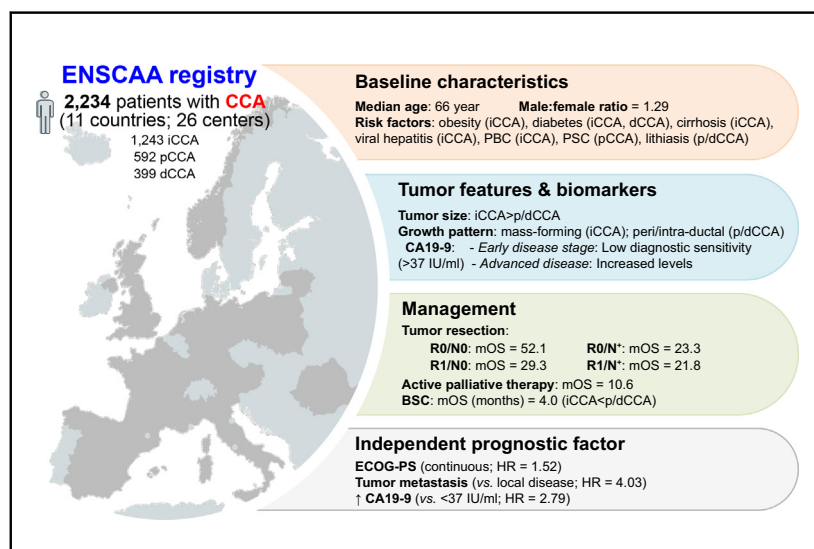


Cholangiocarcinoma landscape in Europe: Diagnostic, prognostic and therapeutic insights from the ENSCCA Registry

Graphical abstract



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Lay summary

This is, to date, the largest international (pan-European: 26 hospitals and 11 countries) observational study, in which the course of cholangiocarcinoma has been investigated, comparing the 3 subtypes based on the latest International Classification of Diseases 11th Edition (ICD-11) (*i.e.*, intrahepatic [2C12], perihilar [2C18], or distal [2C15] affected bile ducts), which come into effect in 2022. General and tumor-type specific features at diagnosis, risk factors, biomarker accuracy, as well as patient management and outcomes, are presented and compared, outlining the current clinical state of cholangiocarcinoma in Europe.

Highlights

- CCA subtypes present different risk factors and tumor features.
- CA19-9 shows low sensitivity in early stages but increased sensitivity in advanced disease.
- Under surgery, positive margins and lymph node invasion compromise survival.
- ECOG-PS, disease status and CA19-9 are independent prognostic factors.



Cholangiocarcinoma landscape in Europe: Diagnostic, prognostic and therapeutic insights from the ENSCCA Registry

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Background & Aims: Cholangiocarcinoma (CCA) is a rare and heterogeneous biliary cancer, whose incidence and related mortality is increasing. This study investigates the clinical course of CCA and subtypes (intrahepatic [iCCA], perihilar [pCCA], and distal [dCCA]) in a pan-European cohort.

Methods: The ENSCCA Registry is a multicenter observational study. Patients were included if they had a histologically proven diagnosis of CCA between 2010-2019. Demographic, histomorphological, biochemical, and clinical studies were performed.

Results: Overall, 2,234 patients were enrolled (male/female=1.29). iCCA (n = 1,243) was associated with overweight/obesity and chronic liver diseases involving cirrhosis and/or viral hepatitis; pCCA (n = 592) with primary sclerosing cholangitis; and dCCA (n = 399) with choledocholithiasis. At diagnosis, 42.2% of patients had local disease, 29.4% locally advanced disease (LAD), and 28.4% metastatic disease (MD). Serum CEA and CA19-9 showed low diagnostic sensitivity, but their concomitant elevation was associated with increased risk of presenting with LAD (odds ratio 2.16; 95% CI 1.43-3.27) or MD (odds ratio 5.88; 95% CI 3.69-9.25). Patients undergoing resection (50.3%) had the best outcomes, particularly with negative-resection margin (R0) (median overall survival [mOS] = 45.1 months); however, margin involvement (R1) (hazard ratio 1.92; 95% CI 1.53-2.41; mOS = 24.7 months) and lymph node invasion (hazard ratio 2.13; 95% CI 1.55-2.94; mOS = 23.3 months) compromised prognosis. Among patients with unresectable disease (49.6%), the mOS was 10.6 months for those receiving active palliative therapies, mostly chemotherapy (26.2%), and 4.0 months for those receiving best supportive care (20.6%). iCCAs were associated with worse outcomes than p/dCCAs. ECOG performance status, MD and CA19-9 were independent prognostic factors.

Conclusion: CCA is frequently diagnosed at an advanced stage, a proportion of patients fail to receive cancer-specific therapies, and prognosis remains dismal. Identification of preventable risk factors and implementation of surveillance in high-risk populations are required to decrease cancer-related mortality.

Lay summary: This is, to date, the largest international (pan-European: 26 hospitals and 11 countries) observational study, in which the course of cholangiocarcinoma has been investigated, comparing the 3 subtypes based on the latest International Classification of Diseases 11th Edition (ICD-11) (*i.e.*, intrahepatic [2C12], perihilar [2C18], or distal [2C15] affected bile ducts), which come into effect in 2022. General and tumor-type specific features at diagnosis, risk factors, biomarker accuracy, as well as patient management and outcomes, are presented and compared, outlining the current clinical state of cholangiocarcinoma in Europe. © 2021 The Authors. Published by Elsevier B.V. on behalf of European Association for the Study of the Liver. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

Cholangiocarcinoma (CCA) comprises a heterogeneous group of malignancies that can arise anywhere in the bile ducts. Despite being a rare disease, over the past 20 years, incidence and mortality rates have increased globally (0.3-6 cases per 100,000 inhabitants yearly in Western countries, and >6 cases in some East Asian regions).¹ Although different risk factors have been

identified, many patients have no apparent cause at diagnosis, limiting the ability of early detection by surveillance programs.² Moreover, the oligo/asymptomatic nature of CCA in early stages, its aggressiveness, and drug resistance strongly compromise patient outcomes.

Based on the anatomical origin, CCAs are classified as intrahepatic (iCCA), perihilar (pCCA), or distal (dCCA).^{1,3} Although these subtypes are hypothesized to have different risk factors, pathobiology, clinical presentation, management, and prognosis,¹ large datasets defining these differences are limited.⁴ Accordingly, a coherent international study to enhance granularity of the global clinical situation of CCA is pivotal to better understand the disease course, define the similarities and/or differences between CCA subtypes, describe outcomes after selected treatments, and identify challenges for future prospective analyses. This study aims to address this unmet need by describing the disease presentation, risk factors, treatment, and outcomes of patients with CCA in a large pan-European cohort.

Patients and methods

Study design and data collection

The ENSCCA Registry, endorsed by the European Network for the Study of Cholangiocarcinoma (ENSCCA), is a multicenter observational study of patient with histologically and/or cytologically confirmed CCA. Patients' data was accrued based on contributions of 26 referral Healthcare Centers from 11 European countries (Austria, France, Germany, Italy, Lithuania, Netherlands, Norway, Poland, Romania, Spain, and United Kingdom) (Fig. S1A). Patients diagnosed with CCA over a 10-year period (January 1st 2010 to December 31st 2019) were included in the registry study. Fig. S1B summarizes the flow and number of patients based on the following inclusion criteria: i) diagnosis following the latest International Classification of Diseases 11th Edition (ICD-11),⁵ in which CCA was categorized as intrahepatic (2C12), perihilar (2C18), or distal (2C15); and ii) histological and/or cytological confirmation of the diagnosis. Individual patient data was obtained from medical records by the participating hospitals. Information on patients' demographics, documented risk factors and medical history, biochemical and clinical parameters, and treatments were included. In the ENSCCA Registry, clinical and pathological parameters were registered at the time of diagnosis using the 7th edition of the American Joint Committee of Cancer (AJCC)/Union for International Cancer Control TNM cancer staging manual.⁶

Data were recorded using a de-identified format in an electronic case report form, collected and managed using the web-based application designed to support data capture for research studies "Research Electronic Data Capture" (REDCapTM) hosted at "Asociación Española de Gastroenterología" (AEG; www.aegastro.es), a non-profit Scientific and Medical Society focused on Gastroenterology research. Data export was performed in February 2020, and subjected to data harmonization and completeness check. Patients were excluded from the study when mandatory epidemiological and/or clinical data (*i.e.*, type of CCA, date of diagnosis, and date of last follow-up or death) were missing. Moreover, patients without tissue-proven CCA (investigator-reported) or with undefined biliary location were also excluded after an internal investigator review process.

