Targeting bile salt-FGF19 signaling

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**Targeting bile salt-FGF19 signaling:
promising therapeutic strategies to promote liver
regeneration and improve intestinal failure**

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**Targeting bile salt-FGF19 signaling:
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regeneration and improve intestinal failure**

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Chapter 1

General introduction

Introduction

Bile salts are synthesized in the hepatocytes from cholesterol through a complex series of biochemical reactions ^{1, 2}. The initial and rate-determining step in the main biosynthetic route (i.e. the classic pathway) is catalyzed by the enzyme cholesterol 7- α -hydroxylase CYP7A1 ¹. The end products of the biosynthetic pathways, i.e. bile salts, are conjugated with the amino acid glycine or taurine and secreted into the canalicular lumen via the bile salt export pump (BSEP) ³. Bile and its constituent bile salts are transported through a ductular network also comprising the gallbladder, to the proximal small intestine (duodenum). In the terminal ileum, bile salts are reabsorbed by enterocytes and return to the liver through the portal circulation, completing the enterohepatic circulation (EHC) (**Fig. 1**).

Figure 1. Enterohepatic circulation of bile salts. Bile salts are synthesized in the hepatocytes from cholesterol and conjugated with the amino acid glycine or taurine, and secreted into the canalicular lumen. In the terminal ileum, 95% of bile salts are reabsorbed by enterocytes and return to the liver through the portal circulation (red arrow). Remaining 5% of bile salts are lost in the feces (blue arrow). (This illustration is adapted from BioRender [<https://biorender.com/>]).

During enterohepatic circulation, bile salts activate dedicated receptors (e.g. farnesoid X receptor, FXR; G protein-coupled bile acid receptor, TGR5) in the terminal ileum and liver ³. Activation of bile salt receptors plays important roles in hepatic bile salt

homeostasis, hepatic nutrient metabolism in the postprandial state, small intestinal barrier function, and inflammatory control ³. Hence, an intact enterohepatic circulation of bile salt fulfills an important role in gut-liver health ^{3, 4}.

Surgical patients, especially patients with hepatobiliary and pancreatic diseases, often suffer from a compromised EHC, which has detrimental effects on gut-liver health ⁵. For examples, patients with perihilar cholangiocarcinoma (pCCA) or head of pancreas tumors have an obstructed EHC, because of malignant obstruction and/or compression of the bile duct. These patients present with obstructive jaundice and elevated systemic markers of cholestatic liver injury ⁶. Furthermore, for patients who underwent a partial hepatectomy, especially when accompanied by concomitant removal of the gallbladder during extended resection procedures, the EHC can be temporarily disrupted due to removal of the 'source' of bile salts. Major liver resection can lead to post-hepatectomy liver failure (PHLF) and death, especially in patients with pCCA ⁷. In addition, patients with a temporary double enterostomy in the jejunum have a disrupted EHC due to outflow of succus entericus in the stoma bag ⁸. They can develop intestinal failure (IF) and intestinal failure-associated liver disease ⁸. To understand the pathophysiology of EHC disturbances in these disease contexts further research is needed, ultimately aiming at targeting affected signaling pathways to improve patient outcomes.

Biliary obstruction and drainage

Disturbed enterohepatic circulation

Under biliary obstruction (e.g. pCCA or head of pancreas tumors), the EHC of bile salts is disturbed. Bile fails to flow towards the intestine. Under these conditions, the transport of bile salts in and out of hepatocytes can undergo adaptive changes. Na⁺-taurocholate cotransporting polypeptide (NTCP, mediating uptake of bile salts from the circulation) is downregulated, while basolateral organic solute transporter a/β (OSTa/β), and multidrug resistance-associated protein 3 (MRP3) and MRP4 are upregulated, thereby releasing bile salts into the sinusoidal circulation ^{9, 10}. The elevated expression of BSEP (bile salt exporter) can result in an unfavored increase of biliary pressure ^{11, 12}. Impaired canalicular bile salt secretion and obstructed bile flow can lead to accumulation of bile salts in hepatocytes and attendant hepatotoxicity ³.

Cholestatic influences on cholangiocytes and hepatocytes

Biliary obstruction induces adaptive changes in the biliary tree. The canalicular network undergoes volumetric expansion by an increase in diameter, with increased number of spine-like or bleb-like protrusions intruding into the hepatocyte cytoplasm¹⁰. In the bile duct ligation (BDL) model of cholestasis, the disruption of tight junctional barrier and the periductular cytoskeleton, may explain the change of canalicular morphology and leakage of bile into the parenchyma¹⁰. Additionally, increased hydrostatic pressure in the biliary tree, which is dependent on the presence of BSEP, may lead to liver injury after BDL¹³.

Elevated intracellular bile salt concentrations can lead to hepatocyte injury through various mechanism. Bile salts can stimulate the mitochondrial respiratory chain to generate reactive oxygen species, which can damage mitochondrial membranes and promote the release of apoptosis-inducing factor, ultimately leading to hepatocyte apoptosis¹⁴. The biological and physicochemical properties of bile salts are determined by the ionization of the side chain carboxylic acid group, and by the number, position and spatial orientation of hydroxyl groups in the steroid nucleus¹⁵ (**Fig. 2**). In general, hydrophobic bile salts are more toxic because of increased propensity to disrupt membrane integrity by solubilizing membrane lipids¹⁵. Hydrophobic bile salts can also induce endoplasmic reticulum (ER) stress, subsequently activate c-Jun N-terminal kinase (JNK) signaling and, thus, contributing to hepatocyte cell death in the context of cholestasis¹⁶. Death receptors are another factor mediating cholestasis-related hepatocyte apoptosis¹⁷.

Figure 2. Chemical structure of primary bile salt cholic acid. In general, bile salts have a steroid nucleus containing one or multiple hydroxyl groups, and a side chain containing a carboxyl group. This illustration is a modification from a picture of Hofmann *et al.* (2008)¹⁸.

Inflammation

Bile salt toxicity induces cell death and an attendant inflammatory response ¹⁹. Neutrophils are the predominant infiltrating immune cells in the early stages of cholestatic liver injury ²⁰. The periportal neutrophil infiltration is positively correlated with the degree of liver injury in BDL mice and cholestatic patients ^{20, 21}. Macrophages also participate in cholestatic liver injury. In pathological concentration, extracellular bile salts act as a danger signal to activate the NLRP3 inflammasome in macrophages ²². Treatment with gadolinium chloride, an inhibitor of Kupffer cell phagocytic function, could ameliorate liver injury and fibrosis in BDL rats ²³.

Preoperative biliary drainage

Due to above harmful effects of biliary obstruction, preoperative biliary drainage (BD) is commonly used to decompress the biliary tree and restore EHC in patients with obstructive jaundice ^{24, 25}. Through internal BD, the natural route of bile flow towards the small intestine is restored. Gene expression profiling revealed that BD improved cholestasis-associated fibrogenic and inflammatory gene signatures in the liver of patients with pCCA ²⁶. Preoperative BD was associated with decreased mortality in patients with pCCA after liver resection ²⁷. However, preoperative BD can lead to drainage-related complications such as cholangitis, pancreatitis and haemobilia ²⁸. Some studies do not recommend preoperative BD for pCCA, due to lack of benefits on reducing postoperative complications and mortality ^{29, 30}. Currently, there is no consensus on the routine use of, and indications for preoperative BD in patients with resectable pCCA.

Percutaneous transhepatic biliary drainage (PTBD) and endoscopic biliary drainage (EBD) are the two main drainage procedure (**Fig. 3**). The best preoperative drainage procedure in resectable pCCA is under debate ³¹⁻³⁵. PTBD is relatively invasive and may be associated with seeding metastasis ³¹. In addition, some studies reported poor postoperative survival in patients undergoing PTBD ^{31, 36}. EBD is performed by a retrograde route from the ampulla of Vater in the proximal small intestine to the (intrahepatic) bile duct, which avoids the potential disadvantages of PTBD. However, as EBD causes a direct connection between intestinal microbes and the biliary system, it can result in ascending cholangitis, a risk for postoperative mortality ²⁵. The first randomized controlled trial that compared PTBD with EBD in resectable pCCA was prematurely stopped because of higher all-cause mortality in the PTBD group ³⁷. A

possible explanation is that external drainage of bile results in intestinal bile salt deficiency and accordingly insufficient enterohepatic bile salt signaling, which negatively affects liver regeneration ³⁷.

Figure 3. Preoperative biliary drainage in perihilar cholangiocarcinoma. Percutaneous transhepatic biliary drainage (PTBD) is performed through punctures of the skin into the intrahepatic bile duct (left panel). Endoscopic biliary drainage (EBD) is achieved by retrograde route via the proximal small intestine and biliary tract (right panel). This illustration is a modification from a picture of Tavakkoli *et al.* (2021) ³⁸.

Bile salt homeostasis and liver regrowth

Bile salt-FGF19 signaling

Partial hepatectomy (PH) for hepatobiliary tumors is relatively safe and often the only curative treatment option. The robust capacity of the liver to regenerate permits up to 70% removal of the liver mass ³⁹. However, after (extended) partial hepatectomy, especially when accompanied by cholecystectomy, the EHC can be disrupted due to the loss of the 'source' of bile salts. Bile salts are involved in liver regeneration after PH via activation of the FXR ⁴⁰. FXR activation in the ileum stimulates the expression of fibroblast growth factor 19 (FGF19, Fgf15 in rodents) in enterocytes ⁴¹. After binding to the fibroblast growth factor receptor 4 (FGFR4) and β -Klotho (KLB) complex at the surface of hepatocytes, FGF19 inhibits synthesis of bile salts via suppressing the expression of *CYP7A1* ⁴¹ (**Fig. 4**). FGF19 also exerts mitogenic properties through activation of FGFR4 signaling ⁴². Delayed liver regrowth and high mortality was observed in *Fxr* or *Fgf15* deficient mice after PH, which is linked with bile salt toxicity ^{40, 43}. In patients undergoing PH, serum bile salt levels were positively associated with liver regeneration ⁴⁴.

Figure 4. Enterohepatic bile salt-FGF19 signaling. In the ileum, bile salts bind and activate nuclear receptor farnesoid X receptor (FXR) and this stimulates the expression of fibroblast growth factor 19 (FGF19). In the liver, FGF19 binds to its receptor fibroblast growth factor receptor 4 (FGFR4), which activates a downstream signaling pathway involving MAP kinases that causes repression of CYP7A1, thus suppressing bile salt synthesis. This illustration is a modification from a picture of Schaap *et al.* (2014) ³.

Liver regrowth induced by portal vein embolization

For a safe liver resection, the future liver remnant (FLR) volume must be >30% in uncompromised livers. However, liver resections are commonly performed in patients with underlying liver disease. Portal vein embolization (PVE) is the gold standard procedure to increase the FLR volume in advance of major hepatectomy ⁴⁵. PVE induces hypertrophy of the FLR by depriving the embolized segments of nutrients, which provides a trigger for compensatory expansion of the FLR ⁴⁶. Moreover, PVE treatment can increase circulating interleukin-6 (IL-6), hepatocyte growth factor (HGF), transforming growth factor-α (TGF-α), and epidermal growth factor (EGF), which stimulate hepatic DNA synthesis and hepatocellular proliferation in rats ⁴⁶. In patients, PVE can induce an increase in FLR volume of 20–50% within 3-7 weeks ⁴⁷. Studies have shown that PVE has a beneficial effect on decreasing liver failure and death rates in patients undergoing major liver resection ^{48, 49}. However, relatively long waiting time between PVE and resection puts patients at risk of tumor progression ⁵⁰. In addition,

PVE may induce insufficient hypertrophy of FLR to allow safe resection. Therefore, identifying factors affecting the proliferative effect of PVE is needed.

Bile salt signaling may play a role in the liver growth induced by PVE. After PVE, plasma bile salt levels were rapidly increased in both humans and in experimental PVE^{51, 52}. Bile salt concentrations at day 3 predict FRL volume increase at day 7 in a standardized rabbit model of PVE⁵¹. Furthermore, bile salt levels at 5 h after PVE were positively correlated with hypertrophy of the FLR and liver function in patients with CRLM⁵². We recently demonstrated that obeticholic acid (OCA), a first-in-class selective and potent agonist of FXR, augments the volume gain and function of the non-embolized liver lobe in a rabbit model of PVE⁵³. The underlying mechanisms remain unknown.

Double jejunal enterostomy and chyme reinfusion

Interrupted enterohepatic circulation in double jejunal enterostomy

In the enterohepatic circulation, bile salts exert direct antimicrobial activity through disrupting cell membrane integrity^{54, 55}. Indirectly, bile salts can induce Paneth cell production of antimicrobial peptides to inhibit gut microbial overgrowth through FXR-dependent manners^{54, 55}. The interaction between gut microbiota and bile salts is mutual. The gut microbiota can deconjugate and further metabolize primary bile salts into secondary bile salts, thereby affecting their biological and signaling properties⁵⁵.

During digestive surgery, a number of clinical (emergency) situations may lead to the establishment of a temporary double enterostomy in the jejunum, including small bowel resection and related anastomosis protection, peritonitis and enterocutaneous fistula⁸. This leads to an interrupted EHC of bile salts due to leakage of the succus entericus in the stoma bag, rather than entering the (terminal) ileum. A double jejunal enterostomy can, thus, disturb the FXR/FGF19 feedback loop that controls hepatic bile salt synthesis, while bile salt deficiency in the distal small intestine can lead to intestinal dysbiosis⁵⁴. In mice, secondary bile salt deficiency, due to the loss of contact between bile salt-metabolizing bacteria in the colon and bile salt substrates, can promote intestinal inflammation by reduced anti-inflammatory signaling through bile salt receptor *Tgr5* in immune cells⁵⁶. Compromised gut barrier allows microorganisms and microorganism-derived products to translocate to the liver, causing liver inflammation

and subsequent immune-mediated injury ⁵⁴.

Chyme reinfusion

Chyme reinfusion (CR) is an enteral nutritional technique, which re-establishes the anatomical continuity of the small intestine in patients with double jejunal enterostomy, through extracorporeal circulation of chyme, likely leading to restoration of the EHC of bile salts as well ⁸. Previous studies have shown that reinfusion of proximal intestinal secretions normalized serum bilirubin, and levels of cholestatic injury markers alkaline phosphatase (ALP) and γ -glutamyl transferase (GGT) in patients with high-output stomas or fistulas ^{57, 58}. Furthermore, CR can increase plasma citrulline levels, a marker for the absorptive function of the remnant small intestine, in adult IF patients with a double enterostomy ⁸. After CR initiation, 126 of 139 patients (91%) could be weaned of parenteral nutrition within a median of 2 days ⁸. Despite these promising findings, the mechanisms underlying the beneficial effects of CR are incompletely understood. But it has been postulated that bile salt signaling is involved ⁸.

Aims and outline of this thesis

In this thesis, we **1)** summarize the current knowledge of the effects and route of preoperative biliary drainage in patients with resectable pCCA, **2) a.** explore the suitability of precision-cut liver slices (PCLS) as an *ex vivo* model to study liver regeneration, **b.** study the mechanisms underlying OCA-accelerated liver regrowth following PVE in a rabbit model, **c.** investigate the influence of cholestasis on PVE-induced FLR hypertrophy in patients, and **3)** explore a contribution of the bile salt-FGF19 signaling axis in beneficial effects of CR in intestinal failure patients.

In **Part I**, a systematic review of systematic reviews is conducted to assess the effect of preoperative BD *versus* no preoperative BD and to evaluate the superiority of EBD *versus* PTBD on perioperative and long-term outcomes in patients with resectable pCCA (**Chapter 2**). In **Part II**, we describe a model, precision-cut human liver slices (PCLS), as an *ex vivo* tool to study interventions aimed at promoting liver growth (**Chapter 3**). Further, the mechanisms underlying OCA-accelerated hypertrophy of non-embolized liver lobe is examined in a standardized rabbit model of PVE (**Chapter 4**). The findings from this animal study led us to investigate the influence of cholestasis on FLR growth in clinical patients who underwent PVE. We compared PVE-induced

hypertrophy of the FLR in patients with CRLM (non-cholestatic) and pCCA (cholestatic or post-cholestatic) and analyzed the effect of different sides of biliary drainage (unilateral or bilateral) on FLR hypertrophy (**Chapter 5**). In **Part III**, it is investigated whether beneficial effects of chyme reinfusion in intestinal failure patients with a temporary double enterostomy, are in part mediated by the bile salt-FGF19 signaling axis (**Chapter 6**). In **Chapter 7** key findings from **Part I**, **Part II**, and **Part III** are summarized and integrally discussed, along with potential clinical implications and future research directions.

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Part I

Preoperative biliary drainage in perihilar
cholangiocarcinoma

Chapter 2

The effect and route of preoperative biliary drainage in patients with resectable perihilar cholangiocarcinoma: a systematic review of systematic reviews

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Abstract

Background: The beneficial effect, and route of preoperative biliary drainage (BD) via percutaneous transhepatic biliary drainage (PTBD) or endoscopic biliary drainage (EBD), in resectable perihilar cholangiocarcinoma (pCCA) are heavily debated. This study aimed to clarify the effect and route of preoperative BD on perioperative and long-term outcomes in patients with resectable pCCA.

Methods: PubMed, Embase, Cochrane Library, and KSR Evidence were searched from inception to March 31, 2020, to identify systematic reviews (SRs) with or without meta-analysis. Risk Of Bias In Systematic reviews (ROBIS) assessment tool was used.

Results: Eleven SRs with meta-analysis including 5950 patients were identified. All but one original studies in the SRs were retrospective and at risk of bias. Ten of eleven SRs had high risk of bias. For preoperative BD *versus* no preoperative BD, all SRs showed no statistical differences in postoperative mortality (odds ratios (ORs) from 0.70 to 1.06). Preoperative BD was associated with increased postoperative major morbidity in 'simple criteria' patients receiving BD only based on the presence of jaundice (OR 1.57 95% CI 1.10-2.25). For EBD *versus* PTBD, three of four SRs showed that the postoperative mortality was not significantly different between two groups (ORs from 0.47 to 0.63). EBD was associated with higher drainage-related overall morbidity, cholangitis and pancreatitis rates in three of four, three of five, and four of four SRs, respectively (ORs from 2.23 to 3.13, 4.58 to 5.41, 7.46 to 11.52, respectively). PTBD was associated with higher seeding metastasis rates and worse postoperative overall survival (ORs from 0.35 to 0.46, hazard ratios (HR) 0.67 95% CI 0.53-0.85, respectively).

Conclusion: This study does not end the debate on the significance and preferred route of preoperative BD. We showed that most available evidence has high risk of bias. The preoperative BD might need to be performed in strictly selected patients in terms of cholangitis, bilirubin levels and future liver remnant volume to avoid increased postoperative major morbidity. EBD might be associated with higher short-term drainage-related morbidity but more favorable long-term oncological outcomes.

Introduction

Perihilar cholangiocarcinoma (pCCA) is the most common type of bile duct malignancy, accounting for 50% to 60% of all cholangiocarcinoma cases ^{1, 2}. Some 90% of patients with pCCA clinically present with (painless) obstructive jaundice ³. Surgery with complete resection represents the only curative opportunity, with approximately 75% of patients eligible for surgical resection ⁴. However, the prognosis of patients after surgery remains poor and 5-year survival rates are around 30% ⁵. Moreover, liver resection in patients with hyperbilirubinemia and (resolved) cholangitis carries a high postoperative risk of severe complications and mortality ⁶.

Preoperative biliary drainage (BD) is employed to decompress the biliary tree, treat cholangitis, and improve (future remnant) liver function ^{7, 8}. BD can be performed unilaterally or bilaterally, and principally drains the future liver remnant ⁹. BD is performed via a percutaneous procedure (PTBD: percutaneous transhepatic biliary drainage) or an endoscopic biliary drainage (EBD) procedure. Studies have demonstrated that preoperative BD can reverse the cholestasis-associated pro-fibrotic and inflammatory status of the liver, and enhance the ability of the liver to regenerate ^{10, 11}. However, the value of preoperative BD in resectable pCCA is under debate. Some studies argued against the utility of preoperative BD, finding no benefits in reducing postoperative complications and mortality ¹²⁻¹⁴. Patients receiving preoperative BD even had higher mortality after left hemi-hepatectomy ¹⁰.

In addition, the optimal drainage route still needs to be determined. PTBD can lead to the diversion of bile, subsequent disturbed enterohepatic circulation (EHC), and impaired liver regeneration ⁹. Also, PTBD can be complicated by seeding metastasis, affecting patients' survival ^{15, 16}. EBD, including endoscopic biliary stenting (EBS, internal) and endoscopic nasobiliary drainage (ENBD, external) modes, is considered less invasive and avoids the potential disadvantages of PTBD. However, EBD can result in cholangitis, as it creates a direct connection between the proximal intestine and the biliary system, a risk for postoperative death ^{8, 17}.

Several systematic reviews (SRs) ¹⁸⁻²⁸ addressed the effects of preoperative BD *versus* no preoperative BD, as well as PTBD *versus* EBD on drainage-related and/or postoperative outcomes in patients with resectable pCCA. However, these reviews ¹⁸⁻²⁸ reported inconsistent results on the same outcomes indicators. For example, two SRs ^{18, 19} showed significantly higher overall postoperative morbidity in patients with

preoperative BD. In contrast, two other SRs ^{20, 21} found no significant difference in overall postoperative morbidity between drained and undrained patients. Moreover, the methodological quality of published SRs is undetermined, and risk of bias may exist. These limitations of previous SRs make it difficult for clinicians to decide whether or not to perform BD and which method to employ to minimize morbidity for patients with resectable pCCA.

This review, therefore, aims to systematically study all SRs with available evidence to assess the effect of preoperative BD *versus* no preoperative BD, and the superiority of EBD *versus* PTBD on perioperative and long-term outcomes in patients with resectable pCCA. Particularly, we also aimed to evaluate the risk of bias (study quality) of available SRs.

Methods

This study was reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement ²⁹. After a preliminary literature survey, the protocol was written and registered at the International Prospective Register of Systematic Reviews (PROSPERO) with registration number CRD42019141412

(https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42019141412).

Search strategy

We used a combination of Medical Subject Headings (MeSH) and free-text terms to search PubMed, Embase (Ovid), Cochrane Library, and KSR Evidence (<https://ksrevidence.com>, it is a database that includes all systematic reviews and meta-analyses published since 2015) from inception until May 31, 2019. We also searched PROSPERO and ClinicalTrials.gov to identify any unpublished studies. The search strategy was developed in collaboration with a certified librarian and further refined by another experienced reviewer (JK) using the following conceptual groups: (1) biliary drainage, (2) perihilar cholangiocarcinoma, and (3) mortality/survival (Appendix S2.1, Supplementary file). No language restrictions were applied in any of the databases. Searches were updated on March 31, 2020. We checked the references in included papers for further studies.

Study selection

Two investigators (XC and HS) independently screened the titles and abstracts and then full texts to identify eligible studies for inclusion. Any disagreements were resolved by discussion. We selected the most recent version in the case of duplicate reports.

We included studies which met the following criteria: (1) studies included adult patients (≥ 18 years old) with resectable pCCA; (2) studies investigated whether or not to perform preoperative BD or the effect of EBD compared with PTBD in pCCA; (3) studies reported on at least one of these outcomes: drainage-related morbidity (e.g. cholangitis, pancreatitis, portal vein injury, cancer seeding, and bleeding), postoperative morbidity (e.g. liver failure, sepsis, and bile leakage), postoperative mortality, survival; (4) studies were SRs, with or without meta-analysis.

We excluded studies involving patients with obstructive jaundice not due to pCCA, such as autoimmune diseases (e.g. IgG4-related autoimmune cholangitis or primary sclerosing cholangitis) or bile duct stones. Also, we excluded narrative reviews (other than SRs) due to the absence of pre-specified eligibility criteria and systematic methodology, as well as SRs of SRs, commentaries, conference proceedings, and editorials.

Data collection

Two reviewers (XC and HS) independently extracted data using pre-specified data collection forms. Discrepancies were resolved through discussion. Data extraction included SR characteristics (e.g. the inclusion criteria, intervention type, numbers of included studies and participants, types of study design, quality assessment of studies, analytical approach, and conclusions) and original study characteristics included in the SRs (e.g. numbers of participants and events, and intervention type). The primary outcome was postoperative mortality. The secondary outcomes were overall drainage-related morbidity, drainage-related cholangitis and pancreatitis, overall postoperative morbidity, postoperative major morbidity (Clavien-Dindo grade III-IV), infectious morbidity, liver failure, seeding metastasis, and long-term survival. It should be noted that for this study, the setting of the outcome indicator 'long-term survival' was modified from the registered protocol (CRD42019141412). The final literature survey revealed that long-term survival was reported in a single SR only (comparing BD with no BD), hence, it was deemed appropriate to include long-term survival as a secondary outcome measure.

Risk-of-bias assessment

Two investigators (XC and HS) independently assessed the risk of bias for each included SR using the Risk Of Bias In Systematic reviews (ROBIS) tool ³⁰, a reliable and widely used appraisal instrument ^{31, 32}. All disagreements were solved by discussion. The ROBIS tool includes 3 phases ³⁰, of which the first phase assesses relevance (optional). The second phase covers 4 domains (21 items): study eligibility criteria, identification and selection of studies, data collection and study appraisal, and synthesis and findings. The third phase evaluates the overall risk of bias in the SRs. The items are rated as *yes*, *probably yes*, *probably no*, *no*, and *no information*. As PRISMA ³³ and ROBIS ³⁰ recommended, we assigned a rating of “low risk of bias”, “high risk of bias”, or “unclear risk of bias” to the overall risk of bias, instead of a summary score, because the latter may mask critical weaknesses decreasing the confidence in the results of a SR ^{33, 34}.

Data synthesis

Data were evaluated using qualitative synthesis. Descriptive statistics were reported as frequency (percentage) when possible. We reported summary estimates of preoperative BD (EBD and/or PTBD) effects on primary and secondary outcomes, as relative risk (RR) or odds ratio (OR) with 95% confidence interval (CI) for dichotomous data, and hazard ratios (HR) with 95% CIs for time-to-event data. Because many included SRs comprised data from overlapping studies, a meta-analysis could not be performed.

Results

Study selection

The database searches identified 2626 records including 5950 patients/2478 citations from the initial search (May 31, 2019) and an additional 148 abstracts from the update (March 31, 2020). After de-duplicating, screening titles and abstracts, 2586 records were excluded. Through completely reviewing the full text of the remaining 40 articles, we included 11 eligible SRs with meta-analyses. The included 11 SRs are published in English language, and no studies in a language other than English, were eligible for inclusion in the present study. No additional studies were eligible for inclusion after checking the references of included studies. Of eleven included SRs, four compared

preoperative BD with no preoperative BD, while seven compared EBD with PTBD in patients with pCCA (**Fig. 1**). The excluded full-text reports with reasons are presented in **Supplementary Table S1**.

Figure 1. PRISMA flow chart showing selection of studies for review. BD, biliary drainage; EBD, endoscopic biliary drainage; PTBD, percutaneous transhepatic biliary drainage.

Comparison of effects between preoperative BD and no preoperative BD in resectable pCCA

Study characteristics

Characteristics of the four SRs¹⁸⁻²¹ evaluating the effects of preoperative BD are shown in **Table 1**. These SRs¹⁸⁻²¹ reported 33 unique studies, ranging from 9 to 19 studies with 711 to 2178 participants per individual SR. Eleven studies were repeatedly included in at least two SRs. Three^{18, 19, 21} of four SRs only included participants who underwent resection. The other SR²⁰ also included participants where metastatic spread prevented actual resection. All original studies included were retrospective except for a single prospective cohort study³⁵. The quality of included original studies was low to moderate¹⁹⁻²¹. The SR by Liu *et al.*¹⁸ reported 252 (54.1%) participants

receiving external drainage, 196 (42.1%) internal drainage, and 18 (3.8%) receiving a combination of internal and external drainage among 466 drained participants. Two SRs reported mean preoperative total bilirubin levels of 9.6¹⁸ and 4.6 mg/dL²⁰ in the drained patients, and 16.3¹⁸ and 15.8 mg/dL²⁰ in the patients without drainage, respectively. The mean duration between BD initiation and hepatic resection was 22.8¹⁸ and 30.8 days²⁰, respectively.

Risk of bias of included systematic reviews

All SRs¹⁸⁻²¹ were rated as “at high risk of bias” using ROBIS assessment (**Supplementary Table S2**). The Cohen’s kappa coefficient for inter-rater agreement between the two reviewers was 0.76. One SR¹⁸ was classified as “high risk of bias” mainly due to lacking quality assessment of original studies. The high risk of bias rating in the remaining SRs¹⁹⁻²¹ was due to a number of concerns, such as language restrictions, lack of search window, not considering bias of primary studies in the synthesis, and incomplete reporting of independence in studies identification and data extraction.

Besides, the numbers of included studies and abstracted participants and events were variable across the SRs¹⁸⁻²¹ (**Supplementary Table S3, Table S4**), reflecting a combination of difference in period, inclusion criteria, or carelessness.

Effects of preoperative BD on primary outcome

Postoperative mortality

All SRs¹⁸⁻²¹ reported postoperative mortality in patients with pCCA (**Table 2**). These SRs consistently showed no statistically significant difference in postoperative mortality between patients with and without preoperative BD (OR 0.70, 95% CI 0.41-1.19¹⁸; RR 0.935, 95% CI 0.612-1.429¹⁹; OR 0.94, 95% CI 0.54-1.63²⁰; and OR 1.06, 95% CI 0.70-1.61²¹). However, these findings were derived from unadjusted ORs or RR. The definitions of mortality were variable and ranged from postoperative day 30 to 90^{10, 14, 36-38}.

Table 1 Characteristics of included systematic reviews for comparison between preoperative BD and no preoperative BD in patients with resectable pCCA.

Inclusion period	Liu <i>et al.</i> , 2011 ¹⁸	Celotti <i>et al.</i> , 2017 ¹⁹	Yan <i>et al.</i> , 2018 ²⁰	Mehrabi <i>et al.</i> , 2020 ²¹
Patients inclusion based on surgical resection	1996-2010	1996-2013	2006-2016	1996-2019
Databases used and search window	Only patients undergoing resection	Only patients undergoing resection	All patients scheduled for resection	Only patients undergoing resection
Country	Medline, Embase, published between 1966 and January, 2010; The Chinese BioMedical Literature on disc, Chinese Medical Current Contents, published between 1978 and January, 2010	PubMed, Embase, Cochrane Library, published between 1980 and 2016	PubMed, Cochrane Library, search window not reported	PubMed, Web of Science, published up to March 2019
Study type included	China, the Netherlands, Japan, USA, UK, Spain	Italy, China, the Netherlands, USA, France/Belgium, Spain, Egypt	Japan, Italy, China, France/Belgium, the Netherlands, USA, Korea, Egypt	Italy, China, the Netherlands, Japan, USA, UK, France/Belgium, Spain, Egypt
Articles included (n)	Retrospective	Retrospective	Retrospective and prospective (non-randomized) ^b	Retrospective
Total number of patients analyzed ^a	11	9	19	16
Common outcomes	466/245	501/391	1434/744	1386/775
Quality assessment	Overall postoperative morbidity, postoperative infectious morbidity and mortality	Overall postoperative morbidity, postoperative mortality	Overall postoperative morbidity, infectious morbidity, postoperative mortality and long-term survival	Overall postoperative morbidity, postoperative major morbidity and mortality
Publication bias	NA	Newcastle-Ottawa Scale, the scores ranged from 5 to 8	14 of 19 studies were considered low quality based on the extent of selection bias	MINORS for quality of study: low to moderate; GRADE for quality of evidence: low
	No	NA	No	NA

^a The total number of patients is presented as preoperative BD/no preoperative BD. ^b Note that, Yan *et al.*²⁰ classified a prospective study³⁵ as retrospective study in their systematic review. BD, biliary drainage; pCCA, perihilar cholangiocarcinoma; MINORS, Methodological index for non-randomized studies; GRADE, Grading of Recommendations Assessment, Development, and Evaluation; NA, not applicable.

