From the Department of Clinical Science,
Intervention and Technology, Division of Surgery
Karolinska Institutet, Stockholm, Sweden

IMMUNOLOGICAL ASPECTS ON PROGNOSIS IN RESECTABLE CHOLANGIOCELLULAR CANCER

Hannes Jansson

Karolinska Institutet

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IMMUNOLOGICAL ASPECTS ON PROGNOSIS IN RESECTABLE CHOLANGIOCELLULAR CANCER
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Hannes Jansson

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Principal Supervisor:
Docent Ernesto Sparrelid
Karolinska Institutet
Department of Clinical Science,
Intervention and Technology
Division of Surgery

Co-supervisor(s):
Docent Niklas Björkström
Karolinska Institutet
Department of Medicine, Huddinge
Center for Infectious Medicine

Docent Christian Sturesson
Karolinska Institutet
Department of Clinical Science,
Intervention and Technology
Division of Surgery

Professor Annika Bergquist
Karolinska Institutet
Department of Medicine, Huddinge
Gastroenterology and Rheumatology Unit

PhD Martin Cornillet
Karolinska Institutet
Department of Medicine, Huddinge
Center for Infectious Medicine

Opponent:
Professor Ewen Harrison
University of Edinburgh
Usher Institute of Population Health
Centre for Medical Informatics

Examination Board:
Docent Mirna Abraham-Nordling
Karolinska Institutet
Department of Molecular Medicine and Surgery

Professor Marie Carlson
Uppsala University
Department of Medical Sciences

Docent Hanna Eriksson
Karolinska Institutet
Department of Oncology-Pathology

To all patients, family, friends affected by biliary tract cancer

POPULAR SCIENCE SUMMARY OF THE THESIS

Cancers of the biliary tract, cholangiocarcinoma and gallbladder cancer, are rare malignancies in many regions of the world, but is a group of malignancies with a high mortality. Most patients are diagnosed late, with unresectable tumours. Even after tumour resection surgery however, a majority of patients suffer recurrence of cancer, with only approximately half of all patients operated surviving more than three years. Previous research has indicated how preoperative systemic inflammatory markers can be associated with survival prognosis.

This doctoral research project aimed to identify and analyse prognostic factors for patients undergoing surgery for biliary tract cancer. If available before surgery, such factors could help to better select and target treatment and evaluate risks.

In four studies, prognostic associations for (I) inflammatory markers in preoperative blood plasma, (II) the underlying chronic inflammatory condition primary sclerosing cholangitis in perihilar cholangiocarcinoma, (III) immune-related proteins in blood plasma and (IV) number and distribution of multiple liver tumour lesions in intrahepatic cholangiocarcinoma, were investigated for patients undergoing liver and bile duct resection due to biliary tract cancers.

Results in brief: (I) common blood tests for inflammation were indicated as strong prognostic factors for survival in biliary tract cancer, (II) patients with perihilar cholangiocarcinoma and underlying primary sclerosing cholangitis had a similar median survival after surgery as patients without primary sclerosing cholangitis, (III) three immune-related proteins were prognostic factors in biliary tract cancer with two intrahepatic cholangiocarcinoma-specific factors demonstrated in tumour cells and immune cells in tumour tissue, (IV) multiple tumours, both close to a primary lesion and at a further distance from each other, were negative prognostic factors in intrahepatic cholangiocarcinoma.

With regard to prognostic value and any potential role in the disease process, confirmatory diagnosis-specific studies in other settings and further tissue analyses are needed.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Gallvägscancer, cancer i gallgångarna eller gallblåsan, är ovanliga cancerformer i många delar av världen, men utgör en grupp maligniteter med hög dödlighet. De flesta patienter får diagnos sent, med tumörer som inte är åtkomliga för kirurgi. Men även efter kirurgi i botande syfte drabbas majoriteten av patienterna av återkommande cancer. Endast omkring hälften av alla patienter överlever mer än tre år efter operation. Tidigare forskning har antytt att preoperativa systemiska inflammationsmarkörer kan vara förknippade med prognos för överlevnad.

Detta doktorandprojekt har syftat till att identifiera och analysera prognosfaktorer för patienter som opereras för gallvägscancer. Om fler sådana faktorer fanns tillgängliga före operation kunde de bidra till att bättre välja och inrikta behandling, och utvärdera risk.

I fyra studier undersöktes prognostiska associationer för (I) inflammationsmarkörer i plasma från preoperativa blodprov, (II) förekomst av den kroniska inflammatoriska sjukdomen primär skleroserande kolangit vid operation för perihilär gallgångscancer, (III) immun-anknutna proteiner i blodplasma och (IV) antal och fördelning av multipla tumörförändringar i levern vid intrahepatisk gallgångscancer.

I korthet visades: (I) att vanliga inflammationsmarkörer i rutinblodprov var starkt förknippade med överlevnadsprognos vid gallvägscancer, (II) en liknande medianöverlevnad efter kirurgi för perihilär gallgångscancer för patienter med och utan underliggande primär skleroserande kolangit, (III) att tre immunrelaterade proteiner var prognostiska faktorer vid gallvägscancer med två faktorer specifika för intrahepatisk gallgångscancer påvisade i tumörceller respektive immunceller i tumörvävnad, (IV) att multipla tumörer både i närhet till en primär förändring och på större avstånd från varandra var negativa prognosfaktorer vid intrahepatisk gallgångscancer.

Med avseende på prognostisk betydelse och eventuell roll i själva sjukdomsprocessen behövs bekräftande diagnosspecifika studier i andra sammanhang och ytterligare vävnadsanalyser.

ABSTRACT

Background: Biliary tract cholangiocellular cancers, cholangiocarcinoma (CCA) and gallbladder cancer (GBC), are malignancies with poor prognosis. Even after resection surgery with curative intent, a majority of patients suffer recurrence, and median overall survival (OS) remains limited to approximately three years. To improve long-term survival, a better understanding of prognostic factors is needed to individualize treatment.

Aims: *Paper I* – To evaluate the prognostic value of two preoperative inflammation-based prognostic scores, the Glasgow prognostic score (GPS) and the Modified Glasgow prognostic score (mGPS). *Paper II* – To evaluate prognostic factors and outcomes after hepatobiliary resection for perihilar CCA (pCCA) in patients with underlying primary sclerosing cholangitis (PSC), a chronic hepatobiliary inflammatory condition. *Paper III* – To identify specific immunologic prognostic markers and to further characterize the immune response in resectable biliary tract cancer. *Paper IV* – To systematically review the prognostic influence of multiple hepatic lesions in patients undergoing resection for intrahepatic CCA (iCCA), with stratification according to distribution and number of lesions.

Methods: *Paper I* was a retrospective single-centre study, including patients undergoing surgery for iCCA, pCCA or GBC (Karolinska University Hospital 2009-2017). The primary outcome was OS, secondary outcome complications. Survival was analysed by the Kaplan-Meier method and Cox regression.

Paper II was a retrospective multicentre cohort study including patients undergoing resection for pCCA at 21 centres (Europe, United States 2000-2020). The primary outcome variable was OS, secondary outcomes disease-free survival and postoperative complications. Survival was analysed by Kaplan-Meier method and Cox regression.

Paper III was a retrospective single-centre cohort study, including patients undergoing surgery for suspected biliary tract cancer at Karolinska University Hospital (2009-2017). The primary outcome variable was OS. Plasma expression of immune-related proteins was analysed in prospectively collected biobank samples by Proximity Extension Assay. Survival associations were analysed by Cox regression. Tissue expression of identified markers and receptors/ligands was analysed in independent public cohorts.

Paper IV was a systematic review and meta-analysis (Medline [Ovid] and Embase, 2010-2021). Original articles with data on OS stratified for tumour distribution (satellite lesions/other multiple lesions) and/or tumour number were included. The study was pre-registered in a public prospective register of systematic reviews and PRISMA 2020 reporting guidelines were followed.

Results: In *paper I*, the GPS and the mGPS were independent prognostic factors for overall survival after resection for biliary tract cancer (GPS≥1 hazard ratio [HR] 2.35, 95% confidence interval [CI] 1.41-3.93, mGPS≥1 HR 1.68, 95% CI 1.05-2.68). The GPS, but not the mGPS, identified an intermediate risk group.

In *paper II*, median OS was 33 months (95% CI 10-54 months) for patients with PSC-associated pCCA and 29 months (95% CI 26-32 months) for patients without underlying PSC. Patients with PSC-associated pCCA had a lower rate of well-differentiated tumours (3% vs. 16%, p=0.043), a higher rate of postoperative complications (71% vs. 44%, p=0.003) and similar 90-day mortality (12% vs 13%, p=1.00)

In *paper III*, three proteins in preoperative plasma were independently associated with OS: TRAIL/TNFSF10 (HR 0.30, 95% CI 0.16-0.56), TIE2/TEK (HR 2.78, 95% CI 1.20-6.48) and CSF1/M-CSF (HR 4.02, 95% CI 1.40-11.59). TRAIL/TNFSF10 was a positive prognostic factor in iCCA and pCCA. CSF1/M-CSF was a negative prognostic factor in iCCA and GBC. TIE2/TEK was a negative prognostic factor in GBC. In CCA tissue analysis, TRAIL-R1/TNFSFR10A receptor expression was higher in tumour and TRAIL/TNFSF10 was expressed by intratumoral lymphocytes, NK-cells and monocytes. CSF1/M-CSF was expressed by tumour-infiltrating CD8+ T-cells.

In *paper IV*, OS was decreased for iCCA patients with satellite lesions (HR 1.89, 95% CI 1.67-2.13) and multiple lesions other than satellites (HR 2.41, 95% CI 1.72-3.37). Data stratified for tumour number was limited, but indicated increased risk per additional lesion.

Conclusions: Preoperative systemic inflammatory markers were independent prognostic factors for OS after resection for BTC.

Median OS after resection for pCCA was similar for patients with and without underlying PSC. Patients with PSC had a lower rate of well-differentiated tumours and a higher rate of complications.

Three specific immunological protein markers in preoperative plasma were associated with OS in BTC, with disease-specific differences on subgroup analysis. iCCA-specific markers TRAIL/TNFSF10 and CSF1/M-CSF were expressed in tumour-infiltrating immune-cells.

Satellite lesions, as well as multiple lesions other than satellites were negative prognostic factors in iCCA. The number of lesions was suggested to be a prognostic factor within the multiple lesion group.

LIST OF SCIENTIFIC PAPERS

I. **Prognostic value of preoperative inflammatory markers in resectable biliary tract cancer - Validation and comparison of the Glasgow Prognostic Score and Modified Glasgow Prognostic Score in a Western cohort.**
Jansson H, Cornillet M, Björkström NK, Sturesson C, Sparrelid E.
Eur J Surg Oncol. 2020 May;46(5):804-810. doi: 10.1016/j.ejso.2019.12.008. Epub 2019 Dec 14. PMID: 31848078.

II. **Outcome after resection for perihilar cholangiocarcinoma in patients with primary sclerosing cholangitis: an international multicentre study.**
Jansson H, Olthof PB, Bergquist A, Ligthart MAP, Nadalin S, Troisi RI, Groot Koerkamp B, Alikhanov R, Lang H, Guglielmi A, Cescon M, Jarnagin WR, Aldrighetti L, van Gulik TM, Sparrelid E; Perihilar Cholangiocarcinoma Collaboration Group. HPB (Oxford). 2021 Nov;23(11):1751-1758. doi: 10.1016/j.hpb.2021.04.011. Epub 2021 Apr 28. PMID: 33975797.

III. **Preoperative immunological plasma markers predict survival after resection for biliary tract cancer**
Jansson H, Cornillet M, Sun D, Filipovic I, Sturesson C, O'Rourke C, Andersen JB, Björkström NK, Sparrelid E.
In manuscript

IV. **Prognostic influence of multiple hepatic lesions in resectable intrahepatic cholangiocarcinoma: a systematic review and meta-analysis**
Jansson H, Villard C, Nooijen LE, Ghorbani P, Erdmann JI, Sparrelid E.
Submitted manuscript

CONTENTS

LIST OF ABBREVIATIONS

AJCC	American Joint Committee on Cancer
ANGPT	Angiopoetin
ASA	American Society of Anesthesiologists
BTC	Biliary tract cancer
CCA	Cholangiocarcinoma
CA 19-9	Carbohydrate antigen 19-9
CEA	Carcinoembryonic antigen
CI	Confidence interval
CRP	C-reactive protein
CSF	Colony-stimulating factor
CT	Computerized tomography
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
dCCA	Distal cholangiocarcinoma
DFS	Disease-free survival
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
ERC	Endoscopic retrograde cholangiography
FGFR	Fibroblast growth factor receptor
GBC	Gallbladder cancer
GPS	Glasgow prognostic score
HER2	Human epidermal growth factor receptor 2
HR	Hazard ratio
iCCA	Intrahepatic cholangiocarcinoma
IDH1	Isocitrate dehydrogenase 1
IQR	Interquartile range
ISGLS	International Study Group of Liver Surgery
MEK	MAPK/ERK kinase
mGPS	Modified Glasgow Prognostic Score
MMP12	Matrix metallopeptidase 12
MRI	Magnetic resonance imaging

N1	Lymph node metastasis
NLR	Neutrophil lymphocyte ratio
OS	Overall survival
pCCA	Perihilar cholangiocarcinoma
PCR	Polymerase chain reaction
PD1	Programmed cell death protein 1
PEA	Proximity extension assay
PET	Positron emission tomography
PGF	Placental growth factor
PLR	Platelet lymphocyte ratio
PSC	Primary sclerosing cholangitis
PVE	Portal vein embolisation
R1	Microscopically tumour positive resection margin
RNA	Ribonucleic acid
T	Tumour extension
TIE2	Tyrosine kinase with immunoglobulin-like and EGF-like domains 2
TNM	Tumour, Nodes, Metastasis; TNM classification of malignant tumours
TRAIL	TNF-related apoptosis-inducing ligand
UICC	Union for International Cancer Control
VEGFR	Vascular endothelial growth factor receptor

1 INTRODUCTION

This thesis presents the results of a doctoral research project on prognostic factors in cholangiocellular cancers: cholangiocarcinoma and gallbladder cancer. While these cancers have low incidence, with the help of regional/national specialisation, through international collaboration and by meta-analysis, we have aimed to further understand which factors could indicate or potentially affect long-term survival outcomes for patients undergoing hepatobiliary resection surgery. What motivated this project was a situation where our patients may face a combination of high surgical risk and poor long-term outcome.

With recent research indicating a prognostic value of a systemic inflammatory response in malignancy, we have had a particular focus on immunological aspects, and more broadly not only on tumour characteristics but also on patient-specific factors and possible host responses to malignancy.

The next chapter provides a literature review on current diagnosis and staging, therapeutic options and prognostic factors for patients with cancers of the biliary tract. In the subsequent chapters, the constituent research papers of the thesis are presented in detail. In the concluding chapters, the results and possible implications of this research are further discussed.

Without regional, national and international collaboration, this project would not have been possible. Our hope for the future of our patients is continued improvements in diagnostics and therapeutics.

2 LITERATURE REVIEW

2.1 BACKGROUND: CLASSIFICATION AND EPIDEMIOLOGY

Biliary tract cholangiocellular cancer: i.e. cholangiocarcinoma (CCA) and gallbladder cancer (GBC), are tumours of low incidence in many regions of the world, but is a group of malignancies with high mortality. As earlier stages of biliary tumours typically are asymptomatic, patients are often diagnosed with biliary tract cancer in an advanced stage (1-3). Only a minority of patients are diagnosed with a surgically resectable tumour (1-5), and prognosis after curative intent resection remains poor. A majority of resected patients will suffer recurrence within five years after surgery (2-10).

2.1.1 Classification of biliary tract cancer

Biliary tract cancers are classified depending on anatomic location in the biliary duct system. Intrahepatic cholangiocarcinoma (iCCA) arises from the intrahepatic bile ducts, perihilar cholangiocarcinoma (pCCA) from the right-, left or common hepatic duct, and distal cholangiocarcinoma (dCCA) from the common bile duct (1, 3, 11). Gallbladder cancers arise from the gallbladder or cystic duct (11). **Figure 1** illustrates the anatomic classification of CCA and GBC.