Patients diagnosed from 2010 to 2017 (n = 1,962) were considered for survival analysis to ensure a minimum 2-year follow-up. The ENSCCA Registry Study protocol was approved by the Ethic Committee of Euskadi, Spain (Code: PI2016137), as coordinating Center. Additionally, each participating Center obtained a local ethical approval (or equivalent).

Data analysis

Patients were classified according to the anatomical location of the primary tumor within the bile ducts (i.e., iCCA, pCCA or dCCA) following the ICD-11⁵ criteria and the experience of investigators within multidisciplinary teams. Positive lymph node invasion and/or tumor metastasis were identified by either histology or imaging techniques. As a result, patients were categorized by the disease status at diagnosis, as: i) local disease (LD), ii) locally advanced disease (LAD), or iii) metastatic disease (MD).

LAD was stated as positive regional lymph node tumor invasion measuring above 1.5 cm in diameter (short axis) and classified as N+ (i.e., N1 for iCCA and dCCA; N1 and N2 for pCCA). According to the aforementioned staging guidelines, MD indicated distant involvement (M1), with the exception for liver dissemination of iCCA that is classified as multiple tumors (T2b), and thus, as LD. Based on local multidisciplinary team discussions, patients were divided into 2 groups, those with resectable vs. unresectable CCA following widely accepted international guidelines (e.g., from the European Society for Medical Oncology and/or the International Liver Cancer Association),^{7,8} and taking into account multiparametric criteria based on performance status, tumor stage, underlying diseases, and comorbidities, among others. Accordingly, treatments were categorized as: 1) surgery (i.e., tumor resection or liver transplantation subdivided into i) resection margin R0 [negative margin tumor resection], ii) resection

Table 1. Baseline patient characteristics and concomitant conditions.

	iCCA	pCCA	dCCA	p value ^a	CCA (overall)
Age, median (IQR)	65 (56–72)	66 (59–73)	68 (59–73)	<0.01	66 (58–73)
Sex, n (%)					
Males	655 (52.7)	352 (59.5)	252 (63.2)	<0.001	1,259 (56.4)
Females	588 (47.3)	240 (40.5)	147 (36.8)		975 (43.6)
Caucasian ethnicity, n (%) [n = 1,738]	996 (96.6)	319 (96.1)	364 (97.1)	n.s. ^b	1,679 (96.6)
Laboratory tests ^c , median (IQR)					
ALT [n = 1,598]	32.0 (21–61)	99.0 (53–199)	66.0 (26–149)	<0.0001	47.0 (24–111)
AST [n = 1,931]	37.0 (25–64)	72.0 (41–135)	38.0 (25–78)	<0.0001	43.0 (27–86)
GGT [n = 1,946]	160.0 (71–419)	497.5 (233–945)	159.0 (54–482)	<0.0001	224.0 (86–587)
ALP [n = 1,670]	148.0 (94–294)	305.0 (187–513)	189.0 (113–339)	<0.0001	178.5 (103–352)
Albumin [n = 902]	4.1 (3.6–4.4)	3.8 (3.4–4.2)	4.0 (3.6–4.3)	<0.0001	4.0 (3.6–4.3)
Bilirubin [n = 1,979]	0.6 (0.4–1.1)	3.3 (0.9–10.6)	0.8 (0.4–3.1)	<0.0001	0.8 (0.5–2.9)
Tumor markers, median (IQR)					
CEA [n = 1,015]	2.53 (1.4–5.25)	2.85 (1.6–7.0)	3.1 (1.8–5.42)	n.s.	2.8 (1.5–5.5)
CA19-9 [n = 1,299]	34.7 (9–213)	215.7 (37–1,069)	78.0 (22–310)	<0.0001	59.0 (13–372)
AFP [n = 524]	3.5 (2.0–7.2)	2.8 (2.1–5.1)	2.6 (2.0–4.1)	<0.01	3.2 (2.0–6.1)
Comorbidities, n (%)					
Obesity [n = 1,973]					
Normal weight (<25)	461 (41.5)	252 (51.1)	172 (46.6)	<0.0001	885 (44.9)
Overweight (≥25)	393 (35.4)	172 (34.9)	140 (37.9)		705 (35.7)
Obese (≥30)	257 (23.1)	69 (14.0)	57 (15.4)		383 (19.4)
Diabetes [n = 1,904]	257 (25.6)	86 (15.6)	85 (24.3)	<0.0001	428 (22.5)
Obesity + diabetes [n = 1,722]	166 (17.9)	45 (9.6)	47 (14.4)	<0.001	258 (15.0)
Arterial hypertension [n = 2,011]	455 (41.8)	198 (36.3)	138 (36.7)	n.s.	791 (39.3)
Metabolic conditions [n = 2,011]					
Hypertriglyceridemia	41 (3.8)	15 (2.7)	27 (7.2)	<0.01	83 (4.1)
Low HDL cholesterol	16 (1.5)	17 (3.1)	9 (2.4)	n.s.	42 (2.1)
Biliary conditions [n = 1,569]					
PSC	34 (3.8)	33 (8.8)	4 (1.3)	<0.0001	71 (4.5)
PBC	45 (5.1)	2 (0.5)	4 (1.3)	<0.0001	51 (3.3)
IBD	28 (3.1)	21 (5.6)	10 (3.3)	n.s.	59 (3.8)
PSC + IBD	12 (1.3)	17 (4.5)	3 (1.0)	<0.001	32 (2.0)
Bile duct stones	35 (3.9)	29 (7.7)	31 (10.3)	<0.001	95 (6.1)
Cholecystitis	14 (1.6)	5 (1.3)	5 (1.7)	n.s.	24 (1.5)
Liver diseases					
Viral hepatitis [n = 1,594]	89 (10.4)	20 (4.4)	11 (3.9)	<0.0001	120 (7.5)
Cirrhosis [n = 1,568]	112 (12.6)	5 (1.3)	6 (2.0)	<0.0001	123 (7.8)
Toxic exposure [n = 1,805]					
Alcohol	206 (19.9)	88 (21.6)	64 (17.7)	n.s.	356 (19.8)
Tobacco	322 (31.1)	160 (39.2)	120 (33.2)	<0.05	602 (33.4)

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CA19.9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; dCCA, distal cholangiocarcinoma; GGT, gamma-glutamyl transferase; HDL, high density lipoprotein; IBD, inflammatory bowel disease; iCCA, intrahepatic cholangiocarcinoma; PBC, primary biliary cholangitis; pCCA, perihilar cholangiocarcinoma; PSC, primary sclerosing cholangitis.

^aStatistic analyses (one-way ANOVA or Kruskal-Wallis tests for continuous variables, and Pearson's Chi-square test for categorical variables) were performed by comparing the 3 cholangiocarcinoma subtypes (iCCA vs. pCCA vs. dCCA).

^bStatistical test considered Caucasian vs. other ethnicities (Asian, African, Hispanic, Caribbean).

^cClinical thresholds for laboratory variables were the highest reported in the literature: ALT 45 U/L, AST 40 U/L, GGT 71 U/L, ALP 129 U/L, Albumin 5.2 g/dl, Bilirubin 1.3 mg/dl.

margin R1 [microscopic residual disease], and iii) resection margin R2 [gross residual disease], and (2) active palliative treatment (*i.e.*, chemotherapy, hepatic artery-based therapies, radiation therapy, and/or ablation). Patients receiving staging laparoscopy or exploratory laparotomy were classified according to the subsequent therapeutic strategy.

Statistical analysis

Baseline demographics and risk factors were summarized using descriptive statistics. Continuous data were described as median (IQR), while categorical variables were summarized as n (%). Probability was calculated excluding cases with unknown information. Shapiro-Wilk test was used to test continuous variables for normal distribution. For multiple comparisons, parametric or non-parametric data were compared using one-way ANOVA or Kruskal-Wallis tests, respectively, and followed by Bonferroni *post hoc* test. Pairwise comparisons were calculated using Dunn's method. Pearson's χ^2 test was used to compare categorical variables between the 3 subgroups. For pairwise comparison between CCA subtypes of categorical data, Fisher's exact test was performed. Logistic regression analysis was carried out in variables previously dichotomized as "normal" vs. "high" based on the normality threshold to assess the risk of disease dissemination. Overall survival (OS) was assessed as the time from diagnosis to death or last medical visit, while post-treatment survival considered the treatment start date. Relapse-free survival was calculated as the time from tumor resection to the event of relapse or death. Patients with no information on survival, lost to follow-up or alive at last medical visit were censored at the date of the latest record. Survival analysis was performed with the Kaplan-Meier method and Cox regression (univariate and multivariable analysis including variables statistically significant in the univariate analysis, defined as $p < 0.05$). The Log-rank test was used for

comparisons of survival in Kaplan-Meier curves. Prognostic factors were related to hazard ratio (HR), 95% CIs, and p values.

