 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*, *KRAS* and *SMAD4*, *FGFR2* receptor fusions, or *ERBB* family gene amplifications [116], which, **[Au: please clarify what is meant by 'in part' here]** 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 **[Au: change to 'only in the past few years'?]** , with the development 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 (**Supplementary Table 2**) [117-119]. This has enabled the selection of cell lines with specific genetic alterations that represent 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**[Au: guidelines on what, specifically?]** [120], and potential misclassification of the origin of some cell lines mght 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 [121,122], but they are classified as a gallbladder carcinoma cell line. Thus, results that were acquired using this cell line should be considered for patients with this specific type of tumour. **[Au: please rephrase this sentence – it's not clear what 'results … should be considered' means]**

In general, the well-established cell lines **[Au: such as?]** represent an easy model to explore mechanisms of tumorigenesis and to 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 that favour the accumulation of new genomic alterations as seen in many other long-term cultured cell lines

[Au: please further clarify why this is a limitation and whether this has been observed in CCA cell lines specifically] [123-126]. New mutations obviously are unwanted to study 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 **[Au: please reference]** . In addition, cell cultures grown as a monolayer might **[Au: might or can?]** 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 **[Au: human?] human** CCA tissue have been established [127-130]. The overall success rate for CCA cell line isolation and establishment is relatively low (approximately 10%) **[Au: please reference]** , partly due to insufficient numbers of tumour cells in resected tissues. Notably, contaminating non-tumour cells such as **[Au: here, does 'i.e.' mean 'that is' or 'such as' or 'specifically'?]** 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, thereby 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. Including the extracellular matrix and/or stromal cells would benefit the model. **[Au: which components of the TME would it be most useful for these models to contain?]** . 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 (that is, cancer-associated fibroblasts (CAFs), and 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 publications the origin of any cell line (previously established or new) according to the new CCA classification (intrahepatic, perihilar, or distal). 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 sub-culturing methods). **[Au: light edits to paragraph OK?]**

[H2] 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 **[Au:OK?]** 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 [5,138], whereas organoids self-organize in a matrix-rich 3D environment with which they interact [139,140, 6,141]. 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.

[H3] Spheroids

Tumour spheroids, which are typically **[Au: by 'mostly', do you mean '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 [5,138]. 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 due to the complex culture conditions. **[Au: why is this?]**

[H3] Organoids

Robust protocols for deriving biliary organoids from both mouse and human primary tissue explants or biopsy samples have been established [6,140], and are complemented by methods that enable the derivation and propagation of organoids from induced pluripotent stem cells [143] or cells collected from bile [144,145]. In addition to 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 from different patient tumours should be at least 25% **[Au: as in, this is a consensus that has been reached as part of this Consensus Statement? Or is this a general comment? If the latter, please reference]** . 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 **[Au:OK?]** . Using specific tumour enrichment medium [148], resorting to hand picking non-malignant or tumour organoids to clean up the culture, and xenotransplantations are ways to address this challenge. It is agreed that tumorigenicity needs to be confirmed for all 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, as well as *FGFR2* fusions and MYC overexpression) were engineered in vitro in either human hepatocyte organoids [149,150] **[Au: CCA specifically? Or were these hepatocyte studies?]** or mouse [151]. 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, gave rise to CCA upon subcutaneous or orthotopic transplantation into mice. This approach is therefore suitable for modelling genetically-defined cholangiocarcinogenesis in bipotent liver precursors and for generating models for precision oncology research [10].

Based on the available data and a unanimous agreement, the expert panel strongly suggests (Box 1) **[Au:OK?]** : 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 **[Au: please replace DOI with reference citation number]**) 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

publications; to characterize every organoid culture before clinical applications such as drug screening. **[Au: we are unable to include bullet points in the main text, so I have separated these recommendations with a semi-colon. OK?]**

[H1] Complex 3D culture systems [Au: should this be a 2nd tier heading? Underneath 'In vitro models'?]

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) not fully comprising human or tumour ECM **[Au: please reference]** . Moreover, additional stromal cells such as fibroblasts and immune cells are generally lacking in these cultures. The tumour microenvironment has 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 to be vital to mimic tumour composition, cell-cell and cell-matrix interaction [156], 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 bio-printed scaffolds that recapitulate the anatomy of the biliary system **[Au: please reference]**. This includes mmune 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 **[Au: please check grammatical and punctuation changes to this sentence – is this the intended meaning? Or are these three separate current efforts?]** . The challenges reside in the co-culture of autologous cell types derived from the same patient, as each cell type will have a unique **[Au: by 'peculiar', do you mean 'unique' or 'characteristic'?]** 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.

[H1] Addressing clinical needs [Au: please shorten heading to 38 characters including spaces]

The experimental models described previously will facilitate the translation from experimental and preclinical work to the clinical setting. Whereas some models provide relevant insights into the basic

mechanisms of cancer progression, unravelling pathway and cell signaling analysis, and cell-cell or tumour-microenvironment interactions **[Au: please rephrase this – I think there's a word missing in this sentence]** , 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 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 RAS-ERK signalling is necessary and sufficient to support the oncogenic activity of *FGFR2* fusions in PDXs [157], **[Au: in the reference list, please complete details for reference 157]** GEMMs [158], and organoid-based iCCA models [151]; and that combination therapies capable of more robust and durable suppression of RAS-ERK improved the therapeutic efficacy of clinically approved FGFR tyrosine kinase inhibitors **[Au:OK?]** [151,157,158]. Likewise, *IDH1/KRAS*-driven **[Au: are you referring to mouse genes or human genes here? Or proteins?]** 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 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 [159,160], are already in clinical use and will push the field forward. Finally, additional pharmacodynamic biomarkers that are able to track disease evolution more accurately than the carbohydrate antigen 19-9 (CA 19-9), which is a tumor marker used in the management of biliary and pancreatic cancer **[Au: this has not yet been mentioned in this article – could you briefly clarify for our nonspecialist readers why CA 19-9 is relevant here?]** and that can reveal the emergence of drug resistance are warranted [161], as shown for FGFR2 resistance **[Au: please provide further details – how has this been shown for FGFR2 resistance?]** [162]. By exposing FGFR2-mutated cells to an irreversible FGFR inhibitor (TAS-120), a clinical benefit is found in resistant patients.

CCA organoids have proven helpful for understanding fundamental mechanisms of cancer progression and biomarker discovery [7]. Although successful derivation of CCA organoids has lagged behind some other tumour types, organoids have high potential as tools for improving CCA research and therapy [163]. **[Au: please complete reference details in the reference list for ref 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 extracellular matrix dynamics, which are constantly remodelled by tumour cells, CAFs and tumor-associated macrophages, create a desmoplastic environment **[Au:OK?]** . 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 the in vivo efficacy, resulting in newly developed drugs failing in phase I to phase 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, 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.