The different types of biliary tract cancer can be further subdivided depending on macroscopic and microscopic appearance. iCCA is the tumour type showing the highest degree of microscopic heterogeneity and is classified into two main subgroups: small bile duct type (mixed histological type) and large bile duct type (mucinous histological type) iCCA (2, 3, 12). Macroscopically, iCCA can be described as mass-forming, periductal infiltrating and intraductal growing. While small bile duct type iCCA presents as mass-forming tumours, iCCA of the large bile duct type can have periductal and intraductal growth patterns, thus exhibiting micro- and macroscopic similarities to pCCA and dCCA (3).

pCCA is anatomically categorized according to modified Bismuth-Corlette class, by location and extent of the tumour in the right-, left and common hepatic ducts (13). Bismuth-Corlette type 1 tumours are located in the common hepatic duct; type 2 tumours reach, but do not extend above the confluence of the right- and left hepatic ducts; type 3a and 3b tumours extend into the right- and left hepatic ducts, respectively; while type 4 tumours engage both the right and left hepatic ducts (13-15).

dCCA occurs in the common bile duct, below the confluence of the cystic duct with the common hepatic duct. However, common bile duct tumours not engaging the upper pancreatic border have also been described as ‘mid-bile duct cholangiocarcinoma’ (16, 17), a sub-group that some authors have proposed could include Bismuth-Corlette type 1 pCCA tumours (16).

While over 95% of biliary tract cancers are of a cholangiocellular type, other histologic tumour types which occur rarely, such as mesenchymal tumours and squamous cell

carcinomas, are not further described in this literature review (15). Ampullary carcinomas, occurring below the confluence of the common bile duct and the pancreatic duct and displaying intestinal, pancreatobiliary or mixed-type histology (18, 19), were also beyond the scope of the project and review of this thesis.

2.1.2 Epidemiology of biliary tract cancer

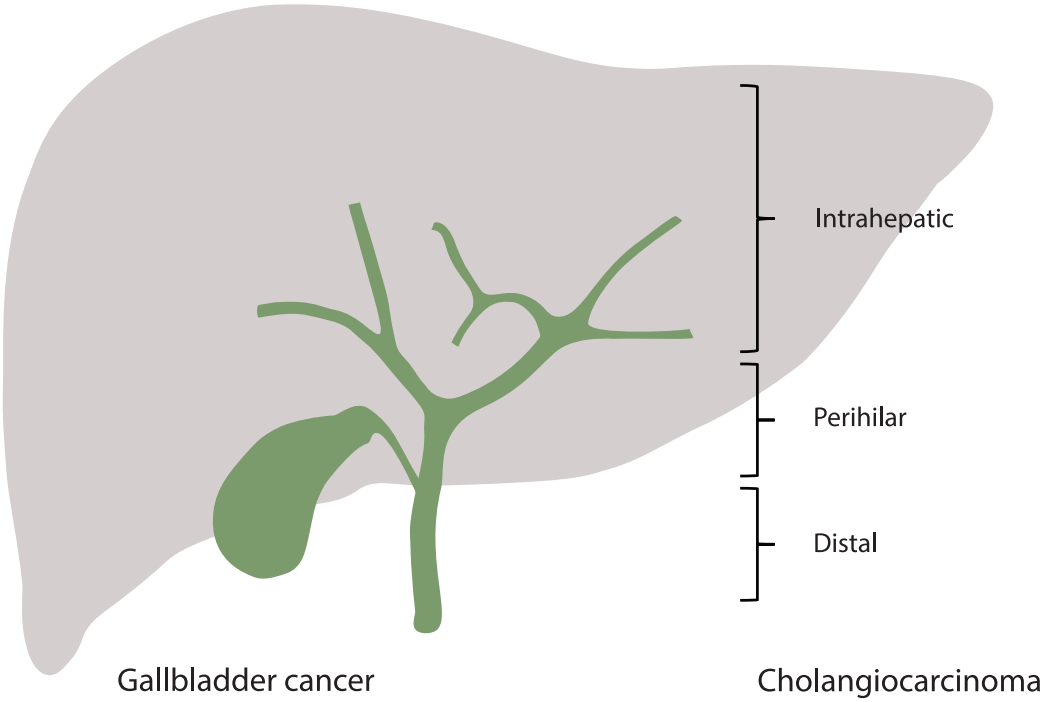
Due to endemic risk factors, such as liver flukes in Southeast Asian countries (20) and viral hepatitis in parts of Asia and Africa (3, 21-23), biliary tract cancers are more common in some countries than in others. In Central and Northern Thailand, incidence of cholangiocarcinoma is over 10 cases per 100 000 persons per year, due to chronic parasitic infection with the Opistorchis Viverrini liver fluke (3, 15). In regions of China and South Korea, chronic hepatitis B virus infection is prevalent, with an incidence of cholangiocarcinoma of 7-9 cases per 100 000 persons per year (3, 23).

Gallbladder cancer is associated to cholelithiasis and chronic inflammation (15, 24, 25). Due to this association to gallstones, gallbladder cancer is more common among women than men (24, 25). Gallbladder cancer is also more common in populations with a high prevalence of gallstones and chronic biliary infection with Salmonella typhi (15, 25). This is the case in regions of Chile, were the incidence of gallbladder cancer is the highest in the world (12 cases per 100 000 persons per year among men, and 27 cases per 100 000 persons per year among women) (24, 25).

In North America and Europe, biliary tract cancer incidence is below 6 cases per 100 000 persons per year (3, 26), making it a rare cancer in these parts of the world (27). Incidence of cholangiocarcinoma in North America and in European countries is reported in ranges from approximately 0.5-3 cases per 100 000 individuals per year (3). Globally averaged, incidence of gallbladder cancer is reported below 2 per 100 000 individuals per year (26). In Sweden, incidence of biliary tract cancer is approximately 4 cases per 100 000 individuals per year among men, and 5 cases per 100 000 individuals per year among women (28).

Globally, the chronic inflammatory liver disease primary sclerosing cholangitis (PSC) is a very strong risk factor for cholangiocarcinoma and gallbladder cancer (29, 30). PSC patients have a life-time risk of cholangiocarcinoma of up to 20%, i.e. up to 400 times the risk in the general population (29, 31). However, even in regions of high PSC prevalence like Scandinavia, PSC prevalence is below 0,02% in the population, and most cases of biliary tract cancer are unassociated to PSC (3, 29, 32). PSC prevalence is highest in northern Europe (up to 0,016% in Sweden) and lower in southern Europe and Asia (29). In North America, PSC prevalence has been reported in ranges from 0,004-0,014%, with equal prevalence among African Americans and white Americans (29).

Figure 1: *Biliary tract cancers – cholangiocarcinoma and gallbladder cancer*



2.2 DIAGNOSIS AND STAGING

2.2.1 Presenting symptoms and diagnosis

The most common presenting symptom in biliary tract cancers is biliary obstruction with jaundice (15). In more distal biliary tumours, this can occur earlier, while proximal tumours of the gallbladder or intrahepatic bile ducts typically only cause symptom-giving obstruction in later stages (15). iCCA is more strongly associated to chronic liver disease than other biliary tract cancers (3). Approximately 20-25% of iCCA cases are found incidentally (3), e.g. with diagnostic or follow-up imaging and laboratory assessment of asymptomatic patients (15). Later stages of biliary tract cancer may debut with general symptoms: fatigue, malaise, weight loss and pain; this presentation however does not necessarily indicate metastasized disease (3, 15).

iCCA most often appears as a nodular mass on imaging, with a solitary tumour or multiple lesions (3, 11). Contrast-enhanced magnetic resonance imaging (MRI) and computerized tomography (CT) can be used for staging and preliminary diagnosis, and to attempt to radiographically differentiate iCCA from hepatocellular carcinoma (1, 3). As biopsies of liver masses may be inconclusive, and theoretically could cause tumour seeding, a strategy of surgical resection on suspicion of iCCA is often recommended in patients fit for surgery (3, 33).

pCCA and dCCA typically occur as bile duct strictures, requiring cholangiography (3, 15, 34). Strictures are best non-interventionally characterized by MRI with a cholangiopancreatography protocol (MRCP) (3). Biliary obstruction can be relieved with endoscopic retrograde cholangiopancreatography (ERCP) or percutaneous transhepatic cholangiography (PTC). Cytologic or histologic diagnosis can be sought with ERCP. Sensitivity of cytology, histology and imaging is however low, and in more than one third of patients, surgical resection is undertaken without a confirmed diagnosis of malignancy (3).

2.2.2 Preoperative imaging and staging

Radiographic staging of biliary tract tumours, with assessment of the vascular and biliary anatomy of the liver, portal and arterial circulation, as well as regional and distant lymph nodes, can be made with contrast-enhanced CT and/or MRI (3, 15, 33). While CT with multiphase contrast is a standard method for staging and to evaluate vascular involvement, MRI with MRCP can have the potential to provide additional information about the biliary extension of the tumour (35-38). The role of positron emission tomography (PET) combined CT or MRI (PET-CT, PET-MRI) in preoperative staging of biliary tract cancer, remains an area of research. Previous studies have indicated that while PET-imaging is more sensitive compared to CT alone in detecting distant metastasis (39, 40), the benefit of general preoperative PET-imaging is unclear (41). Inconsistent data has been presented on whether PET-imaging is more sensitive than CT alone in detecting regional lymph node metastasis (39, 40, 42). PET-MRI could have some advantages compared to PET-CT with regard to hepatobiliary imaging and staging, but any clinical added value of this novel technology remains to be demonstrated and cost-benefit analyses to be performed (43).

Staging systems aim to incorporate information on the extent of a malignancy, and thus convey prognostic information to guide therapy, preoperatively (clinical staging) and postoperatively (pathological staging). The American Joint Committee on Cancer (AJCC)/Union for International Cancer Control (UICC) staging system is the most widely used standard to establish a classification of tumour stage, according to primary tumour extension (T), presence and extent of regional lymph node metastasis (N) and presence of distant metastasis (M) (11, 44). Examples of other systems, also employing TNM categories, are the classifications of extrahepatic BTC from the Japanese Society of Hepato-Biliary-Pancreatic Surgery (JSHBPS) (45) and of iCCA from the JSHBPS or from the Liver Cancer Study Group of Japan (46-49). For categorisation of tumour extension in iCCA, these different systems all incorporate tumour size, number of lesions and degree of invasion of adjacent structures; with differences between the systems in requirements, combinations and cut offs for each T category. While the two Japanese iCCA staging systems use a 20 mm size category cut off (46, 49), the AJCC/UICC system has a size limit of 50 mm (11). For the separate T-categorisations of pCCA, dCCA and GBC, the AJCC/UICC system and the JSHBPS system differ in how depth and extent of invasion is described, with differences both in regard to subcategories (pCCA) and to categories/subcategories (dCCA, GBC) (11, 45). Concerning tumour extension in pCCA, the Memorial Sloan-Kettering Cancer Center

proposed a T-categorisation on the basis of radiological signs of vessel involvement and biliary extension beyond the right/left hepatic ducts (50-52). A more recent, comprehensive classification for pCCA, including biliary and vascular extension, was proposed by the International Cholangiocarcinoma group in 2011 (53).

2.3 SURGICAL RESECTION

2.3.1 Surgical resection of cholangiocarcinoma

Patients with resectable iCCA are operated with hepatic or (in cases with a more central tumour) hepatobiliary resection, with the goal of achieving a microscopically radical resection margin while leaving a sufficient remnant liver (35). In pCCA, a major hepatectomy (hemihepatectomy or extended hemihepatectomy) with resection of the extrahepatic bile duct is generally necessary (35, 54, 55). In highly selected patients with a more extensively ductally infiltrating pCCA, a combined hepatobiliary and pancreatoduodenal resection may be considered, although associated with a considerably increased morbidity and mortality (56, 57). Patients with resectable dCCA are operated with pancreatoduodenectomy (58). While selected patients with a mid-common bile duct cholangiocarcinoma can be considered for isolated resection of the extrahepatic bile duct (16, 17), a multicentre study from Korea and Japan indicated a possible survival benefit with pancreatoduodenectomy even in early stage tumours (17).

To secure an adequate future liver function, a future liver remnant volume of 25-30% is generally sought before hepatic resection. However, in patients with biliary tract cancer, cholestasis and/or pre-existing liver disease may decrease liver function and thus require a larger remnant volume (35, 59). A preoperative future liver remnant volume of at least 40% has been suggested for patients with pCCA (35, 60). Portal vein embolization of the hemiliver that is to be resected, is the standard approach to increase the volume of the contralateral future liver remnant. Importantly, patients suffering from significant cholestasis and decreased liver function also have impaired regeneration, necessitating adequate biliary drainage of the future liver remnant to be undertaken prior to portal vein embolization (35).

The role and appropriate extent of lymphadenectomy in cholangiocarcinoma is debated. While lymph node metastasis is a negative prognostic factor in iCCA, lymphadenectomy has not been associated to improved survival in national registry data, multicentre cohorts or in a meta-analysis of retrospective studies (61-63). Instead, lymphadenectomy is described mainly as a staging procedure (35), providing additional information, especially for patients with radiologically node-negative disease before surgery (63, 64). To the degree that an upstaging of patients from radiological N0 to pathological N1 can direct additional efficacious therapy (e.g. adjuvant chemotherapy), survival benefits have been proposed (63, 64). Additionally, a possible survival benefit with lymphadenectomy has been suggested in subgroup analyses and in studies using multivariable analyses or matching, with the purpose to adjust for baseline differences in other prognostic factors between patient groups selected for hepatobiliary resection with and without lymphadenectomy (62, 63, 65). The situation is

similar for patients with pCCA, where lymphadenectomy primarily has been described as an important part of adequate staging (35). With intraoperative frozen section, the presence and extent of regional and non-regional lymph node metastasis can be evaluated, with the purpose to guide decision-making about resection for patients with borderline resectable tumours and/or a high surgical risk (55).

2.3.2 Surgical resection of gallbladder cancer

In gallbladder cancer, the extent of resection is determined by the tumour extension stage. While early tumours, diagnosed incidentally after cholecystectomy, may be radically resected with the index operation, a re-resection including regional lymphadenectomy is recommended for patients with incidental tumours stage T1b or above (66, 67). Apart from removing any potential residual tumour, re-resection with lymphadenectomy can provide adequate staging data to upstage a patient with T1bNX after index surgery and T1bN1 after re-resection from TNM stage I to TNM stage IIIb (68). Improved staging for such patients could improve survival by guiding adjuvant therapy choices (68). Possibly, an extended clearing of regional metastatic nodes could also reduce the risk of nodal recurrence, a common recurrence site in resected early stage gallbladder cancer (67-69). For patients with gallbladder cancer of a higher tumour extension stage, extended hepatobiliary resections as in pCCA can be indicated (70, 71). However, outcomes after extended resections including pancreatoduodenectomy for gallbladder cancer are poor, with survival inferior to that of patients with cholangiocarcinoma (56, 57).

2.4 MULTIMODAL TREATMENT: ADJUVANT AND NEOADJUVANT THERAPY IN BTC

The evidence base for indications and suitable treatment regimens of adjuvant chemotherapy in BTC is growing. In 2019, results from the randomized controlled BILCAP trial were published (72). BILCAP was a United Kingdom multicentre trial, comparing oral capecitabine (a fluoropyrimidine prodrug, antimetabolite agent, established in gastrointestinal oncologic treatment) to observation only (72). 447 patients were included: 19% had iCCA, 29% pCCA, 35% dCCA and 18% GBC. Disease types were equally distributed in the treatment and control arms. Inclusion criteria were: macroscopically radical resection and physical performance status 0 (fully active) or 1 (capable of light work/housework) according to the World Health Organization/Eastern Cooperative Oncology Group scale (73). Exclusion criteria were unresolved biliary obstruction, any previous chemotherapy for BTC and incomplete recovery after surgery. The primary endpoint was overall survival (OS) according to intention-to-treat analysis. Secondary endpoints were OS according to per-protocol analysis, recurrence-free survival (RFS) and quality of life. There was no significant difference between groups for the primary endpoint, with a median OS of 51 months (95% confidence interval 35-59 months) in the treatment group, and 36 months (95% confidence interval 30-45 months) in the control group ($p = 0.097$). There was a significant difference in intention-to-treat analysis of RFS ($p = 0.033$), and in per-protocol analysis of OS ($p = 0.028$). Power and sample size calculations for the BILCAP study had assumed a 2-year survival rate

of 20%, but at 2-year follow-up OS in the control group was 60%, indicating fewer events and a lower statistical power than anticipated. Serious adverse events were approximately twice as common in the capecitabine arm (21%), with the most common events being toxic skin reaction with dysesthesia, diarrhoea and fatigue. In quality of life measures, patients in the treatment group had significantly inferior scores for social functioning and more symptoms of peripheral neuropathy.