Statistical analyses were performed with IBM SPSS Statistics Version 22.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 6.0 for Microsoft Windows, (GraphPad Software, La Jolla California, USA). All p values were obtained in 2-tailed tests and $p < 0.05$ was considered statistically significant.

Results

Patients' characteristics and CCA features at diagnosis

From the 3,039 patients initially included in the ENSCCA Registry, 2,234 (73.5%) were selected and further analyzed after fulfilling the inclusion criteria (Fig. S1B), including 1,243 (55.6%) with iCCA, 592 (26.5%) with pCCA and 399 (17.9%) with dCCA. Baseline patient characteristics, including laboratory tests and comorbidities, are listed in Table 1. The majority of patients were Caucasian (96.6%) with a median age at diagnosis of 66 years (IQR 58-73) and slight overrepresentation of males (56.4%). Most patients showed, at diagnosis, increased serum levels of alanine aminotransferase (ALT), markers of cholestasis (gamma-glutamyltransferase [GGT] and alkaline phosphatase [ALP]) and carbohydrate antigen 19-9 (CA19-9), particularly patients with pCCA or dCCA (Table 1). No significant abnormalities were observed in specific hematological and metabolic blood test measures (Table S2).

Considering patients' comorbidities (Table 1), 55.1% were overweight/obese (BMI 25-30 kg/m² [35.7%] or BMI ≥ 30 kg/m² [19.4%]) at the time of diagnosis, this feature being more frequent in patients with iCCA (Table S1); 22.5% had diabetes, observed more frequently in patients with iCCA or dCCA compared to pCCA, and 39.9% had arterial hypertension. Of note, 15% of the patients with CCA were obese and diabetic. In addition, patients suffered from underlying biliary or liver diseases predisposing to CCA development, including primary biliary

Table 2. Tumor presentation at diagnosis.

	iCCA	pCCA	dCCA	<i>p</i> value ^a	CCA (overall)
ECOG-PS [n = 1,984]					
0	564 (51.1)	226 (40.8)	83 (24.4)	n.s. ^b	873 (44.0)
1	359 (32.5)	220 (40.7)	196 (57.6)		775 (39.1)
2	129 (11.7)	74 (13.7)	44 (12.9)		247 (12.4)
3	46 (4.2)	20 (3.7)	15 (4.4)		81 (4.1)
4	5 (0.5)	1 (0.2)	2 (0.6)		8 (0.4)
Tumor size [n = 1,268]					
≤3 cm	117 (13.4)	147 (56.8)	105 (76.6)	<0.0001	369 (29.1)
>3 cm	487 (55.8)	90 (34.7)	23 (16.8)		600 (47.3)
Multiple lesions	268 (30.7)	22 (8.5)	9 (6.6)		299 (23.6)
Pattern of growth [n = 1,108]					
Mass-forming	700 (92.8)	50 (27.0)	57 (33.7)	<0.0001	807 (72.8)
Periductal infiltrating	21 (2.8)	105 (56.8)	93 (55.0)		219 (19.8)
Intraductal growth	8 (1.1)	27 (14.6)	17 (10.1)		52 (4.7)
Mixed pattern	25 (3.4)	3 (1.6)	2 (1.2)		30 (2.8)
Differentiation grade [n = 1,245]					
Not assessed (Gx)	66 (8.9)	15 (5.7)	19 (7.9)	<0.0001	100 (8.0)
Well (G1)	89 (12.0)	54 (20.5)	60 (24.8)		203 (16.3)
Moderate (G2)	378 (51.2)	150 (56.8)	108 (44.6)		636 (51.1)
Poor (G3)	200 (27.1)	45 (17.0)	55 (22.7)		300 (24.1)
Undifferentiated (G4)	6 (0.8)	0 (0.0)	0 (0.0)		6 (0.5)
Regional lymph node invasion (N+) [n = 1,630]	419 (50.3)	229 (45.3)	145 (49.8)	n.s.	793 (48.7)
Distant metastasis (M1) [n = 2,043]	276 (23.9)	140 (27.3)	78 (20.9)	n.s.	494 (24.2)

Data are presented as n (%).

dCCA, distal cholangiocarcinoma; ECOG, Eastern Cooperative Oncology Group performance status; iCCA, intrahepatic cholangiocarcinoma; IG, intraductal growth; MF, mass-forming; OS, overall survival; pCCA, perihilar cholangiocarcinoma; PI, periductal infiltrating.

^aStatistic analyses (Pearson's Chi-square test) were performed by comparing the 3 cholangiocarcinoma subtypes (iCCA vs. pCCA vs. dCCA).

^bStatistical test considered ECOG 0-1 vs. ≥ 2 .

cholangitis (PBC: 3.3%, mainly iCCA), primary sclerosing cholangitis (PSC: 4.5%; mainly pCCA), bile duct stones (6.1%; mainly pCCA and dCCA), viral hepatitis (2.8% hepatitis C virus, 4.6% hepatitis B virus, and 0.1% concomitant infection; mainly iCCA) and cirrhosis (7.8%; mainly iCCA). In this registry cohort there was also a history of smoking or alcohol consumption in 33% and 19.8% of patients, respectively.

Table 2 summarizes patients' fitness, measured as Eastern Cooperative Oncology Group performance status (ECOG-PS), and tumor-related features at diagnosis. The majority of patients with CCA had ECOG-PS of 0 (44.0%) or 1 (39.1%). Regarding tumor size and growth pattern, iCCAs were frequently larger lesions (>3 cm or multifocal) with a mass-forming pattern compared to

pCCA and dCCA that in general were smaller lesions (<3 cm) with periductal infiltration (Table S3). Moderate grade of tumor differentiation was the most frequently observed in the 3 CCA subtypes. From 1,998 patients with available information on imaging, 6.2% had initial tumor staging based on MRI and/or magnetic resonance cholangiopancreatography (MRCP), 47.4% with CT, and 54.9% with both approaches (Table S4). Of note, 32.3% and 4.0% of all patients with MRI/MRCP/CT-based staging had an additional ultrasound or PET evaluation, respectively. Imaging findings elucidated that regional lymph node invasion and disseminated disease were present in 48.7% and 24.2% of patients, respectively. CCAs preferentially metastasized to lung, liver, distant lymph nodes, bone and peritoneum – including