Table 2 Assessment of meta-analytical methods and results in systematic reviews comparing preoperative BD in patients with resectable pCCA.

	Liu <i>et al.</i> , 2011 ¹⁸	Celotti <i>et al.</i> , 2017 ¹⁹	Yan <i>et al.</i> , 2018 ²⁰	Mehrabi <i>et al.</i> , 2020 ²¹
ROBIS rating	High risk of bias	High risk of bias	High risk of bias	High risk of bias
Overall conclusion regarding BD	Not recommended for routine use	Preoperative BD was associated with higher postoperative morbidity	Preoperative BD may improve clinical outcomes in some jaundiced patients	Not recommended for routine use
Primary outcome^a				
Postoperative mortality	<ul style="list-style-type: none"> • 50/422 <i>versus</i> 29/238 • OR 0.70 (0.41-1.19) 	<ul style="list-style-type: none"> • 48/457 <i>versus</i> 38/384 • RR 0.935 (0.612-1.429) 	<ul style="list-style-type: none"> • 77/727 <i>versus</i> 45/445 • OR 0.85 (0.54-1.31) 	<ul style="list-style-type: none"> • 94/1040 <i>versus</i> 50/687 • OR 1.06 (0.70-1.61)
Secondary outcomes^a				
Overall postoperative morbidity	<ul style="list-style-type: none"> • 274/442 <i>versus</i> 112/233 • OR 1.67 (1.17-2.39) 	<ul style="list-style-type: none"> • 339/501 <i>versus</i> 228/391 • RR 1.266 (1.039-1.543) 	<ul style="list-style-type: none"> • 468/788 <i>versus</i> 250/475 • OR 1.51 (0.94-2.43) 	<ul style="list-style-type: none"> • 621/1015 <i>versus</i> 293/537 • OR 1.31 (0.94-1.82)
Postoperative major morbidity (CD III-IV)	NA	NA	NA	<ul style="list-style-type: none"> • 341/875 <i>versus</i> 131/533 • OR 1.51 (1.14-2.00)
Postoperative infectious morbidity	<ul style="list-style-type: none"> • 51/134 <i>versus</i> 26/122 • OR 2.17 (1.24-3.80) 	NA	<ul style="list-style-type: none"> • 84/193 <i>versus</i> 45/151 • OR 0.95 (0.30-3.02) 	NA
Postoperative survival	NA	NA	HR 0.94 (0.66-1.34)	NA
Meta-analytical approach	ORs with fixed-effects model for all outcomes	<ul style="list-style-type: none"> • RRs with fixed-effects model: mortality • RRs with random-effects model: overall postoperative morbidity 	<ul style="list-style-type: none"> • Mantel-Haenszel ORs with random-effects model: overall postoperative morbidity, infectious morbidity, mortality • Inverse variance method: survival 	Mantel-Haenszel ORs with random-effects model for all outcomes
Sensitivity analyses	NA	NA	NA	NA

Table 2 (Continued)

Subgroup analyses	Liu <i>et al.</i> , 2011 ¹⁸	Celotti <i>et al.</i> , 2017 ¹⁹	Yan <i>et al.</i> , 2018 ²⁰	Mehrabani <i>et al.</i> , 2020 ²¹
	NA	NA	<ul style="list-style-type: none"> Overall postoperative morbidity: studies in 2006-2011 OR 2.67 (1.71-4.16), studies in 2012-2016 OR 0.86 (0.52-1.42); Postoperative mortality: jaundiced patients OR 0.70 (0.33-1.45), partially jaundiced patients OR 0.94 (0.54-1.63) 	<ul style="list-style-type: none"> Postoperative mortality: simple selected patients OR 0.81 (0.47-1.40), strict selected patients OR 1.44 (0.49-4.26); Overall postoperative morbidity: simple selected patients OR 1.16 (0.58-2.33), strict selected patients OR 0.87 (0.57-1.32); Postoperative major morbidity: simple selected patients OR 1.57 (1.10-2.25), strict selected patients OR 0.51 (0.18-1.42)

^a Outcomes are presented as numbers of events/numbers of participants and preoperative BD versus no preoperative BD. The effect measures are presented as point estimates with the 95% confidence intervals. BD, biliary drainage; pCCA, perihilar cholangiocarcinoma; ROBIS, Risk Of Bias In Systematic reviews; OR, odds ratio; RR, relative risk; HR, hazard ratios; CD, Clavien-Dindo grade; NA, not applicable.

Effects of preoperative BD on secondary outcomes

Overall postoperative morbidity

All SRs ¹⁸⁻²¹ reported on overall postoperative morbidity (**Table 2**). Of these, two demonstrated that preoperative BD was associated with significantly higher overall postoperative morbidity compared to immediate surgery (OR 1.67, 95% CI 1.17-2.39 ¹⁸ and RR 1.266, 95% CI 1.039-1.543 ¹⁹), however, the other two SRs ^{20, 21} found no significant difference between two groups. Through subgroup analysis, Yan *et al.* ²⁰ showed higher overall postoperative morbidity in the preoperative BD group in the early 5 years' studies (2006-2011) (OR 2.67, 95% CI 1.71-4.16), but not in the late 5 years' studies (2012-2016).

Postoperative major morbidity

Postoperative major morbidity was reported by one SR ²¹ revealing significantly higher major morbidity in the preoperative BD group (OR 1.51, 95% CI 1.14-2.00) (**Table 2**). However, due to different indications for BD in the original studies, Mehrabi *et al.* ²¹ performed a subgroup analysis. Patients were classified into a simple criteria group where preoperative BD was routinely used in all jaundiced patients, and a strict criteria group where preoperative BD was performed in patients with cholangitis and/or total bilirubin levels ≥ 15.0 mg/dL and/or inadequate future liver remnant (FLR) volume in addition to jaundice ²¹. They found that preoperative BD was associated with increased postoperative major morbidity in 'simple criteria' patients (OR 1.57, 95% CI 1.10-2.25), but not in strictly selected patients (OR 0.51, 95% CI 0.18-1.42) ²¹.

Postoperative infectious morbidity

Of the two SRs that reported on postoperative infectious morbidity, one ¹⁸ showed a significant increase of infectious morbidity in patients receiving preoperative BD (OR 2.17, 95% CI 1.24-3.80) (**Table 2**). However, the infectious morbidity was similar in the other SR ²⁰. Celotti *et al.* ¹⁹ evaluated postoperative wound infections, showing significantly higher infection rates in the preoperative BD group (RR 2.035, 95% CI 1.041-3.977).

Postoperative long-term survival

Postoperative long-term survival was assessed by one SR ²⁰ and no difference between drained and undrained patients was observed (**Table 2**).

Comparison of effects between EBD and PTBD in resectable pCCA

Study characteristics

Characteristics of the seven SRs ²²⁻²⁸ investigating the effect of EBD compared to PTBD are presented in **Table 3**. These SRs included 24 original studies in total, ranging from 4 to 15 studies with 433 to 1230 participants per SR. Eight studies were repeatedly included in at least two SRs. Three ^{22, 26, 27} of seven SRs only included participants who underwent resection. All original studies included were retrospective studies. The quality of included original studies was moderate to high ²⁴⁻²⁸. One SR ²⁷ reported preoperative total bilirubin levels, ranging from 5.2 to 9.6 mg/dL and 8.4 to 12.0 mg/dL in the EBD and PTBD group, respectively. One SR reported median duration between drainage and surgery of 19 and 15 days in the EBD and PTBD group, respectively ²². Patients receiving PTBD had more advanced tumors compared to those receiving EBD in terms of Bismuth type IV ^{27, 28} and American Joint Committee on Cancer classification T3/4 stage ^{26, 28}.

Risk of bias of included systematic reviews

All but one SR ²²⁻²⁷ were classified as “at high risk of bias” using the ROBIS tool (**Supplementary Table S5**). A single SR ²⁸ was rated as “at low risk of bias”. The Cohen’s kappa coefficient for inter-rater agreement was 0.77. Four SRs ²⁴⁻²⁷ received the rating at high risk of bias mainly because of the restrictions in publication date and language. One SR ²² was assessed as high risk of bias due to the absence of quality assessment of the included studies and restrictions in publication date. The remaining SR ²³ rated as high risk of bias was due to the restriction in language and a lack of appropriate range of search databases and sensitivity analysis.

In addition, the numbers of studies incorporated and participants and outcomes abstracted were reported inconsistently across the SRs ²²⁻²⁸ (**Supplementary Table S6, Table S7**).

Comparison of effects between EBD and PTBD on primary outcomes

Postoperative mortality

Four SRs ^{22, 23, 25, 28} compared the effect of EBD *versus* PTBD on postoperative mortality (**Table 4**). Of these, three showed no statistically significant difference between the EBD group and the PTBD group (OR 0.47, 95% CI 0.17-1.24 ²³; OR 0.63,

95% CI 0.19-2.10²⁵; and OR 0.61, 95% CI 0.31-1.22²⁸). The remaining SR²² reported the percentage of deaths following resection, viz. 2% (6/281) and 6% (23/416) in EBD group and PTBD group, respectively ($p = 0.028$).

Comparison of effects between EBD and PTBD on secondary outcomes

Overall drainage-related morbidity

Four SRs^{22, 23, 25, 28} evaluated overall drainage-related morbidity. Three SRs demonstrated significantly higher drainage-related morbidity following an EBD procedure compared to PTBD (OR 2.23, 95% CI 1.39-3.57²³; OR 3.13, 95% CI 1.96-5.01²⁵; and OR 2.24, 95% CI 1.38-3.63²⁸) (**Table 4**). However, this significant difference was not noted in the SR by Hameed *et al.*²².

Drainage-related cholangitis

Of the five SRs^{22-25, 28} assessing drainage-related cholangitis, three pointed towards significantly higher cholangitis rates in the EBD group compared to the PTBD group (OR 5.41, 95% CI 2.75-10.63²³; RR 0.49, 95% CI 0.31-0.76 (PTBD *versus* EBS)²⁴; and OR 4.58, 95% CI 2.20-9.52²⁸) (**Table 4**). However, cholangitis rates were not affected by drainage procedure in the two other SRs^{22, 25}.

Drainage-related pancreatitis

Drainage-related pancreatitis was assessed by three SRs^{23, 25, 28}. The pancreatitis rates were consistently significantly higher in the EBD group compared to the PTBD group (OR 7.46, 95% CI 3.02-18.44²³; OR 11.52, 95% CI 2.59-51.30²⁵; and OR 8.90, 95% CI 1.74-45.44²⁸) (**Table 4**).

Overall postoperative morbidity

Two SRs^{23, 25} reported overall postoperative morbidity, which was not significantly different between the two drainage procedures (**Table 4**).

Postoperative major morbidity

One SR²⁸ assessed postoperative major morbidity and revealed no significant differences between EBD with PTBD (**Table 4**).

Table 3 Characteristics of included systematic reviews for the comparison between EBD and PTBD in patients with resectable pCCA.

	Hameed <i>et al.</i> , 2016 ²²	Al Mahjoub <i>et al.</i> , 2017 ²³	Tang <i>et al.</i> , 2017 ²⁴	Liu <i>et al.</i> , 2018 ²⁵	Wang <i>et al.</i> , 2019 ²⁶	Wang <i>et al.</i> , 2019 ²⁷	Hajibandeh <i>et al.</i> , 2020 ²⁸
Inclusion period	1996-2014	2010-2017	2010-2017	2010-2017	2014-2017	2011-2017	2010-2018
Patients inclusion based on surgical resection	Only patients undergoing resection	All patients scheduled for resection	All patients scheduled for resection	All patients scheduled for resection	Only patients undergoing resection	Only patients undergoing resection	All patients scheduled for resection
Databases used and search window	Medline, Embase, published between 1995 and December 2014	Medline, Cochrane Database, published by June 2016	Medline, Embase, Web of Science, published between 2000 and November 8, 2016	Medline, Embase, PubMed, Chinese Biological Medicine Database, CNKI, published between January, 1, 1990 and October, 31, 2017	PubMed, Medline, Cochrane Library, Web of Knowledge, published between January 1, 1990 and May 31, 2018	PubMed, Medline, Cochrane Library, Web of Knowledge, published between November, 1990 and March, 2018	Medline, Embase, CINAHL, CENTRAL, last search run on December 15, 2018
Country	Japan, China, the Netherlands, Germany, France, Spain, Canada, China (Taiwan)	Korea, the Netherlands, Japan	Korea, Japan, the Netherlands, Canada	the Netherlands, Korea, China, Japan	Japan, the Netherlands/USA, Korea	Japan, the Netherlands/USA, Korea	Japan, the Netherlands, Korea, USA
Study type included	Retrospective	Retrospective	Retrospective	Retrospective	Retrospective	Retrospective	Retrospective
Articles included (n)	15	4	7	6	4	6	9
Total number of patients analyzed ^a	536/494	275/158	548/273	379/286	427/385	601/629	758/678

Table 3 (Continued)

	Hameed <i>et al.</i> , 2016 ²²	Al Mahjoub <i>et al.</i> , 2017 ²³	Tang <i>et al.</i> , 2017 ²⁴	Liu <i>et al.</i> , 2018 ²⁵	Wang <i>et al.</i> , 2019 ²⁶	Wang <i>et al.</i> , 2019 ²⁷	Hajibandeh <i>et al.</i> , 2020 ²⁸
Common outcomes	Overall drainage-related morbidity, cholangitis, pancreatitis, liver failure, postoperative mortality and survival	Overall drainage-related morbidity, cholangitis, pancreatitis, overall postoperative morbidity, postoperative mortality	Drainage-related cholangitis, pancreatitis	Overall drainage-related morbidity, cholangitis, pancreatitis, overall postoperative morbidity, postoperative mortality	Postoperative overall survival	Seeding metastasis	Overall drainage-related morbidity, cholangitis, pancreatitis, postoperative major morbidity, seeding metastasis, postoperative mortality, 5-year survival
Quality assessment	NA	NA	Newcastle-Ottawa Scale, average medium quality, the scores ranged from 5 to 6	Newcastle-Ottawa Scale, the scores ranged from 5 to 6	Newcastle-Ottawa Scale, all studies were high quality	Newcastle-Ottawa Scale, all studies were high quality	Newcastle-Ottawa Scale, low to moderate risk of bias
Publication bias	NA	NA	No	No	NA	NA	NA

^a The total numbers of patients are presented as EBD/PTBD. EBD, endoscopic biliary drainage; PTBD, percutaneous transhepatic biliary drainage; pCCA, perihilar cholangiocarcinoma; CNKI, China National Knowledge Infrastructure; CINAHL, Cumulative Index to Nursing and Allied Health Literature; CENTRAL, Cochrane Central Register of Controlled Trials; NA, not applicable.

Table 4 Assessment of meta-analytical methods and results in systematic reviews comparing EBD with PTBD in patients with resectable pCCA

	Hameed <i>et al.</i> , 2016 ²²	Al Mahjoub <i>et al.</i> , 2017 ²³	Tang <i>et al.</i> , 2017 ²⁴	Liu <i>et al.</i> , 2018 ²⁵	Wang <i>et al.</i> , 2019 ²⁶	Wang <i>et al.</i> , 2019 ²⁷	Hajibandeh <i>et al.</i> , 2020 ²⁸
ROBIS rating	High risk of bias	High risk of bias	High risk of bias	High risk of bias	High risk of bias	High risk of bias	Low risk of bias
Overall conclusion	PTBD may be superior in short term morbidity; EBD is not inferior to PTBD in long term	PTBD was associated with lower drainage-related morbidity	PTBD was associated with lower drainage-related morbidity	PTBD may be superior in short term morbidity; no difference in long term	It is less convincing that EBD was superior to PTBD in overall survival	EBD was superior to PTBD in the prophylaxis of seeding metastasis	EBD was associated with higher drainage-related morbidity
Primary outcomes^a							
Postoperative mortality	<ul style="list-style-type: none"> • 6/281 <i>versus</i> 23/416 • 2% <i>versus</i> 6% 	<ul style="list-style-type: none"> • 7/185 <i>versus</i> 11/147 • OR 0.47 (0.17-1.24) 	NA	<ul style="list-style-type: none"> • 5/68 <i>versus</i> 8/85 • OR 0.63 (0.19-2.10) 	NA	NA	<ul style="list-style-type: none"> • 12/359 <i>versus</i> 25/416 • OR 0.61 (0.31-1.22)
Secondary outcomes^a							
Overall drainage-related morbidity	<ul style="list-style-type: none"> • 59/167 <i>versus</i> 26/90 • RR 1.26 (0.86-1.84) 	<ul style="list-style-type: none"> • 82/185 <i>versus</i> 39/147 • OR 2.23 (1.39-3.57) 	NA	<ul style="list-style-type: none"> • 125/232 <i>versus</i> 51/199 • OR 3.13 (1.96-5.01) 	NA	NA	<ul style="list-style-type: none"> • 123/207 <i>versus</i> 45/164 • OR 2.24 (1.38-3.63)
Drainage-related cholangitis	<ul style="list-style-type: none"> • 83/257 <i>versus</i> 11/101 • RR 3.36 (0.66-17.19) 	<ul style="list-style-type: none"> • 93/275 <i>versus</i> 12/158 • OR 5.41 (2.75-10.63) 	<ul style="list-style-type: none"> • 143/424 <i>versus</i> 55/347^b • RR 0.49 (0.31-0.76)^b 	<ul style="list-style-type: none"> • 89/334 <i>versus</i> 51/244 • OR 2.13 (0.31-14.52) 	NA	NA	<ul style="list-style-type: none"> • 67/207 <i>versus</i> 14/164 • OR 4.58 (2.20-9.52)
Drainage-related pancreatitis	NA	<ul style="list-style-type: none"> • 25/275 <i>versus</i> 0/158 • OR 7.46 (3.02-18.44) 	NA	<ul style="list-style-type: none"> • 21/177 <i>versus</i> 0/156 • OR 11.52 (2.59-51.30) 	NA	NA	<ul style="list-style-type: none"> • 18/207 <i>versus</i> 0/164 • OR 8.90 (1.74-45.44)
Overall postoperative morbidity	NA	<ul style="list-style-type: none"> • 40/185 <i>versus</i> 39/147 • OR 0.79 (0.36-1.76) 	NA	<ul style="list-style-type: none"> • 31/112 <i>versus</i> 40/147 • OR 0.87 (0.49-1.55) 	NA	NA	NA

Table 4 (Continued)

	Hameed <i>et al.</i> , 2016 ²²	Al Mahjoub <i>et al.</i> , 2017 ²³	Tang <i>et al.</i> , 2017 ²⁴	Liu <i>et al.</i> , 2018 ²⁵	Wang <i>et al.</i> , 2019 ²⁶	Wang <i>et al.</i> , 2019 ²⁷	Hajibandeh <i>et al.</i> , 2020 ²⁸
Postoperative major morbidity	NA	NA	NA	NA	NA	NA	<ul style="list-style-type: none"> • 173/511 versus 225/579 • OR 0.78 (0.60-1.01) NA
Postoperative liver failure	<ul style="list-style-type: none"> • 22/194 versus 56/432 • 11% versus 13% 	NA	NA	NA	NA	NA	NA
Seeding metastasis	NA	NA	NA	NA	NA	<ul style="list-style-type: none"> • 64/649 versus 120/685 • OR 0.35 (0.17-0.74) NA 	<ul style="list-style-type: none"> • 40/419 versus 80/404 • OR 0.46 (0.30-0.71) • 185/427 versus 129/385 • OR 1.62 (1.21-2.17)
Postoperative survival	<ul style="list-style-type: none"> • 1-year 91% versus 73% • 5-year 46% versus 30% 	NA	NA	NA	<ul style="list-style-type: none"> • HR 0.67 (0.53-0.85) 	NA	NA
Meta-analytical approach	Mantel-Haenszel RRs with random-effects model for overall drainage-related morbidity, cholangitis	<ul style="list-style-type: none"> • Mantel-Haenszel ORs with fixed-effects model: overall drainage-related morbidity, cholangitis, mortality • Mantel-Haenszel OR with random effects model: overall postoperative morbidity • Peto OR with fixed-effects model: pancreatitis 	Mantel-Haenszel RRs with fixed-effects model	<ul style="list-style-type: none"> • Inverse-variance method with fixed-effects model: overall drainage-related morbidity, pancreatitis, overall postoperative morbidity, mortality • DerSimonian-Laird OR with random-effects model: cholangitis 	Inverse-variance method with fixed-effects model	Mantel-Haenszel OR with random effects model	Mantel-Haenszel ORs with fixed-effects model for all outcomes

Table 4 (Continued)

	Hameed <i>et al.</i> , 2016 ²²	Al Mahjoub <i>et al.</i> , 2017 ²³	Tang <i>et al.</i> , 2017 ²⁴	Liu <i>et al.</i> , 2018 ²⁵	Wang <i>et al.</i> , 2019 ²⁶	Wang <i>et al.</i> , 2019 ²⁷	Hajibandeh <i>et al.</i> , 2020 ²⁸
Sensitivity analyses	NA	NA	No change in the direction of the effect size	NA	NA	NA	No change in the direction of the effect sizes: postoperative mortality, postoperative major morbidity NA
Subgroup analyses	NA	NA	ENBD <i>versus</i> EBS: cholangitis RR 1.96 (0.96- 4.01), pancreatitis RR 1.62 (0.76- 3.47)	NA	NA	NA	NA

^a Outcomes are presented as numbers of events/numbers of participants and EBD *versus* PTBD. The effect measures are presented as point estimates with the 95% confidence intervals. ^b Tang *et al.*²⁴ presented and calculated drainage-related cholangitis rates based on PTBD *versus* EBD. EBD, endoscopic biliary drainage; PTBD, percutaneous transhepatic biliary drainage; pCCA, perihilar cholangiocarcinoma; ENBD, endoscopic nasobiliary drainage; EBS, endoscopic biliary stenting; OR, odds ratio; RR, relative risk; HR, hazard ratios; NA, not applicable.

Postoperative liver failure

Postoperative liver failure rates were evaluated in one SR ²², where similar incidence rates were observed, 11% (22/194) and 13% (56/432) in the EBD and PTBD group, respectively ($p = 0.570$) (**Table 4**).

Seeding metastasis

Two SRs reported the incidence of drainage-related seeding metastasis and unanimously showed lower rates in the EBD group (OR 0.27, 95% CI 0.13-0.56 ²⁷ and OR 0.46, 95% CI 0.30-0.71 ²⁸) (**Table 4**).

Postoperative long-term survival

Of the three SRs ^{22, 26, 28} assessing postoperative survival, Wang *et al.* ²⁶ and Hajibandeh *et al.* ²⁸ revealed that patients receiving EBD had longer overall survival (HR 0.67, 95% 0.53-0.85) and 5-year survival (OR 1.62, 95% CI 1.21-2.17), respectively, than those receiving PTBD (**Table 4**). Hameed *et al.* ²² showed that median 1-year postoperative survival was 91% and 73%, 5-year survival 46% and 30% in EBD and PTBD group, respectively.

Discussion

We performed a SR of SRs to assess the effect and route of preoperative BD in patients with resectable pCCA. The available evidence reflected conflicting results, and identified substantial variation in data abstraction and statistical methods, and high risk of bias in most included SRs. Preoperative BD may need to be used in strictly selected patients with resectable pCCA. EBD might be associated with more short-term drainage-related morbidity compared to PTBD in patients with resectable pCCA. However, EBD might be related with better long-term outcomes after surgery.

All but one SR included in the present study are rated as high risk of bias according to ROBIS assessment. Most SRs ^{19, 20, 22-27} had restrictions in publication date and language. Three SRs ^{18, 22, 23} did not report quality assessment of original studies. Additionally, there is considerable variability in the inclusion of studies and the numbers of participants and events abstracted across the SRs. These differences could be caused by variable period of study inclusion, inclusion criteria, and data extraction errors. The errors in data extraction procedure are a vital source of bias in SRs ³⁹. The

influence of risk of bias on pooled results could underestimate or overestimate the actual intervention effects, resulting in a limitation of the validity of the conclusions ⁴⁰.

The indications for preoperative BD have not reached unanimity. Elevated serum bilirubin levels are generally used as indicator for BD. A recent study demonstrated that preoperative BD should be performed when serum bilirubin ≥ 6.0 mg/dL and not recommended when bilirubin levels < 2.5 mg/dL ⁶. Imaging of the liver plays an important role in the management of pCCA and the use of BD ⁴¹. FLR volume assessment by computed tomography (CT) or magnetic resonance imaging (MRI) is another vital determinant to perform BD ⁴². For patients with FLR volumes $< 50\%$, preoperative drainage can be beneficial to decrease mortality, but not for large FLR volumes $> 50\%$ ⁴². Normalization of bile duct diameters in the FLR on ultrasound examination and 20% or more decrease of total bilirubin levels after 7 days, are considered indicators of therapeutic success of BD ⁹. The SR of Mehrabi *et al.* ²¹ revealed that preoperative BD was not associated with postoperative major morbidity in strictly selected patients with cholangitis and/or high bilirubin levels (≥ 15.0 mg/dL) and/or inadequate FLR. In contrast, increased postoperative major morbidity was observed in simple criteria patients where preoperative BD was routinely used in all jaundiced patients ²¹.

We found that postoperative mortality was similar between drained and undrained patients. This finding seems consistent with previous studies ^{43, 44}, which concluded that preoperative BD does not improve postoperative mortality. However, the latter conclusions were derived from unadjusted odds ratios (ORs) or relative risk (RR), without considering tumor features and resection methods. Hence, we cannot decisively conclude whether preoperative BD has effects on postoperative mortality.

EBD can be associated with high drainage-related morbidity caused by retrograde bacterial contamination from the proximal small intestine ^{15, 28}. Our study demonstrated that overall drainage-related morbidity and pancreatitis rates were higher in the EBD group than in the PTBD group (ORs from 2.23 to 3.13, 7.46 to 11.52, respectively). Hameed *et al.* ²² indicated no differences in overall drainage-related morbidity between the two drainage routes. However, they included fewer original articles in their SR, which was therefore more susceptible to publication bias. Although PTBD seems to induce less drainage-related morbidity, other major complications must be considered, including tumor seeding. We found that PTBD was associated with

significantly higher incidence of seeding metastasis, which was consistent with other studies ^{16, 45}.

Our analysis showed that mortality was not statistically different in EBD *versus* PTBD in most included SRs. However, a recently published Dutch randomized controlled trial (RCT) ⁹ demonstrated significantly higher mortality in PTBD group compared to EBD group ⁹. Importantly, in the RCT both perioperative and postoperative mortality were assessed. PTBD significantly increased perioperative mortality, with 3 of 11 fatalities occurring before surgery in patients receiving PTBD ⁹.

Most included SRs did not state whether internal or external drainage was used. However, internal drainage elicits a different physiological response compared to external drainage. The latter drainage mode abrogates the digestive, signaling and antimicrobial roles of bile salts in EHC. Diminished activation of the ileal bile salt receptor farnesoid X receptor (FXR) can result in the loss of negative feedback control of hepatic bile salt synthesis and attendant bile salt overload and hepatotoxicity ⁴⁶. Importantly, bile salt signaling and maintained bile salt homeostasis is required for proper regrowth of the remnant liver ^{47, 48}. Liver regeneration volumes and rates are positively associated with serum bile salts levels in patients undergoing major hepatectomy ⁴⁹. However, bile can be easily given back orally with external drainage mode ⁵⁰.

Overall, preoperative BD has merits and limitations. Preoperative BD can decompress the biliary obstruction, mitigate intrahepatic bile salt overload, relieve cholangitis, and improve liver function ^{8, 10, 11}. Moreover, the restoration of bile flow to the small intestine may improve epithelium function of gut and decrease bacterial translocation ⁵¹. However, preoperative BD can result in complications. For example, PTBD is related to bile leakage and vascular complications including haemobilia and pseudoaneurysm ⁵². In addition, tumor seeding metastasis might be another potential risk ²⁷. EBD can be associated with drainage-related cholangitis and pancreatitis, which may have nonspecific clinical manifestations ^{23, 28}.

Our SR has certain strengths. First, a pre-specified protocol was followed in the review procedure, and a comprehensive and robust search strategy by two information experts was used to identify studies. Second, we used a validated instrument, ROBIS tool, to specifically assess the risk of bias in the included SRs. Finally, identification of studies, data collection, and study appraisal was performed by two reviewers

independently to minimize potential bias.

Our SR also has several limitations, mainly caused by limitations in the included studies. First, eight ^{19, 20, 22-27} of eleven SRs had restrictions in publication date and/or English language. All but one SR did not include unpublished or grey literature in their selection criteria ¹⁸⁻²⁷. This could narrow the breadth of data sources and exclude potentially eligible studies. Second, because many original studies were repeatedly included in at least two SRs and there was heterogeneity in the definitions of outcomes, we could not calculate overall pooled estimates. Third, the ORs and/or RRs of primary and secondary outcomes were only reported in only few SRs, therefore, the results need to be interpreted with caution. Finally, all but one of the original studies included in the SRs ¹⁸⁻²⁸ had a retrospective design, resulting in selection bias.

It is unclear from the SRs ²²⁻²⁸ if in PTBD procedures bile was given back to the patients (to restore functionality of the EHC). In the Dutch RCT, external drainage of bile may be a reason for the increased mortality in patients receiving PTBD ⁹. Pre-clinical studies have demonstrated FXR-mediated acceleration of liver growth in absence ⁵³ and presence ⁴⁸ of partial hepatectomy. Recent clinical trials have also shown that FXR activation can improve cholestasis in patients with primary biliary cirrhosis ⁵⁴ and primary sclerosing cholangitis ⁵⁵, and liver histological features in patients with primary biliary cholangitis ⁵⁶. Bile salt supplementation or FXR agonism may have the potential to replace preoperative BD to improve perioperative outcomes in patients with pCCA, considering the activation of pathways involved in bile salt homeostasis and liver regeneration ⁴⁶⁻⁴⁸.

Conclusion

The present SR of SRs comprehensively assesses the effectiveness of PBD *versus* no PBD, and the superiority of EBD *versus* and PTBD on perioperative and long-term outcomes in patients with resectable pCCA. The preoperative BD might need to be performed in strictly selected patients in terms of cholangitis, bilirubin levels and future liver remnant volume to avoid increased postoperative major morbidity. EBD might be associated with higher short-term drainage-related morbidity compared to PTBD. But EBD might be related with more favorable postoperative long-term outcomes. The available evidence has high risk of bias. Large sample sizes and/or international

multicentre RCTs are urgently needed to assess the value of preoperative BD in surgical management of patients with resectable pCCA.

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Supplementary file

Appendix S1 Search strategy used for electronic databases.

PubMed

- #1 Klatskin tumor[MeSH Terms]
- #2 Klatskin tumor*[ti/ab]
- #3 Klatskin's tumor*[ti/ab]
- #4 (bile duct neoplasms[MeSH Terms]) OR cholangiocarcinoma[MeSH Terms]
- #5 bile duct[ti/ab]
- #6 (((tumor*[ti/ab]) OR tumour*[ti/ab]) OR carci*[ti/ab]) OR cancer*[ti/ab]) OR neoplas*[ti/ab]
- #7 #5 AND #6
- #8 cholangiocarcinoma*[ti/ab]
- #9 #4 OR #7 OR #8
- #10 ((hilar[ti/ab]) OR perihilar[ti/ab]) OR proximal[ti/ab]
- #11 #9 AND #10
- #12 hepatic duct[ti/ab]
- #13 #6 AND #12
- #14 bile duct bifurcation carcinoma*[ti/ab]
- #15 #1 OR #2 OR #3 OR #11 OR #13 OR #14
- #16 cholangiopancreatography, endoscopic retrograde[MeSH Terms]
- #17 (((bile drain*[ti/ab]) OR bile duct drain*[ti/ab]) OR bile tract drain*[ti/ab]) OR biliary drain*[ti/ab] OR nasobiliary drain*[ti/ab]
- #18 (biliary decompression[ti/ab]) OR biliary stent*[ti/ab]
- #19 ((((((endoscopic retrograde cholangiopancreatography[ti/ab]) OR endoscopic retrograde cholangiopancreatographies[ti/ab]) OR endoscopic retrograde cholangiography[ti/ab]) OR endoscopic cholangiography[ti/ab]) OR endoscopic cholangiopancreatography[ti/ab]) OR endoscopic pancreatocholangiography[ti/ab]) OR ERCP[ti/ab]
- #20 #16 OR #17 OR #18 OR #19
- #21 ((morbidity[MeSH Terms]) OR mortality[MeSH Terms]) OR survival[MeSH Terms]
- #22 (((morbidity[ti/ab]) OR complicat*[ti/ab]) OR mortality[ti/ab]) OR death rate[ti/ab]) OR survival[ti/ab]
- #23 #21 OR #22
- #24 #15 AND #20 AND #23

Embase

- #1 Klatskin tumor/
- #2 ('Klatskin tumor' or 'Klatskin tumors' or 'Klatskin's tumor' or 'Klatskin's tumors').ab,ti.
- #3 exp bile duct tumor/
- #4 bile duct'.ab,ti.