A multinational randomized controlled trial comparing intravenous gemcitabine (a pyrimidine prodrug, antimetabolite agent) and cisplatin (a platinum-based, alkylating like agent) to capecitabine as adjuvance is now in recruitment phase (ACTiCCA-1) (74). Two smaller randomized controlled trials studying gemcitabine as adjuvance, alone or in combination therapy, have yielded negative results: the French PRODIGE 12-ACCORD 18 trial (gemcitabine and oxaliplatin) (75), and the Japanese BCAT trial (gemcitabine) (76). Protocols including gemcitabine had previously been established for palliative oncological treatment of unresectable BTC (77).

Neoadjuvant therapy for cholangiocarcinoma has been established in the highly selective Mayo liver transplantation protocol for pCCA (78), but not for resection surgery (79). According to the Mayo protocol, chemoradiation (radiotherapy and intravenous fluorouracil, a fluoropyrimidine, followed by oral capecitabine) is given for patients fulfilling pCCA-specific criteria for transplantation, i.e. early stage tumours in PSC, or small but unresectable tumours (tumour size < 3 cm, no evidence of lymph node metastasis) (78). A retrospective observational study comparing outcomes for patients resected and patients included for transplant within the Mayo protocol in 10 United States institutions, showed a survival benefit for neoadjuvant treatment and transplantation, also in intention-to-treat analysis and when comparing patients with tumours < 3 cm and lymph node negative-disease. Importantly, this study was observational and retrospective, with risk of selection bias, and the resection and transplantation arms differed in several characteristics, among them age (resected patients were older). To study neoadjuvant chemotherapy for resectable BTC, a randomized controlled multicentre phase 3 trial of gemcitabine and cisplatin before resection, compared to up-front resection, is currently in recruitment phase in Germany (80).

2.5 ONCOLOGICAL THERAPY FOR ADVANCED OR RECURRING BTC

In the setting of locally advanced or metastatic BTC, systemic chemotherapy with gemcitabine and cisplatin was established as first-line treatment by the ABC-02 phase-III trial in 2010 (77). With single agent gemcitabine, median OS was 8.1 months, while with gemcitabine plus cisplatin a median OS of 11.7 months was reached ($p<0.001$). Serious adverse events occurred in 69 percent of patients with single agent gemcitabine and 71 percent of patients treated with gemcitabine plus cisplatin. Hematologic toxicity, abnormal liver function, infection and fatigue were the most common adverse events (77). The same year, a smaller single-centre phase-III trial evaluating gemcitabine and oxaliplatin (a platinum-based alkylating like agent) in patients with advanced GBC, showed a median OS of 9.5 months for patients with gemcitabine plus oxaliplatin and 4.5 months for patients

with best supportive care (p=0.039) (81). A systematic review of phase-II and -III studies indicated that cisplatin in unresectable BTC could confer a survival advantage compared to oxaliplatin, but with increased toxicity (82). Recently, the ABC-06 phase-III trial evaluated fluorouracil with folinic acid plus oxaliplatin and active symptom control in comparison to active symptom control alone, in patients with disease progression on first-line treatment with gemcitabine and cisplatin for advanced BTC. Median OS was 6.2 months in the treatment group (95% confidence interval 5.4-7.6) and 5.3 months (95% confidence interval 4.1-5.8) in the active symptom control group, with a statistical significance when adjusting analysis for prespecified stratification factors: disease stage, platinum sensitivity and serum albumin concentration (p=0.031) (83). Severe adverse events occurred in 69% of patients in the treatment group and 52% of patients with active symptom control (83). A phase-III trial is now ongoing, comparing gemcitabine plus cisplatin to fluorouracil with folinic acid, irinotecan plus oxaliplatin as first-line therapy (84). Also, with regard to first-line therapy in advanced BTC, a phase-II trial recently evaluated adding nanoparticle albumin-bound paclitaxel (nab-paclitaxel) to gemcitabine and cisplatin, with a median OS of 19.2 months and serious adverse events in 58% of patients (85). The treatment protocol in this later trial was adjusted with dose-reductions due to the rate of hematologic adverse events (85). A phase-III trial comparing gemcitabine and cisplatin with or without nab-paclitaxel as first-line therapy is now ongoing (86).

For advanced iCCA without distant metastases, addition of locoregional therapy by hepatic artery infusion chemotherapy (HAI), has been investigated in a single-centre phase-II trial, with HAI administration of floxuridine (a fluoropyrimidine prodrug) plus systemic gemcitabine and oxaliplatin (87). Out of patients undergoing HAI pump implantation surgery in this trial, 9.5% could not initiate HAI treatment due to hyperbilirubinemia, disease progression or hepatic artery dissection. Median OS in the per-protocol cohort of patients starting HAI treatment was 25 months, and 11% of patients withdrew due to grade IV adverse events (portal hypertension, gastroduodenal artery aneurysm, subcutaneous pump pocket infection). In the same study, results from a smaller single-centre phase-I/II trial was reported with two-year OS 40% (no median OS reported), and where 30% of patients withdrew due to grade IV adverse events (gastroduodenal artery aneurysm, HAI catheter extravasation and hyperbilirubinemia) (87). Only low-moderate level evidence is available for locoregional therapies in advanced iCCA, with HAI, transarterial chemo-embolisation and selective internal radiation therapy studied in the settings of second-line treatment or first-line combination treatment (88). Ablation and external beam radiotherapy have been investigated in unresectable iCCA, with observational studies reporting ablation in the setting of small lesions (lesion size range 15-44 mm, 11 studies) and in a majority of patients in the setting of recurrence after surgery (51%, 10 studies) (88). With external beam radiotherapy, a majority of patients were treated with concomitant systemic chemotherapy (73%, 6 studies) (88).
A phase-II trial of external radiotherapy with high-dose proton beam for patients with locally advanced or recurrent iCCA without distant metastases (lesion size range 22-109 mm),

showed a median OS of 22.5 months, with 7.7% of patients suffering severe radiation related toxicity (liver failure, hyperbilirubinemia, gastric ulcer) (89).

2.6 TARGETED THERAPIES IN BTC

Through genetic mutational profiling and genome sequencing, actionable mutations have recently been found in a subset of patients with iCCA. With regional variations in frequency, approximately 10-15% of patients with iCCA have been found to have isocitrate dehydrogenase 1 (IDH1) mutations and fibroblast growth factor receptor 2 (FGFR2) gene alterations respectively (90-94). In approximately 5% of patients these mutations have been found to overlap (95).

In the setting of advanced CCA with progression on second line therapy and with IDH1-mutations on mutation profiling, a randomized placebo-controlled trial of the mutant-IDH1 inhibitor ivosidenib showed significantly prolonged median progression-free survival in intention-to-treat analysis (2.7 vs. 1.4 months, $p<0.0001$) (96). There was no significant difference in median overall survival in the intention-to-treat population (10.8 vs. 9.7 months, $p=0.06$), while in a pre-specified analysis, adjusting for cross-over from the placebo group to the active group, a significant overall survival difference was seen (10.8 vs. 6.0 months, $p=0.0008$) (96). Serious adverse events (most commonly hyperbilirubinemia, electrolyte disturbances and ascites) occurred among 30% of patients in the active group and 22% of patients in the placebo group (96).

A phase-II trial of the FGFR-inhibitor pemigatinib in the setting of advanced CCA with progression on systemic therapy, found an objective radiological response in 35.5% of patients with FGFR2 fusions/rearrangements and stable disease in 47% of patients (97). The median follow-up for the subgroup with actionable FGFR2 fusions/rearrangements was 15.4 months, and the median duration of response 7.5 months (97). Among all patients, with or without an actionable FGFR2 fusion/rearrangement, 45% had a serious adverse event, most commonly abdominal pain, fever, cholangitis and pleural effusion (97).

While these two trials were open to patients with both intra- and extrahepatic CCA, the majority of patients with an actionable mutation had iCCA (99% in the IDH1-inhibitor trial and 98% in the FGFR-inhibitor trial) (96, 97). Currently, a randomized controlled trial of pemigatinib FGFR-inhibition versus gemcitabine plus cisplatin as first-line treatment for patients with advanced CCA and FGFR2 rearrangement, is in recruitment phase (98). A selective FGFR2-inhibitor, infigratinib, is also being studied in a randomized controlled trial in the same setting (99).

Previously, other targeted therapies including EGFR-, VEGFR-, MEK-, HER-, tyrosine kinase- and immune-checkpoint inhibitors have been tested in advanced BTC with mixed results, and limited evidence of effect in subgroups of patients (2, 100). In the KEYNOTE-028 and KEYNOTE-158 phase I/II trials of the PD1-inhibitor pembrolizumab, the response rate for patients with advanced BTC was 6% and 13% respectively (101). Previously, immune-checkpoint inhibitors have been demonstrated to give a better response in patients

with DNA mismatch repair deficient tumours, as evidenced by micro satellite instability (102). Only one out of 128 BTC patients in the KEYNOTE-028 and KEYNOTE-158 trials had tumours with high micro satellite instability (101). In a United States multicentre phase II trial, patients undergoing second line treatment with the PD1-inhibitor nivolumab had a response rate of 11% on central independent review, with significantly better progression-free survival seen in the subgroup of patients with PD1-ligand 1 expression in tumours ($p<0.001$), compared to patients with PD1-ligand 1 negative tumours (103). Similarly, in a Japanese multicentre phase I trial of nivolumab, as second-line monotherapy or combined with gemcitabine plus cisplatin as first-line therapy, indications of higher response rates were seen for patients with PD1-ligand 1 positivity, however not statistically significant (104, 105). In this later trial, only one out of 60 patients had tumours with high microsatellite instability, and response rates were 3% with monotherapy and 37% with combination therapy. Ninety percent of patients with the combination therapy suffered severe adverse events, most often neutropenia, compared to 10% of patients with nivolumab monotherapy (105). In a multicentre phase I study from Japan, Korea and Taiwan, a combination therapy of PD1-inhibitor durvalumab with cytotoxic T-lymphocyte-associated protein 4 (CTLA4) inhibitor tremelimumab showed a response rate of 11% as a secondary endpoint (106). The primary endpoint was safety, with 15 of 65 patients (23%) experiencing severe adverse events deemed treatment related, including one death due to drug-induced liver injury (106). In a Korean phase II trial of combination first-line therapy with durvalumab and gemcitabine plus cisplatin, with or without addition of tremelimumab, similar response rates were seen in the groups with (70%) and without (72%) addition of the CTLA4 inhibitor (107). In this study, 77% of patients required dose-reduction of chemotherapy, and 53% of patients suffered neutropenia as a severe adverse event, with no mortality (107). An interim analysis of the ongoing international phase III randomized placebo-controlled TOPAZ-1 trial, of first-line combination therapy with gemcitabine plus cisplatin with addition of durvalumab or placebo, showed a statistically significant increase in overall survival with the addition of durvalumab (HR 0.80, 95% CI 0.66-0.97, $p=0.021$), during a median follow-up of 14 months for the durvalumab group and 13 months for the placebo group (108). There was no significant difference in median overall survival (durvalumab 12.8 months, 95% CI 11.1-14.0; placebo 11.5 months, 95% CI 10.1-12.5) (108). The response rates were 27% in the durvalumab group and 19% in the placebo group. The rates of severe adverse events and discontinuation of treatment were similar in the durvalumab (76% severe adverse events, 9% discontinuation) and placebo groups (78% severe adverse events, 11% discontinuation) (108).

2.7 REPEATED RESECTION FOR RECURRING BTC

The majority of studies reporting outcomes after re-resection for recurrent BTC have been single-centre case series with less than 20 patients, and with most studies reporting re-resection in the setting of iCCA recurrence (109, 110). Two recent retrospective studies have reported multicentric outcomes after re-resection for recurring iCCA (111, 112). In an international study including 15 centres from North America, Europe, Asia and Australia, median OS for 88 patients undergoing curative-intent re-resection was 48.6 months, while

median OS for all other patients with recurrence, including patients receiving best supportive care was 9.7 months (111). Approximately half of all patients with recurrence in this study suffered isolated hepatic recurrence, either at the resection margin or at a separate intrahepatic location. A survival analysis stratified for recurrence pattern indicated similar median OS for patients with recurrence in the resection margin as for patients with a distant recurrence (18.8 months and 23.5 months respectively), whereas patients with iCCA recurrence at a separate hepatic location had a significantly higher median OS of 51.5 months ($p<0.001$). The authors interpreted such hepatic non-margin recurrences as possible de novo recurrence on the background of underlying liver disease (111). In a German multicentre study with 18 participating centres (2008-2017), outcomes were reported for 113 patients undergoing re-resection for iCCA recurrence (90). Median OS after re-resection was 65.2 months, while median OS in patients with iCCA recurrence found unresectable at re-exploration was 46.7 months ($p=0.002$). The rate of complications Clavien-Dindo Grade III or higher was 24%, and the postoperative mortality after re-resection was 3.5% (112).

In a retrospective single-centre study (2007-2011) comparing outcomes after re-resection (n=32) or ablation (n=77) for patients with hepatic recurrence after surgical resection for iCCA, median OS was 20.3 months after re-resection and 21.3 months after ablation ($p=0.996$) (113). In the subgroup of patients with a recurrent lesion larger than 30 mm, survival was better after re-resection compared to ablation ($p=0.037$, no median OS reported). An increased rate of major complications was reported after re-resection (47% vs. 4%) (113).

Regarding re-resection in the setting of recurrent BTC of other types than iCCA, three single-centre studies including at least 10 patients with extrahepatic CCA or GBC have been reported (114-116). In the largest of these retrospective single-centre studies, three- and five-year OS for 74 patients treated with re-resection after recurrence of BTC (1991-2010) was 37% and 14% respectively, compared to 3% and 0.3% for patients with recurrence and no re-resection (116). The median time to recurrence from the primary resection was longer in the re-resection group (16.8 months vs. 9.6 months, $p<0.001$). Seventy-three percent of patients undergoing re-resection had a recurrence of CCA (iCCA 9.5%, pCCA 46%, dCCA 17.5%), while 27% had a recurrence of GBC. No statistically significant difference in survival after re-resection was seen when comparing patients with recurrence of CCA and recurrence of GBC ($p=0.939$), however, sample size was small and five-year OS limited (CCA 18%, GBC 6%) (116). A smaller single-centre study (2000-2014), reported outcomes for 27 patients operated with re-resection for recurrence of extrahepatic CCA (n=18) or GBC (n=9) (114). Median OS was 21.6 months after re-resection compared to 9.5 months for patients with recurrence and no re-resection ($p<0.01$). The rate of complications Clavien-Dindo grade III or higher after re-resection in this study was 7%, with no postoperative mortality. No statistically significant difference was seen when comparing survival for patients with recurrence of extrahepatic CCA and GBC ($p=0.26$), however with a clearly limited sample size and with low five-year OS in the CCA group (extrahepatic CCA 12%, GBC 43%) (114). In the third retrospective single-centre study (1995-2010), 27 patients were treated surgically for recurrent CCA (iCCA 18.5%, pCCA 33.3%, dCCA 48.1%) with a median OS of 18.9

months, compared to a median OS of 7.7 months for patients with recurrence and no re-resection (115). The median time to recurrence from the primary resection had been 15 months in the surgically treated recurrence group, and 10 months in the group with no re-resection (p=0.118). The proportion of patients with concomitant chemoradiotherapy was higher in the re-resection group, with no statistically significant difference in survival in a subgroup analysis stratified for oncological therapy, however clearly limited by small sample size. The survival analysis in this last study was not stratified for CCA subtype (115).

2.8 LONG-TERM SURVIVAL AFTER SURGERY

In a systematic review from 2014, including a total of more than 4500 patients resected for iCCA from both Eastern and Western centres, median OS was 28 months (8). In a recent United States national registry study, for the period 2009-2014 (1263 patients), median OS after resection for iCCA was 39 months, with evidence of improvements in survival over time when compared to earlier time periods (1992-2002: median OS 22 months, 2000-2008: 33 months) (117). In a review from 2016 of reports from both Western and Eastern centres, including a total of more than 4000 patients resected for pCCA, median OS was 34 months (118). In a 2017 meta-analysis on outcomes after resection for dCCA including 970 patients (data on median OS lacking), 5-year OS was 38% (119). The largest of the included dCCA cohorts from that meta-analysis (370 patients from 24 Japanese institutions, 2001-2010), showed a median OS of 41 months (120). The largest Western dCCA cohort in the study (229 patients, United States, 1973-2004), showed a median OS of 18 months, without a survival difference in dCCA when comparing the early and later time periods within that cohort (121). In a recent meta-analysis of more than 20000 patients, median OS after curative intent resection of GBC was 31.5 months with surgery plus adjuvant chemotherapy/ chemoradiotherapy and 19.3 months with surgery only (122).