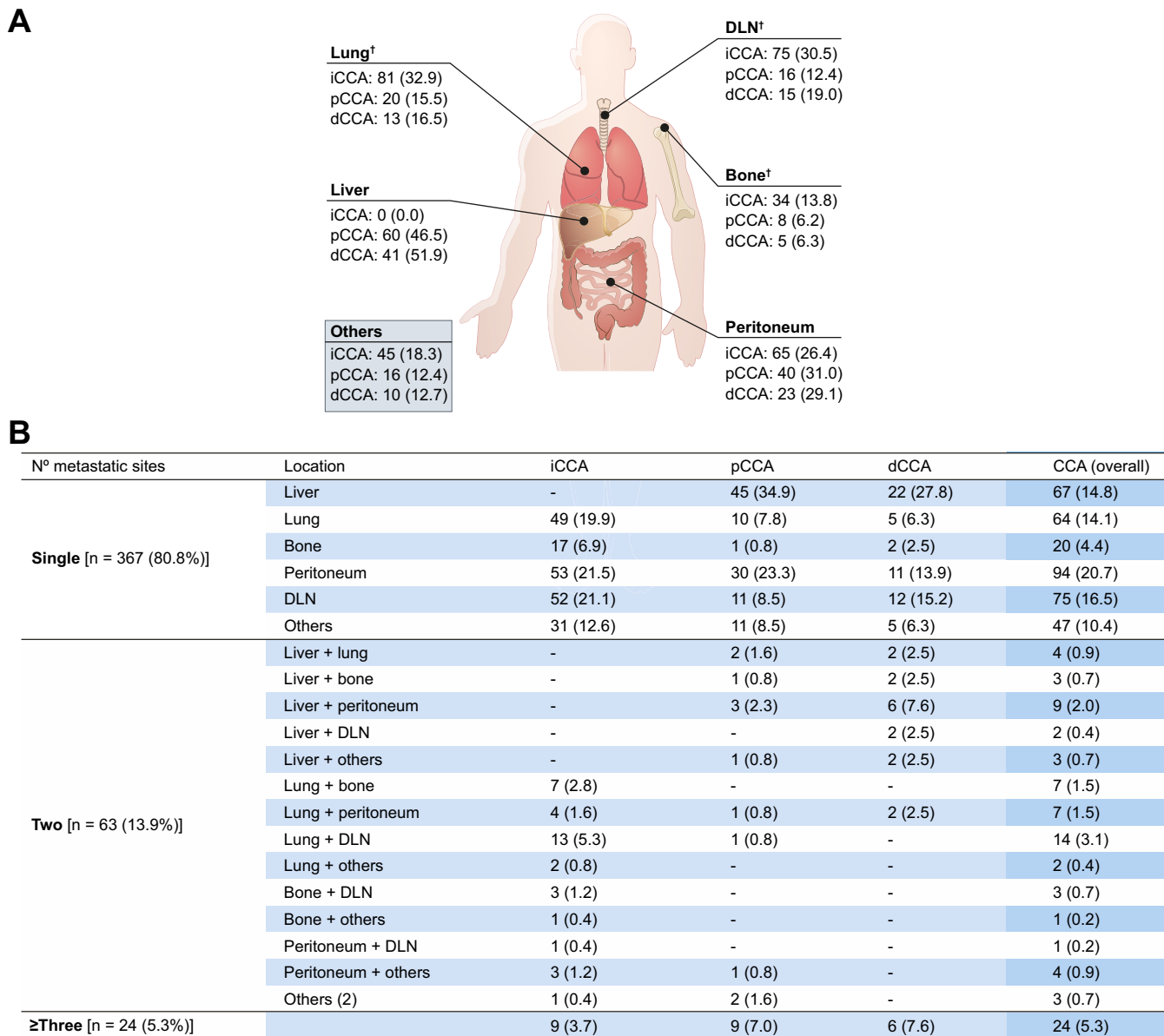


Fig. 1. Preferential metastatic locations of cholangiocarcinoma. (A) Most commonly found metastatic locations stratified by CCA subtype, expressed as number and percentage [n (%)], and (B) classification of patients with disseminated CCA depending on their sites of metastasis, as single, two, three or more sites of metastasis. †Significant Pearson's chi-squared test for Bone ($\chi^2 p < 0.05$): iCCA vs. pCCA, $p < 0.05$; DLN ($\chi^2 p < 0.001$): iCCA vs. pCCA, $p < 0.0001$; Lung ($\chi^2 p < 0.001$): iCCA vs. pCCA, $p < 0.001$, and iCCA vs. dCCA, $p < 0.01$. CCA, cholangiocarcinoma; dCCA, distal CCA; DLN, distant lymph nodes; iCCA, intrahepatic CCA; pCCA, perihilar CCA. (This figure appears in color on the web.)

omentum – with significant differences between subtypes. iCCA was mainly found to disseminate into lung, distant lymph nodes, and bone, whereas pCCA and dCCA mainly metastasized into the liver or to the peritoneum (Fig. 1A). In most patients with MD at presentation, a single site of metastasis was found (80.8%). Fig. 1B shows the frequency of each metastatic site based on the CCA subtype.

Sensitivity of serum carcinoembryonic antigen (CEA) and CA19-9 tumor biomarkers

The sensitivity of CEA (cut-off value: 5 IU/ml) and CA19-9 (≥ 37 IU/ml) was evaluated according to the disease stage. Serum CEA was above the upper reference limit in 30.9% of patients, correlating with disease severity (for LAD: odds ratio [OR] 1.71; 95% CI 1.16-2.51; for MD: OR 3.03; 95% CI 2.11-4.35) (Fig. 2B and Table S5-6). Increased CA19-9 was found in 59.1% of cases, particularly with LAD or MD (Fig. 2B and Table S5). Of note, CA19-9 above the cut-off value was associated with an increased risk of tumor spread (for LAD: OR 1.99; 95% CI 1.47-2.70; for MD: OR 3.04; 95% CI 2.21-4.17) (Fig. 2B and Table S6).

The elevation of a single serum marker (i.e., CEA or CA19-9) was slightly associated with LAD (OR 1.72; 95% CI 1.16-2.53) or MD (OR 2.53; 95% CI 1.56-4.10) at diagnosis, whereas the concomitant elevation of both considerably increased the odds for LAD (OR 2.16; 95% CI 1.43-3.27) and for MD (OR 5.86; 95% CI 3.69-9.25) (Fig. 2C).

Management and outcome of patients with CCA

Patients with CCA often present with tumor-mediated biliary obstruction, requiring biliary drainage prior to starting any therapeutic regimen. In particular, 40.3% of the patients received biliary drainage, from whom 42.4% required subsequent stenting, with a median time interval of 1.8 months (Table S7). Fig. 3A represents a flow chart summarizing the first therapeutic strategy for patients with CCA following initial diagnosis. Of note, biliary drainage was performed prior to surgery or systemic therapy in 32.2% and 35.0% of patients, respectively (Fig. 3A). Moreover, 61.8% of all patients not receiving anti-cancer therapy had biliary drainage as part of best supportive care (BSC).