Chapter 2

- #5 (tumor or tumors or tumour or tumours or cancer or cancers or carci* or neoplas*).ab,ti.
- #6 #4 and #5
- #7 cholangiocarcinoma*.ab,ti.
- #8 #3 or #6 or #7
- #9 (hilar or perihilar or proximal).ab,ti.
- #10 #8 and #9
- #11 hepatic duct'.ab,ti.
- #12 #5 and #11
- #13 #1 or #2 or #10 or #12
- #14 exp biliary tract drainage/
- #15 endoscopic retrograde cholangiopancreatography/
- #16 ('bile drainage' or 'bile duct drainage' or 'bile tract drainage' or 'biliary drainage' or 'nasobiliary drainage' or 'biliary decompression' or 'biliary stent' or 'biliary stenting').ab,ti.
- #17 ('endoscopic retrograde cholangiopancreatography' or 'endoscopic retrograde cholangiopancreatographies' or 'endoscopic retrograde cholangiography' or 'endoscopic cholangiography' or 'endoscopic cholangiopancreatography' or 'endoscopic pancreatocholangiography' or ERCP).ab,ti.
- #18 #14 or #15 or #16 or #17
- #19 exp morbidity/
- #20 exp complication/
- #21 exp mortality/
- #22 exp survival/
- #23 (morbidity or complicat* or mortality or 'death rate' or survival).ab,ti.
- #24 #19 or #20 or #21 or #22 or #23
- #25 #13 and #18 and #24

Cochrane Library

- #1 MeSH descriptor: [Klatskin Tumor] explode all trees
- #2 ('Klatskin tumor*' or 'Klatskin's tumor*'):ti,ab,kw
- #3 MeSH descriptor: [Bile Duct Neoplasms] explode all trees
- #4 MeSH descriptor: [Cholangiocarcinoma] explode all trees
- #5 (bile duct):ti,ab,kw
- #6 (tumor* or tumour* or carci* or cancer* or neoplas*):ti,ab,kw
- #7 #5 and #6
- #8 (cholangiocarcinoma*):ti,ab,kw
- #9 #3 or #4 or #7 or #8
- #10 (hilar or perihilar or proximal):ti,ab,kw
- #11 #9 and #10
- #12 (hepatic duct):ti,ab,kw

- #13 #6 and #12
- #14 (bile duct bifurcation carcinoma*):ti,ab,kw
- #15 #1 or #2 or #11 or #13 or #14
- #16 MeSH descriptor: [Cholangiopancreatography, Endoscopic Retrograde] explode all trees
- #17 ('bile drain*' or 'bile duct drain*' or 'bile tract drain*' or 'biliary drain*' or 'nasobiliary drain*'):ti,ab,kw
- #18 ('biliary decompression' or 'biliary stent*'):ti,ab,kw
- #19 ('endoscopic retrograde cholangiopancreatography' or 'endoscopic retrograde cholangiopancreatographies' or 'endoscopic retrograde cholangiography' or 'endoscopic cholangiography' or 'endoscopic cholangiopancreatography' or 'endoscopic pancreatocholangiography' or ERCP):ti,ab,kw
- #20 #16 or #17 or #18 or #19
- #21 MeSH descriptor: [Morbidity] explode all trees
- #22 MeSH descriptor: [Mortality] explode all trees
- #23 MeSH descriptor: [Survival] explode all trees
- #24 (morbidity or complicat* or mortality OR death rate OR survival):ti,ab,kw
- #25 #21 or #22 or #23 or #24
- #26 #15 and #20 and #25

Table S1 List of excluded full text reviewed studies with reasons.

Authors, Year	Title	Exclusion reason
Takada <i>et al.</i> , 2001 ¹	Is preoperative biliary drainage necessary according to evidence-based medicine?	Not a systematic review
Belghiti <i>et al.</i> , 2005 ²	Preoperative optimization of the liver for resection in patients with hilar cholangiocarcinoma	Not a systematic review
Sakata <i>et al.</i> , 2005 ³	Catheter tract implantation metastases associated with percutaneous biliary drainage for extrahepatic cholangiocarcinoma	Not a systematic review
Briggs <i>et al.</i> , 2007 ⁴	Investigation and management of obstructive jaundice	Not a systematic review
Maguchi <i>et al.</i> , 2007 ⁵	Preoperative biliary drainage for hilar cholangiocarcinoma	Not a systematic review
Nagino <i>et al.</i> , 2008 ⁶	Preoperative biliary drainage for biliary tract and ampullary carcinomas	Not a systematic review
Nimura <i>et al.</i> , 2008 ⁷	Preoperative biliary drainage before resection for cholangiocarcinoma (Pro)	Not a systematic review
van Delden <i>et al.</i> , 2008 ⁸	Percutaneous drainage and stenting for palliation of malignant bile duct obstruction	Not a systematic review
Kawakami <i>et al.</i> , 2011 ⁹	Preoperative biliary drainage for hilar cholangiocarcinoma: which stent should be selected?	Not a systematic review
Parodi <i>et al.</i> , 2012 ¹⁰	Endoscopic management of hilar cholangiocarcinoma	Not a systematic review
Iacono <i>et al.</i> , 2013 ¹¹	Role of preoperative biliary drainage in jaundiced patients who are candidates for pancreatoduodenectomy or hepatic resection: highlights and drawbacks	Not a systematic review
Webb <i>et al.</i> , 2013 ¹²	Endoscopic Management of Malignant Bile Duct Strictures	Not a systematic review
Yasuda <i>et al.</i> , 2013 ¹³	Unilateral versus bilateral endoscopic biliary stenting for malignant hilar biliary strictures	Not a systematic review
Zhimin <i>et al.</i> , 2013 ¹⁴	Advances in diagnosis and treatment of hilar cholangiocarcinoma - A review	Not a systematic review
Fang <i>et al.</i> , 2013 ¹⁵	Meta-analysis of randomized clinical trials on safety and efficacy of biliary drainage before surgery for obstructive jaundice	Not appropriate participants
Leng <i>et al.</i> , 2014 ¹⁶	Percutaneous transhepatic and endoscopic biliary drainage for malignant biliary tract obstruction: a meta-analysis	Not appropriate participants
Paik <i>et al.</i> , 2014 ¹⁷	Preoperative biliary drainage in hilar cholangiocarcinoma: When and how?	Not a systematic review
Soares <i>et al.</i> , 2014 ¹⁸	Hilar cholangiocarcinoma: diagnosis, treatment options, and management	Not a systematic review
Park <i>et al.</i> , 2015 ¹⁹	Endoscopic ultrasound-guided biliary drainage of hilar biliary obstruction	Not a systematic review
Poruk <i>et al.</i> , 2015 ²⁰	Perioperative Management of Hilar Cholangiocarcinoma	Not a systematic review
Rodarte-Shade <i>et al.</i> , 2015 ²¹	Stent placement as a bridge to surgery in malignant biliary obstruction (pancreatic cancer, distal bile duct cancer, and hilar tumors)	Not a systematic review
Rustagi <i>et al.</i> , 2015 ²²	Endoscopic treatment of malignant biliary strictures	Not a systematic review
Saxena <i>et al.</i> , 2015 ²³	Preoperative biliary drainage	Not a systematic review
Tsuchikawa <i>et al.</i> , 2015 ²⁴	Advances in the surgical treatment of hilar cholangiocarcinoma	Not a systematic review

Table S1 (continued)

Authors, Year	Title	Exclusion reason
Umeda <i>et al.</i> , 2015 ²⁵	Current status of preoperative biliary drainage	Not a systematic review
Jo <i>et al.</i> , 2017 ²⁶	Best options for preoperative biliary drainage in patients with Klatskin tumors	Not a systematic review
Tringali <i>et al.</i> , 2017 ²⁷	Endoscopic vs percutaneous preoperative biliary drainage in hilar cholangiocarcinoma: A systematic review and meta-analysis	Abstract only
Tang <i>et al.</i> , 2018 ²⁸	The clinicopathological factors associated with prognosis of patients with resectable perihilar cholangiocarcinoma: A systematic review and meta-analysis	No outcomes of interest
Lee <i>et al.</i> , 2020 ²⁹	Biliary stenting for hilar malignant biliary obstruction	Not a systematic review

Table S2 Assessment with ROBIS for risk of bias of systematic reviews comparing preoperative BD with no preoperative BD in patients with resectable pCCA.

	Phase 2											
	Domain 1: Study eligibility criteria					Domain 2: Identification and selection of studies						
	1.1 Did the review adhere to pre-defined objectives and eligibility criteria?	1.2 Were the eligibility criteria appropriate for the review question?	1.3 Were eligibility criteria unambiguous?	1.4 Were all restrictions in eligibility criteria based on study characteristics appropriate (e.g. date, sample size, study quality, outcomes measured)?	1.5 Were any restrictions in eligibility criteria based on sources of information appropriate (e.g. publication status or format, language, availability of data)?	Concerns	2.1 Did the search include an appropriate range of databases/electronic sources for published and unpublished reports?	2.2 Were methods additional to database searching used to identify relevant reports?	2.3 Were the terms and structure of the search strategy likely to retrieve as many eligible studies as possible?	2.4 Were restrictions based on date, publication format, or language appropriate?	2.5 Were efforts made to minimise error in selection of studies?	Concerns
Review												
Liu <i>et al.</i> , 2011 ³⁰	PY	Y	PN	PY	Y	High	N	Y	PN	PN	NI	High
Celotti <i>et al.</i> , 2017 ³¹	PY	PN	PN	NI	N	High	PY	PY	PY	N	PY	High
Yan <i>et al.</i> , 2018 ³²	PY	PN	PN	PY	Y	High	PN	NI	NI	NI	NI	High
Mehrabi <i>et al.</i> , 2020 ³³	PY	Y	Y	Y	Y	Low	PN	Y	Y	PY	Y	High

Table S2 (continued)

Phase 2												
Domain 3: Data collection and study appraisal							Domain 4: Synthesis and findings					
3.1 Were efforts made to minimise error in data collection?	3.2 Were sufficient study characteristics available for both review authors and readers to be able to interpret the results?	3.3 Were all relevant study results collected for use in the synthesis?	3.4 Was risk of bias (or methodological quality) formally assessed using appropriate criteria?	3.5 Were efforts made to minimise error in risk of bias assessment?	Concerns	4.1 Did the synthesis include all studies that it should?	4.2 Were all pre-defined analyses reported or departures explained?	4.3 Was the synthesis appropriate given the nature and similarity in the research questions, study designs and outcomes across included studies?	4.4 Was between-study variation (heterogeneity) minimal or addressed in the synthesis?	4.5 Were the findings robust, e.g. as demonstrated through funnel plot or sensitivity analyses?	4.6 Were biases in primary study minimised or addressed in the synthesis?	Concerns
Review	Y	PY	PY	N	PN	High	Y	NI	PY	PY	N	High
	NI	PN	Y	Y	Y	High	Y	NI	PY	PY	PN	High
	Y	Y	PY	Y	NI	Low	Y	NI	Y	PY	PN	High
	NI	Y	Y	Y	Y	Low	Y	PY	Y	N	PN	High
Liu <i>et al.</i> , 2011 ³⁰	Y	PY	PY	N	PN	High	Y	NI	PY	PY	N	High
Celotti <i>et al.</i> , 2017 ³¹	NI	PN	Y	Y	Y	High	Y	NI	PY	PY	PN	High
Yan <i>et al.</i> , 2018 ³²	Y	Y	PY	Y	NI	Low	Y	NI	Y	PY	PN	High
Mehrabi <i>et al.</i> , 2020 ³³	NI	Y	Y	Y	Y	Low	Y	PY	Y	N	PN	High

Table S2 (continued)

Review	Phase 3			
	Risk of bias in the review			Overall risk of bias in the review
	A. Did the interpretation of findings address all of the concerns identified in Domains 1 to 4?	B. Was the relevance of identified studies to the review's research question appropriately considered?	C. Did the reviewers avoid emphasizing results on the basis of their statistical significance?	
Liu <i>et al.</i> , 2011 ³⁰	N	Y	Y	High
Celotti <i>et al.</i> , 2017 ³¹	N	Y	Y	High
Yan <i>et al.</i> , 2018 ³²	N	Y	Y	High
Mehrabi <i>et al.</i> , 2020 ³³	N	Y	Y	High

ROBIS, Risk Of Bias In Systematic reviews; BD, biliary drainage; pCCA, perihilar cholangiocarcinoma; Y, Yes; PY, Probably yes; PN, Probably no; N, No; NI, No information.

Table S3 Number of studies and participants in systematic reviews for the comparison between preoperative BD and no preoperative BD in patients with resectable pCCA.

	Studies included in systematic review				Number of participants (preoperative BD/no preoperative PBD)			
	Liu <i>et al.</i> , 2011 ³⁰	Celotti <i>et al.</i> , 2017 ³¹	Yan <i>et al.</i> , 2018 ³²	Mehrabi <i>et al.</i> , 2020 ³³	Liu <i>et al.</i> , 2011 ³⁰	Celotti <i>et al.</i> , 2017 ³¹	Yan <i>et al.</i> , 2018 ³²	Mehrabi <i>et al.</i> , 2020 ³³
Su <i>et al.</i> , 1996 ³⁴	✓	✓	-	✓	33/16	33/16	-	33/16
Takada <i>et al.</i> , 1996 ³⁵	✓	-	-	-	24/12	-	-	-
Hochwald <i>et al.</i> , 1999 ³⁶	✓	✓	-	-	42/29	42/29	-	-
Parks <i>et al.</i> , 2000 ³⁷	✓	-	-	✓	20/27	-	-	20/27
Gerhards <i>et al.</i> , 2000 ³⁸	✓	-	-	✓	93/18	-	-	93/18
Figueras <i>et al.</i> , 2000 ³⁹	✓	✓	-	✓	11/9	11/9	-	11/9
Dinant <i>et al.</i> , 2006 ⁴⁰	✓	✓	✓	✓	83/14	83/14	85/14	83/14
Sano <i>et al.</i> , 2006 ⁴¹	-	-	✓	-	-	-	65/37	-
Chen <i>et al.</i> , 2007 ⁴²	✓	-	-	-	31/27	-	-	-
Ferrero <i>et al.</i> , 2009 ⁴³	✓	✓	✓	✓	30/30	30/30	30/30	30/30
Li <i>et al.</i> , 2009 ⁴⁴	✓	-	-	-	55/56	-	-	-
Sakata <i>et al.</i> , 2009 ⁴⁵	-	-	✓	-	-	-	71/10	-
El-Hanafy <i>et al.</i> , 2010 ⁴⁶	-	✓	✓	✓	-	46/54	46/54	46/54
Ercolani <i>et al.</i> , 2010 ⁴⁷	✓	✓	✓	✓	44/7	44/7	44/7	44/7
Hirano <i>et al.</i> , 2010 ⁴⁸	-	-	✓	-	-	-	131/15	-
Rocha <i>et al.</i> , 2010 ⁴⁹	-	-	✓	-	-	-	38/22	-
Grandadam <i>et al.</i> , 2010 ⁵⁰	-	-	✓	-	-	-	12/26	-
Regimbeau <i>et al.</i> , 2011 ⁵¹	-	-	✓	-	-	-	38/18	-
Nuzzo <i>et al.</i> , 2012 ⁵²	-	-	✓	-	-	-	252/47	-
Cho <i>et al.</i> , 2012 ⁵³	-	-	✓	-	-	-	84/21	-
Farges <i>et al.</i> , 2013 ⁵⁴	-	✓	✓	✓	-	180/186	180/186	180/186
Ratti <i>et al.</i> , 2013 ⁵⁵	-	-	✓	-	-	-	55/39	-

Table S3 (continued)

	Studies included in systematic review				Number of participants (preoperative BD/no preoperative PBD)			
	Liu <i>et al.</i> , 2011 ³⁰	Celotti <i>et al.</i> , 2017 ³¹	Yan <i>et al.</i> , 2018 ³²	Mehrabi <i>et al.</i> , 2020 ³³	Liu <i>et al.</i> , 2011 ³⁰	Celotti <i>et al.</i> , 2017 ³¹	Yan <i>et al.</i> , 2018 ³²	Mehrabi <i>et al.</i> , 2020 ³³
Yu <i>et al.</i> , 2013 ⁵⁶	-	-	✓	-	-	-	56/60	-
Xiong <i>et al.</i> , 2013 ⁵⁷	-	✓	✓	✓	-	32/46	32/46	32/46
Furusawa <i>et al.</i> , 2014 ⁵⁸	-	-	✓	-	-	-	122/22	-
Yan <i>et al.</i> , 2014 ⁵⁹	-	-	✓	-	-	-	64/67	-
Ribero <i>et al.</i> , 2016 ⁶⁰	-	-	-	✓	-	-	-	98/35
Ito <i>et al.</i> , 2016 ⁶¹	-	-	✓	-	-	-	29/23	-
Cai <i>et al.</i> , 2017 ⁶²	-	-	-	✓	-	-	-	55/163
Higuchi <i>et al.</i> , 2017 ⁶³	-	-	-	✓	-	-	-	163/45
Kimura <i>et al.</i> , 2017 ⁶⁴	-	-	-	✓	-	-	-	153/30
Zhang <i>et al.</i> , 2018 ⁶⁵	-	-	-	✓	-	-	-	196/44
Ratti <i>et al.</i> , 2019 ⁶⁶	-	-	-	✓	-	-	-	149/51

BD, biliary drainage; pCCA, perihilar cholangiocarcinoma.

Table S4 Number of abstracted events included in systematic reviews for the comparison between preoperative BD and no preoperative BD in patients with resectable pCCA.

	Postoperative mortality				Overall postoperative morbidity				Postoperative infectious morbidity			
	Celotti et al., 2017 ³¹		Yan et al., 2018 ³²		Liu et al., 2011 ³⁰		Celotti et al., 2017 ³¹		Liu et al., 2011 ³⁰		Celotti et al., 2017 ³¹	
	Liu et al., 2011 ³⁰	Mehrabian et al., 2020 ³³	Liu et al., 2018 ³²	Mehrabian et al., 2020 ³³	Liu et al., 2011 ³⁰	Mehrabian et al., 2020 ³³	Liu et al., 2017 ³¹	Mehrabian et al., 2020 ³³	Liu et al., 2011 ³⁰	Mehrabian et al., 2020 ³³	Liu et al., 2017 ³¹	Mehrabian et al., 2020 ³³
Su et al., 1996 ³⁴	5/0	5/0	-	5/0	17/6	17/6	17/6	17/6	-	-	-	-
Takada et al., 1996 ³⁵	3/6	-	-	-	-	-	-	-	-	-	-	-
Hochwald et al., 1999 ³⁶	2/4	2/4	-	-	36/19	42/23	42/23	-	22/8	-	-	-
Parks et al., 2000 ³⁷	1/1	-	-	1/1	11/11	-	-	5/3	5/6	-	-	-
Gerhards et al., 2000 ³⁸	16/3	-	-	16/3	59/13	-	-	59/13	-	-	-	-
Figueras et al., 2000 ³⁹	1/2	1/2	-	1/2	11/6	11/6	11/6	11/6	2/0	-	-	-
Dinant et al., 2006 ⁴⁰	14/2	14/2	14/2	14/2	56/6	56/6	56/6	56/6	-	-	-	-
Sano et al., 2006 ⁴¹	-	-	-	-	-	-	-	36/15	-	-	-	-
Chen et al., 2007 ⁴²	3/3	-	-	-	18/14	-	-	-	10/7	-	-	-
Ferrero et al., 2009 ⁴³	1/3	1/3	1/3	1/3	21/19	21/19	21/19	21/19	12/5	-	12/5	-
Li et al., 2009 ⁴⁴	4/5	-	-	-	20/16	-	-	-	-	-	-	-
Sakata et al., 2009 ⁴⁵	-	-	9/0	-	-	-	-	-	-	-	54/7	-
El-Hanafy et al., 2010 ⁴⁶	-	5/3	5/3	5/3	-	27/11	27/11	7/11	-	-	-	-
Ercolani et al., 2010 ⁴⁷	-	-	-	-	25/2	25/2	25/2	25/2	-	-	-	-
Hirano et al., 2010 ⁴⁸	-	-	-	-	-	-	-	53/3	-	-	-	-
Rocha et al., 2010 ⁴⁹	-	-	-	-	-	-	-	-	-	-	-	-
Grandadam et al., 2010 ⁵⁰	-	-	0/4	-	-	-	-	-	-	-	0/11	-
Regimbeau et al., 2011 ⁵¹	-	-	-	-	-	-	-	-	-	-	-	-
Nuzzo et al., 2012 ⁵²	-	-	27/7	-	-	-	-	-	-	-	-	-
Cho et al., 2012 ⁵³	-	-	-	-	-	-	-	-	-	-	-	-
Farges et al., 2013 ⁵⁴	-	17/22	17/22	17/22	-	123/128	123/128	123/128	-	-	-	-
Ratti et al., 2013 ⁵⁵	-	-	-	-	-	-	-	-	-	-	-	-
Yu et al., 2013 ⁵⁶	-	-	1/2	-	-	-	-	14/20	-	-	5/11	-
Xiong et al., 2013 ⁵⁷	-	3/2	3/2	3/2	-	17/27	17/27	17/27	-	-	13/11	-

Table S4 (continued)

	Postoperative mortality				Overall postoperative morbidity				Postoperative infectious morbidity			
	Liu <i>et al.</i> , 2011 ³⁰	Celotti <i>et al.</i> , 2017 ³¹	Yan <i>et al.</i> , 2018 ³²	Mehrabi <i>et al.</i> , 2020 ³³	Liu <i>et al.</i> , 2011 ³⁰	Celotti <i>et al.</i> , 2017 ³¹	Yan <i>et al.</i> , 2018 ³²	Mehrabi <i>et al.</i> , 2020 ³³	Liu <i>et al.</i> , 2011 ³⁰	Celotti <i>et al.</i> , 2017 ³¹	Yan <i>et al.</i> , 2018 ³²	Mehrabi <i>et al.</i> , 2020 ³³
Furusawa <i>et al.</i> , 2014 ⁵⁸	-	-	-	-	-	-	92/13	-	-	-	-	-
Yan <i>et al.</i> , 2014 ⁵⁹	-	-	-	-	-	-	-	-	-	-	-	-
Ribero <i>et al.</i> , 2016 ⁶⁰	-	-	-	12/3	-	-	-	78/24	-	-	-	-
Ito <i>et al.</i> , 2016 ⁶¹	-	-	-	-	-	-	4/6	-	-	-	-	-
Cai <i>et al.</i> , 2017 ⁶²	-	-	-	0/7	-	-	-	-	-	-	-	-
Higuchi <i>et al.</i> , 2017 ⁶³	-	-	-	6/0	-	-	-	-	-	-	-	-
Kimura <i>et al.</i> , 2017 ⁶⁴	-	-	-	-	-	-	-	-	-	-	-	-
Zhang <i>et al.</i> , 2018 ⁶⁵	-	-	-	13/2	-	-	-	133/20	-	-	-	-
Ratti <i>et al.</i> , 2019 ⁶⁶	-	-	-	-	-	-	-	69/28	-	-	-	-

BD, biliary drainage; pCCA, perihilar cholangiocarcinoma.

Table S5 Assessment with ROBIS for risk of bias of systematic reviews comparing EBD with PTBD in patients with resectable pCCA.

Phase 2												
	Domain 1: Study eligibility criteria						Domain 2: Identification and selection of studies					
	1.1 Did the review adhere to pre-defined objectives and eligibility criteria?	1.2 Were the eligibility criteria appropriate for the review question?	1.3 Were eligibility criteria unambiguous?	1.4 Were all restrictions in eligibility criteria based on study characteristics appropriate (e.g. date, sample size, study quality, outcomes measured)?	1.5 Were any restrictions in eligibility criteria based on sources of information appropriate (e.g. publication status or format, language, availability of data)?	Concerns	2.1 Did the search include an appropriate range of databases/electronic sources for published and unpublished reports?	2.2 Were methods additional to database searching used to identify relevant reports?	2.3 Were the terms and structure of the search strategy likely to retrieve as many eligible studies as possible?	2.4 Were restrictions based on date, publication format, or language appropriate?	2.5 Were efforts made to minimise error in selection of studies?	Concerns
Review												
Hameed <i>et al.</i> , 2016 ⁶⁷	NI	PY	PY	PY	PY	Low	PY	Y	NI	N	NI	High
Al Mahjoub <i>et al.</i> , 2017 ⁶⁸	NI	PY	PY	PY	PY	Low	N	Y	NI	N	Y	High
Tang <i>et al.</i> , 2017 ⁶⁹	NI	PY	PN	PY	PY	High	Y	Y	NI	N	NI	High
Liu <i>et al.</i> , 2018 ⁷⁰	NI	Y	Y	Y	Y	Low	PN	Y	NI	N	NI	High
Wang <i>et al.</i> , 2019 ⁷¹	Y	Y	Y	PN	PY	High	Y	Y	NI	N	Y	High
Wang <i>et al.</i> , 2019 ⁷²	Y	PY	PY	PY	PY	Low	PN	PN	NI	N	Y	High
Hajibandeh <i>et al.</i> , 2020 ⁷³	Y	Y	Y	PY	PY	Low	Y	Y	PY	PY	Y	Low

Table S5 (continued)

Phase 2													
	Domain 3: Data collection and study appraisal					Domain 4: Synthesis and findings							
	3.1 Were efforts made to minimise error in data collection?	3.2 Were sufficient study characteristics available for both review authors and readers to be able to interpret the results?	3.3 Were all relevant study results collected for use in the synthesis?	3.4 Was risk of bias (or methodological quality) formally assessed using appropriate criteria?	3.5 Were efforts made to minimise error in risk of bias assessment?	Concerns	4.1 Did the synthesis include all studies that it should?	4.2 Were all pre-defined analyses reported or departures explained?	4.3 Was the synthesis appropriate given the nature and similarity in the research questions, study designs and outcomes across included studies?	4.4 Was between-study variation (heterogeneity) minimal or addressed in the synthesis?	4.5 Were the findings robust, e.g. as demonstrated through funnel plot or sensitivity analyses?	4.6 Were biases in primary studies minimal or addressed in the synthesis?	Concerns
Review													
Hameed et al, 2016 ⁶⁷	Y	Y	Y	N	N	High	Y	NI	Y	Y	PY	N	High
Al Mahjoub et al, 2017 ⁶⁸	NI	Y	Y	PY	NI	Low	Y	NI	Y	Y	N	N	High
Tang et al, 2017 ⁶⁹	Y	Y	Y	PY	NI	Low	Y	NI	Y	Y	Y	PY	Low
Liu et al, 2018 ⁷⁰	PY	PN	Y	PY	NI	High	Y	NI	Y	Y	PY	PY	Low
Wang et al, 2019 ⁷¹	Y	Y	Y	Y	NI	Low	Y	Y	Y	Y	PY	PY	Low
Wang et al, 2019 ⁷²	Y	Y	Y	Y	NI	Low	Y	Y	PY	PY	PY	Y	Low
Hajibandeh et al, 2020 ⁷³	Y	Y	Y	Y	Y	Low	Y	Y	Y	Y	Y	Y	Low

Table S5 (continued)

Review	Phase 3			
	Judging risk of bias			Overall risk of bias in the review
	A. Did the interpretation of findings address all of the concerns identified in Domains 1 to 4?	B. Was the relevance of identified studies to the review's research question appropriately considered?	C. Did the reviewers avoid emphasizing results on the basis of their statistical significance?	
Hameed <i>et al.</i> , 2016 ⁶⁷	N	Y	Y	High
Al Mahjoub <i>et al.</i> , 2017 ⁶⁸	PN	Y	PY	High
Tang <i>et al.</i> , 2017 ⁶⁹	PN	Y	Y	High
Liu <i>et al.</i> , 2018 ⁷⁰	N	Y	Y	High
Wang <i>et al.</i> , 2019 ⁷¹	PN	Y	Y	High
Wang <i>et al.</i> , 2019 ⁷²	PN	Y	Y	High
Hajjbandeh <i>et al.</i> , 2020 ⁷³	Y	Y	Y	Low

ROBIS, Risk Of Bias In Systematic reviews; EBD, endoscopic biliary drainage; PTBD, percutaneous transhepatic biliary drainage; pCCA, perihilar cholangiocarcinoma; Y, Yes; PY, Probably yes; PN, Probably no; N, No; NI, No information.

Table S6 Number of studies and participants in systematic reviews for the comparison between EBD and PTBD in patients with resectable pCCA.