2.9 PROGNOSTIC FACTORS

2.9.1 Clinicopathological factors

In a meta-analysis published in 2014 (57 reported cohorts, 4756 patients), negative prognostic factors for OS after resection for iCCA were: age, tumour size, multiple tumours, lymph node metastasis, vascular invasion and poor tumour differentiation (grade 3) (8). The strongest prognostic factor was lymph node metastasis (N1), with hazard ratio 2.09 in pooled univariable analysis (8).

Negative prognostic factors for OS after resection for pCCA, in a meta-analysis from 2018 (24 reported cohorts, 4599 patients) were: age, regional lymph node metastasis, lymphovascular invasion, perineural invasion, microscopic radicality of resection, vascular resection, tumour stage T3 or T4 and tumour grade 2 or 3 (123). Lymph node metastasis (N1) and tumour positive resection margin (R1) were the strongest risk factors for shorter OS in this pooled univariable analysis, with hazard ratios 1.78 and 1.77 respectively (123). For resected pCCA patients with N1-disease, long-term overall survival is limited, with a 5-year survival of 13% in a multi-institutional Western cohort (119 N1 patients) (6), and with no

patient surviving to 7 years of follow-up without recurrence in a smaller two-centre study (78 N1 patients) (9). In the former study, on multivariable analysis, negative prognostic factors for pCCA patients with N1-disease were R1 resection status and lymphovascular invasion (LV1) (6). An Italian multicentre study (70 N1 patients), including the ratio of tumour-positive lymph nodes (lymph node ratio, LNR) but not lymphovascular invasion in multivariable analysis, found LNR to be the only prognostic factor for the N1 subgroup (124).

For patients with dCCA, negative prognostic factors for OS after resection in a meta-analysis from 2017 (23 reported cohorts, 2063 patients) were: lymph node metastasis, perineural invasion, resection margin status and tumour grade 2 or 3 (119). The strongest prognostic factor in this pooled univariable analysis was R1 resection status (119).

In resected GBC, analysis of outcomes in a United States multicentre cohort from 2016 (10 institutions, 217 patients), showed resection margin status, tumour grade, tumour stage, lymphovascular and perineural invasion to be prognostic factors for overall survival on univariable analysis (10). The strongest negative prognostic factor was advanced tumour extension (T3) (10). In a large single-centre study from 2019 (South Korea, 272 patients) also providing multivariable analysis, T3 tumour extension was the most important prognostic factor for OS, with a hazard ratio of 5.6 compared to patients with T1 tumours (70).

Other than age, the prognostic factors analysed in the above-mentioned studies are postoperative, determined by pathological staging. The predictive value of preoperative radiological staging in BTC has so far been limited, because of difficulty in determining presence and extent of lymph node metastases (125).

2.9.2 Preoperative prognostic markers

The two laboratory tumour markers most established in BTC are carbohydrate antigen 19-9 (CA 19-9) and carcinoembryonic antigen (CEA) (15, 126, 127). CA 19-9 is the marker most widely used in clinical practice (126). However, the specificity of these tumour markers is limited, as levels can be increased in benign conditions and affected by cholestasis (126). As a diagnostic marker for cholangiocarcinoma, with a cut-off value of 100 U/ml for malignancy, the sensitivity of CA 19-9 has been reported to be limited to approximately 50% (126). In a large retrospective United States national registry study, increased CA 19-9 (cut-off value > 37 U/ml) was a negative prognostic factor for OS in resected iCCA (128). Similarly, in a retrospective United States national registry study, increased CA 19-9 (cut-off value > 38 U/ml) was a negative prognostic factor for OS in resected pCCA and dCCA (129). In a large single-centre study, CEA but not CA 19-9 was an independent prognostic factor for OS after resection for BTC (proportion of BTC subtypes in cohort: pCCA 43%, iCCA 41%, dCCA 9%, GBC 8%) (130).

Other laboratory prognostic markers for patients with BTC are being investigated, with circulating tumour cells, extracellular vesicles, nucleic acids (cell-free DNA, microRNA, long non-coding RNA), proteins and metabolites under exploration (127, 131, 132).

Detection of circulating cell-free DNA or tumour cells could allow the diagnosis of specific prognostic genetic alterations, such as FGFR2 mutations in iCCA (127). Prognostic value has been indicated for several microRNAs (133), with a systemic review including one study where serum miRNA-21 had similar negative prognostic association as tumour tissue-expressed miRNA-21 for patients with CCA (132, 134). Examples of circulating proteins reported to have prognostic associations in BTC are CYFRA 21-1 (135-137) and osteopontin (138, 139), however with conflicting data for osteopontin in a third study (140, 141).

Improving the prognostic understanding of preoperative staging radiology is another area of ongoing investigation. Multi-phase contrast enhanced computed tomography is commonly used in surgical planning for patients with resectable BTC, with hypovascularity and periductal infiltration as suggested risk markers for poor survival (hypovascularity) and for presence of lymph node metastasis (hypovascularity plus periductal infiltration) in patients with iCCA (142-145). Furthermore, using magnetic resonance imaging, distinct enhancement patterns (peripheral rim enhancement, diffuse hypoenhancement) and quantitative diffusion values (proportion of tumour volume with diffusion restriction, apparent diffusion coefficient) have been suggested as possible prognostic markers in resectable iCCA (142, 146-148). As previously mentioned, PET-imaging can be more sensitive for the detection of metastasis, but could also have further prognostic value. A higher metabolic tumour volume on PET-CT in patients with iCCA and a higher PET-CT maximal standard uptake value for patients with BTC have been associated with worse long-term prognosis (39, 142, 149).

Preoperative markers have been combined into prognostic models and scoring systems, to improve predictive ability (127, 141, 150). Different prognostic scoring systems including preoperative laboratory tumour markers have been proposed, but the prognostic performance in validation cohorts has been limited (4, 150). In the setting of iCCA, the Wang nomogram (151), which incorporates both laboratory (CEA, CA 19-9) and clinicopathological variables has been externally validated, however with inclusion of lymph node metastasis and invasive growth as evaluated by postoperative histopathology (150, 152). The Fudan iCCA prognostic score (153), incorporating preoperative imaging parameters (tumour size, number of lesions, tumour boundary) together with preoperative CA 19-9 and alkaline phosphatase concentrations, performed worse than the Wang nomogram according to a recent meta-analysis of validation studies, as did all other eleven models analysed and notably also the three latest versions of the postoperative AJCC/TNM staging system (150).

Preoperative inflammation-based prognostic scores have been another focus of research in different types of malignancies. Such scores, measuring inflammatory markers, are intended to also evaluate the host response to tumour disease and oncological therapy (154). A prognostic value of the so called Glasgow Prognostic Score (GPS) or modified Glasgow Prognostic Score (mGPS), calculated from albumin and CRP levels, has been validated in prospective trials for colorectal and prostate cancer patients (154). In Eastern retrospective cohorts, a prognostic value of the GPS or modified GPS in BTC has previously been shown (155-159). Examples of other inflammation-based scores are the neutrophil lymphocyte ratio

(NLR) and the platelet lymphocyte ratio (PLR). In a retrospective United States multicentre study, NLR was a significant prognostic factor in resected GBC, but not in pCCA and dCCA (160). In a smaller retrospective single-centre study from the United Kingdom, NLR was a prognostic factor for OS also after resection for iCCA (161).

2.10 CONCLUSION

In conclusion, even after curative intent resection, median overall survival for patients with cholangiocarcinoma and gallbladder cancer remains limited to approximately 30-40 months. Long-term survival outcomes depend on the specific diagnosis, risk factors and also to some degree on whether multimodal treatment (such as adjuvant chemotherapy) is given.

To improve long-term survival in BTC after resection, a better understanding of prognostic factors is needed. If available preoperatively, such factors could help to improve risk assessments, and allow stratification of patients for a more individualized treatment.

3 RESEARCH AIMS

I.

To evaluate and compare the prognostic value of two preoperative inflammation-based prognostic scores, the Glasgow prognostic score and the Modified Glasgow prognostic score for overall survival after resection for biliary tract cancer.

II.

To compare prognostic factors and outcomes after hepatobiliary resection for perihilar cholangiocarcinoma in patients with and without underlying primary sclerosing cholangitis.

III.

To identify specific immunologic prognostic markers and to further characterize the immune response in resectable biliary tract cancer.

IV.

To systematically review the prognostic influence of multiple hepatic lesions on overall survival in patients undergoing resection for intrahepatic cholangiocarcinoma, with stratification according to distribution and number of lesions.

4 MATERIALS AND METHODS

4.1 STUDY DESIGN, OUTCOMES AND PATIENT COHORTS

4.1.1 Paper I

Paper I reports a retrospective single-centre cohort study including patients undergoing surgical exploration with a diagnosis of perihilar cholangiocarcinoma, intrahepatic cholangiocarcinoma or gallbladder cancer at Karolinska University Hospital (2009-2017). The primary outcome variable was overall survival, with surgical complications as secondary outcome. Survival was analysed by the Kaplan-Meier method and Cox regression, assessed for all patients included and for patients undergoing resection. Association of preoperative inflammation-based scores with survival was assessed in multivariable models.

4.1.2 Paper II

Paper II presents a retrospective multicentre cohort study including patients undergoing hepatobiliary resection for perihilar cholangiocarcinoma at 21 centres in Europe and the United States (2000-2020). The primary outcome variable was overall survival, with disease-free survival and postoperative complications as secondary outcome measures. Survival was analysed by the Kaplan-Meier method and Cox regression. Prognostic factors and postoperative outcomes were compared between patients with and without underlying primary sclerosing cholangitis.

4.1.3 Paper III

Paper III presents a retrospective single-centre cohort study including patients undergoing surgical exploration for suspected biliary tract cancer at Karolinska University Hospital (2009-2017). The primary outcome variable was overall survival. Protein expression was analysed in prospectively collected preoperative plasma samples. Association with survival was analysed by Cox regression. The expression of identified markers and receptors/ligands in tumour tissue was further analysed in independent public cohorts.

4.1.4 Paper IV

Paper IV reports a systematic review and meta-analysis of the English language research literature (2010-2021) on outcomes after resection for intrahepatic cholangiocarcinoma. The primary outcome variable was overall survival. Systematic database searches of Medline (Ovid) and Embase were performed. Original research articles with overall survival data stratified for tumour distribution (satellite lesions/other multiple lesions) and/or tumour number, were included for review and assessed for meta-analysis. Two authors independently screened the records. The quality of studies and risk of bias were assessed with the Newcastle-Ottawa scale (162), and the GRADE framework for prognostic studies was used to assess the confidence of pooled risk estimates (163). The study was pre-registered in a public prospective register of systematic reviews and PRISMA 2020 reporting guidelines were followed (164).

4.2 DEMOGRAPHIC, CLINICOPATHOLOGICAL AND BIOCHEMICAL DATA

4.2.1 Demographic and clinicopathological variables

Demographic and clinicopathological data were retrospectively collected from electronic health records and clinical quality registries. Variables included were: age, sex, body mass index, comorbidities including PSC, diabetes (Paper III) and cirrhosis (Paper III), preoperative plasma concentrations of albumin and CRP, pre- and postoperative plasma concentrations of bilirubin, postoperative prothrombin-International Normalized Ratio, preoperative interventions such as PVE and ERCP/PTC (Paper I and II), preoperative physical status classification according to the American Society of Anesthesiologists (165), extent of hepatobiliary resection, postoperative complications according to the Clavien-Dindo classification (166), posthepatectomy liver failure as per the applied Balzan 50:50 criteria (Paper I) (167, 168), postoperative liver failure as per the International Study Group of Liver Surgery (ISGLS) criteria (Paper II) (169), postoperative bile leakage according to the ISGLS criteria (Paper II) (170), preoperative cholangitis according to the DRAINAGE trial definition (Paper II) (171), staging according to the 7th edition of the AJCC/TNM guidelines and histopathological tumour grade according to the College of American Pathologists (172-174).

4.2.2 Glasgow prognostic score/Modified Glasgow prognostic score

The Glasgow prognostic score (GPS) and the Modified Glasgow prognostic score (mGPS) were calculated from preoperative plasma CRP and albumin concentrations according to the descriptions from McMillan et al. (175). The GPS is defined as: GPS 0 if CRP ≤10 mg/L and albumin ≥35 g/L; GPS 1 if CRP>10 mg/L or albumin <35 g/L; GPS 2 if CRP >10 mg/L and albumin <35 g/L. Thus, the mGPS differs from the GPS in that it does not award a score for isolated hypoalbuminemia.

4.2.3 Immunoassay analysis of plasma protein expression

Proximity Extension Assay-analysis (PEA) uses paired oligonucleotide coupled antibodies to detect analytes in a multiplexed immunoassay (92 analytes, Immuno-Oncology I panel) (176-178). Relative quantification of protein expression is performed by polymerase chain reaction (PCR). Plasma samples for PEA were thawed on ice with 20 microliters transferred to 96 well plates. PEA was performed at a university core facility (Clinical Biomarker Facility, SciLifeLab, Uppsala University) that was blinded to all outcome data. Intraplate variability was assessed with assay-specific protein, antibody and oligonucleotide controls. Interplate variability was assessed with a panel of 92 oligonucleotide duplexes. Protein expression was expressed in Log2 scale as Normalized Protein Expression units (NPX) after normalization of PCR quantification cycle values, according to the intraplate detection and interplate controls. The analytical precision of the PEA has been validated for hyperbilirubinemia and hyperlipidaemia corresponding to approximately 8-10 times the normal upper reference values, and the Immuno-Oncology I panel has been validated for preserved precision in the

detection of 84 out of the 92 proteins also in the presence of haemolysis of up to 5-10% (178).

4.2.4 Analysis of public tumour tissue gene expression data

Differential gene expression analysis was performed in R 4.1.1 (R Foundation for Statistical Computing) and RStudio 1.4.1717 (RStudio Inc, Boston, USA) with the limma 3.50.0 package for bulk tissue microarray data (179), DESeq2 1.34.0 for bulk tissue sequencing data (180) and Seurat 4.0.4 for single-cell sequencing data (181, 182). The gene expression datasets included in Paper III were: GSE107943 (183), GSE138709 (184), GSE89749 (185), GSE26566 (186), EGAD00001001693 (187), E-MTAB-6389 (188), OEP001105 (189) and phs001404.v1.p1 (190). For GSE138709 single-cell data analysis, normalization and integration of samples was performed after filtering out cells with a percentage of mitochondrial genes above 5 and cells with gene counts less than 500 or above 3000. Cells were clustered and visualized by principle component analysis with uniform manifold approximation and projection. Annotation of clusters was performed by: mapping to CITE-seq immune cell reference data (181), use of hepatocyte, cholangiocyte, fibroblast and endothelial cell markers (184), and for malignant cells by annotation according to copy number variation scores calculated using InferCNV 1.8.1 (191), with a cut point of three for malignancy.

4.3 STATISTICAL ANALYSIS

4.3.1 Descriptive statistics, comparisons of proportions and distributions

Statistical analyses were performed using R (R 3.5.3 and 4.1.1, R Foundation for Statistical Computing; RStudio 1.1.463, 1.4.1717 and 2021.09.0, RStudio Inc, Boston, USA), SPSS Statistics v25 and v28 (IBM, New York, USA) and Olink Insights Stat Analysis (Olink Proteomics, Uppsala Sweden). Categorical variables were reported with whole numbers and proportions, continuous variables with medians and interquartile range (IQR). Proportions were compared by the Chi square test or the Fisher exact test. Distributions were compared with the Mann-Whitney U test. Significance tests were two-sided and p-values below 0.05 considered statistically significant. In volcano plot analysis of differential protein expression independent t-tests were performed, with additional non-parametric testing by Mann-Whitney U test. Volcano plot independent t-test p-values were corrected for multiple testing using the Benjamini-Hochberg method (192). In Paper III, for visualization with a correlation matrix, correlations among independent variables were assessed by Spearman's rank correlation and a hierarchical clustering of variables performed according to degree of correlation (193).

