# Distal cholangiocarcinoma - from novel biomarkers to clinical management and outcome

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# Distal cholangiocarcinoma

## From novel biomarkers to clinical management and outcome

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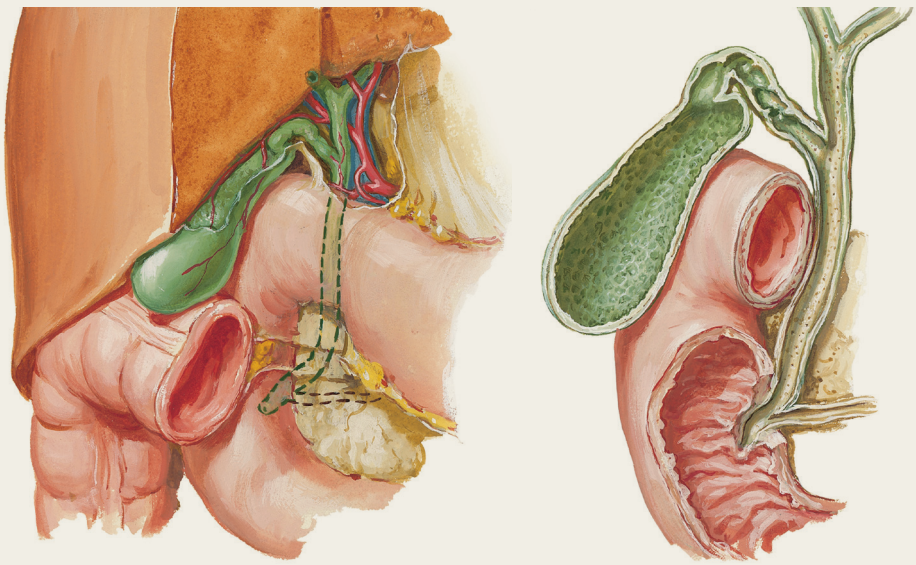

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## From novel biomarkers to clinical management and outcome

Johannes Byrling

LUND
UNIVERSITY

DOCTORAL DISSERTATION

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**Title and subtitle** Distal cholangiocarcinoma – from novel biomarkers to clinical management and outcome


**Abstract**

Cholangiocarcinoma is an aggressive malignacy arising from the biliary tree. Anatomical subtypes of cholangiocarcinoma differs in tumor biology and clinical management. Distal cholangiocarcinoma (dCCA) originates from the common bile duct. Radical resection is the only curative treatment, for dCCA it entails a pancreatoduodenectomy (Whipple procedure). Other periampullary cancers treated with pancreatoduodenectomy include pancreatic cancer (PC), ampullary cancer (AC) and duodenal cancer (DC). There is a high rate of recurrence after resection for dCCA. This thesis aimed to evaluate the clinical management of dCCA but also improve understanding of the tumor biology and identify novel biomarkers.

In paper I, the outcome and prognostic factors of patients treated with pancreatoduodenectomy for dCCA from 2008 through 2015 at Skane University Hospital were evaluated. We found the median survival to be 22 months which was worse than most previous studies. The presence of lymph node metastasis was confirmed as an important prognostic factor.

In paper II, the expression of secreted protein acidic and rich in cysteine (SPARC) in resected dCCA speciemns, paired lymph node metastases and normal bile ducts were evaluated using immunohistochemistry (IHC). We found SPARC to be expressed in the stromal compartment of dCCA in 80% of samples. Stromal expression was retained in 68% of lymph node metastases. There was no significant correlation between SPARC expression and survival.

In paper III, bottom-up mass spectrometry (MS) followed by verification using parallel reaction monitoring (PRM) was used to identify differentially expressed proteins between dCCA samples and normal bile ducts. Bioinformatic analysis highlighted stromal alterations in dCCA. Forty-six proteins were verified using PRM. Thrombospondin-2 (THBS2) was further validated using IHC. We found THBS2 to be upregulated in dCCA epithelial and stromal compartments. Stromal THBS2 expression was present in 72% of paired lymph node metastases. There was a correlation between stromal THBS2 expression and poor disease-free survival.

In paper IV, we studied the utility of serum THBS2 as a diagnostic biomarker for dCCA and PC. THBS2 levels were similar in dCCA and PC. THBS2 + CA 19–9 had an area under the curve of 0.92 in differentiating dCCA + PC from healthy donors. THBS2 did not provide utility is discriminating benign disease however, it was diagnosis dependent.

In paper V, we used Swedish National Registry for Pancreatic and Periampullary Cancer to study national trends in frequency of tumor origin, survival, histopathological evaluation and diagnostic accuracy for patients with periampullary cancers. We found PC diagnosis to be more common in unresected patients. Survival was better for AC and DC then dCCA or PC. Median survival was 33 months for dCCA. Regional differences in tumor origin frequency and histopathological outcomes were identified. Clinical rate of misdiagnosis was 15 % for PC and 23% for non-pancreatic periampullary cancers.



**Key words** distal cholangiocarcinoma, biliary tract cancer, periampullary cancer, pancreatoduodenectomy, lymph node metastasis, stroma, SPARC, THBS2, biomarker, mass spectrometry, diagnosis, histopathology

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*"In the realm of ideas, everything depends on enthusiasm. In the real world, all rests of perseverance."*

-Johann Wolfgang von Goethe

# Table of Contents

# List of papers and manuscripts

This thesis is based on the following papers. They will be referred to in the text by the corresponding roman numerals.

I. Byrling J, Andersson R, Sasor A, Lindell G, Ansari D, Nilsson J, Andersson B. Outcome and evaluation of prognostic factors after pancreaticoduodenectomy for distal cholangiocarcinoma. Annals of gastroenterology. 2017; 30:571-577.

II. Byrling J, Sasor A, Nilsson J, Said Hilmersson K, Andersson R, Andersson B. Expression of peritumoral SPARC during distal cholangiocarcinoma progression and correlation with outcome, Scandinavian Journal of Gastroenterology. 2020; 55:725-731

III. Byrling J, Kristl T, Hu D, Pla I, Sanchez A, Sasor A, Andersson R, Marko-Varga G, Andersson B. Mass spectrometry-based analysis of formalin-fixed, paraffin-embedded distal cholangiocarcinoma identifies stromal thrombospondin-2 as a potential prognostic marker. Journal of translational medicine. 2020; 18:343.

IV. Byrling J, Said Hilmersson K, Ansari D, Andersson R, Andersson B. Thrombospondin-2 as a diagnostic biomarker for distal cholangiocarcinoma and pancreatic ductal adenocarcinoma. Clin Transl Oncol. Epub 2021 Jul 29.

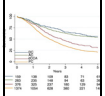
V. Byrling J, Ghazi S, Andersson B. National trends in tumor origin, diagnostic accuracy and histopathological evaluation in patients with periampullary cancer. Manuscript.

Related papers not included in this thesis:

Byrling J, Andersson B, Marko-Varga G, Andersson R. Cholangiocarcinoma – current classification and challenges towards personalized medicine. Scandinavian journal of gastroenterology. 2016; 51:641-3.

Byrling J, Sasor A, Nilsson J, Said Hilmersson K, Andersson R, Andersson B. Expression of fibroblast activation protein and the clinicopathological relevance in distal cholangiocarcinoma. Scandinavian journal of gastroenterology. 2020; 55:82-9.

# Thesis at a glance

| Paper | Objective | Methods | Results | Conclusions |
|---|---|---|---|---|
| I  | To evaluate survival and prognostic factors of surgically treated dCCA patients. | Clinicopathological data from patients treated with pancreatoduodenectomy for dCCA between 2008 and 2015 at SUS (N=54) were collected. | Median postoperative survival was 22.2 months. Lymph node metastasis was a negative prognostic factor. | Survival after surgery for dCCA was worse than previously presented. The presence of lymph node metastasis was prognostic. |
| II  | To investigate SPARC expression and prognostic impact in dCCA. | SPARC expression was evaluated by IHC in 59 resected dCCA samples and paired lymph node metastases. | SPARC expression was present in the stromal compartment of dCCA in 80% of patients and in 68% of lymph node metastases. There was no correlation with survival. | Stromal SPARC expression is common in dCCA and frequently retained during metastatic spread to lymph nodes. |
| III  | To identify dysregulated proteins in dCCA. | Mass spectrometry was used to analyze protein expression in resected dCCA specimens and normal bile ducts. THBS2 was validated using IHC. | Forty-six proteins were found to be dysregulated. Stromal THBS2 expression correlated with poor DFS. | Several dysregulated proteins not previously implicated in dCCA carcinogenesis were identified. |
| IV  | To analyze serum THBS2 expression and diagnostic biomarker properties in dCCA and PC. | THBS2 and CA 19-9 was measured in preoperative serum samples from patients with dCCA (N=51), PC (N=52), benign diseases (N=27) and healthy donors (N=52) using ELISA. | THBS2 levels were similar in dCCA and PC. THBS2+CA 19-9 had an AUC of 0.92 in differentiating dCCA+ PC from healthy donors. | Serum THBS2 together with CA 19-9 has potential as a diagnostic biomarker for dCCA and PC. |
| V  | To evaluate national trends in tumor origin, survival, histopathological evaluation and diagnostic accuracy for patients with periampullary cancers in Sweden. | Data from patients diagnosed with periampullary cancer from 2010-2019 (N=9143) was retrieved from the Swedish National Registry for Pancreatic and Periampullary Cancer. | PC diagnosis was more common in palliative patients. Survival was better for DC and AC patients than dCCA or PC patients. Regional differences in histopathological outcomes were found. Rate of misdiagnosis was 15 and 23% for PC and non-pancreatic periampullary cancers respectively. | In palliative patients, non-pancreatic periampullary cancers are likely underdiagnosed. There is a need for further harmonization of histopathological evaluation methodology. The clinical rate of misdiagnosis for periampullary cancers needs to be considered. |

Abbreviations: AC; ampullary cancer. dCCA; distal cholangiocarcinoma. DC; duodenal cancer. DFS; disease free survival. ELISA; enzyme-linked immunosorbent assay. IHC; immunohistochemistry. PC; pancreatic cancer. SPARC; secreted protein acidic and rich in cysteine. SUS; Skane University Hospital. THBS2; thrombospondin-2.

# Populärvetenskaplig sammanfattning

Gallvägscancer är en ovanlig cancersjukdom, men förödande för dem som drabbas. Radikal kirurgi är det enda botande behandlingsalternativet, dessvärre har en majoritet av patienterna ej botbar sjukdom vid diagnostillfället. Det finns undergrupper av gallvägscancer som baseras på den anatomiska utgångspunkten i gallträdet, dessa undergrupper kräver olika kirurgiskt omhändertagande men uppvisar även skillnader i tumörbiologi. Distal gallvägscancer uppkommer från den gemensamma gallgången. Den gemensamma gallgången sträcker sig från infästningen av gallblåsegången, genom bukspottskörtelns huvud ner till Vaters ampull. Vaters Ampull mynnar därefter i tolvfingertarmen. Det finns få studier på de olika undergrupperna av gallvägscancer, framförallt på distal gallvägscancer. I min doktorsavhandling har jag fokuserat på distal gallvägscancer och forskningen har syftat både till att förbättra det kliniska omhändertagandet men också att bättre förstå tumörbiologin och identifiera nya molekylära markörer för sjukdomen.

För de patienter som har en resekabel tumör utan fjärrspridning rekommenderas kirurgi med så kallad pankreatoduodenektomi, även känt som Whipples operation. Det är en stor operation och även stor risk för återfall efter kirurgi. Tidigare studier har visat stor variation i återfallsrisk och överlevnad efter operation. I min första studie analyserade vi utfallet för patienter som genomgått pankreatoduodenektomi för distal gallvägscancer vid Skånes Universitetssjukhus mellan 2008 och 2015 (54 patienter). Medianöverlevnaden (tiden till hälften av patienterna har avlidit) var 22 månader, detta var lägre än i de flesta tidigare studier. Förekomst av tumörspridning i bortopererade lymfkörtlar bekräftades som en viktig negativ prognostisk faktor.

Utöver cancercellerna består en tumör även av flera andra celltyper, exempelvis bindvävsceller och immunförsvarsceller. I tumören ingår även stödjevävnad. Vid distal gallvägscancer har man funnit att stora delar av tumörmassan utgörs av ärrbildningsliknande stödjevävnad. Denna ”mikromiljö” har visats samverka med cancercellerna och bidra till cancerns utveckling. Proteinet SPARC, som framförallt återfinns i stödjevävnad, har visats kunna bidra till cancerutveckling och korrelera med dålig prognos i flera cancerformer. I den andra studien i avhandlingen analyserade vi uttrycket av SPARC i bortopererade distala gallvägscancrar (59 patienter). Vi fann att SPARC inte uttrycktes i normala gallvägar men uttrycktes i stödjevävnaden i distal gallvägscancer i 80% av tumörerna. SPARC uttrycktes även av stödjevävnad kring cancerspridning i bortopererade lymfkörtlar hos 68%. SPARC uttryck var inte signifikant korrelerat med överlevnad. Resultaten talar för att SPARC har en roll under cancerutvecklingen och spridningsprocessen vid distal gallvägscancer.

Nästan alla funktioner i kroppen styrs av proteiner. Vid cancerutveckling sker skador i arvsmassan. Detta leder till ändrat uttryck och funktion hos proteiner, vilket får cancercellerna att ändra sitt beteende jämfört med en frisk cell. Masspektrometri

är en metod för att studera proteinuttryck i biologiska system och kan användas för att analysera ett stort antal proteiner samtidigt. I studie III använde vi masspektrometri för att analysera proteinuttryck i operationspreparat och jämföra distala gallvägscancrar och friska gallvägar. Vi identifierade 46 protein där nivåerna skiljde sig åt, flera av dessa proteiner uttrycks av bindvävsceller eller i stödjevävnad. Vi valde att studera proteinet THBS2 vidare och fann att det uttrycktes av både tumörceller och stödjevävnad i distala kolangiocarcinom men ej i normala gallvägar. Uttryck av THBS2 i tumörstödjevävnad korrelerade med högre risk för återfall. Liksom för SPARC sågs THBS2 uttryck frekvent i stödjevävnad kring cancerspridning i bortopererade lymfkörtlar. Huvudresultatet av studien var identifikationen av flertal protein utan tidigare koppling till distal gallvägscancer som kan studeras vidare. Resultaten talar även för att THBS2 har en roll vid distal gallvägscancers utveckling och korrelerar med sämre överlevnad.