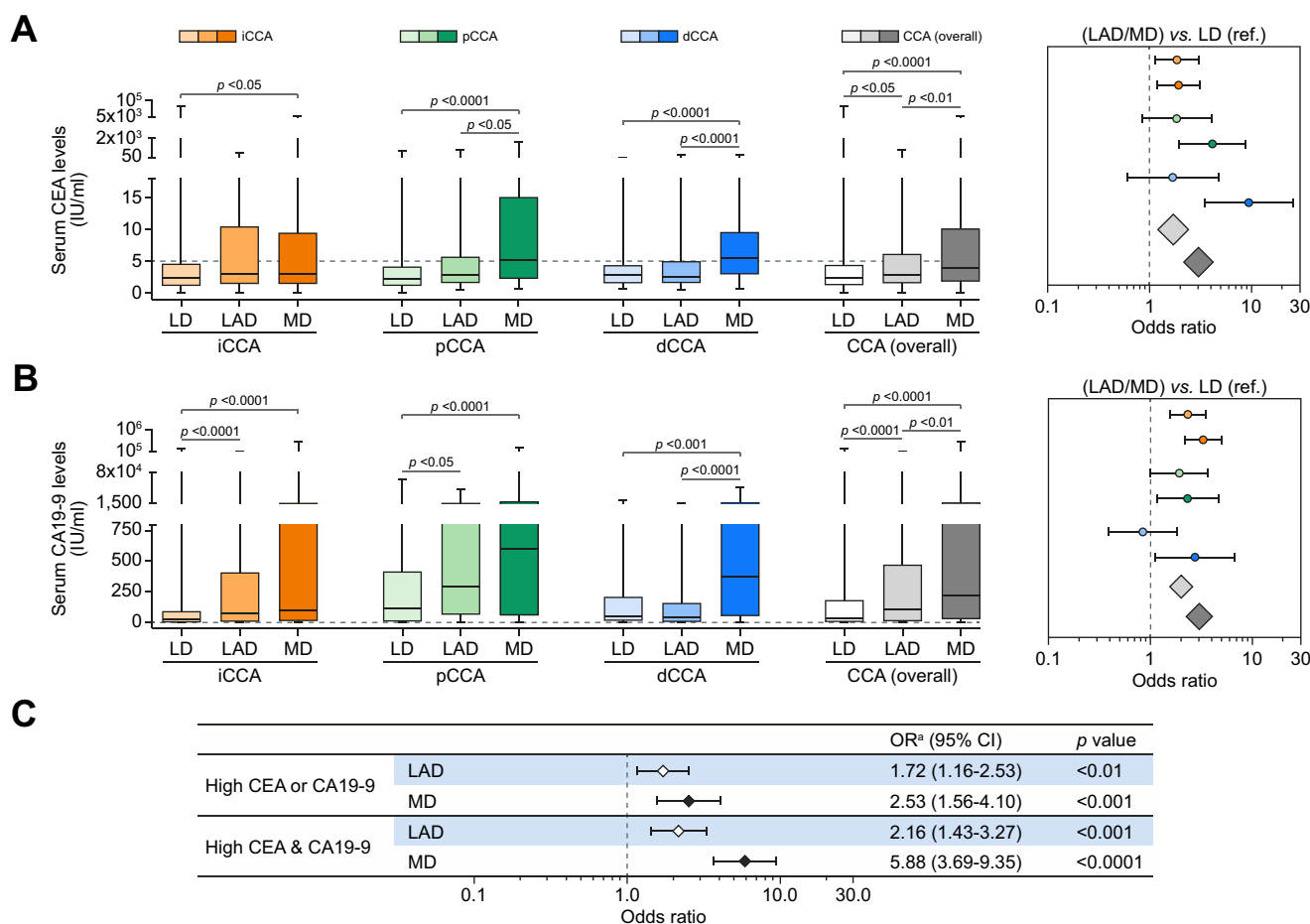
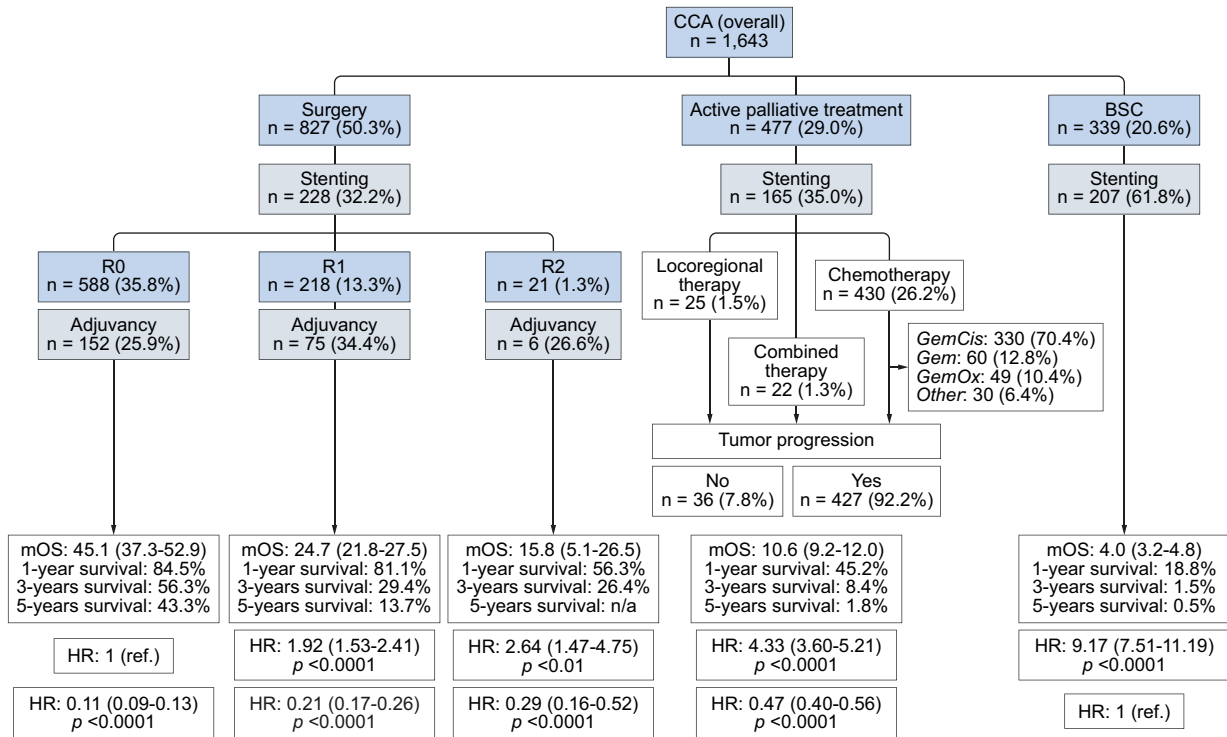
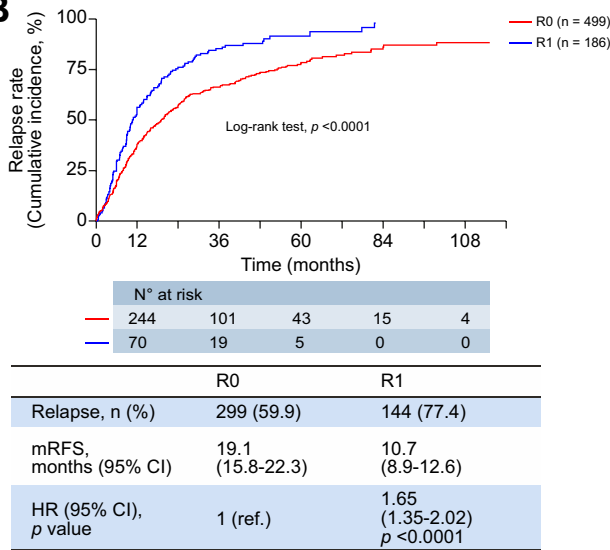


Fig. 2. Serum tumor markers in cholangiocarcinoma. (A) Serum CEA levels depicted for disease stage (i.e., LD, LAD, or MD) for overall CCAs and subtypes. Odds value of CEA as potential tumor biomarker in the identification of tumor spread compared to LD. CEA cut-off value established at 5 IU/ml. (B) Serum CA19-9 levels depicted for tumor spread stage (i.e., LD, LAD, or MD) for overall CCAs and subtypes. Odds value of CA19-9 as potential tumor biomarker in the identification of tumor spread compared to LD. CA19-9 cut-off value established at 37 IU/ml. (C) Patients were classified into 3 comparison groups: (1) with both circulating tumor biomarkers (CA19-9 and CEA) below the established threshold, (2) one of both biomarkers over the cut-off value, or (3) both, CA19-9 and CEA, over the cut-off value. Odds value of the combination of CA19-9 and CEA in the prediction of CCA staging compared to LD. One-way ANOVA Kruskal-Wallis test was used for multiple comparisons. CA19-9, carbohydrate antigen 19-9; CCA, cholangiocarcinoma; CEA, carcinoembryonic antigen; dCCA, distal CCA; iCCA, intrahepatic CCA; LAD, locally advanced disease; LD, local disease; MD, metastatic disease; OR, odds ratio; pCCA, perihilar CCA. (This figure appears in color on the web.)

A



B



C

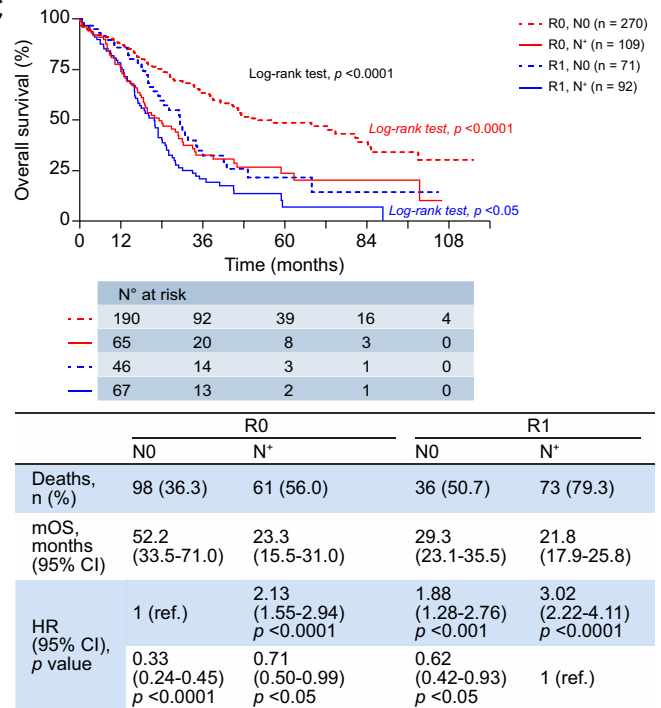


Fig. 3. Cholangiocarcinoma clinical management and outcome. (A) Diagram of classification of patients with CCA divided by type of treatment strategy (i.e. surgery, active palliative treatment or BSC) together with the corresponding median overall survival and Cox regression analysis between groups. Kaplan-Meier analysis and multivariable Cox regression models for the assessment of long-term outcome of patients with CCA after tumor resection, with (B), tumor relapse as primary endpoints of patients after tumor resection, and (C), lymph node invasion-associated mortality for patients under tumor resection. BSC, best supportive care; CCA, cholangiocarcinoma; Cis, cisplatin; dCCA, distal CCA; Gem, gemcitabine; HR, hazard ratio; iCCA, intrahepatic CCA; mOS, median overall survival; mRFS, median relapse-free survival; N, lymph node invasion; Ox, oxaliplatin; pCCA, perihilar CCA; R0, null margin tumor resection; R1, microscopic residual disease tumor resection; R2, gross residual disease tumor resection. (This figure appears in color on the web.)