4.3.2 Survival analysis

Survival was analysed by Kaplan-Meier estimate with log-rank test and by uni- and multivariable Cox regression. In R, the survival 3.1-8/3.2-13 and rms 6.2-0 packages were employed, with the rms package used to display nomograms. The survminer 0.4.6/0.4.9 and the ggplot2 3.3.5 packages were used to display graphs. The proportional hazards assumption

was tested with time dependent covariates or assessed graphically and tested by scaled Schoenfeld residuals (194-196). Variables with significant non-proportionality of hazards were included in Cox regression models as time-dependent. Median follow-up time was calculated according to the reverse Kaplan-Meier method. In Paper II, to account for small sample size and censoring, 95% confidence intervals were calculated with the beta product confidence procedure for right censored data (197).

4.3.3 Multivariable regression models

Multivariable Cox regression was used to adjust relative risk estimates for possible confounding factors, and for multivariable prognostic models. In Paper III, to evaluate the predictive value of a multivariable regression model, concordance index (c-index) was used according to Harrell (198). For prediction of time-to-event outcomes, the c-index can be described as an equivalent to the area under the receiver operating curve (AUROC) statistic for binary outcomes: a c-index of 0.50 would indicate no predictive ability, whereas a c-index of 1.00 would indicate perfect predictive ability (198).

The complexity of a time-to-event regression model, i.e. the number of variables included, is limited by the number of events. While an increasing number of variables will provide apparently improved risk estimates within the development cohort, such a model will suffer in generalizability by being perfectly tailored to random variability in a limited number of observations, a phenomenon called over-fitting (199). To take over-fitting into account in the development of a regression model, resampling techniques can be employed to provide adjusted measures of discrimination and calibration. In Paper III, bootstrap resampling was performed with 600 resamples to provide bootstrap corrected c-indices, and bootstrap corrected calibration curves were used to assess the accuracy of predictions at specific time-points (198). The use of resampling procedures to incorporate a measure of bias due to over-fitting can be termed internal validation, in contrast to external validation i.e. replication in a second cohort.

In Paper I and Paper III, variable selection was performed on the basis of univariable significance with backward elimination applied to the multivariable model. The stopping criterion for backward elimination was p=0.10 in Paper I and p=0.157 (equivalent to the Akaike information criterion (200), uncorrected for multiple comparisons) in Paper III. Among significance based variable selection-strategies, backward elimination has been described as less biased than forward selection, however while still introducing bias with each step of selection (200, 201). In Paper III, to account for multiple comparisons in evaluation of univariable prognostic associations, Bonferroni-Holm correction of p-values was performed (202) and variables with a corrected p-value below 0.20 were included in multivariable analysis. In Paper II and III, imputation of missing data for independent variables was performed by multivariate imputation (203). For proteomics data, missing values below the limit of detection were imputed by a quantile regression method as left-censored data missing not at random (204, 205).

4.3.4 Meta-analysis

Meta-analysis was performed in Review Manager (RevMan) v.5.4 (The Cochrane Collaboration). The random effects model and inverse variance method were used to calculate pooled hazard ratios (206). Log hazard ratios with standard errors were calculated from published summary statistics according to Parmar et al. (207). From studies reporting survival proportions and relative risks as survival curves, the log hazard ratio with standard error was estimated according to Williamson et al. (207, 208). Publication bias was assessed with funnel plots and heterogeneity quantified with the I^2 statistic (209).

4.4 ETHICAL CONSIDERATIONS

The studies presented in Paper I-III in this doctoral project comprise the collection and handling of data on health as well as the analysis of biological patient tissue samples, with ethical permit granted from the Regional Ethical Review Board of Stockholm and the Swedish Ethical Review Authority. All data on health from clinical quality registries and health records has been collected and stored in pseudonymised form. Code lists containing personal identifiers were encrypted and stored separately from the data in a locked location. The patients in these studies are not expected to have any direct medical benefit from their participation in this research. Inclusion in research biobanks was made with informed consent. Biobank sample collection has been performed in connection with the collection of clinical diagnostic samples, without expected extra risk of harm for the patient. Inclusion, sample collection and curation of biobanks was performed by authorised research nurses. To assure relevant use of collected biobank samples; statistical power- and sample size calculations were performed before any analyses were initiated and analysis methods were tailored to expend as little biobank material as possible. Paper IV, as a systematic review of published literature, did not require ethical approval.

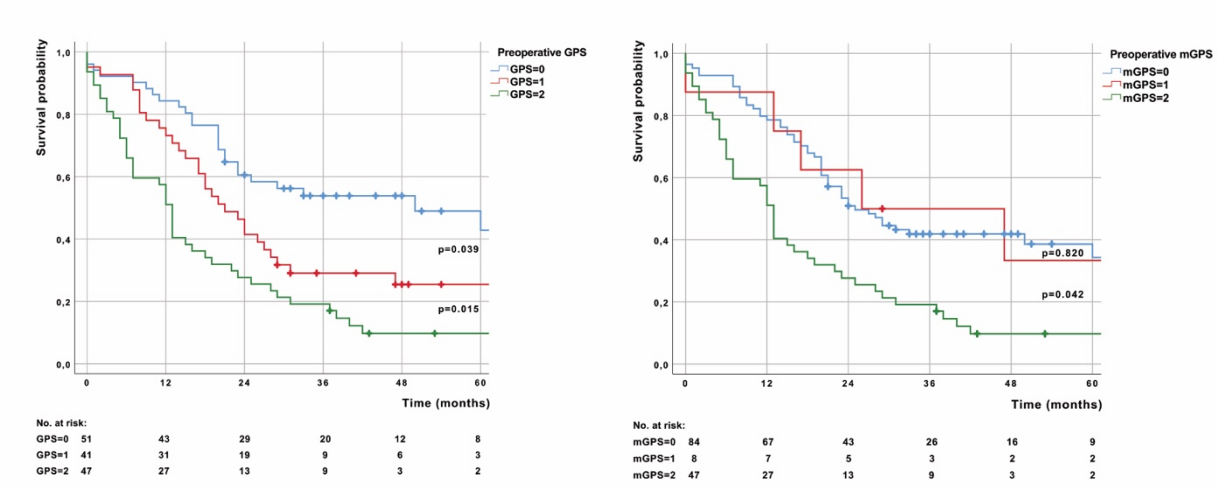
5 RESULTS

5.1 PAPER I

Two-hundred and sixteen patients with perihilar cholangiocarcinoma, intrahepatic cholangiocarcinoma and gallbladder cancer underwent surgery during the study period (January 2009 to January 2017) and were assessed for inclusion. One-hundred and sixty-eight patients, with preoperative CRP and albumin values available, were included in the study. Baseline characteristics and median overall survival were similar in the study cohort and in the group of patients excluded due to missing laboratory data. In the study cohort, 139 patients (83%) underwent resection, whereas 29 patients (17%) underwent exploration with diagnosis of unresectable disease.

The median follow-up time was 48 months (IQR 36-71 months). Median overall survival was 21 months (95% CI 17-25 months) for resected patients and 7 months (95% CI 5-9 months) for patients with unresectable disease.

Figure 2: *Overall survival stratified by GPS (left panel) and mGPS (right panel) (resected patients)*



Adapted from Paper I, reference (210): Figure 2C-D, doi: 10.1016/j.ejso.2019.12.008.
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Kaplan-Meier survival curves stratified according to prognostic scores, together with the distribution of scores among resected patients, are presented in **Figure 2** (left panel GPS, right panel mGPS, Figure 2C-D in Paper I). While both the GPS and the mGPS were associated with overall survival after resection, only the GPS identified an intermediate risk group. Median overall survival was 50 months for patients with a preoperative GPS of 0, 21 months for patients with a preoperative GPS of 1, and 13 months for patients with a preoperative GPS of 2 (GPS1 vs. GPS2: log rank p = 0.015; GPS1 vs. GPS0: log rank p=0.039). On multivariable analyses including postoperatively available risk factors (tumour

extension, lymph node metastasis) and preoperative interventions (ERC/PTC, PVE), both the GPS (hazard ratio [HR] 2.35, 95% confidence interval [CI] 1.41-3.93) and the mGPS (HR 1.68, 95% CI 1.05-2.68) remained negatively associated to overall survival (**Table 1**, Supplemental Table B in Paper I).

Table 1: *Multivariable Cox regression analyses for overall survival, including preoperative factors (resected patients)*

Multivariable analysis with GPS			**Multivariable analysis with mGPS**		
	HR (95% CI)	p-value		HR (95% CI)	p-value
GPS≥1	2.35* (1.41-3.93)	0.001	**mGPS≥1**	1.68* (1.05-2.68)	0.03
T≥3	1.91* (1.23-2.96)	0.004	**T≥3**	1.87* (1.20-2.93)	0.006
N1	1.61* (1.03-2.51)	0.04	**N1**	1.50 (0.96-2.34)	0.08
ERC/PTC	0.92 (0.53-1.58)	0.76	**ERC/PTC**	1.06 (0.62-1.83)	0.82
PVE	1.72 (0.94-3.14)	0.08	**PVE**	1.57 (0.86-2.89)	0.15
Bilirubin (>25 μmol/L)	1.11 (0.54-2.25)	0.78	**Bilirubin (>25 μmol/L)**	0.98 (0.47-2.05)	0.96

CI: confidence interval; ERC/PTC: endoscopic retrograde cholangiography and/or percutaneous transhepatic cholangiography; GPS: Glasgow prognostic score; HR: hazard ratio; mGPS: modified Glasgow prognostic score; N1: lymph node metastasis; PVE: portal vein embolization; umol/L: micromole per litre.
* P-value < 0.05.

Adapted from Paper I, reference (210): Supplemental Table B, doi: 10.1016/j.ejso.2019.12.008.

No significant association was found between GPS and postoperative complications (all complications Clavien-Dindo ≥ 3a: p=0.27; posthepatectomy liver failure: p=0.09; in-hospital mortality: p=0.19).

5.2 PAPER II

One-thousand five-hundred and twenty-four patients resected for pCCA during the study period (January 2000 to January 2020) were assessed. PSC status was available for 1230 patients (81%). One-hundred and two patients, operated with bile duct resection only, were excluded. A total of 1128 patients operated with hepatobiliary resection, with pCCA confirmed by postoperative pathology and data on PSC status, were included. The median follow-up time was 50 months.

When comparing demographic/clinicopathological data and surgical characteristics, patients with PSC-associated pCCA were found to be younger than patients with non-PSC pCCA (median age difference 17 years, p<0.001). The proportion of patients with well-differentiated tumours was lower in the PSC-pCCA group compared to non-PSC patients (3% vs. 16%, p=0.043). PSC-pCCA patients had a higher rate of preoperative biliary interventions (ERC 71% vs. 52%, p=0.029), and more often required preoperative portal vein embolization and extended resections (PVE 32% vs. 16%, p=0.013; extended resection 59% vs. 39%, p=0.019). The clinical characteristics of patients with PSC pCCA and non-PSC pCCA are presented in **Table 2** (Table 1 in Paper II).

Table 2: *Clinical characteristics of patients with PSC-associated pCCA and non-PSC pCCA.*

	Missing data n PSC / n Non-PSC	PSC n = 34	Non-PSC n = 1094	p-value
Age, years, median (IQR)	-/8	48 (36–63)***	65 (57–72)	<.001
Sex, male	–/–	23 (68%)	663 (61%)	.407
BMI, median (IQR)	5/215	23 (21–29)	25 (23–27)	.182
ASA class III or higher	1/64	11 (33%)	381 (37%)	.668
Preoperative ERC	-/2	24 (71%)*	563 (52%)	.029
Preoperative cholangitis	2/67	10 (31%)	247 (24%)	.350
Bismuth-Corlette IV	-/9	8 (24%)	242 (22%)	.866
PVE	–/–	11 (32%)*	177 (16%)	.013
Extended resection	–/–	20 (59%)*	425 (39%)	.019
Tumour size, cm, median (IQR)	9/192	3 (1.9–3.7)	2.5 (2.0–3.5)	.678
T-stage 3 or 4	2/16	13 (41%)	403 (37%)	.709
Lymph node positive	1/32	18 (55%)	445 (42%)	.148
Perineural invasion	2/146	27 (84%)	692 (73%)	.152
R1 margin status	-/10	11 (32%)	370 (34%)	.829
Moderate or poor tumour differentiation	1/71	32 (97%)*	859 (84%)	.043

Data expressed as n (%) unless otherwise stated.
pCCA: perihilar cholangiocarcinoma; PSC: primary sclerosing cholangitis; IQR: interquartile range; BMI: body mass index; ASA: American Society of Anesthesiologists physical status classification; Preoperative ERC: preoperative biliary drainage by endoscopic retrograde cholangiography (alone or in combination with percutaneous drainage); PVE: portal vein embolisation; R1: microscopically tumour-positive resection margin.
*p < .05 (Chi square test), ***p< .001 (Mann–Whitney U test).

Comparing complication rates, PSC patients more often suffered any complication Clavien-Dindo grade III or above. Postoperative percutaneous abdominal drainage due to abscess or ascites was more commonly performed in the PSC group. There were no significant differences in the rates of grade B/C posthepatectomy liver failure (21% vs. 17%, p=0.530), biliary leakage (26% vs. 20%, p=0.367) or 90-day mortality (12% vs. 13%, p=1.000) between PSC pCCA and non-PSC pCCA patients. Postoperative complications for the two groups are presented in **Table 3** (Table 2 in Paper II).

Table 3: *Postoperative complications and mortality in patients with PSC-associated pCCA and non-PSC pCCA*

	Missing data n PSC / n Non-PSC	PSC n = 34	Non-PSC n = 1094	p-value
Any complication Clavien-Dindo grade III or higher	-/4	24 (71%)*	484 (44%)	.003
Drained abscess/ascites	-/8	16 (47%)*	254 (23%)	.001
Liver failure ISGLS grade B/C	-/28	7 (21%)	176 (17%)	.530
Biliary leakage ISGLS grade B/C	-/27	9 (26%)	215 (20%)	.367
90-day mortality	–/–	4 (12%)	139 (13%)	1.000 †

Data expressed as n (%) unless otherwise stated.
pCCA: perihilar cholangiocarcinoma; PSC: primary sclerosing cholangitis; ISGLS: International Study Group of Liver Surgery.
*p < .05 (Chi square test), † Fisher's exact test.

Adapted from Paper II, reference (211) Table 2, doi: 10.1016/j.hpb.2021.04.011.
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The median overall survival was 29 months (95% CI 26-32 months) in non-PSC pCCA patients and 33 months (95% CI 10-54 months) in PSC pCCA patients (log-rank p=0.630). For non-PSC pCCA patients three- and five-year overall survival rates were 43% (95% CI 39-46%) and 27% (95% CI 24-31%), while for PSC pCCA patients three- and five-year overall survival was 39% (95% CI 19-60%) and 19% (6-41%). The Kaplan-Meier overall survival curves for PSC pCCA patients (blue) and non-PSC pCCA patients (red) are presented in **Figure 3** (Figure 2A in Paper II). Recurrence data was incomplete, with recurrence status registered for 61% of patients. Median disease-free survival was 22 months (95% CI 19-25 months) in non-PSC pCCA patients and 20 months (95% CI 11-38 months) in PSC pCCA patients (log-rank p=0.741). There was no significant association for PSC-status with survival in univariable or multivariable Cox regression analysis (univariable HR 1.11 [95% CI 0.73-1.70], age-adjusted HR 1.08 [95% CI 0.70-1.67]).