	Studies included in systematic review							Number of participants (EBD/PTBD)						
	Hameed <i>et al.</i> , 2016 ⁶⁷	Al Mahjoub <i>et al.</i> , 2017 ⁶⁸	Tang <i>et al.</i> , 2017 ⁶⁹	Liu <i>et al.</i> , 2018 ⁷⁰	Wang <i>et al.</i> , 2019 ⁷¹	Wang <i>et al.</i> , 2019 ⁷²	Hajibandeh <i>et al.</i> , 2020 ⁷³	Hameed <i>et al.</i> , 2016 ⁶⁷	Al Mahjoub <i>et al.</i> , 2017 ⁶⁸	Tang <i>et al.</i> , 2017 ⁶⁹	Liu <i>et al.</i> , 2018 ⁷⁰	Wang <i>et al.</i> , 2019 ⁷¹	Wang <i>et al.</i> , 2019 ⁷²	Hajibandeh <i>et al.</i> , 2020 ⁷³
Su <i>et al.</i> , 1996 ³⁴	✓	-	-	-	-	-	-	0/33	-	-	-	-	-	-
Neuhaus <i>et al.</i> , 1999 ⁷⁴	✓	-	-	-	-	-	-	37/3	-	-	-	-	-	-
Nimura <i>et al.</i> , 2000 ⁷⁵	✓	-	-	-	-	-	-	0/133	-	-	-	-	-	-
Figueras <i>et al.</i> , 2000 ³⁹	✓	-	-	-	-	-	-	0/18	-	-	-	-	-	-
Arakura <i>et al.</i> , 2009 ⁷⁶	✓	-	-	-	-	-	-	62/0	-	-	-	-	-	-
Li <i>et al.</i> , 2009 ⁴⁴	✓	-	-	-	-	-	-	0/55	-	-	-	-	-	-
Yi <i>et al.</i> , 2010 ⁷⁷	✓	-	-	-	-	-	-	1/13	-	-	-	-	-	-
Kloek <i>et al.</i> , 2010 ⁷⁸	✓	✓	✓	✓	-	-	✓	80/8	90/11	90/11	90/11	-	-	90/11
Grandadam <i>et al.</i> , 2010 ⁵⁰	✓	-	-	-	-	-	-	0/12	-	-	-	-	-	-
Kawakami <i>et al.</i> , 2011 ⁷⁹	✓	✓	✓	✓	-	✓	✓	80/48	80/48	80/48	20/48	-	80/48	60/48
Cai <i>et al.</i> , 2011 ⁸⁰	-	-	-	✓	-	-	-	-	-	-	23/35	-	-	-
Hwang <i>et al.</i> , 2012 ⁸¹	-	-	-	-	-	✓	-	-	-	-	-	-	62/17 1	-
Walter <i>et al.</i> , 2013 ⁸²	✓	-	✓	-	-	-	-	31/18	-	87/42	-	-	-	-

Table S6 (continued)

	Studies included in systematic review							Number of participants (EBD/PTBD)						
	Hameed <i>et al.</i> , 2016 ⁶⁷	Al Mahjoub <i>et al.</i> , 2017 ⁶⁸	Tang <i>et al.</i> , 2017 ⁶⁹	Liu <i>et al.</i> , 2018 ⁷⁰	Wang <i>et al.</i> , 2019 ⁷¹	Wang <i>et al.</i> , 2019 ⁷²	Hajibandeh <i>et al.</i> , 2020 ⁷³	Hameed <i>et al.</i> , 2016 ⁶⁷	Al Mahjoub <i>et al.</i> , 2017 ⁶⁸	Tang <i>et al.</i> , 2017 ⁶⁹	Liu <i>et al.</i> , 2018 ⁷⁰	Wang <i>et al.</i> , 2019 ⁷¹	Wang <i>et al.</i> , 2019 ⁷²	Hajibandeh <i>et al.</i> , 2020 ⁷³
Kawashima <i>et al.</i> , 2013 ⁸³	✓	-	-	-	-	-	-	164/0	-	-	-	-	-	-
Yu <i>et al.</i> , 2013 ⁵⁶	✓	-	-	-	-	-	-	0/56	-	-	-	-	-	-
Xiong <i>et al.</i> , 2013 ⁵⁷	✓	-	-	-	-	-	-	5/23	-	-	-	-	-	-
Hirano <i>et al.</i> , 2014 ⁸⁴	✓	-	✓	-	✓	✓	✓	74/67	-	74/67	-	74/67	74/67	74/67
Kim <i>et al.</i> , 2015 ⁸⁵	-	✓	✓	✓	✓	-	✓	-	44/62	44/62	44/62	44/62	-	44/62
Wiggers <i>et al.</i> , 2015 ⁸⁶	-	-	-	✓	✓	✓	✓	-	-	-	157/88	157/88	157/88	157/88
Kawakubo <i>et al.</i> , 2016 ⁸⁷	-	-	✓	-	-	-	-	-	-	118/0	-	-	-	-
Jo <i>et al.</i> , 2017 ²⁶	-	✓	✓	✓	-	-	✓	-	61/37	55/43	55/43	-	-	13/43
Higuchi <i>et al.</i> , 2017 ⁶³	-	-	-	-	-	✓	✓	-	-	-	-	-	76/87	76/87
Komaya <i>et al.</i> , 2017 ⁸⁸	-	-	-	-	✓	✓	✓	-	-	-	-	152/168	152/168	152/168
Zhang <i>et al.</i> , 2018 ⁶⁵	-	-	-	-	-	-	✓	-	-	-	-	-	-	92/104

EBD, endoscopic biliary drainage; PTBD, percutaneous transhepatic biliary drainage; pCCA, perihilar cholangiocarcinoma.

Table S7 Number of abstracted events included in systematic reviews for the comparison between EBD and PTBD in patients with resectable pCCA.

	Postoperative mortality						Drainage-related cholangitis							
	Hameed <i>et al.</i> , 2016 ⁶⁷	Al Mahjoub <i>et al.</i> , 2017 ⁶⁸	Tang <i>et al.</i> , 2017 ⁶⁹	Liu <i>et al.</i> , 2018 ⁷⁰	Wang <i>et al.</i> , 2019 ⁷¹	Wang <i>et al.</i> , 2019 ⁷²	Hajiban deh <i>et al.</i> , 2020 ⁷³	Hameed <i>et al.</i> , 2016 ⁶⁷	Al Mahjou b <i>et al.</i> , 2017 ⁶⁸	Tang <i>et al.</i> , 2017 ⁶⁹	Liu <i>et al.</i> , 2018 ⁷⁰	Wang <i>et al.</i> , 2019 ⁷¹	Wang <i>et al.</i> , 2019 ⁷²	Hajiban deh <i>et al.</i> , 2020 ⁷³
Su <i>et al.</i> , 1996 ³⁴	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Neuhaus <i>et al.</i> , 1999 ⁷⁴	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nimura <i>et al.</i> , 2000 ⁷⁵	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Figueras <i>et al.</i> , 2000 ³⁹	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arakura <i>et al.</i> , 2009 ⁷⁶	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Li <i>et al.</i> , 2009 ⁴⁴	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Yi <i>et al.</i> , 2010 ⁷⁷	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Kloek <i>et al.</i> , 2010 ⁷⁸	-	-	-	-	-	-	-	43/1	43/1	43/1	43/1	-	-	43/1
Grandadam <i>et al.</i> , 2010 ⁵⁰	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Kawakami <i>et al.</i> , 2011 ⁷⁹	-	1/3	-	1/3	-	-	0/3	18/1	18/1	25/5	13/5	-	-	6/1
Cai <i>et al.</i> , 2011 ⁸⁰	-	-	-	-	-	-	-	-	-	-	4/15	-	-	-
Hwang <i>et al.</i> , 2012 ⁸¹	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Walter <i>et al.</i> , 2013 ⁸²	-	-	-	-	-	-	-	22/9	-	22/9	-	-	-	-

Table S7 (continued)

	Postoperative mortality						Drainage-related cholangitis							
	Hameed <i>et al.</i> , 2016 ⁶⁷	Al Mahjoub <i>et al.</i> , 2017 ⁶⁸	Tang <i>et al.</i> , 2017 ⁶⁹	Liu <i>et al.</i> , 2018 ⁷⁰	Wang <i>et al.</i> , 2019 ⁷¹	Wang <i>et al.</i> , 2019 ⁷²	Hajiban <i>et al.</i> , 2020 ⁷³	Hameed <i>et al.</i> , 2016 ⁶⁷	Al Mahjoub <i>et al.</i> , 2017 ⁶⁸	Tang <i>et al.</i> , 2017 ⁶⁹	Liu <i>et al.</i> , 2018 ⁷⁰	Wang <i>et al.</i> , 2019 ⁷¹	Wang <i>et al.</i> , 2019 ⁷²	Hajiban <i>et al.</i> , 2020 ⁷³
Kawashima <i>et al.</i> , 2013 ⁸³	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Yu <i>et al.</i> , 2013 ⁵⁶	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xiong <i>et al.</i> , 2013 ⁵⁷	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hirano <i>et al.</i> , 2014 ⁸⁴	-	-	-	-	-	-	2/3	-	-	9/14	-	-	-	-
Kim <i>et al.</i> , 2015 ⁸⁵	-	2/3	-	-	-	-	2/3	-	16/5	16/5	16/5	-	-	16/5
Wiggers <i>et al.</i> , 2015 ⁸⁶	-	-	-	-	-	-	-	-	-	-	13/25	-	-	-
Kawakubo <i>et al.</i> , 2016 ⁸⁷	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Jo <i>et al.</i> , 2017 ²⁶	-	4/5	-	4/5	-	-	0/5	-	16/5	14/7	-	-	-	2/7
Higuchi <i>et al.</i> , 2017 ⁶³	-	-	-	-	-	-	3/3	-	-	-	-	-	-	-
Komaya <i>et al.</i> , 2017 ⁸⁸	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Zhang <i>et al.</i> , 2018 ⁶⁵	-	-	-	-	-	-	5/8	-	-	-	-	-	-	-

Table S7 (continued)

	Overall drainage-related morbidity						Drainage-related pancreatitis						
	Hameed <i>et al.</i> , 2016 ⁶⁷	Al Mahjoub <i>et al.</i> , 2017 ⁶⁸	Tang <i>et al.</i> , 2017 ⁶⁹	Liu <i>et al.</i> , 2018 ⁷⁰	Wang <i>et al.</i> , 2019 ⁷¹	Hajiban deh <i>et al.</i> , 2020 ⁷³	Hameed <i>et al.</i> , 2016 ⁶⁷	Al Mahjoub <i>et al.</i> , 2017 ⁶⁸	Tang <i>et al.</i> , 2017 ⁶⁹	Liu <i>et al.</i> , 2018 ⁷⁰	Wang <i>et al.</i> , 2019 ⁷¹	Wang <i>et al.</i> , 2019 ⁷²	Hajiban deh <i>et al.</i> , 2020 ⁷³
Su <i>et al.</i> , 1996 ³⁴	-	-	-	-	-	-	-	-	-	-	-	-	-
Neuhaus <i>et al.</i> , 1999 ⁷⁴	-	-	-	-	-	-	-	-	-	-	-	-	-
Nimura <i>et al.</i> , 2000 ⁷⁵	-	-	-	-	-	-	-	-	-	-	-	-	-
Figueras <i>et al.</i> , 2000 ³⁹	-	-	-	-	-	-	-	-	-	-	-	-	-
Arakura <i>et al.</i> , 2009 ⁷⁶	-	-	-	-	-	-	-	-	-	-	-	-	-
Li <i>et al.</i> , 2009 ⁴⁴	-	-	-	-	-	-	-	-	-	-	-	-	-
Yi <i>et al.</i> , 2010 ⁷⁷	-	-	-	-	-	-	-	-	-	-	-	-	-
Kloek <i>et al.</i> , 2010 ⁷⁸	-	-	-	59/4	-	74/4	-	7/0	-	7/0	-	-	7/0
Grandadam <i>et al.</i> , 2010 ⁵⁰	-	-	-	-	-	-	-	-	-	-	-	-	-
Kawakami <i>et al.</i> , 2011 ⁷⁹	36/15	36/15	-	15/19	-	23/15	-	3/0	-	1/0	-	-	2/0
Cai <i>et al.</i> , 2011 ⁸⁰	-	-	-	7/2	-	-	-	-	-	4/0	-	-	-
Hwang <i>et al.</i> , 2012 ⁸¹	-	-	-	-	-	-	-	-	-	-	-	-	-
Walter <i>et al.</i> , 2013 ⁸²	23/11	-	-	-	-	-	-	-	-	-	-	-	-

Table S7 (continued)

	Overall drainage-related morbidity						Drainage-related pancreatitis							
	Hameed <i>et al.</i> , 2016 ⁶⁷	Al Mahjoub <i>et al.</i> , 2017 ⁶⁸	Tang <i>et al.</i> , 2017 ⁶⁹	Liu <i>et al.</i> , 2018 ⁷⁰	Wang <i>et al.</i> , 2019 ⁷¹	Wang <i>et al.</i> , 2019 ⁷²	Hajiban deh <i>et al.</i> , <i>al.</i> , 2020 ⁷³	Hameed <i>et al.</i> , 2016 ⁶⁷	Al Mahjoub <i>et al.</i> , 2017 ⁶⁸	Tang <i>et al.</i> , 2017 ⁶⁹	Liu <i>et al.</i> , 2018 ⁷⁰	Wang <i>et al.</i> , 2019 ⁷¹	Wang <i>et al.</i> , 2019 ⁷²	Hajiban deh <i>et al.</i> , <i>al.</i> , 2020 ⁷³
Kawashima <i>et al.</i> , 2013 ⁸³	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Yu <i>et al.</i> , 2013 ⁵⁶	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xiong <i>et al.</i> , 2013 ⁵⁷	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hirano <i>et al.</i> , 2014 ⁸⁴	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Kim <i>et al.</i> , 2015 ⁸⁵	-	24/14	-	24/14	-	-	24/14	-	9/0	-	9/0	-	-	9/0
Wiggers <i>et al.</i> , 2015 ⁸⁶	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Kawakubo <i>et al.</i> , 2016 ⁸⁷	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Jo <i>et al.</i> , 2017 ²⁶	-	22/10	-	20/12	-	-	2/12	-	6/0	-	-	-	-	0/0
Higuchi <i>et al.</i> , 2017 ⁶³	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Komaya <i>et al.</i> , 2017 ⁸⁸	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Zhang <i>et al.</i> , 2018 ⁶⁵	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table S7 (continued)

	Overall postoperative morbidity						Seeding metastasis						
	Hameed <i>et al.</i> , 2016 ⁶⁷	Al Mahjoub <i>et al.</i> , 2017 ⁶⁸	Tang <i>et al.</i> , 2017 ⁶⁹	Liu <i>et al.</i> , 2018 ⁷⁰	Wang <i>et al.</i> , 2019 ⁷¹	Hajibandeh <i>et al.</i> , 2020 ⁷³	Hameed <i>et al.</i> , 2016 ⁶⁷	Al Mahjoub <i>et al.</i> , 2017 ⁶⁸	Tang <i>et al.</i> , 2017 ⁶⁹	Liu <i>et al.</i> , 2018 ⁷⁰	Wang <i>et al.</i> , 2019 ⁷¹	Wang <i>et al.</i> , 2019 ⁷²	Hajiban deh <i>et al.</i> , 2020 ⁷³
Su <i>et al.</i> , 1996 ³⁴	-	-	-	-	-	-	-	-	-	-	-	-	-
Neuhaus <i>et al.</i> , 1999 ⁷⁴	-	-	-	-	-	-	-	-	-	-	-	-	-
Nimura <i>et al.</i> , 2000 ⁷⁵	-	-	-	-	-	-	-	-	-	-	-	-	-
Figueras <i>et al.</i> , 2000 ³⁹	-	-	-	-	-	-	-	-	-	-	-	-	-
Arakura <i>et al.</i> , 2009 ⁷⁶	-	-	-	-	-	-	-	-	-	-	-	-	-
Li <i>et al.</i> , 2009 ⁴⁴	-	-	-	-	-	-	-	-	-	-	-	-	-
Yi <i>et al.</i> , 2010 ⁷⁷	-	-	-	-	-	-	-	-	-	-	-	-	-
Kloek <i>et al.</i> , 2010 ⁷⁸	-	-	-	-	-	-	-	-	-	-	-	-	-
Grandadam <i>et al.</i> , 2010 ⁵⁰	-	-	-	-	-	-	-	-	-	-	-	-	-
Kawakami <i>et al.</i> , 2011 ⁷⁹	-	7/9	-	1/9	-	-	-	-	-	-	-	0/3	-
Cai <i>et al.</i> , 2011 ⁸⁰	-	-	-	-	-	-	-	-	-	-	-	-	-
Hwang <i>et al.</i> , 2012 ⁸¹	-	-	-	-	-	-	-	-	-	-	-	0/7	-
Walter <i>et al.</i> , 2013 ⁸²	-	-	-	-	-	-	-	-	-	-	-	-	-

Table S7 (continued)

	Overall postoperative morbidity						Seeding metastasis							
	Hameed <i>et al.</i> , 2016 ⁶⁷	Al Mahjoub <i>et al.</i> , 2017 ⁶⁸	Tang <i>et al.</i> , 2017 ⁶⁹	Liu <i>et al.</i> , 2018 ⁷⁰	Wang <i>et al.</i> , 2019 ⁷¹	Wang <i>et al.</i> , 2019 ⁷²	Hajibandeh <i>et al.</i> , 2020 ⁷³	Hameed <i>et al.</i> , 2016 ⁶⁷	Al Mahjoub <i>et al.</i> , 2017 ⁶⁸	Tang <i>et al.</i> , 2017 ⁶⁹	Liu <i>et al.</i> , 2018 ⁷⁰	Wang <i>et al.</i> , 2019 ⁷¹	Wang <i>et al.</i> , 2019 ⁷²	Hajiban deh <i>et al.</i> , 2020 ⁷³
Kawashima <i>et al.</i> , 2013 ⁸³	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Yu <i>et al.</i> , 2013 ⁵⁶	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xiong <i>et al.</i> , 2013 ⁵⁷	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hirano <i>et al.</i> , 2014 ⁸⁴	-	-	-	-	-	-	-	-	-	-	-	-	3/20	-
Kim <i>et al.</i> , 2015 ⁸⁵	-	16/16	-	16/16	-	-	-	-	-	-	-	-	-	0/2
Wiggers <i>et al.</i> , 2015 ⁸⁶	-	-	-	-	-	-	-	-	-	-	-	-	25/14	4/3
Kawakubo <i>et al.</i> , 2016 ⁸⁷	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Jo <i>et al.</i> , 2017 ²⁶	-	17/14	-	14/15	-	-	-	-	-	-	-	-	-	-
Higuchi <i>et al.</i> , 2017 ⁶³	-	-	-	-	-	-	-	-	-	-	-	-	11/31	11/31
Komaya <i>et al.</i> , 2017 ⁸⁸	-	-	-	-	-	-	-	-	-	-	-	-	25/45	25/44
Zhang <i>et al.</i> , 2018 ⁶⁵	-	-	-	-	-	-	-	-	-	-	-	-	-	-

EBD, endoscopic biliary drainage; PTBD, percutaneous transhepatic biliary drainage; pCCA, perihilar cholangiocarcinoma.

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Part II

The role of bile salt signaling in
liver regeneration

Chapter 3

Precision-cut liver slices as a model to study
human liver regeneration

Xinwei Chang, Steven W.M. Olde Damink, Frank G. Schaap

Abstract

Background: Fibroblast growth factor 19 (FGF19) activates signal transducer and activator of transcription 3 (STAT3) signaling in hepatocytes through induction of interleukin 6 (IL6) production by innate immune cells, driving malignant proliferation of mouse hepatocytes. Here we aimed to explore whether FGF19 activates the IL6/STAT3 axis to promote hepatocyte proliferation in the human precision-cut liver slices (hPCLS) model, and to assess the suitability of hPCLS as an *ex vivo* model to study human liver regeneration.

Methods: hPCLS were derived from tumor-distal liver tissue from patients who underwent partial liver resection for colorectal liver metastases and incubated with vehicle, lipopolysaccharide (LPS), recombinant FGF19, nuclear bile salt receptor agonists (OCA, FXR agonist; CITCO, CAR agonist; rifampicin, PXR agonist). 5C and Geltrex were used to assess their effectiveness in maintaining expression of genes related to a differentiated hepatocyte phenotype. mRNA expression of *IL6*, FXR/FGF19 pathway-related genes and proliferation-related genes were assessed. IL6 and FGF19 concentrations in the conditioned medium were determined.

Results: FGF19 did not induce *IL6* mRNA expression in hPCLS. In addition, marked phosphorylation of ERK1/2 was already observed in untreated liver slices. FGF19 had no additional effect on levels of phosphorylated ERK1/2. Expression of FGF19 pathway-related genes (CYP7A1, FGFR4, KLB, PCK1) rapidly declined upon hPCLS culturing. The use of 5C and Geltrex did not maintain expression of these genes. Furthermore, nuclear receptors agonists OCA and rifampicin did not induce cell proliferation in hPCLS.

Conclusions: FGF19 and CAR treatment did not result in cell proliferation in hPCLS. However, the efficacy of FGF19 and CAR treatment could not be ascertained in these exploratory experiments. Hence, questions regarding the involvement of the FGF19/IL6 axis in hepatocellular proliferation, and augmentation of liver growth by CAR agonism could not be answered with the current method of hPCLS source, preparation and culturing.

Introduction

The robust capacity of the liver to regenerate allows a large removal (70%) of liver mass for the curative goal of liver tumors. Insufficient liver remnant after hepatic resection can lead to small for size syndrome, where patients present with impaired liver function ^{1, 2}. The remnant liver volume and its functionality are important for successful regeneration, especially when there is pre-existing damaged liver parenchyma ^{3, 4}. An effective pharmaceutical therapy to accelerate liver regeneration in patients undergoing liver resection is currently lacking.

Bile salts activate a dedicated nuclear receptor farnesoid X receptor (FXR) to maintain bile salt homeostasis through regulation of hepatic synthesis and transport of bile salts ⁵. Dysregulation of bile salt homeostasis can result in hepatic bile salt accumulation and attendant hepatocellular toxicity. Bile salts are also involved in the process of liver regeneration after partial hepatectomy (PH), as liver regeneration is delayed in *Fxr* deficient mice ⁶. One role of FXR in liver regeneration might be the release of mitogenic fibroblast growth factor 19 (FGF19, Fgf15 in rodents). *Fgf15*^{-/-} mice showed delayed liver regeneration and considerable mortality (about 70%) after 70% PH ⁷.

The cytokine interleukin 6 (IL-6) activates JAK/STAT, MAPK and PI3K/AKT signaling cascades, to control the early regenerative response after PH ⁸. FGF19 can activate signal transducer and activator of transcription 3 (STAT3) signaling in hepatocytes through induction of IL-6 production by non-parenchymal cells, likely Kupffer cells. This non-cell autonomous FGF19-IL6 axis was shown to drive malignant proliferation of mouse hepatocytes ⁹. It is conceivable that effects of bile salts on liver regeneration are via bile salt homeostatic actions, as well as via an FXR-FGF19-IL6 signaling axis.

The liver contains parenchymal and non-parenchymal cells. The latter consists of various immune cells (e.g. macrophages, natural killer cells, dendritic cells), hepatic stellate cells, bile duct epithelial cells and other cell types ¹⁰. Although isolated hepatocytes are widely used for the research of liver processes, it fails to imitate the cell-cell interactions occurring *in situ*, whereas this interaction is important for liver regeneration ^{11, 12}. For example, angiocrine factors derived from the liver sinusoidal endothelial cells trigger hepatocyte growth in mice ¹³. Precision-cut liver slices (PCLS) preserve all cell types, where the cell-cell interaction and cell heterogeneity remain in the original liver matrix ^{14, 15}. PCLS were first used to study liver regeneration by Tschuor *et al.*, who demonstrated increased numbers of hepatocytes positive for the

proliferative marker phosphorylated histone 3, following treatment of murine and human PCLS with an agonist of the constitutive androstane receptor (CAR)². Thus far, these initial findings (note that observations were based on PCLS from a single patient) -and use of PCLS as *ex vivo* model of liver regeneration- have not been confirmed or extended.

Most studies on the role of FGF19 and bile salts in liver regeneration employed animal models, and the empirical support for conservation of these functions in human is scarce^{6, 7, 16}. In this study we aimed to explore whether FGF19 activates the IL6/STAT3 axis to promote hepatocyte proliferation in the human PCLS (hPCLS) model. Further aim was to reproduce and extend the potential of specific nuclear receptor agonists, including FXR activators, to stimulate hepatocyte growth in hPCLSs. These were exploratory studies, with main goal to assess the suitability of hPCLS as an *ex vivo* model to study liver regeneration and pharmaceutical modulation thereof.

Materials and Methods

Patient materials

Tumor-distal liver tissue (ca. 4×3×2 cm) was obtained from patients who underwent partial liver resection for colorectal liver metastases, at the beginning of the resection procedure (open or laparoscopic). The edge of segment 4 was sampled by the surgeon using surgical scissors and judged by the surgeon to be macroscopically devoid of tumor. Liver specimens of a total of 10 patients were processed to hPCLS for below exploratory studies. All patients provided written informed consent. This study was approved by the Medical Ethical Committee of Maastricht University Medical Center (METC# 14-4-153). Following excision of liver tissue in the operating room, the liver tissues were immediately stored in ice-cold University of Wisconsin organ preservation solution (UW) and transported to the laboratory.

Chemicals

Lipopolysaccharide (LPS, from *Escherichia coli* O55:B5, #L2880, Sigma, Darmstadt, Germany), FGF19 (#969-FG, R&D Systems, Minnesota, US), obeticholic acid (OCA) (Intercept Pharmaceuticals, New York, USA), CITCO (6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime) (R&D Systems, Minnesota, US), and rifampicin (Sigma, Darmstadt, Germany) were obtained from the

indicated suppliers. Forskolin (FSK) (Sigma, Darmstadt, Germany), transforming growth factor β inhibitor SB431542 (SB43) (Tocris, Abingdon, UK), DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) (Sigma, Darmstadt, Germany), IWP2 (N-(6-Methyl-2-benzothiazolyl)-2-[(3,4,6,7-tetrahydro-4-oxo-3-phenylthieno[3,2-d]pyrimidin-2-yl)thio]-acetamide) (Tocris, Abingdon, UK), bone morphogenetic protein inhibitor LDN193189 (Tocris, Abingdon, UK), PMA (phorbol 12-myristate 13-acetate) (R&D Systems, Minnesota, US), and dimethyl sulfoxide (DMSO) (molecular biology grade, D8418, Sigma, Darmstadt, Germany) were purchased from the indicated suppliers.

Preparation of precision cut liver slices

hPCLS were prepared as described previously¹⁵. Briefly, cylindrical cores of the freshly sampled liver (6 mm diameter) were made using a dermal biopsy punch. Liver cores were sectioned to slices of approximately 250 μ m thickness with a Krumdieck tissue slicer (Alabama Research and Development, Munford, AL, USA) filled with oxygenated ice-cold Krebs-Henseleit Buffer (KHB, 5 mM NaCl, 118 mM KCl, 1.1 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 2.5 mM CaCl₂, 25 mM D-Glucose, 9.0 mM HEPES in ultrapure water, pH 7.42). Liver slices were cultured in William's E Medium (Gibco, Paisley, Scotland) supplemented with 2.75 mg/mL D-glucose and 50 μ g/mL gentamicin in 12-well plates at 37°C in an incubator containing 95% O₂ and 5% CO₂. Plates were continuously shaken on a rocking platform (80 rpm, transversal motion). After 1h of recovery from the hPCLS procedure, medium was refreshed and the liver slices were exposed to various treatments. Prior studies revealed that liver slices remain viable (i.e. ATP levels were maintained) for at least 120 hrs in William's E Medium¹⁷.

Treatments consisted of vehicle, 100 ng/mL LPS (dissolved in PBS/0.1%BSA), 1-100 ng/ml FGF19 (dissolved in PBS/0.1%BSA), 10 μ M obeticholic acid (OCA, dissolved in DMSO; FXR agonist), 10 μ M CITCO (dissolved in DMSO, CAR agonist), 25 μ M rifampicin (dissolved in DMSO, PXR agonist) for the indicated time. For the treatment with these nuclear receptor agonists, reported experimental conditions were exactly replicated and included culturing hPCLS in 6 well plates on 0.4 μ m inserts (#PICM0RG50, 30 mm diameter, Millicell) as described². Supernatants of conditioned medium (centrifugation 1000 rpm, 5 minutes) were collected and stored at -20°C for eventual analysis by ELISA. hPCLS were snap frozen in liquid nitrogen and stored at -80 °C for RNA and protein extraction.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from hPCLS using TRIzol reagent. A total of 750 ng RNA was used for reverse transcription with SensiFAST™ cDNA Synthesis Kit. For the qPCR, we used SensiFAST™ SYBR Hi-ROX kit, primers, and cDNA as template using a LightCycler® 480 SW 1.5 system according to the manufacturer's protocol. LinRegPCR program was used to analyze qPCR data ¹⁸. Gene expression levels were normalized using *36B4* as reference gene.

Enzyme linked immunosorbent assay (ELISA)

IL6 levels in the conditioned medium were determined by sandwich ELISA as described ¹⁹. Briefly, microtiter plates were coated with mouse anti-human IL-6 antibody. Samples and recombinant human IL6 standards were diluted in PBS containing 0.1% bovine serum albumin. Biotinylated rabbit anti-human IL6 antibody was used to detect captured antigen and streptavidin-horseradish peroxidase as a secondary detection reagent. We used tetramethylbenzidine as chromogenic substrate. FGF19 levels in the conditioned medium were assessed by sandwich ELISA as reported previously ²⁰.

Western blotting

For testing the activation (i.e. phosphorylation) of the ERK pathway, hPCLS were treated with vehicle (0.1% DMSO), 100 nM PMA, and 100 ng/mL FGF19 for different time points. Phosphorylated ERK1/2 protein expression was assessed by Western blotting. hPCLS were lysed using RIPA lysis buffer containing protease inhibitor cOmplete Mini (#30496700, Roche, Mannheim, Germany) and phosphatase inhibitor PhosSTOP (#4906845001, Roche, Mannheim, Germany) in a mini bead-beater (SUNON, China) in 2.0 mL screw cap vials containing glass beads, for three cycles of 10 seconds at maximum speed. Tissue homogenates were spun down for 1 min at 10000g, and supernatants were transferred to fresh tube. Total protein in homogenates was determined by BCA protein assay with bovine serum albumin as standard. A total of 100 µg protein was separated on a 10% sodium dodecyl sulfate polyacrylamide gel. Proteins were transferred to nitrocellulose membrane which was incubated with primary antibody (cat nr# 4695, Cell Signaling Technology, Massachusetts, US) and secondary detection antibody. Immunoreactive bands were visualized with SuperSignal West Pico Chemiluminescent Substrate Kit (Thermo Scientific, Rockford,

US).

Statistical analysis

Data are presented as median [IQR]. Nonparametric Mann–Whitney U test or Kruskal–Wallis test was used to test for differences between groups. Longitudinal changes were evaluated by Friedman test, with Dunn’s multiple comparison test to compare differences between baseline and subsequent individual time points. Four slices were included per treatment condition or time point. Experiments were repeated three times for treatment with FGF19 and CITCO in hPCLS. A p value of less than 0.05 was considered statistically significant. All statistical analyses were performed using IBM SPSS 24.0, and all graphs were made using GraphPad Prism 8.

Results

FGF19 does not increase IL6 mRNA expression in hPCLS

To address whether FGF19 induces IL6 production in the hPCLS model, slices were treated with FGF19 and LPS as a positive control, for 20 and 44 hrs. LPS stimulation increased the mRNA expression of *IL6* after 20 h and 44h ($P < 0.05$, $P < 0.01$, respectively) (**Fig. 1A-B**). Consistently, the IL6 levels in the medium were elevated after LPS treatment for 20 h and 44h ($P < 0.05$, $P < 0.01$, respectively) (**Fig. 1C-D**). FGF19 did not affect the mRNA expression of *IL6*. To evaluate whether FGF19 induces cell proliferation in hPCLS, we tested expression of STAT3 downstream targets, including genes related to cell proliferation (*CCND1* and *PCNA*) and suppression of apoptosis (*BIRC5*). FGF19 did not alter the expression of *CCND1*, *PCNA* and *BIRC5* (**Fig. 1A-B**). The FGF19 target gene *CYP7A1* was not detectable after culturing 20 h and 44 h, and could not be used as a transcriptional read-out to verify proper FGF19 action. In addition, FGF19 did not increase IL6 levels in the conditioned medium after 20 h and 44 h incubation (**Fig. 1C-D**). IL6 levels induced by LPS treatment were apparently insufficient for paracrine activation of STAT3 signaling, as inferred from lack of effects on *CCND1*, *PCNA* and *BIRC5*.

Figure 1. FGF19 does not induce *IL6* mRNA expression in hPCLS after 20 hrs incubation.

hPCLS were incubated with vehicle, FGF19 or LPS for 20 and 44 hrs. Conditioned media were harvested and assayed for IL6 protein level. RNA was isolated from hPCLS and gene expression was analyzed by RT-qPCR. LPS treatment increased *IL6* mRNA expression and protein levels after 20 h (**A, C**) and 44 h (**B, D**). FGF19 does not induce *IL6* mRNA expression (**A, B**). Four slices were included per condition. The experiment for LPS/FGF19 treatment was replicated three times using slices derived from different patients (**A, B**). The dose-response experiment was performed once (**C, D**). A representative experiment is depicted. Differences among groups were analyzed with Kruskal-Wallis test, with Dunn's multiple comparison test to compare differences between control and specific treatment. Data is depicted as median [IQR]. Asterisks indicate significance levels: * $P < 0.05$.

FGF19 does not induce enhanced activation of ERK1/2

Since FGF19 treatment did not result in altered expression of the tentative target gene *IL6*, we next tested responsiveness of slices to FGF19 treatment using a non-transcriptional read-out, i.e. activation of the extracellular signal-related kinase [ERK]1/2 pathway, a mediator of FGF19 signaling⁹. In unstimulated slices, marked phosphorylation of ERK1/2 was already observed. FGF19 treatment for 5 to 60 minutes did not result in enhanced phosphorylation of ERK1/2 (**Fig. 2**). Likewise, the positive control PMA did not lead to higher levels of pERK1/2 in comparison to the vehicle control (DMSO). This suggests that the time to recover from slice preparation

stress is insufficient to inactivate (e.g. dephosphorylate) the extracellular signal-regulated kinase ERK1/2, and cellular 'stress' is still being experienced.