[H1] Study strengths and limitations [Au:OK?]

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 anti-tumour therapies, and for deciphering therapeutic resistance mechanisms. The panoply of CCA experimental models is wide, ranging from simple, practical and inexpensive to

more complex models resembling human cancer biology, albeit with a more challenging implementation process **[Au: a more challenging implementation process?]** and higher costs **[Au:OK?]** . 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, 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 advantages or 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, results gathered using different models are highly recommended to provide a comprehensive tumour mimic,. 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

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 a brief overview of currently available models, highlighting the advantages and disadvantages that scientists should be aware of **[Au:OK?]** . Importantly, this Consensus Statement has been prepared on the basis of the expertise of both researchers and clinicians from different specialties (cell biologists, molecular

biologists, oncologists, hepatologists and pathologists), thus ensuring the relevance of these statements and recommendations for a broad range of scientific communities **[Au:OK?]** , from healthcare professionals **[Au: professionals? Is 'medical healthcare' your intended meaning here?]** to scientists who are directly investigating this fatal cancer.

Acknowledgements

The authors thank the European Network for the Study of Cholangiocarcinoma (ENS-CCA) and the European H2020 COST Action CA18122. The authors also acknowledge the valuable contributions of the external advisory panel **[Au: which external advisory panel?]** . M.M.A.V. and L.J.W.v.d.L. are supported by Medical Delta Regenerative Medicine 4D: Generating complex tissues with stem cells and printing technology and TKI-LSH grant: EMC-LSH19002. S.V. is supported by Ministerio de Ciencia, Innovación y Universidades, Convocatoria 2019 para incentivar la Incorporación Estable de Doctores (IED2019-001007-I), by FEDER/Ministerio de Ciencia, Innovación y Universidades - Agencia Estatal de Investigación (PID2020-116344-RB-100) and by the Government of Navarra-Health Research Department (58; 2018). J.V. is funded by Ministerio de Ciencia e Innovación, which is part of Agencia Estatal de Investigación (AEI), through the Retos Investigación grant, number PID2019-108651RJ-I00 / DOI: 10.13039/501100011033. The authors thank CERCA Programme / Generalitat de Catalunya for institutional support. R.I.R.M. and J.J.G.M. are supported by Instituto de Salud Carlos III, Spain (PI20/00189, PI19/00819) co-funded by the European Union. L.F. belongs to a team supported by the Fondation pour la Recherche Médicale (Equipe FRM 2020 n°EQU202003010517). L.F. thanks Yves Chrétien for designing the figures . **[Au: please clarify what is meant by 'the graphic support']**

Author contributions

L.F. coordinated the workgroups and the process of generating and editing the manuscript **[Au: please clarify what is meant by 'the review']** M.V. and R.C. coordinated the Delphi questionnaire, and all authors contributed equally to the writing **[Au:please clarify what is meant by 'redaction']** and final revision of the manuscript.

Competing interests

C.B. receives honoraria from Incyte and Servier. A.F. received consultancy fees from Bayer, AstraZeneca, Roche, Boston, Exact Science, and Guerbert. J.W.V. reports honoraria from Agios, AstraZeneca, Genoscience Pharma, Incyte, Mundipharma EDO, Mylan, QED, Servier, SIRTex, Zymeworks and honoraria, and non-financial support from NuCana; all outside the submitted work.

The other authors declare no competing interests.

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

Peer review information

Nature Reviews XXX thanks [Referee#1 name], [Referee#2 name] and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. **[Au: added as a placeholder for now]**

Supplementary information

Supplementary information is available for this paper at https://doi.org/10.1038/s415XX-XXX-XXXX-X

[Au: For references that are particularly worth reading (5-10% of the total), please provide a single bold sentence that indicates the significance of the work. (This is optional)]

Bibliography

1. Marin, J. J. G., Herraez, E., Lozano, E., Macias, R. I. R. & Briz, O. Models for Understanding Resistance to Chemotherapy in Liver Cancer. *Cancers (Basel)* **11** (2019).
2. Banales, J. M. et al. Cholangiocarcinoma 2020: the next horizon in mechanisms and management. *Nat Rev Gastroenterol Hepatol* **17**, 557-588 (2020).
3. Zach, S., Birgin, E. & Rückert, F. Primary Cholangiocellular Carcinoma Cell Lines. *J Stem Cell Res Transplant* **2**, 1013 (2015).
4. Martinez-Becerra, P. et al. No correlation between the expression of FXR and genes involved in multidrug resistance phenotype of primary liver tumors. *Mol Pharm* **9**, 1693-1704 (2012).
5. Raggi, C. et al. Cholangiocarcinoma stem-like subset shapes tumor-initiating niche by educating associated macrophages. *J Hepatol* **66**, 102-115 (2017).
6. Marsee, A. et al. Building consensus on definition and nomenclature of hepatic, pancreatic, and biliary organoids. *Cell Stem Cell* **28**, 816-832 (2021).
7. Broutier, L. et al. Human primary liver cancer-derived organoid cultures for disease modeling and drug screening. *Nat Med* **23**, 1424-1435 (2017).
8. Weiswald, L. B., Bellet, D. & Dangles-Marie, V. Spherical cancer models in tumor biology. *Neoplasia* **17**, 1-15 (2015).
9. Nuciforo, S. et al. Organoid Models of Human Liver Cancers Derived from Tumor Needle Biopsies. *Cell Rep* **24**, 1363-1376 (2018).
10. Saborowski, A. et al. Murine Liver Organoids as a Genetically Flexible System to Study Liver Cancer In Vivo and In Vitro. *Hepatol Commun* **3**, 423-436 (2019).
11. Wang, J. et al. Loss of Fbxw7 synergizes with activated Akt signaling to promote c-Myc dependent cholangiocarcinogenesis. *J Hepatol* **71**, 742-752 (2019).
12. Primrose, J. N. et al. Capecitabine compared with observation in resected biliary tract cancer (BILCAP): a randomised, controlled, multicentre, phase 3 study. *Lancet Oncol* **20**, 663-673 (2019).
13. Jusakul, A. et al. Whole-Genome and Epigenomic Landscapes of Etiologically Distinct Subtypes of Cholangiocarcinoma. *Cancer Discov* **7**, 1116-1135 (2017).
14. Abou-Alfa, G. K. et al. Ivosidenib in IDH1-mutant, chemotherapy-refractory cholangiocarcinoma (ClarIDHy): a multicentre, randomised, double-blind, placebo-controlled, phase 3 study. *Lancet Oncol* **21**, 796-807 (2020).
15. Abou-Alfa, G. K. et al. Pemigatinib for previously treated, locally advanced or metastatic cholangiocarcinoma: a multicentre, open-label, phase 2 study. *Lancet Oncol* **21**, 671-684 (2020).
16. Roskoski, R., Jr. Properties of FDA-approved small molecule protein kinase inhibitors: A 2022 update. *Pharmacol Res* **175**, 106037 (2022).
17. Izquierdo-Sanchez, L. et al. Cholangiocarcinoma landscape in Europe: diagnostic, prognostic and therapeutic insights from the ENSCCA Registry. *J Hepatol* (2021).