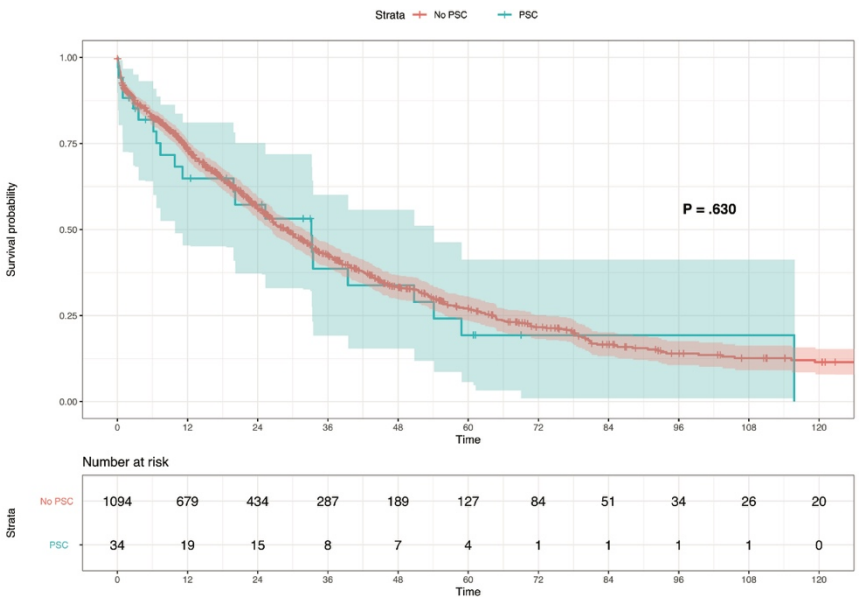

Figure 3: *Overall survival in PSC pCCA patients (blue) compared to non-PSC pCCA patients (red).*

Kaplan-Meier estimates with 95% confidence intervals (shaded bands). Time in months. P-value by log-rank test. pCCA: perihilar cholangio-carcinoma; PSC: primary sclerosing cholangitis.

From Paper II, reference (211) Figure 2A, doi: 10.1016/j.hpb.2021.04.011.
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5.3 PAPER III

One-hundred and two patients resected for pCCA, iCCA or GBC during the study period (January 2009 to January 2017) were included in the development and internal validation cohort. Selected for inclusion were all patients resected for pCCA with plasma available in biobank, together with random samples from all patients operated for iCCA and GBC (resected pCCA n=46, resected iCCA n=27, resected GBC n=29). Also included for analysis of plasma protein expression by proximity extension assay (PEA) were 27 patients with unresectable BTC at surgical exploration (unresected pCCA n=13, unresected iCCA n=5, unresected GBC n=9) and 32 patients with a benign lesion on final postoperative histopathology after resection for BTC (suspected pCCA n=10, suspected iCCA n=11, suspected GBC n=11).

Median follow-up for resected patients was 67 months (IQR 50-87 months), while all patients undergoing surgical exploration with diagnosis of an unresectable tumour were followed to death. Median overall survival after resection for BTC was 23 months (95% CI 17-29 months). Median overall survival for patients with unresectable tumours was 7 months (95% CI 0-14 months).

On univariable Cox regression analysis with correction for multiple testing, six proteins were found to be associated with overall survival with an adjusted p-value < 0.20 (unadjusted $p<0.005$). On multivariable analysis with backward elimination, three of these proteins remained independently associated with survival: TNF-related apoptosis-inducing ligand (TRAIL) HR 0.30, 95% CI 0.16-0.56, tyrosine kinase with immunoglobulin-like and EGF-like domains 2 (TIE2) HR 2.78, 95% CI 1.20-6.48 and colony-stimulating factor 1 (CSF1) HR 4.02, 95% CI 1.40-11.59. TRAIL (also: TNF superfamily member 10, TNFSF10) is one of the effector mechanisms of cytotoxic lymphocytes, natural killer cells and macrophages, CSF1 (also: macrophage CSF, M-CSF) is a regulator of monocyte proliferation, differentiation and function, TIE2 (also: tyrosine kinase endothelial, TEK) is an angiopoietin receptor and regulator of angiogenesis.

The three markers, TRAIL, CSF1 and TIE2, were not internally strongly correlated, with separation on hierarchical clustering analysis, nor were they strongly correlated to any clinicopathological variables. The strongest such association to a clinicopathological or prognostic factor was seen for CSF1 and TIE2, which were found to be moderately correlated with GPS (CSF1 Spearman's r = 0.49, TIE2 Spearman's r = 0.42), where GPS and TIE2 were grouped adjacently on hierarchical clustering analysis according to degree of correlation. In multivariable analyses including pre- and postoperative factors, the three identified markers remained significantly associated to survival, except for TIE2 in the second analysis including GPS (**Table 4**, Supplemental Table 7 in Paper III).

The predictive value for overall survival after resection for BTC, as assessed with bootstrap corrected concordance-index (c-index), for the multivariable regression model with TRAIL, TIE2 and CSF1 was 0.70. The corrected c-index for a model with postoperative pathological

risk factors (T≥3, N1, Pn1, LV1, Grade 2-3, R1) was 0.66. Calibration analyses showed that the preoperative model with TRAIL, TIE2 and CSF1 overestimated one-year survival, while underestimating survival at three and five years for survival predictions below 60% (three years) and 40% (five years). In analysis of subgroup differences, TRAIL was a prognostic factor in pCCA and iCCA, TIE2 was prognostic in GBC and CSF1 was prognostic in iCCA and GBC. Subgroup-specific prognostic models for pCCA (TRAIL, TIE2 and Glasgow prognostic score [GPS]), iCCA (TRAIL, CSF1 and GPS) and GBC (TIE2, CSF1) were evaluated with corrected c-indices 0.78 for iCCA, 0.65 for pCCA and 0.74 for GBC.

While no significant differences were found in plasma protein expression between patients with resectable and unresectable tumours, expression levels for 25 out of 78 proteins were significantly higher in samples from patients with BTC as compared to patients with benign lesions. The three proteins that had the highest area under the receiver operating curve (AUROC) value for prediction of malignancy on logistic regression were CSF1, PGF and MMP12 (AUROC=0.69 for each of the three proteins).

Table 4: *Multivariable Cox regression analyses including pre- and postoperative prognostic factors*

Variable	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**
TRAIL	0.30 (0.14-0.63)	0.001*	0.23 (0.10-0.55)	<0.001*	0.22 (0.10-0.53)	<0.001*
CSF1	5.88 (1.51-22.88)	0.011*	8.96 (1.67-48.17)	0.011*	10.61 (2.37-47.47)	0.002*
TIE2	3.69 (1.39-9.80)	0.009*	1.32 (0.36-4.84)	0.672		
T≥3	1.15 (0.62-2.13)	0.653	1.39 (0.68-2.82)	0.367	1.47 (0.76-2.82)	0.249
N1	2.28 (1.22-4.27)	0.010*	1.83 (0.91-3.68)	0.092	1.77 (0.89-3.51)	0.103
LV1	0.52 (0.20-1.34)	0.176	0.44 (0.15-1.24)	0.118	0.44 (0.15-1.25)	0.123
Pn1	0.54 (0.18-1.59)	0.263	0.98 (0.24-4.10)	0.981	1.01 (0.24-4.18)	0.989
R1	1.20 (0.52-2.76)	0.668	1.27 (0.49-3.24)	0.623	1.34 (0.54-3.31)	0.528
Grade≥2	3.84 (1.44-10.27)	0.007*	1.92 (0.60-6.17)	0.274	1.78 (0.58-5.47)	0.311
ASA≥3	1.92 (0.99-3.69)	0.052	2.20 (1.01-4.80)	0.059	2.18 (1.00-4.77)	0.051
Age (years)	1.00 (0.98-1.02)	0.876	0.99 (0.97-1.02)	0.489	0.99 (0.97-1.02)	0.508
Gender (female)	1.13 (0.62-2.06)	0.696	0.80 (0.36-1.77)	0.579	0.75 (0.36-1.58)	0.451
GPS≥1			2.28 (0.97-5.36)	0.059	2.36 (1.02-5.46)	0.044*

ASA≥3: American Society of Anesthesiologists physical status class 3-4; CI: confidence interval; Grade≥2: moderate or low tumor differentiation; GPS≥1: Glasgow prognostic score 1-2; HR: hazard ratio; LV1: lymphovascular invasion; N1: lymph node metastasis; Pn1: perineural invasion; R1: microscopically tumor positive resection margin; T≥3: Tumor extension stage 3-4; * p<0.05

With analysis of public gene expression data for bulk cholangiocarcinoma and surrounding liver tissue samples from two cohorts with paired samples (GSE107943, bulk tissue sequencing, iCCA n=30; GSE26566, tissue microarray, iCCA+pCCA n=59), three proteins were found to be consistently differentially expressed. TRAIL-receptor 1 and ANGPT2 expression was higher in tumour in both datasets, whereas TIE2 expression was lower in tumour compared to surrounding liver tissue.

On differential gene expression analysis with public single-cell RNA sequencing data for patients with iCCA, TRAIL was found to be expressed by malignant cells, monocytes, T-cells and endothelial cells. Comparing TRAIL expression in intratumoural immune cells to levels in immune cells of the same type from surrounding liver tissue, higher expression was seen in intratumoural B-cells and CD4+ T-cells, while lower TRAIL expression was seen in intratumoural monocytes. TRAIL-receptors 1 and 3 showed increased expression in malignant cells, while TRAIL-receptors 2 and 4 were expressed at higher levels in endothelial cells. TIE2 was expressed primarily in endothelial cells, while the TIE2 ligands ANGPT1 and ANGPT2 were expressed chiefly by fibroblasts. CSF1 expression was highest in CD8+ T-cells, fibroblasts and endothelial cells. Comparing intratumoural and surrounding liver immune cells, higher CSF1 expression was seen in intratumoural CD8+ T-cells and NK-cells.

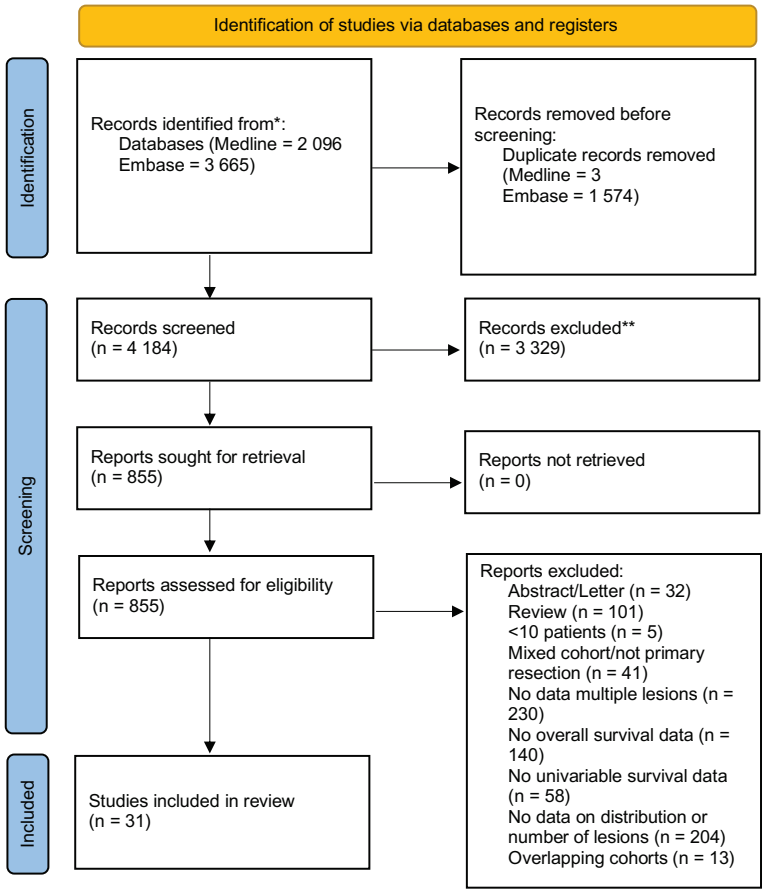
Association between the tumour tissue expression of identified markers or their receptors/ligands and survival was analysed in four public iCCA datasets (GSE107943: iCCA n=30; EGAD00001001693: iCCA n=112; E-MTAB-6389: iCCA n=72; OEP001105: iCCA n=224). TRAIL-receptor expression was significantly positively associated with disease-free survival, but not overall survival, in the GSE107943 cohort (TRAIL-R2 DFS p=0.02 and TRAIL-R4 DFS p=0.02). In the E-MTAB-6389 cohort, expression of TRAIL-R1 (OS p=0.03) and TRAIL-R4 (OS p=0.006) was significantly positively associated to overall survival. However, in the OEP001105 cohort, a significant negative association was seen between TRAIL-receptor expression and overall survival (TRAIL-R1 OS p=0.005 and TRAIL-R4 OS p=0.04). In the EGAD00001001693 cohort no association with survival was seen for TRAIL-receptors. CSF1 expression was negatively associated with disease-free survival in the GSE107943 cohort (p=0.02) and with overall survival in the EGAD00002002693 cohort (p=0.047). No other significant associations were seen.

5.4 PAPER IV

Thirty-one original research articles reporting outcomes after primary resection for iCCA, with univariable overall survival data for patients with multiple lesions and stratification according to tumour distribution or number of lesions, were included after screening of 4184 records and assessment of 855 reports. All studies included were retrospective cohort studies, 21 articles reported single-centre data (148, 151, 212-228), nine studies reported multicentre data (229-237) and one study reported a national survey (238). The PRISMA 2020 diagram for the study is presented in **Figure 4** (Figure 1 in Paper IV).

The proportion of patients with multiple lesions in the studies ranged from 15 to 47 percent. The proportion of patients with satellite lesions ranged from eight to 44 percent. The proportion of multiple lesions not including satellites ranged from three to 17 percent. Five studies reported the proportion of patients with two lesions or two to three lesions, representing 40 to 71 percent of all patients within the multiple lesion subgroup (220, 224, 228, 235, 238). Median follow-up ranged from 18 to 55 months and median overall survival for all resected patients ranged from 17 to 53 months.

Figure 4: *Prisma 2020 flow diagram*



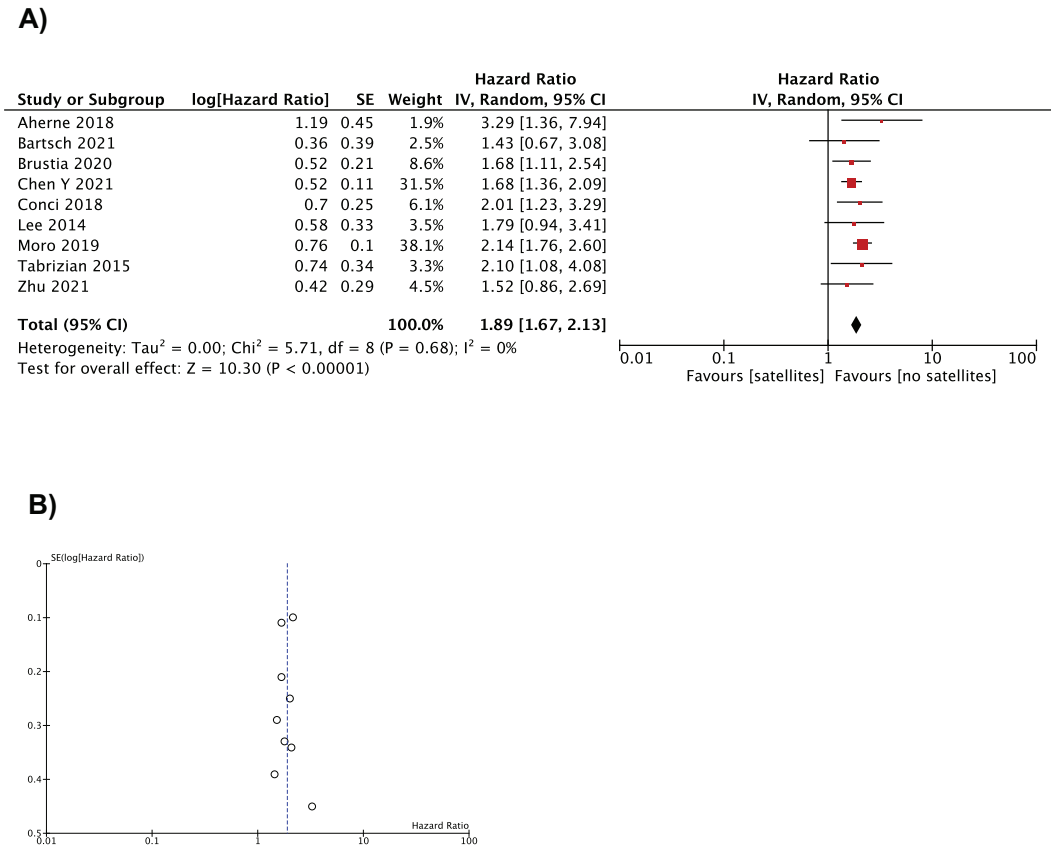
From: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. doi: 10.1136/bmj.n71

While ten studies reported overall survival outcomes with stratification according to number of lesions, heterogeneity in categorisations precluded meta-analysis (151, 220, 221, 224, 228, 230, 234, 235, 238, 239). Five studies reported relative risks for patients with two, or two to three lesions, as compared to patients with one lesion (151, 224, 228, 230, 239), with one of the five studies finding a statistically significant risk increase (2-3 lesions vs. 1 lesion, HR 1.75, 95% CI 1.39-2.19) (230). One study compared survival for patients with more than five lesions and patients with a single lesion, reporting a statistically significant difference in survival (HR 2.83, 95% CI 1.50-5.36), while not finding a statistically significant risk

increase for patients with 2-3 lesions (HR 1.68, 95% CI 0.93-3.05) or 4-5 lesions (HR 1.70, 95% CI 0.77-3.79), as compared to patients with a single lesion (228). One report, of outcomes in a cohort of patients with underlying cirrhosis resected for iCCA, presented the additional risk increase per lesion, with a hazard ratio of 1.44 (95% CI 1.29-1.60) (215).