THBS2 har tidigare studerats som en diagnostisk markör för bukspottkörtelcancer. Distal gallvägscancer och bukspottkörtelcancer har flera biologiska likheter. Vi valde därför att studera huruvida THBS2 i blodprover (51 patienter) kunde användas för att diagnosticera distal gallvägscancer. Vi fann att nivåerna av THBS2 var förhöjda i distal gallvägscancer liknade vid bukspottkörtelcancer. THBS2 kunde tillsammans med en redan använd markör (CA 19–9) med god precision användas för att skilja cancerpatienter från friska individer, inklusive patienter med tidig sjukdom. Kombinationen presterade sämre på att skilja godartade sjukdomar från cancer, men det såg ut att vara diagnosberoende. THBS2 har potential att diagnosticera distal gallvägscancer och bukspottkörtelcancer. Ytterligare större studier inkluderande metodoptimering, kartläggning av nivåerna vid olika godartade tillstånd och andra cancerformer krävs innan THBS2 kan användas i kliniken.

En förklaring till skillnaden mellan olika studier avseende förekomst och utfall för patienter med distal gallvägscancer är den metodik som används för att undersöka operationspreparat efter pankreatoduodenektomi. Under det sista årtiondet har en standardiserad undersökningsmetod införts gradvis i Sverige. I den sista studien i avhandlingen användes data från det nationella kvalitetsregistret för tumörer i pankreas och periampullärt. Periampullär cancer är ett samlingsbegrepp för tumörer som uppkommer kring Vaters ampull och inbegriper bukspottkörtelcancer, distal gallvägscancer, ampullär cancer samt tolvfingertarmcancer. De behandlas alla kirurgiskt med pankreatoduodenektomi. I studie V användes registerdata för att jämföra andelen av de olika tumörerna samt skillnader över tid och mellan regioner i Sverige. Vi fann att det var vanligare att patienter som ej kunde opereras diagnosticerades med bukspottkörtelcancer. Standardiseringen korrelerade med att fler tumörer bedömdes som bukspottkörtelcancer samt att andelen med mikroskopiskt icke radikal resektion och förekomst av cancerspridning till lymfkörtlar ökade. Vi fann omotiverat stora skillnader mellan olika regioner avseende bedömt tumörursprung, icke radikal resektion och lymfkörtelspridning. Överlevnaden nationellt var betydligt bättre för tolvfingertarm- och ampullär cancer

än för distal gallvägscancer och bukspottkörtelcancer. Medianöverlevnaden för distal gallvägscancer var 33 månader, vilket var bättre än i första studien. Slutligen fann vi när vi jämförde diagnosen före och efter operation att 15% av patienterna med bukspottkörtelcancer hade annan periampullärcancer diagnos före operation och 23% av patienterna med distal gallvägscancer, ampullär- eller tolvfingertarmscancer hade bukspottkörtelcancerdiagnos före operation. Studien påtalar vikten av fortsatt arbete för att harmonisera undersökningsmetodiken av pankreatoduodenektomipreparat i Sverige (och sannolikt resten av världen) samt att man bör ta hänsyn till osäkerheten i diagnostiken, framförallt om preoperativ cytostatikabehandling övervägs då preparatval skiljer sig mellan de olika cancerformerna.

Min förhoppning med avhandlingen är att bidra med några pusselbitar i det stora pussel som är förståelsen av tumörbiologin vid distal gallvägscancer. Det är även att de kliniska delarna kan användas för att förbättra dagens omhändertagande. Jag hoppas på så sätt att avhandlingen kan bidra till att hjälpa de patienter som drabbas av denna svåra sjukdom.

# Abbreviations

| | |
|---|---|
| AC | Ampullary Cancer |
| AJCC | American Joint Committee on Cancer |
| AUC | Area under the curve |
| BilIN | Biliary intraepithelial neoplasm |
| BTC | Biliary tract cancer |
| CA 19–9 | Carbohydrate antigen 19–9 |
| CCA | Cholangiocarcinoma |
| CT | Computed tomography |
| CPH | Cox proportional hazards regression |
| dCCA | Distal cholangiocarcinoma |
| DC | Duodenal cancer |
| DEPs | Differentially expressed proteins |
| DFS | Disease-free survival |
| eCCA | Extrahepatic cholangiocarcinoma |
| ELISA | Enzyme-linked immunosorbent assay |
| ERCP | Endoscopic retrograde cholangiopancreatography |
| EUS-FNA | Endoscopic ultrasound-guided fine-needle aspiration |
| ECM | Extracellular matrix |
| FDA | Food and drug administration |
| FGFR | Fibroblast growth factor receptor |
| FISH | Fluorescence in situ hybridization |
| FFPE | Formalin-fixed paraffin-embedded |
| FOLFOX | 5-fluorouracil + leucovorin and oxaliplatin |
| GBC | Gallbladder cancer |
| GEMCIS | Gemcitabine cisplatin |
| GEMOX | Gemcitabine oxaliplatin |
| GO | Gene ontology |
| HR | Hazard ratio |

| | |
|---|---|
| iCCA | Intrahepatic cholangiocarcinoma |
| IDH1 | Isocitrate dehydrogenase 1 |
| IPNB | Intraductal papillary neoplasm of the bile duct |
| IHC | Immunohistochemistry |
| KM | Kaplan-Meier |
| LC | Liquid chromatography |
| LC-MS/MS | Liquid phase separation on-line with tandem mass spectrometry |
| MMR | Mismatch repair |
| MRCP | Magnetic resonance cholangiopancreatography |
| MS | Mass spectrometry |
| NCCN | National comprehensive cancer network |
| NTRK | Neurotrophic receptor tyrosine kinase |
| OS | Overall survival |
| PBD | Preoperative biliary drainage |
| PC | Pancreatic cancer |
| pCCA | Perihilar cholangiocarcinoma |
| PET | Positron emission tomography |
| PRM | Parallel reaction monitoring |
| PSC | Primary sclerosing cholangitis |
| PTC | Percutaneous transhepatic cholangiography |
| ROC | Receiver operating characteristics |
| R1 resection | Non-radical resection |
| SPARC | Secreted protein acidic and rich in cysteine |
| SUS | Skane University Hospital |
| THBS2 | Thrombospondin-2 |
| 5-FU | 5-fluorouracil + leucovorin |

# Introduction

## Anatomy and physiology

The primary role of the biliary tract (Figure 1) is the transportation, modulation and storage of bile. Bile flows from the liver to the duodenum. The biliary tract originates at the microscopic interaction surface between hepatocytes and cholangiocytes named the canals of Hering. The small bile ductules in the liver then merge gradually to interlobular, septal and segmental ducts. The segmental ducts, which drain the corresponding liver segments join to form the right and left hepatic ducts. The confluence of the right and left hepatic duct occurs at the liver hilum and the common hepatic duct is formed. The cystic duct originates from the gallbladder, where bile is stored and concentrated, then join the common hepatic duct to form the common bile duct. The common bile ducts transverses the pancreatic head and join with the main pancreatic duct leading to the ampulla of Vater which, in turn, opens into the duodenum (1). In the ampullary region, a smooth muscle sphincter called the sphincter of Oddi surround the terminal common bile- and pancreatic ducts.

The main functions of bile are the excretion of waste products and the digestion of fat. Bile components include bile acids, bilirubin, phospholipids, steroids, electrolytes, endogenous and exogenous lipophilic compounds (2). Approximately 600 ml of bile is produced daily in humans (3). The green to yellow colour typical of bile is contributed to the pigment bilirubin which is a by-product from the degradation of red blood cells. Under physiological conditions, the majority of bile is stored and concentrated in the gallbladder and a coordinated release of bile into the duodenum is performed in response to food intake. The coordination is mediated by hormones such as cholecystokinin which induce gallbladder contraction and sphincter of Oddi relaxation (3).

### Nomenclature

Cholangiocarcinoma (CCA) is a malignancy arising from the epithelial lining of the biliary tree. Anatomically, CCA is subtyped based on anatomical location as intrahepatic cholangiocarcinoma (iCCA) or extrahepatic cholangiocarcinoma (eCCA). ICCA arises above the second order bile ducts in the liver. ECCA is subclassified as perihilar cholangiocarcinoma (pCCA), sometimes referred to as

Klatskin tumor, which arises between the second order bile ducts and the insertion of the cystic duct or distal cholangiocarcinoma (dCCA) which arises along the common bile duct from the cystic insertion to the ampulla of Vater (Figure 1) (4). Approximately 10-20% of CCAs are iCCA, 50-60% pCCA and 20-30% dCCA (5). Biliary tract cancer (BTC) encompasses all subtypes of cholangiocarcinoma and additionally gallbladder cancer (GBC). Furthermore, cancers originating in the anatomical proximity of the ampulla of Vater are referred to as periampullary cancers and includes pancreatic cancer (PC), dCCA, ampullary cancer (AC) and duodenal cancer (DC) (6).

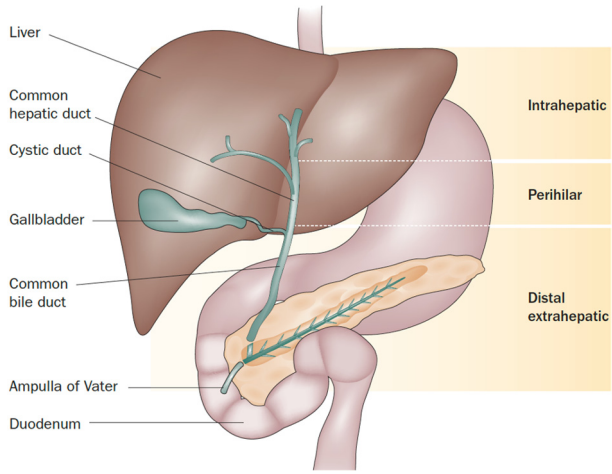



**Figure 1.** Anatomy of the biliary tract. Anatomical origins of cholangiocarcinoma subtypes are highlighted. Reproduced from Blechacz et al. (7) with permission from Springer Nature.

# Epidemiology and risk factors

CCA accounts for approximately 3% of all gastrointestinal cancers (8) and has a slight male predominance. CCA is most commonly diagnosed in the seventh decade of life and is uncommon before the fifth decade of life (9). The incidence trends and risk factors depend on subtype and additionally, a large geographical variation is seen in the incidence of CCA. The incidence is low in western countries (0.35-2 per 100 000 person years) with substantially higher incidences (>14 per 100 000 person years) in some eastern countries (10). The incidence of iCCA has been increasing substantially in western countries during the last decades with the incidence of eCCA unchanged or slightly decreased (10). The epidemiological trends in CCA need to be interpreted with caution given problems with coding and classification of CCA subtypes. PCCA has been classified as intrahepatic in previous versions of the international classification of disease (ICD), with no separate coding for dCCA

prior to the 11th version (not yet implemented) (11). Epidemiological data for dCCA only is limited. In a national cohort from the Netherlands, the incidence of dCCA was 0.55-0.9 per 100 000 person years from 2009 through 2016 (12).

The majority of CCA cases in western countries arise in the absence of identifiable risk factors. However, patients with primary sclerosing cholangitis (PSC) have a substantially elevated lifetime risk of CCA development and the presence of benign biliary strictures which presents a diagnostic challenge (13). The highest incidence rate of CCA in the world (84 per 100 000 person-years) is seen in regions of Thailand where the liver flukes *Opisthorchis viverrini* and *Clonorchis sinensis* are endemic (5). Other risk factors associated with CCA include biliary diseases (bile duct cysts, hepatolithiasis, cholecystolithiasis, choledocholithiasis), liver diseases (cirrhosis, hepatitis, hemochromatosis), digestive diseases (inflammatory bowel disease, chronic pancreatitis, peptic ulcers), metabolic disorders (diabetes mellitus type 2, obesity, non-alcoholic fatty liver disease/steatohepatitis), lifestyle/environmental exposure (smoking, alcohol consumption, thorotrast, asbestos, 1,2-dichloropropane) as well as some genetic polymorphisms (Table 1) (11).

