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Criteria for preclinical models of cholangiocarcinoma: scientific and medical relevance

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Abstract

Cholangiocarcinoma (CCA) is a rare malignancy developing at any point along the biliary tree. CCA has a poor prognosis, its clinical management remains challenging, and effective treatments are lacking. Preclinical research, therefore, is of pivotal importance and necessary to acquire a deeper understanding of CCA and improve therapeutic outcomes. Preclinical research involves developing and managing complementary experimental models from *in vitro* assays using primary cells or cell lines cultured in 2D or 3D to *in vivo* models with engrafted material, chemically-induced CCA, or genetically-engineered models. All are valuable tools with well-defined advantages and limitations. The choice of preclinical model is guided by the question(s) to be addressed, and ideally, results should be recapitulated in independent approaches. Here, a task force of 45 experts in CCA molecular and cellular biology, clinicians, including pathologists, from 10 countries, provides recommendations on the minimal criteria for preclinical models to provide a uniform approach. These recommendations are based on two rounds of questionnaires completed by 37 (first round) and 45 (second round) experts to reach a consensus with 13 statements. An agreement was defined when at least 90% of the participants voting anonymously agreed with a statement. The ultimate goal is to transfer (basic) laboratory research to the clinics through increased disease understanding and develop clinical biomarkers and innovative therapies for patients with CCA.

97 During the last decade, we witnessed considerable advances in understanding the molecular
98 pathogenesis of cholangiocarcinoma (CCA). However, early diagnosis and effective treatments for this
99 aggressive cancer lag behind other fields. To accelerate the development of novel clinical strategies,
100 preclinical models of CCA are essential [1]. Critical points to consider when using or developing these
101 tools are the tumour anatomical origin (i.e., intrahepatic, perihilar, or distal CCA), the cell(s) of origin
102 (e.g., preneoplastic lesions), and the histomorphological tumour features (e.g., large vs. small bile duct
103 type) [2].
104 Historically, 2D cell cultures have been widely used as *in vitro* model of CCA. In addition to
105 experimentally-immortalized or primary cultures of normal cholangiocytes derived from normal bile
106 ducts, over 50 CCA-derived cell lines have been established [3]. A limitation of these models is the lack
107 of resemblance to the original tumours upon the continuous culturing, making it difficult to infer which
108 therapeutics would have been efficient to treat the original neoplasm [4]. Moreover, 2D mono-cultures do
109 not accurately mimic the characteristic features of biliary tumours, namely the three-dimensional
110 architecture, cell-to-cell, and cell-to-matrix interactions, cellular heterogeneity, and the effect of the
111 tumour microenvironment in cancer progression. To overcome these limitations, multicellular 3D
112 models, such as spheroids and organoids, have been developed. Although they constitute valuable
113 models to study CCA [5], spheroids usually do not precisely recapitulate the native tissue architecture and
114 function of the tissue of origin [6]. In contrast, organoids maintain a higher and more predictable physical
115 order in the cellular self-assembly and display a marked interaction with the extracellular matrix, thereby
116 retaining most of the histological and malignant characteristics of the original neoplasm [6-9]. In addition
117 to cell culture-based models, different *in vivo* CCA models have been developed. CCA induction through
118 administering hepatocarcinogens or liver fluke infestation has the advantage of mimicking cancer
119 pathogenesis. However, animal studies are time-consuming, expensive, ethically challenging, and
120 sometimes, hepatocellular carcinoma (HCC) rather than CCA preferentially develops. To give *in vivo*
121 context to 2D cell lines, CCA cells have been used to generate subcutaneous or orthotopic xenografts
122 in mice. However, these approaches remain limited by poor rates of tumor engraftment. Technological
123 advancements have made it possible to grow liver organoids, i.e., 3D cultures of bipotent liver
124 precursors, and therefore develop mouse models based on transplantation of genetically modified liver
125 organoids that undergo *in vivo* oncogenic transformation along the cholangiocellular lineage [10].

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

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

135

136 **Methods**

137 ***Panel of experts***

138 A core group of 8 core group members, all active researchers with significant contributions to the CCA
139 field, initiated and led a Delphi study to define recommendations on the minimal criteria for experimental
140 CCA models to provide a uniform approach for future studies. Furthermore, core group members
141 identified 27 additional experts to be invited to join the steering committee and to be actively involved in
142 implementing the Delphi process. These core and steering team members filled the initial Delphi
143 questionnaire and are listed authors, and they proposed 10 additional experts to fill the second and final
144 questionnaire. These 10 experts, not actively involved in writing the recommendations but providing
145 their precious input by filling the second questionnaire, are listed as one collaborative author; CCA Model
146 consortium. Thus, the final panel consisted of 45 individual experts from 10 countries located in Europe,
147 Asia, and USA. **Supplementary Table S1** summarizes the expert panel's names, institutes, and
148 demographics.

149 ***Building consensus***

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

153 ***Questionnaires***

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

156 Part 1.Defining minimal and advanced criteria for experimental models, Part 2.*In vivo* model for CCA,
157 Part 3.*In vitro* models for CCA, and Part 4.Preclinical models for CCA. Based on questionnaire 1
158 (Supplemental data 1), a second questionnaire was designed, including 13 statements, of which 12
159 could be solely answered with 'yes' or 'no' (**Table 1**). All experts could comment on every question. Both
160 questionnaires and summaries of the outcome are shared in Supplementary Data 1. Through the
161 consensus of experts in the field, we propose overarching criteria to be used when establishing or using
162 preclinical models of CCA and linking this to the clinic (**Figure 1**). From the second questionnaire, core
163 recommendations were edited (new **Box 1**).

164 **Clinical features to consider when using experimental models**

165 ***Clinics***

166 Experimental models of CCA must reflect the natural history of the known subtypes of CCA, their
167 molecular heterogeneity, and the impact of clinical or therapeutic interventions. In ICD11, published in
168 2022, CCA is classified according to its origin as intrahepatic (iCCA) and extrahepatic (eCCA)
169 (https://icd.who.int). iCCA arises from intrahepatic bile ducts, i.e., it grows in the liver. Consequently, it
170 is more often surgically resectable than perihilar CCA (pCCA), the latter arising at the liver hilum where
171 the likelihood of local vascular invasion is greater [12]. The impact of tumour biology on local invasion is
172 poorly understood and requires further examination.

173 The biology of CCA subtypes also differs significantly. Approximately 50% of iCCAs have actionable
174 molecular alterations, and targeted therapies against *FGFR2* fusions and *IDH1* mutation-driven cancers
175 are already approved [13-16]. The reason why iCCAs are more molecularly heterogeneous than p/dCCAs
176 is not fully understood and requires detailed examination. In addition, the influence of biology on the
177 natural history of iCCA and its impact on surgical, local, and systemic treatment options necessitate
178 further studies [17]. dCCA more closely resembles pCCA, but, again, the effect of both anatomy and
179 biology on outcome has not been fully elucidated. However, many tools only seek to mimic iCCA, and
180 there is a critical absence of pCCA and dCCA models.

181 A second essential requirement of an experimental model is to reflect the interventional outcome.
182 Although chemotherapy remains the standard of care, the increasing use of targeted therapies requires
183 a deeper examination of molecular mechanisms and critical mechanisms of resistance [18-21]. As such,
184 any model must reflect molecular changes in the patient that can be measured to provide hypotheses

185 to overcome this commonly occurring resistance. Furthermore, such resistance mechanisms should be
186 unraveled to develop and assess novel interventions to overcome resistance before clinical testing.

187 ***Pathology***

188 Separate classifications (UICC, AJCC, WHO) exist for iCCA, pCCA, and dCCA. Macroscopic features
189 divide iCCA into two subtypes: large duct and small duct [22]. Large duct iCCAs typically arise near large
190 central ducts and grow along the ductal wall. Small duct iCCAs are usually peripheral mass-forming
191 tumours in the hepatic parenchyma. Four patterns of growth are described for CCA: mass-forming,
192 periductal infiltrating, intraductal, and mixed types [23].

193 ***Histopathology.*** Small duct iCCAs are typically non-mucin-secreting adenocarcinomas with a ductular
194 or tubular pattern. Large duct iCCAs are generally mucin-secreting tubular adenocarcinomas resembling
195 perihilar and distal CCAs [24]. Most p/dCCAs are adenocarcinomas with pancreaticobiliary morphology,
196 comprising glandular structures and/or small groups of cells within the desmoplastic stroma [24].

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

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

211 ***Molecular profiling***

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

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

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

As the three anatomical CCA subtypes differ in their molecular alterations [36] and potentially in the cell-of-origin [44-47], the CCA subtypes should be studied in separate experimental models [2]. However, the step-wise progression of human CCA and thus the accumulation of a wide variety of molecular alterations may not be reflected in the most rapid mouse models. Furthermore, the available experimental models represent specific subsets of patients with CCA, and it is essential to consider the molecular heterogeneity of patients with CCA when using these models. With this in mind, integrative transcriptomics may represent a relevant strategy to define the best-fit models as previously demonstrated for HCC [48,49].

In vivo CCA models

Engrafted models

Xenograft. Xenografts consist of transplanting tissues or cells from a different species into an immunodeficient host [50]. Xenograft CCA models are generated by either implanting human neoplastic CCA cells subcutaneously into the flanks of immunodeficient or athymic mice (ectopic grafts) or directly in the liver (orthotopic grafts). These experimental animal models help evaluate the therapeutic efficacy and safety of novel candidate drugs or physical-based therapies for treating CCA *in vivo*. They are highly reproducible, cost-efficient, technically easy and feasible, with limited adverse effects related to the procedure, and they only require short periods for evaluation [50-53]. Furthermore, when engrafted subcutaneously, the generated tumours are easily accessible throughout the duration of the *in vivo*

245 model, which enables the real-time measurement of tumour volume growth with a caliper. Several
246 studies have investigated the therapeutic efficacy and safety of different compounds [54 55-58]. Additionally,
247 the role of various proteins [59-64] and miRNAs [65-69] were evaluated in ectopic xenograft models by
248 implanting genetically-manipulated CCA cells. Nevertheless, ectopic xenografts also have intrinsic
249 limitations. Xenografts usually reflect advanced tumour stages, growing rapidly, and making the study
250 of early CCA challenging. At the same time, distinct CCA cell lines display different implantation rates,
251 with some not generating tumours after injection. Furthermore, these tumours are implanted in a non-
252 physiological site, seldom metastasize, and may lose the molecular heterogeneity characteristic of
253 human CCA. Most importantly, they do not allow the study of the crosstalk between tumour cells, the
254 multicellular microenvironment milieu, and the immune system [50-53].

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

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

275 relatively low, depending on the primary tumour itself and the experimental design for tumor engraftment.
276 Thus, they constitute a time and resource-intense model and may require several months for successful
277 implantation [50]. Based on the available data and unanimous agreement, the expert panel strongly
278 suggests that the type of CCA should be defined by a pathologist for PDX models, with the histology of
279 the tumor shown in the publication (Box1).