A meta-analysis including nine studies comparing survival after resection for patients with satellite lesions and patients without satellites lesions (213, 214, 218, 223, 227, 229, 231, 233, 237), indicated a significant decrease in overall survival for the group with satellites (HR 1.89, 95% CI 1.67-2.13). Forest (A) and funnel plots (B) for the meta-analysis are presented in **Figure 5** (Figure 2 in Paper IV). Statistical heterogeneity was limited among studies, and no publication bias was evident from funnel plot analysis.

Figure 5: *Meta-analysis of satellite lesions as prognostic factor for overall survival after resection for iCCA, A) forest plot B) funnel plot*

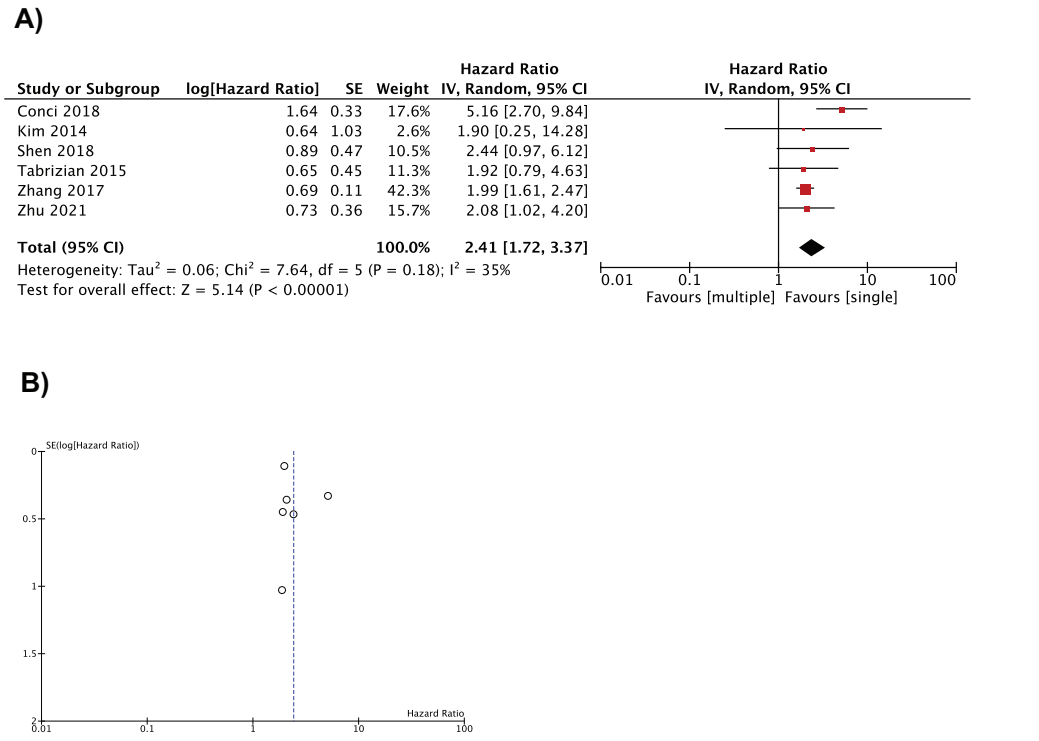


CI: confidence interval; df: degrees of freedom; iCCA: intrahepatic cholangiocarcinoma; IV: inverse variance; SE: standard error

A meta-analysis including six studies comparing survival for patients with and without multiple lesions not including satellites (217, 222, 223, 227, 231, 240), indicated significantly worse overall survival for the group with multiple lesions (HR 2.41, 95% CI 1.72-3.37). Forest (top panel) and funnel plots (bottom panel) for the meta-analysis are presented in **Figure 6** (Figure 3 in Paper IV). Low to moderate heterogeneity was present together with funnel plot asymmetry, suggesting possible bias.

In sensitivity analyses, excluding studies with the lowest quality assessment scores (Newcastle-Ottawa scale <5), similar pooled relative risks were found (satellite lesions vs. no satellite lesions HR 2.05, 95% CI 1.75-2.39; multiple lesions other than satellites vs. no multiple lesions other than satellites HR 2.53, 95% CI 1.64-3.91).

Figure 6: *Meta-analysis of multiple lesions (not including satellites) as prognostic factor for overall survival after resection for iCCA, A) forest plot B) funnel plot*



CI: confidence interval; df: degrees of freedom; iCCA: intrahepatic cholangiocarcinoma; IV: inverse variance; SE: standard error

6 DISCUSSION

6.1 INTRODUCTION

As a majority of patients operated with curative intent resection for cholangiocarcinoma or gallbladder cancer suffer recurrence, with a median overall survival of only approximately three years after surgery, an improved understanding of prognostic factors is important to guide future attempts to improve treatment. In this doctoral thesis research project, factors associated to long term outcomes after resection were analysed in data from retrospective cohort studies.

In Paper I, a strong association was found between general systemic inflammatory plasma markers (CRP and albumin) and overall survival after resection for biliary tract cancer. In this single-centre cohort study, the combination of hypoalbuminemia and increased CRP was an independent negative prognostic factor, also when adjusting for postoperative pathological risk factors.

In Paper II, a retrospective multicentre cohort study, no significant association was found between the underlying chronic inflammatory disease primary sclerosing cholangitis and median overall survival after surgery for perihilar cholangiocarcinoma. Patients with underlying primary sclerosing cholangitis more often had tumours of a higher histopathological grade, indicating risk of worse long-term outcomes.

In Paper III, three immunological proteins in preoperative plasma were found to be strongly associated to survival outcomes after resection for biliary tract cancer in a single-centre cohort. The predictive value of the preoperative markers was similar to that of postoperative histopathological risk factors and with independent prognostic value on multivariable analysis. Differences were found between diagnostic subgroups, with TRAIL positively associated to survival for patients resected for cholangiocarcinoma, CSF1 negatively associated to survival in intrahepatic cholangiocarcinoma and gallbladder cancer and with TIE2 negatively associated to survival after resection for gallbladder cancer.

In Paper IV, with a systematic review and meta-analysis of published retrospective data, the presence of multiple hepatic lesions, with stratification according to distribution and number of lesions, was studied as a prognostic factor for overall survival after resection for intrahepatic cholangiocarcinoma. In meta-analyses, both the presence of satellite lesions and other multiple lesions not including satellites was found to be negatively associated to survival. On systematic review, there was an indication also of an association between number of lesions and survival.

6.2 GENERAL METHODOLOGICAL DISCUSSION

6.2.1 Bias in observational research

All data analysed in this research project came from observational research, i.e. non-randomized and non-controlled studies. With regard to bias, there are several important

aspects to consider. Bias can occur during study accrual (selection bias, intervention bias), data collection (information bias) and during analysis and interpretation of data (confounding) (241). Randomized controlled trials are considered high-level evidence as they can permit the evaluation of treatment response in two groups that are balanced in respect to background characteristics, minimizing confounding from non-random selection (242). However, as participation in clinical trials can be regulated by pre-defined inclusion and exclusion criteria (i.e. age, concurrent disease), trial patient cohorts can be significantly different from the general patient population of interest. Observational research on the other hand can provide prognostic data for a broader population. However, any potentially improved generalizability of data from observational research will be dependent on the study design and the quality of data registration and data collection (163). Retrospective inclusion of patients and retrospective collection of data may introduce bias as records can be incomplete and non-standardized, whereas prospective registration can have the potential to be both more complete and better standardized. Furthermore, a prospective research database can be specifically designed to address predefined research questions, while retrospective studies are limited to what data is available and thus at risk for post hoc rationalizations and data driven research (243).

6.2.2 Missing data

Incompleteness of records, as mentioned in the previous section, is one reason for missing data. In regression analyses, missing data points among independent variables will limit the number of individuals included in analysis. Missing data across several variables and study subjects in a multivariable regression model can be an important methodological limitation. Excluding incomplete cases from analysis can limit not only statistical power, but will also induce bias if missing cases differ from complete cases (244, 245). Rather than missing completely at random, observational research data points are often missing because of other underlying individual factors. In such cases, imputation of missing data can limit bias, compared to complete-case analysis (244). A second type of missingness occurs when data is missing in a systematic way due to the registration itself, with thresholds for detection in biochemical analyses as one example. In such cases, all values below a limit of detection will be missing. Also here, with data missing not at random, imputation may help to reduce bias (204).

6.2.3 Association

A further important factor in prognostic and observational research, is the distinction between association and causation. While a factor may be associated to an outcome of interest and found valuable for risk stratification, it may or may not be linked to the outcome by a causal relationship (246). To delineate potential causal relationships for further study, possible confounding factors can be investigated and sought to be accounted for in statistical models. However, residual bias will always be an issue in observational non-randomized studies, precluding strong inferences about causation (247, 248).

6.2.4 Variable selection

As the number of variables that can be included in a multivariable time-to-event regression model is limited by the number of events, different methods can be employed to select variables to reduce the impact of over-fitting a model to the study sample cohort. While univariable and step-wise significance based variable selection strategies have been used, they introduce bias. Variables without apparent univariable association with the outcome can be clinically important and relevant to adjust for, or have relevant correlations to other factors that would influence variance and bias. A limited number of events, due to small sample size and/or a short follow-up period, will further increase the possible bias from selecting variables on the basis of significance testing (200, 201). Apart from selection on the basis of statistical significance or information criteria such as Akaike's information criteria (AIC) and Schwartz's Bayesian information criteria (BIC), selection methods can take the strength of regression coefficients into account also to introduce weighted penalties to the model (200, 201). The Lasso method, Elastic Net method and Adaptive Lasso are examples of such 'penalized likelihood' selection methods (201). Importantly, all variable selection methods will have the potential to introduce bias, and any data-driven selection of variables can produce an uncertain final model where regression coefficients can be skewed. Analysing the variance in the selection process with bootstrap resampling methods, and interpreting the regression coefficients from non-selective models, have been proposed as measures to account for variable selection bias (200, 201).

6.2.5 Categorisation of continuous variables

Regarding methodology, a number of continuous variables in the regression analyses in Paper I were categorised. Importantly, such categorisation will ignore the complete data for these variables and order model predictions on a step function (201). Instead of calculating so called 'optimal cut points' adapted from the dataset being analysed, it has been recommended to use recognised predefined cut-offs if categorisation is undertaken, but also to rather avoid categorisation when first building a statistical model (201). In Paper I, the predefined upper normal reference value was used as cut point for plasma bilirubin. For calculation of the prognostic scores, the predefined cut-offs according to McMillan et al. were used (175), i.e. the lower normal reference value for plasma albumin and a cut-off of 10 mg/L for CRP.

6.3 DISCUSSION OF THE CONSTITUENT PAPERS

6.3.1 Paper I

In Paper I, a statistically significant association with overall survival was found for both of the evaluated preoperative inflammation-based scores: the GPS and the mGPS. This association was seen in the whole cohort of patients undergoing surgical exploration, and in the group of patients undergoing curative intent resection. The association with survival was seen in all three subgroups of BTC: iCCA, pCCA and GBC. Both scores were independent prognostic factors for survival in multivariable models including tumour extension stage and

lymph node metastasis. The GPS, but not the mGPS, could stratify patients into three separate risk groups according to score.

While preoperatively increased plasma bilirubin and preoperative biliary interventions were included in multivariable models, residual confounding from preoperative cholangitis cannot be excluded. Preoperative cholangitis has previously been indicated as a negative prognostic factor for both short- (morbidity, mortality) and long-term (DFS, OS) postoperative outcomes after hepatobiliary resection for perihilar cholangiocarcinoma (249-252), albeit with variability between reports regarding impact on morbidity and mortality (253). In Paper I, 18% of pCCA patients had preoperative hyperbilirubinemia, without any significant association to GPS. A second relevant point, with regard to a possible influence of preoperative cholangitis, is the previous demonstration of an association between cholangitis and tumour specific risk factors (tumour extension, lymph node metastasis) (249). Other possible confounding or mediating factors for which associations with the prognostic scores were not evaluated were tumour characteristics and the extent of surgery.

In Paper I, the findings from previous reports from Asian centres of a negative association between the GPS/mGPS and OS in patients with resectable BTC were confirmed. Additionally, in this cohort study, the GPS was found to stratify patients into three separate risk groups. Such a separation of the GPS=1 group as an intermediate risk group was not seen in two previous studies with patients resected for pCCA/dCCA (155) and GBC (157). While a later multi-institutional study in the setting of iCCA has reported a similar prognostic implication of increased GPS, no separate analysis was presented for the GPS=1 group (254). A recent smaller study in resectable iCCA analysed CRP and albumin as separate variables, together with other inflammatory and nutritional laboratory markers and several scoring systems including the mGPS, as prognostic factors for disease-specific survival (255). Among laboratory variables, CRP, CEA, neutrophil count and total white blood cell count were significant prognostic factors, while albumin alone was not statistically significant ($p=0.067$). mGPS and several other derived scores (neutrophil-to-lymphocyte ratio, lymphocyte-to-monocyte ratio, lymphocyte-to-CRP score, prognostic nutritional index, CRP-to-albumin ratio) were found to have statistically significant associations to disease-specific survival (255). With regard to avoiding categorization of continuous variables to retain prognostic information, use of the CRP-to-albumin ratio, or the recently proposed CRP-lymphocyte-albumin-index first described for patients with hepatocellular carcinoma (256, 257), rather than the GPS or mGPS which both rely on dichotomization, could be advantageous (258). Furthermore, while several other studies have been able to combine inflammation-based scores with established tumour markers such as CEA and CA 19-9 to improve predictive value (151, 254, 257), these two markers were inconclusively employed and reported in the present study cohort with a majority of missing data, precluding a similar analysis. Lastly, as no imputation of missing data was used, with analysis limited to complete cases, the frequency of missing data is a possible source of bias.

While plasma albumin historically often has been interpreted as a nutritional marker, current studies in malignancy and chronic disease have emphasized albumin as an inflammatory marker and negative acute phase reactant (259-262). The GPS, which awards a score of 1 for isolated hypoalbuminemia, identified an intermediate risk group and a more delimited low risk group compared to the mGPS. In the study cohort, 23% of BTC patients had a preoperative hypoalbuminemia without an increase of CRP >10 mg/L. In the colorectal cancer cohort originally described by McMillan et al. when developing the mGPS, only 5% of patients had isolated hypoalbuminemia (175). To what degree the high rate of hypoalbuminemia seen here mirrors a stronger general systemic inflammatory response or reflects BTCs as primary hepatobiliary malignancies is not clear from this data.

6.3.2 Paper II

In Paper II, presenting prognostic factors and outcomes for the largest series of patients undergoing hepatobiliary resection for PSC-associated pCCA reported to date to our knowledge, a similar median OS was seen for patients with PSC pCCA and non-PSC pCCA. The patients with PSC-associated pCCA were younger and with a lower rate of well-differentiated tumours. While complications were more frequent in the PSC pCCA group, mortality was similar in the two groups.

Compared to results reported for liver transplantation after neoadjuvant chemoradiation (263), the postoperative 90-day mortality risk of 12% and five-year OS of 19%, indicates how PSC pCCA patients if eligible in accordance with Mayo protocol criteria (32), could benefit from neoadjuvant therapy and transplantation.