# Pathobiology

## Pathological classification

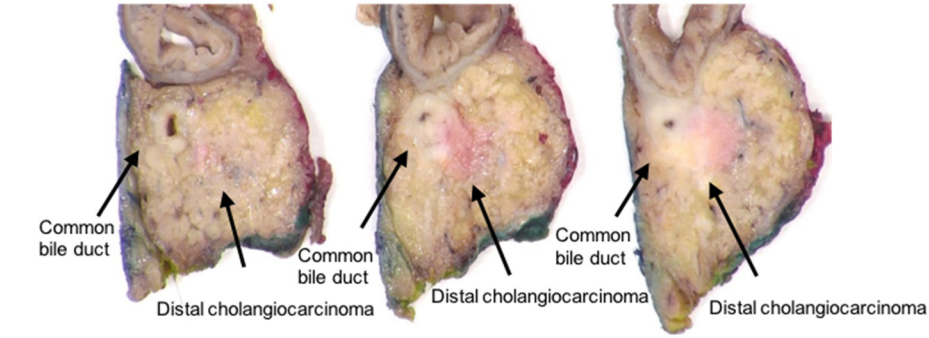
DCCA typically presents as a nodular sclerosing or flat lesion, or less common as an intraductal growing papillary tumor, tubular lesion or superficial spreading tumor (14). The absolute majority are conventional adenocarcinomas (Figure 2). Rare histological variants including squamous or adenosquamous carcinoma, mucinous/signet ring, clear cell, undifferentiated and lymphoepithelial can also occur (14). Periampullary adenocarcinomas can present an intestinal or pancreaticobiliary phenotype, a distinction with prognostic/predictive importance which is done primarily in AC (15, 16). The majority of dCCAs have a pancreatobiliary phenotype. However, approximately 10% have an intestinal phenotype (17). The nodular sclerosing subtype of dCCA is derived from the precursor lesion biliary intraepithelial neoplasia (BilIN), and the rare papillary subtype from intraductal papillary neoplasm of the bile duct (IPNB) (18). BilIN is classified according to the world health organization (19) as BilIN-1 (low-grade dysplasia), BilIN-2 (intermediate grade dysplasia) and BilIN-3 (high-grade dysplasia) (19). The gradual development from low to high grade dysplasia and eventually invasive cancer is believed to represent sequentially acquired genetic alterations in analogy to the well characterized dysplasia-carcinoma sequence in

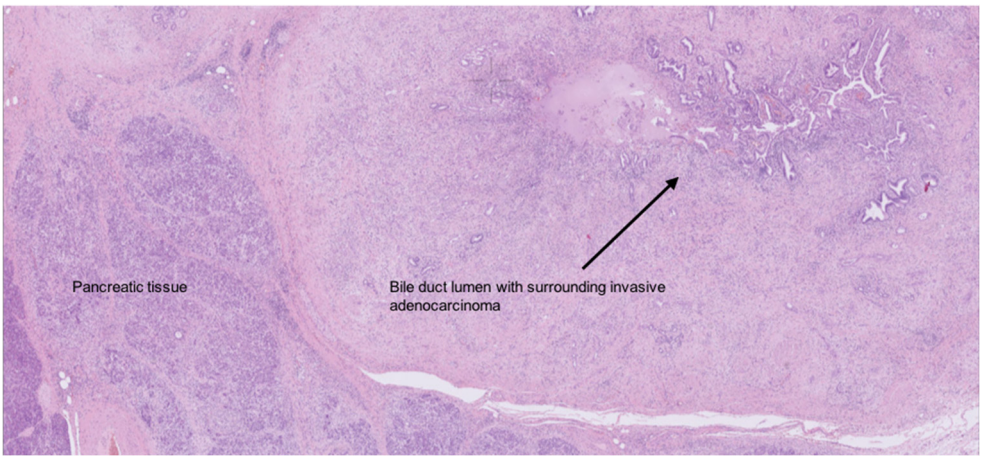
PC (20). The specific sequence of genetic alterations during the development from BilIN to invasive dCCA is poorly characterized (21).

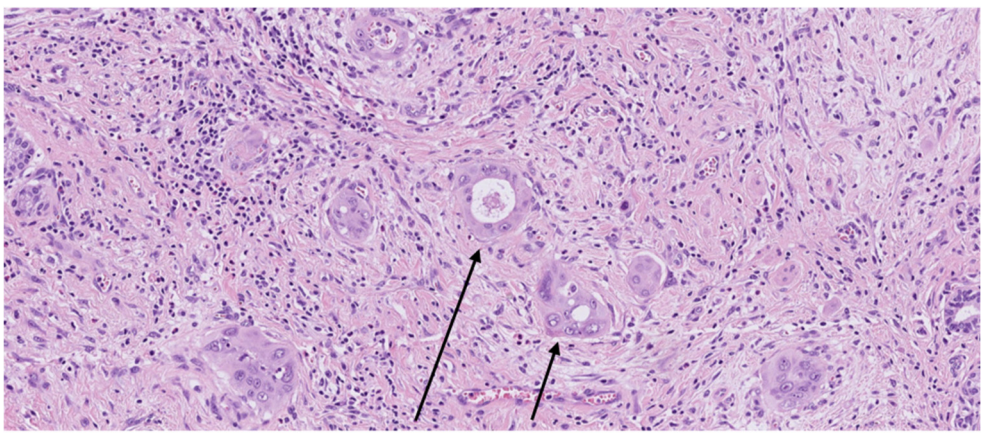
**Table 1.** Risk factors for intra- and extrahepatic cholangiocarcinoma. Reproduced from Kahn et al. (11) with permission from John Wiley and Sons.

| Risk factor | Strenght of the association in iCCA | Strenght of the association in eCCA |
|---|---|---|
| Bile duct cysts | ++++ | ++++ |
| Caroli's disease | ++++ | ++++ |
| PSC/Cholangitis | ++++a | ++++a |
| Hepatolithiasis | +++ | No association |
| Cholelithiasis/ choledocholithiasis | ++/+++ | ++++ |
| Cirrhosis | +++/++++ | ++/+++ |
| HBV | ++/+++ | + |
| HCV | ++/+++ | +/++ |
| Hemochromatosis ++ | ++ | No association |
| Wilson's disease | No association | No association |
| IBD | ++ | +/++ |
| Chronic pancreatitis | ++ | +++ |
| Duodenal/gastric ulcer | + | + |
| Opisthorchis viverrini | +++a | +++a |
| Clonorchis sinensis | +++a | +++a |
| Diabetes type II | + | + |
| Obesity | +a | +a |
| NAFLD/NASH | +++ | ++ |
| Alcohol | ++ | No association |
| Cigarette smoking | + | + |
| Thorotrast | ++++a | ++++a |
| 1,2-dichloropropane | ++++a | ++++a |
| Asbestos +++ +/++ | +++ | +/++ |

+, weak/modest association (OR: 1-1.7); ++, moderate association (OR:1.7-3); +++, strong association (OR: 3-8); ++++, very strong association (OR > 8). a, Available studies did not distinguish between iCCA and eCCA. Abbreviations: ECCA; extrahepatic cholangiocarcinoma.HBV; hepatitis B virus. HCV; hepatitis C virus. IBD; inflammatory bowel disease. ICCA; intrahepatic cholangiocarcinoma. NASH; non-alcoholic steatohepatitis. NAFLD; non-alcoholic fatty liver disease. PSC; primary sclerosing cholangitis.



Nests of cancer cells surrounded by desmoplastic stroma

**Figure 2.** Macroscopic picture of a distal cholangiocarcinoma from serial axial sections through the pancreatic head and microscopic pictures from the same patient showing a poorly differentiated adenocarcinoma originating from the common bile duct with an ample desmoplastic reaction. Image courtesy of Dr Agata Sasor.

## Molecular characterization

Sequencing studies have elucidated the mutational panorama of CCA. Initial sequencing studies analysed mixed or iCCA cohorts. Clear differences in the mutational spectrum of CCA subtypes were identified (22, 23). The most commonly mutated genes identified in eCCA are KRAS, TP53, ARID1A and SMAD4 (24). Mutations in potentially druggable targets such as EGFR/HER2, PIK3CA, BRCA, ELF3, NTRK, BRAF and mismatch repair (MMR) proteins have been identified in approximately 25% of eCCA patients (24, 25). Few studies have presented genomic data for the dCCA subtype. In 42 dCCA patients subjected to whole exome sequencing the most frequent mutations were TP53, KRAS, SMAD4, and CDKN2A (17). In another study using targeted sequencing of 30 dCCA patients; TP53, KRAS, CDKN21, SMARCA4 and SMAD4 were the most frequently mutated genes (26). Clustering analysis of transcriptomic data has identified 4 molecular subclasses of eCCA. These subclasses serve as a framework for understanding the molecular pathogenesis and also helps identify potential therapeutical targets. The proliferation class is characterized by enrichment of MYC signaling, HER2 overexpression/amplification and mTOR pathway activation. The immune class is characterized by immune infiltrates and overexpression of immune checkpoint molecules. Further, the mesenchymal class is characterized by expression of epithelial-mesenchymal transition markers and TGF-ß signaling. Finally, the metabolic class is characterized by overexpression of genes involved in bile- and fatty acid signaling and show hepatocyte features (24). In dCCA, the proliferation class was the most frequent (58%), followed by the immune type (12%), the mesenchymal type (11%) and the metabolic type (4%) (Figure 3).

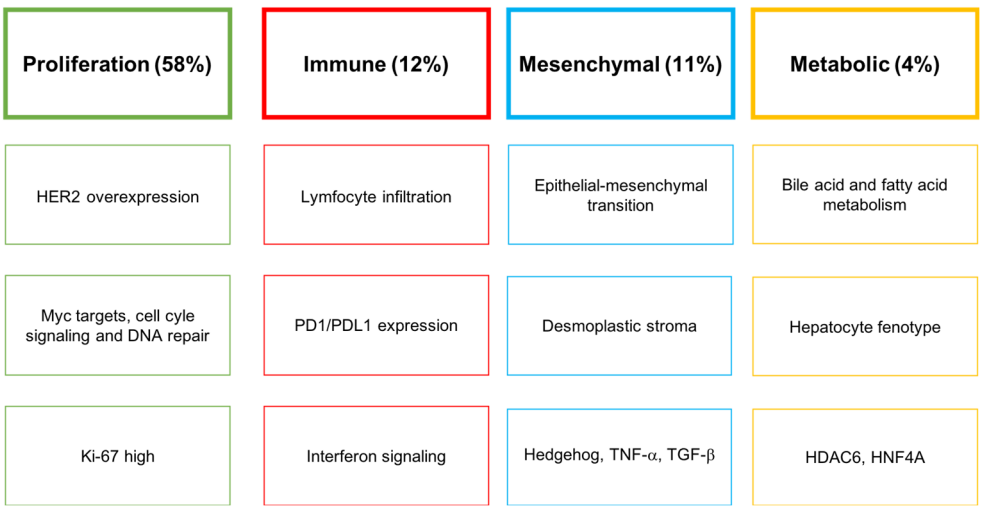



**Figure 3**. Molecular features of eCCA subclasses adopted from Montal et al. (24). The frequency of subclasses in dCCA is presented.

### Tumor microenvironment

During recent decades, the major importance of the interplay between cancerous cells and components of the tumor microenvironment such as fibroblasts, endothelial cells and immune cell populations have been elucidated (27). CCA is characterized by an extensive stromal reaction, often with extensive fibrosis which has been shown to play an important role during CCA carcinogenesis (28). Stromal components in the tumor microenvironment has been shown to contribute to CCA proliferation, dissemination and development of treatment resistance (29, 30). One study found 58% of dCCA samples to have a tumor-stroma percentage (percentage stroma/tumor cells) above 70% with a higher stroma proportion correlated with worse survival (31).

## Diagnosis and prognosis

### Clinical presentation and diagnostic workup

The most common presentation of dCCA is obstructive jaundice with or without cholangitis. Other more unspecific symptoms typically indicative of advanced disease are abdominal pain, weight loss, malaise, night sweats, loss of appetite and fatigue (32). Routine chemistry can show elevated liver enzymes with a cholestatic pattern. Initial workup in the case of jaundice include ultrasound and in the absence of a benign explanation for obstruction (gallstone disease) a computed tomography (CT) scan is performed. The CT scan can characterize the tumor and surrounding structures, and additionally identify distant metastases. A dominant biliary stricture without associated tumor is the most common finding. Some patients present with a pancreatic head tumor (7). Further, non-invasive characterization of the biliary tree in the case of unclear diagnosis or surveillance of patients with PSC can be performed using magnetic resonance cholangiopancreatography (MRCP) (33). Positron emission tomography (PET) is not routinely used due to subpar diagnostic performance for the primary tumor but can be considered for improved staging in cases of indeterminate lymph node involvement or suspicion of other metastatic locations (34).

The majority of dCCA patients require alleviation of biliary obstruction with preoperative biliary drainage (PBD) prior to surgery. Notably, PBD compared to upfront surgery is associated with increased risk of complications (35). However, waiting times, very high bilirubin levels and cholangitis (36) are reasons to perform PBD prior to surgery and in clinical routine it’s performed in most patients. Endoscopic retrograde cholangiopancreatography (ERCP) is the preferred method with percutaneous transhepatic cholangiography (PTC) as a secondary option (37). During intervention, brush cytology can be obtained to confirm malignancy.

Cytology suffers from limited sensitivity (38), although it has been substantially improved by the use of complementary fluorescence in situ hybridization (FISH). FISH detects specific chromosomal aberrations associated with malignancy (39). In strictures with suspected malignancy and negative cytology/FISH, alternatives such as cholangioscopy with targeted biopsies using the Spyglass system (40) or endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) (41, 42) are available. Notably, in patients with a clear suspicion of malignancy such as a solid pancreatic head mass which is upfront resectable, biopsy confirmation is not required before proceeding to surgery (43). In patients with unresectable/metastatic disease, percutaneous biopsies from the primary tumor or available metastases are options to histopathologically verify the cancer diagnosis prior to palliative therapy.

## Biomarkers

The National Institutes of Health has defined a biomarker as *"a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic response to a therapeutic intervention"* (44). A biomarker can be diagnostic (used to detect a condition), prognostic (used to indicate the likelihood of an event) or predictive (used to predict response to a treatment). Serum Carbohydrate 19–9 (CA 19–9) is the primary diagnostic biomarker used for CCA and PC. Obstructive jaundice regardless of diagnosis can cause CA 19–9 elevation and 5-10% in a Caucasian population do not genetically express CA 19–9 (45, 46) which limit utility. The diagnostic sensitivity and specificity are approximately 72% and 84% respectively in CCA (47). The low diagnostic performance, in particularly at detecting early-stage disease means CA 19–9 isn't suitable for screening purposes and the primary use is in monitoring disease progression (48). The colorectal marker carcinoembryonic antigen (CEA) and ovarian cancer marker carbohydrate antigen 125 has lower diagnostic performance than CA 19–9 for CCA diagnosis and monitoring and are infrequently used clinically (49).

Large efforts have been directed towards novel diagnostic biomarkers and prognostic biomarkers for CCA, but none have reached the clinic yet (5). Most evaluated diagnostic candidates have been blood or bile markers. Cell-free DNA, circulating tumor cells, microRNA, extracellular vesicles, proteomic and metabolomic biomarkers/biomarker panels have shown promise (50). Selected potential diagnostic protein biomarkers include MMP-7 (51), osteopontin (52), interleukin-6 (53) and CYFRA (21-1) (54). A meta-analysis of prognostic biomarkers evaluated by immunohistochemistry (IHC) in patients with resected CCA included 4126 patients and found expression of fascin, EGFR, MUC1, MUC4 and p27 to associate with prognosis (55).