18. Goyal, L. et al. Polyclonal Secondary FGFR2 Mutations Drive Acquired Resistance to FGFR Inhibition in Patients with FGFR2 Fusion-Positive Cholangiocarcinoma. *Cancer Discov* **7**, 252-263 (2017).
19. Lowery, M. A. et al. Safety and activity of ivosidenib in patients with IDH1-mutant advanced cholangiocarcinoma: a phase 1 study. *Lancet Gastroenterol Hepatol* **4**, 711-720 (2019).
20. Saborowski, A., Vogel, A. & Segatto, O. Combination therapies for targeting FGFR2 fusions in cholangiocarcinoma. *Trends Cancer* **8**, 83-86 (2022).
21. Wu, M. J., Shi, L., Merritt, J., Zhu, A. X. & Bardeesy, N. Biology of IDH mutant cholangiocarcinoma. *Hepatology* (2022).
22. Cancer, I. A. f. R. o. in *WHO Blue Books* Vol. 1 (ed WHO Classification of Tumours) 635 (World Health Organization, 2019).
23. Guglielmi, A. et al. Intrahepatic cholangiocarcinoma: prognostic factors after surgical resection. *World J Surg* **33**, 1247-1254 (2009).
24. Kendall, T. et al. Anatomical, histomorphological and molecular classification of cholangiocarcinoma. *Liver Int* **39 Suppl 1**, 7-18 (2019).
25. Zen, Y., Quaglia, A., Heaton, N., Rela, M. & Portmann, B. Two distinct pathways of carcinogenesis in primary sclerosing cholangitis. *Histopathology* **59**, 1100-1110 (2011).
26. Zen, Y. et al. Biliary intraepithelial neoplasia: an international interobserver agreement study and proposal for diagnostic criteria. *Mod Pathol* **20**, 701-709 (2007).
27. Fujikura, K. et al. Comparative clinicopathological study of biliary intraductal papillary neoplasms and papillary cholangiocarcinomas. *Histopathology* **69**, 950-961 (2016).
28. Komori, T. et al. CT imaging comparison between intraductal papillary neoplasms of the bile duct and papillary cholangiocarcinomas. *Eur Radiol* **29**, 3132-3140 (2019).
29. Quigley, B. et al. Hepatobiliary Mucinous Cystic Neoplasms With Ovarian Type Stroma (So-Called "Hepatobiliary Cystadenoma/Cystadenocarcinoma"): Clinicopathologic Analysis of 36 Cases Illustrates Rarity of Carcinomatous Change. *Am J Surg Pathol* **42**, 95-102 (2018).
30. Zen, Y. et al. Mucinous cystic neoplasms of the liver: a clinicopathological study and comparison with intraductal papillary neoplasms of the bile duct. *Mod Pathol* **24**, 1079-1089 (2011).
31. Chan-On, W. et al. Exome sequencing identifies distinct mutational patterns in liver fluke-related and non-infection-related bile duct cancers. *Nat Genet* **45**, 1474-1478 (2013).
32. Farshidfar, F. et al. Integrative Genomic Analysis of Cholangiocarcinoma Identifies Distinct IDH-Mutant Molecular Profiles. *Cell Rep* **19**, 2878-2880 (2017).
33. Fujimoto, A. et al. Whole-genome mutational landscape of liver cancers displaying biliary phenotype reveals hepatitis impact and molecular diversity. *Nat Commun* **6**, 6120 (2015).
34. Gao, Q. et al. Activating mutations in PTPN3 promote cholangiocarcinoma cell proliferation and migration and are associated with tumor recurrence in patients. *Gastroenterology* **146**, 1397-1407 (2014).
35. Nepal, C. et al. Genomic perturbations reveal distinct regulatory networks in intrahepatic cholangiocarcinoma. *Hepatology* **68**, 949-963 (2018).
36. Nakamura, H. et al. Genomic spectra of biliary tract cancer. *Nat Genet* **47**, 1003-1010 (2015).

37. Ong, C. K. et al. Exome sequencing of liver fluke-associated cholangiocarcinoma. *Nat Genet* **44**, 690-693 (2012).
38. Zou, S. et al. Mutational landscape of intrahepatic cholangiocarcinoma. *Nat Commun* **5**, 5696 (2014).
39. Lawrence, M. S. et al. Mutational heterogeneity in cancer and the search for new cancer-associated genes. *Nature* **499**, 214-218 (2013).
40. Andersen, J. B. et al. Genomic and genetic characterization of cholangiocarcinoma identifies therapeutic targets for tyrosine kinase inhibitors. *Gastroenterology* **142**, 1021-1031 e1015 (2012).
41. Sia, D. et al. Integrative molecular analysis of intrahepatic cholangiocarcinoma reveals 2 classes that have different outcomes. *Gastroenterology* **144**, 829-840 (2013).
42. Goeppert, B. et al. Integrative Analysis Defines Distinct Prognostic Subgroups of Intrahepatic Cholangiocarcinoma. *Hepatology* **69**, 2091-2106 (2019).
43. Montal, R. et al. Molecular classification and therapeutic targets in extrahepatic cholangiocarcinoma. *J Hepatol* **73**, 315-327 (2020).
44. Marquardt, J. U., Andersen, J. B. & Thorgeirsson, S. S. Functional and genetic deconstruction of the cellular origin in liver cancer. *Nat Rev Cancer* **15**, 653-667 (2015).
45. Moeini, A., Haber, P. K. & Sia, D. Cell of origin in biliary tract cancers and clinical implications. *JHEP Rep* **3**, 100226 (2021).
46. Holczbauer, A. et al. Modeling pathogenesis of primary liver cancer in lineage-specific mouse cell types. *Gastroenterology* **145**, 221-231 (2013).
47. Guest, R. V. et al. Cell lineage tracing reveals a biliary origin of intrahepatic cholangiocarcinoma. *Cancer Res* **74**, 1005-1010 (2014).
48. Lee, J. S. et al. Application of comparative functional genomics to identify best-fit mouse models to study human cancer. *Nat Genet* **36**, 1306-1311 (2004).
49. Lee, J. S. et al. A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells. *Nat Med* **12**, 410-416 (2006).
50. Massa, A. et al. Evolution of the Experimental Models of Cholangiocarcinoma. *Cancers (Basel)* **12** (2020).
51. Loeuillard, E., Fischbach, S. R., Gores, G. J. & Rizvi, S. Animal models of cholangiocarcinoma. *Biochim Biophys Acta Mol Basis Dis* **1865**, 982-992 (2019).
52. Mohr, R. et al. In Vivo Models for Cholangiocarcinoma-What Can We Learn for Human Disease? *Int J Mol Sci* **21** (2020).
53. Cadamuro, M. et al. Animal models of cholangiocarcinoma: What they teach us about the human disease. *Clin Res Hepatol Gastroenterol* **42**, 403-415 (2018).
54. Fava, G. et al. gamma-Aminobutyric acid inhibits cholangiocarcinoma growth by cyclic AMP-dependent regulation of the protein kinase A/extracellular signal-regulated kinase 1/2 pathway. *Cancer Res* **65**, 11437-11446 (2005).
55. Hu, M. H. et al. Targeting SHP-1-STAT3 signaling: A promising therapeutic approach for the treatment of cholangiocarcinoma. *Oncotarget* **8**, 65077-65089 (2017).