280 ***Allograft (syngeneic).*** Syngeneic models have the advantage of implanting murine CCA cells into an
281 immunocompetent host, displaying a fully-functional immune system. The first syngeneic model was
282 developed when 2 rat CCA cell lines (BDEneu and BDEsp) were directly implanted in the biliary tract of
283 Fisher 344 rats. While BDEsp engraftment induces the development of non-metastatic iCCA, BDEneu-
284 derived tumours were more aggressive, with the rapid and consistent formation of CCA lesions and
285 metastases [80,81]. This model was used to elucidate the mechanisms underlying tumour progression and
286 evaluate the efficacy of novel drug candidates [81-85]. More recently, a novel syngeneic murine model was
287 reported by engrafting the malignant mouse cell lines SB1-7, obtained from a bile-duct ligation and
288 transposon-based CCA model into mice [86,87]. The obtained cell lines were successfully implanted,
289 leading to CCA lesions resembling human CCAs [87]. In addition, foetal liver cells obtained from
290 genetically-modified mouse embryos may also be implanted in the mouse liver, inducing CCA formation
291 [88]. Furthermore, the cells mentioned above can be genetically manipulated before engraftment,
292 revealing insights into the mechanisms governing cholangiocarcinogenesis and allowing the
293 implantation of the cells in already established knockout mice strains, thus permitting the study of
294 alterations in specific genes in the tumour stroma [89]. In this line, unpublished observations from the SB1
295 orthotopic model indicate that extending 2 weeks the frequently used endpoint (4 weeks) allows the
296 formation of extrahepatic metastases in the lung. Therefore, further characterization of this timeline in a
297 genetically malleable immunocompetent host, coupled with the isolation of tumor cells from the original
298 site of injection and the metastatic sites, could provide an excellent model to understand, and perhaps
299 even prevent, a rather understudied process such as CCA metastatic spreading. Overall, these models
300 may overcome xenograft limitations, such as the absence of the immune system, are ideal for studying
301 tumour-stroma interactions, and are an excellent alternative to test immunotherapy-based strategies.
302 Still, they require microsurgical procedures, increasing the probability of procedure-related
303 complications.

304 **Chemically-induced models**

305 High levels of inflammation, fibroblast activation, and rich extracellular matrix deposition in the tumour
306 typify CCA in patients [90]. In some cases, these tumours develop in the context of chronic diseases, and
307 the cells associated with these pre-cancerous conditions contribute to cancer formation. Several
308 chemical models that generate chronic and iterative injury, leading to tumour formation, have been
309 developed to recapitulate this complex microenvironment in CCA.

310 Early work demonstrated that administering thiourea or thioacetamide (TAA) to rats triggers liver cancer
311 formation over two years [91]. TAA is a potent hepatotoxin that induces hepatic fibrosis and cirrhosis in
312 rodents owing to progressive damage of hepatocytes and biliary epithelium. TAA-induced biliary
313 damage reproduces the typical dysplasia-carcinoma sequence, ultimately evolving to invasive iCCA [92].
314 Consequently, the use of TAA to induce tumour-initiating injury in rodents has become a cornerstone of
315 CCA research. However, as detailed in this early work, CCA formation in TAA-treated rats is very
316 variable, with only ~50% of animals developing frank carcinomas. Results are even more variable in
317 wild-type mice. TAA is not mutagenic *per se*; instead, the initiation of chronic sclerosing inflammation
318 and continuous regeneration drives the spontaneous accumulation of mutations in biliary cells, which
319 then become cancerous, akin to what is observed in patients with chronic cholangiopathies. Therefore,
320 combined with bile duct ligation (BDL), a classical model of obstructive cholestasis and subsequent bile
321 duct proliferation, TAA accelerates the formation of biliary tumours [93]. Different from TAA, several
322 mutagenic models have also been developed to induce CCA in rodents. For instance, diethylnitrosamine
323 (DEN) and dimethylnitrosamine (DMN) generate DNA adducts in the liver and suffice for liver
324 carcinogenesis [94], and in combination with inflammatory injury (BDL or *O. viverrini* infection), drive CCA
325 development in mice and hamsters [95-97]. Furan is a potent mutagen capable of initiating CCA in rats [98].
326 Long-term furan treatment is currently the only chemically-induced model of CCA with nearly 100% of
327 tumour incidence, which results in multi-organ metastases and closely recapitulates the primary and
328 secondary pathologies of human CCA. Available models are summarised in **Table 2** and **Figure 2**.

329 Although many rat and mouse models driven by chemical insults reflect both the pre-cancerous disease
330 history and molecular and histopathological features of human CCA, their use is becoming less popular,
331 primarily due to their long latency, cost, and variability (both in terms of tumour penetrance and high
332 molecular heterogeneity). Recent work has focused on combining the disease-inducing aspects of these
333 models, such as inflammation and fibrosis, with GEMMs, discussed in more detail in the following
334 section. A critical point to consider is the control tissue that should be compared with malignant biliary

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

337 **Genetically Engineered Mouse Models (GEMMs)**

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

344 General concerns precluding the use of GEMMs are their high cost, tumour latency, and embryonic Cre
345 expression in non-inducible models that may compromise translation to human disease. However,
346 adopting CRISPR/Cas9 strategies to generate new GEMM strains and the development of tamoxifen-
347 inducible, organ-specific Cre-recombinase strains circumvented some of these limitations. A summary
348 of selected GEMMs is provided herein.

349 Most CCA GEMMs incorporate common oncogenic alterations found in humans, including inactivation
350 of tumour suppressor genes (*PTEN*, *SMAD4*, *P53*) or induction of oncogenes (*KRAS*, *IDH1/2*, *AKT1*,
351 *NOTCH1*) to investigate the consequences of cell-autonomous effects on cholangiocarcinogenesis. In
352 the first reported CCA GEMM, ablation of *Pten* and *Smad4* in fetal bipotential hepatic progenitors (liver
353 progenitor cells, LPCs) was achieved during embryogenesis using an Albumin Cre (*Alb-Cre*) strain [100].
354 *Alb-Cre*; *Smad4*$^{flox/flox}$; *Pten*$^{flox/flox}$ mice displayed the histopathological stages detected in human
355 disease, from bile duct hyperplasia and dysplasia to carcinoma *in situ* and invasive CCA.

356 Another model closely recapitulating human cholangiocarcinogenesis consists of the concomitant *Trp53*
357 abrogation and *KrasG12D* expression in the Alb-Cre mouse background [101]. This model features
358 premalignant biliary lesions (intraductal papillary neoplasms and Von Meyenburg complexes), leading
359 to invasive carcinoma and distal metastases. To directly probe the cell of origin in this model, *Kras*$^{LSL-
360 G12D/+}$; *Tp53*$^{flox/flox}$ mice were bred to the tamoxifen-inducible *Sox9-Cre*$^{ERT2+}$ strain (targeting
361 cholangiocytes) or intravenously administered the AAV8 vector expressing Cre under the thyroxine-
362 binding protein (targeting adult hepatocytes) [102]. *KrasG12D* activation and *Trp53* loss in adult
363 hepatocytes required co-administration of DDC-diet to form tumours (iCCA and HCC with a similar
364 incidence, in addition to combined HCC/CCA), highlighting the role of inflammation on liver cancer

365 formation. By contrast, activation of the transgenes in the adult ductal compartment in the *Sox9-Cre*$^{ERT2+}$
366 accelerated the development of hepatic tumours, mainly iCCA, from preneoplastic lesions (not found in
367 AAV8-injected mice) without the need for inflammatory cues.
368 Targeting *KrasG12D* activation and *Pten* deletion triggered the fastest GEMM in Alb-Cre mice [103]. In
369 Kras$^{LSL\text{-}G12D/+}$; Pten$^{flox/flox}$; Alb-Cre mice, early hyperplastic biliary foci were detected by 4 weeks of age,
370 and mice died by 7 weeks. Tumours were multifocal, stroma-rich localized iCCA. Interestingly, mice with
371 heterozygous *Pten* deletion and *KrasG12D* activation developed tumours after longer latency, showing
372 hepatocyte and cholangiocyte differentiation features. By using Alb-Cre^{ERT2+} or K19Cre$^{ERT/+}$ mouse
373 strains to activate the oncogenic alterations in adult hepatocytes or cholangiocytes, respectively, the
374 authors reported the development of HCC and HCC-precursor lesions, but not iCCA, in 8-week old Alb-
375 Cre^{ERT2+}; *Kras*$^{LSL\text{-}G12D}$; *Pten*$^{flox/flox}$ mice, while tamoxifen injection on day 10 elicited iCCA. The formation
376 of iCCA in Alb-Cre^{ERT2+}; *Kras*$^{LSL\text{-}G12D}$; *Pten*$^{flox/flox}$ mice might be because Alb-Cre is still active in biliary
377 cells at 10 days of age and indicates that cholangiocytes are the cell of origin of CCA in these models,
378 which was later independently confirmed using similar approaches [104].
379 IDH1/2 oncogene modelling in mice was employed [105,106]. Breeding of *Idh2*$^{LSL\text{-}R172K}$ and *Kras*$^{LSL\text{-}G12D}$ mice
380 in the *Alb-Cre* background yielded multifocal iCCA-like liver masses with invasive growth and metastatic
381 capacity. Furthermore, adjacent to the tumours, oval cell expansion and biliary intra-epithelial neoplasia-
382 like lesions, suggestive of preneoplastic stages, occurred. In more recent work, the same group
383 generated *Idh1*^{LSLR132C} mice that developed iCCA upon crossing with *Kras*$^{LSL\text{-}G12D}$ mice in the *Alb-Cre*
384 background [107]. Another oncogene investigated in *Alb-Cre* mice was *Notch1*, via a mouse strain
385 expressing the Notch 1 intracellular domain (NICD) from the *Rosa26* locus [108]. By 8 months post-birth,
386 malignant foci were detected, leading to CCA formation in transplanted immunodeficient mice.
387 Two GEMMs highlighted the importance of a pro-inflammatory environment in cholangiocarcinogenesis.
388 In the first model, severe liver damage by inflammatory cues originating from mitochondrial dysfunction
389 characterized *Hspd1*$^{flox/flox}$ mice bred to the *Alb-Cre* strain [109]. Mice developed hepatocyte and
390 cholangiocyte regenerative foci, the latter resembling human biliary intra-epithelial neoplasia. The
391 lesions arose in the context of an injured microenvironment and not through cell-autonomous
392 mechanisms, as most regenerative liver foci exhibited Hspd1 expression. In the second model,
393 *KrasG12D* expression and deletion of both *Tgfβr2* and *Cdh1* (E-cadherin) were achieved in adult CK19^{+}
394 biliary cells, leading to early-onset metastatic tumours in the extrahepatic and hilar bile duct [110]. Dying

cholangiocytes in response to E-cadherin ablation released IL-33 to foster a proliferative phenotype in biliary epithelial cells that contributed to neoplastic transformation. However, after 4 weeks of tamoxifen administration, mice succumbed to liver and/or respiratory failure. In these models, transplantation of liver tissues in immunodeficient mice [109] or derivation of tumour organoids from mice [110] allowed follow-up experiments otherwise limited by the mice's short life span.

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

Orthotopic or subcutaneous allografts models of premalignant liver cells (LPCs or adult liver organoids) or GEMM-derived CCA cell lines provide an alternative experimental strategy to time-consuming GEMMs [10,64,88,107]. These cellular models are amenable to gene editing, and their orthotopic transplantation in syngeneic mice enables tumour growth in an immune-competent microenvironment. Additionally, the plasticity of LPCs and liver organoids to originate CCA- or HCC-like tumours, depending on the genetic context, is preserved.