## Staging and survival

As with most cancers the most important prognostic factor is the stage. The TNM system describes the extent of disease of the primary tumor (T), regional lymph nodes (N) and distant metastasis (M) which are then combined to a prognostic stage grouping which also dictate treatment alternatives. With the introduction of the 7th version of the American Joint Committee on Cancer (AJCC) staging system in 2010, the first separate TNM staging criteria for dCCA was introduced. In 2018 the 8th edition of the AJCC staging manual was introduced which changed the T stages to dept of invasion rather than invasion of specific bile duct structures as well as an expanded N stage category (Table 2). Regional lymph node stations for dCCA include nodes located at the superior/splenic artery (station 11), anterior and posterior pancreatoduodenal nodes (station 17 and 13), nodes in the hepatoduodenal ligament (station 12), upper mesenteric nodes (station 14), common hepatic artery (station 8) and coeliac nodes (station 9) (56). Common locations for distant metastasis are liver, non-regional lymph nodes, peritoneum and lungs (57).

The lack of early symptoms and aggressive tumor biology translate to a majority of patients with CCA presenting at an advanced disease stage. Approximately one third of patients are considered for surgical treatment which is the only treatment with curative potential (58). Locally advanced disease (T4) or spread beyond regional lymph nodes disqualify surgical treatment of dCCA

The survival for patients with locally advanced or metastatic dCCA at presentation is dismal. In a national cohort from the Netherlands the median survival for locally advanced unresectable dCCA was 6.7 months with a 5-year survival rate of 3% and for metastatic dCCA 3.6 months with a 5-year survival rate of 0% (12). The rate of recurrence after surgery with curative intent for dCCA is high with an overall median postoperative survival of 33 months and a 5-year survival rate ranging from 13% to 54% according to a meta-analysis of 3258 dCCA patients from 2017 (59). There is a substantial heterogeneity in the reported survival after resection for dCCA in the literature. The majority of studies have historically been from Asian high-volume centers. Postoperative median survival in selected larger western cohorts have ranged from 18 to 40 months (12, 60-65).

**Table 2.** The TNM staging system for dCCA according to the American Joint Committee on Cancer (AJCC).

| | AJCC 7th edition | AJCC 8th edition |
|---|---|---|
| **Primary tumor (T)** | | |
| TX | Primary tumor cannot be assessed | Primary tumor cannot be assessed |
| T0 | No evidence of primary tumor | No evidence of primary tumor |
| Tis | Carcinoma in situ | Carcinoma in situ |
| T1 | Tumor confined to the bile duct histologically | Tumor invades the bile duct wall with a depth less than 5 mm |
| T2 | Tumor invades beyond the wall of the bile duct | Tumor invades the bile duct wall with a depth of 5-12 mm |
| T3 | Tumor invades the gallbladder, pancreas, duodenum, or other adjacent organs without involvement of the celiac axis, or the superior mesenteric artery | Tumor invades the bile duct wall with a depth greater than 12 mm |
| T4 | Tumor involves the celiac axis, or the superior mesenteric artery | Tumor invades the celiac axis, superior mesenteric artery or common hepatic artery |
| **Regional lymph nodes (N)** | | |
| NX | Regional lymph nodes cannot be assessed | Regional lymph nodes cannot be assessed |
| N0 | No regional lymph node metastasis | No regional lymph node metastasis |
| N1 | Regional lymph node metastasis | Metastasis in one to three regional lymph nodes |
| N2 | Not applicable | Metastasis in four or more regional lymph nodes |
| **Distant metastasis (M)** | | |
| M0 | No distant metastasis | No distant metastasis |
| M1 | Distant metastasis | Distant metastasis |
| **Stage grouping** | | |
| | 0: TisN0M0 | 0: TisN0M0 |
| | IA: T1N0M0 | I: T1N0M0 |
| | IB: T2N0M0 | IIA: T1N1M0 or T2N0M0 |
| | IIA: T3N0M0<br>IIB: T1-3, N1M0 | IIB: T2N1M0 or T3,N0-1, M0<br>IIIA: T1-3, N2M0 |
| | III: T4, any N, M0 | IIIB: T4, N0-2, M0 |
| | IV: Any T, any N, M1 | IV: IV: Any T, any N, M1 |

## Clinical prognostic factors

The overall most influential prognostic factors for patients that undergo resection for dCCA are N stage and presence of non-radical resection (R1 resection) (10). Additional routine histopathological variables associated with prognosis include T stage, histologic grade (well-differentiated, moderately differentiated, poorly differentiated), presence of perineural invasion, lymphatic invasion, venous invasion, pancreatic and peripancreatic fat invasion (59). Preoperative levels of biomarkers CA 19–9 and CEA and postoperative non-normalization has been associated with prognosis (66). A prognostic nomogram based on the preoperative

variable's neutrophil to lymphocyte ratio, peak total bilirubin and major vascular resection has been developed to predict early recurrence (67). Other selected clinical variables that's been associated with prognosis in resected dCCA patients include PBD (68), postoperative complications (69) and blood transfusions (68, 70).

### Impact of histopathological evaluation

There is a large discrepancy in the literature regarding the relative frequency of periampullary cancers, R1 resection rate and nodal involvement. The methodology of histopathological evaluation has been shown to influence these assessments (71, 72). Differences in histopathological evaluation could contribute to the heterogenous study results with regard to frequency of dCCA, survival estimates and prognostic factors. The use of standardized histopathological methodology has been shown to increase the rate of non-pancreatic tumor origin, R1 resection and detected lymph node metastases substantially (73). Two main approaches have been developed; axial sectioning as described by Verbeke et al. (74) and bivalving as described by Adsay et al. (75). Both techniques give comparable outcomes (76). In Sweden a standardized protocol based on axial sectioning has been implemented during the 2010s (56). Given that different adjuvant and palliative oncological treatments are used for the individual periampullary cancers, correct identification of tumor origin is of outmost importance.

## Treatment

### Surgery

For patients with dCCA and other periampullary cancers, the surgical procedure of choice entails a pancreatoduodenectomy, also called the Whipple procedure (77). During the procedure the gallbladder, common bile duct, pancreatic head, duodenum and the distal stomach is removed *en bloc*. The procedure can also be done as a pylorus-preserving resection, which show similar outcome (78). Reconstruction is done with a hepaticojejunostomy, gastroenterostomy and a pancreaticogastrostomy or pancreaticojejunostomy. Some reconstructions also include an entero-entero anastomosis. Venous resection and reconstruction can be performed at high volume centers without increased mortality or morbidity (79). Arterial resection is associated with a high mortality and morbidity but considered in highly selected patients (80). Pancreatoduodenectomy is one of the most extensive surgical procedures used in modern medicine and has historically been associated with a substantial postoperative mortality. Advances in surgical technique and perioperative care have improved the results. The 30-day and 90-day

postoperative mortality after pancreatoduodenectomy in Sweden was 1.5% and 3.5% respectively during the 2010s (81). The postoperative morbidity is still substantial with complications grade 3 or higher according to the Clavien-Dindo classification (82) occurring in approximately 15% of patients (81). In addition to common post-operative complications such as wound infection, thrombosis etc, procedure specific complication include leakage from the pancreatic anastomosis (postoperative pancreatic fistula) (83), delayed gastric emptying (84) and postpancreatectomy hemorrhage (85). DCCA compared to PC has been associated with a higher frequency of pancreatic fistula, which in turn has been associated with worse survival (86). This is presumably due to a higher frequency of soft pancreatic tissue in dCCA compared with PC (87, 88).

## Palliative oncological treatment

For the majority of patients with unresectable/metastatic disease palliative chemotherapy is the only treatment option. During the 1990s and 2000s several, mostly smaller, non-randomized trials evaluated the role of chemotherapy in advanced BTC. The primary compounds with evidence of efficacy were the antimetabolites gemcitabine and 5-fluorouracil/leucovorin (5-FU) (leucovorin is a reduced folate which enhances 5-fluorouracil effectiveness) as well as the platinum compounds cisplatin and oxaliplatin (89). In 2010 the randomized ABC-02 trial, which compared first-line gemcitabine and cisplatin (GEMCIS) with gemcitabine singlet established a new standard of care. Treatment with the combination chemotherapy resulted in a significantly improved overall survival (OS) (11.7 vs 8.1 months) (90). The treatment benefit was similar across anatomical subtypes. The toxicity profile of cisplatin (oto/neurotoxicity, high emetogenicity, renal toxicity) has led many centers to substitute cisplatin for the less toxic platinum compound oxaliplatin. No randomized comparisons of GEMCIS and gemcitabine+oxaliplatin (GEMOX) regimens exist. A meta-analysis found a slight survival benefit for GEMCIS (11.7 vs 9.7 months) at the expense of higher toxicity (91). A randomized trial comparing GEMCIS and GEMOX in first line advanced GBC showed similar outcomes, with the numerical survival slightly favoring GEMOX (9 vs 8.3 months) (92). In Sweden the GEMOX regimen is generally preferred and real-world data have shown similar survival to comparable clinical trials (93). A minority of patients receive second line treatment (94), the ABC-06 trial was the first randomized study in the second line setting and compared 5-FU and oxaliplatin (FOLFOX) versus active symptom control after progression on first line GEMCIS treatment. The FOLFOX arm had a modestly improved survival compared to active symptom control (6.2 vs 5.3 months) (95). Slightly lower treatment benefit of FOLFOX was observed in the eCCA patients compared with other locations. Radiotherapy is rarely used for advanced BTC in Sweden but can be considered for symptomatic relief of painful lesions and in highly selected patients for treatment of localized disease.

The poor survival with current treatments options and identification of potentially targetable molecular alterations have led to a multitude of targeted therapies and immunotherapies investigated for the treatment of CCA (96). The most encouraging results have been found with fibroblast growth factor receptor (FGFR) inhibitors in FGFR-2 gene fusion CCA and isocitrate dehydrogenase-1 (IDH1) inhibitors in IDH1 mutant CCA (97). However, these mutations occur almost exclusively in iCCA. No targeted therapy for dCCA is currently used in clinical routine in Sweden. Notable emerging therapies include the immune checkpoint inhibitor pembrolizumab which has been Food and Drug Administration (FDA) approved for solid tumors with palliative treatment intention and MMR deficiency or microsatellite instability. The approval is based on results from basket trials including BTC patients (98, 99). MMR deficiency occurs in approximately 2% of all BTCs. Tumors with neurotrophic receptor tyrosine kinase (NTRK) fusions have shown a high response rate to NTRK inhibitors in basket trials (100-102), getting FDA and European Medicines Agency approval for treatment agnostic of tumor origin in patients without alternative treatment options. NTRK fusions are present in <1% of BTC patients (103). HER2 overexpression/amplification is present in 17% of eCCA, a phase II trial has shown promising results with anti-HER2 antibodies trastuzumab and pertuzumab in pre-treated patients (104) with randomized data eagerly awaited.

## Adjuvant oncological treatment

Because of the high rate of recurrence after surgery with curative intent, several adjuvant strategies of chemotherapy or chemoradiotherapy have been evaluated. Until the 2010s, mostly small non-randomized or retrospective studies had been performed which meant adjuvant treatment were given inconsistently and with various regimes in different centres (105). The European Study Group for Pancreatic Cancer (ESPAC)-3 trial enrolled 428 patients with resected periampullary cancers, 96 of which had dCCA. Patients were allocated to 3 arms: observation, 5-FU and gemcitabine. The study did not find differences in the primary outcome of OS, however, a secondary multivariable analysis adjusting for prognostic factors revealed a survival benefit of adjuvant chemotherapy (hazard ratio (HR)= 0.75) with a favourable safety profile of gemcitabine compared to 5-FU (106). The study was not powered for subgroup analysis. Notably, among dCCA patients the survival was not numerically improved with a median survival in the observation, 5-FU and gemcitabine arms of 27.2, 18.3 and 19.5 months respectively. The BCAT trial randomized patients after resection for eCCA to gemcitabine or observation, no significant difference in RFS or OS were seen (107). The PRODIGE-12/ACCORD-18 trial randomized patients after resection to GEMOX or observation, with no significant difference in RFS or OS observed (108). The BILCAP trial was published in 2019 and although somewhat controversial, established a new standard of care for adjuvant treatment in BTC. BTC patients were randomized after

resection to capecitabine (an oral prodrug to 5-FU) for 6 months or observation. The study did not meet the primary endpoint of improved OS in the intention to treat population (numerical survival 52 vs 36 months favouring capecitabine). A pre-specified sensitivity analysis of the intention to treat population with adjustment for prognostic factors revealed a significant benefit from capecitabine treatment (HR=0.71) (109). Based on the results of the BILCAP trial capecitabine is used for adjuvant treatment of BTC in Sweden. Based on the national comprehensive cancer network (NCCN) guidelines, gemcitabine can be offered to patients for whom capecitabine is not an option (110). Chemoradiotherapy in the adjuvant setting is generally not used in Sweden. Based on non-randomized phase II data, American society of clinical oncology and NCCN guidelines suggest it can be considered in patients with non-radical resection or high-risk features (110-112). Neoadjuvant treatment for dCCA is not used outside of clinical trials (113).