In this study, as in previous observational studies on outcomes for PSC pCCA patients after resection or transplantation without neoadjuvance (264-266), a possible source of bias when considering outcomes is the time of diagnosis, both of PSC and of pCCA. In transplantation on the indication of PSC with liver failure, an incidental postoperative de novo diagnosis of pCCA can sometimes be made postoperatively. In the setting of resection surgery on the indication of pCCA, a de novo PSC diagnosis can sometimes be made during pre- or postoperative diagnostic work up. In this current retrospective cohort, data on time of PSC diagnosis, liver function or any inclusion in surveillance programs was lacking.

Even if PSC is a strong risk factor for CCA, both PSC and pCCA are conditions of a low incidence, with PSC-associated pCCA being diagnosed infrequently, except in specialized high-volume centres. Moreover, a majority of patients diagnosed with pCCA have an unresectable tumour at presentation, precluding resection and at times indicating transplantation within strict criteria. Thus, being limited to retrospective resection data accrued over a longer time-period and yet with a small sample size, the ability to draw strong conclusions or inferences is clearly restricted. With a selected surgical resection cohort, no data was available for patients with the same diagnoses undergoing other therapies. Data on recurrence status was missing for more than one third of all patients in this retrospective dataset. Also, data on adjuvant or neoadjuvant oncological therapy was unavailable. Lastly, in

similarity to Paper I, with regard to PSC status as an independent variable, only patients with non-missing PSC data (81% of all patients) were analysed, a possible source of bias and information loss (267). While it sometimes has been recommended to avoid imputation when a considerable proportion of the data is incomplete, the use of imputation regardless of the proportion of missingness may decrease bias and increase statistical power (267, 268).

6.3.3 Paper III

In Paper III, three immunological protein markers in plasma were found to be associated with survival in patients undergoing resection for BTC. The tumour and cell-type specific expression of the three markers and their receptors or ligands were further investigated in public gene expression datasets. Plasma TRAIL/TNFSF10, was identified as a positive prognostic factor in CCA, with expression in intratumoural lymphocytes, monocytes and NK-cells seen in analysis of single-cell iCCA data. The expression of the agonistic TRAIL-R1/TNFSFR10A receptor was higher in tumour as compared to surrounding tissue in three diverse CCA datasets employing different methods of gene expression analysis (microarray, bulk tissue sequencing, single-cell sequencing). In the single-cell iCCA dataset, TRAIL-R1 expression was seen specifically in malignant cells. Plasma CSF1/M-CSF was identified as a negative prognostic factor in iCCA and GBC. In the iCCA single-cell analysis, CSF1 expression was seen in tumour-infiltrating CD8+ T-cells. Plasma TIE2/TEK was identified as a significant negative prognostic factor in GBC.

With regard to the study cohort, the sample size was limited, especially for subgroup analyses. There was also a further selection bias present due to a limited number of samples randomly selected to undergo core facility analysis by PEA. There was a relative overrepresentation of pCCA patients and underrepresentation of GBC patients, as all pCCA patients with available samples were included, approximately two thirds of iCCA patients, whereas only approximately half of the patients with GBC were selected for inclusion. The limited number of patients in subgroup analyses increases the risk of not only committing type II errors due to restricted sample size and statistical power, but also of type I errors as a consequence of overfitting statistical models to a specific dataset. Furthermore, the use of variable selection to build models will incur a risk of skewed risk ratios in the final model. While found statistically significant, the degree of uncertainty in the estimates of prognostic influence was illustrated by wide confidence intervals. Thus, the presented findings will need to be reproduced in further series and external cohorts and should also be investigated in a prospective setting to be validated. In such validation studies, a quantification of plasma protein concentration could permit further development of preoperative prognostic models, as compared to the relative quantification data for protein expression presented by PEA.

Concerning the analyses of identified markers and their ligands or receptors in tissue, limited sample size was also an important factor, notably in the case of the single-cell iCCA dataset, with tumour samples from only four patients, two of whom had underlying hepatitis B. Apart from differences in geographic location and included diagnostic groups, the variable

frequency of chronic hepatitis B/C among cohorts was a noticeable factor underscoring the heterogeneity not only between, but also within BTC diagnostic subgroups. Data on infectious hepatitis was not available in Paper III, but the frequency of hepatitis in this cohort could be expected to be similar to that reported in other studies including European cohorts (186, 188). In a previous study including patients with both resectable and unresectable BTC from our institution, a prevalence of chronic hepatitis B and C below 5% was found (269).

With growing evidence supporting multimodal therapy in BTC, to consider both the use and the sequencing of chemoradiotherapy and of targeted therapies, will be increasingly important in surgical research. In Paper III, as in the two previous papers, data on oncological therapy was missing. In two recent large multicentre studies (229, 233), six and seven percent respectively of iCCA patients undergoing resection received neoadjuvant therapy, while 28 and 21 percent of patients received adjuvant therapy. When considering prognostic factors and outcomes after surgery, receipt of systemic and/or regional oncological therapy is a relevant and increasingly important factor to adjust analyses for, e.g. by stratification or multivariable analysis. While neoadjuvant therapy is a possible confounding factor in analysis of preoperative prognostic markers, the use of a neoadjuvant strategy before resection has been very limited in BTC, with reported frequencies of neoadjuvant therapy ranging from zero to nine percent among all studies reviewed in Paper IV (the median reported proportion of patients receiving neoadjuvance among the 12 studies including the variable was 3.5%, with five of these studies reporting no use of neoadjuvant treatment).

While the plasma protein expression of the prognostic markers was associated with survival in the development and internal validation cohort, divergent results were seen when investigating associations between tissue gene expression of markers and ligands or receptors and survival in the public gene expression cohorts. Whether no reproducible associations exist between gene expression in the tumour tissue and outcomes, or if underlying differences in study cohorts and datasets could explain these results, remains to be further explored. Importantly, a prognostic association of a soluble factor could reflect both a biological process in tumour or peritumoral tissue, as well as a systemic inflammatory response to malignancy or a concomitant condition. The possible roles and prognostic implications of tumour TRAIL expression in CCA, and of lymphoid and myeloid immune cell infiltration and CSF1/M-CSF signalling, need to be further studied. Furthermore, TIE2/TEK which was identified as a prognostic factor in the GBC subgroup has recently been implicated in BTC with regard to the effect of VEGFR inhibition therapy (270), and in other malignancies with a proangiogenic subset of TIE2/TEK-expressing tumour associated macrophages however with an unclear role in BTC (271-274).

6.3.4 Paper IV

In Paper IV, a systematic review with meta-analyses including 2737 and 1589 patients respectively, satellite lesions as well as multiple lesions other than satellites were found to be negative prognostic factors in resectable iCCA. Data stratified for tumour number was limited but indicated increased risk per additional tumour lesion.

Previously, divergent data has been reported on the prognostic impact of satellite lesions. While one recent study reported decreased survival for patients with satellite lesions compared to patients with a single lesion (212), a second study found no significant survival difference between patients with satellite lesions and patients with a single tumour (275).

In the meta-analysis of overall survival outcomes for patients with/without satellite lesions, the statistical heterogeneity was low and no apparent publication bias was seen. In the meta-analysis on prognosis for patients with/without multiple lesions other than satellites, the degree of heterogeneity was higher with possible underlying bias, indicating that pooled results should be interpreted cautiously. On assessment of evidence according to GRADE domains (163), serious limitations were seen in one out of five areas (incomplete data) for the meta-analysis on satellite lesions and in three out of five areas (incomplete data, imprecise pooled estimate, possible bias) for the meta-analysis on other multiple lesions.

While the adapted GRADE approach for assessment of observational studies (163) can permit a structured presentation of limitations, the synthesis of data from observational studies will be at inherent risk of incorporating biased results with underlying confounding factors (276). Moreover, all observational studies included were of a retrospective nature, increasing the risk of both selection and information bias. Different criteria to delimit satellite lesions by histopathology or proximity to a main lesion on radiology were used among studies (223, 227, 237, 275, 277, 278). Importantly, most studies included in this review did not specify which modality was employed to diagnose multiple lesions. Lastly, the included studies represented surgical cohorts where patients with multiple lesions had been included despite this being a possible contra-indication to resection, representing possible selection bias. However, the fact that several of the included studies were broad multicentric collaborations between reference centres, that 15% of all patients undergoing resection in a recent national survey had multiple lesions (279), and also that a considerable proportion of all patients with multiple lesions have been reported to undergo surgery (26% in a recent United States national registry study, 28% in a multicentre European registry) (280, 281), could be seen as indications that the reported findings reflect data from current practice.

In Paper IV, a comprehensive search was performed to provide data on relative risk according to tumour distribution and number of lesions, and to present granular data specifically for the substantial subgroup of patients with iCCA that are diagnosed with multiple hepatic lesions. Two previous systematic reviews have analysed prognostic factors for patients undergoing resection for iCCA, both with unstratified data for patients with multiple tumours and indicating multiple lesions as a negative prognostic factor (8, 282). The first review, published in 2014, analysed overall survival outcomes and included five studies with data on multiple lesions in a pooled analysis (8). A more recent systematic review focused on early recurrence and included only three studies in a meta-analysis (282).

Concerning the question of whether or to what degree a prognostic factor reflects tumour characteristics and/or host responses, a negative association between tumour multiplicity in iCCA and peritumoural non-tumour cell expression of programmed cell death 1-receptor

ligand (PD-L1) has previously been indicated in one analysis (283), while no association was seen between intratumoural tumour/stroma/lymphocyte PD-L1 expression and tumour multiplicity in a second study (284). The interplay between immune cell activity and the invasiveness of tumour cells and extension of the tumour lesions in iCCA remains to be further explored.

7 CONCLUSIONS

7.1 STUDY I.

Two preoperative inflammation-based scores, the Glasgow prognostic score and the Modified Glasgow prognostic score, were independent prognostic factors for overall survival after resection for biliary tract cancer.

The association with survival was seen both in patients with gallbladder cancer and cholangiocarcinoma.

The Glasgow prognostic score, which compared to the Modified Glasgow prognostic score weights isolated hypoalbuminemia higher, could identify an intermediate risk group.

7.2 STUDY II.

Median overall survival after hepatobiliary resection for perihilar cholangiocarcinoma was similar in patients with and without underlying primary sclerosing cholangitis.

Patients with underlying primary sclerosing cholangitis were found to have a lower rate of well-differentiated tumours, a difference in tumour grade that may negatively impact long-term survival.

The five-year overall survival for patients undergoing hepatobiliary resection for perihilar cholangiocarcinoma with underlying primary sclerosing cholangitis was 19%, indicating how patients, if eligible in accordance with Mayo protocol criteria, could benefit from neoadjuvant chemoradiotherapy and transplantation.

Patients with perihilar cholangiocarcinoma and underlying primary sclerosing cholangitis had a higher rate of postoperative complications, a finding that emphasizes the importance of a careful preoperative evaluation and optimization in this patient group.

7.3 STUDY III.

Three specific immunological protein markers in preoperative plasma were found to be associated with survival in patients undergoing resection for biliary tract cancer. Plasma TRAIL/TNFSF10 was a positive prognostic factor in both intrahepatic and perihilar cholangiocarcinoma. Plasma CSF1/M-CSF was a negative prognostic factor in intrahepatic cholangiocarcinoma and gallbladder cancer. Plasma TIE2/TEK was a negative prognostic factor in gallbladder cancer.

In analysis of public gene expression data from tumour tissue and surrounding liver tissue in patients with cholangiocarcinoma, expression of the agonistic TRAIL-R1/TNFSFR10A receptor was higher in tumour and specifically higher in intrahepatic cholangiocarcinoma tumour cells. TRAIL/TNFSF10 was expressed by intratumoural lymphocytes, NK-cells and monocytes in intrahepatic cholangiocarcinoma.

CSF1/M-CSF was expressed by tumour-infiltrating CD8+ T-cells in intrahepatic cholangiocarcinoma.

In intrahepatic cholangiocarcinoma, TIE2/TEK was lower in tumour and mainly expressed by endothelial cells. The TIE2/TEK ligands ANGPT1 and ANGPT2 were mainly expressed by fibroblasts.

7.4 STUDY IV.

Satellite lesions, as well as multiple lesions other than satellites, were found to be negative prognostic factors in resectable intrahepatic cholangiocarcinoma.

Data stratified for tumour number was limited, but indicated increased risk per additional lesion.

In further studies, number of tumours, presence of satellite lesions and presence of multiple lesions other than satellites should be separately reported, to allow analysis as possible additive risk factors and to improve risk stratification for patients diagnosed with multiple intrahepatic cholangiocarcinoma.

8 POINTS OF PERSPECTIVE

Since first starting this project in the fall of 2017, more evidence to improve therapy for patients with biliary tract cancer has been established, and new clinical trials have been launched, as briefly described in the previous literature review in chapter 2. The BILCAP trial published in 2019 provided evidence for prolonged recurrence-free survival with adjuvant chemotherapy (72). In 2020, IDH1 inhibition was shown to prolong progression-free survival as a targeted therapy for the subgroup of cholangiocarcinoma patients with advanced disease and tumour IDH1-mutations (96). Recently, an interim analysis of the TOPAZ-1 trial suggested improvements in overall survival with combination therapy including a PD1-inhibitor with first-line gemcitabine-cisplatin chemotherapy for patients with advanced BTC (108).

While biliary tract cancers remain malignancies with a high mortality, such developments bring hope of further steady incremental gains with respect of finding the best combination of therapies for each patient and of improvements in outcomes.

While surgery sometimes can offer the hope of radical cure, an important point when looking at advances in cancer surgery is to see the role of surgery as a part in a multimodal treatment and to establish and tailor the sequencing of such treatments.

That is why not only anatomical, pathological or physiological surgical risk factors are of importance, but also the disease-specific and perhaps patient-specific biological risk factors. With the hope of continuing improvements in both short- and long-term outcomes after surgery for biliary tract cancers, comes the hope of having more factors to direct therapy and predict therapy response rather than only prognostic associations; of seeing a prolonged overall survival and decreased disease-specific mortality – making disease-free survival a more important end-point to study; and of improving both quality-of-life and survival for patients with recurring disease.

Much like the recent studies that have established oncological therapy in advanced disease and in the adjuvant setting for biliary tract cancer, the studies in this thesis project have included patients with different diagnoses: intrahepatic cholangiocarcinoma, perihilar cholangiocarcinoma and gallbladder cancer. While sometimes the extent of surgery and outcomes may be similar for patients with these different diagnoses, to further improve therapy, diagnosis-specific characteristics need to be addressed and more diagnosis-specific studies need to be performed. As is underscored by contrasting the findings in Paper I and the subgroup analyses in Paper III, while general systemic inflammatory markers may have similar prognostic associations in different BTC subgroups, the associations of more specific plasma markers can differ between patients with different diagnoses.

This broadly means that as much as possible, therapeutic trials must address heterogeneity and be specific in inclusion criteria, stratification factors and power analyses, to make it

possible to discern for future trials which specific patient subgroup could represent the identified “five-year survival rate”, “median response rate” or “median survival time”.

Such specificity however, must also be the goal in observational studies. This thesis presents four studies limited to retrospectively collected observational data. Study protocols for data collection, standardized terminology, and multi-centre collaboration to increase statistical power and decrease selection bias, are issues just as pertinent to observational research as to randomized controlled trials.

National data, e.g. from quality registries, can provide a way to further study such questions of outcomes and prognostic factors in surgical oncology as those in Study II and IV, ideally with prospectively registered data and higher coverage than multi-institutional cohort studies. However, such efforts can be hampered by a limited granularity of quality registry data.

With advances in biochemistry, simultaneous analysis of many analytes in many samples is possible. The resolution of the ‘magnification glasses’ we use to look closely at the world has improved. It is possible to not only investigate tissue expression of genes, but also the spatial distribution of transcripts and subcellular networks of transportation. It is also possible to investigate not only mutations, but why a cell delineates, what silences a part of the genome or may lead a gene to over-express. The combination of perspectives, to look from different angles at a clinical problem, can bring both new answers and new questions.

While screening of samples for a large number of markers can increase the risk of spurious findings, analysis of more than single factors can at its best provide data on pathways, signatures or patterns reflecting biological processes to study in detail. Previous research in intrahepatic cholangiocarcinoma and BTC has implicated tumour-infiltrating myeloid cells and subgroups of lymphocytes. The prognostic association of plasma CSF1/M-CSF and TRAIL/TNFSF10 seen in Study III can be further investigated by external validation and a possible correlate in the tumour-microenvironment can be investigated in tumour tissue.

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