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

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

- The invasion of the basement membrane and tumorigenic capacity of isolated cells engrafted subcutaneously in immune-deficient mice are the most critical malignant features of CCA.
- Morphological examination by H&E and immunohistochemistry should be conducted to characterize an early-stage tumour in the preclinical CCA model.

424 - Immunohistochemistry of at least one biliary cytokeratin (CK7 or CK19) should always be
425 performed to characterize a lesion as CCA in the absence of hepatobiliary primary lesions in a
426 preclinical model.
427 - Three histopathological features of human CCA must be assessed in a preclinical model: (a.)
428 intra-tumoral heterogeneity (high stroma, inflammatory response, epithelial phenotype), (b)
429 pattern of growth (mass-forming, periductal infiltration, intraductal growth), and (c)
430 immunopositivity for CK7 or CK19.
431 - The expert panel recommends classifying preclinical CCA models as intrahepatic, perihilar, and
432 distal CCA, and suggests that focal desmoplastic stroma is a morphological feature required to
433 classify a lesion as CCA in a preclinical model.
434 - A drug should be tested in more than one model.
435
436 Lastly, to adopt a shared tool for defining the CCA experimental models homogeneously, an
437 "experimental model sheet" was generated based on an initial expert discussion done in a physical
438 ad hoc meeting (Malta meeting 20189; WG1 meeting) (**Table 4**) to provide complete information on
439 animal experimentations to the scientific community through publications.
440

441 ### *In vitro* CCA models

442 #### *2D-culture with cell lines or primary cells*

443 The urgent need to understand the biological processes of CCA progression and drug resistance has
444 led to the widespread use of *in vitro* models represented by human and animal primary cultures and
445 established cell lines. In 1985, the first CCA cell line - HChol-Y1 - was established from a patient with
446 iCCA and characterized [115]. Later, an assortment of CCA cell lines of intrahepatic and extrahepatic origin
447 was generated from primary tumours, ascites, metastases, and patient-derived xenografts
448 (**Supplementary Table 2**). Besides human CCA cells, several lines derived from mouse, rat, and
449 hamster models have been described (**Supplementary Table 2**). As proper control cells, primary
450 cultures of normal cholangiocytes should be used.
451 Molecular studies performed in human CCA tissues have uncovered recurring genomic alterations in
452 specific genes such as mutations in *TP53*, *IDH1*, *KRAS*, and *SMAD4* genes, *FGFR2* receptor fusions,
453 or *ERBB* family gene amplifications [116], which, in part, qualify as targets for molecular approaches.

454 Although most described CCA cell lines have been studied in terms of phenotypic and functional
455 characterization of some parameters, only recently, with the development of high-throughput
456 sequencing techniques, three studies have used exome sequencing or RNA-seq analyses to perform
457 deep molecular phenotyping of some of the most widely used CCA cell lines (**Supplementary Table 2**)
458 [117-119]. This has allowed the selection of cell lines with specific genetic alterations representing valuable
459 drug screening tools, particularly for targeted therapy.
460 Most cell lines were established before the release of the latest WHO guidelines [120], and potential
461 misclassification of the origin of some cell lines may impact the clinical translation of some molecular
462 and functional studies. For instance, Mz-ChA-1 cells have been traditionally used as a CCA cell line
463 [121,122], but they are classified as a gallbladder carcinoma cell line. Thus, results extrapolated from this
464 cell line should be considered for patients with this specific type of tumour.
465 In general, the well-established cell lines represent an easy model to explore mechanisms of
466 tumourigenesis and gain high experimental reproducibility mainly due to their long-term growth ability,
467 short replication doubling time, and low maintenance costs. However, several significant weaknesses
468 have been described, such as long-term serum-based culture conditions favouring the accumulation of
469 new genomic alterations [123-126]. Furthermore, *in vitro* maintenance often supports the selection of cell
470 clones that are not representative of the genetic heterogeneity of the original tumor. In addition, cell
471 cultures grown as a monolayer may lack polarization and realistic cell-cell contacts within the tumour
472 bulk. Finally, the absence of cancer stromal cells and cell-matrix interactions do not recreate the
473 fundamental interaction with the tumour microenvironment [3,123].
474 In addition to immortalized 2D cell lines, primary cultures of CCA tissue were established [127-130]. The
475 overall success rate for CCA cell line isolation and establishment is relatively low (around 10%), partly
476 due to insufficient numbers of tumour cells in resected tissues. Notably, contaminating non-tumour cells
477 (*i.e.,* fibroblasts) must be removed. Primary cultures are grown under serum-free and growth factor-
478 enhanced conditions, which better resemble the *in vivo* tumour condition. Also, primary CCA cultures
479 can be used shortly after derivation, retaining more of the morphological and functional characteristics
480 of their tissue of origin [131]. Primary cultures constrain cell differentiation and partially preserve the stem-
481 like component, thus reflecting tumour heterogeneity. However, the short time window to reach
482 senescence hampers long-term experiments and their reproducibility.

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

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

3D-culture recapitulating tumour organization

To facilitate personalized/precision medicine, patient material is used to study treatment responses. While 2D CCA models are a step closer to the *in vivo* situation in the patient compared to the established CCA cell lines, 3D culture models, including spheroids and organoids, resemble physiological conditions even more thoroughly. Spheroids are 3D aggregates of cells grown without a predefined culture substrate to adhere to [5,138], while organoids self-organize in a matrix-rich 3D environment with which they interact [139,140] [6,141]. While traditional organoids represent an epithelial cell culture, there is a consensus that 3D models should ideally be upgraded to include epithelial stem cells, cells from the tumour microenvironment (e.g., fibroblasts and/or immune cells), and extracellular matrix components to enable the analysis of cell-cell and cell-matrix interactions.

Spheroids

Tumour spheroids, mostly generated as 3D multicellular aggregates from 2D-grown adherent cells, sometimes including stromal cells such as fibroblasts and endothelial cells, are used to model tumour biology [5,138]. They can be grown in natural and/or synthetic hydrogels [141,142], and the increased

513 complexity of the model enhances the understanding of tumour pathobiology, including tumour
514 homeostasis and organization. In contrast to 2D cultures, tumour spheroids inherently recapitulate the
515 gradient of oxygen supply and drug diffusion occurring within the tumour. However, their use as high-
516 throughput, robust platforms is still limited.

517 ***Organoids***

518 Robust protocols for deriving biliary organoids from both mouse and human primary tissue explants or
519 biopsies have been established [6,140], and complemented by methods that allow for the derivation and
520 propagation of organoids from iPS cells [143], or cells collected from bile [144,145]. Apart from organoids
521 derived from healthy donors, the successful establishment of organoid cultures from tumour tissues
522 [6,7,9,146,147] can substantially add to the toolbox of preclinical and translational CCA research. The overall
523 consensus in the field is that the efficiency of establishing these CCA organoids (CCAOs) from different
524 patient tumours should be at least 25%. Efficiency should reach over 50% to guarantee the applicability
525 of organoids to personalized medicine. Working with CCAOs inevitably has limitations, including the
526 overgrowth by non-malignant cholangiocyte organoids. Using specific tumour enrichment medium [148],
527 resort to hand picking non-malignant or tumour organoids to clean up the culture, and
528 xenotransplantations are ways to address this challenge. It is agreed upon that tumourigenicity needs
529 to be confirmed for all CCAO lines, preferably done by mutation analysis (stand-alone or as part of whole
530 genomic profiling). Proof of organoid tumourigenicity in immunocompromised mice and
531 histopathological analysis are additional tests that can be performed. A shortcoming of CCAOs is that
532 an established line does not fully reflect the polyclonal nature of the original tumour. This might hamper
533 insights into drug sensitivity or clonal regrowth of treated CCA tumours.

534 In addition to fully transformed CCAOs, non-malignant cholangiocyte organoids can be a genetically
535 flexible platform to functionally annotate the influence of specific genetic alterations on CCA
536 pathobiology. Thus, recurrent iCCA genetic alterations (such as *BAP1*, *NF1*, *SMAD4*, *PTEN*, *KRAS*,
537 *AKT*, and *IDH1/2* mutations, as well as FGFR2 fusions and MYC overexpression) were engineered *in*
538 *vitro* in either human [149,150] or mouse [151]. Collectively, these studies provided convincing evidence that
539 liver organoids, in which few genetic hits were introduced to recapitulate recurrent patterns of putative
540 iCCA driver mutations, gave rise to CCA upon sub-cutaneous or orthotopic transplantation in mice. This
541 approach is therefore suitable for modelling genetically-defined cholangiocarcinogenesis in bipotent
542 liver precursors and generating models for precision oncology research [10].

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

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

Complex 3D culture systems

Although a hydrogel-based extracellular matrix (ECM) is used to support the 3D growth of cells for both spheroids and organoids, this is typically a mouse tumour-derived basement membrane extract (Matrigel or BME) not fully comprising human or tumour ECM. Moreover, additional stromal cells such as fibroblasts and immune cells are generally lacking in these cultures. The tumour microenvironment plays a crucial role in the initiation, progression, and invasion of CCA through a complex interaction between tumour cells, stromal cells, and the extracellular matrix [152]. Targeting this desmoplastic, stroma-rich tumour microenvironment might be essential to overcome chemoresistance [153-155]. Thus, including the CCA extracellular environment *in vitro* seems vital to mimic tumour composition, cell-cell and cell-matrix interaction [156], morphology, and tumour architecture more closely.

Current efforts is focussed on the generation of future complex models (assembloids) that integrate the epithelial CCA component with 3D bio-printed scaffolds that recapitulate the anatomy of the biliary system; immune cells that shape tumour growth and drug sensitivity through direct- or paracrine-interaction; stromal cells that create a physical barrier for drug delivery in addition to a pro-tumorigenic microenvironment. The challenges reside in the co-culture of autologous cell types derived from the same patient, as each cell type will have a peculiar growth dynamic and timeline. The use of cryopreservation protocols and human iPSC-derived generation of cell types from the same background cell may overcome these issues.

How can clinical needs be addressed using currently available experimental models

572 The experimental models described here will facilitate the translation from experimental and preclinical
573 work to the clinical setting. While some models provide relevant insights into the basic mechanisms of
574 cancer progression, unraveling pathway and cell signaling analysis, cell-cell, or tumour-
575 microenvironment interactions, others provide results that can be cautiously translated into the design
576 of more effective treatments for CCA or the development of new human clinical trials. A few recent
577 studies indicate that genetically defined cellular and animal models can advance the discovery of
578 actionable vulnerabilities associated with druggable iCCA oncogenic drivers. Specifically, three
579 independent studies reported that a) RAS-ERK signalling is necessary and sufficient to support the
580 oncogenic activity of FGFR2 fusions in PDX [157], GEMMs [158], and organoid-based iCCA models [151]; b)
581 combination therapies capable of providing for more robust and durable suppression of RAS-ERK,
582 improved the therapeutic efficacy of clinically approved FGFR tyrosine kinase inhibitors [151,157,158].
583 Likewise, Idh1/Kras-driven models revealed that pharmacological targeting of mutated Idh1 sensitized
584 iCCA to host-mediated immune responses, which could be enhanced by concomitant administration of
585 immune checkpoint inhibitors [107].
586 The increasing availability of novel circulating biomarkers beyond the conventional serum tumour
587 markers warrants validation for specific uses. Additional prognostic biomarkers may allow for a more
588 accurate patient risk assessment and stratification in clinical trials. Predictive biomarkers for selecting
589 the optimal therapy, such as ctDNA-based assays for FGFR2 fusions and IDH-1 mutations [159,160], are
590 already in clinical use and will push the field forward. Finally, additional pharmacodynamic biomarkers
591 able to track disease evolution more accurately than the carbohydrate antigen (CA) 19-9 and that can
592 reveal the emergence of drug resistance are warranted [161], as shown for FGFR2 resistance [162].