# Biomarker investigation

## Applications of mass spectrometry in cholangiocarcinoma

Proteomics is the large-scale study of proteins involved in a biological condition. Proteins are the functional unit of the cell and often suitable as biomarkers. Mass spectrometry (MS) is the most frequently employed technique for proteomics (114). The basic principle of MS is the ionization of compounds and subsequent separation based on the mass to charge ratio of the generated ions and finally ion detection. Several different MS technologies for analysis of biological compounds have been developed. Commonly used methods such as 2-dimensional gel electrophoresis followed by MS has downsides such as limited reproducibility and difficulty to upscale (115). A rapid technological development during the last decades has enabled the simultaneous identification and quantification of proteomes in complex biological systems such as tissues (116). In particular, the development of bottom-up or “shotgun” proteomics has shown promise for biomarker discovery (117). Several previous MS based proteomics studies of resected CCA specimens have been performed using different analytical approaches (118-131). The majority of these have investigated iCCA or a mixed CCA/BTC cohorts. The first proteomic characterization of an eCCA cohort was done by Maeda et al. (120). They used a bottom-up mass spectrometry workflow to analyse resected formalin-fixed paraffin-embedded (FFPE) specimens and performed sequential targeted verification and IHC validation. The discovery experiment identified 136 overexpressed proteins, 10 of which could be validated as upregulated in eCCA. Takenami et al. used bottom-up proteomics follow by IHC validation to identify novel biomarkers to differentiate dCCA and PC. Eighteen differentially expressed proteins (DEPs) were identified, 5 proteins were validated by IHC as potential novel biomarkers (132).

## Secreted protein acidic and rich in cysteine

Secreted protein acidic and rich in cysteine (SPARC) is a 32 kDa matricellular (non-structural) protein component of the extracellular matrix (ECM). SPARC is composed of three structural domains, the acidic N-terminal which has low calcium affinity, the cystine rich follastin-like domain and the C-terminal calcium binding domain (133). SPARC has a diverse functionality and impacts several biological functions such as ECM-remodelling, cell-ECM interaction, cell differentiation, angiogenesis, bone remodelling and wound healing (134-139). SPARC has been studied in various cancer models and a functional role in tumor associated stroma remodelling, tumor growth, migration and apoptosis has been found. Additionally, SPARC can contribute to development of chemotherapy resistance (133). The function of SPARC in cancer is seemingly context dependent and both pro- and antitumoral effects have been identified (140). SPARC has been proposed as a treatment predictive biomarker for treatment with gemcitabine (141) and nab-paclitaxel (142) where contradictory results exist (143). In two cohorts of patients treated with resection for BTC, stromal SPARC expression was associated with poor survival, with the negative prognostic effect enhanced in gemcitabine treated patients suggesting treatment resistance (144, 145).

## Thrombospondin-2

Thrombospondin-2 (THBS2) has a mass of 150 kDa and is composed of an N-terminal domain, a disulphide link, a procollagen domain, types I-III repeats and a C terminus. Structurally THBS2 form homotrimers (146). Like SPARC, THBS2 is a matricellular protein with an important role in ECM remodelling. THBS2 is required for normal fibroblast function and can induces dysfunctional activation of matrix metalloproteinases (147). The ECM modulating function of THBS2 has a potent anti-angiogenic role during tissue homeostasis (148). The function of THBS2 during carcinogenesis seems dependent on its cellular context. In a PC model, cancer cell expression of THBS2 inhibited invasiveness through downregulation of matrix metalloproteinase-9 and urokinase-type plasminogen activator (149). In a coculture model, THBS2 expression in stromal pancreatic stellate cells was found to promote invasiveness (150). Kim et al. identified THBS2 as a secreted protein from a PC precursor lesion model using MS. It was further validated in several cohorts and proposed as a novel biomarker for the early diagnosis of PC (151).

# Aims of the thesis

The general aim of this thesis was to investigate strategies for improving the management of patients with dCCA. This included the evaluation of clinical outcomes, diagnostic process as well as identification of novel protein biomarkers.

The specific aims of each paper are listed below

I. To investigate clinical outcome and clinicopathological prognostic factors for patients treated with pancreatoduodenectomy at Skane University Hospital from 2008 through 2015

II. To examine the expression of SPARC in resected dCCA including primary tumors and paired lymph node metastases and relationship with survival.

III. To identify novel tissue biomarkers for dCCA using discovery mass spectrometry and targeted verification

IV. To evaluate the expression of serum THBS2 in dCCA, PC, patients with benign diseases and healthy individuals and evaluate the diagnostic and prognostic biomarker potential.

V. To investigate national trends in the frequency of periampullary tumor origin, survival, histopathological evaluation and diagnostic accuracy.

# Methodological considerations

The detailed methods are presented in the original papers. The methods are briefly presented below.

## Patient cohorts

This thesis includes three different patient cohorts. The first cohort was identified from institutional records and included all patients treated with pancreatoduodenectomy for dCCA at Skane University Hospital (SUS) between 2000 and 2015 (N=64), 59 of which had (FFPE) tissues available. A histopathological re-evaluation of available material was performed in order to ensure a standardized assessment, staging was done according to AJCC 7th edition. Demographical, clinical and follow up was collected retrospectively from patient charts. For paper I, the time period was restricted to 2008-2015 (N=54) in order to evaluate a modern time span during which SUS served as a tertiary referral centre (152). Resected tumor specimens from the full cohort (N=59) were used for molecular analysis in papers II and III.

A prospective biobank of patients scheduled for pancreatic resection at SUS was started in 2012. Preoperative serum samples were obtained from patients that underwent pancreatoduodenectomy, in addition healthy controls were obtained from the local blood donation centre. Serum aliquots were stored at -80 °C until analysis. For paper IV, serum samples from all patients with a histopathological dCCA diagnosis treated with surgery from 2012 through 2019 were analysed (N=51). PC patients (N=52) from the cohort were identified and matched to the best extent possible in a 1:1 ratio to the N- and T-stage distribution in the dCCA samples. Available patients from the cohort with benign pancreatic diseases (N=27) were included as well as 51 age- and gender matched healthy donors.

The Swedish National Registry for Pancreatic and Periampullary Cancer was established in 2009. The registry prospectively gathers data from patients with periampullary cancers and patients that undergo pancreatic surgery irrespective of diagnosis. The registry is organized into six parts: registration, preoperative- and intraoperative data, postoperative data, histopathology, oncology and follow up. The registry has been validated with a good coverage against the Swedish Cancer

Registry (81). For paper V, all patients with periampullary tumors between 2010 and 2019 were included (N=9143).

# Immunohistochemistry

IHC is widely used in biology and clinical pathology to visualize antigen expression and localization in tissues (Figure 4). Most commonly, the antigen is a protein. When applied to FFPE-tissues the first steps include mounting on a glass slide, deparaffinization and rehydration through incubation with xylene and graded ethanol solutions. Next, in order to break the methylene bridges that cross link proteins and "mask" antigens an "antigen retrieval" is performed, in this thesis a heat-induced antigen retrieval was used throughout. An antibody targeting the antigen of interest (primary antibody) is then incubated on the tissue specimen. The primary antibody can be either monoclonal, which means it targets one specific epitope of the antigen, such as the SPARC antibody used in paper II, or polyclonal, such as the THBS2 antibody used in paper III, where different epitopes of the same antigen are targeted. Benefits of monoclonal antibodies include lower background, and low lot-to-lot variation, whilst polyclonal antibodies are less sensitive to fixation and processing (153). After washing a secondary antibody targeting the primary antibody is applied. The secondary antibody is labelled, typically with a polymer-linked enzyme (paper II) or biotin, which forms an avidin-biotin-enzyme complex in an additional step (paper III). Finally, the enzyme catalyses a reaction after the addition of a chromogenic substrate producing a visible colour, the intensity of which correlate to the presence of antigen in the tissue (154).

The evaluation of IHC consists of quality assessment, including the evaluation of adequate external positive and negative controls, and any available internal controls (cell types such as immune cells or endothelial cells predicted to express or not express the antigen of interest in the stained section). A valuation of the expression includes assessment of which cell types express the antigen such as cancer cells, stromal fibroblasts, endothelial cells or immune populations. A valuation of the cellular compartment (membranous, cytoplasmic or nuclear) and whether consistent with expected antigen expression compartment should be performed. The staining is by nature semi quantitative and both the intensity and percentage can be quantified. Different scoring systems have been developed and validated. Commonly used scoring systems include the Allred, H-score and immunoreactive score, additionally various statistical methods can be used to identify the optimal cutpoint for prognostic differentiation (155). For the evaluation of SPARC, a previously used scoring system was employed for dichotomization into high and low expression (156). There was no previously described scoring system for THBS2, and in paper III the prognostic correlation was done between samples with

and without THBS2 expression rather than performing data-driven cut point optimization in the relatively small cohort.

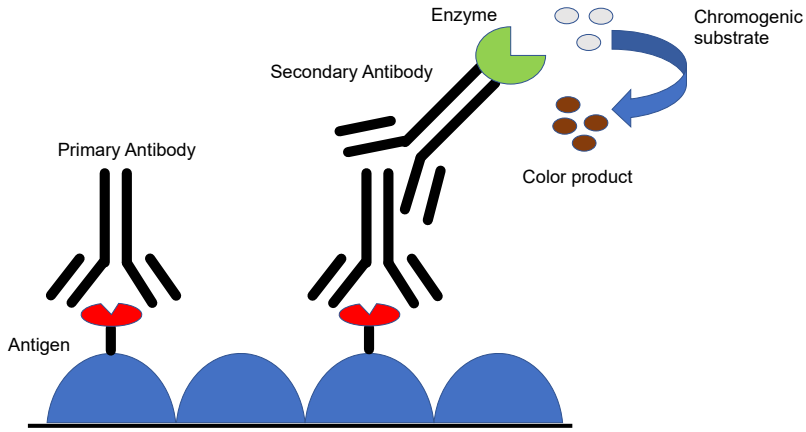



**Figure 4.** Schematic illustration of immunohistochemistry. A primary antibody binds to an antigen in a tissue. A secondary antibody with a conjugated enzyme then binds the primary antibody. The enzyme catalyzes a chemical reaction in which a visible precipitate is generated, the intensity of which correlates to the presence of the antigen in the sample.

# Mass spectrometry

In a bottom-up proteomic workflow proteins are extracted and digested into peptides enzymatically. Peptides are then separated using liquid chromatography (LC) and injected into the MS instrument where they are ionized and separated based on their mass/charge ratio. The most intense precursors are fragmented and analyzed in a second MS run. The resulting fragmentation ions are used to perform peptide sequencing and subsequent in-silico protein prediction. Quantification can be performed either based on label free or labelled methods. Previously, MS has mostly been used to analyze fresh frozen tissues. However, protocols for the analysis of FFPE tissues have been developed (157, 158). In paper III, a “bottom-up” proteomics workflow using LC on-line with tandem mass spectrometry (LC-MS/MS) was used to compare the proteomes of resected dCCA FFPE specimens (N=20) and FFPE normal bile ducts (N=6). Specimens were macrodissected. Protein extraction, deparaffinization and protein cross-link breakage was done using heat induced antigen retrieval similar to IHC. Proteins were digested to peptides using trypsin. The peptides were separated using an LC on-line to the MS instrument

(Q Exactive Plus, Thermofisher Scientific). Protein identification and quantification was done using the software Proteome discoverer and alternative software programs (MaxQuant and OpenMS). Label free quantification was performed using a precursor ion area detector.

Limitations of bottom-up proteomics include quantitative imprecision, limited sensitivity and difficulty in validating a large number of identified DEPs (159, 160). In order to overcome this, a verification of DEPs was done using targeted MS. Most commonly used targeted protemics methods are multiple-reaction monitoring and parallel reaction monitoring (PRM). PRM is performed using a quadrupole Orbitrap MS such as the Q Exactive Plus used for the discovery bottom-up proteomics. Specific peptides are selected for identification and the instrument is set to identify the specific precursor ions and a full MS/MS scan of precursor and fragment ions are analyzed. PRM is able to detect peptides at a lower concentration and with improved accuracy compared to discovery bottom-up proteomics (161, 162). In paper III, new material from 16 dCCA specimens and 9 controls were analyzed using PRM (the same sample preparation and MS as during the discovery study was used) in order to verify selected DEPs from the discovery study. Quantification based on precursor ion intensity was done using the Skyline software (163).

## Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) is frequently used in biological and clinical research for quantitative measurement of antigens in fluids such as serum/plasma, bile, urine, cellular extracts and more. Like IHC, ELISA uses specific antibodies to bind the antigen of interest. The most commonly used version is the "sandwich" ELISA. Two antibodies targeting different epitopes of an antigen are used (Figure 5). In paper IV, a commercial sandwich ELISA (DTSP20, R&D Systems, Minneapolis, MN, USA) was used to measure THBS2 concentration in serum samples. The ELISA was selected as it was used in the majority previous studies evaluating THBS2 as a diagnostic biomarker (151, 164-166). Principally, the samples are incubated in a welled plate with the first antibody (capture antibody) coated. After washing, the second antibody (detection antibody) with a conjugated enzyme is added to the wells. After additional washing a chromogen is added. The enzyme catalyses the chromogenic reaction and a change in colour takes place proportional to the presence of the antigen. A microplate reader is used to measure the density of each well at a specified wavelength. Standards of a known concentration are included, and a standard curve is calculated. For THBS2 concentration calculation a four-parameter logistic regression standard curve was calculated using MyAssays online data analysis tool.

Serum CA 19–9 concentrations were measured in a clinical laboratory using an accredited Chemiluminescent immunoassay (Ref.11776193 122 Cobas/Roche).

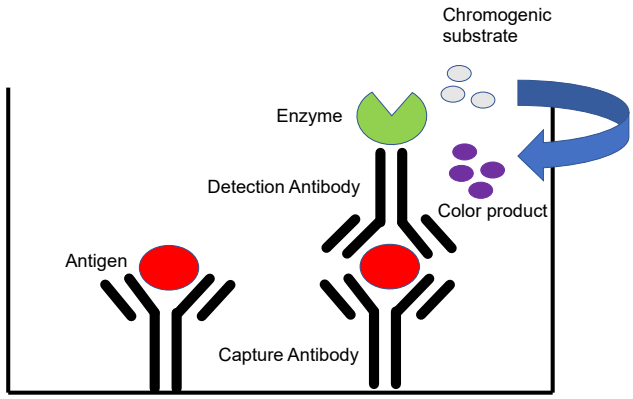



**Figure 5.** Schematic illustration of a sandwich ELISA. The antigen binds to the coated capture antibody. A detection antibody with a conjugated enzyme then binds a different epitope of the antigen. The enzyme catalyzes a chemical reaction in which a colored product is generated, the intensity of which correlates to the presence of the antigen in the sample.