Figure 2. FGF19 does not enhance phosphorylation of ERK1/2 in hPCLS. hPCLS were incubated with vehicle, FGF19 or PMA for 0-60 minutes. Phosphorylation of ERK1/2 was determined by Western blotting. FGF19 (100 ng/mL), DMSO (0.1%) (vehicle control), PMA (100 nM) (positive control) were used to treat hPCLS. The experiment was replicated two times using slices derived from two patients.

Change of FGF19 pathway-related genes expression during culture in hPCLS

Since no effects of FGF19 on *IL6* mRNA expression or phosphorylation of ERK1/2 were observed, we next explored if mRNA expression of FGF19 pathway-related genes altered during slice culturing. hPCLS were cultured in the medium with no additions for up to 19 h. During the first 1 h of pre-incubation (i.e. the recovery period), the expression of *FXR*, *FGFR4* and *KLB* (FGF19 receptors), *CYP7A1* and *PCK1* (both negatively regulated by FGF19) rapidly declined (**Fig. 3A**). In contrast, FGF19 was initially below detection limits, but was readily detectable at the mRNA and protein level (data not shown) after 19 hrs. The expression of *CYP7A1* further decreased after 9 h, and became undetectable (i.e. could not be reliably quantified due to $C_q > 35$) after 19 h. The median C_q of tested genes at different time points are summarized in **Fig. 3B**.

Use of 5C and Geltrex does not improve FXR/FGF19 related genes expression

In the experiment reported in **Fig. 3**, we observed a rapid decline of genes related to a differentiated hepatocyte phenotype such as the bile salt synthetic gene *CYP7A1*, which we use as a read-out for FGF19 signaling and becomes undetectable after 19 hrs of culturing. Dedifferentiated human hepatocytes lose some of the mature liver

Figure 3. Time course of FGF19 pathway-related genes during culturing. hPCLS were cultured in the medium with no additions for up to 19 h. RNA was isolated from hPCLS and gene expression was analyzed by RT-qPCR. T0=start recovery, T1=end recovery and medium refreshment. The change of FGF19 pathway-related genes during culturing (**A**). The median Cq of genes at different time points (**B**). Data was derived from a single patient, with 4 slices per time point.. Longitudinal changes were evaluated by Friedman test, with Dunn's multiple comparison test to compare differences between baseline (T0) and subsequent individual time points. Data is depicted as median [IQR]. Asterisks indicate significance levels: *P<0.05, **P<0.01.

functions which may limit their usefulness as an *in vitro* model to study liver pathophysiology ^{21, 22}. It has been reported that a cocktail of five chemicals (5C) preserves differentiated function in isolated human hepatocytes ²³. To evaluate effectiveness of this cocktail in preserving function in hPCLS, we compared slices cultured under regular and 5C-supplemented conditions. Likewise, we explored whether embedding in basement membrane matrix (i.e. Geltrex) improved expression of genes associated with a differentiated phenotype, in particular genes regulated by FGF19 signaling. Geltrex, also employed in 3D cell culture models, can maintain

viability of primary human hepatocytes and prolong metabolizing enzyme activities (e.g. CYP3A4, CYP1A2) ²⁴.

We thus tested whether 5C and/or Geltrex maintained expression of FXR/FGF19 pathway-related genes during culturing for 24 hrs. Unfortunately, mRNA expression of *CYP7A1* was still not detectable in any of the groups (**Fig. 4**), whereas its expression was present in parent liver tissue (Cq=30.0). Similar to the earlier experiment (**Fig. 3**), a general decrease in expression of tested genes was observed when compared with parent liver tissue not subjected to slicing/culturing (**Fig. 4**). The experimental treatments (5C, Geltrex) failed to prevent this general decline, although expression of the drug metabolizing enzyme *CYP3A4* was induced 2.0 fold by 5C.

Figure 4. Use of 5C and Geltrex did not prevent the decline of FXR/FGF19-pathway related genes. hPCLS were cultured with vehicle, 5C or Geltrex for 24 h. RNA was isolated from hPCLS and gene expression was analyzed by RT-qPCR. Four slices were included per condition, the experiment was conducted once. Data is depicted as median [IQR]. Asterisks indicate significance levels: *P<0.05. n.d., not detectable.

Selected nuclear receptors agonists do not induce hepatocytes proliferation in hPCLS

Activation of CAR was previously reported to stimulate hepatocyte proliferation in PCLS derived from mouse liver, as well as from a single patient ². To replicate and extend this finding, hPCLS were treated with agonists for nuclear bile salt receptors FXR (OCA), PXR (Rifampicin) and CAR (CITCO), the latter two being promiscuous receptors that can be activated by a limited number of bile salt species. As all three

receptors are ligand-activated transcription factors, a transcriptional response was used as read-out for receptor activation, as reported previously ². Rifampicin induced mRNA expression of the PXR target gene *CYP3A4* (**Fig. 5A**). No notable induction of CAR target genes *CYP2E1* was observed upon treatment with CITCO (**Fig. 5A**). FXR target *FGF19* was induced at transcriptional and protein levels by OCA treatment (**Fig. 5A-B**). Forkhead box M1 (*FOXM1B*) is a transcription factor involved in control of cell proliferation, and can regulate the expression of cell cycle gene cyclin D1 (*CCND1*). Treatment of hPCLS with OCA, Rifampicin or CITCO did not induce the mRNA expression of proliferation related genes *FOXM1B*, *CCND1* and *PCNA* (**Fig. 5C**).

Figure 5. Selected nuclear receptors agonists do not induce cellular proliferation in hPCLS. hPCLS were cultured with vehicle, rifampicin, OCA, or CITCO for 44 h. RNA was isolated from hPCLS and gene expression was analyzed by RT-qPCR. Conditioned media were harvested and assayed for FGF19 protein level. Rifampicin induced mRNA expression of *CYP3A4* (**A**). OCA induced FGF19 expression at transcriptional and protein levels (**A-B**). Treatment of hPCLS with OCA, Rifampicin or CITCO did not induce the mRNA expression of *FOXM1B*, *CCND1* and *PCNA* (**C**). Four slices were included per condition, the experiment was replicated three times using slices derived from different patients. A representative experiment is depicted. Differences among four groups were analyzed with Kruskal-Wallis test, with Dunn's multiple comparison test to compare differences between control and specific treatment. FGF19 levels between control and OCA group was compared with the Mann-Whitney U test. Data is depicted as median [IQR]. Asterisks indicate significance levels: *P<0.05, **P<0.01.

Discussion

In the current study, responsiveness of hPCLS to FGF19 could not be verified at the level of ERK1/2, a kinase that is activated following binding of FGF19 to its receptor FGFR4/KLB. ERK1/2 activation, assessed by detection of its phosphorylated form, was already maximal without addition of FGF19. This likely results from external triggers during harvesting and/or processing of the liver tissue for hPCLS preparation. At the time, we did not investigate whether 'spontaneous' activation of ERK was sustained until the time (18-22 hrs) of harvesting of PCLS for RT-qPCR analysis, and if this could provide an explanation for lack of FGF19 responsiveness and low/undetectable expression of known FGF19 target genes. Expression of FGF19 receptors *FGFR4* and *KLB* was somewhat lowered during hPCLS culturing, but remaining expression after >18 hrs was deemed high enough to allow FGF19 signaling. Unfortunately, the expression of FGF19-regulated genes could not be used as a read-out of FGF19 activity, as *CYP7A1* was undetectable after >19 hrs of culturing and *PCK1* was drastically reduced. Although employed recombinant FGF19 was biologically active at the tested dose (100 ng/mL) in an unrelated experimental setting (e.g. HepG2 cells), we could not verify FGF19 responsiveness in hPCLS. Hence, we cannot draw a conclusion with regard to involvement of a FGF19/IL6 axis in driving hepatocellular proliferation in a human model system.

In our study, we found marked increase of IL6 mRNA expression and protein levels (in medium) in LPS-treated hPCLS. This may be because LPS induces innate immune response through activation of TLR4 in Kupffer cells. This is consistent with Wu *et al*¹⁴, who report that hPCLS can be a valuable model to study human innate immunity. Despite marked increase of IL6 protein in the medium, the cell proliferation (*CCND1*, *PCNA*) and inhibition of apoptosis (*BIRC5*) related genes remained unchanged with LPS treatment. An explanation may be that absolute IL6 levels were not high enough for efficient activation of STAT3. Measuring STAT3 phosphorylation or direct testing of the effect of exogenous IL6 appeared useful to verify suitability of hPCLS to study liver regeneration.

In its current form, the hPCLS model is not suitable for studying FGF19 actions, and improvements to maintain a fully differentiated state of hepatocytes seems pivotal. A combination of five small molecules, 5C, synergistically regulated multiple signaling pathways to maintain global gene expression pattern of human functional hepatocytes

over 4 weeks in vitro (2D culture)²³. We used the same 5C condition to culture hPCLS. However, 5C treatment failed to prevent the general decrease of FXR/FGF19 pathway related genes in our study, including expression of *NTCP* that was maintained for 4 weeks in isolated hepatocytes²³. Similar to 5C, Geltrex could not maintain expression of genes related to the FXR/FGF19 pathway. The absence of effectiveness of 5C in hPCLS model may be due to the differences between 2D and 3D culture system. Optimized concentrations of the five chemicals and culture time can be further investigated for hPCLS culture. In addition, as the targets of 5C components were not tested, the activity of 5C was undetermined.

Our current study has limitations. Ideally, 'healthy' liver tissue should be used to prepare hPCLS. The pathological state (e.g. chemotherapy-associated liver injury) of the liver tissue was not assessed before the preparation of PCLS. Full evaluation of histological status of liver tissue may be needed prior to PCLS culture. In addition, as loss of a differentiated hepatocyte phenotype is observed during PCLS culture, as well as in isolated hepatocytes, culture conditions should be optimized to prevent dedifferentiation. Furthermore, perfusion of PCLS in a microfluidic system may be a promising strategy, which allows a constant delivery of nutrients and oxygen and a continuous removal of waste products^{25, 25}.

CAR activation by CITCO was reported to induce hepatocyte proliferation in PCLS, including slices prepared from the liver of a patient². However, despite carefully replicating experimental conditions we could not reproduce the proliferative effect of CITCO in hPCLS from three distinct patients. Since induction of CAR target gene *CYP2E1* by CITCO was not observed in hPCLS, we cannot draw a conclusion on CAR action.

In summary, while treatment with LPS, OCA or rifampicin elicited clear transcriptional responses in hPCLS, indicative for overall cellular viability and biological responsiveness, efficacy of FGF19 and CAR treatment could not be ascertained in these exploratory experiments. Hence, questions regarding the involvement of the FGF19/IL6 axis in hepatocellular proliferation, and augmentation of liver growth by CAR agonism could not be answered. Systematic evaluation of patient factors (background liver disease, prior chemotherapy, ischemic time, etc), and culturing conditions (e.g. optimized recovery time, strategies to prevent dedifferentiation etc.) should be considered in optimizing the hPCLS as model to study -and therapeutically

modulate- liver regeneration.

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Chapter 4

Obeticholic acid accelerates portal vein
embolization-induced liver hypertrophy by
improving bile salt homeostasis

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Abstract

Background: Farnesoid X receptor (FXR) agonist obeticholic acid (OCA) accelerated liver growth following portal vein embolization (PVE) in rabbits. The aim of this study was to study the underlying mechanisms.

Methods: Materials were analyzed from rabbits treated by oral gavage with OCA (10 mg/kg, n=11) or vehicle (control group, n=11) prior to, and during the course of, PVE. Serum total bile salts (TBS), bile acid composition, C4 (marker of bile acid synthesis), and liver synthetic function were determined before, after 3 hrs, and at day 1, day 3, and day 7 after PVE. Ileal and hepatic genes expression were analyzed through targeted and untargeted approaches at day three after PVE, when the growth-enhancing effect of OCA was maximal. For correlation analyses, volumes of (non)embolized liver segments, and %volume gain, were previously determined by CT scan.

Results: We previously reported that PVE induced a larger volume gain of the non-embolized liver lobe in animals treated with OCA. Effects of OCA were largest at 3 days after PVE (volume gain: 56.1% vs. 26.1% in OCA- and vehicle-treated animals, respectively). OCA treatment led to earlier normalization of serum TBS levels and lower bile salt content of the non-embolized liver lobe after PVE. OCA decreased mRNA expression of *Cyp7a1* (involved in bile salt synthesis) and promoted expression of *Slc51a/b* (involved in bile salt export) in the non-embolized liver lobe, likely contributing to the observed improved serum and liver bile salt homeostasis. Levels of lithocholic acid (LCA), a hepatotoxic bile salt, were lower in the OCA-treated animals compared to the control animals at day 1 and day 3 ($P=0.002$ and $P=0.031$, respectively). LCA levels were strongly related with caudal (i.e. non-embolized) liver volume (CLV) increase and the numbers of Ki-67 positive hepatocytes in the non-embolized lobe ($\rho = -0.726$, $P < 0.001$; $\rho = -0.718$, $P = 0.009$, respectively). Both targeted and untargeted transcript analysis revealed associations with genes engaged in bile salt homeostasis and liver growth, adding to the notion that OCA-mediated improvement of bile salt homeostasis was in part responsible for enhanced PVE-induced liver growth. Additional factors candidate for mediating growth-stimulating effects of OCA include the secreted factors Bmp3 and Ihh.

Conclusions: OCA improved bile salt homeostasis, which partly contributed to augmented hypertrophy of the non-embolized liver lobe in rabbits. OCA may be

promising to increase the efficacy of PVE in patients with small future liver remnant planned for (extended) partial liver resection.

Introduction

Partial hepatectomy (PH) presents the only curative treatment for malignant hepatobiliary tumors. The robust regenerative capacity of the liver allows up to 70% resection of liver mass ¹. However, post-hepatectomy liver failure (PHLF) is a major concern and the main cause of postoperative morbidity and mortality ². A small future liver remnant (FLR) volume is associated with higher risk of PHLF and postoperative mortality ³. Portal vein embolization (PVE) is the gold standard procedure to induce hypertrophy of the FLR if volume is deemed insufficient for direct resection, increasing resectability and safety of liver resection at a later stage ⁴. However, relatively long waiting time (around 3 to 4 weeks) between PVE and resection puts patients at risk of tumor progression ⁵. Pharmacological strategies to accelerate PVE-induced liver growth are lacking in clinical practice.

Bile salt signaling is involved in liver regeneration triggered by PH, as well as in liver growth in the absence of a surgical stimulus *viz.* bile salt feeding ⁶. The nuclear bile salt receptor farnesoid X receptor (FXR) and FXR-regulated mediators (e.g. fibroblast growth factor 19, FGF19, termed *Fgf15* in rodents), are key players in these processes. Mice deficient in either *Fxr* or *Fgf15* have impaired liver regeneration and high mortality rates following PH ^{6, 7}. *Fxr* and FGF19/*Fgf15* are essential to maintain bile salt homeostasis, which is achieved by (transcriptional) regulation of hepatic synthesis and import/export of bile salts. Dysregulation of bile salt homeostasis, resulting in hepatic bile salt accumulation and attendant cytotoxicity, as well as diminished action of bile salt-regulated mitogens, underlies defective regeneration and mortality in these mice strains. In patients undergoing PH, serum bile salt levels are positively associated with liver regeneration ⁸⁻¹⁰. Above findings highlight the importance of maintained bile salt homeostasis in the remnant liver, thus balancing beneficial signaling actions and detrimental consequences of bile salt overload.

In patients undergoing PVE, we observed that serum bile salt levels after PVE were early predictors of post-PVE liver volume and function ⁹. This suggested that transient elevation of serum bile salts after PVE, provides a signal to increase liver mass. To directly test whether bile salt/FXR signaling was involved in PVE-induced liver growth, we previously examined the effects of obeticholic acid (OCA) in a rabbit model of PVE ¹¹. OCA is a first-in-class selective and potent agonist of FXR, which is currently evaluated in clinical trials in patients with cholestatic or metabolic liver disease ¹²⁻¹⁴. In

our experimental model, we noted that OCA accelerated PVE-induced growth of the non-embolized liver segment, with the largest effect seen in the initial three days after PVE. Augmented volume gain was due to enhanced proliferation of hepatocytes, and was accompanied by a functional increase in secretory function. In the current study, we sought to determine the molecular pathways beyond the hyperplastic action of OCA in this model. To this end, we tested the hypothesis that improved bile salt homeostasis is involved. Furthermore, molecular players were examined by targeted and untargeted transcript analysis.

Methods

Animal specimens

For the current study we analyzed plasma samples and tissue specimens that were collected at the time of the original study ¹¹. In brief, rabbits (n=12 per group, note that in each group, one animal had to be euthanized prior to scheduled sacrifice) were pre-treated for one week (day -7 and day -1) by daily gavage of OCA (10 mg/kg) or vehicle, before undergoing embolization of the three cranial lobes (**Supplementary Fig. 1**). The respective treatments were continued after PVE, with blood samples taken after 3 hrs, day 1, day 3, and day 7. Animals were sacrificed after day 3 (n=6 per group) or day 7 (n=5 per group), and terminal ileum, caudal (non-embolized) and cranial (embolized) liver lobes were harvested, snap-frozen and stored at -80°C. For correlation analyses, volumes of (non)embolized liver segments, and %volume gain, were previously determined by CT scan.

Quantification of bile salts

Bile salts were extracted from homogenates of (non)embolized liver segments in 75% ethanol as described ¹⁵. Total bile salt (TBS) levels in serum and liver extracts were determined through an enzymatic cycling method according to the manufacturer's protocol (Diazyme Laboratories, CA, USA). Liver bile salts were normalized to wet weight of liver specimens. Serum bile salt composition was determined by LC-MS as will be detailed elsewhere (van Eijk, Lenicek, Olde Damink and Schaap, manuscript in preparation). Serum 7- α -hydroxy-4-cholesten-3-one (C4, a serum marker of bile salt synthesis) was assessed simultaneously. For calculation of fractional bile salt composition (**Fig. 3A**), unconjugated (e.g. DCA) and conjugated forms (e.g. GDCA and

TDCA) of a particular species were summed.

Targeted transcript analyses

cDNA synthesis and targeted analysis of expression levels by RT-qPCR was previously described ¹¹. Heretofore unreported transcripts were analyzed for the present study. Employed primer sequences are provided in **Supplementary Table S1**.

RNA sequencing

Total RNA was extracted from (non)embolized liver lobes and terminal ileum tissue from animals sacrificed at day 3, with the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). RNA concentration and quality were determined on a 2100 Bioanalyzer (Agilent, CA, US). All samples for RNA sequencing had a RNA integrity number (RIN) of 7.1 or higher. cDNA library preparation, next-generation sequencing (Illumina sequencing platform, paired-end reads of 150 bp, >30 million reads per sample), and mapping to the rabbit genome, were conducted by Eurofins Genomics (Ebersberg, Germany). Rabbit genes were converted into human orthologues using biomaRt R package (v2.48.1) based on Ensembl gene ID. Gene wise read counts were collected from the samples to be compared, and genes receiving less than 10 reads on average across all the samples, were removed. Raw gene counts were normalized separately for ileal and hepatic samples using R package DESeq2 (v1.32.0) ¹⁶. A fold change of ≥ 2 in either direction, an average normalized gene count >100, and a Benjamini-Hochberg false discovery rate (FDR) corrected $p < 0.10$ was used for identifying differentially expressed genes (DEGs) using DESeq2 (v1.32.0) with default settings (independent filtering = TRUE, $\alpha = 0.10$). Pathway analysis was performed using Metascape program ¹⁷.

ELISA

Plasma levels of coagulation factor II, a measure of liver synthetic function, was determined by ELISA per manufacturers protocol (cat.nr abx053007, Abbexa, Cambridge, UK).

Statistical analysis

Data are presented as median [interquartile range] or mean $\pm$ standard error of mean (SEM) when appropriate. The mean of day -7 and day -1 blood measurements was taken as baseline value. Repeated measures analysis of variance (ANOVA) or a mixed

effect model (for blood parameters from baseline to day 7) was used to evaluate longitudinal changes, with Dunn's multiple comparison test to compare differences between baseline and subsequent individual time points. Differences between OCA-treated and control animals were analyzed with the Mann-Whitney U test. Correlations were assessed by Spearman's correlation coefficient (ρ). The correlation coefficients between liver growth and differentially expressed genes that were identified in all changed pathways were corrected with Benjamini-Hochberg false discovery rate (FDR) method. P values < 0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software Inc., CA, USA), SPSS 24.0 (IBM SPSS Inc, Chicago, Illinois, USA) and R Statistical Software 4.0.5 (R Foundation for Statistical Computing).

Results

We previously reported that PVE induced a larger volume gain of the non-embolized liver lobe in animals treated with OCA ¹¹. As effects of OCA were largest at 3 days after PVE (volume gain: 56.1% vs. 26.1% in OCA- and vehicle-treated animals, respectively), attempts to find a molecular explanation focused on this time point. We hypothesized that improved bile salt homeostasis contributes to augmented liver volume gain in OCA-treated animals.

Improved bile salt homeostasis in OCA-treated animals

First, we assessed serum TBS levels during the course of PVE. Bile salt levels did not alter during the pre-treatment phase, and were not affected by OCA treatment (data not shown). PVE induced a rapid elevation of serum TBS, with peak levels on average 8.2-9.3 fold higher than baseline values (**Fig. 1A**). This increase likely reflects limited spare capacity of the non-embolized lobe (ca. 24% of total liver volume) to extract bile salts from the portal circulation, resulting in their spill-over in the systemic circulation. Maximum elevation and time to reach this peak level were different between groups. TBS levels peaked at day 1 in controls, but already after 3h in OCA-treated animals ($P=0.012$). The trend in time tended to be different between experimental groups ($P_{\text{time*group}}=0.063$), as reflected by lower TBS levels in the OCA group at day 1 ($P=0.023$) and apparent normalization of serum levels after day 3 (vs. day 7 in controls) (**Fig. 1A-B**).

Next, we examined hepatic bile salt content at 3 days after PVE. In the non-embolized (hyperplastic) lobe, bile salts levels were 1.7 fold lower upon OCA treatment (67 [60.3-77.4] vs. 110.6 [88.6-169.6] nmol/g liver; $P=0.015$) (**Fig. 1C**). In line with abrogated portal supply, bile salt content of the embolized lobes was lower relative to the non-embolized lobe ($P=0.002$), with a tendency ($P=0.055$) to further lowering by OCA (**Fig. 1C**). Hence, both circulating and hepatic bile salt levels were reduced in OCA-treated animals, indicating improved bile salt homeostasis.

Figure 1. Improved bile salt homeostasis in OCA-treated animals. (A) Serum TBS levels peaked earlier in OCA-treated animals ($n=11$ per group). (B) Serum TBS levels normalized faster in OCA-treated animals. (C) Non-embolized hepatic lobe TBS levels at day 3 were lower in OCA-treated animals than controls, and TBS levels were lower in embolized than non-embolized lobes within control animals ($n=6$ per group). Longitudinal changes were evaluated by mixed effect model. Differences between OCA-treated animals and control animals were analyzed with the Mann-Whitney U test. Differences between embolized and non-embolized hepatic lobes within control animals were compared with the Wilcoxon signed-rank test. Data is depicted as mean $\pm$ SEM. P values are depicted. Asterisks indicate significance levels: * $P<0.05$, ** $P<0.01$. TBS, total bile salt.

Regulation of genes involved in bile salt homeostasis by OCA

To find an explanation for reduced hepatic bile salt content in OCA-treated animals, expression of ileal and hepatic genes engaged in bile salt homeostasis was examined at day 3. Note that expression of some of these genes was previously reported ¹¹, and this data has been duplicated to allow a comprehensive overview of factors involved in hepatic bile salt homeostasis. At the level of the terminal ileum, OCA target engagement was evident by induction of Fxr target genes *Slc51a* and *Shp* (**Fig. 2A**). Note that we could not detect expression of Fxr target *Fgf19* in the terminal ileum. This endocrine factor plays an important role in repression of bile salt synthesis by targeting

hepatic *Cyp7a1* expression. Of note, *Cyp7a1* expression in the non-embolized (i.e. caudal) lobe was markedly lower (-6.9 fold, $P=0.004$) in OCA-treated animals (**Fig. 2B**). *Fgf19* may be involved in this repression by binding to its hepatic receptor composed of *Fgfr4* (maintained expression) and β -*Klotho* (slightly reduced expression). Caudal expression of *Shp*, another Fxr-induced negative regulator of *Cyp7a1* expression, was not affected by OCA treatment. Hepatic Fxr targets that were upregulated included *Fabp1*, a bile salt-binding protein implicated in sequestering potentially harmful hydrophobic bile salts, and bile salt transporter subunits *Slc51a/b* (**Fig. 2B**). With maintained expression of bile salt uptake protein *Ntcp*, reduced bile salt levels in the non-embolized lobe of OCA-treated animals may be explained by reduced bile salt synthesis (*Cyp7a1*) combined with enhanced basolateral export of bile salts (*Slc51a/b*). Unexpectedly, the lower expression of *Cyp7a1* mRNA in the non-embolized lobe in the OCA group, was not accompanied by reduced levels of C4 in the systemic circulation at day 3 (**Fig. 2C**). C4 is a serum marker for bile salt synthesis. Its validity as a biomarker has been examined mainly in rodent models and healthy subjects. Serum C4 levels did not change during the course of PVE in control animals ($P_{\text{time}}=0.609$), whereas a transient drop was noted in the OCA-treated animals ($P_{\text{time}}=0.013$) (**Fig. 2C**). The trend in time did not differ between groups ($P_{\text{time*group}} = 0.728$). For reasons unknown, a correlation between *Cyp7a1* mRNA and serum C4 was not apparent in this study (**Fig. 2D**).

Figure 2. Regulation of genes involved in bile salt homeostasis by OCA. (A) Ileal

expression of *Shp* and *Slc51a* were upregulated in OCA-treated animals (n=6 per group). **(B)** Expression of *Cyp7a1*, *Cyp8b1* and β -*Klotho* were downregulated, and expression of *Slc51a/b* and *Fabp1* were upregulated in the non-embolized liver lobe in OCA-treated animals (n=6 per group). **(C)** Serum C4 levels showed a transient drop in OCA-treated animals, but not in control animals (n=6 per group). **(D)** Decreased mRNA expression of *Cyp7a1* in the non-embolized liver lobe was not accompanied by reduced levels of C4 in the systemic circulation at day 3 (n=12). Differences between OCA-treated animals and control animals were analyzed with the Mann-Whitney U test. Correlations were evaluated with Spearman correlation coefficient (ρ). Longitudinal changes were evaluated by mixed effect model. Data is depicted as mean $\pm$ SEM. P values are depicted. Asterisks indicate significance levels: *P<0.05, **P<0.01. C4, 7- α -hydroxy-4-cholesten-3-one.

Effect of OCA on serum bile salt composition

Individual bile salt species differ in their signaling activities and cytotoxic potential. We next evaluated if the composition of systemic bile salts was affected during the course of PVE, and by treatment with OCA. In rabbits, deoxycholic acid (DCA) and conjugated forms thereof, are the dominant bile salt species, in this study representing ca. 65% of circulating bile salts at baseline (**Fig. 3A**). Note that OCA and its conjugated forms amounted to ca. 10% of circulating bile salt species at baseline in OCA-treated animals. With exception of HDCA and OCA in the OCA-treated animals, fractional serum bile salt composition did not show major shifts during the course of PVE (**Fig. 3A**). Mole fraction of HDCA was lower in the OCA-treated animals than controls after PVE ($P_{\text{group}}=0.001$), but the downward trend in time was similar between groups ($P_{\text{time*group}}=0.954$) (**Fig. 3B**). Interestingly, mole fraction of OCA was highest in the period (i.e. between day 1 and 3) where the volume of the non-embolized liver lobe increased most steeply.

Bile salt hydrophobicity index (HI) is a measure associated with bile salt-induced cytotoxicity. We calculated the HI of serum bile salts using the molar fraction and the previously reported hydrophobicity of individual bile salts¹⁸. Note that OCA was unknown at the time the HI concept was proposed. For calculating contributions of OCA (conjugates) we used the mean value reported for chenodeoxycholic acid (CDCA) (conjugates) and lithocholic acid (LCA) (conjugates). Chromatographic behavior of OCA (a.k.a. 6-ethyl-CDCA) on the employed reverse phase column justifies this approach (data not shown). The overall HI of serum bile salts was increased after PVE

in both control and OCA-treated animals, but the trend in time was similar between groups ($P_{\text{time} \times \text{group}} = 0.231$, **Fig. 3C**). This is indicative for a more cytotoxic composition after PVE.

Next, we investigated the change of absolute levels of bile salt species during the course of PVE. Only species with a significant trend in time and a significant association between levels and liver growth parameters at day 3, are discussed. Serum levels of LCA, a minor but well-known hepatotoxic bile salt ¹⁹, were transiently elevated after PVE, reaching peak levels that were on average 13 and 10-fold higher compared to baseline values in control and OCA-treated animals, respectively ($P < 0.001$ and $P = 0.002$, respectively) (**Fig. 3D**). During the course of PVE, LCA showed a faster normalization in the OCA-treated animals compared to control animals ($P_{\text{time} \times \text{group}} = 0.012$). LCA levels were lower in the OCA-treated animals compared to the controls at day 1 and day 3 ($P = 0.002$ and $P = 0.031$, respectively) (**Fig. 3D**). As mirrored by lower fractional composition, HDCA levels were reduced in OCA-treated animals following PVE ($P_{\text{time}} = 0.005$), and lower relative to controls from 3h onwards (P values between < 0.001 and 0.009) (**Fig. 3E**). In addition, DCA levels showed a trend towards faster return to baseline values after PVE ($P_{\text{time} \times \text{group}} = 0.054$) in the OCA group (**Fig. 3F**). Longitudinal changes of the other bile salt species were similar in controls and OCA-treated animals or showed no relation with liver growth. (**Supplementary Fig. S2, Supplementary Table 2**).

Associations between bile salt-related parameters and liver growth

To evaluate the associations between bile salt parameters and liver growth-related parameters, we performed Spearman correlation analyses using day 3 values. Serum TBS levels were negatively correlated ($\rho = -0.518$, $P = 0.014$) with volume gain of the non-embolized lobe (i.e. CLV) (**Fig. 4A**). Remarkably, LCA levels were strongly correlated with CLV increase and the numbers of Ki-67 positive (Ki-67⁺) hepatocytes in this segment ($\rho = -0.726$, $P < 0.001$; $\rho = -0.718$, $P = 0.009$, respectively) (**Fig. 4B-C**). Additionally, serum levels of HDCA and DCA were both negatively correlated with CLV increase ($\rho = -0.491$, $P = 0.020$; $\rho = -0.527$, $P = 0.012$, respectively) (**Fig. 4D-E**). No significant correlations between liver growth parameters and the levels of hepatic TBS or serum C4 were observed. All correlations are summarized in **Supplementary Table S2**.

LCA induces cholestasis and impairs biliary bile salt secretion in rats ²⁰. We assessed

Figure 3. Effect of OCA on serum bile salt composition. (A-B) Mole fractions of HDCA was lower in the OCA-treated animals, and other bile salt species did not show major shifts during the course of PVE by OCA treatment. **(C)** Hydrophobicity index of serum bile salt were similar between controls and OCA-treated animals. Absolute levels of **(D)** LCA and **(E)** HDCA normalized faster in OCA-treated animals. **(F)** Absolute levels of DCA trend towards faster return to baseline levels in the OCA-treated animals. n=11 per group for serum bile salt composition analyses. Longitudinal changes were evaluated by mixed effect model. Differences between OCA-treated animals and control animals were analyzed with the Mann-Whitney U test. Data is depicted as mean $\pm$ SEM. P values are depicted. Asterisks indicate significance levels: *P<0.05, **P<0.01, ***P<0.001.

the relations between LCA levels and genes involved in bile salt homeostasis. Serum LCA levels were positively correlated with the expression of *Cyp7a1*, and negatively associated with expression of *Slc51a* in the non-embolized liver lobe ($\rho = +0.585$, $P = 0.046$; $\rho = -0.706$, $P = 0.015$, respectively) (**Fig. 4F-G**).