593 CCA organoids have proven helpful for understanding fundamental mechanisms of cancer progression
594 and biomarker discovery [7]. Though successful derivation of CCA organoids has lagged behind some
595 other tumour types, organoids hold high potential as tools for improving CCA research and therapy [163].
596 With further improvement of clinical applicability, through continued advances in stem cell biology,
597 organoid culture, and single-cell sequencing, a possible golden era for CCA organoids in personalized
598 medicine is within reach. A common limitation of experimental models is their inability to fully mimic all
599 aspects of the tumour biology and personalized cancer features of individual patients. For example, the
600 tumour microenvironment is a complex mix of cancerous and non-cancerous cells. The ECM dynamics
601 constantly remodeled by tumour cells, CAFs, and tumor-associated macrophages create a desmoplastic

environment. In addition, there is considerable heterogeneity within and between tumours. It is challenging to capture this in experimental models but essential in assessing drug resistance and tumour progression. Due to the lack of the tumour microenvironment, drug screenings performed *in vitro* do not fully reflect the *in vivo* efficacy, resulting in newly developed drugs failing in phase I-III clinical trials [164]. Finally, common risk factors and co-existing diseases characterizing human CCA (primary sclerosing cholangitis, liver flukes, chronic viral hepatitis, liver cirrhosis, etc.) are generally absent in the existing models. Thus, generating new models that combine established risk factors and concomitant morbidities for the human tumour with specific genetic alterations such as those reported above might recapitulate human CCA more accurately.

Consensus strengths and limitations

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

Experimental models are essential for a better understanding of carcinogenesis and tumour progression, testing anti-tumour therapies, and deciphering therapeutic resistance mechanisms. The panoply of CCA experimental models is wide, from simple, practical, and inexpensive to more complex models resembling human cancer biology, with a more challenging implementation and higher costs. The choice of the model depends on what is requested of it, its accessibility, and, most importantly, its ability to answer a well-defined scientific question. 2D cultures and engrafted subcutaneous murine models are the most used to dissect signalling pathways, identify therapeutic targets, and investigate drug resistance mechanisms. Depending on the type of research, *in vivo* orthotopic implantation models are preferred over ectopic CCA models. Both have advantages and limitations, as reviewed above. GEMMs appear to mimic pathobiological features of human tumourigenesis more closely, despite being

632 complex and expensive. Regarding *in vitro* models, tremendous progress has been made in better
633 recapitulating the tumour 3D structure. The difficulty in employing these models includes not only the
634 relatively high costs to set up the culture but also the availability of starting material (human CCA tissue).

635 In addition to providing an inventory, including evaluating (dis)advantages, of the most accurate
636 experimental models currently available to the CCA scientific community, we present recommendations
637 on minimal criteria for using these models. Using a Delphi-based process, a panel of experts in the field
638 reached a consensus on these criteria as proposed herein. Obviously, disease models should ultimately
639 lead to knowledge transfer from (basic) laboratory research to the clinic, to better understand the disease
640 and offer innovative therapies. As the choice of model is highly dependent on the research question, to
641 provide a comprehensive tumour mimic, results gathered using different models are highly
642 recommended. This fosters the consolidation of scientific data with well-defined minimal criteria before
643 validating them on humans by manipulating *ex vivo* samples or clinical trials.
644

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

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

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672

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674 LF coordinated the workgroups and the process of generating the manuscript, the review, MV and RC
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677

678 **Competing interests**

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684

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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).

825 19. Lowery, M. A. et al. Safety and activity of ivosidenib in patients with IDH1-mutant advanced
826 cholangiocarcinoma: a phase 1 study. *Lancet Gastroenterol Hepatol* **4**, 711-720 (2019).
827 20. Saborowski, A., Vogel, A. & Segatto, O. Combination therapies for targeting FGFR2 fusions in
828 cholangiocarcinoma. *Trends Cancer* **8**, 83-86 (2022).
829 21. Wu, M. J., Shi, L., Merritt, J., Zhu, A. X. & Bardeesy, N. Biology of IDH mutant cholangiocarcinoma.
830 *Hepatology* (2022).
831 22. Cancer, I. A. f. R. o. in *WHO Blue Books* Vol. 1 (ed WHO Classification of Tumours) 635 (World
832 Health Organization, 2019).
833 23. Guglielmi, A. et al. Intrahepatic cholangiocarcinoma: prognostic factors after surgical resection.
834 *World J Surg* **33**, 1247-1254 (2009).
835 24. Kendall, T. et al. Anatomical, histomorphological and molecular classification of
836 cholangiocarcinoma. *Liver Int* **39 Suppl 1**, 7-18 (2019).
837 25. Zen, Y., Quaglia, A., Heaton, N., Rela, M. & Portmann, B. Two distinct pathways of carcinogenesis
838 in primary sclerosing cholangitis. *Histopathology* **59**, 1100-1110 (2011).
839 26. Zen, Y. et al. Biliary intraepithelial neoplasia: an international interobserver agreement study and
840 proposal for diagnostic criteria. *Mod Pathol* **20**, 701-709 (2007).
841 27. Fujikura, K. et al. Comparative clinicopathological study of biliary intraductal papillary neoplasms
842 and papillary cholangiocarcinomas. *Histopathology* **69**, 950-961 (2016).
843 28. Komori, T. et al. CT imaging comparison between intraductal papillary neoplasms of the bile duct
844 and papillary cholangiocarcinomas. *Eur Radiol* **29**, 3132-3140 (2019).
845 29. Quigley, B. et al. Hepatobiliary Mucinous Cystic Neoplasms With Ovarian Type Stroma (So-Called
846 "Hepatobiliary Cystadenoma/Cystadenocarcinoma"): Clinicopathologic Analysis of 36 Cases
847 Illustrates Rarity of Carcinomatous Change. *Am J Surg Pathol* **42**, 95-102 (2018).
848 30. Zen, Y. et al. Mucinous cystic neoplasms of the liver: a clinicopathological study and comparison
849 with intraductal papillary neoplasms of the bile duct. *Mod Pathol* **24**, 1079-1089 (2011).
850 31. Chan-On, W. et al. Exome sequencing identifies distinct mutational patterns in liver fluke-related
851 and non-infection-related bile duct cancers. *Nat Genet* **45**, 1474-1478 (2013).
852 32. Farshidfar, F. et al. Integrative Genomic Analysis of Cholangiocarcinoma Identifies Distinct IDH-
853 Mutant Molecular Profiles. *Cell Rep* **19**, 2878-2880 (2017).
854 33. Fujimoto, A. et al. Whole-genome mutational landscape of liver cancers displaying biliary
855 phenotype reveals hepatitis impact and molecular diversity. *Nat Commun* **6**, 6120 (2015).
856 34. Gao, Q. et al. Activating mutations in PTPN3 promote cholangiocarcinoma cell proliferation and
857 migration and are associated with tumor recurrence in patients. *Gastroenterology* **146**, 1397-1407
858 (2014).
859 35. Nepal, C. et al. Genomic perturbations reveal distinct regulatory networks in intrahepatic
860 cholangiocarcinoma. *Hepatology* **68**, 949-963 (2018).
861 36. Nakamura, H. et al. Genomic spectra of biliary tract cancer. *Nat Genet* **47**, 1003-1010 (2015).
862 37. Ong, C. K. et al. Exome sequencing of liver fluke-associated cholangiocarcinoma. *Nat Genet* **44**,
863 690-693 (2012).

864 38. Zou, S. et al. Mutational landscape of intrahepatic cholangiocarcinoma. *Nat Commun* **5**, 5696
865 (2014).
866 39. Lawrence, M. S. et al. Mutational heterogeneity in cancer and the search for new cancer-associated
867 genes. *Nature* **499**, 214-218 (2013).
868 40. Andersen, J. B. et al. Genomic and genetic characterization of cholangiocarcinoma identifies
869 therapeutic targets for tyrosine kinase inhibitors. *Gastroenterology* **142**, 1021-1031 e1015 (2012).
870 41. Sia, D. et al. Integrative molecular analysis of intrahepatic cholangiocarcinoma reveals 2 classes
871 that have different outcomes. *Gastroenterology* **144**, 829-840 (2013).
872 42. Goeppert, B. et al. Integrative Analysis Defines Distinct Prognostic Subgroups of Intrahepatic
873 Cholangiocarcinoma. *Hepatology* **69**, 2091-2106 (2019).
874 43. Montal, R. et al. Molecular classification and therapeutic targets in extrahepatic
875 cholangiocarcinoma. *J Hepatol* **73**, 315-327 (2020).
876 44. Marquardt, J. U., Andersen, J. B. & Thorgeirsson, S. S. Functional and genetic deconstruction of
877 the cellular origin in liver cancer. *Nat Rev Cancer* **15**, 653-667 (2015).
878 45. Moeini, A., Haber, P. K. & Sia, D. Cell of origin in biliary tract cancers and clinical implications.
879 *JHEP Rep* **3**, 100226 (2021).
880 46. Holczbauer, A. et al. Modeling pathogenesis of primary liver cancer in lineage-specific mouse cell
881 types. *Gastroenterology* **145**, 221-231 (2013).
882 47. Guest, R. V. et al. Cell lineage tracing reveals a biliary origin of intrahepatic cholangiocarcinoma.
883 *Cancer Res* **74**, 1005-1010 (2014).
884 48. Lee, J. S. et al. Application of comparative functional genomics to identify best-fit mouse models to
885 study human cancer. *Nat Genet* **36**, 1306-1311 (2004).
886 49. Lee, J. S. et al. A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic
887 progenitor cells. *Nat Med* **12**, 410-416 (2006).
888 50. Massa, A. et al. Evolution of the Experimental Models of Cholangiocarcinoma. *Cancers (Basel)* **12**
889 (2020).
890 51. Loeuillard, E., Fischbach, S. R., Gores, G. J. & Rizvi, S. Animal models of cholangiocarcinoma.
891 *Biochim Biophys Acta Mol Basis Dis* **1865**, 982-992 (2019).
892 52. Mohr, R. et al. In Vivo Models for Cholangiocarcinoma-What Can We Learn for Human Disease?
893 *Int J Mol Sci* **21** (2020).
894 53. Cadamuro, M. et al. Animal models of cholangiocarcinoma: What they teach us about the human
895 disease. *Clin Res Hepatol Gastroenterol* **42**, 403-415 (2018).
896 54. Fava, G. et al. gamma-Aminobutyric acid inhibits cholangiocarcinoma growth by cyclic AMP-
897 dependent regulation of the protein kinase A/extracellular signal-regulated kinase 1/2 pathway.
898 *Cancer Res* **65**, 11437-11446 (2005).
899 55. Hu, M. H. et al. Targeting SHP-1-STAT3 signaling: A promising therapeutic approach for the
900 treatment of cholangiocarcinoma. *Oncotarget* **8**, 65077-65089 (2017).
901 56. Samukawa, E. et al. Angiotensin receptor blocker telmisartan inhibits cell proliferation and tumor
902 growth of cholangiocarcinoma through cell cycle arrest. *Int J Oncol* **51**, 1674-1684 (2017).