# Statistics

Survival analysis was used in all papers included in the thesis. Survival outcomes included disease-free survival (DFS; time to recurrence or death) and OS. While OS is considered the golden standard for cancer research, DFS is a commonly used surrogate endpoint that can identify statistically relevant differences at a shorter follow up time (167). The Kaplan-Meier (KM) method was used to estimate the survival of patients. The KM method uses the exact time to event or censoring (loss to follow up) and incorporates the number of patients at risk in the calculation of survival. For an adequate estimation, it is important that censoring is not related to the groups investigated. The KM survival estimate is combined with the log-rank test to compare differences between groups. The log-rank test compares a weighted average of recorded events and expected events at all time points. When interpreting KM survival curves the precision in the survival estimate is reduced when few patients at risk remain and a presentation of number at risk and confidence intervals are recommended (168). Although survival calculation is performed using all

available data, the graphical presentation of survival in Paper I could have been shorter since few patients remained past 3 years.

A regression model is a statistical way of estimating the relationship between an outcome variable and one or more predictor variables. In this thesis, the relationship between predictor variables and survival was performed using Cox proportional hazard regression (CPH) (paper I, III) and predictor variables and diagnosis using logistic regression (paper IV). The CPH model calculates the HR of predictor variables. The HR is the difference in the likelihood of the outcome event occurring when the predictor variable is present compared to not present (categorical variables) or when increased by one unit (continuous variables). With inclusion of multiple covariables (multivariable regression) the HR estimates are adjusted for one another which is used to adjust for confounding and identify variables independently associated with survival. CPH-models are built on the assumption that the hazard associated with a variable does not vary over time. Other important aspects are to avoid overfitting and collinearity (169). The selection of variables for a multivariable CPH can be performed using different methods (170). In paper I, the goal was to identify the strongest predictor for survival. Backward elimination was used which step- by step remove variables that do not contribute to model prediction. In paper III the goal was to adjust for confounders of THBS2 association with survival and variable selection of clinically relevant confounders were done manually.

The statistical analysis of MS data in paper III was done using the dedicated software Perseus (171). Important analytical steps included data filtering (only proteins quantified in 50% of samples in each group was used), normalization, imputation and group comparison with adjustment for multiple testing. Normalization was done to account for variations of cellularity in the samples. The normalization in the discovery experiment was based on median protein intensity in each sample. In the PRM verification, only pre specified proteins were quantified and normalization was done using common housekeeping proteins glyceraldehyde 3-phosphate dehydrogenase and tubulin beta chain. DEPs were identified using t-tests with a permutation based false discovery rate adjustment. In order to further describe the biological properties of identified DEPs bioinformatic analysis was performed. The Gene ontology (GO) project has standardized the description of genes and gene products. Specifically, the cellular component, molecular function and biological process are annotated (172). PANTHER database (173) was used to perform GO classification of identified DEPS and identify enrichment. Different ways of annotating signaling pathways have been developed and enrichment analyses of pathways were done using additional reference databases KEGG (174) and REACTOME (175). Protein-protein interaction patterns were analysed using the STRING database (176).

Diagnostic accuracy of biomarkers THBS2 and CA 19–9 was evaluated in paper IV. Central properties include the sensitivity (proportion of patients with a condition

correctly classified by the biomarker) and specificity (proportion of patients without the condition correctly classified by the biomarker). In clinical practice, the actual performance of a biomarker is also highly dependent on the prevalence of the condition investigated (177). Receiver operating characteristics (ROC) analysis is performed by plotting the sensitivity on the y axis and (1-specificity) on the x-axis of a two-dimensional plot. It provides a visual representation of the trade-off between sensitivity and specificity at different cutpoints which can aid in cutpoint selection. Additionally, the area under the curve (AUC) provides a summary statistic of diagnostic performance. Logistic regression models were used to generate AUCs of THBS2 and CA 19–9 individually and combined.

# Ethics

The studies in this thesis were approved by the regional ethics committee at Lund University. Dnr 2015-392 (papers I-III). Dnr 2010/684, 2012/661, 2015/266 (Paper IV). Dnr 2015/392, 2021/00622 (Paper V). None of the studies included in this thesis had an impact on medical treatment of the patients. All data was anonymized and analysed on group level.

# Results summary

## Paper I

The first paper of this thesis evaluated outcome and prognostic factors in patients treated with pancreatoduodectomy for dCCA at SUS between 2008-2015 ((N=54). The mean age was 68 ± 7.7 years and 38% were women. The most common presenting symptoms were jaundice (72%), abdominal pain (35%) and weight loss (19%). PBD was performed in 98% of patients. Postoperative complications ≥3 according to the Clavien-Dindo classification occurred in 17% of patients. After histopathological re-evaluation, 72% of patients had lymph node metastasis. Sixty-three percent of patients had R1 resections. Chemotherapy was administered in 52% of patients with the most common regimen being gemcitabine monotherapy. The median time to recurrence was 13.2 months and 52% and 82% of patients had recurrence at year 1- and 3 respectively. The most common location of recurrence was the liver (31%), followed by local recurrence (24%). The median survival was 22.2 months. The OS at year 1 was 80%, year 3 (21%) and year 5 (9.2%).

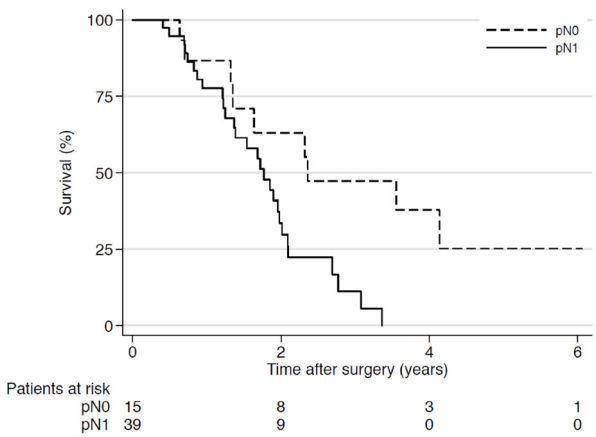



**Figure 6.** Overall survival after resection for distal cholangiocarcinoma stratified by lymph node status. Survival is estimated using the Kaplan-Meier method.

Using univariable CPH, the presence of lymph node metastasis (categorical) HR 2.9 (P=0.016) and lymph node metastases (continuous) HR 1.08 (P=0.017) were significantly associated with survival. A CPH model with stepwise backward selection found no other variable to be significantly associated with survival when adjusted for lymph node metastasis. Analysing number of lymph node metastases or the lymph node ratio did not improve the prediction of the model compared to presence of lymph node metastasis alone. Survival of dCCA patients stratified by lymph node status is presented in Figure 6.

# Paper II

The second paper evaluated the expression pattern and prognostic significance of SPARC in patients with dCCA treated with pancreatoduodenectomy between 2000-2015 (N=59) using IHC. Both primary tumors and paired lymph node metastases were evaluated. No SPARC immunoreactivity was detected in normal bile ducts. Tumor cell immunoreactivity was weak and focal and thus was not quantified further. Stromal immunoreactivity intensity was absent in 20%, weak in 10%, intermediate in 39% and strong in 31% of patients. The proportion of SPARC positive stroma was absent in 20%, 11-25% in 1.7% of patients, 26-50% in 15% of patients, 51-75% in 25% of patients and ≥75% in 37% of patients (Figure 7). An intermediate or strong staining in >25% of stromal cells was classified as high expression which entailed 68% of patients. Stromal (non-lymphoid) SPARC expression was present in 68% of lymph node metastases. When comparing paired primary tumors and paired lymph node metastases 96% and 68% (P=0.016) respectively had stromal SPARC expression present. High stromal SPARC expression was associated with presence of lymph node metastasis (78 vs 47%; P=0.013). No significant association between SPARC expression and survival was found.

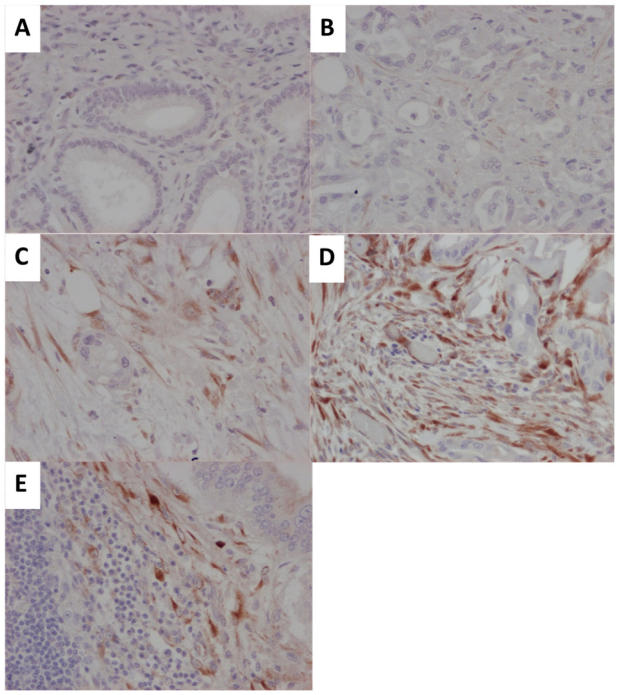



**Figure 7.** Representative immunohistochemical images at 200 x magnification of SPARC expression in the stromal compartment of distal cholangiocarcinoma (A), negative expression, (B) weak expression, (C) moderate expression, (D) strong expression, (E) Strong peritumoral stromal SPARC expression in a paired lymph node metastasis.

# Paper III

Paper III encompasses a proteomic profiling of resected FFPE dCCA specimens and normal bile ducts using discovery bottom-up MS, followed by verification using PRM and further validation of THBS2 expression using IHC. A total of 3037 proteins were identified. Eighty-seven DEPs were identified, 31 proteins were upregulated and 56 downregulated in dCCA compared to normal bile ducts. Bioinformatic analysis of DEPs from the discovery study revealed enrichment of several extracellular components and signaling pathways such as integrin signaling, ECM-receptor interaction and collagen degradation.

In the PRM verification, 122 peptides mapping to 79 proteins could be quantified. In total, 65 peptides were found to be differentially expressed corresponding to 46 proteins. Twenty-eight proteins were upregulated and 18 downregulated. Several previously described tentative CCA biomarkers (S100-P, CECAM-6, cytokeratin 17, thymidine phosphorylase and heat-shock protein 90) were correctly identified as upregulated and several novel proteins without previous association to CCA

biology were identified. The identified peptides/ proteins and their previous association with CCA/BTC are presented in Table 3.

IHC was used to further investigate the expression of THBS2 in dCCA, paired lymph node metastases and normal bile ducts. THBS2 was predominantly negative in normal biliary epithelium but weak focal expression was detected. THBS2 immunoreactivity was seen in dCCA cancer cells with a membranous/cytoplasmic staining pattern and in the stroma. Epithelial immunoreactivity was absent in 10%, 1+ in 51%, 2+ in 32% and 3+ in 7%. The stromal intensity was absent in 8%, 1+ in 31%, 2+ in 42% and 3+ in 11% (Figure 8). When comparing primary tumors and paired lymph node metastases, epithelial THBS2 expression was present in 96% and 54% (P=0.001) respectively. Stromal THBS2 expression was present in 96% and 72% (P=0.031) respectively. Epithelial THBS2 expression was not associated with survival. Stromal THBS2 was not significantly associated with DFS (P=0.105) or OS (P=0.079) using KM analysis. After adjustment for confounders in a multivariable CPH, stromal THBS2 was significantly associated with poor DFS (HR 3.95, P = 0.037) and a trend was seen for OS (HR 3.34, P =0.062).

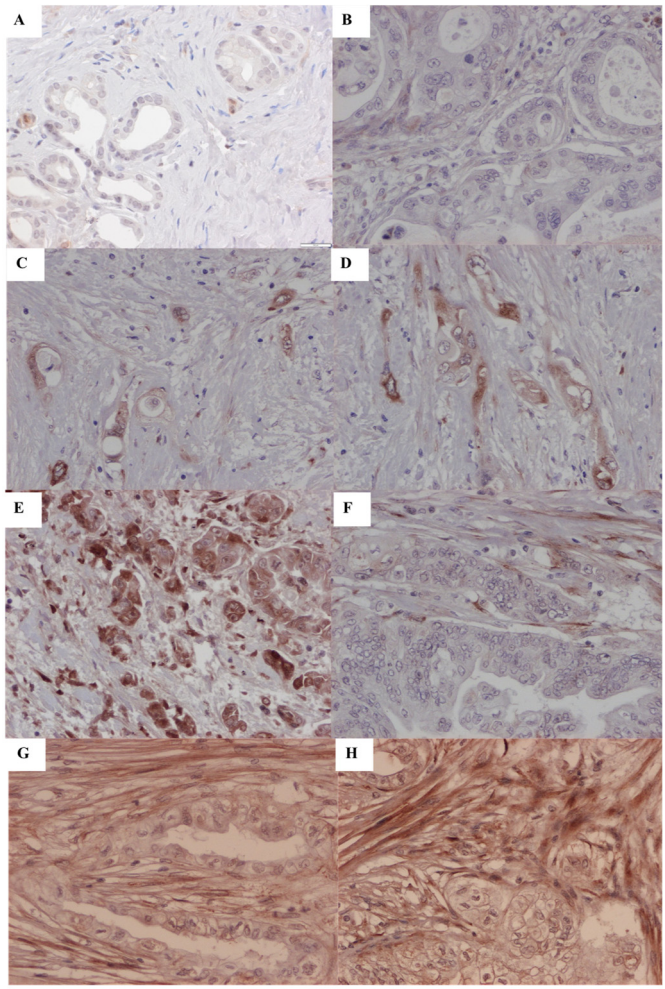



**Figure 8.** Representative immunohistochemical images of THBS2 expression at at 200 x magnification. Normal bile duct; (A) no expression. Distal cholangiocarcinoma; (B) no expression, (C) weak epithelial expression, (D) moderate epithelial expression, (E) strong epithelial expression, (F) weak stromal expression, (G) moderate stromal expression and (H) strong stromal expression.