56. Samukawa, E. et al. Angiotensin receptor blocker telmisartan inhibits cell proliferation and tumor growth of cholangiocarcinoma through cell cycle arrest. *Int J Oncol* **51**, 1674-1684 (2017).
57. Pawar, P. et al. Molecular mechanisms of tamoxifen therapy for cholangiocarcinoma: role of calmodulin. *Clin Cancer Res* **15**, 1288-1296 (2009).
58. Colyn, L. et al. Dual Targeting of G9a and DNA Methyltransferase-1 for the Treatment of Experimental Cholangiocarcinoma. *Hepatology* **73**, 2380-2396 (2021).
59. Hou, Y. J. et al. Inhibition of active autophagy induces apoptosis and increases chemosensitivity in cholangiocarcinoma. *Lab Invest* **91**, 1146-1157 (2011).
60. Merino-Azpitarte, M. et al. SOX17 regulates cholangiocyte differentiation and acts as a tumor suppressor in cholangiocarcinoma. *J Hepatol* **67**, 72-83 (2017).
61. Meng, F., Yamagiwa, Y., Ueno, Y. & Patel, T. Over-expression of interleukin-6 enhances cell survival and transformed cell growth in human malignant cholangiocytes. *J Hepatol* **44**, 1055-1065 (2006).
62. Lobe, C. et al. Zinc Finger E-Box Binding Homeobox 1 Promotes Cholangiocarcinoma Progression Through Tumor Dedifferentiation and Tumor-Stroma Paracrine Signaling. *Hepatology* **74**, 3194-3212 (2021).
63. Gentilini, A. et al. Extracellular Signal-Regulated Kinase 5 Regulates the Malignant Phenotype of Cholangiocarcinoma Cells. *Hepatology* **74**, 2007-2020 (2021).
64. Vallejo, A. et al. FOSL1 promotes cholangiocarcinoma via transcriptional effectors that could be therapeutically targeted. *J Hepatol* **75**, 363-376 (2021).
65. Olaru, A. V. et al. MicroRNA down-regulated in human cholangiocarcinoma control cell cycle through multiple targets involved in the G1/S checkpoint. *Hepatology* **54**, 2089-2098 (2011).
66. Zhang, J., Han, C. & Wu, T. MicroRNA-26a promotes cholangiocarcinoma growth by activating beta-catenin. *Gastroenterology* **143**, 246-256 e248 (2012).
67. Zhu, H., Han, C., Lu, D. & Wu, T. miR-17-92 cluster promotes cholangiocarcinoma growth: evidence for PTEN as downstream target and IL-6/Stat3 as upstream activator. *Am J Pathol* **184**, 2828-2839 (2014).
68. Zhu, H. et al. Neuropilin-1 regulated by miR-320 contributes to the growth and metastasis of cholangiocarcinoma cells. *Liver Int* **38**, 125-135 (2018).
69. Han, S. et al. Suppression of miR-16 promotes tumor growth and metastasis through reversely regulating YAP1 in human cholangiocarcinoma. *Oncotarget* **8**, 56635-56650 (2017).
70. McVeigh, L. E. et al. Development of orthotopic tumour models using ultrasound-guided intrahepatic injection. *Sci Rep* **9**, 9904 (2019).
71. Erice, O. et al. Differential effects of FXR or TGR5 activation in cholangiocarcinoma progression. *Biochim Biophys Acta Mol Basis Dis* **1864**, 1335-1344 (2018).
72. Cardinale, V. et al. Profiles of cancer stem cell subpopulations in cholangiocarcinomas. *Am J Pathol* **185**, 1724-1739 (2015).
73. Wu, Z. et al. Significance of S100P as a biomarker in diagnosis, prognosis and therapy of opisthorchiasis-associated cholangiocarcinoma. *Int J Cancer* **138**, 396-408 (2016).