903 57. Pawar, P. et al. Molecular mechanisms of tamoxifen therapy for cholangiocarcinoma: role of
904 calmodulin. *Clin Cancer Res* **15**, 1288-1296 (2009).
905 58. Colyn, L. et al. Dual Targeting of G9a and DNA Methyltransferase-1 for the Treatment of
906 Experimental Cholangiocarcinoma. *Hepatology* **73**, 2380-2396 (2021).
907 59. Hou, Y. J. et al. Inhibition of active autophagy induces apoptosis and increases chemosensitivity in
908 cholangiocarcinoma. *Lab Invest* **91**, 1146-1157 (2011).
909 60. Merino-Azpitarte, M. et al. SOX17 regulates cholangiocyte differentiation and acts as a tumor
910 suppressor in cholangiocarcinoma. *J Hepatol* **67**, 72-83 (2017).
911 61. Meng, F., Yamagiwa, Y., Ueno, Y. & Patel, T. Over-expression of interleukin-6 enhances cell
912 survival and transformed cell growth in human malignant cholangiocytes. *J Hepatol* **44**, 1055-1065
913 (2006).
914 62. Lobe, C. et al. Zinc Finger E-Box Binding Homeobox 1 Promotes Cholangiocarcinoma Progression
915 Through Tumor Dedifferentiation and Tumor-Stroma Paracrine Signaling. *Hepatology* **74**, 3194-
916 3212 (2021).
917 63. Gentilini, A. et al. Extracellular Signal-Regulated Kinase 5 Regulates the Malignant Phenotype of
918 Cholangiocarcinoma Cells. *Hepatology* **74**, 2007-2020 (2021).
919 64. Vallejo, A. et al. FOSL1 promotes cholangiocarcinoma via transcriptional effectors that could be
920 therapeutically targeted. *J Hepatol* **75**, 363-376 (2021).
921 65. Olaru, A. V. et al. MicroRNA down-regulated in human cholangiocarcinoma control cell cycle
922 through multiple targets involved in the G1/S checkpoint. *Hepatology* **54**, 2089-2098 (2011).
923 66. Zhang, J., Han, C. & Wu, T. MicroRNA-26a promotes cholangiocarcinoma growth by activating
924 beta-catenin. *Gastroenterology* **143**, 246-256 e248 (2012).
925 67. Zhu, H., Han, C., Lu, D. & Wu, T. miR-17-92 cluster promotes cholangiocarcinoma growth:
926 evidence for PTEN as downstream target and IL-6/Stat3 as upstream activator. *Am J Pathol* **184**,
927 2828-2839 (2014).
928 68. Zhu, H. et al. Neuropilin-1 regulated by miR-320 contributes to the growth and metastasis of
929 cholangiocarcinoma cells. *Liver Int* **38**, 125-135 (2018).
930 69. Han, S. et al. Suppression of miR-16 promotes tumor growth and metastasis through reversely
931 regulating YAP1 in human cholangiocarcinoma. *Oncotarget* **8**, 56635-56650 (2017).
932 70. McVeigh, L. E. et al. Development of orthotopic tumour models using ultrasound-guided
933 intrahepatic injection. *Sci Rep* **9**, 9904 (2019).
934 71. Erice, O. et al. Differential effects of FXR or TGR5 activation in cholangiocarcinoma progression.
935 *Biochim Biophys Acta Mol Basis Dis* **1864**, 1335-1344 (2018).
936 72. Cardinale, V. et al. Profiles of cancer stem cell subpopulations in cholangiocarcinomas. *Am J Pathol*
937 **185**, 1724-1739 (2015).
938 73. Wu, Z. et al. Significance of S100P as a biomarker in diagnosis, prognosis and therapy of
939 opisthorchiasis-associated cholangiocarcinoma. *Int J Cancer* **138**, 396-408 (2016).
940 74. Cadamuro, M. et al. Low-Dose Paclitaxel Reduces S100A4 Nuclear Import to Inhibit Invasion and
941 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).

981 93. Lozano, E. et al. Cocarcinogenic effects of intrahepatic bile acid accumulation in
982 cholangiocarcinoma development. *Mol Cancer Res* **12**, 91-100 (2014).
983 94. Verna, L., Whysner, J. & Williams, G. M. N-nitrosodiethylamine mechanistic data and risk
984 assessment: bioactivation, DNA-adduct formation, mutagenicity, and tumor initiation. *Pharmacol*
985 *Ther* **71**, 57-81 (1996).
986 95. Yang, H. et al. A mouse model of cholestasis-associated cholangiocarcinoma and transcription
987 factors involved in progression. *Gastroenterology* **141**, 378-388, 388 e371-374 (2011).
988 96. Thamavit, W. et al. Promotion of cholangiocarcinogenesis in the hamster liver by bile duct ligation
989 after dimethylnitrosamine initiation. *Carcinogenesis* **14**, 2415-2417 (1993).
990 97. Thamavit, W., Pairojkul, C., Tiwawech, D., Shirai, T. & Ito, N. Strong promoting effect of
991 Opisthorchis viverrini infection on dimethylnitrosamine-initiated hamster liver. *Cancer Lett* **78**, 121-
992 125 (1994).
993 98. Maronpot, R. R., Giles, H. D., Dykes, D. J. & Irwin, R. D. Furan-induced hepatic
994 cholangiocarcinomas in Fischer 344 rats. *Toxicol Pathol* **19**, 561-570 (1991).
995 99. Erice, O. et al. Genetic Mouse Models as In Vivo Tools for Cholangiocarcinoma Research. *Cancers*
996 *(Basel)* **11** (2019).
997 100. Xu, X. et al. Induction of intrahepatic cholangiocellular carcinoma by liver-specific disruption of
998 Smad4 and Pten in mice. *J Clin Invest* **116**, 1843-1852 (2006).
999 101. O'Dell, M. R. et al. Kras(G12D) and p53 mutation cause primary intrahepatic cholangiocarcinoma.
1000 *Cancer Res* **72**, 1557-1567 (2012).
1001 102. Hill, M. A. et al. Kras and Tp53 Mutations Cause Cholangiocyte- and Hepatocyte-Derived
1002 Cholangiocarcinoma. *Cancer Res* **78**, 4445-4451 (2018).
1003 103. Ikenoue, T. et al. A novel mouse model of intrahepatic cholangiocarcinoma induced by liver-specific
1004 Kras activation and Pten deletion. *Sci Rep* **6**, 23899 (2016).
1005 104. Lin, Y. K. et al. Combination of Kras activation and PTEN deletion contributes to murine
1006 hepatopancreatic ductal malignancy. *Cancer Lett* **421**, 161-169 (2018).
1007 105. Saha, S. K. et al. Mutant IDH inhibits HNF-4alpha to block hepatocyte differentiation and promote
1008 biliary cancer. *Nature* **513**, 110-114 (2014).
1009 106. Dang, L. et al. Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. *Nature* **465**, 966
1010 (2010).
1011 107. Wu, M. J. et al. Mutant IDH Inhibits IFNgamma-TET2 Signaling to Promote Immunoevasion and
1012 Tumor Maintenance in Cholangiocarcinoma. *Cancer Discov* **12**, 812-835 (2022).
1013 108. Zender, S. et al. A critical role for notch signaling in the formation of cholangiocellular carcinomas.
1014 *Cancer Cell* **23**, 784-795 (2013).
1015 109. Yuan, D. et al. Kupffer Cell-Derived Tnf Triggers Cholangiocellular Tumorigenesis through JNK due
1016 to Chronic Mitochondrial Dysfunction and ROS. *Cancer Cell* **31**, 771-789 e776 (2017).
1017 110. Nakagawa, H. et al. Biliary epithelial injury-induced regenerative response by IL-33 promotes
1018 cholangiocarcinogenesis from peribiliary glands. *Proc Natl Acad Sci U S A* **114**, E3806-E3815
1019 (2017).

1020 111. Guest, R. V. et al. Notch3 drives development and progression of cholangiocarcinoma. *Proc Natl*
1021 *Acad Sci U S A* **113**, 12250-12255 (2016).
1022 112. Farazi, P. A. et al. Chronic bile duct injury associated with fibrotic matrix microenvironment
1023 provokes cholangiocarcinoma in p53-deficient mice. *Cancer Res* **66**, 6622-6627 (2006).
1024 113. Younger, N. T. et al. In vivo modeling of patient genetic heterogeneity identifies new ways to target
1025 cholangiocarcinoma. *Cancer Res* (2022).
1026 114. Fan, B. et al. Cholangiocarcinomas can originate from hepatocytes in mice. *J Clin Invest* **122**, 2911-
1027 2915 (2012).
1028 115. Yamaguchi, N., Morioka, H., Ohkura, H., Hirohashi, S. & Kawai, K. Establishment and
1029 characterization of the human cholangiocarcinoma cell line HChol-Y1 in a serum-free, chemically
1030 defined medium. *J Natl Cancer Inst* **75**, 29-35 (1985).
1031 116. Valle, J. W., Lamarca, A., Goyal, L., Barriuso, J. & Zhu, A. X. New Horizons for Precision Medicine
1032 in Biliary Tract Cancers. *Cancer Discov* **7**, 943-962 (2017).
1033 117. Lau, D. K. et al. Genomic Profiling of Biliary Tract Cancer Cell Lines Reveals Molecular Subtypes
1034 and Actionable Drug Targets. *iScience* **21**, 624-637 (2019).
1035 118. Scherer, D. et al. RNA Sequencing of Hepatobiliary Cancer Cell Lines: Data and Applications to
1036 Mutational and Transcriptomic Profiling. *Cancers (Basel)* **12** (2020).
1037 119. Ghandi, M. et al. Next-generation characterization of the Cancer Cell Line Encyclopedia. *Nature*
1038 **569**, 503-508 (2019).
1039 120. Nagtegaal, I. D. et al. The 2019 WHO classification of tumours of the digestive system.
1040 *Histopathology* **76**, 182-188 (2020).
1041 121. Knuth, A. et al. Biliary adenocarcinoma. Characterisation of three new human tumor cell lines. *J*
1042 *Hepatol* **1**, 579-596 (1985).
1043 122. Fausther, M. et al. Establishment and characterization of rat portal myofibroblast cell lines. *PLoS*
1044 *One* **10**, e0121161 (2015).
1045 123. Kaur, G. & Dufour, J. M. Cell lines: Valuable tools or useless artifacts. *Spermatogenesis* **2**, 1-5
1046 (2012).
1047 124. Domcke, S., Sinha, R., Levine, D. A., Sander, C. & Schultz, N. Evaluating cell lines as tumour
1048 models by comparison of genomic profiles. *Nat Commun* **4**, 2126 (2013).
1049 125. Ertel, A., Verghese, A., Byers, S. W., Ochs, M. & Tozeren, A. Pathway-specific differences between
1050 tumor cell lines and normal and tumor tissue cells. *Mol Cancer* **5**, 55 (2006).
1051 126. Gillet, J. P. et al. Redefining the relevance of established cancer cell lines to the study of
1052 mechanisms of clinical anti-cancer drug resistance. *Proc Natl Acad Sci U S A* **108**, 18708-18713
1053 (2011).
1054 127. Massani, M. et al. Isolation and characterization of biliary epithelial and stromal cells from resected
1055 human cholangiocarcinoma: a novel in vitro model to study tumor-stroma interactions. *Oncol Rep*
1056 **30**, 1143-1148 (2013).
1057 128. Fraveto, A. et al. Sensitivity of Human Intrahepatic Cholangiocarcinoma Subtypes to
1058 Chemotherapeutics and Molecular Targeted Agents: A Study on Primary Cell Cultures. *PLoS One*
1059 **10**, e0142124 (2015).