**Table 3.** Proteins found to be differentially expressed using parallel reaction monitoring ($q < 0.05$, FC ≥ 2 or ≤ 0.5) between distal cholangiocarcinoma samples and normal bile ducts. Proteins are ordered by descending FC. Abbreviations: CA; cancer. CCA; cholangiocarcinoma. C0; control. FC; fold change.

| # | Accession | Gene | Protein Name | Peptide sequence | Valid Cancer | Valid Control | q-value | FC Ca/Co | Literature CCA |
|---|---|---|---|---|---|---|---|---|---|
| 1 | P35442 | TSP2 | Thrombospondin-2 | LVFNPDQEDLDGDGR | 16 | 9 | < 0.0001 | 29.3 | |
| 2 | P35442 | TSP2 | Thrombospondin-2 | FDYIPPVNADDLSK | 16 | 9 | < 0.0001 | 8.3 | |
| 3 | Q6UX06 | OLFM4 | Olfactomedin-4 | LLEYYR | 16 | 8 | < 0.0001 | 7.8 | (120, 178) |
| 4 | P25815 | S100P | Protein S100-P | YSGSEGSTQTLTK | 16 | 9 | < 0.0001 | 7.3 | (179, 180) |
| 5 | P40199 | CEAM6 | Carcinoembryonic antigen-related cell adhesion molecule 6 | IGYSWYK | 16 | 9 | 0.0007 | 7.2 | 181 |
| 6 | P25815 | S100P | Protein S100-P | ELPGFLQSGK | 16 | 8 | < 0.0001 | 6.4 | (179, 180) |
| 7 | Q04695 | K1C17 | Keratin, type I cytoskeletal 17 | ASLEGNLAETENR | 16 | 9 | 0.0044 | 5.5 | (182, 183) |
| 8 | Q99439 | CNN2 | Calponin-2 | GLQSGVDIGVK | 16 | 8 | < 0.0001 | 4.8 | |
| 9 | Q96CG8 | CTHR1 | Collagen triple helix repeat-containing protein 1 | VLFSGSLR | 16 | 9 | < 0.0001 | 4.5 | |
| 10 | P08238 | HS90B | Heat shock protein HSP 90-beta | NPDDITQEEYGEFYK | 16 | 9 | < 0.0001 | 4.2 | (184) |
| 11 | P19971 | TYPH | Thymidine phosphorylase | MLAAQGVDPGLAR | 16 | 9 | < 0.0001 | 4.1 | (185, 186) |
| 12 | P31949 | S10AB | Protein S100-A11 | DGYNYTLSK | 16 | 9 | < 0.0001 | 3.8 | (187) |
| 13 | P19971 | TYPH | Thymidine phosphorylase | VAAALDDGSALGR | 16 | 9 | < 0.0001 | 3.6 | (185, 186) |
| 14 | P19827 | ITIH1 | Inter-alpha-trypsin inhibitor heavy chain H1 | AAISGENAGLVR | 16 | 9 | 0.0030 | 3.5 | |
| 15 | Q96HE7 | ERO1A | ERO1-like protein alpha | LGAVDESLSEETQK | 16 | 9 | < 0.0001 | 3.0 | |
| 16 | Q01518 | CAP1 | Adenylyl cyclase-associated protein 1 | VENQENVSNLVIEDTELK | 16 | 9 | < 0.0001 | 2.9 | |
| 17 | Q9UBR2 | CATZ | Cathepsin Z | NVDGVNYASITR | 16 | 9 | 0.0004 | 2.7 | |
| 18 | P50454 | SERPH | Serpin H1 | AVLSAEQLR | 16 | 9 | < 0.0001 | 2.7 | (188) |

| # | Accession | Gene | Protein Name | Peptide sequence | Valid Cancer | Valid Control | q-value | FC Ca/Co | Literature CCA |
|---|---|---|---|---|---|---|---|---|---|
| 19 | P21291 | CSRP1 | Cysteine and glycine-rich protein 1 | GYGYGQGAGTLSTDK | 16 | 9 | 0.0018 | 2.7 | |
| 20 | Q9UBR2 | CATZ | Cathepsin Z | NSWGEPWGER | 16 | 9 | 0.0007 | 2.6 | |
| 21 | P02792 | FRIL | Ferritin light chain | ALFQDIK | 16 | 9 | 0.0002 | 2.6 | |
| 22 | Q01518 | CAP1 | Adenylyl cyclase-associated protein 1 | LSDLLAPISEQIK | 16 | 9 | < 0.0001 | 2.5 | |
| 23 | P42224 | STAT1 | Signal transducer and activator of transcription 1-alpha/beta | TELISVSEVHPSR | 14 | 4 | 0.0033 | 2.5 | (189) |
| 24 | Q9NZM1 | MYOF | Myoferlin | ANVTVLDTQIR | 16 | 6 | 0.0020 | 2.4 | |
| 25 | Q96CG8 | CTHR1 | Collagen triple helix repeat-containing protein 1 | IIIEELPK | 16 | 6 | 0.0020 | 2.4 | |
| 26 | P00338 | LDHA | L-lactate dehydrogenase A chain | SADTLWGIQK | 16 | 9 | < 0.0001 | 2.4 | (190, 191) |
| 27 | P00338 | LDHA | L-lactate dehydrogenase A chain | VTLTSEEEAR | 16 | 9 | < 0.0001 | 2.3 | |
| 28 | O75369 | FLNB | Filamin-B | FNDEHIPESPYLVPVIAPSDDAR | 16 | 9 | 0.0005 | 2.3 | |
| 29 | P43490 | NAMPT | Nicotinamide phosphoribosyltransferase | STQAPLIIRPDSGNPLDTVLK | 15 | 8 | < 0.0001 | 2.2 | |
| 30 | Q9NZM1 | MYOF | Myoferlin | GPVGTVSEAQLAR | 16 | 9 | < 0.0001 | 2.2 | |
| 31 | P40121 | CAPG | Macrophage-capping Protein | ANAQAAALYK | 16 | 6 | 0.0006 | 2.2 | (130) |
| 32 | P38606 | VATA | V-type proton ATPase catalytic subunit A | TVISQSLSK | 16 | 9 | < 0.0001 | 2.2 | |
| 33 | P14618 | KPYM | Pyruvate kinase PKM | LDIDSPPITAR | 16 | 9 | < 0.0001 | 2.1 | (192) |
| 34 | P08238 | HS90B | Heat shock protein HSP 90-beta | ALLFIPR | 14 | 5 | 0.0021 | 2.1 | (184) |

| # | Accession | Gene | Protein Name | Peptide sequence | Valid Cancer | Valid Control | q-value | FC Ca/Co | Literature CCA |
|---|---|---|---|---|---|---|---|---|---|
| 35 | P27348 | 1433 T | 14–3-3 protein theta | YLIANATNPESK | 16 | 9 | 0.0078 | 2.1 | (118, 193) |
| 36 | P07900 | HS90A | Heat shock protein HSP 90-alpha | NPDDITNEEYGEFYK | 16 | 9 | 0.0092 | 2.1 | (184) |
| 37 | P43490 | NAMPT | Nicotinamide phosphoribosyltransferase | AVPEGFVIPR | 16 | 7 | < 0.0001 | 2.1 | |
| 38 | P14618 | KPYM | Pyruvate kinase PKM | GDLGIEIPAEK | 16 | 9 | < 0.0001 | 2.1 | (192) |
| 39 | P10809 | CH60 | 60 kDa heat shock protein, Mitochondrial | LVQDVANNTNEEAGDGTTTATVLAR | 16 | 9 | 0.0408 | 2.0 | (194) |
| 40 | P00352 | AL1A1 | Retinal dehydrogenase 1 | TIPIDGNFFTYTR | 16 | 9 | 0.0457 | 0.46 | (195) |
| 41 | P04040 | CATA | Catalase | ADVLTTGAGNPVGDK | 16 | 9 | < 0.0001 | 0.34 | (196) |
| 42 | P04040 | CATA | Catalase | FNTANDDNVTQVR | 16 | 9 | < 0.0001 | 0.32 | (196) |
| 43 | P09525 | ANXA4 | Annexin A4 | GLGTDDNTLIR | 16 | 9 | 0.0005 | 0.32 | (126) |
| 44 | Q16853 | AOC3 | Membrane primary amine Oxidase | YQLAVTQR | 16 | 9 | < 0.0001 | 0.29 | |
| 45 | O95994 | AGR2 | Anterior gradientprotein 2 Homolog | LPQTLSR | 16 | 9 | < 0.0001 | 0.29 | (197) |
| 46 | O60218 | AK1BA | Aldo–keto reductase family 1-member B10 | SGDDLFPK | 16 | 9 | 0.0152 | 0.27 | |
| 47 | Q13228 | SBP1 | Methanethiol oxidase | IYVVDVGSEPR | 16 | 9 | < 0.0001 | 0.26 | (126) |
| 48 | P00167 | CYB5 | Cytochrome b5 | FLEEHPGGEEVLR | 16 | 9 | < 0.0001 | 0.25 | (126) |
| 49 | Q13228 | SBP1 | Methanethiol oxidase | LVLPSLISSR | 14 | 5 | 0.0004 | 0.24 | (126) |
| 50 | P51884 | LUM | Lumican | ISNIPDEYFK | 16 | 9 | < 0.0001 | 0.18 | |
| 51 | Q9UBX5 | FBLN5 | Fibulin-5 | DQPFTILYR | 16 | 9 | < 0.0001 | 0.18 | (198) |
| 52 | Q07507 | DERM | Dermatopontin | YFESVLDR | 16 | 9 | < 0.0001 | 0.18 | (199) |
| 53 | P51884 | LUM | Lumican | ILGPLSYSK | 16 | 9 | < 0.0001 | 0.16 | |
| 54 | P55083 | MFAP4 | Microfibril-associated glycoprotein 4 | GFYYSLK | 16 | 9 | < 0.0001 | 0.16 | (200) |
| 55 | P07585 | PGS2 | Decorin | VSPGAFTPLVK | 16 | 9 | < 0.0001 | 0.16 | (201) |

| # | Accession | Gene | Protein Name | Peptide sequence | Valid Cancer | Valid Control | q-value | FC Ca/Co | Literature CCA |
|---|---|---|---|---|---|---|---|---|---|
| 56 | P51888 | PRELP | Prolargin | NQLEEVPSALPR | 16 | 9 | < 0.0001 | 0.15 | |
| 57 | Q07507 | DERM | Dermatopontin | GATTTFSAVER | 16 | 9 | < 0.0001 | 0.14 | (1992) |
| 58 | P55083 | MFAP4 | Microfibril-associated glycoprotein 4 | WTVFQK | 14 | 5 | < 0.0001 | 0.12 | (200) |
| 59 | P00325 | ADH1B | Alcohol dehydrogenase 1B | AAVLWEVK | 14 | 5 | < 0.0001 | 0.12 | |
| 60 | P07585 | PGS2 | Decorin | ASYSGVSLFSNPVQYWEIQPSTFR | 16 | 9 | < 0.0001 | 0.11 | (201) |
| 61 | P08294 | SODE | Extracellular superoxide dismutase | VTGVVLFR | 14 | 5 | < 0.0001 | 0.11 | |
| 62 | P23141 | EST1 | Liver carboxylesterase 1 | FTPPQPAEPWSFVK | 16 | 9 | < 0.0001 | 0.08 | (126, 198) |
| 63 | P20774 | MIME | Mimecan | LTLFNAK | 16 | 9 | < 0.0001 | 0.07 | (198) |
| 64 | P23141 | EST1 | Liver carboxylesterase 1 | TVIGDHGDELFSVFGAPFLK | 16 | 9 | < 0.0001 | 0.06 | (126, 198) |
| 65 | P20774 | MIME | Mimecan | DFADIPNLR | 16 | 9 | < 0.0001 | 0.05 | (198) |

# Paper IV

The utility of serum THBS2 as a biomarker for the diagnosis of dCCA (N=51) and PC (N=52) was further explored in paper IV. There was no significant difference in THBS2 levels between dCCA and PC. Unlike CA 19–9, THBS2 levels did not correlate with stage. The combination of THBS2 and CA 19–9 (≥35) had a discriminatory power (AUC) of 0.94 in separating dCCA and healthy controls (Figure 9). THBS2 + CA 19–9 (≥35) had an AUC of 0.92 for the discrimination of dCCA+ PC from healthy controls. When THBS2 was dichotomized at 51 ng/ml THBS2 (≥ 51) + CA 19–9 (≥35) had a sensitivity of 79% with a specificity of 96% for the discrimination of dCCA and PC from healthy controls. THBS2 provided benefit compared to CA 19–9 alone in early- stage disease. In N0 dCCA patients the AUC increased from 0.69 to 0.87 with the addition of THBS2. For N0 PC patients, the AUC increased from 0.70 to 0.81. THBS2 did not provide value to CA 19–9 in discriminating dCCA and PC from the benign pancreatic disease's cohort (N=27). Notably, large heterogeneity within the benign cohort was noted 3/5

patients with autoimmune pancreatitis had substantially elevated THBS2 levels. No correlation between THBS2 levels and prognosis was found.