74. Cadamuro, M. et al. Low-Dose Paclitaxel Reduces S100A4 Nuclear Import to Inhibit Invasion and Hematogenous Metastasis of Cholangiocarcinoma. *Cancer Res* **76**, 4775-4784 (2016).
75. Peraldo Neia, C. et al. Gene and microRNA modulation upon trabectedin treatment in a human intrahepatic cholangiocarcinoma paired patient derived xenograft and cell line. *Oncotarget* **7**, 86766-86780 (2016).
76. Peraldo-Neia, C. et al. Anti-cancer effect and gene modulation of ET-743 in human biliary tract carcinoma preclinical models. *BMC Cancer* **14**, 918 (2014).
77. Wang, Y. et al. Antitumor effect of FGFR inhibitors on a novel cholangiocarcinoma patient derived xenograft mouse model endogenously expressing an FGFR2-CCDC6 fusion protein. *Cancer Lett* **380**, 163-173 (2016).
78. Saha, S. K. et al. Isocitrate Dehydrogenase Mutations Confer Dasatinib Hypersensitivity and SRC Dependence in Intrahepatic Cholangiocarcinoma. *Cancer Discov* **6**, 727-739 (2016).
79. Kabashima, A. et al. Fibroblast growth factor receptor inhibition induces loss of matrix MCL1 and necrosis in cholangiocarcinoma. *J Hepatol* **68**, 1228-1238 (2018).
80. Sirica, A. E. et al. A novel "patient-like" model of cholangiocarcinoma progression based on bile duct inoculation of tumorigenic rat cholangiocyte cell lines. *Hepatology* **47**, 1178-1190 (2008).
81. Fingas, C. D. et al. A smac mimetic reduces TNF related apoptosis inducing ligand (TRAIL)-induced invasion and metastasis of cholangiocarcinoma cells. *Hepatology* **52**, 550-561 (2010).
82. Blechacz, B. R. et al. Sorafenib inhibits signal transducer and activator of transcription-3 signaling in cholangiocarcinoma cells by activating the phosphatase shatterproof 2. *Hepatology* **50**, 1861-1870 (2009).
83. Smoot, R. L. et al. A Bax-mediated mechanism for obatoclax-induced apoptosis of cholangiocarcinoma cells. *Cancer Res* **70**, 1960-1969 (2010).
84. Fingas, C. D. et al. Targeting PDGFR-beta in Cholangiocarcinoma. *Liver Int* **32**, 400-409 (2012).
85. Mertens, J. C. et al. Therapeutic effects of deleting cancer-associated fibroblasts in cholangiocarcinoma. *Cancer Res* **73**, 897-907 (2013).
86. Yamada, D. et al. IL-33 facilitates oncogene-induced cholangiocarcinoma in mice by an interleukin-6-sensitive mechanism. *Hepatology* **61**, 1627-1642 (2015).
87. Rizvi, S. et al. YAP-associated chromosomal instability and cholangiocarcinoma in mice. *Oncotarget* **9**, 5892-5905 (2018).
88. Saborowski, A. et al. Mouse model of intrahepatic cholangiocarcinoma validates FIG-ROS as a potent fusion oncogene and therapeutic target. *Proc Natl Acad Sci U S A* **110**, 19513-19518 (2013).
89. Loeuillard, E. et al. Targeting tumor-associated macrophages and granulocytic myeloid-derived suppressor cells augments PD-1 blockade in cholangiocarcinoma. *J Clin Invest* **130**, 5380-5396 (2020).
90. Fabris, L., Sato, K., Alpini, G. & Strazzabosco, M. The Tumor Microenvironment in Cholangiocarcinoma Progression. *Hepatology* **73 Suppl 1**, 75-85 (2021).
91. Fitzhugh, O. G. & Nelson, A. A. Liver Tumors in Rats Fed Thiourea or Thioacetamide. *Science* **108**, 626-628 (1948).

92. Al-Bader, A. et al. Cholangiocarcinoma and liver cirrhosis in relation to changes due to thioacetamide. *Mol Cell Biochem* **208**, 1-10 (2000).
93. Lozano, E. et al. Cocarcinogenic effects of intrahepatic bile acid accumulation in cholangiocarcinoma development. *Mol Cancer Res* **12**, 91-100 (2014).
94. Verna, L., Whysner, J. & Williams, G. M. N-nitrosodiethylamine mechanistic data and risk assessment: bioactivation, DNA-adduct formation, mutagenicity, and tumor initiation. *Pharmacol Ther* **71**, 57-81 (1996).
95. Yang, H. et al. A mouse model of cholestasis-associated cholangiocarcinoma and transcription factors involved in progression. *Gastroenterology* **141**, 378-388, 388 e371-374 (2011).
96. Thamavit, W. et al. Promotion of cholangiocarcinogenesis in the hamster liver by bile duct ligation after dimethylnitrosamine initiation. *Carcinogenesis* **14**, 2415-2417 (1993).
97. Thamavit, W., Pairojkul, C., Tiwawech, D., Shirai, T. & Ito, N. Strong promoting effect of Opisthorchis viverrini infection on dimethylnitrosamine-initiated hamster liver. *Cancer Lett* **78**, 121-125 (1994).
98. Maronpot, R. R., Giles, H. D., Dykes, D. J. & Irwin, R. D. Furan-induced hepatic cholangiocarcinomas in Fischer 344 rats. *Toxicol Pathol* **19**, 561-570 (1991).
99. Erice, O. et al. Genetic Mouse Models as In Vivo Tools for Cholangiocarcinoma Research. *Cancers (Basel)* **11** (2019).
100. Xu, X. et al. Induction of intrahepatic cholangiocellular carcinoma by liver-specific disruption of Smad4 and Pten in mice. *J Clin Invest* **116**, 1843-1852 (2006).
101. O'Dell, M. R. et al. Kras(G12D) and p53 mutation cause primary intrahepatic cholangiocarcinoma. *Cancer Res* **72**, 1557-1567 (2012).
102. Hill, M. A. et al. Kras and Tp53 Mutations Cause Cholangiocyte- and Hepatocyte-Derived Cholangiocarcinoma. *Cancer Res* **78**, 4445-4451 (2018).
103. Ikenoue, T. et al. A novel mouse model of intrahepatic cholangiocarcinoma induced by liver-specific Kras activation and Pten deletion. *Sci Rep* **6**, 23899 (2016).
104. Lin, Y. K. et al. Combination of Kras activation and PTEN deletion contributes to murine hepatopancreatic ductal malignancy. *Cancer Lett* **421**, 161-169 (2018).
105. Saha, S. K. et al. Mutant IDH inhibits HNF-4alpha to block hepatocyte differentiation and promote biliary cancer. *Nature* **513**, 110-114 (2014).
106. Dang, L. et al. Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. *Nature* **465**, 966 (2010).
107. Wu, M. J. et al. Mutant IDH Inhibits IFNgamma-TET2 Signaling to Promote Immunoevasion and Tumor Maintenance in Cholangiocarcinoma. *Cancer Discov* **12**, 812-835 (2022).
108. Zender, S. et al. A critical role for notch signaling in the formation of cholangiocellular carcinomas. *Cancer Cell* **23**, 784-795 (2013).
109. Yuan, D. et al. Kupffer Cell-Derived Tnf Triggers Cholangiocellular Tumorigenesis through JNK due to Chronic Mitochondrial Dysfunction and ROS. *Cancer Cell* **31**, 771-789 e776 (2017).