1060 129. Carnevale, G. et al. Activation of Fas/FasL pathway and the role of c-FLIP in primary culture of
1061 human cholangiocarcinoma cells. *Sci Rep* **7**, 14419 (2017).
1062 130. Lustri, A. M. et al. TGF-beta signaling is an effective target to impair survival and induce apoptosis
1063 of human cholangiocarcinoma cells: A study on human primary cell cultures. *PLoS One* **12**,
1064 e0183932 (2017).
1065 131. Pastor, D. M. et al. Primary cell lines: false representation or model system? a comparison of four
1066 human colorectal tumors and their coordinately established cell lines. *Int J Clin Exp Med* **3**, 69-83
1067 (2010).
1068 132. Vaquero, J. et al. The IGF2/IR/IGF1R Pathway in Tumor Cells and Myofibroblasts Mediates
1069 Resistance to EGFR Inhibition in Cholangiocarcinoma. *Clin Cancer Res* **24**, 4282-4296 (2018).
1070 133. Zhou, Z. et al. Tumor-associated neutrophils and macrophages interaction contributes to
1071 intrahepatic cholangiocarcinoma progression by activating STAT3. *J Immunother Cancer* **9** (2021).
1072 134. Okabe, H. et al. Hepatic stellate cells may relate to progression of intrahepatic cholangiocarcinoma.
1073 *Ann Surg Oncol* **16**, 2555-2564 (2009).
1074 135. Nicolas-Boluda, A. et al. Photothermal Depletion of Cancer-Associated Fibroblasts Normalizes
1075 Tumor Stiffness in Desmoplastic Cholangiocarcinoma. *ACS Nano* **14**, 5738-5753 (2020).
1076 136. Cadamuro, M. et al. Platelet-derived growth factor-D enables liver myofibroblasts to promote tumor
1077 lymphangiogenesis in cholangiocarcinoma. *J Hepatol* **70**, 700-709 (2019).
1078 137. Li, L. et al. Extracellular vesicles carry microRNA-195 to intrahepatic cholangiocarcinoma and
1079 improve survival in a rat model. *Hepatology* **65**, 501-514 (2017).
1080 138. Fennema, E., Rivron, N., Rouwkema, J., van Blitterswijk, C. & de Boer, J. Spheroid culture as a
1081 tool for creating 3D complex tissues. *Trends Biotechnol* **31**, 108-115 (2013).
1082 139. Sato, T. et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal
1083 niche. *Nature* **459**, 262-265 (2009).
1084 140. Huch, M., Boj, S. F. & Clevers, H. Lgr5(+) liver stem cells, hepatic organoids and regenerative
1085 medicine. *Regen Med* **8**, 385-387 (2013).
1086 141. Sato, K. et al. Organoids and Spheroids as Models for Studying Cholestatic Liver Injury and
1087 Cholangiocarcinoma. *Hepatology* **74**, 491-502 (2021).
1088 142. Girard, Y. K. et al. A 3D fibrous scaffold inducing tumoroids: a platform for anticancer drug
1089 development. *PLoS One* **8**, e75345 (2013).
1090 143. Wu, F. et al. Generation of hepatobiliary organoids from human induced pluripotent stem cells. *J
1091 Hepatol* **70**, 1145-1158 (2019).
1092 144. Soroka, C. J., Assis, D. N. & Boyer, J. L. Patient-Derived Organoids from Human Bile: An In Vitro
1093 Method to Study Cholangiopathies. *Methods Mol Biol* **1981**, 363-372 (2019).
1094 145. Roos, F. J. M. et al. Human Bile Contains Cholangiocyte Organoid-Initiating Cells Which Expand
1095 as Functional Cholangiocytes in Non-canonical Wnt Stimulating Conditions. *Front Cell Dev Biol* **8**,
1096 630492 (2020).
1097 146. Lampis, A. et al. MIR21 Drives Resistance to Heat Shock Protein 90 Inhibition in
1098 Cholangiocarcinoma. *Gastroenterology* **154**, 1066-1079 e1065 (2018).

1099 147. Li, L. et al. Human primary liver cancer organoids reveal intratumor and interpatient drug response
1100 heterogeneity. *JCI Insight* **4** (2019).
1101 148. Broutier, L. et al. Culture and establishment of self-renewing human and mouse adult liver and
1102 pancreas 3D organoids and their genetic manipulation. *Nat Protoc* **11**, 1724-1743 (2016).
1103 149. Artegiani, B. et al. Probing the Tumor Suppressor Function of BAP1 in CRISPR-Engineered Human
1104 Liver Organoids. *Cell Stem Cell* **24**, 927-943 e926 (2019).
1105 150. Sun, L. et al. Modelling liver cancer initiation with organoids derived from directly reprogrammed
1106 human hepatocytes. *Nat Cell Biol* **21**, 1015-1026 (2019).
1107 151. Cristinziano, G. et al. FGFR2 fusion proteins drive oncogenic transformation of mouse liver
1108 organoids towards cholangiocarcinoma. *J Hepatol* **75**, 351-362 (2021).
1109 152. Leyva-Illades, D., McMillin, M., Quinn, M. & Demorrow, S. Cholangiocarcinoma pathogenesis: Role
1110 of the tumor microenvironment. *Transl Gastrointest Cancer* **1**, 71-80 (2012).
1111 153. Hogdall, D., Lewinska, M. & Andersen, J. B. Desmoplastic Tumor Microenvironment and
1112 Immunotherapy in Cholangiocarcinoma. *Trends Cancer* **4**, 239-255 (2018).
1113 154. Cadamuro, M. et al. The deleterious interplay between tumor epithelia and stroma in
1114 cholangiocarcinoma. *Biochim Biophys Acta Mol Basis Dis* **1864**, 1435-1443 (2018).
1115 155. Mertens, J. C., Rizvi, S. & Gores, G. J. Targeting cholangiocarcinoma. *Biochim Biophys Acta Mol
1116 Basis Dis* **1864**, 1454-1460 (2018).
1117 156. Tanimizu, N. et al. Generation of functional liver organoids on combining hepatocytes and
1118 cholangiocytes with hepatobiliary connections ex vivo. *Nat Commun* **12**, 3390 (2021).
1119 157. Wu, Q. et al. EGFR inhibition potentiates FGFR inhibitor therapy and overcomes resistance in
1120 FGFR2 fusion-positive cholangiocarcinoma. *Cancer Discovery* (2022).
1121 158. Kendre, G. et al. The Co-mutational Spectrum Determines the Therapeutic Response in Murine
1122 FGFR2 Fusion-Driven Cholangiocarcinoma. *Hepatology* **74**, 1357-1370 (2021).
1123 159. Wintachai, P. et al. Diagnostic and Prognostic Value of Circulating Cell-Free DNA for
1124 Cholangiocarcinoma. *Diagnostics (Basel)* **11** (2021).
1125 160. Csoma, S. L. et al. Circulating Cell-Free DNA-Based Comprehensive Molecular Analysis of Biliary
1126 Tract Cancers Using Next-Generation Sequencing. *Cancers (Basel)* **14** (2022).
1127 161. Varghese, A. M. et al. Noninvasive Detection of Polyclonal Acquired Resistance to FGFR Inhibition
1128 in Patients With Cholangiocarcinoma Harboring FGFR2 Alterations. *JCO Precis Oncol* **5** (2021).
1129 162. Goyal, L. et al. TAS-120 Overcomes Resistance to ATP-Competitive FGFR Inhibitors in Patients
1130 with FGFR2 Fusion-Positive Intrahepatic Cholangiocarcinoma. *Cancer Discov* **9**, 1064-1079 (2019).
1131 163. van Tienderen, G. S. et al. Hepatobiliary tumor organoids for personalized medicine: a multicenter
1132 view on establishment, limitations, and future directions. *Cancer Cell* (2022).
1133 164. Wong, C. H., Siah, K. W. & Lo, A. W. Estimation of clinical trial success rates and related
1134 parameters. *Biostatistics* **20**, 273-286 (2019).
1135 165. Lozano, E. et al. Enhanced antitumour drug delivery to cholangiocarcinoma through the apical
1136 sodium-dependent bile acid transporter (ASBT). *J Control Release* **216**, 93-102 (2015).
1137 166. Katz, S. F. et al. Disruption of Trp53 in livers of mice induces formation of carcinomas with bilineal
1138 differentiation. *Gastroenterology* **142**, 1229-1239 e1223 (2012).

1139 167. Tschaharganeh, D. F. et al. p53-dependent Nestin regulation links tumor suppression to cellular
1140 plasticity in liver cancer. *Cell* **158**, 579-592 (2014).
1141 168. El Khatib, M. et al. Activation of Notch signaling is required for cholangiocarcinoma progression
1142 and is enhanced by inactivation of p53 in vivo. *PLoS One* **8**, e77433 (2013).
1143 169. Ikenoue, T. et al. Establishment and analysis of a novel mouse line carrying a conditional knockin
1144 allele of a cancer-specific FBXW7 mutation. *Sci Rep* **8**, 2021 (2018).
1145 170. Manieri, E. et al. JNK-mediated disruption of bile acid homeostasis promotes intrahepatic
1146 cholangiocarcinoma. *Proc Natl Acad Sci U S A* **117**, 16492-16499 (2020).
1147 171. Cubero, F. J. et al. Loss of c-Jun N-terminal Kinase 1 and 2 Function in Liver Epithelial Cells
1148 Triggers Biliary Hyperproliferation Resembling Cholangiocarcinoma. *Hepatol Commun* **4**, 834-851
1149 (2020).
1150 172. Marsh, V., Davies, E. J., Williams, G. T. & Clarke, A. R. PTEN loss and KRAS activation cooperate
1151 in murine biliary tract malignancies. *J Pathol* **230**, 165-173 (2013).
1152 173. Falcomata, C. et al. Genetic Screens Identify a Context-Specific PI3K/p27(Kip1) Node Driving
1153 Extrahepatic Biliary Cancer. *Cancer Discov* (2021).
1154 174. Sekiya, S. & Suzuki, A. Intrahepatic cholangiocarcinoma can arise from Notch-mediated
1155 conversion of hepatocytes. *J Clin Invest* **122**, 3914-3918 (2012).
1156 175. Crawford, D. R. et al. Characterization of liver injury, oval cell proliferation and
1157 cholangiocarcinogenesis in glutathione S-transferase A3 knockout mice. *Carcinogenesis* **38**, 717-
1158 727 (2017).