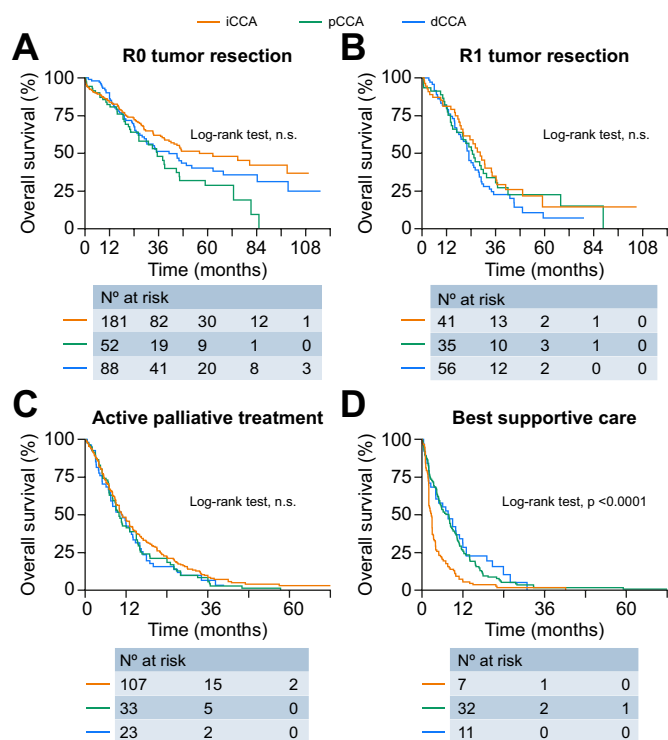


Fig. 4. Therapeutic approaches and outcomes in cholangiocarcinoma subtypes. Kaplan-Meier analysis for the assessment of long-term outcome of patients with CCA after (A) R0 tumor resection, (B) R1 tumor resection, (C) active palliative treatment, and (D) best supportive care. dCCA, distal cholangiocarcinoma; iCCA, intrahepatic cholangiocarcinoma; pCCA, perihilar cholangiocarcinoma; R0, null margin tumor resection; R1, microscopic residual disease tumor resection. (This figure appears in color on the web.)

Surgical resection was performed in 50.3% of patients showing a median OS (mOS) of 33.4 months. A total of 35.8% of patients had a negative-resection margin (R0) after surgery; these patients achieved a mOS of 45.1 months and 1-, 3-, 5-year survival rates of 84.5%, 56.3% and 43.3%, respectively.

Microscopic residual disease (R1) after tumor resection was associated with an increased risk of relapse compared to R0 (HR 1.65; 95% CI 1.35-2.02), with a median relapse-free survival of 10.7 and 19.1 months, respectively (Fig. 3B). Moreover, R1 surgery achieved a mOS of 24.7 months and 1-, 3-, 5-year survival rates of 81.1%, 29.4% and 13.7%, respectively. Patients with R1 after surgery were at an increased risk of death compared to patients who had R0 resection (HR 1.92; 95% CI 1.53-2.41), despite not showing survival differences compared to those with gross residual disease (R2) (HR 1.37; 95% CI 0.76-2.48).

Lymph node invasion (N+) also compromised the OS of patients after resection (Fig. 3C). Worse outcomes were observed in patients with N+ compared to N0, both after R0 or R1 tumor resections (HR 2.13; 95% CI 1.55-2.94, and HR 1.61; 95% CI 1.08-2.38), respectively. These differences were also observed in mOS: 52.2 months for R0/N0, 23.3 months for R0/N+, and 29.3 months and 21.8 months for R1/N0 and R1/N+, respectively. Notably, 25.9% of R0- and 34.4% of R1-resected patients received adjuvant treatment, which did not improve the mOS when compared to patients not receiving any adjuvant therapy (Fig. 3A, Fig. S2).

Out of the 816 (49.6%) patients with unresectable disease at diagnosis, the majority (477; 29.0%) received active palliative therapy (i.e. chemotherapy (26.2% of whole cohort), locoregional therapy (1.5%) and combined chemo- and locoregional therapies (1.3%)), with mOS and 1- and 3-year survival rates from time of treatment initiation of 10.6 months, and 45.2% and 8.4%, respectively. In total, 92.2% of patients under palliative treatment showed tumor progression before death. In patients receiving palliative chemotherapy, gemcitabine plus cisplatin was the most common regimen used (70.4%) and led to a significantly reduced risk of death compared to BSC (HR 2.24; 95% CI 1.87-2.67) or gemcitabine alone (HR 1.66; 95% CI 1.22-2.28)(Fig. S3). Patients under active palliative treatment had reduced mOS compared to those undergoing surgery with curative intent (R0/R1) (for R0: HR 4.33; 95% CI 3.60-5.21; for R1: HR 2.25; 95% CI 1.82-2.77) (Fig. 3C). No significant survival differences were found between active palliative therapy and R2 tumor resection (HR 1.62; 95% CI

Table 3. Univariable and multivariable Cox regression analysis of variables at diagnosis.

Covariables	Deaths, n (%)	Univariable			Multivariable ^a		
		HR	95% CI	p value	HR	95% CI	p value
Subtype of CCA, (vs. pCCA)	1,348 (68.7)						
iCCA		0.74	0.65–0.84	<0.0001	1.48	0.74–2.97	n.s.
dCCA		0.67	0.57–0.78	<0.0001	1.31	0.50–3.44	n.s.
Age, ≥65 (vs. <65)	1,348 (68.7)	1.28	1.15–1.42	<0.0001	1.24	0.70–2.22	n.s.
Sex, male (vs. female)	1,348 (68.7)	1.12	1.00–1.24	<0.05	0.99	0.58–1.70	n.s.
ECOG-PS, (continuous)	1,247 (72.2)	1.66	1.56–1.78	<0.0001	1.52	1.01–2.31	<0.05
Disease status, (vs. local disease)	1,098 (72.9)						
Locally advanced disease		1.91	1.65–2.22	<0.0001	1.68	0.87–3.25	n.s.
Metastatic disease		3.46	2.98–4.02	<0.0001	4.03	1.82–8.92	<0.01
CEA, ≥5 (vs. <5)	487 (62.0)	2.02	1.67–2.43	<0.0001	1.19	0.65–2.19	n.s.
CA19-9, ≥37 (vs. <37)	660 (61.1)	2.02	1.70–2.37	<0.0001	2.79	1.46–5.33	<0.01
ALT, ≥45 (vs. <45)	853 (63.5)	1.15	1.00–1.31	<0.05	1.26	0.62–2.59	n.s.
AST, ≥40 (vs. <40)	1,180 (69.8)	1.43	1.27–1.61	<0.0001	0.48	0.21–1.09	n.s.
GGT, ≥71 (vs. <71)	1,189 (70.1)	1.96	1.68–2.28	<0.0001	1.51	0.69–3.31	n.s.
ALP, ≥129 (vs. <129)	1,014 (70.2)	1.80	1.57–2.06	<0.0001	1.24	0.57–2.71	n.s.
Albumin, <5.2 (vs. ≥5.2)	556 (71.5)	0.26	0.08–0.82	<0.05	0.28	0.03–2.64	n.s.
Bilirubin, ≥1.3 (vs. <1.3)	1,209 (70.0)	1.41	1.26–1.58	<0.0001	0.98	0.49–1.95	n.s.

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CA19.9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; dCCA, distal cholangiocarcinoma; ECOG, Eastern Cooperative Oncology Group performance status; GGT, gamma-glutamyl transferase; HR, hazard ratio; iCCA, intrahepatic cholangiocarcinoma; pCCA, perihilar cholangiocarcinoma; PS, performance status.

^aMultivariable analysis, number of events, n = 66 (63.5%).

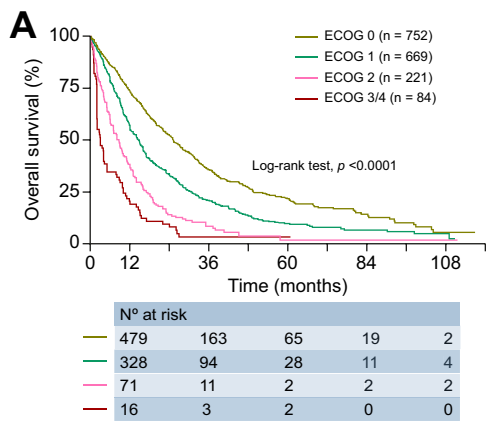
0.91-2.89). Of note, 20.6% of patients received only BSC resulting in a mOS of 4.0 months.

This comparative analysis of patient management and outcomes revealed certain differences between the 3 CCA subtypes (Fig. 4). In particular, significant differences in survival were observed between CCA subtypes receiving BSC (Fig. 4D). Patients with pCCA received BSC more often (37.3%); however, patients with iCCA showed the poorest prognosis with mOS of 2.8 months compared to the 7.0 and 7.7 months found in pCCA and dCCA, respectively (Table S7). On the other hand, comparable mOS were obtained between CCA subtypes in patients undergoing tumor resection or active palliative treatment (Fig. 4A-C, Table S7).