Figure 4. Associations between individual serum bile salt species and liver growth. (A) Serum TBS levels were negatively correlated with CLV increase. Serum LCA levels were negatively correlated with CLV increase (B) and the numbers of Ki-67 positive hepatocytes in this segment (C). Serum (D) HDCA and (E) DCA levels were negatively correlated with CLV increase. Serum LCA levels were positively correlated with the expression of *Cyp7a1* (F) and negatively correlated with the expression of *Slc51a* in the non-embolized liver lobe (G). Correlations were evaluated with Spearman correlation coefficient (ρ). P values are depicted. TBS, total bile salt; CLV, caudal liver volume (non-embolized liver volume); LCA, lithocholic acid; HDCA, hyodeoxycholic acid.

OCA does not affect liver synthetic function

We previously reported that OCA enhanced liver transport function at day 3 after PVE, as assessed by uptake of mebrotfenin ¹¹. To assess whether OCA treatment affects the

synthetic function of the liver as well, plasma coagulation factor II levels were determined. Factor II levels did not change after PVE in either group (**Supplementary Fig. S3**).

Analyses of genes involved in cell cycle regulation

Apart from bile salt homeostatic actions, Fxr has been implicated in regulation of cell proliferation, including direct upregulation of *Foxm1b*, which is essential for cell cycle re-entry of (quiescent) hepatocytes after PH ⁶. *Foxm1b* expression was notably higher in the non-embolized liver lobe, but no further modulation by OCA was observed (**Fig. 5A**). However, *Foxm1b* target gene *Cdc25b* was induced by OCA specifically in the non-embolized lobe ($P=0.006$) (**Fig. 5B**). Gene expression of the proliferation marker *Mki67* and proliferating cell nuclear antigen (*Pcna*) was not affected by OCA treatment (**Supplementary Fig. S4**). *Cdc25b* expression was negatively correlated with expression of *Cyp7a1* ($\rho=-0.601$, $P=0.039$), and positively correlated with expression of Fxr targets *Slc51a* ($\rho=+0.745$, $P=0.008$) and *Slc51b* ($\rho=+0.804$, $P=0.002$), the number of Ki-67⁺ hepatocytes ($\rho=+0.701$, $P=0.011$) and the volume gain of non-embolized liver lobe ($\rho=+0.566$, $P=0.055$) (**Fig. 5C-G**).

RNA sequencing

For systematic identification of FXR-regulated genes that may underlie the augmented volume gain of the non-embolized liver lobe, we conducted RNAseq analysis of terminal ileum, and embolized and non-embolized liver lobes harvested at day 3 after PVE. On average around 33 (range 30-74) million reads were produced per sample. Approximately 74% of the reads could be mapped to the rabbit genome.

OCA-induced changes in the terminal ileum

Ileum-derived FGF19 regulates hepatic bile salt synthesis and may act as a direct mitogen for hepatocytes ⁷. Ileal factors are potentially involved in OCA-augmented liver growth and were studied by differential gene expression analysis and correlative analysis. Note that textual description of results from correlation analysis focuses on secreted signaling factors for which autocrine, endocrine or paracrine modulation of cell growth is easy to conceptualize.

OCA treatment resulted in 67 differentially expressed genes (DEGs, 34 up and 33 down) in the terminal ileum (**Fig. 6A**). FXR target *Shp/Nr0b2* is one of the most highly

Figure 5. Analyses of genes involved in cell cycle regulation. OCA did not affect mRNA expression of (A) *Foxm1b*, whereas induced the mRNA expression of (B) *Cdc25b*. *Cdc25b* expression was negatively correlated with expression of (C) *Cyp7a1*, and positively correlated with the expression of Fxr targets (D) *Slc51a* and (E) *Slc51b*, (F) the number of Ki-67 positive hepatocytes and (G) the volume gain of non-embolized liver lobe (i.e. CLV). Differences between OCA-treated animals and control animals were analyzed with the Mann-Whitney U test. Correlations were evaluated with Spearman correlation coefficient (ρ). P values are depicted. Asterisks indicate significance levels: ** $P < 0.01$.

upregulated genes, which is consistent with RT-qPCR results. Additionally, OCA induced expression of secreted factors *C1qa*, *C1qb* and *C1qc*, which are components of the complement system that participates in liver regeneration ²¹. The top 10 of induced and repressed genes can be found in **Supplementary Table 3**. We performed pathway enrichment analysis using all DEGs. The top 3 of ileal pathways affected by

OCA were 'C1q complex', 'regeneration', and 'cell-substrate adhesion' (**Fig. 6B**). Genes in the affected pathways are presented in **Supplementary Table 4**. We used Spearman correlation coefficient to evaluate associations between liver growth and DEGs identified in the changed pathways. Significant associations are marked in the heatmap (**Fig. 6C**). CLV increase was negatively correlated with the expression of the signaling factor bone morphogenetic protein 3 (*Bmp3*) ($\rho=-0.601$, $P=0.039$), with OCA repressing *Bmp3* expression 2 fold. Bmp signaling participates in adult tissue homeostasis including liver regeneration ²². The numbers of Ki-67⁺ hepatocytes were positively correlated with the expression of *C1qa* ($\rho=+0.760$, $P=0.004$).

Figure 6. OCA-induced changes in the terminal ileum. (A) Differentially expressed genes induced by OCA in terminal ileum (n=6 per group). **(B)** Identification of changed pathway by OCA treatment. **(C)** Heatmaps of Spearman correlation analysis between CLV increase, the number of Ki67 positive hepatocytes and genes present in changed pathways. Asterisks indicate significance levels: *P<0.05, **P<0.01. N.S., not significant. CLV, caudal liver volume (i.e. non-embolized liver volume).

PVE-induced changes in the control liver

PVE elicited broad transcriptional changes in the liver of vehicle-treated animals, with a total of 467 genes differentially expressed in atrophic and hypertrophic lobes (261 up and 206 down in the non-embolized liver lobe) (**Fig. 7A**). *Slc51b* and cell cycle-related genes, such as *Cdk1*, *Ccnb1*, *Ccnb2* and *Cdca3*, were among the most highly induced genes in the proliferating liver segment (**Supplementary Table 3**). Furthermore, the expression of signaling factor *Bmp10* was elevated (2 fold) in the non-embolized lobe. BMP10 was previously shown to negatively regulate cell proliferation in multiple tumors^{23, 24}, but has thus far not been linked with compensatory liver growth. Here, the CLV increase was negatively correlated with the expression of *Bmp10* ($\rho=-0.829$, $P=0.042$). Additionally, connective tissue growth factor (*Ctgf*) was downregulated 2-fold in the non-embolized lobe. CTGF plays an important role in liver regeneration after partial hepatectomy or ethanol/carbon tetrachloride (CCl_4)-induced liver injury^{25, 26}. The CLV increase was strongly -but negatively- correlated with the expression of *Ctgf* ($\rho=-1.000$, $P<0.001$). Through pathway enrichment analyses, 'Cell cycle, Mitotic' was identified as the most significantly changed pathway, followed by 'Condensation of Prometaphase Chromosomes' and 'monocarboxylic acid metabolic process' (**Fig. 7B**, **Supplementary Table 4**). The numbers of Ki-67⁺ hepatocytes strongly correlated with the expression of *Cdk1*, *Ccnb1* and *Ccnb2* ($\rho=+0.829$, $P=0.042$; $\rho=+0.829$, $P=0.042$; $\rho=+0.943$, $P=0.005$, respectively) (**Fig. 7C**). Note that for reasons of graphical clarity, only significant correlations between DEGs and liver growth are depicted in **Fig. 7C**, with the full set of correlations depicted in **Supplementary Fig. S5**.

OCA-induced changes in the non-embolized liver lobe

OCA treatment resulted in 58 DEGs (33 up and 25 down) in the non-embolized proliferating liver lobe (**Fig. 8A**). Of note, and in line with RT-qPCR findings (**Fig. 2B**), FXR targets *Slc51b* and *Cyp7a1* were the most highly upregulated and downregulated genes, respectively, in the non-embolized liver segment (**Supplementary Table 3**). Moreover, secreted factor apolipoprotein D (*APOD*) expression was increased 2 fold. *APOD* has been related to cell proliferation in bone metabolism and tumors^{27, 28}. CLV increase was positively correlated with the expression of *APOD* ($\rho=+0.580$, $P=0.048$). Pathway enrichment analyses showed that 'steroid metabolic process' was the pathway most significantly affected by OCA treatment (**Fig. 8B**). Additionally, 'Biological oxidations' and 'positive regulation of steroid biosynthetic process' were

Figure 7. PVE-induced changes in the control liver. (A) Differentially expressed genes induced by PVE in non-embolized and embolized liver lobes (n=6 per group). **(B)** Identification of changed pathway by PVE. **(C)** Heatmaps of Spearman correlation analysis between CLV increase, the number of Ki67 positive hepatocytes and genes present in changed pathways. Asterisks indicate significance levels: *P<0.05, **P<0.01, ***P<0.001. N.S., not significant. CLV, caudal liver volume (i.e. non-embolized liver volume).

among the changed pathways. Remarkably, CLV increase was negatively correlated with the expression of *Cyp7a1* and positively with *Slc51b* expression ($\rho=-0.601$, $P=0.039$; $\rho=+0.629$, $P=0.029$, respectively) (**Fig. 8C**). Furthermore, the numbers of Ki-67⁺ hepatocytes were positively correlated with *Slc51b* expression and tended to be negatively correlated with *Cyp7a1* expression ($\rho=+0.662$, $P=0.019$; $\rho=-0.539$, $P=0.070$, respectively). Above correlations suggest that improved bile salt homeostasis directly contributes to OCA-accelerated liver hypertrophy. However, it cannot be excluded that improved bile salt homeostasis is not the cause but the consequence of augmented non-embolized liver volume (and function). Although serum levels of certain bile salt species at day 1 were predictive for non-embolized liver volume at day 3, further

empirical examination of above cause-effect relationship is required.

Figure 8. OCA-induced changes in the non-embolized liver lobe. (A) Differentially expressed genes induced by OCA in the non-embolized liver lobe (n=6 per group). (B) Identification of changed pathway by OCA. (C) Heatmaps of Spearman correlation analysis between CLV increase, the number of Ki67 positive hepatocytes and genes present in changed pathways. Asterisks indicate significance levels: *P<0.05, **P<0.01, ***P<0.001. N.S., not significant. CLV, caudal liver volume (i.e. non-embolized liver volume).

OCA-induced changes in the embolized liver lobes

It is conceivable that signals from the embolized (i.e. atrophic) liver lobes are engaged in compensatory growth of the non-embolized lobe. A total of 63 DEGs (36 up and 27 down) were observed after OCA treatment (**Fig. 9A**). *Cyp7a1* was the most downregulated gene and *Slc51b* was the most upregulated gene in OCA-treated animals (**Supplementary Table 3**). In addition, OCA induced expression of Indian hedgehog (*Ihh*, 2 fold), a secreted signaling factor that is implicated in liver regeneration²⁹ and induced following standard PH as well in experimental two-staged hepatectomy (associating liver partition and portal vein ligation for staged hepatectomy, ALPPS)³⁰. The significantly changed pathways in the embolized liver lobes are shown in **Fig. 9B** and **Supplementary Table 4**, and include 'steroid metabolic process', 'lipid localization' and 'cellular response to low-density lipoprotein particle stimulus'. CLV

increase was negatively correlated with the cranial (i.e. embolized lobes) expression of *Cyp7a1* ($\rho=-0.608$, $P=0.036$). The numbers of Ki-67⁺ hepatocytes in the non-embolized lobe were positively correlated with cranial *Slc51b* expression, and tended to be positively correlated with *Ihh* expression ($\rho=+0.715$, $P=0.009$; $\rho=+0.560$, $P=0.058$, respectively) (**Fig. 9C**).

Figure 9. OCA-induced changes in the embolized liver lobes. (A) Differentially expressed genes induced by OCA in the embolized liver lobes (n=6 per group). (B) Identification of changed pathway by OCA. (C) Heatmaps of Spearman correlation analysis between CLV increase, the number of Ki67 positive hepatocytes and genes present in changed pathways. Asterisks indicate significance levels: *P<0.05, **P<0.01. N.S., not significant. CLV, caudal liver volume (i.e. non-embolized liver volume).

Discussion

We previously reported that the FXR agonist OCA promotes liver growth following PVE in rabbits. In this study, we demonstrate that OCA improves bile salt homeostasis after PVE, likely through regulating hepatic synthesis and export of bile salts. Opposite relationships between hepatic transcripts involved in bile salt synthesis (*Cyp7a1*, negatively correlated) and bile salt export (*Slc51b*, positively correlated) and CLV gain

were apparent. Our findings support a contribution of restored homeostatic control of bile salt levels, in OCA-augmented liver growth in rabbits undergoing PVE. Furthermore, candidate secreted factors (e.g. *Bmp3*, *lhh*) that may be involved in enhanced liver growth were identified on the basis of transcriptional regulation by OCA and correlation with liver growth parameters.

The PVE procedure redistributes the portal blood flow to the non-embolized liver lobe which is accordingly exposed to the entire pool of bile salts returning from the intestines. Three hours after PVE, serum bile salts levels were elevated 8 to 9-fold, and this likely reflects systemic spill-over from the portal circulation due to limited spare capacity for bile salt extraction by the non-embolized lobe that comprises only 24% of total liver volume. Earlier normalization of total and individual bile salt levels after PVE was apparent in OCA-treated animals (**Figure 1 and 3**). The reason may be that OCA accelerates liver growth, consequently enhancing the capacity of the non-embolized liver lobe to extract portal bile salts. Improved bile salt homeostasis by OCA was also reflected by reduced bile salt content in the non-embolized liver lobe, which may be caused by decreased hepatic synthesis (downregulated expression of *Cyp7a1*) and enhanced basolateral export (upregulated expression of *Slc51a/b*) of bile salt. These findings are in line with reported actions of OCA, including observations in patients with cholestatic liver disease ^{31, 32}, and result in a shorter exposure of hepatocytes to potentially harmful bile salts such as LCA.

Serum levels of LCA were lower after PVE in OCA-treated animals, and showed a strong inverse correlation with CLV gain and number of Ki67⁺ hepatocytes. Likewise, serum levels of HDCA were consistently lower following PVE in the OCA group (**Fig. 3**). HDCA was previously reported to inhibit proliferation of epithelial cells in the intestine by interfering with the phosphoinositide 3-kinase (PI3K)/AKT pathway that controls the cell cycle, and is also a critical player in early events after liver regeneration triggered by PH ^{33, 34}. Fractional bile salt composition of individual bile salt species did not show major shifts during the course of PVE, except for HDCA that became a minor fraction after PVE in OCA-treated animals. It is worth exploring whether this is accompanied by enhanced hepatic PI3K signaling in follow-up studies. After PVE, the overall hydrophobicity index of serum bile salts increased in both groups to the same extent. This is indicative for a more cytotoxic bile salt composition after PVE. Taking absolute levels into account, potential detrimental effects of bile salt amount and composition, are expected to be less in OCA-treated animals. The aggregated findings

indicate that OCA-mediated improvement of bile salt homeostasis contributes to augmented PVE-induced liver growth.

Targeted transcript analysis revealed that OCA induces expression of *Cdc25b* in the non-embolized lobe but not in the embolized lobes (**Fig. 5B**). *Cdc25b* is a phosphatase that is required for entry into mitosis, and its expression is under control of *Foxm1b*, an FXR target gene. In order to identify additional candidate factors that may mediate OCA-augmented liver growth after PVE, RNA isolated from the terminal ileum, the proliferating caudal liver lobe, and the atrophic embolized liver lobes was subjected to RNA sequencing. Among differentially expressed genes (DEGs), candidates were identified based on correlations with liver growth parameters (CLV gain, number of Ki67⁺ hepatocytes) and an extracellular location of the gene product (e.g. a secreted factor with preferably a signaling activity).

Pathway enrichment analyses indicated that the 'C1q complex' was most significantly affected by OCA in the ileum, with each of the three subunits of this complex upregulated by OCA treatment. Of these, *C1qa* was positively correlated with hepatocyte proliferation (**Fig 6C**). The C1q complex was reported to activate mitogen-activated protein kinases (MAPKs) and PI3K/AKT signaling, and resulted in malignant progression of HepG2 hepatoma cells ³⁵. C1q is also a constituent of the C1 complex of the complement system, a system that is intimately linked with liver repair after injury and liver regeneration after PH ²¹. In addition, we observed an inverse association between signaling factor *Bmp3* and liver growth. *Bmp3* is a member of the bone morphogenetic protein (BMP) family that is linked to the regenerative process of the liver ²². BMP family members are transcriptionally inhibited after PH in mice ³⁶. Furthermore, BMP receptor type 1A (BMPRI1A) deficient mice display an augmented hepatocyte proliferation after PH ³⁷.

In the non-embolized lobe, 'steroid metabolic process' and 'positive regulation of steroid biosynthetic process' were the pathways most significantly affected by OCA treatment. These pathways contain genes (e.g. *Cyp7a1* and *Slc51b*) that were also included in our targeted transcript analysis, and showed a strong correlation with liver growth parameters. In the embolized lobe, OCA induced expression of *Ihh* that tended to be positively related to hepatocyte proliferation in the non-embolized lobe ($p=0.058$). *Ihh* is a cell signaling protein of the hedgehog family that plays a central role mediating liver regeneration in an experimental ALPPS model ³⁰. *Ihh* administration combined

with portal vein ligation induced ALPPS-like acceleration of liver growth, however, blocking Ihh signaling markedly inhibited ALPPS-induced liver regeneration ³⁰.

This study makes it conceivable that improved bile salt homeostasis contributes to OCA-induced acceleration of liver hypertrophy after PVE. Clinically, OCA may be beneficial to increase FLR volume gain in patients before partial liver resection, thus decreasing the risks of postoperative liver failure and death. In addition, patients scheduled for liver surgery often have damaged liver parenchyma. OCA has already been shown to improve histological features (hepatic steatosis and fibrosis, hepatocellular ballooning) and secretory function in patients with metabolic or cholestatic liver disease^{32, 38}. A large body of safety data in humans is available for OCA from ongoing clinical trials, including long-term safety IV studies ^{14, 31, 38}. Application of OCA in surgical patients holds promise. However, an essential additional step would be the demonstration of lack of effects of OCA on tumor progression in the embolized segments in a preclinical model.

In conclusion, OCA restores bile salt homeostasis, likely through regulating hepatic bile salt synthesis and export, and this contributes to augmented liver hypertrophy induced by PVE. By accelerating growth of a small FLR, OCA may be promising to shorten the interval between PVE and partial liver resection in patients scheduled for extended liver resection.

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Supplementary Table S1. Primer sequences.

Gene	Forward sequence	Reverse sequence
<i>36B4</i> [†]	CCTCGTGAGAGTGACATCGT	CGCCCACGATGAAGCATTTT
<i>HPRT</i> [†]	GACCAGTCAACAGGGGACAT	ATCCAACAAAGTCTGGCCTGT
<i>GAPDH</i> [†]	CCACTACATGGTCTACATGTTCC	TCACCCCACTTGATGTTGGC
<i>FXR</i>	ACAAGTGACGTCGACAACGA	AGGTCTGAAACCCTGGCAAC
<i>SHP</i>	GCCCCAAGGAATACGCCTAC	CCGGAATGGACTTGAGGGTG
<i>NTCP</i>	CCTTGGCCATCTTGGTCTGT	ATCCCCAGGGCTAAGAAGGT
<i>ASBT</i>	TGGTACAGGTGCCGAACAG	GACTCCGGCTCTGTTTTGGT
<i>FGFR4</i>	GAAAACCAGCAATGGCCGC	GAGCCCCCAAGTGTGAAGAT
<i>KLB</i> [*]	GAGAACGGCTGGTTCACAGA	TCGAAGCCATCCAGGAGAGA
<i>CYP7A1</i>	ATATGATGAGGAGCTCTGAAGC	GGGACTCCTTGATGATGCTGT
<i>CYP8B1</i>	CACCTGATGGGACATGGCTT	CAGGTAGCCAGCCTTGAACA
<i>SLC51A</i>	CCCCACGGTGGTGTCTATAA	GCCTTCCACCATGACCATCA
<i>SLC51B</i>	TGGGAACAGGAGCCAGAAAC	CGTCAGGGCAAGGATGGAAT
<i>SLCO1B3</i>	AGATGCTGGGGAAAGGTTGT	TGCAAAGCCAATGATAGGGC
<i>LFABP</i>	ATGGGGAAGGTCAAGACAGT	ATCCGCTTGCTGATTGCTT
<i>FOXO1B</i>	CCGTCACAACCTGTCTCTCC	GCTGCTGTGATTCCAAGTGC
<i>CDC25B</i>	GACTTTTGCCCGTCTCAGGA	GGAGCCCCCTAGGACACTAA
<i>MKI67</i>	CGCCTGCTCGTTCGGAAG	TATACAACACTGCCTCCTGCTC
<i>PCNA</i>	ACCTCACCAGCATGTCCAAAAT	GGTCTCGGCATATACGTGCAA

[†]Reference genes. ^{*}Synonyms: KLB, β -Klotho.

Supplementary Table S2. Correlations between bile salt-related parameters and liver growth-related parameters at day 3.

Variables	CLV increase (%)		Ki67 ⁺ hepatocytes (%) [*]	
	ρ	P value	ρ	P value
Non-embolized liver lobe TBS (nmol/g)	-0.357	0.255	-0.123	0.704
Embolized liver lobes TBS (nmol/g)	-0.364	0.245	-0.091	0.778
Serum TBS (μ mol/L)	-0.518	0.014	+0.056	0.863
C4 (ng/mL)	-0.007	0.983	-0.200	0.534
DCA (nmol/L)	-0.527	0.012	-0.340	0.280
GDCA (nmol/L)	-0.373	0.087	-0.172	0.594
HDCA (nmol/L)	-0.491	0.020	-0.532	0.075
CDCA (nmol/L)	-0.377	0.084	-0.077	0.812
CA (nmol/L)	-0.315	0.154	-0.119	0.712
GCA (nmol/L)	-0.266	0.232	-0.172	0.594
LCA (nmol/L)	-0.726	<0.001	-0.718	0.009
GLCA (nmol/L)	-0.139	0.536	-0.189	0.556
UDCA (nmol/L)	-0.220	0.326	-0.014	0.966
OCA (nmol/L)	-0.536	0.089	-0.696	0.125
GOCA (nmol/L)	-0.109	0.750	-0.812	0.050
TOCA (nmol/L)	+0.214	0.610	+0.500	0.667
Hydrophobicity index	+0.220	0.326	+0.235	0.463

^{*}Correlations are based on values derived from 12 animals, except for OCA (n=6). CLV, caudal liver volume (non-embolized liver volume); TBS, total bile salt; C4, 7- α -hydroxy-4-cholesten-3-one.

Supplementary Table S3. The 10 highest **upregulated** and **downregulated** genes.

Ileum (effect OCA)				Control liver (effect PVE)				Non-embolized liver lobes (effect OCA)				Embolized liver lobe (effect OCA)			
Gene	Read counts	Fold change		Gene	Read counts	Fold change		Gene	Read counts	Fold change		Gene	Read counts	Fold change	
LPH	400	+26		SLC51B	288	+44		SLC51B	288	+11		SLC51B	288	+15	
LCT	656	+20		DHRS9	2795	+24		NAT1	383	+4		MMP12	461	+15	
TM4SF4	10869	+16		CA14	508	+22		C18orf32	104	+3		FABP4	1279	+11	
CD19	151	+15		CDK1	516	+15		SECISBP2L	126	+3		DHRS9	2795	+7	
CPO	148	+7		SPAG5	153	+15		PCF11	624	+2		LPL	731	+7	
POU2AF1	1194	+6		CCNB1	474	+14		ANKRD26	146	+2		GPNMB	1664	+6	
PLB1	4340	+6		CCNB2	269	+14		APOD	226	+2		LGALS3	700	+5	
MYO1G	466	+6		SLC16A11	181	+13		CEP290	107	+2		CD9	231	+5	
NR0B2	126	+5		CDC20	358	+13		RBBP6	916	+2		SLC37A2	148	+4	
MAB21L3	308	+5		TCF19	242	+12		FBNP4	135	+1		MT1A	531	+4	
MAGIX	618	-3		LGALS3	700	-12		ELF3	858	-1		DDX60	492	-1	
RNF186	311	-3		CD1C	130	-16		PIM1	134	-1		CYP51A1	645	-2	
FZD4	156	-5		FABP5	220	-17		IFIT5	355	-2		SC5D	816	-2	
NR4A3	131	-6		CHI3L1	306	-19		RPL17	4525	-2		PPARGC1A	186	-2	
PPARGC1A	217	-6		SLAMF7	124	-24		TMEM132A	361	-2		SQLE	534	-2	
CA12	1522	-7		FABP4	1279	-31		CYP26A1	1051	-2		TCN1	338	-2	
IFIT5	266	-8		TREM2	207	-44		TCN1	338	-2		CYP8B1	4886	-3	
LRBA	499	-8		SPP1	5181	-55		EXTL1	2598	-2		HMGCS1	836	-4	
AQP8	3318	-19		MMP12	461	-58		NR1D1	383	-3		TMEM14A	3608	-4	
HOXB13	976	-101		ATP6V0D2	160	-63		CYP7A1	1771	-7		CYP7A1	1771	-12	

Supplementary Table S4. Changed pathways through enrichment analyses.

Tissue	Pathways	Genes
Ileum (effect OCA)	CORUM:6418: C1q complex	C1QA,C1QB,C1QC,CD19,HLA-DPB1, HMOX1, NR4A3, MYO1G, POU2AF1, APOD, CSF1R, EFNA1, BMP3, MMP1, EGLN3, PPARGC1A
	GO:0031099: regeneration	ADM,APOD,HMOX1,TM4SF4,NR4A3,NR0B2,MMP1,PLD1
	GO:0031589: cell-substrate adhesion	APOD,COL8A1,EFNA1,ID1,FZD4,NTN4,MYO1G,ADM,HMOX1, HOXB13,HLA-DPB1,NR4A3,CSF1R,ELMO1
	M182: PID IL3 PATHWAY	CSF2RB,ID1,CSF1R,HMOX1,MMP1,HLA-DPB1
	GO:0002253: activation of immune response	C1QA,C1QB,C1QC,CD19,HLA-DPB1,NR4A3,ELMO1,MYO1G
	hsa04640: Hematopoietic cell lineage	CD19,CSF1R,HLA-DPB1, IL3RA, EFNA1, FZD4, NTN4, DUSP5, NEK2,PPARGC1A,CDON
	GO:0043392: negative regulation of DNA binding	HMOX1,ID1,NEK2,ADM,EFNA1,NR0B2,CDON,NR4A3,CSF1R
	GO:0019216: regulation of lipid metabolic process	ADM,APOD,CD19,NR4A3,PPARGC1A,HMOX1,EGLN3,C1QA,NR0B2,KCNMA1,PLB1
	GO:0032922: circadian regulation of gene expression	ID1,NR0B2,PPARGC1A,NR4A3,HMOX1,FZD4,APOD,CSF1R,CDON,SLC2A12,AQP8,IFIT1
	GO:0010817: regulation of hormone levels	ADM,FZD4,NR0B2,PPARGC1A,TM7SF3,PLB1
	GO:0032642: regulation of chemokine production	APOD,CSF1R,HMOX1,HLA-DPB1, POU2AF1, NR4A3, MMP1, MYO1G
	GO:0048598: embryonic morphogenesis	ADM,COL8A1,DUSP5,EFNA1,NR4A3,CDON
	hsa05200: Pathways in cancer	CSF1R,MMP1,PLD1,FZD4,EGLN3
	GO:1904659: glucose transmembrane transport	NR4A3,MFSD4A,SLC2A12,SLC9A2,APOL1
	GO:0022612: gland morphogenesis	CSF1R,HOXB13,NTN4,C1QA,ADM,FZD4
	GO:0008203: cholesterol metabolic process	NR0B2,APOL1,PLB1

Supplementary Table S4 (continued)

Tissue	Pathways	Genes
Ileum (effect OCA)	GO:0007264: small GTPase mediated signal transduction	HMOX1,PLD1,ELMO1,G3BP1,CDON
	GO:0098771: inorganic ion homeostasis	ADM,CA12,CD19,HMOX1,KCNMA1,SLC9A2
	GO:0150063: visual system development	C1QA,COL8A1,FZD4,CDON
	R-HSA-69278: Cell Cycle, Mitotic	CCNA2,CCNB1,CCND2,CDK1,CDC25B,CDKN2C,CENPE,CSNK2A2,LMNA,MCM2,MCM3,MCM4,MCM5,NEK2,PCNA,PLK1,POLD2,RFC5,TOP2A,TYMS,DYNLL1,CCNB2,PTTG1,NCAPD2,SMC4,KIF20A,PSMD14,TUBA1B,NDC80,SMC2,TPX2,NCAPH,NCAPG2,CENPN,KNL1,NCAPG,CENPU,NUF2,SGO2,SKA2,CENPS,EPB41,FGF1,HELLS,KIF11,KIFC1,STMN1,VEGFA,KIF20B,SPAG5,RACGAP1,GPSM2,PDGFD,CDCA3,RAB11FIP4,ASPM,PCBP4,DCN,MCU,DIAPH3,FMNL3,MYH11,WAS,CAV1,CPD,AKAP12,ARHGAP11A,ARHGAP25,CKAP4,PREX2,ARHGAP30
Control liver (effect PVE)	R-HSA-2514853: Condensation of Prometaphase Chromosomes	CCNB1,CDK1,CSNK2A2,CCNB2,NCAPD2,SMC4,SMC2,NCAPH,NCAPG,STMN1,KIF20A,NDC80,RACGAP1,TOP2A,NCAPG2,PLK1,LMNA
		ACAT1,ACAT2,APOA1,BPGM,CAV1,CD74,CYP1B1,FABP5,FGFR4,GATM,GCKR,GLO1,HADHB,HAL,LIPC,LPL,PDK2,PFKFB1,PKLR,PROX1,SCD,SLC16A1,OGT,ABCB11,CES2,RGN,DHRS9,SDS,SLC27A3,ANGPTL3,ACAD9,HSD17B12,ELOVL2,ACSM5,ACSS2,GBA2,EL
		OVL5,ABHD1,AMDHD1,ACSM2B,AKR1B1,ATF3,CDO1,FGF1,NME1,ENPP3,ALDH18A1,S
	GO:0032787: monocarboxylic acid metabolic process	NCA,SP1,SRD5A2,AASS,SLC25A13,SEC14L2,GNMT,CSAD,CSNK2A2,PLPP1,ST3GAL5,SYNJ2,PRKD3,DHRS11,GPAT3,SDR42E1,SLC44A3,AK4,NQO1,RORC,PSMD14,TREM2,AKR1B10,APOD,CCL21,TNFAIP8L3

Supplementary Table S4 (continued)