110. Nakagawa, H. et al. Biliary epithelial injury-induced regenerative response by IL-33 promotes cholangiocarcinogenesis from peribiliary glands. *Proc Natl Acad Sci U S A* **114**, E3806-E3815 (2017).
111. Guest, R. V. et al. Notch3 drives development and progression of cholangiocarcinoma. *Proc Natl Acad Sci U S A* **113**, 12250-12255 (2016).
112. Farazi, P. A. et al. Chronic bile duct injury associated with fibrotic matrix microenvironment provokes cholangiocarcinoma in p53-deficient mice. *Cancer Res* **66**, 6622-6627 (2006).
113. Younger, N. T. et al. In vivo modeling of patient genetic heterogeneity identifies new ways to target cholangiocarcinoma. *Cancer Res* (2022).
114. Fan, B. et al. Cholangiocarcinomas can originate from hepatocytes in mice. *J Clin Invest* **122**, 2911-2915 (2012).
115. Yamaguchi, N., Morioka, H., Ohkura, H., Hirohashi, S. & Kawai, K. Establishment and characterization of the human cholangiocarcinoma cell line HChol-Y1 in a serum-free, chemically defined medium. *J Natl Cancer Inst* **75**, 29-35 (1985).
116. Valle, J. W., Lamarca, A., Goyal, L., Barriuso, J. & Zhu, A. X. New Horizons for Precision Medicine in Biliary Tract Cancers. *Cancer Discov* **7**, 943-962 (2017).
117. Lau, D. K. et al. Genomic Profiling of Biliary Tract Cancer Cell Lines Reveals Molecular Subtypes and Actionable Drug Targets. *iScience* **21**, 624-637 (2019).
118. Scherer, D. et al. RNA Sequencing of Hepatobiliary Cancer Cell Lines: Data and Applications to Mutational and Transcriptomic Profiling. *Cancers (Basel)* **12** (2020).
119. Ghandi, M. et al. Next-generation characterization of the Cancer Cell Line Encyclopedia. *Nature* **569**, 503-508 (2019).
120. Nagtegaal, I. D. et al. The 2019 WHO classification of tumours of the digestive system. *Histopathology* **76**, 182-188 (2020).
121. Knuth, A. et al. Biliary adenocarcinoma. Characterisation of three new human tumor cell lines. *J Hepatol* **1**, 579-596 (1985).
122. Fausther, M. et al. Establishment and characterization of rat portal myofibroblast cell lines. *PLoS One* **10**, e0121161 (2015).
123. Kaur, G. & Dufour, J. M. Cell lines: Valuable tools or useless artifacts. *Spermatogenesis* **2**, 1-5 (2012).
124. Domcke, S., Sinha, R., Levine, D. A., Sander, C. & Schultz, N. Evaluating cell lines as tumour models by comparison of genomic profiles. *Nat Commun* **4**, 2126 (2013).
125. Ertel, A., Verghese, A., Byers, S. W., Ochs, M. & Tozeren, A. Pathway-specific differences between tumor cell lines and normal and tumor tissue cells. *Mol Cancer* **5**, 55 (2006).
126. Gillet, J. P. et al. Redefining the relevance of established cancer cell lines to the study of mechanisms of clinical anti-cancer drug resistance. *Proc Natl Acad Sci U S A* **108**, 18708-18713 (2011).
127. Massani, M. et al. Isolation and characterization of biliary epithelial and stromal cells from resected human cholangiocarcinoma: a novel in vitro model to study tumor-stroma interactions. *Oncol Rep* **30**, 1143-1148 (2013).

128. Fraveto, A. et al. Sensitivity of Human Intrahepatic Cholangiocarcinoma Subtypes to Chemotherapeutics and Molecular Targeted Agents: A Study on Primary Cell Cultures. *PLoS One* **10**, e0142124 (2015).
129. Carnevale, G. et al. Activation of Fas/FasL pathway and the role of c-FLIP in primary culture of human cholangiocarcinoma cells. *Sci Rep* **7**, 14419 (2017).
130. Lustri, A. M. et al. TGF-beta signaling is an effective target to impair survival and induce apoptosis of human cholangiocarcinoma cells: A study on human primary cell cultures. *PLoS One* **12**, e0183932 (2017).
131. Pastor, D. M. et al. Primary cell lines: false representation or model system? a comparison of four human colorectal tumors and their coordinately established cell lines. *Int J Clin Exp Med* **3**, 69-83 (2010).
132. Vaquero, J. et al. The IGF2/IR/IGF1R Pathway in Tumor Cells and Myofibroblasts Mediates Resistance to EGFR Inhibition in Cholangiocarcinoma. *Clin Cancer Res* **24**, 4282-4296 (2018).
133. Zhou, Z. et al. Tumor-associated neutrophils and macrophages interaction contributes to intrahepatic cholangiocarcinoma progression by activating STAT3. *J Immunother Cancer* **9** (2021).
134. Okabe, H. et al. Hepatic stellate cells may relate to progression of intrahepatic cholangiocarcinoma. *Ann Surg Oncol* **16**, 2555-2564 (2009).
135. Nicolas-Boluda, A. et al. Photothermal Depletion of Cancer-Associated Fibroblasts Normalizes Tumor Stiffness in Desmoplastic Cholangiocarcinoma. *ACS Nano* **14**, 5738-5753 (2020).
136. Cadamuro, M. et al. Platelet-derived growth factor-D enables liver myofibroblasts to promote tumor lymphangiogenesis in cholangiocarcinoma. *J Hepatol* **70**, 700-709 (2019).
137. Li, L. et al. Extracellular vesicles carry microRNA-195 to intrahepatic cholangiocarcinoma and improve survival in a rat model. *Hepatology* **65**, 501-514 (2017).
138. Fennema, E., Rivron, N., Rouwkema, J., van Blitterswijk, C. & de Boer, J. Spheroid culture as a tool for creating 3D complex tissues. *Trends Biotechnol* **31**, 108-115 (2013).
139. Sato, T. et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* **459**, 262-265 (2009).
140. Huch, M., Boj, S. F. & Clevers, H. Lgr5(+) liver stem cells, hepatic organoids and regenerative medicine. *Regen Med* **8**, 385-387 (2013).
141. Sato, K. et al. Organoids and Spheroids as Models for Studying Cholestatic Liver Injury and Cholangiocarcinoma. *Hepatology* **74**, 491-502 (2021).
142. Girard, Y. K. et al. A 3D fibrous scaffold inducing tumoroids: a platform for anticancer drug development. *PLoS One* **8**, e75345 (2013).
143. Wu, F. et al. Generation of hepatobiliary organoids from human induced pluripotent stem cells. *J Hepatol* **70**, 1145-1158 (2019).
144. Soroka, C. J., Assis, D. N. & Boyer, J. L. Patient-Derived Organoids from Human Bile: An In Vitro Method to Study Cholangiopathies. *Methods Mol Biol* **1981**, 363-372 (2019).
145. Roos, F. J. M. et al. Human Bile Contains Cholangiocyte Organoid-Initiating Cells Which Expand as Functional Cholangiocytes in Non-canonical Wnt Stimulating Conditions. *Front Cell Dev Biol* **8**, 630492 (2020).