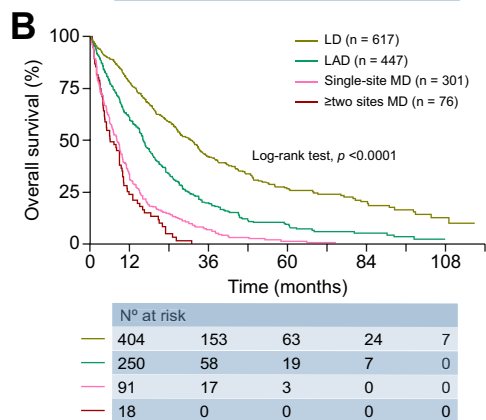
Prognostic factors

The univariable analysis between clinical and demographic variables at diagnosis and OS showed significant associations for CCA subtype, age, sex, ECOG-PS, disease status, and the serum levels of CA19-9, CEA, ALT, aspartate aminotransferase, GGT, ALP, albumin, or bilirubin (Table 3). However, a stepwise multivariable Cox regression analysis indicated that ECOG-PS, MD, and elevated CA19-9 levels were independent prognostic factors (HRs 1.52, 4.03, 2.79, respectively; Table 3). Thus, patient outcomes based on these 3 independent variables were further depicted.

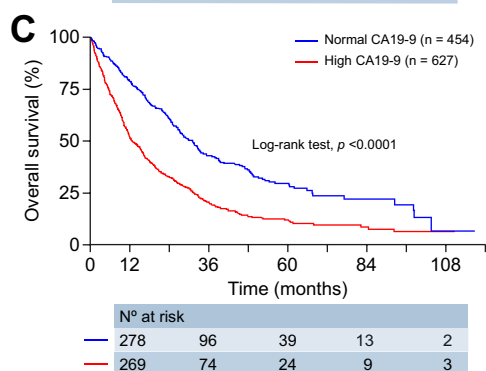
Fig. 5A represents the OS for patients stratified according to ECOG-PS scores (0, 1, 2, 3-4) at diagnosis with pronounced



	ECOG 0	ECOG 1	ECOG 2	ECOG 3/4
Deaths, n (%)	477 (63.4)	512 (76.5)	182 (82.4)	76 (90.5)
mOS, months (95% CI)	25.2 (22.7-27.7)	14.8 (13.2-16.3)	8.7 (7.0-10.4)	3.0 (1.6-4.4)
HR (95% CI), p value	1 (ref.)	1.57 (1.38-1.78) <0.0001	2.76 (2.32-3.28) <0.0001	4.65 (3.64-5.95) <0.0001
HR (95% CI), p value	0.22 (0.17-0.28) p <0.0001	0.34 (0.26-0.43) p <0.0001	0.59 (0.45-0.78) p <0.0001	1 (ref.)



	LD	LAD	MD	
			Single-site	≥two sites
Deaths, n (%)	343 (55.6)	355 (79.4)	273 (90.7)	71 (93.4)
mOS, months (95% CI)	30.9 (27.7-34.0)	16.2 (14.8-17.5)	8.1 (6.9-9.4)	6.1 (3.7-8.6)
HR (95% CI), p value	1 (ref.)	1.94 (1.67-2.26) <0.0001	3.75 (3.18-4.42) <0.0001	5.02 (3.86-6.54) <0.0001
HR (95% CI), p value	0.20 (0.15-0.26) <0.0001	0.39 (0.30-0.50) <0.0001	0.75 (0.57-0.97) <0.05	1 (ref.)



	Normal CA19-9	High CA19-9
Deaths, n (%)	218 (48.0)	442 (70.5)
mOS, months (95% CI)	31.0 (27.5-34.5)	12.7 (10.8-14.5)
HR (95% CI), p value	1 (ref.)	2.02 (1.71-2.37) p <0.0001

Fig. 5. Independent prognostic value of ECOG performance status, tumor dissemination and CA19-9 in CCA. Kaplan-Meier analysis and multivariable Cox regression models for the assessment of long-term outcomes of patients with CCA, with all-cause mortality as primary endpoints for (A) ECOG, (B) disease status, and (C) CA19-9 serum tumor biomarker. CA19-9, carbohydrate antigen 19-9; CCA, cholangiocarcinoma; ECOG, Eastern Cooperative Oncology Group; HR, hazard ratio; LAD, locally advanced disease; LD, local disease; MD, metastatic disease; mOS, median overall survival. (This figure appears in color on the web.)

differences between groups and with a mOS of 25.2 months (reference), 14.8 months (HR 1.57; 95% CI 1.38-1.78), 8.7 months (HR 2.76; 95% CI 2.32-3.28) and 3.0 months (HR 4.65 (95% CI 3.64-5.95), respectively. Besides, the disease stage (LAD, single-site MD, ≥ 2 -site MD) progressively increased the risk of death compared to patients with LD (reference: mOS = 30.9 months), with mOS of 16.2 months (HR 1.94; 95% CI 1.67-2.26) for LAD, 8.1 months (HR 3.75 (95% CI 3.18-4.42) for single-site MD, and 6.1 months (HR 5.02; 95% CI 3.86-6.54) for ≥ 2 -site MD (Fig. 5B). Moreover, CA19-9 had intrinsic prognostic value (HR 2.02; 95% CI 1.71-2.37), with mOS of 31.0 and 12.7 months for normal or elevated (>37 IU/ml) CA19-9 levels, respectively (Fig. 5C).

Discussion

To our knowledge, this study constitutes the largest and most comprehensive international analysis evaluating the presentation, clinical management, and outcome of patients with CCA, with special focus on differences between CCA subtypes.

Although a significant proportion of CCAs arise within an apparently healthy liver, population-based studies have identified different risk factors.^{1,2,9} Herein, we show that more than 50% of patients with CCA were overweight/obese and 20% were diabetic at diagnosis. Evidence suggests obesity, and the metabolic syndrome in particular, as a major risk factor for cancer in general, but also for CCA.² In fact, the obesity pandemic in the adult population has grown rapidly since the 1970s, which preceded the increased incidence of iCCA observed since the 1980s.¹⁰⁻¹² Moreover, recent studies suggest that non-alcoholic fatty liver disease (NAFLD), part of the metabolic syndrome, might be a major risk factor, alone or in association with obesity, for cancer, and in particular for hepatocellular carcinoma and CCA.¹³⁻¹⁵ Based on our results and on a meta-analysis of 24 studies showing a pooled prevalence of NAFLD of 77.87% in diabetic patients with obesity,¹⁶ we could expect a considerable prevalence of NAFLD in our patients with CCA. In addition, other pathologic conditions that have traditionally been associated with CCA development seem to have a subtype-dependent impact. For instance, viral hepatitis B and C infections inferred greater risk for iCCA than p/dCCA. Of note, during the last decades, the prevalence of viral hepatitis has decreased due to vaccination programs or new effective treatments,¹⁷ while metabolic-associated conditions are altering the epidemiological setting of CCA. This raises the need for the involvement of primary care to carry out awareness policies based on lifestyle prevention.

Furthermore, 12.6% of the patients with iCCAs arose on a cirrhotic background, most probably in association with the previously reported viral infection, alcohol consumption, or NAFLD. Interestingly, and according to our data, although end-stage cirrhosis in patients with PBC is a well-known risk factor for the development of hepatocellular carcinoma, it may also predispose to iCCA development. This confirms previous data from the Surveillance, Epidemiology and End Results (SEER) program of the National Cancer Institute in the United States, in which PBC was associated with iCCA, but not extrahepatic CCA.¹⁸ The well-known association of PSC with pCCA,¹⁵ and of bile duct stones with p/dCCA² was confirmed in our study. These data support the need to raise CCA awareness and for specific screening programs for clearly identified high-risk populations (*i.e.*, choledochal cysts, biliary stones, cirrhosis, biliary diseases [Caroli's, PSC], viruses [HBV, HCV]).²

Beyond the features shared by all CCAs, increasing evidence indicates that CCA subtypes might differ in clinical presentation, etiology, natural history, management, and prognosis and thus, should be regarded as distinct entities.^{9,19} This study suggests that perihilar and distal tumors are detected earlier than intrahepatic lesions, mainly because they usually cause obstructive jaundice at an early stage. Consequently, iCCAs appeared as larger or multifocal lesions, and predominantly as moderate-to-poorly differentiated tumors. Most iCCAs showed a mass-forming growth pattern, whereas pCCA and dCCA were mostly flat or periductal infiltrating, and less frequently intraductal, supporting previous observations.²⁰ Nonetheless, no differences in disease stage were found between CCA subtypes. This may be, at least in part, because hepatic dissemination of iCCA is not formally considered metastasis according to the current AJCC guidelines. In this regard, we have recently shown that patients with iCCA and cancer spread within the liver, with or without lymph node invasion, have worse prognosis than patients with local iCCA, strongly encouraging the establishment of a new specific code (M1) for these patients.²¹