Tissue	Pathways	Genes
Control liver (effect PVE)	WP2446: Retinoblastoma Gene in Cancer	CCNA2,CCNB1,CDK1,CDC25B,HMGB2,STMN1,MCM3,MCM4,PCNA,RFC5,RRM1,SMARCA2, TOP2A, TYMS,CCNB2,SMC2,CCND2,CDKN2C,MCM2,MCM5,PLK1,PTTG1,GADD45G,PSMD14,POLD2,WDHD1,FAM111A,PRIMPOL,CENPS,DCLRE1B,WAS,BTG2,FAN1,SMG1,PARBP,NAV2,KPNA2,PTPRC,LMNA
	GO:1901137: carbohydrate derivative biosynthetic process	ACAT1,AK4,AMPD2,ATP7A,VCAN,DCN,DDOST,HBEGF,STT3A,LUM,NME1,OGN,PKD2,PAS,PKLR,RPN2,RRM1,SCD,CCL21,SDC1,TYMS,OGT,ST3GAL5,ITM2A,SLC25A13,SDF2L1,HSD17B12,NANS,TREM2,TET2,ELOVL2,ACSS2,OSTC,ELOVL5,GALNT11,SLC51B,B3GALNT2,CDO1,GSTM5,SP1,GSTO1,CSAD,GLO1,AASS,GNMT,ACSM5,GSTA5,ACSM2B,BPGM,GCKR,PFKFB1,ENPP3,ACAT2,HADHB,APOA1,CSNK2A2,FABP5,SNCA,SYNJ2,GPAT3,SLC44A3,SLC27A3,CD74,CYP1B1,CES2,ACAD9
	GO:0030198: extracellular matrix organization	ATP7A,CAPG,CAV1,CD44,COL3A1,COL6A2,COL15A1,VCAN,CCN2,CTSK,CYP1B1,DCN,FGFR4,LAMA4,LCP1,LUM,MMP2,MMP12,MYH11,TNFRSF11B,SPP1,TIMP1,VWF,ST7,ITGA8,HSD17B12,LOXL4,PPIB,SDC1,BMP10,OGN,SPARCL1,PRG4
	R-HSA-109582: Hemostasis	ANXA5,APOA1,ATP2A2,CAV1,CD9,CD44,CD48,CD74,CENPE,CLU,F11,HGF,JCHAIN,KIF11,KIFC1,MPL,SERPINE2,PLAU,PTPN6,SDC1,SLC16A1,TIMP1,VEGFA,VWF,MANF,CD84,KIF20B,KIF20A,TUBA1B,SLC7A8,SCG3,RACGAP1,EHD3,JMJD1C
M176: PID FOXM1 PATHWAY		CCNA2,CCNB1,CDK1,CDC25B,LAMA4,MMP2,NEK2,PLK1,SP1,CCNB2,PCNA,GADD45G,AIF1,CCND2,CDKN2C,CDKN3,CENPE,MCM2,MCM3,MCM4,MCM5,ABCB1,PTPN6,TYMS,BTG2,DYNLL1,PSMD14,NDC80,GPNMB,TPX2,KMT2E,PCBP4,PTPRC,CSNK2A2,CCN2,KIF11,PKHD1,PTTG1,KIF20B,KIF20A,SPAG5,RACGAP1,GPSM2,SLC25A33,RAB11FIP4,PROX1,SNCA,TUBA1B,LMNA,DCLRE1B, TOP2A,CD44,CD74,PRR11,PDCCD4,SGK1,TNFSF10,HOMER1,PLPP1,ECRG4,PKD2,RFC5

Supplementary Table S4 (continued)

Tissue	Pathways	Genes
Control liver (effect PVE)	GO:0045859: regulation of protein kinase activity	CAV1,CBLB,CCNA2,CCNB1,CCND2,CD74,CDK1,CDC25B,CDKN2C,CDKN3,CENPE,CHI3L1,CLU,CSF1R,HBEGF,FGF1,HGF,PLK1,PROX1,PTPN6,PTPRC,RGS2,CCL21,SNCA,VEGFA,FZD4,RGN,CCNB2,MAP3K13,GADD45G,TPX2,PDCD4,NCAPG2,HEG1,PDGFD,SRGIN1,MAP3K21,FGFR4,NEK2,PCNA,PFKFB1,RFC5,ARRDC3,TNFAIP8L3,APOA1,ATF3,CD44,CCN2,LGALS9,PKHD1,AKAP12,GPNMB,TREM2
	GO:0008202: steroid metabolic process	ACAT1,ACAT2,AKR1B1,APOA1,CETP,CYP1B1,FGF1,FGFR4,PROX1,RORC,SCD,SP1,SP1,SRD5A2,ABCB11,DHRS9,SEC14L2,ANGPTL3,HSD17B12,GBA2,DHRS11,SDR42E1,P
	GO:0045055: regulated exocytosis	CSK9,ATP7A,CTSK,SNCA,PLPP1,SYNJ2,DUOX2,ACSS2,AKR1B10,SEC24D
	GO:0051272: positive regulation of cellular component movement	ANXA5,APOA1,ATP2A2,CD9,CD44,CD53,CHI3L1,CLU,DDOST,FABP5,FCGR2A,HGF,HP,LGALS3,LGALS9,LYZ,MNDA,PIGR,PLAU,PTPN6,PTPRC,SNCA,TCN1,TIMP1,VEGFA,VWF,MANF,DYNLL1,CD84,PSMD14,CKAP4,CORO1A,SCG3,BIN2,SYT15,ATP7A,CD74,HLA-DRA,LCP1,ENPP3,RORC,ITM2A,TREM2,NFKBIZ,AIF1,KMT2E
	GO:1901652: response to peptide	AIF1,ATP7A,CAV1,CD74,CSF1R,HBEGF,FGF1,HGF,STMN1,LGALS3,LGALS9,PLAU,PROX1,PTPRC,CCL21,SP1,VEGFA,PLA2G7,KIF20B,AKAP12,GPNMB,CORO1A,ANGPTL3,CD274,DUOX2,TREM2,PDGFD,MCU,CCBE1,FOLR2,HMGB2,PPIB,BIN2,APOA1,LECT2,NGFR,SPTBN2,SH3KBP1,APOD,WAS,LPL,MMP12,MNDA,SNCA,PSMD14,PDCD4,NFKBIZ,HLA-DRA
		AKR1B1,CA2,CAV1,CCNA2,CDO1,CCN2,INHBB,IRF5,LPL,MMP2,MMP12,NGFR,PKD2,SERPINF1,PFKFB1,PKLR,PTPRN,SP1,TIMP1,UCP2,BTG2,OGT,TNFSF10,TREM2,SLC25A33,ATP6V0D2,PCSK9,AQP1,ATP7A,FOLR2,MEF2C,SNCA,PDGFD,IFIT1B,RXRG,SGK1,UCP1,PLPP1

Supplementary Table S4 (continued)

Tissue	Pathways	Genes
Control liver (effect PVE)	M129: PID PLK1 PATHWAY	CCNB1, CDK1, CDC25B, CENPE, PLK1, KIF20A, NDC80, TPX2, CENPU, NEK2, SPAG5, RACGA P1, KNL1, NUF2, CSNK2A2, TOP2A, PTTG1, CENPS, KIFC1, LMNA, MCM2, VEGFA, OGT, SMG1 , KMT2E
	GO:0051383: kinetochore organization	CENPE, SMC4, NDC80, SMC2, CENPN, NUF2, CENPS, CCNB1, HELLS, HMGB2, MCM2, MCM3, MCM4, MCM5, RFC5, TOP2A, NCAPD2, NCAPH, NCAPG2, KNL1, NCAPG, CENPU, NAV2, CCN A2, PCNA, POLD2, SMARCA2
	R-HSA-2132295: MHC class II antigen presentation	CD74, CENPE, CTSE, CTSK, HLA-DQB1, HLA-DRA, KIF11, SPTBN2, DYNLL1, SEC24D, KIF20A, TUBA1B, RACGAP1, TREM2, CCL21, WAS, PSMD14, KIFC1, KIF20B, CBLB, COL3A1, P TPN6, PTPRC, CD274, SH3KBP1, KLHL5, SLAMF7, EHD3, JMJD1C, APOA1, CPD, HBEGF, HP, J CHAIN, FZD4, SYNJ2, SEC22C, STAB2
	GO:0000302: response to reactive oxygen species	APOD, AQP1, ATP7A, CCNA2, CYP1B1, NQO1, HGF, HP, MMP2, PCNA, PDK2, PTPRN, SDC1, U CP1, UCP2, ZNF277, PDGFD, AIF1, ATP2A2, SNCA, SP1, PDLIM1, DUOX2, SRXN1, AKR1B1, CA V1, CDO1, CCN2, VEGFA
	GO:0009611: response to wounding	ANXA5, APCS, APOA1, APOD, ATP7A, CAV1, CD9, CD44, COL3A1, CCN2, DCN, HBEGF, F11, F GF1, INHBB, LCP1, MMP12, MPL, SERPINE2, PLAU, PTPN6, SDC1, SPP1, TIMP1, VEGFA, VWF, WAS, EHD3, DUOX2, JMJD1C, AKR1B1, AQP1, CDO1, SLC29A1, STMN1, NME1, HEG1
	ko05205: Proteoglycans in cancer	CAV1, CBLB, CD44, DCN, HBEGF, ELK1, HGF, LUM, MMP2, PLAU, PTPN6, SDC1, VEGFA, WNT2 , FZD4, PDCD4, CCN2, EPB41, MEFC2, MYH11, SMARCA2, RCAN2, SDF2L1, BMP10, DLL4, LR RC59, FMNL3, CSF1R, EIF4A2, HLA-DQB1, HLA-DRA, IRF5, KPNA2, LCP1, LGALS9, MT2A, TNFRSF11B, RORC, SNRPA1, TIMP1, GSTO1, PSMD14

Supplementary Table S4 (continued)

Tissue	Pathways	Genes
Control liver (effect PVE)	M5885: NABA MATRISOME ASSOCIATED	ANXA5, CTSE, CTSK, HBEGF, FGF1, GDF9, HGF, INHBB, LGALS3, LGALS9, MMP2, MMP12, SERPINE2, PLAU, S100A6, CCL21, SDC1, SERPINA7, TIMP1, VEGFA, WNT2, CHRD, NFSF10, BMP10, ANGPTL3, HHIP, PDGFD, LOXL4, EGLN3, CCBE1, CLEC9A
Non-embolized liver lobes (effect OCA)	GO:0008202: steroid metabolic process	CYP7A1, CYP26A1, HMGCS1, HSD3B1, APOL1, NR1D1, PPARGC1A, CYP4V2, CES2
	R-HSA-211859: Biological oxidations	NAT1, CYP7A1, CYP26A1, CES2, AKR7L, CYP4V2
	GO:0010893: positive regulation of steroid biosynthetic process	CYP7A1, NR1D1, PPARGC1A, HMGCS1, HSD3B1, CYP26A1, GATM, CES2, CYP4V2, ARRDC3, PIM1, IFIT1B, ANKRD26, APOD, GNM1, ETNPPL, SLC51B
	GO:0005977: glycogen metabolic process	NR1D1, RB1CC1, GNM1, PPARGC1A, APOD
	GO:0033273: response to vitamin	CYP26A1, HMGCS1, PIM1, HSD3B1, PPARGC1A, NR1D1
	GO:0034440: lipid oxidation	APOD, PPARGC1A, CYP4V2
	GO:0070201: regulation of establishment of protein localization	APOD, NR1D1, CEP290, CEP295, SLC51B, CYP7A1, APOL1
Embolized liver lobe (effect OCA)	GO:0008202: steroid metabolic process	CYP7A1, CYP51A1, HMGCS1, HSD3B1, IDI1, SC5D, SQLE, SOAT2, APOL1, DHRS9, PPARGC1A, LPL, GPCPD1
	GO:0010876: lipid localization	CYP7A1, LPL, SLC1A2, SQLE, SOAT2, APOL1, EEPD1, SLC51B, SLC16A11, CA14
	GO:0071404: cellular response to low-density lipoprotein particle stimulus	CD9, HMGCS1, LPL, IDI1, DHRS9, GPCPD1, INHBB, PPARGC1A

Supplementary Table S4 (continued)

Tissue	Pathways	Genes
Embolized liver lobe (effect OCA)	GO:0008209: androgen metabolic process	HSD3B1,DHRS9,PPARGC1A,INHBB
	WP430: Statin Pathway	CYP7A1,LPL,SQLE,SOAT2,HMGCS1,PPARGC1A,CES2,GPCPD1,DHRS9,HSD3B1
	GO:0007162: negative regulation of cell adhesion	CD9,IHH,LGALS3,MMP12,GPNMB,MPL,PPARGC1A
	R-HSA-211859: Biological oxidations	NAT1,CYP7A1,CYP51A1,CES2,AOX1
	GO:0032963: collagen metabolic process	CYP7A1,IHH,MMP12
	M5885: NABA MATRISOME ASSOCIATED	ANXA4,IHH,INHBB,LGALS3,MMP12,CPAMD8,LPL,GPNMB
	R-HSA-196854: Metabolism of vitamins and cofactors	AOX1,LPL,TCN1
	GO:0006612: protein targeting to membrane	RPL17,RPL34,SLC51B

Supplementary Figure S1. Portal vein embolization of liver in rabbit. Caudal liver lobe (CL) was non-embolized and cranial liver lobes (RL, LM, and LL) were embolized.

Supplementary Figure S2. Change of serum individual bile acid species during the course of experiment. Longitudinal changes were evaluated by mixed effect model (n=11 per group). Differences between OCA-treated animals and control animals were analyzed with the Mann-Whitney U test. Data is depicted as mean $\pm$ SEM. P values are depicted. Asterisks indicate significance levels: **P<0.01, ***P<0.001. HDCA, deoxycholic acid; CA, cholic acid; UDCA, ursodeoxycholic acid; GDCA, glycodeoxycholic acid; GCA, glycocholic acid; GLCA, glycolithocholic acid; OCA, obeticholic acid.

Supplementary Figure S3. OCA does not affect liver synthetic function. Plasma coagulation factor II levels were not different between OCA-treated animals and control animals during the experiment (n=11 per group). Longitudinal changes were evaluated by mixed effect model. Data is depicted as mean $\pm$ SEM. P values are depicted.

Supplementary Figure S4. OCA does not induce mRNA expression of *Mki67* and *Pcna*. Differences between OCA-treated animals and control animals were analyzed with the Mann-Whitney U test (n=6 per group).

Supplementary Figure S5. Heatmaps of Spearman correlation analysis between CLV increase, the number of Ki67 positive hepatocytes and genes present in changed pathways in control liver. Asterisks indicate significance levels: *P<0.05, **P<0.01, *P<0.001. N.S., not significant. CLV, caudal liver volume (i.e. non-embolized liver volume).**

Chapter 5

No influence of cholestasis on portal vein
embolization-induced hypertrophy of the future
liver remnant

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Submitted

Abstract

Background: In the pre-clinical setting, hepatocellular bile salt accumulation impairs liver regeneration following partial hepatectomy. Here, we study the impact of cholestasis on portal vein embolization (PVE)-induced hypertrophy of the future liver remnant (FLR).

Methods: Patients were enrolled with perihilar cholangiocarcinoma (pCCA) or colorectal liver metastases (CRLM) undergoing PVE before a (extended) right hemihepatectomy. Volume of segments II/III was considered as FLR and assessed on pre- and post-embolization CT scans. The degree of hypertrophy (DH, percentual increase), and kinetic growth rate (KGR, percentage/week) were used to assess PVE-induced hypertrophy.

Results: A total of 50 patients (31 CRLM, 19 pCCA) were included. The DH and KGR were similar in patients with pCCA and CRLM, and in pCCA patients with or without hyperbilirubinemia. For patients with pCCA, unilateral drainage in FLR induced a higher DH than bilateral drainage (6.7 [4.9-7.9] versus 2.7 [1.5-4.2] %, $p=0.012$). C-reactive protein before PVE was negatively correlated with DH and KGR in patients with pCCA.

Conclusion: There was no influence of cholestasis on FLR hypertrophy in patients undergoing PVE. Unilateral drainage of the FLR resulted in higher liver hypertrophy than bilateral drainage. Elevated inflammation response was associated with impaired liver growth in patients with pCCA.

Introduction

Most major hepatectomies are performed to achieve a complete margin-free resection with a curative intention, in patients with hepatobiliary tumors. However, in patients undergoing resection, a small future liver remnant (FLR) volume is associated with a higher incidence of post-hepatectomy liver failure and death ^{1,2}. In order to achieve a safe liver resection, the FLR volume must be >30% in uncompromised livers, >40% in chemotherapy-damaged or steatotic livers, and >50% in cirrhotic livers ^{3,4}. In practice, liver resections are commonly performed in patients with underlying liver disease, and an insufficient FLR volume may preclude surgery ⁵.

The current standard procedure to induce hypertrophy of the non-embolized liver segments is portal vein embolization (PVE), where the aim is to increase FLR volume prior to resection ⁶. PVE has been associated with an increase in FLR volume of 20–50% within 3-7 weeks ^{7,8}. Studies have shown that PVE-induced hypertrophy of the FLR has a beneficial effect on reducing the rates of liver failure and death after major liver resection ^{2,9}. PVE induces insufficient hypertrophy of the FLR (i.e. failure to proceed to hepatectomy) in approximately 30% patients with colorectal liver metastasis (CRLM) ¹⁰, which might be linked to the formation of collateral blood vessels in embolized segments. For this reason, new techniques are being investigated to induce hypertrophy of the FLR. A novel procedure, i.e. combined Portal and Hepatic Vein Embolization (PVE/HVE), aims at preventing collateral formation and is currently being evaluated by the international DRAGON consortium ¹¹.

Besides more effective hypertrophy-inducing techniques, investigations are also being conducted to identifying those factors that negatively or positively affect cell growth in the non-embolized liver lobe ^{12,13}. In that respect, cholestasis is frequently present in patients with hepatobiliary tumors, especially in patients with perihilar cholangiocarcinoma (pCCA). Previous studies have demonstrated that during cholestasis excessive hepatic bile salt levels occur, which impair the proliferative capacity of hepatocytes ^{14,15}. By contrast, stimulation of bile salt signaling by agonistic activation of the bile salt receptor Farnesoid X Receptor (FXR) was found to promote liver growth in a rabbit PVE model ¹³. A wealth of animal studies have stressed the importance of maintained bile salt homeostasis in the proper progression of liver growth following partial hepatectomy ¹⁶⁻¹⁸.

A few studies have examined the impact of cholestasis on FLR hypertrophy in patients undergoing PVE, but their findings have been inconclusive ^{8, 19}. One recent study, for example, reported similar hypertrophy of the FLR in patients with a primary biliary malignancy and normal or elevated (i.e. $\geq 50 \mu\text{mol/L}$) serum bilirubin ¹⁹. The aim of the present study was to test the hypothesis that cholestasis impairs FLR hypertrophy in patients undergoing PVE. To this end, PVE-induced FLR hypertrophy was analyzed based on serum bilirubin levels. In addition, we investigated the associations between routine serum biochemical tests and/or biliary drainage, and FLR hypertrophy.

Methods

Study design

This study is a bi-center retrospective cohort study. Adult patients (≥ 18 years) with pCCA or CRLM were considered who had undergone PVE prior to (extended) right hepatectomy between January 01, 2016 and December 31, 2019 in the Maastricht University Medical Center (the Netherlands) or in the Uniklinik Aachen (Germany). Patients who had missing liver computed tomography (CT) scans, partial resection of the FLR between PVE and liver volume assessment, or only inadvertent drainage of the tumor bearing segments, were excluded. This study was approved by the institutional review boards of both centers (METC2019-1375 and EK 434/19).

Assessment of liver volume

Since all included patients were planned for (extended) right hepatectomy, liver segments II and III were consistently part of the FLR, and the volume of these segments was taken as the measure of FLR. Total liver volumes (TLV) and FLR volumes were determined from routine CT scans both prior to and after PVE. If multiple PVE procedures had been attempted to induce sufficient FLR volume gain, the liver volumetry after the last PVE was used. The diameters of the left and right hepatic bile duct were measured on pre-embolization scans, with bile duct dilation indicative for obstructive cholestasis. Additionally, the degree of bile duct dilation was assessed visually as: no, mild, moderate, or severe. CT scans were locally de-identified and volumetry was performed using Syngo.via Liver Analysis, Siemens Healthineers. All volumetries were performed by an investigator (XC), checked by another investigator (RK), and both were proctored by a local interventional radiologist (CL).

Definitions and data collection

FLR increase was defined as the difference in FLR volume before (FLR1) and after PVE (FLR2). FLR share (FLR%) was calculated as the FLR volume divided by TLV. %Hypertrophy was defined as the difference in FLR volume before and after PVE (FLR2-FLR1) divided by the FLR volume before PVE (FLR1). The degree of hypertrophy (DH) was defined as the difference in FLR share before (FLR1%) and after PVE (FLR2%) ($DH = FLR2\% - FLR1\%$). Kinetic growth rate (KGR) was calculated as DH divided by the time elapsed in weeks between PVE and the CT volumetry after PVE.

The following main clinical and laboratory variables were retrieved from the patient files: tumor type, histological status of liver parenchyma, occurrence of drainage prior to PVE, and presence of cholangitis prior to PVE. Routine serum biochemistry parameters (bilirubin, albumin, international normalized ratio [INR], gamma-glutamyl transferase [GGT], alkaline phosphatase [ALP], alanine aminotransferase [ALT], aspartate aminotransferase [AST], C-reactive protein [CRP] and white blood cell count) were gathered prior to PVE and -if applicable- prior to biliary drainage. Bilirubin level $\geq 50.0 \mu\text{mol/L}$ was used as a clinical marker of cholestasis. Confirmation bias was prevented by having a separate researcher (BO) collect the data from the Electronic Health Records. All data were gathered in CASTOR EDC, Amsterdam, a Good Clinical Practice-accepted online data capturing and monitoring system.

Statistical analyses

All statistical analyses were performed using SPSS 24.0 (IBM, Armonk, New York, USA). Data are presented as median [interquartile range] or frequency (percentage) when appropriate. For continuous data, differences between two groups were compared using the Mann-Whitney U test. Serum biochemistry parameters, assessed sequentially in patients with pCCA before drainage and before PVE, were compared using a Wilcoxon signed rank test. Categorical data was compared using chi-square test or Fisher's exact test as appropriate. Correlations were evaluated by Spearman rank correlation coefficients (ρ). Statistical significance was considered at $p < 0.05$.

Results

Patient characteristics

A total of 50 patients (CRLM, n=31; pCCA, n=19) were enrolled and their characteristics are shown in **Table 1**. Patients diagnosed with CRLM had a higher median Charlson comorbidity index than those with pCCA, but were otherwise similar. All patients underwent embolization of the right portal branch, with the intention to undergo (extended) right hemihepatectomy. Four patients received an extended segment IV embolization (**Table 1**). The technical success of the PVE procedure (successful obstruction of the right portal branch) was achieved in all patients. Two patients with CRLM had more than one PVE procedure. Twenty-nine out of thirty-one patients with CRLM received chemotherapy before PVE, whereas none of the patients with pCCA received chemotherapy.

None of the patients with CRLM were drained, whereas seventeen out of nineteen patients with pCCA underwent biliary drainage prior to PVE. Of those seventeen patients, eleven received unilateral drainage of the FLR, with the remaining six receiving simultaneous drainage of the right hepatic lobe.

pCCA patients remain cholestatic at the time of PVE

To assess the efficacy of biliary drainage in patients with pCCA, serum biochemistry parameters prior to and after biliary drainage were compared (**Supplementary Table 1**). Thirteen out of seventeen patients had hyperbilirubinemia before drainage, with bilirubin data missing for one patient. Serum bilirubin levels were reduced 2.6-fold after drainage, but remained above the normal range in 10 patients. After drainage, ALT levels were decreased, and there was a trend towards a reduction of AST levels ($p=0.026$ and $p=0.087$, respectively). Prior to the PVE procedure, patients with pCCA had higher serum levels of bilirubin, GGT, and ALP than patients with CRLM (**Supplementary Table 1**), reflecting cholestatic liver injury and its incomplete resolution by drainage.

Liver growth after PVE

The median time from the initial PVE procedure to volumetric assessment post-PVE was 22 [19-29] days and 20 [16-22] days in patients with CRLM and pCCA, respectively ($p=0.141$, **Table 2**). The absolute FLR volume increased from 263 [208-327] to 363 [322-439] mL after PVE in patients with CRLM, and from 326 [220-437] to 394 [363-542] mL in patients with pCCA (**Supplementary Fig. 1A**). Accordingly, the FLR% increased significantly after PVE in both groups (**Supplementary Fig. 1B**).

Table 1. Baseline characteristics of participants.

Characteristics	CRLM (n=31)	pCCA (n=19)	p value
Age (years)	65 [56-73]	70 [67-76]	0.070
Gender			0.276
Female	7 (23%)	7 (37%)	
Male	24 (77%)	12 (63%)	
BMI (kg/m ²)	25.2 [22.8-28.4]	23.4 [22.3-26.3]	0.448
Diabetes	6 (19%)	4 (21%)	1.000
Charlson comorbidity index	8 [7-9]	6 [5-8]	< 0.001
Nontumoral liver parenchyma*			0.946
Normal	18 (62%)	11 (65%)	
Fibrosis	5 (17%)	2 (12%)	
Cirrhosis	1 (4%)	1 (6%)	
Steatosis	5 (17%)	3 (17%)	
Cholangitis	0 (0%)	3 (16%)	
Bismuth-Corlette classification			
I	-	1 (5%)	
II	-	2 (11%)	
IIIA	-	5 (26%)	
IIIB	-	1 (5%)	
IV	-	10 (53%)	
Chemotherapy			< 0.001
No	2 (6%)	31(100%)	
FOLFOX/XELOX	23 (74%)		
FOLFIRI/FOLFOXIRI	5 (16%)		
Other	1 (4%)		
Type of biliary drainage			< 0.001
No	31 (100%)	2 (11%)	
PTBD	-	2 (11%)	
EBD	-	9 (47%)	
Both	-	6 (31%)	
Side of biliary drainage			
Unilateral	-	11 (65%)	
Bilateral	-	6 (35%)	
Hepatic bile duct diameter (mm)			
Left	<3.0	6.3 [5.3-10.0]	< 0.001
Right	<3.0	7.3 [5.4-10.7]	< 0.001
Hepatic bile duct dilation (left or right)			< 0.001
No	31 (100%)	0 (0%)	
Mild+Moderate	-	8 (42%)	
Severe	-	11 (58%)	
Type of PVE			0.629
Right PVE	29 (94%)	17 (90%)	
Right PVE extended to segment IV	2 (6%)	2 (10%)	

Data are presented as median [interquartile range] or frequency (percentage). * Histological information is not available for two patients in each group. CRLM, colorectal liver metastasis; pCCA, perihilar cholangiocarcinoma; BMI, body mass index; PTBD, percutaneous transhepatic biliary drainage; EBD, endoscopic biliary drainage; FLR, future liver remnant; PVE, portal vein embolization.

The absolute FLR volume increase and %hypertrophy were not different in CRLM and pCCA groups ($p=0.522$ and $p=0.897$, respectively) (**Table 2**). In addition, patients with CRLM and pCCA had similar DH (5.2 [3.3-6.9] versus 5.7 [3.2-7.4] %, respectively; $p = 0.960$) and KGR (1.4 [0.9-2.5] versus 1.9 [1.0-2.4] %/week, respectively; $p = 0.742$) after PVE (**Table 2**).

As patients with CRLM had a higher Charlson comorbidity index, a sensitivity analysis was performed, which was done by 1:1 matching based on age, gender, BMI, Charlson comorbidity index, cirrhosis, and presence of diabetes. The characteristics of the matched patients ($n=10$ in each group) are presented in **Supplementary Table 2**. Both DH and KGR were still comparable between patients with CRLM and pCCA (DH: 5.9 [3.8-7.8] versus 5.8 [1.8-7.1] %, respectively; $p = 0.597$; KGR: 1.6 [0.9-2.8] versus 1.6 [0.5-2.4] %/week, respectively; $p = 0.597$).

Table 2. Assessment of FLR hypertrophy after PVE between patients with CRLM and pCCA.

Variable	CRLM (n=31)	pCCA (n=19)	p value
Number of days after PVE	22 [19-29]	20 [16-22]	0.141
FLR increase (mL)	88 [53-139]	120 [49-165]	0.522
FLR increase per day (mL/day)	3.5 [1.9-6.9]	4.5 [2.4-8.8]	0.299
%Hypertrophy	30 [19-59]	38 [14-65]	0.897
%Hypertrophy per week	9.2 [6.0-18.3]	10.6 [5.2-19.8]	0.667
DH (%)	5.2 [3.3-6.9]	5.7 [3.2-7.4]	0.960
KGR (% per week)	1.4 [0.9-2.5]	1.9 [1.0-2.4]	0.742

Data are presented as median [interquartile range]. CRLM, colorectal liver metastasis; pCCA, perihilar cholangiocarcinoma; PVE, portal vein embolization; FLR, future liver remnant; DH, Degree of hypertrophy; KGR, kinetic growth rate.

Cholestasis does not affect PVE-induced liver growth in pCCA

After biliary drainage, ten patients with pCCA still had hyperbilirubinemia. These patients had comparable DH and KGR to patients with bilirubin levels lower than 50 $\mu\text{mol/L}$ (DH: 5.6 [3.0-7.5] versus 5.7 [2.4-7.0] %, respectively, $p=0.806$; KGR: 1.7 [1.0-2.4] versus 1.9 [0.8-2.4] %/week, respectively, $p=1.000$) (**Table 3**). Spearman correlation analyses also showed that serum bilirubin levels before drainage (pCCA group: $p=-0.129$, $p=0.633$) or before PVE (entire cohort: $p=-0.023$, $p=0.879$) were not correlated with the DH (**Fig. 1A-B, Supplementary Table 4**). Likewise, serum bilirubin levels before drainage (pCCA group: $p=+0.097$, $p=0.72$) or before PVE (entire cohort: $p=+0.148$, $p=0.321$) were unrelated to KGR.

In the pCCA group, there were no differences in DH and KGR between patients with mild-moderate bile duct dilation (n=8) and those with severe bile duct dilation (n=11) (DH: 6.0 [4.5-8.5] versus 4.5 [2.4-7.0] %, respectively, $p=0.283$; KGR: 2.0 [1.1-2.6] versus 1.6 [1.0-2.3] %/week, respectively, $p=0.509$). Additionally, the diameter of bile ducts was not correlated with the DH and KGR ($p=-0.396$, $p=0.084$; $p=-0.269$, $p=0.252$, respectively). In the CRLM group, the DH and KGR were similar in patients with cirrhosis or liver fibrosis (n=6) and those with normal background liver (n=18) (6.4 [3.0-9.3] versus 4.6 [3.0-6.4] %, respectively, $p=0.205$; KGR: 1.8 [0.9-2.6] versus 1.2 [0.8-2.1] %/week, respectively, $p=0.351$).

Table 3. Assessment of FLR hypertrophy after PVE in pCCA patients with and without hyperbilirubinemia.

Variable	Hyperbilirubinemia group (n=10)	Without hyperbilirubinemia group (n=9)	p value
Age (years)	70 [67-72]	70 [64-76]	0.563
Gender			0.650
Female	3 (30%)	4 (44%)	
Male	7 (70%)	5 (56%)	
BMI (kg/m ²)	22.8 [22.0-26.1]	25.6 [23.2-27.0]	0.102
Charlson comorbidity index	6 [5-8]	7 [4-8]	0.934
Cholangitis	2 (20%)	1 (11%)	1.000
Serum biochemistry before PVE			
Bilirubin (μmol/L)	63.9 [56.4-95.5]	25.8 [10.0-39.4]	< 0.001
GGT (U/L)	535 [333-1029]	339 [106-753]	0.191
ALP (U/L)	369 [271-725]	255 [128-420]	0.083
CRP (mg/L)	42.1 [16.4-94.7]	20.6 [7.0-89.0]	0.637
Number of days after PVE	22 [16-26]	20 [17-22]	0.652
FLR increase (mL)	131 [68-189]	91 [28-144]	0.253
FLR increase per day (mL/day)	6.2 [2.2-11.0]	4.1 [1.5-7.2]	0.288
%Hypertrophy	56 [15-67]	30 [8-51]	0.191
%Hypertrophy per week	16.4 [6.0-27.0]	10.4 [2.8-16.1]	0.221
DH (%)	5.6 [3.0-7.5]	5.7 [2.4-7.0]	0.806
KGR (% per week)	1.7 [1.0-2.4]	1.9 [0.8-2.4]	1.000

Data are presented as median [interquartile range]. pCCA, perihilar cholangiocarcinoma; PVE, portal vein embolization; FLR, future liver remnant; GGT, gamma-glutamyl transferase; ALP, alkaline phosphatase; CRP, C-reactive protein; DH, Degree of hypertrophy; KGR, kinetic growth rate.

The FLR hypertrophy response was analyzed according to the side (unilateral, bilateral) of biliary drainage prior to PVE in seventeen patients with pCCA. Patients with unilateral drainage (n=11) had a higher DH than those with bilateral drainage (n=6) (6.7 [4.9-7.9] versus 2.7 [1.5-4.2] %, $p = 0.012$) (**Fig. 2**). The characteristics of pCCA

patients with unilateral and bilateral biliary drainage were similar (**Supplementary Table 3**).