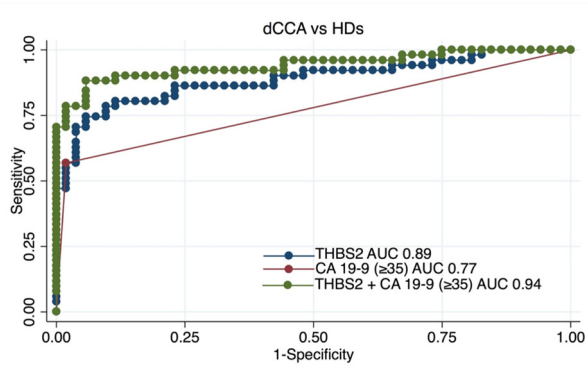



**Figure 9.** ROC curves for THBS2, CA 19–9 and THBS2 + CA 19–9 combined for dCCA vs healthy donors.

# Paper V

The inspiration for the final paper of this thesis came from the findings in paper I and the large discrepancies in the literature regarding dCCA frequency and outcome after surgery. The Swedish National Registry for Pancreatic and Periampullary Cancer was used to evaluate national trends in assessment of periampullary tumor origin, diagnostic accuracy, histopathological evaluation and outcome in Sweden. The frequency of individual periampullary cancers in patients with unresectable/metastatic disease at diagnosis (N=4428) was PC in 84%, dCCA in 4%, AC in 5% and DC 7%. Among patients treated with pancreatoduodenectomy (N=2760) the histopathological diagnosis was PC in 63%, dCCA in 13%, AC in 17% and DC in 7%. Comparing before and after histopathological standardization 57% vs 64% of patients was diagnosed with PC, 15% vs 12% dCCA, 20% vs 16 % AC and 9% vs 7% DC. When stratified by region, the frequency range of PC was 47-71%, dCCA 9-25%, AC 14-21% and DC 3-9%. The rate of R1 resection increased after standardization (32% vs 45%) and large regional differences were seen (12-65%). A similar pattern was found for nodal stage. Standardization increased the frequency of N1 stage (62% vs 77%) and regional differences (52-82%) were found. Median and 5-year OS was 24 months and 21% for PC, 33 months and 31% for dCCA. Median survival was not reached in AC or DC and 5-year survival rates were 54% and 56% respectively (Figure 10). Among patients with PC on final histology 15% had a preoperative diagnosis of non-pancreatic

periampullary tumor. Among patients with non-pancreatic periampullary cancer 23% had a preoperative diagnosis of pancreatic tumor.

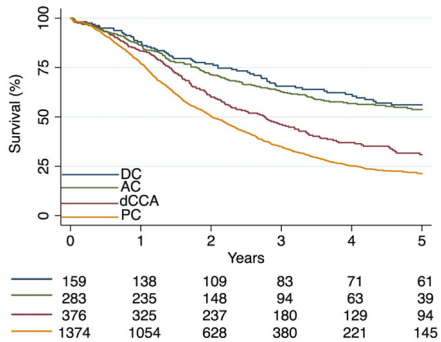



**Figure 10.** Postoperative survival for patients treated with pancreatoduodenectomy for periampullary cancer stratified by tumor origin.

# Discussion

## Aspects of clinical management and outcome

In paper I, we found the survival following pancreatoduodenectomy for dCCA (median survival 22.2 months) to be worse than most previous studies (59). A high frequency of negative prognostic factors, such as lymph node metastasis and R1 resection were present, which could have contributed. In paper V, which presented outcome in a national cohort during a comparable time period, the median survival was 33.3 months, which is a substantially better. There were large regional differences with respect to frequency of identified tumor origin in paper V, (9%-25%) for dCCA. It is possible that samples which would have been classified as PC in other centers/studies were included in the paper I cohort, contributing to worse survival. The large discrepancy in frequency of individual periampullary cancers in the literature (72) have most likely influenced survival estimates and contributed to the heterogeneity seen. Further research in dCCA and other periampullary cancers would benefit from reporting the methodology of histopathological evaluation and frequency of individual periampullary cancers in the underlying cohort. The fact that differences in histopathological evaluation of pancreatoduodenectomy specimens exists in Sweden, where a national protocol for evaluation exists, suggest additional quality efforts are warranted to ensure consistency. We believe a similar problem exists globally.

In paper V, we found a diagnosis of PC to be more common in patients with non-resectable periampullary cancer. Non-pancreatic origin was found in 37% of resected patients and 16% of non-resected patients. Although a lower rate of resectable tumors for patients with PC could contribute, this result is likely due to the difficulty in diagnosing individual periampullary cancers clinically. When comparing the pre-and postoperative diagnosis of patients, 15% of PC patients and 23% of non-pancreatic periampullary cancers had an incorrect preoperative diagnosis. The clinical rate of misdiagnosis was similar to a recent study from the Netherlands (202). Distal bile duct tumors had the worst diagnostic accuracy, the concordance between pre- and postoperative diagnosis was 37%. The use of neoadjuvant therapy is emerging primarily for PC (203) but also in CCA (113). In today's oncological treatment algorithm, tumor origin dictates chemotherapy regimens used. Admittedly, there is an overlap in the regimens used for periampullary cancers, which makes the impact of misclassification difficult to

estimate. Several studies have suggested the morphology (intestinal or pancreatobiliary differentiation) to better reflect prognosis and treatment prediction than anatomical tumor origin in periampullary cancers (15-17). Further research is required to define a treatment predictive, biologically relevant tumor stratification and improve diagnostic precision in periampullary cancers. The rate of misdiagnosis should be considered when neoadjuvant therapy is an option and in the design of clinical trials.

In paper I, the presence of lymph node metastasis was confirmed as an important prognostic factor, in concordance with the literature (204-208). Number of lymph node metastases or lymph node ratio did not improve the prediction. Since the publication of Paper I, the AJCC 8th edition was published which redefined the N stage in dCCA (Table 2). The optimal predictive lymph node variable for prognosis is still under debate with heterogeneous study results, probably due to differences in patient selection, surgical technique and histopathological evaluation influencing results (209, 210).

### Methodological considerations

Paper I had a retrospective design and a small sample size. Consequently, there was a limiting precision of survival estimates and low statistical power. Paper V was based on a large, validated, prospectively collected national cohort. Limitations of paper V are inherent to registry data, such as quality of registration and availability of variables. We did not have the possibility of comparing diagnostic precision in patients based on preoperative histopathological verification or certainty of preoperative diagnosis. We did also not have data on intestinal or pancreatobiliary differentiation and limited oncological treatment data.

## Aspects of tumor biology and biomarkers

In paper III, which represents the first quantitative comparison of proteomes in dCCA and normal bile ducts, bioinformatic analysis of DEPs from the discovery experiment revealed enrichment of the extracellular compartment and extracellular signalling pathways. Studies on the functional properties of the tumor stroma in CCA have been done mostly in iCCA cohorts (211). Given the drastically different microenvironments of the liver and extrahepatic biliary tree, finding from iCCA cannot be directly extrapolated to dCCA. Tumor microenvironment scores based on the amount of desmoplasia and infiltration of immune cell subsets have shown to correlate with prognosis in dCCA. However, few studies on the unique microenvironment in dCCA has been performed (31). One study characterized the tumor-stroma fraction in 16 solid malignancies found pancreatobiliary type

periampullary cancer to have the highest stroma proportion. A high stromal fraction was correlated with better survival (212). Studies on stromal biology in PC have highlighted the importance of tumor-stroma interactions for cancer development, but also the impact of stromal heterogeneity. Tumor suppressive functions of stromal components have been described. Stromal therapeutic targeting has been notoriously difficult, but promising candidates are in development (213). A more thorough description of individual proteins identified in paper III is available in the discussion of the manuscript. Further studies on the unique functional properties of the stroma in dCCA is merited.

Both SPARC and THBS2 are matricellular ECM proteins. In paper II, we found SPARC expression to be frequent in dCCA stroma and also frequently maintained in paired lymph node metastases. Previous studies in BTC cohorts have identified a negative prognostic impact of stromal SPARC expression (144, 145), which was not found in the present study. In paper III, we found THBS2 to be expressed in dCCA stromal, but also epithelial compartments. THBS2 had not previously been associated with CCA. A negative prognostic correlation between stromal THBS2 and DFS was seen. Previously, THBS2 secreted from stromal stellate cells has been shown to promote PC cell invasion in vitro (150). One hypothesis could be that a similar mechanism is in play in dCCA and that THBS2 secreted in dCCA microenvironment contributes to cancer invasiveness and poor prognosis. Like SPARC, stromal THBS2 expression was frequently maintained in lymph node metastases. Previous studies have found primary tumors to prime distant microenvironments and stromal reestablishment as a mechanism driving metastasis outgrowth (214). Pre-metastatic lymph node alterations with increased immunosuppressive signaling and following metastasis, lymph node remodelling are important parts of the metastatic process (215). The microenvironment of metastatic CCA is largely unexplored. The functional roles of SPARC and THBS2 in dCCA and role during metastatic development merits further investigation.

The main finding in paper IV was that serum THBS2 was elevated in dCCA, similarly to PC. THBS2 could aid in identifying early-stage disease compared with CA 19–9 alone in the preoperative setting. There was no benefit of THBS2 in separating dCCA and PC from benign controls. However, heterogeneity was seen in the benign cohort. Three out of five patients with autoimmune pancreatitis had substantially elevated levels of THBS2. After the initiation of this study, Large et al. evaluated THBS2 as a diagnostic biomarker for dCCA and found comparable diagnostic performance (216). Similarly, they found no additive value of THBS2 to CA 19–9 in discriminating benign diseases as a group, however, it did improve performance in patients with high bilirubin. This was a hypothesis we could not test in our study since we had few jaundiced patients in the cohort. Another study evaluated THBS2 expression in prospectively collected pre-diagnostic samples. Unfortunately, THBS2 failed to identify presymptomatic PC development and as

such won't be suitable for screening of asymptomatic individuals (217). Whether this is also the case for dCCA remains to be elucidated, although plausible.

Given the rarity of CCA, and even more dCCA, screening of asymptomatic individuals is not realistic. Any clinical application of a new biomarker would be in the screening of high- risk individuals such as PSC patients or as differential diagnostic tools against benign conditions after a suspicious finding. Evaluations of THBS2 utility in larger cohorts (preferentially including other CCA subtypes) representing these clinical scenarios, either alone or as part of a panel, should be the next step. If utility is found, further method optimization and sequential validation is required prior to clinical use (218).

## Methodological considerations

The cohorts used for IHC analysis in papers II and III are retrospectively acquired and relatively small, especially with regards to paired lymph nodes. Thus, the statistical power for detection of prognostic markers is low. The difference in prognostic implication of SPARC expression between the current study and previous studies could in addition to patient selection and the inclusion of all subtypes of BTC in the other studies be due to low power, antibody specificity or cutpoint selection. An additional weakness is that the IHC scoring was done by a single person.

Limitations in the MS analysis in paper III include low sample size. The control group was normal bile ducts from separate patients rather than the more commonly used normal adjacent to tumor cells. Although this can introduce bias, it can also help identify additional proteins since normal appearing cells adjacent to tumors can be influenced by signaling from the tumor "field cancerization" (219). Samples were not similarly orientated to the bile duct. Discovery and verification were done using mostly overlapping samples. Due to practical considerations no additional pre-fractionation of samples was performed, which could have been used to further improve analytical performance. A housekeeping gene normalization was used for the PRM verification. Although housekeeping protein are presumably stably expressed this is not always the case and introduces a source of bias (220).

In paper IV there are some imbalances in the sample matching with cancers patients being slightly older than benign and normal cohorts. Additionally, PC samples had higher N stages than the dCCA cohort. A benefit of the benign group was that it represented "realistic" benign samples with a high enough suspicion of malignancy to motivate surgery, however this also meant it was small and heterogenous with very few individual benign diagnoses. Additionally, no relevant benign biliary controls were included. Preoperative bilirubin levels were obtained from patient charts at variating time points from surgery (and PBD) as well as THBS2/CA 19-9 measurements limiting utility. The THBS2 level in the healthy controls were higher

than previous studies due to unknown technical variation. However, the diagnostic performance was quite similar.

# Main conclusions

•Lymph node metastasis is an important prognostic factor after resection for dCCA.

•SPARC is upregulated in dCCA stroma and frequently maintained in lymph node metastases

•Several previously unknown differentially expressed proteins were identified in dCCA. Bioinformatic analysis highlighted stromal alterations in dCCA. THBS2 was validated as upregulated in dCCA cancer cells and stroma. Stromal THBS2 expression is a potential negative prognostic marker. Stromal THBS2 expression was also frequently retained in lymph node metastases

•Serum THBS2 has potential as a diagnostic biomarker for dCCA together with CA 19–9.

•PC diagnosis is more frequent in patients that do not undergo surgical resection for periampullary cancer. Standardization has improved histopathological evaluation of pancreatoduodenectomy specimens, but unmotivated regional differences exist. AC and DC has a significantly better survival following pancreatoduodenectomy than dCCA or PC. The rate of clinical misdiagnosis should be considered when preoperative therapy is an option.

•Further knowledge about the tumor biology in dCCA and optimization of clinical management is necessary to improve survival for patients with this dismal disease.

# Future perspectives

The research in CCA has taken large steps forward during the last decades, with an increased understanding of the genetic and molecular alterations involved in cancer development, and improvements in surgical technique and oncological treatment options. Despite this, the prognosis of patients remains poor. This thesis highlights areas of improvement in the clinical management of dCCA, where further research hopefully can contribute to better diagnostic precision, both in the pre- and postoperative setting. The studies of protein expression have highlighted the importance of the stromal compartment in dCCA. Further knowledge regarding the function of stroma during dCCA development and metastatic spread is merited. There is an unmet need for novel diagnostic and prognostic/predictive biomarkers for CCA/dCCA but prior to implementation of a biomarker, extensive method development and validation are required. In the short term, no new biomarkers are likely to be approved. Hopefully, some of the proteins in this thesis can be further studied to elucidate their function during and dCCA development and their biomarker potential, either alone or as part of a panel.

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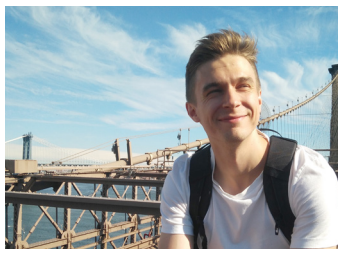
# Distal cholangiocarcinoma



**Johannes Byrling, MD,** has during his doctoral studies investigated aspects of the rare but devastating cancer distal cholangiocarcinoma - from novel biomarkers to clinical management and outcome.

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