146. Lampis, A. et al. MIR21 Drives Resistance to Heat Shock Protein 90 Inhibition in Cholangiocarcinoma. *Gastroenterology* **154**, 1066-1079 e1065 (2018).
147. Li, L. et al. Human primary liver cancer organoids reveal intratumor and interpatient drug response heterogeneity. *JCI Insight* **4** (2019).
148. Broutier, L. et al. Culture and establishment of self-renewing human and mouse adult liver and pancreas 3D organoids and their genetic manipulation. *Nat Protoc* **11**, 1724-1743 (2016).
149. Artegiani, B. et al. Probing the Tumor Suppressor Function of BAP1 in CRISPR-Engineered Human Liver Organoids. *Cell Stem Cell* **24**, 927-943 e926 (2019).
150. Sun, L. et al. Modelling liver cancer initiation with organoids derived from directly reprogrammed human hepatocytes. *Nat Cell Biol* **21**, 1015-1026 (2019).
151. Cristinziano, G. et al. FGFR2 fusion proteins drive oncogenic transformation of mouse liver organoids towards cholangiocarcinoma. *J Hepatol* **75**, 351-362 (2021).
152. Leyva-Illades, D., McMillin, M., Quinn, M. & Demorrow, S. Cholangiocarcinoma pathogenesis: Role of the tumor microenvironment. *Transl Gastrointest Cancer* **1**, 71-80 (2012).
153. Hogdall, D., Lewinska, M. & Andersen, J. B. Desmoplastic Tumor Microenvironment and Immunotherapy in Cholangiocarcinoma. *Trends Cancer* **4**, 239-255 (2018).
154. Cadamuro, M. et al. The deleterious interplay between tumor epithelia and stroma in cholangiocarcinoma. *Biochim Biophys Acta Mol Basis Dis* **1864**, 1435-1443 (2018).
155. Mertens, J. C., Rizvi, S. & Gores, G. J. Targeting cholangiocarcinoma. *Biochim Biophys Acta Mol Basis Dis* **1864**, 1454-1460 (2018).
156. Tanimizu, N. et al. Generation of functional liver organoids on combining hepatocytes and cholangiocytes with hepatobiliary connections ex vivo. *Nat Commun* **12**, 3390 (2021).
157. Wu, Q. et al. EGFR inhibition potentiates FGFR inhibitor therapy and overcomes resistance in FGFR2 fusion-positive cholangiocarcinoma. *Cancer Discovery* (2022).
158. Kendre, G. et al. The Co-mutational Spectrum Determines the Therapeutic Response in Murine FGFR2 Fusion-Driven Cholangiocarcinoma. *Hepatology* **74**, 1357-1370 (2021).
159. Wintachai, P. et al. Diagnostic and Prognostic Value of Circulating Cell-Free DNA for Cholangiocarcinoma. *Diagnostics (Basel)* **11** (2021).
160. Csoma, S. L. et al. Circulating Cell-Free DNA-Based Comprehensive Molecular Analysis of Biliary Tract Cancers Using Next-Generation Sequencing. *Cancers (Basel)* **14** (2022).
161. Varghese, A. M. et al. Noninvasive Detection of Polyclonal Acquired Resistance to FGFR Inhibition in Patients With Cholangiocarcinoma Harboring FGFR2 Alterations. *JCO Precis Oncol* **5** (2021).
162. Goyal, L. et al. TAS-120 Overcomes Resistance to ATP-Competitive FGFR Inhibitors in Patients with FGFR2 Fusion-Positive Intrahepatic Cholangiocarcinoma. *Cancer Discov* **9**, 1064-1079 (2019).
163. van Tienderen, G. S. et al. Hepatobiliary tumor organoids for personalized medicine: a multicenter view on establishment, limitations, and future directions. *Cancer Cell* (2022).
164. Wong, C. H., Siah, K. W. & Lo, A. W. Estimation of clinical trial success rates and related parameters. *Biostatistics* **20**, 273-286 (2019).
165. Lozano, E. et al. Enhanced antitumour drug delivery to cholangiocarcinoma through the apical sodium-dependent bile acid transporter (ASBT). *J Control Release* **216**, 93-102 (2015).

166. Katz, S. F. et al. Disruption of Trp53 in livers of mice induces formation of carcinomas with bilineal differentiation. *Gastroenterology* **142**, 1229-1239 e1223 (2012).
167. Tschaharganeh, D. F. et al. p53-dependent Nestin regulation links tumor suppression to cellular plasticity in liver cancer. *Cell* **158**, 579-592 (2014).
168. El Khatib, M. et al. Activation of Notch signaling is required for cholangiocarcinoma progression and is enhanced by inactivation of p53 in vivo. *PLoS One* **8**, e77433 (2013).
169. Ikenoue, T. et al. Establishment and analysis of a novel mouse line carrying a conditional knockin allele of a cancer-specific FBXW7 mutation. *Sci Rep* **8**, 2021 (2018).
170. Manieri, E. et al. JNK-mediated disruption of bile acid homeostasis promotes intrahepatic cholangiocarcinoma. *Proc Natl Acad Sci U S A* **117**, 16492-16499 (2020).
171. Cubero, F. J. et al. Loss of c-Jun N-terminal Kinase 1 and 2 Function in Liver Epithelial Cells Triggers Biliary Hyperproliferation Resembling Cholangiocarcinoma. *Hepatol Commun* **4**, 834-851 (2020).
172. Marsh, V., Davies, E. J., Williams, G. T. & Clarke, A. R. PTEN loss and KRAS activation cooperate in murine biliary tract malignancies. *J Pathol* **230**, 165-173 (2013).
173. Falcomata, C. et al. Genetic Screens Identify a Context-Specific PI3K/p27(Kip1) Node Driving Extrahepatic Biliary Cancer. *Cancer Discov* (2021).
174. Sekiya, S. & Suzuki, A. Intrahepatic cholangiocarcinoma can arise from Notch-mediated conversion of hepatocytes. *J Clin Invest* **122**, 3914-3918 (2012).
175. Crawford, D. R. et al. Characterization of liver injury, oval cell proliferation and cholangiocarcinogenesis in glutathione S-transferase A3 knockout mice. *Carcinogenesis* **38**, 717-727 (2017).