CCAs are usually diagnosed by a sequential protocol comprising imaging approaches and assessment of non-specific tumor biomarkers in serum, followed by biopsy or cytology, when feasible.¹ Serum levels of CA19-9 and CEA are frequently determined in clinical practice when CCA is suspected.^{22,23} Nevertheless, the diagnostic accuracy of both tumor markers is controversial.^{24,25} Our data showed that CEA and CA19-9 are increasingly elevated as the disease progresses, supporting previous reports in which preoperative elevation of serum CA19-9 appears as a predictor of nodal invasion.²⁶ Consequently, future prospective studies should determine the potential utility of CEA and CA19-9 to identify patients who would benefit from a more detailed staging, using, for instance, PET with ¹⁸F-fluorodeoxyglucose (FDG-PET). Our results highlight the underuse of FDG-PET for the staging of CCA, even though it may help in the identification of occult nodal and distant metastatic status.²⁷ Of note, the multivariable Cox regression analysis revealed that elevation of CA19-9, but not of CEA, is an independent prognostic factor for CCA, a finding of translational relevance for patient stratification and design of clinical trials. Indeed, our findings showed independent prognostic value not only for CA19-9, but also for both the ECOG-PS, and disease status. These results share some similarities with a previous work that proposed a new clinical-based staging system for pCCA that includes ECOG-PS, tumor size and number, vascular encasement, tumor dissemination and CA19-9 as stratification factors.²⁸

In our dataset, 1/5 patients did not receive active palliative treatment (just BSC), probably due to late diagnosis and deterioration of patients' ECOG-PS. However, our cohort study confirmed longer survival in patients who received some form of anti-cancer treatment for unresectable disease,^{8,29,30} mainly gemcitabine plus cisplatin, highlighting the need to consider these therapies when performance status is suitable.^{8,31} In this regard, future studies should compare locoregional – with current limited experience in CCA – vs. systemic therapies for the treatment of unresectable CCAs. According to our data, tumor resection is the best therapeutic option, even though lymph node involvement is an important determinant of clinical outcome. However, the decision to perform tumor resection is a difficult trade-off between short-term risk (*i.e.*, post-surgical mortality) and potential long-term benefit. According to the CCA subtypes,

patients with iCCA showed the worst OS under BSC, probably due to the progression of associated chronic liver diseases (e.g., cirrhosis, viral hepatitis, NAFLD). In contrast, similar outcomes were shown for all 3 CCA subtypes under active palliative treatment and tumor resection. Altogether, these data reinforce the need for adequate investment in early diagnosis of CCA, the shortening of time to surgery, and the systemic treatment of advanced disease when feasible, as the best strategies to improve the outcome of patients.

This study has several limitations. It shows novel data on the course of CCAs in European reference centers, but it cannot be interpreted as a demographical study; therefore, caution is required when extrapolating the results. Selection bias related to clinical specialties of participating centers (hepatologists, gastroenterologists, medical oncologists, surgeons) could explain the differences between CCA subtypes and disease stages at diagnosis. Besides, the diagnosis and classification by CCA subtypes were based on investigator-reported data following data harmonization. Even though no external audit was performed, an internal review was conducted by each center in order to double-check the included data. Nevertheless, the absence of a central reading should not have a major impact on the conclusions drawn from this work. In fact, the expected number of cases with undistinguishable location would be very low as they are retrieved by investigators affiliated to referral hospitals with large experience in the management of CCA. Clinical approaches related to the diagnostic work-up and disease monitoring programs may diverge between hospitals and specific departments of specialization. In addition, differences in terms of disease phenotype and incidence of risk conditions for CCA may exist between countries. In a separate matter, the percentage of patients receiving BSC is probably underestimated as invasive methods for histological/cytological disease confirmation required patients to be eligible for this study, and thus, are often not performed for those unfit for anti-cancer treatment.

In conclusion, our study including more than 2,200 patients with CCA from 11 European countries provides a comprehensive analysis of diagnostic, prognostic and therapeutic aspects of the complex CCA landscape, showing that CCA is still diagnosed at an advanced stage, a significant proportion of patients fail to receive any cancer-specific therapy, and therefore, the prognosis is dismal. Accordingly, the promotion of awareness campaigns and education programs aimed to prevent lifestyle-related risk factors and the implementation of surveillance for early detection of CCA in high-risk populations are urgently required in order to decrease cancer-related mortality. In this regard, this study represents valuable knowledge for future comparisons with new targeted therapies and for the design of next-generation personalized clinical trials.

Abbreviations

AEG, "Asociación Española de Gastroenterología"; AJCC, American Joint Committee of Cancer; ALP, alkaline phosphatase; ALT, alanine aminotransferase; BSC, best supportive care; CA19-9, carbohydrate antigen 19-9; CCA, cholangiocarcinoma; CEA, carcinoembryonic antigen; dCCA, distal CCA; ECOG-PS, Eastern Cooperative Oncology Group performance status; ENS-CCA, European Network for the Study of CCA; FDG-PET, positron emission tomography with 18F-fluorodeoxyglucose; GGT, gamma-glutamyltransferase; HR, hazard ratio; IBD, inflammatory bowel disease; iCCA, intrahepatic CCA; ICD, International Classification

of Diseases; INR, international normalized ratio; LAD, locally advanced disease; LD, local disease; M, distant metastasis; MD, metastatic disease; mOS, median overall survival; MRCP, magnetic resonance cholangiopancreatography; N, lymph node invasion; NAFLD, non-alcoholic fatty liver disease; OR, odds ratio; OS, overall survival; Ox, oxaliplatin; PBC, primary biliary cholangitis; pCCA, perihilar CCA; PSC, primary sclerosing cholangitis; R0, null resection margin; R1, microscopic residual resection margin; R2, macroscopic residual resection margin.

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Conflict of interest

AF declares lecture fees (from Bayer HealthCare, Gilead, Roche, MSD and Boston Scientific), and consultancy fees (from Bayer HealthCare, AstraZeneca, Roche, Guerbert, SIRTEX and Exact Science). AL reported travel and educational support (from Ipsen, Pfizer, Bayer, Advanced Acceletaro Applications, SirtEx, Novartis, Mylan and Delcath), speaker honoraria (from Merck, Pfizer, Ipsen, Incyte and AAA), advisory honoraria (from EISAI, Nutricia Ipsen, QED and Roche), and she is Member of the Knowledge Network and NETConnect Initiatives funded by Ipsen. ALI declares lecture fees (from Intercept Pharma, Abbvie, Gilead, Alfa-Sigma, and MSD) and consultancy fees (from Intercept and Alfa Sigma). AL-M declares travel and educational support (from Pfizer, Roche, Sanofi, Rovi, Pharma Mar and Merck). CB declares consultancy honoraria (from Incyte). GV declares consultancy fee (from Bayer HealthCare). JBA declares consulting role (for QED Therapeutics and SEALD). JMB declares research grants (from Incyte), personal fees for lecturer (from Bayer and Intercept), and consulting role (for QED Therapeutics, Albireo Pharma and OWL Metabolomics). JWV reports personal fees (from Agios, AstraZeneca, Baxter, Genoscience Pharma, Hutchison Medipharma, Imaging Equipment Ltd (AAA), Incyte, Ipsen, Mundipharma EDO, Mylan, QED, Servier, Sirtex, Zymeworks), and grants, personal

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Authors' contributions

LI-S: study concept and design; analysis and interpretation of data; statistical analysis; drafting of the manuscript. ALI, AL-M, AS, ASc, AV, CB, FL-L, GLG, GV, HJK, JA, JI, JK, JPJ, KE, KU, LB, LF, LK, MK, MM, MP-S, NJ, RB, RIRM, SB, TF, VC, VS, WH, ZS, JIE: data acquisition; proof-reading. AS-L: study concept and design; data acquisition; proof-reading. GC, JBA, JJGM, DA: funding; proof-reading. AF, AL, ALC, BGK, JWV: data acquisition, analysis and interpretation; drafting of the manuscript; proof-reading. JMB: study concept and design; analysis and interpretation of data; statistical analysis; drafting of the manuscript; funding.

Data availability statement

Due to the nature of this research, participants of this study did not agree for their data to be shared publicly, so data should remain confidential.

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Supplementary data

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