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

<table>
<tr><th></th><th>Benefits</th><th>Limitations</th></tr>
<tr><td>In vivo models</td><td></td><td></td></tr>
<tr><td>Engrafted models</td><td></td><td></td></tr>
<tr><td>Xenograft</td><td>- Engraftment of human cells or tissue
- Ectopic engraftment inexpensive and easy to implement
- Easy-to-measure ectopic tumours
- Commonly used for drug testing</td><td>- Defective immune system
- Ectopic allograft poorly relevant
- Rate of human CCA tissue ectopic engraftment (PDX) very low
- Orthotopic engraftment difficult to perform</td></tr>
<tr><td>Allograft</td><td>- Full immune system
- Ideal to study tumour-stroma interplay
- Fully compatible for testing immunotherapy-based therapies</td><td>- Ectopic allograft poorly relevant
- Orthotopic engraftment difficult to perform</td></tr>
<tr><td colspan="3"></td></tr>
<tr><td>Chemically-induced</td><td>- Recapitulate development of CCA (TAA) with pre-cancerous disease history
- Long-term furan treatment induces 100% of tumour incidence</td><td>- Highly variable
- Control tissue: isolated bile duct and not whole liver</td></tr>
<tr><td>GEMM</td><td>- Design to mimic genetic alterations of human CCA
- Model of advanced CCA
- Valuable tool for testing targeted therapies</td><td>- Fast tumour development
- Origin of CCA multiple
- Appearance of mixed HCC/CCA tumour
- Costly</td></tr>
<tr><td colspan="3">RECOMMENDATIONS
Histological assessment (all in vivo models)
1. Invasion of the basement membrane and tumorigenic capacity of isolated cells engrafted subcutaneously in immune-deficient mice are the most important malignant features of CCA (97% and 91%, A).
2. Immunohistochemistry of at least one biliary cytokeratin should always be performed to characterize an early-stage tumour in a preclinical CCA model (90%, A).
3. A classification of preclinical CCA models as intrahepatic, perihilar, and distal CCA is recommended. (93%, A).
4. Focal desmoplastic stroma is a morphological feature required to classify a lesion as CCA in a preclinical model (100%, U).
5. Three histopathological features of human CCA must be assessed in a preclinical model: intra-tumoral heterogeneity (high stroma, inflammatory response, epithelial phenotype) (90%, A), the pattern of growth (mass-forming, periductal infiltration, intraductal growth) (90%, A), and immunopositivity for CK7 or CK19 (100%, U).
Xenograft models, Genetically Engineered Mouse Models (GEMM)
6. The type of CCA should be specified for patient-derived xenograft models (92%, A).
7. Drugs should be tested in more than one model (95%, A).</td></tr>
<tr><td>In vitro models</td><td></td><td></td></tr>
<tr><td></td><td></td><td></td></tr>
<tr><td>2D-culture with cell lines or primary cells</td><td>- Easy and low maintenance costs
- High experimental reproducibility
- Large panels of cell lines commercially available</td><td>- Absence of stromal cells
- Cultures grown as a monolayer</td></tr>
</table>

	- Cells available with genetic alteration(s)	
3D-culture recapitulating a tumour organization		
Spheroids	- Can be patient-derived - Increased complexity through 3D multicellular aggregates of epithelial cells and stromal cells - Recapitulate the gradient of oxygen supply and drug diffusion - Increased complexity	- Limited use for high-throughput analysis - Often made from cell lines - Do not fully reflect the polyclonal nature of a CCA tumour
Organoids	- Increased complexity by 3D tumour cell growth in ECM - Well established protocol - Specific mutations can be introduced in non-tumour organoids to analyse CCA driver mutations	- Low initiation efficiency from human tumours - An established line does not fully reflect the polyclonal nature of the original tumour - Overgrowth of non-tumour cells in culture initiation - Absence of stromal cells

RECOMMENDATIONS

2D cultures

8. Cell culture procedures should be standardised in experiments with cell lines or primary 2D cultures and be reported in publications. Procedures include the choice of plastic support, cell culture medium, and the level of confluence when performing the experiments should be mentioned (88%, 85%, 82%, B).
   The isolation protocol for primary cells, including passaging and sub-culturing methods, should be reported in publications (i.e., enzymatic vs. mechanical dissociation, etc.) (89% and 85%, B).
   The origin of any cell line (previously established or new) should be stated for publication according to the new CCA classification (i.e., intrahepatic, perihilar, distal) (90-99, A)
9. The origin of any cell line (previously established or new) should be presented in a publication according to the new CCA classification (i.e., intrahepatic, perihilar, distal) (97%, A).

3D cultures

10. A specific tumour "enrichment" medium (i.e., tumour initiating medium as described by Broutier et al., 2017, DOI: 10.1038/nm.4438) is recommended to minimize contamination in non-tumour organoids (94%, A).
11. Mutation analysis (targeted genomic profiling using a diagnostic panel) (90%, A), and phenotypic analysis should be done to confirm the malignant origin of established organoid lines and reported in publications (93%, A).
12. Every organoid culture should be characterized before clinical applications such as drug screening (92%, A).
13. The shorter period for patient-organoids initiation, expansion, and analysis has to be less than 3 months (57%, C).

1164 Grading system: U, denotes unanimous (100%) agreement; A, 90–99% agreement; B, 70–89%
1165 agreement; C, 50-69% agreement; and D, <50% agreement.
1166 ECM, extracellular matrix; HCC/CCA, hepatocholangiocarcinoma; TAA, thioacetamide.
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1176 **[Au: please add details of the grading system used as a footnote as each display item must be**

1177 **understood as a standalone item]**

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1179 **Table 1. Consensus statements.**

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#	Statement	Response yes / total responders	Grade
Histological assessment			
1	Which of the following ones are malignant features of biliary tumours?		
	1. Invasion of the basement membrane	31/32	A
	2. Increased nucleus/cytoplasm ratio	18/31	C
	3. Distant metastasis	27/32	B
	4. Tumorigenic capacity of isolated cells after subcutaneous injection in immune-deficient mice	29/32	A
2	What type of histological investigation(s) should always be done to characterize an early-stage tumour in a preclinical CCA model?		
	1. Morphological examination of H&E	32/32	U
	2. Immunohistochemistry	27/30	A
	3. Immunohistochemistry for at least one biliary cytokeratin (e.g., CK19, CK7, pan CK, etc.)	16/25	C
	4. Markers for inflammatory cells and CAFs	12/26	D
	5. PAS reaction for highlighting mucin	13/26	C
	6. A broad panel of markers for hepatobiliary malignancies and metastasis	12/24	C
3	To allow correlation with the anatomical classification of human tumours, a preclinical model of CCA should specifically classify tumours induced as:		
	1. Intrahepatic CCA, perihilar CCA, and distal CCA	25/30	B
	2. Intrahepatic CCA and extrahepatic CCA	12/25	D
	3. No need for such classification	1/23	D
4	Which of the following morphological and/or immunophenotypic features must be present to classify a lesion as CCA in a preclinical model?		
	1. Location within the liver or extrahepatic biliary tree	24/28	B
	2. Absence of an extrahepatic bile duct primary lesion	14/28	C
	3. Epithelial cytological features (cohesive groups or structures and/or pan-cytokeratin immunopositivity)	25/28	B
	4. At least focal gland formation	9/25	D
	5. Absence of hepatocellular differentiation (bile production and canalicular CD10 or BSEP)	14/24	D
	6. Immunopositivity for CK7 or CK19	31/31	U
	7. Focal desmoplastic stroma	22/30	B
	8. Presence of precursor lesions	4/24	D
	9. Primary origin within the intra- or extra-hepatic biliary tree	19/28	D
	10. Absence of primary hepatobiliary lesions	0/28	U
5	What histopathological features of human CCA must be verified in a preclinical model of CCA?		
	1. Intra-tumoral heterogeneity (high stroma, inflammatory response, epithelial phenotype)	27/30	A

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

10	Contaminating non-tumour organoids often grow in CCA organoid cultures. How should selection for tumour organoids be performed?		
	1. Specific tumour "enrichment" medium (i.e., tumour initiating medium (as described by Broutier *et al.,* 2017, DOI: 10.1038/nm.4438)	29/31	A
	2. Hand-picking of organoids with a different phenotype / removing the 'normal-looking' organoids	21/30	B
	3. Xenotransplantation in mice to select for tumour clones	22/30	B
11	Which analyses should be done to confirm the malignant origin of established organoid lines and be reported in publications?		
	1. Full genomic profiling	8/28	D
	2. Mutation analysis (targeted genomic profiling using a diagnostic panel)	28/31	A
	3. Phenotypic analysis	28/30	A
	4. Histological analysis (immunohistochemistry of EpCAM, CK7)	28/32	B
	6. Xenotransplantation in mice	26/32	B
12	Should every organoid culture be characterized (as proposed in Q 11) before clinical applications such as drug screening?	33/36	A
13	Personalized medicine applications such as drug screenings to find the best treatment for the patient, will cost time. How much time is acceptable to initiate, grow and expand the organoids for these analyses? In other words, what is the maximum time acceptable to be relevant to the clinics?		
	<1 month	9/35	D
	<3 months	20/35	C
	<6 months	4/35	D
	Other; the less as possible / <1 mo 1st line treatment and <3 mo 2nd line treatment	2/35	D