[Au: The previous Box 1 was against our house style. I have therefore split it up into a Table of benefits vs limitations (now Table 4, but I can renumber appropriately at a later stage) and a Box (Box 1) of recommendations. Is this OK?]

Box 1. Benefits and limitations of cholangiocarcinoma 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 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
***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 **[Au:OK?]**	- 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
3D culture recapitulating a tumour organization: organoids **[Au:OK?]**	- Increased complexity by 3D tumour cell growth in ECM - Well established protocol	- Low initiation efficiency from human tumours - An established line does not fully reflect the polyclonal nature of the original tumour

	- Specific mutations can be introduced in non-tumour organoids to analyse CCA driver mutations	- Overgrowth of non-tumour cells in culture initiation - Absence of stromal cells

ECM, extracellular matrix; HCC/CCA, hepatocholangiocarcinoma; TAA, thioacetamide.

Box 1 | Recommendations for cholangiocarcinoma experimental models [Au:OK?]

Histological assessment (all *in vivo* models)

- 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).
- 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: 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)

- 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 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)

- 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

- A specific tumour "enrichment" medium (i.e., tumour initiating medium as described by Broutier et al., 2017, DOI: 10.1038/nm.4438 **[Au: please replace the DOI with the reference number from the refs list]**) 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-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.

Table 1. Consensus statements [Au: This Table has been reformatted into our house style – I have split the questions up from their 'answers'. I hope that is OK, please check changes carefully. In addition, please check that my minor edits to this Table, for readability and house style, are OK.]

Number	Question	Statement [Au: as I've put the Questions into a separate column, would there be a more appropriate heading for this column than Statement?]	Response yes / total responders	Grade
Histological assessment [Au: all in vivo models?]				
1	Which of the following are malignant features of biliary tumours? [Au:OK?]	Invasion of the basement membrane	31/32	A
		Increased nucleus:cytoplasm ratio	18/31	C
		Distant metastasis	27/32	B
		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?	Morphological examination of H&E	32/32	U
		Immunohistochemistry	27/30	A
		Immunohistochemistry for at least one biliary cytokeratin (e.g., 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-cytokeratin 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 intra- or extra-hepatic 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?	Intra-tumoral heterogeneity (high stroma, inflammatory response, epithelial phenotype)	27/30	A
		Inter-tumoral heterogeneity (large versus small bile duct tumour in iCCA)	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 iCCA may originate from several cells of	Mature hepatocytes	27/32	B
		Mature cholangiocytes	23/32	B
		Hepatic progenitor/oval cells	32/33	A
		Peribiliary glands	29/30	A

	origin. Which of the following cell types may be the cells-of-origin for iCCA?			
In vivo models [Au:OK?] : 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 [Au:OK?] : 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?	Choice of plastic support (i.e., 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 sub-culturing methods (i.e., enzymatic vs.	29/34	B

		mechanical dissociation, etc.)		
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)	NA **[Au:OK?]**	37/38	A
In vitro models [Au:OK?] : 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 (i.e., tumour initiating medium (as described by Broutier *et al.,* 2017, DOI: 10.1038/nm.4438) **[Au: please replace this 'Broutier et al. (2017)' followed by the reference number]**	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 Q 11) before clinical applications such as drug screening?	NA **[Au:OK?]**	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	Refs
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 [Au: this column split into two. OK?]	Disadvantages	Refs
Alfp-Cre, *Trp53*$^{f/f}$	Advanced HCC/CCA (from LPCs)	*Trp53* mutation found in human CCA	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)	100% tumour penetrance	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)	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	103 104
Alb-Cre, *Idh2*$^{LSL-R172}$, *Kras*$^{LSL-G12D}$	Multifocal liver masses of iCCA (from LPCs)	100% penetrance, splenic invasion and peritoneal metastases	Cre activation during embryogenesis, long tumour latency (33-58 weeks)	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)	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	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)	100% penetrance, development of fibrous or inflammatory microenvironment	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)	Low latency (2 months of age)	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	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	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	iCCA exclusive	~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	Elevated ROS associated with cholangiocellular proliferation	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 **[Au: and what?]**) (from LPCs)	100% penetrance, average latency 16 weeks, metastatic lesions	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)	Recombination event in adult mice, higher CCA frequency in combination with DCC diet (all tumours ICC or mixed HCC/CCA)	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)	100% penetrance, low latency (43 days), tumour development starts in adult mice	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)	100% penetrance, iCCA exclusive, recombination in mature cholangiocytes	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)	Low latency (4 weeks), eCCA exclusive	Concurrent development of lung adenocarcinomas leads	110

			to mice asphyxiation	
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)	eCCA exclusive, only one genetic hit driving CCA	~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	Quick model, orthotopic implantation in the liver, iCCA exclusive, stroma presence	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	Quick model	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	Tumour latency of 6-8 weeks for Kras-mut and Trp53-ko organoids	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	Quick and reproducible model, orthotopic implantation in the liver, iCCA exclusive, stroma presence	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	100% penetrance, iCCA exclusive	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	iCCA exclusive	80% penetrance, long latency (>6 months)	111
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	112
GSTA3$^{-/-}$ mice treated with aflatoxin B1	Macro- and microscopic liver cysts, hepatocellular nodules, cholangiomas, iCCA and oval cell proliferation	**[Au: no advantages?]**	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	Participation of inflammatory insult	No established CCA, long latency	171

BIN: biliary intraepithelial neoplasia; CCl_4: carbon tetrachloride; 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.

[Au: Unfortunately, the previous Table 4 was against house style. We are unable to have blank cells in a Table. Therefore, I have converted this Table into a Box (see below, Box 2) and I have moved this full 'Experimental model sheet' into the Supplementary Information file (please check). OK?]

Box 2 | Experimental model sheet criteria

[Au: please add an introductory sentence to this list. You can include a citation to the Suppl Table (Suppl Table 3) here too.]

- Type of model (in vitro, ex vivo, in vivo)
- Species (mouse, rat, hamster, human, etc.)
- Sex (male, female, both) [Au: edit from 'Gender' to 'Sex' OK?]
- 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)

[Au: Please confirm whether the Figures are entirely original or whether they have previously appeared elsewhere. If they have appeared anywhere previously, we may need to acquire permissions to use them.]

Figure 1. Panel of experimental models provided for cholangiocarcinoma preclinical studies. a | In vitro models. b | In vivo models. **[Au:OK?]**

Figure 2. Schematic summary of available chemical models to initiate cholangiocarcinoma in rodents and induce metastatic dissemination. **[Au: Figure legends require one figure legend heading (in bold) and at least one additional sentence of detail. Please either add a new, shorter title, or add an additional sentence of description.]**