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

1187 **Table 2. Carcinogen-based rodent models of cholangiocarcinoma.**

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

1188 BDL, bile duct ligation; DEN, diethylnitrosamine; DMN, dimethylnitrosamine; TAA, thioacetamide.

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

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

Kras$^{LSLG12D/+}$, *Trp53*$^{f/f}$ infected with AAV8-TBG-Cre	Development of ICC (40%), HCC (40%), mixed HCC/CCA (20%) (from hepatocytes)	A: Recombination event in adult mice, higher CCA frequency in combination with DCC diet (all tumours ICC or mixed HCC/CCA) D: Cre-recombinase administration via adeno-associated virus (AAV), large tumour latency range (12-66 weeks post-AAV infection)	102
AhCre^{ERT}, *Kras*$^{G12V/+}$, *Pten*$^{f/f}$	Multifocal non-invasive papillary neoplasms in the intrahepatic biliary tract (from major interlobular bile ducts to small bile duct radicles in portal tracts)	A: 100% penetrance, low latency (43 days), tumour development starts in adult mice D: Not specific to liver tissue, lack of invasive tumour or metastasis	172
Sox9-Cre^{ERT2}; *Kras*$^{LSL-G12D/+}$, *Trp53*$^{f/f}$	iCCA tumours accompanied by adjacent extensive ductular reactions and desmoplasia, with areas resembling BIN (from cholangiocytes)	A: 100% penetrance, iCCA exclusive, recombination in mature cholangiocytes D: 30 weeks average latency	102
Ck19-Cre^{ER}, *Kras*$^{LSL-G12D}$,*Tgfbr2*$^{flox/flox}$; *Cdh1*$^{flox/flox}$;	Markedly thickened EHBD wall with a swollen gallbladder involving invasive periductal infiltrating-type eCCA and lymphatic metastasis (from biliary cells)	A: Low latency (4 weeks), eCCA exclusive B: Concurrent development of lung adenocarcinomas leads to mice asphyxiation	110
Pdx1-Cre, *Pik3ca*$^{LSLH1047R/+}$	Adult mice develop enlarged extrahepatic bile duct and BIN with complete penetrance leading to eCCA (from well-differentiated, stroma-rich ductal adenocarcinomas to more undifferentiated)	A: eCCA exclusive, only one genetic hit driving CCA B: ~40 weeks average latency, 90% penetrance, wide tumour latency range	173
GEM-based implantation models			
LPCs from *Alb-Cre*, *Kras*$^{LSL-G12D}$, *Trp53*$^{LSL-R172H/lox}$ +/- FIG-ROS fusion	Allografted tumours resemble advanced CCA	A: Quick model, orthotopic implantation in the liver, iCCA exclusive, stroma presence D: Requires technical training to isolate LPC	88
LPCs or cholangiocytic progenitor cells or hepatocytes from *Trp53*$^{-/-}$ mice	Tumours exhibit a high stromal content and a mixed hepatocellular and cholangiocellular differentiation	A: Quick model D: Not CCA exclusive	166
Adult liver organoids from *Kras*$^{LSL-G12D}$, *Trp53*$^{f/f}$ mice	Kras driven organoids lead to CCA while c-Myc expression in wild-type organoids induces HCC formation	A: Tumours latency of 6-8 weeks for Kras mut and Trp53 ko organoids D: Requires training in organoid isolation, growth and manipulation	10
Cholangiocytes from *Kras*$^{LSL-G12D}$, *Trp53*$^{f/f}$ mice	Tumours with a high stromal component expressing CCA markers	A: Quick and reproducible model, orthotopic implantation in the liver, iCCA exclusive, stroma presence D: Requires technical training to isolate mouse cholangiocytes	64
GEM-based carcinogenic models			
Alb-Cre^{ERT2}, *R26*$^{RlacZ/+}$ or *Ck19-Cre*^{ERT2}, *R26*$^{RlacZ/+}$ ^{mice} treated with TAA	Macronodular liver cirrhosis containing cells the typical histology of CCA	A: 100% penetrance, iCCA exclusive D: Long latency (30 weeks)	174
Ck19-Cre$^{ERT/eYFP}$; *Trp53*$^{f/f}$ mice treated with TAA	Treatment with TAA generates oncogenic stress yielding multifocal invasive iCCA	A: iCCA exclusive D: 80% penetrance, long latency (>6 months)	111
Trp53$^{-/-}$ mice treated with CCl_4	Bile duct injury/necrosis, proliferation and fibrosis development triggered by CCl_4	A: Exclusive iCCA D: 50% mice develop tumours, metastatic lesions rarely observed	112

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

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1196 A: Advantages; BIN: biliary intraepithelial neoplasia; CCl_4: carbon tetrachloride; D: disadvantages; DEN:

1197 diethylnitrosamine; GSTA3: glutathione-S-transferase A3; IPBN: intraductal papillary biliary neoplasms;

1198 LPCs: bipotent liver progenitor cells; ROS: reactive oxygen species; TTA: tetradecylthioacetic acid; VMC

1199 Von Meyenburg complexes.

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1201 **Table 4. Experimental model sheet.**
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Experimental model sheet	
1. **Type of model**: (*in vitro, ex vivo, in vivo*)	
2. **Species**: (mouse, rat, hamster, human, etc.)	
3. **Gender**: (male, female, both)	
4. **Strain:**	
5. **Condition of the surrounding liver** (apparently healthy, cirrhosis, fibrosis, etc.) :	
6. **Method of generation**: (spontaneous, carcinogenic, chronic injury, infectious, transgenic, knockout, transposon-mediated, patient-derived xenograft, organoids, isolated from animal tumours, isolated from human tumours, etc.):	
7. **Tumour development:** (fast, slow)	
8. **Metastasis:** (yes, no, locations,...)	
9. **Anatomical location of the lesions (when applicable)**: (intrahepatic, extrahepatic, both)	
10. **Cell of origin (if available)**: (cholangiocyte, stem/progenitor cell, hepatocyte)	
11. **Types of samples and storage conditions for future analyses**	
12. **Presence of preneoplastic lesions**: (yes/no)	
13. **Type of preneoplastic lesions**: (IPNB, IPMN, BilIN, etc.)	
14. **Type of cholangiocarcinoma**: (iCCA, pCCA, dCCA, combined HCC/CCA)	
15. **Histology of tumours**: (large duct type, small duct type, CCA, lymphoepithelioma-like CCA, etc.)	
16. **Microenvironment features**: (presence of stroma/desmoplastic reaction, absence of stroma, immune infiltration yes/no)	
17. **Phenotype of the lesions**: (CK7, CK19, MUC1, MUC2, MUC5AC, MUC6, HNF4A, AFP, markers of stemness, markers of EMT, etc.)	
18. **Control samples used if applicable** (bile duct freshly isolated from liver or cell line)	

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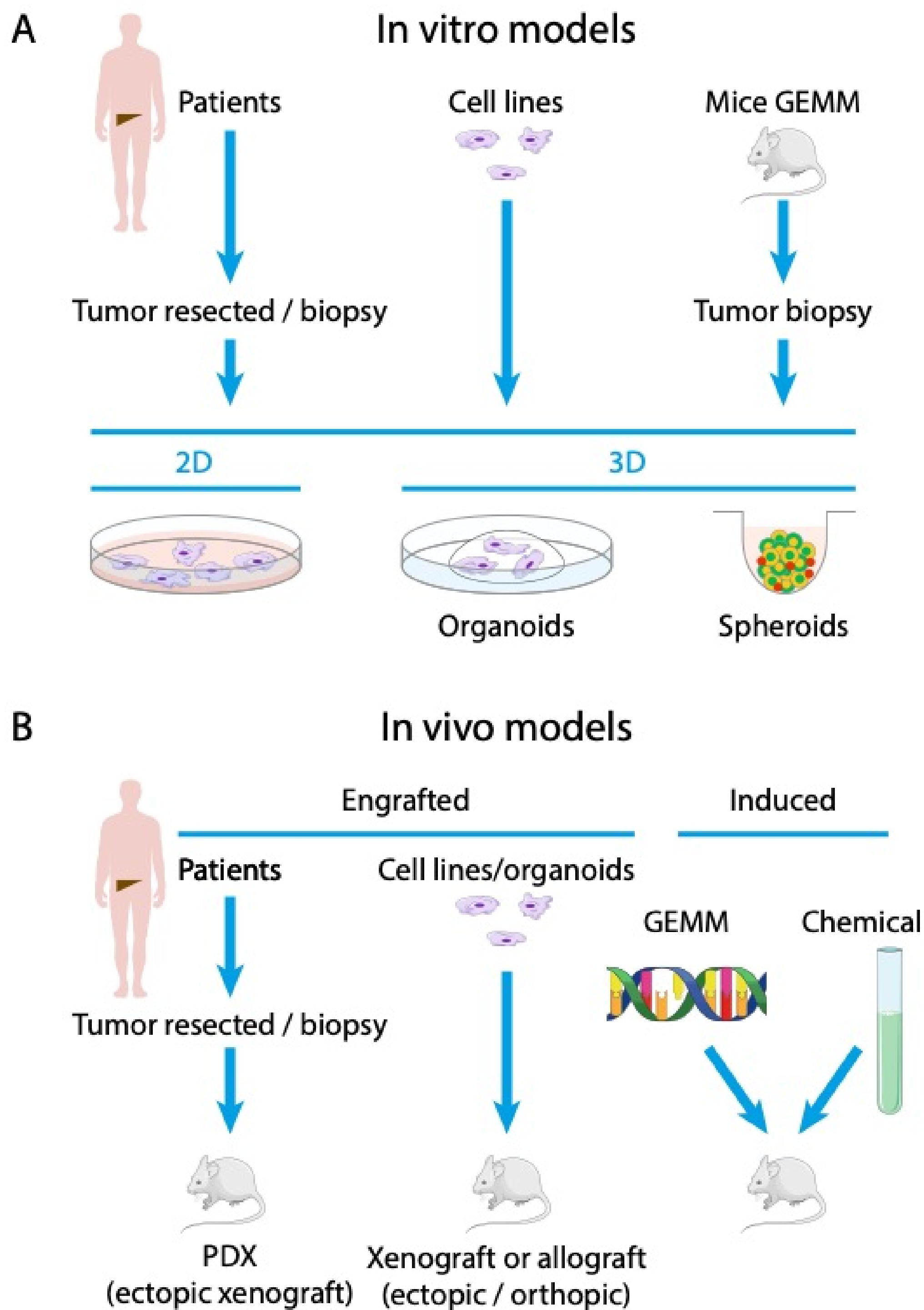

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1208 **Figure 1.** Panel of experimental *in vitro* (A) and *in vivo* (B) models provided for cholangiocarcinoma
1209 preclinical studies.
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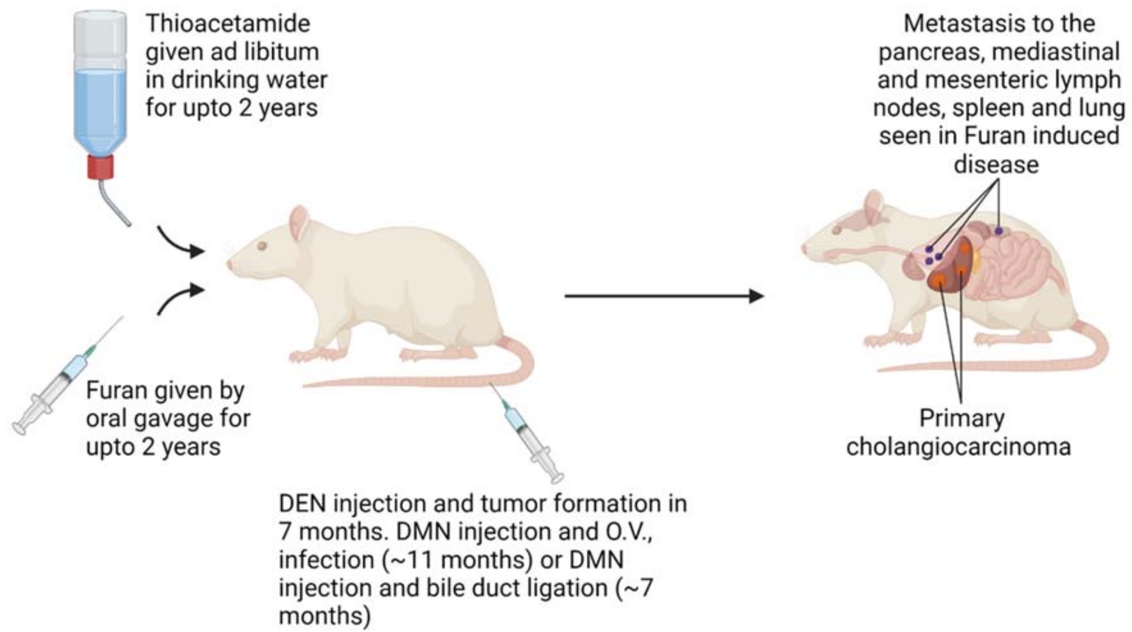

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1215 **Figure 2.** Schematic summary of available chemical models to initiate cholangiocarcinoma in rodents
1216 and induce metastatic dissemination.
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