Figure 1. Serum CRP negatively correlates with hypertrophy of FLR in patients with pCCA. Correlations between degree of hypertrophy of FLR and serum bilirubin levels before drainage (**A**) and before PVE (**B**). Correlations between serum CRP levels and degree of hypertrophy and kinetic growth rate of FLR in patients with pCCA (**C** and **D**, respectively) and CRLM (**E** and **F**, respectively). Correlations were assessed using Spearman’s rank test. CRLM, colorectal liver metastasis; pCCA, perihilar cholangiocarcinoma; CRP, C-reactive protein.

Figure 2. Degree of hypertrophy of the future liver remnant is higher in patients with unilateral biliary drainage. Seventeen out of nineteen patients with perihilar cholangiocarcinoma received biliary drainage for relief of cholestasis prior to portal vein embolization. The future liver remnant (FLR) was

drained in patients receiving unilateral drainage (n=11). Mann-Whitney U test was used to compared degree of hypertrophy of FLR between two groups. Asterisks indicate significance level: *p<0.05.

Correlations between routine blood tests and FLR hypertrophy

A Spearman correlation analysis was performed to evaluate associations between serum biochemistry and FLR hypertrophy parameters. Serum CRP levels before PVE were negatively correlated with the DH ($\rho=-0.401$, $p=0.009$), and showed a strong tendency to be negatively correlated with KGR ($\rho=-0.300$, $p=0.054$). Subgroup analysis revealed that the inverse relationships between CRP and FLR hypertrophy were apparent in patients with pCCA (DH: $\rho=-0.539$, $p=0.038$; KGR: $\rho=-0.532$, $p=0.041$, respectively) (**Fig. 1C-D**), but not in patients with CRLM (DH: $\rho=-0.339$, $p=0.083$; KGR: $\rho=-0.236$, $p=0.237$, respectively) (**Fig. 1E-F**). Additionally, serum albumin levels before PVE were positively correlated with the DH ($\rho=+0.301$, $p=0.045$). All correlations between serum biochemistry parameters and FLR hypertrophy are summarized in **Supplementary Table 4**.

Discussion

This study has evaluated whether cholestasis affects PVE-induced hypertrophy of the future liver remnant. The main findings are that the degree of hypertrophy and kinetic growth rate of the FLR are similar in patients with CRLM and pCCA, and in pCCA patients with or without hyperbilirubinemia. Neither serum bilirubin levels before drainage, nor before PVE, were correlated with indices of FLR hypertrophy. Patients with pCCA receiving unilateral biliary drainage of the FLR had a higher DH than those with bilateral drainage. Elevated inflammatory response was associated with impaired liver growth in patients with pCCA.

In previous studies, cholestasis has been associated with poor regenerative capacity of the liver after partial hepatectomy, with toxicity due to bile salt overload in hepatocytes serving as an underlying mechanism²⁰. Patients with pCCA, characterized by cholestasis in one or both liver halves due to biliary obstruction, have been shown to have a high 90-day mortality rate of up to 14% after partial liver resection, despite routine biliary decompression prior to surgery²¹. In the present study, we observed similar FLR hypertrophy after PVE, in patients with CRLM and pCCA,

which is in line with recent findings elsewhere ²². The majority (89%) of pCCA patients here received drainage prior to PVE. Nine out of nineteen patients with pCCA had serum bilirubin levels lower than 50 $\mu\text{mol/L}$ at the time of PVE. These nine patients had comparable DH and KGR with pCCA patients who had bilirubin levels higher than 50 $\mu\text{mol/L}$. Moreover, serum bilirubin either before drainage or before PVE were not correlated with the DH and KGR. The similar FLR hypertrophy in cholestatic and non-cholestatic patients suggests that the growth-stimulating effect of PVE outweighs any potential negative effects of bile salt accumulation.

Biliary drainage is widely used to decompress the biliary tree and improve cholestatic status before liver resection ^{23, 24}. In our study, seventeen of nineteen patients with pCCA underwent biliary drainage before PVE. Remarkably, the DH was higher in those patients with unilateral drainage of the FLR than in those with bilateral drainage. Our findings are thus consistent with the study of Ishizawa *et al.*, who observed a higher hypertrophy ratio and bilirubin clearance rate of the FLR after unilateral drainage of the FLR group than the bilateral drainage group ²⁵. In our cohort, patients with bilateral drainage had insufficient improvement of cholestasis upon initial biliary drainage. Of note, the occurrence of cholangitis appeared to be higher in the bilateral drainage group (2 out of 6 = 33% versus 1 out of 11 = 9%), although low event rates do not allow statistical back-up. It is nonetheless tempting to speculate that cholangitis and associated inflammation contributed to impaired liver hypertrophy in bilaterally drained patients. It should be noted that serum CRP, which was found to negatively correlate with DH, was not affected by the side of drainage.

Our study has demonstrated a negative relation between serum CRP levels before PVE and the DH and KGR, especially in patients with pCCA. To the best of our knowledge, this is the first study to reveal the association of an inflammation marker and FLR growth in clinical patients. Notably, serum CRP levels were 1.7 fold higher after biliary drainage in patients with pCCA, albeit this did not reach statistical significance ($p=0.167$). The tentative CRP elevation might have been due to the retrograde route through the proximal small intestine in the case of endoscopic biliary drainage (EBD). In support of this notion, 15 out of 17 (88%) drained patients received an EBD or EBD+PTBD (percutaneous transhepatic biliary drainage) procedure.

The question of which molecular pathway could underlie the observed relationship between CRP and FRL hypertrophy remains unclear. However, it is known that

cytokines (e.g. interleukin-8) induced by inflammation can stimulate hepatic infiltration of neutrophils ²⁶. As part of innate immune defenses, neutrophils produce reactive oxygen species and hypochlorous acid that cause cytotoxicity ²⁷. Experimental and human studies have shown that periportal neutrophil infiltration is positively correlated with the degree of liver damage ^{27, 28}, which is recognised as having a negative impact on liver hypertrophy. Serum CRP alterations may have promise as a surrogate marker of PVE-induced FLR hypertrophy in patients with pCCA.

Certain limitations have to be acknowledged. For example, the study's retrospective design makes it impossible to avoid selection bias when including patients. In addition, the sample size of the present cohort is rather small, which means some events appear rare, such as cholangitis, PTBD procedure, and non-drainage of the liver in pCCA. As such, it is impossible to conduct a logistic regression to assess the predictive value of these factors on FLR hypertrophy. Furthermore, nine patients had small metastases in segment II or III, which might have impacted PVE-induced hypertrophy. Nevertheless, the volume of the small metastases was excluded when analyzing FLR volumes.

Besides bilirubin, bile salts are another indicator reflecting cholestasis, although not one routinely assayed in clinical chemistry. Due to the unavailability of serum samples, bile salt levels could not be assessed. Bile salts are involved in liver regeneration after partial hepatectomy via activation of the nuclear bile salt receptor FXR ¹⁶. FXR plays a central role in maintaining bile salt homeostasis, and regulating hepatocyte cell cycle progression ¹⁷. Moreover, we have previously demonstrated that FXR agonist obeticholic acid accelerates liver growth following PVE in rabbits ¹³. Bile salt levels at 5 h after PVE predict FLR volume after 3 weeks in patients scheduled for resection ¹². It would therefore be worthwhile including serum bile salt measurements in larger prospective studies to address the influence of cholestasis on PVE-induced hypertrophy in pCCA patients.

In conclusion, cholestasis was found to have no influence on hypertrophy of the FLR in patients undergoing PVE. And, unilateral biliary drainage of FLR appears to induce more FLR hypertrophy than bilateral drainage. Systematic CRP changes may thus be promising to predict FLR hypertrophy in patients with pCCA.

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Chapter 5

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Supplementary Table 1. Serum biochemistry tests in patients with pCCA and CRLM.

Parameters	pCCA (n=19)		p value ^a	CRLM (n=31)	p value ^b
	Before drainage	Before PVE		Before PVE	
Bilirubin (μmol/L)	137.8 [57.0-191.9]	53.8 [25.8-64.5]	0.001	6.9 [5.2-8.9]	<0.001
Albumin (g/L)	36.3 [33.3-40.8]	35.0 [27.7-37.3]	0.188	36.0 [30.3-42.0]	0.246
INR	1.02 [0.98-1.12]	1.02 [1.00-1.08]	0.364	1.01 [0.97-1.10]	0.732
GGT (U/L)	735 [297-1048]	434 [225-878]	0.300	77 [36-185]	<0.001
ALP (U/L)	438 [249-571]	318 [208-463]	0.173	121 [86-155]	<0.001
ALT (U/L)	133 [76-310]	103 [58-127]	0.026	50 [27-150]	0.235
AST (U/L)	124 [78-169]	74 [42-103]	0.087	39 [26-94]	0.053
CRP (mg/L)	22.7 [11.2-73.8]	38.5 [8.2-88.6]	0.131	38.0 [10.1-111.0]	0.684
WBC count (10 ⁹ /L)	8.7 [5.3-11.1]	8.2 [6.0-10.0]	0.155	6.5 [6.2-8.9]	0.271

Data are presented as median [interquartile range].

^a p values were calculated for comparison between “before drainage” and “before PVE” values, within patients with pCCA.

^b p values were calculated for comparison of “before PVE” values between pCCA and CRLM groups.

pCCA, perihilar cholangiocarcinoma; CRLM, colorectal liver metastasis; INR, international normalized ratio; GGT, gamma-glutamyl transferase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; WBC, white blood cell.

Supplementary Table 2. Characteristics of 10 matched patients with CRLM and pCCA.

Characteristics	CRLM (n=10)	pCCA (n=10)	p value
Age (years)	61 [47-71]	70 [69-73]	0.079
Gender			1.000
Female	4 (40%)	5 (50%)	
Male	6 (60%)	5 (50%)	
BMI (kg/m ²)	24.6 [23.1-29.0]	25.7 [23.1-26.5]	0.705
Diabetes	2 (20%)	2 (20%)	1.000
Charlson comorbidity index	8 [6-9]	8 [7-9]	0.969
Cirrhosis	0 (0%)	0 (0%)	1.000

Data are presented as median [interquartile range] or frequency (percentage). CRLM, colorectal liver metastasis; pCCA, perihilar cholangiocarcinoma; BMI, body mass index.

Supplementary Table 3. Characteristics of 17 pCCA patients with biliary drainage.

Variable	Unilateral drainage (n=11)	Bilateral drainage (n=6)	p value
Age (years)	70 [67-72]	73 [69-77]	0.359
Gender			1.000
Female	5 (46%)	2 (33%)	
Male	6 (54%)	4 (67%)	
BMI (kg/m ²)	24.6 [22.6-27.0]	22.8 [22.1-27.8]	0.615
Diabetes	4 (36%)	0 (0%)	0.237
Charlson comorbidity index	6 [5-8]	7 [5-9]	0.721
Cholangitis	1 (9%)	2 (33%)	0.515
Bismuth-Corlette classification			0.266
I	1 (9%)	0 (0%)	
II	2 (18%)	0 (0%)	
IIIA	3 (27%)	1 (17%)	
IIIB	1 (9%)	0 (0%)	
IV	4 (37%)	5 (83%)	
Serum biochemistry tests before PVE			
Bilirubin (μmol/L)	53.8 [28.6-62.9]	60.8 [36.9-82.7]	0.421
Albumin (g/L)	35.5 [30.0-38.5]	30.5 [22.4-35.3]	0.158
INR	1.01 [0.95-1.12]	1.02 [1.01-1.09]	0.359
GGT (U/L)	719 [414-1083]	342 [281-612]	0.108
ALP (U/L)	379 [220-662]	291 [242-376]	0.396
ALT (U/L)	115 [60-152]	66 [46-98]	0.090
AST (U/L)	82 [42-205]	84 [46-103]	0.914
CRP (mg/L)	45.6 [8.2-112.5]	47.0 [18.2-94.7]	0.775
WBC count (10 ⁹ /L)	8.8 [6.2-12.5]	7.1 [4.9-9.3]	0.289
Number of days after PVE	22 [17-35]	17 [12-20]	0.086

Data are presented as median [interquartile range] or frequency (percentage). pCCA, perihilar cholangiocarcinoma; BMI, body mass index; PVE, portal vein embolization; INR, international normalized ratio; GGT, gamma-glutamyl transferase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; WBC, white blood cell.

Supplementary Table 4. Correlations between serum biochemistry parameters and FLR hypertrophy.

Parameters	DH		KGR	
	ρ	p value	ρ	p value
Entire cohort (n=50)				
Parameters before PVE				
Bilirubin ($\mu\text{mol/L}$)	-0.023	0.879	0.148	0.321
Albumin (g/L)	0.301	0.045	0.110	0.471
INR	-0.189	0.193	-0.120	0.412
GGT (U/L)	0.139	0.345	0.074	0.618
ALP (U/L)	-0.021	0.892	-0.007	0.963
ALT (U/L)	-0.018	0.911	-0.154	0.342
AST (U/L)	-0.120	0.442	-0.039	0.803
CRP (mg/L)	-0.401	0.009	-0.300	0.054
WBC count ($10^9/\text{L}$)	-0.072	0.640	-0.083	0.589
CRLM patients (n=31)				
Parameters before PVE				
Bilirubin ($\mu\text{mol/L}$)	-0.082	0.678	0.215	0.273
Albumin (g/L)	0.176	0.370	0.066	0.738
INR	0.013	0.945	0.034	0.855
GGT (U/L)	0.113	0.560	0.116	0.548
ALP (U/L)	-0.275	0.164	-0.130	0.519
ALT (U/L)	-0.312	0.129	-0.329	0.108
AST (U/L)	-0.304	0.140	-0.077	0.713
CRP (mg/L)	-0.339	0.083	-0.236	0.237
WBC count ($10^9/\text{L}$)	-0.151	0.443	-0.108	0.584
pCCA patients (n=19)				
Parameters before drainage				
Bilirubin ($\mu\text{mol/L}$)	-0.129	0.633	0.097	0.721
Albumin (g/L)	0.302	0.340	0.256	0.422
INR	-0.502	0.057	-0.203	0.469
GGT (U/L)	0.565	0.023	-0.032	0.905
ALP (U/L)	0.391	0.134	0.256	0.339
ALT (U/L)	0.289	0.296	-0.021	0.940
AST (U/L)	0.407	0.149	-0.086	0.771
CRP (mg/L)	-0.218	0.455	-0.345	0.227
WBC count ($10^9/\text{L}$)	-0.135	0.661	-0.030	0.922
Parameters before PVE				
Bilirubin ($\mu\text{mol/L}$)	-0.105	0.668	-0.025	0.920
Albumin (g/L)	0.503	0.039	0.415	0.098
INR	-0.513	0.030	-0.469	0.050
GGT (U/L)	0.481	0.037	-0.016	0.949
ALP (U/L)	0.353	0.165	0.172	0.510
ALT (U/L)	0.579	0.024	0.271	0.328
AST (U/L)	0.298	0.229	0.037	0.884
CRP (mg/L)	-0.539	0.038	-0.532	0.041
WBC count ($10^9/\text{L}$)	0.032	0.903	-0.225	0.384

Spearman correlation analyses were performed to evaluate associations between serum biochemistry parameters and FLR hypertrophy. DH, Degree of hypertrophy; KGR, kinetic growth rate; INR, international normalized ratio; GGT, gamma-glutamyl transferase; ALP, alkaline phosphatase; ALT, alanine

aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; WBC, white blood cell; CRLM, colorectal liver metastasis; pCCA, perihilar cholangiocarcinoma.

Supplementary Figure 1. Volumetric expansion of the future liver remnant by PVE in individual patients. The increase of absolute future liver remnant (FLR) volume (**A**) and FLR share (**B**) after portal vein embolization (PVE) in patients with colorectal liver metastasis (CRLM) and perihilar cholangiocarcinoma (pCCA). Wilcoxon signed rank test was used to compared FLR volume between before and after PVE in each group of patients. Asterisks indicate significance level: *** $p < 0.001$.

Part III

Restored bile salt-FGF19 signaling by chyme
reinfusion in intestinal failure

Chapter 6

Chyme reinfusion restores the regulatory bile salt-FGF19 axis in intestinal failure patients

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Abstract

Introduction: Automated chyme reinfusion (CR) in intestinal failure (IF) patients with a temporary double enterostomy (TDE) restores intestinal function and protects against liver injury, but the mechanisms are incompletely understood. The aim was to investigate whether beneficial effects of CR relate to functional recovery of enterohepatic signaling via the bile salt-FGF19 axis.

Methods: Blood samples were collected from 12 patients, 3 days before, at start, and 1, 3, 5 and 7 weeks after CR initiation. Plasma FGF19, total bile salts (TBS), C4 (marker of bile salt synthesis), citrulline (CIT), bile salt composition, liver tests and nutritional risk indices were determined. Paired small bowel biopsies prior to CR and after 21 days were taken and genes related to bile salt homeostasis and enterocyte function were assessed.

Results: CR induced an increase in plasma FGF19 and decreased C4 levels, indicating restored regulation of bile salt synthesis via endocrine FGF19 action. TBS remained unaltered during CR. Intestinal FXR was upregulated after 21 days of CR. Secondary and deconjugated bile salt fractions were increased after CR, reflecting restored microbial metabolism of host bile salts. Furthermore, CIT and albumin levels were gradually rising after CR, while abnormal serum liver tests normalized after CR, indicating restored intestinal function, improved nutritional status and amelioration of liver injury. CR increased gene transcripts related to enterocyte number, carbohydrate handling and bile salt homeostasis. Finally, the reciprocal FGF19/C4 response after 7 days, predicted the plasma CIT time course.

Conclusions: CR in IF-TDE patients restored bile salt-FGF19 signaling and improved gut-liver function. Beneficial effects of CR are partly mediated by recovery of the bile salt-FGF19 axis and subsequent homeostatic regulation of bile salt synthesis.

Introduction

A temporary double enterostomy (TDE) is most often created to avoid or protect high risk anastomoses in frail or sick patients. TDEs are often used in the acute setting of intestinal ischaemia resulting from e.g. a thrombo-embolic event or herniation. Unfortunately, some patients may develop intestinal failure (IF) due to (functional) short bowel syndrome, requiring parenteral nutrition (PN) and/or intravenous fluid supplementation (IVS) until recovery for surgical re-establishment of intestinal continuity, typically 3 to 6 months after primary surgery¹. Prolonged PN dependence is often necessary, but is associated with risk of increased morbidity and mortality^{2, 3}, mainly related to central venous tract complications, catheter-related infections, central venous thrombosis, or related to catheter replacement. The development of IF-associated liver disease (IFALD) frequently occurs¹. An alternative treatment is chyme (intestinal or fistula secretions) reinfusion (CR), through an extracorporeal enteral nutrition technique, into the distal part of the small bowel. This method results in improved nutritional status, better intestinal absorptive function, recovery from liver disease, reduced intestinal secretions and shorter period to PN independence in retrospective studies⁴⁻⁷. The mechanisms underlying the beneficial effects of chyme reinfusion are incompletely understood, but functional recovery of the enterohepatic circulation of bile salts and attendant signaling has been postulated⁴.

Fibroblast growth factor (FGF19) is a postprandial hormone secreted by ileocytes in a bile salt-stimulated fashion that involves activation of the bile salt-sensing transcription factor Farnesoid X Receptor (FXR)⁸. The key role of FGF19 is to control bile salt homeostasis by negatively regulating bile salt synthesis via repression of the bile salt-synthetic enzyme cholesterol-7 α -hydroxylase (*CYP7A1*) in the liver⁸. Disruption of the negative feedback loop results in unopposed bile salt synthesis. As a result of low circulating FGF19 levels, bile salt overproduction occurs and this has been associated with pediatric and adult IFALD, or primary bile acid diarrhea⁹⁻¹⁵. Currently, pharmacological treatment with FGF19 analogues in patients with hepatobiliary diseases including primary biliary cholangitis or non-alcoholic steatohepatitis has been evaluated clinically and renders promising therapeutic effects^{16, 17}.

In the RESCUE study presented here, we test the hypothesis that functional recovery of the enterohepatic circulation by CR and attendant restoration of the regulatory bile salt-FGF19 axis in IF patients with a TDE, is associated with CR-related beneficial

effects.

Methods

Study design and participants

The RESCUE study (restored enterohepatic signaling: chyme reinfusion therapy) is a prospective study with a pre- and post-intervention design conducted at Rennes University Hospital (CHU Rennes) and Clinique Saint Yves, Rennes, France (a tertiary referral center for intestinal rehabilitation of patients prior to restorative surgery) from January 2017 to July 2018. The study was registered at clinicaltrials.gov (NCT02990195). Eligible participants were adult IF patients aged 18 years or older with a temporary double enterostomy intended to receive CR as primary care. Further inclusion criteria were a minimum of 25 cm of healthy distal small intestine that was suitable for CR. We excluded patients with mental disability, pregnancy or lactation, hepatocellular carcinoma, auto-immune disorders, blood coagulation disorders, or shock of any cause. None of the patients had any known viral or genetic liver disease prior to the double enterostomy. We obtained written informed consent from all participants. This study was approved by the Institutional Review Board of Rennes University Hospital, Rennes, France (2016-A01156-45).

CR was performed at Clinique Saint Yves using the Enteromate II® system (Labodial, Les Clayes Sous Bois, France) as described previously¹⁸. In brief, proximal effluent was continuously pumped into a 30 mL collection container equipped with weight-monitoring. When collected volume exceeded 10 mL, the second pump started and reinfused the collected chyme into the distal part of the small bowel. In patients with inadequate oral intake, an additional enteroclysis was applied to deliver enteral nutrition to the reinfusion tube in the efferent small bowel. CR was initiated in approximately one week after referral of the patient to Clinique Saint Yves and continuously applied for two to three months. Afterwards, patients underwent surgical re-establishment of intestinal continuity. Blood samples were collected at three days before CR (day -3), at the day of CR initiation (day 0), and during the course of CR (after 1, 3, 5 and 7 weeks). Patients underwent endoscopic biopsy of the distal small intestine three days before and three weeks after CR at CHU Rennes by an experienced gastroenterologist. Study design is shown in **Supplementary Figure S1**.

Data collection and definitions

Data were collected and managed using a customized Access Database (Microsoft, Redmond, WA, USA). The following items were recorded: gender, age, height, body weight, body mass index (BMI), underlying diseases, afferent small intestinal length (from duodeno-jejunal flexure to the proximal stoma), efferent small intestinal length (from distal stoma to terminal part of the remnant efferent small intestine), resected length, efferent small intestinal anatomy, energy (kcal/kg) and protein (g/kg) intake (parenteral, oral, and enteral) adjusted by body weight, and duration of CR and PN. We measured proximal stoma or fecal output daily in all patients before and after CR initiation, respectively. Percent weight loss was assessed as follows: $\text{weight loss (\%)} = 100 * (\text{usual weight} - \text{present weight}) / \text{usual weight}$. None of the patients were diagnosed with ascites during CR treatment. Nutritional Risk Index (NRI) was determined as follows: $\text{NRI} = 1.515 * \text{serum albumin (g/L)} + 41.77 * (\text{present weight} / \text{usual weight})^{19}$. NRI-based risk groups were as follows: absence of malnutrition risk ($\text{NRI} > 97.5$), moderate malnutrition risk ($83.5 \leq \text{NRI} \leq 97.5$) and severe malnutrition risk ($\text{NRI} < 83.5$)¹⁹.

Blood samples and analyses

Blood samples were collected from patients in the morning and after an overnight fast in three 8 mL vacuum tubes (serum, EDTA and lithium heparin tubes). Serum tubes were left at room temperature for 30 min, and plasma tubes were put on ice immediately after collection. After 30 min, all blood samples were centrifuged at 4000 rpm for 10 min at 4°C. The supernatant was collected, divided into aliquots, and stored at -80°C until transport to Maastricht University (Maastricht, The Netherlands) for further analysis. Plasma levels of FGF19 and pro-inflammatory cytokines (interleukin-6, IL-6 and tumor necrosis factor- α , TNF- α) were assessed by sandwich ELISA as reported previously²⁰. Plasma citrulline (CIT, a marker of the mass of functional enterocytes) was determined by a high-performance liquid chromatography method^{6, 21}. Plasma 7- α -hydroxy-4-cholesten-3-one (C4, blood marker of bile salt synthesis)²², total bile salts (TBS), and bile salt composition (both in plasma and chyme) were assayed by liquid chromatography-mass spectrometry (Schaap and Olde Damink, manuscript in preparation). Serum liver tests (alanine aminotransferase, ALT; aspartate aminotransferase, AST; alkaline phosphatase, ALP; γ -glutamyl transferase, GGT and total bilirubin), albumin and C-reactive protein (CRP) were evaluated by routine assay

at the Department of Clinical Biochemistry at Maastricht University Medical Center (Maastricht, The Netherlands). For baseline comparisons of TBS, FGF19 and C4 levels, own published data on twelve healthy controls (mean age 55 years, 33% female) was used²³.

Gene expression analysis

Ileal biopsies were obtained through routine endoscopic procedures and immediately preserved in RNeasy Lysis Buffer (Thermo Scientific, Waltham, MA, USA) for 24 hours at 4°C and then stored at -80°C (Rennes University Hospital, Rennes, France). Biopsies were ultimately transported to Maastricht University Medical Center (Maastricht, The Netherlands) for transcript analyses. Hereto, total RNA was isolated using the RNeasy Plus Mini Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). Final RNA concentration and purity were determined by spectrophotometry. A total of 750 ng RNA was converted to cDNA using SensiFAST™ cDNA Synthesis Kit (Bioline, London, UK). For the quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), we used SensiFAST™ SYBR Hi-ROX kit (Bioline, London, UK) and cDNA as template on a LightCycler® 480 SW 1.5 system (Roche, Basel, Switzerland). qRT-PCR data was analyzed using LinRegPCR software²⁴, and gene expression levels were normalized to the geometric mean of 4 reference genes (*36B4*, *PSMB4*, *REEP5*, and *HPRT1*). Primer sequences provided in **Supplementary Table S1**. Paired ileal biopsies were available from seven of twelve patients. In remaining patients, (paired) biopsies could not be gathered for logistic reasons (n = 2), last-minute patient refusal (n = 1), or transcript data was excluded from analyses due to a non-enterocytic transcript signature (n = 2).

Statistical analysis

Initially, twenty patients were planned for enrollment in this study. A planned interim-analysis was performed after enrollment of two thirds of patients (n=13). One patient was not included in the study after enrollment because he received enteral nutrition as enteroclysis into the downstream small bowel, before starting the protocol. On the interim-analysis (n = 12), the primary endpoint (FGF19 level) had significantly changed ($P < .005$) justifying early termination of the study. For blood analyses, five out of seventy-two time points were missing (6.9%). Multiple data imputation was used to handle the missing data. The mean of day -3 and day 0 measurements were taken as baseline values. Data are expressed as mean $\pm$ standard deviation (SD), median

(interquartile range), or frequency (percentage) where appropriate. Graphical data are expressed as mean $\pm$ 95% Confidence Interval (CI). Differences between IF patients and healthy controls were evaluated by Mann-Whitney U test. Correlations were evaluated by the Pearson (r) or Spearman's (ρ) correlation coefficient dependent on data distribution. For continuous variables, changes during CR were evaluated by paired-samples *t* test or Wilcoxon matched-pairs signed ranks sum test dependent on the data distribution. Categorical variables were analyzed using Fisher's exact test. To evaluate longitudinal changes, ANOVA or Friedman's test for repeated measurements, corrected using Dunn's multiple comparisons test, were used. Linear mixed models were performed to identify whether the FGF19 or C4 response predicts the evolution of markers for intestinal absorptive function (CIT) and liver injury (ALP and GGT). The Akaike's Information Criterion was used to compare the best fit of a covariance structure for the linear mixed models. *P* value $<.05$ was considered statistically significant. SPSS statistics version 26.0 (IBM Corporation, Chicago, IL, USA) was used for all statistical analyses.

Results

Patient characteristics

Twelve patients (mean age 68 years, 42% females) were included in the study (**Table 1**). Despite the fact that features of IF -necessitating supplementation with PN- were apparent in all surgical patients, ten patients actually received PN and/or intravenous (IV) hydration at admission. Ten patients remained in the hospital during the 7 weeks course of CR, and two patients received home CR after specific training and education. Major underlying causes for small intestine resection and/or creation of a TDE were mechanical obstruction and intestinal ischemia. Patients underwent small intestinal resection ($n = 8$) or received a terminal colostomy on the transverse ($n = 3$) or right colon ($n = 1$). Small bowel resection was combined with a partial gastrectomy in a single patient. Additional individual patient data are summarized in **Supplementary Table S2**.

Table 1. Patient demographics and surgical characteristics at baseline.

Variable	Study cohort
Patients, n*	12
Age, yrs	67.7 ± 14.6
Male/female	7/5
Underlying etiology, n (%)	
Mechanical occlusion	3 (25)
Ischemia	4 (33)
Peritonitis	5 (42)
PN dependence, n (%)	10 (83)
Jejunal efflux (mL/24 hrs)	2245 [1366 to 2913]
Jejunal efflux (mL•weight⁻¹•24 hrs⁻¹)	40 [22 to 55]
Hospital admission time before referral (days)	28 [18 to 59]
Plasma citrulline ≤ 20 μmol/L, n (%)	7 (58)
SB resection ≥ 30 cm, n (%)	4 (50)
Resection SB length if resection (n = 6), cm	60 [15 to 113]
Upstream SB length (n = 9), cm	120 [88 to 210]
Upstream SB length < 100 cm, n (%)	2 [22]
Downstream SB length, cm	1670 [1300 to 222]
Theoretical total SB length** (n = 98), cm	310 [223 to 350]
Downstream SB anatomy, n (%)	
Ileo-colon-rectum	8 (67)
Ileo-colon-stoma	4 (33)
Serum liver tests (IU/L)	
ALP	216 [163 to 291]
GGT	176 [73 to 252]
ALT	66 [45 to 109]
AST	37 [29 to 57]
Total bilirubin, μmol/L	6.2 [3.7 to 9.3]
Serum CRP (mg/L)	6.3 [2.1 to 36.0]

* If data are missing, numbers in parentheses indicate the number of assessments for a particular factor. Data are depicted as percentages (%), mean ± SD or median [IQR]. Abbreviations: PN, parenteral nutrition; SB, small bowel; ALP, alkaline phosphatase; GGT, γ-glutamyl transferase; ALT, alanine aminotransferase; AST, aspartate aminotransferase. ** Theoretical total SB length = upstream + downstream SB length

Dysregulated bile salt-FGF19 axis in intestinal failure patients with a temporary double enterostomy

First, we investigated whether the bile salt-FGF19 axis was disturbed in IF-TDE patients prior to CR. Baseline levels of TBS, FGF19 and C4 of healthy controls and IF-patients are shown in **Figure 1A**. Note, demographic information on healthy controls

are provided in **Supplementary Table S3**. TBS levels in IF patients were not different compared to healthy controls (1.9 [1.4 to 3.0] vs 1.6 [0.9 to 2.4] $\mu\text{mol/L}$, $P = .288$). In line with earlier observations in patients with chronic IF¹⁴, baseline FGF19 levels were markedly lower compared to healthy controls (0.023 [0.012 to 0.061] vs 0.098 [0.071 to 0.136] ng/mL , $P < .001$) (**Figure 1A**). Conversely, high baseline C4 levels were observed in IF patients compared to controls (95 [38 to 169] vs 19 [10 to 31] ng/mL , $P = .002$), indicating dysregulated control of bile salt synthesis (**Figure 1A**). Indeed, FGF19 showed a strong negative correlation with C4 at baseline ($\rho = -0.86$, $P < .001$) (**Figure 1B**). Furthermore, baseline C4 levels were positively related to 24 hrs jejunal output adjusted by baseline weight ($\rho = +0.80$, $P = .002$) (**Figure 1C**), indicating a choleric effect before CR that is linked to enhanced bile salt synthesis²⁵. FGF19 levels were not related to jejunal efflux ($\rho = -0.44$, $P = .155$) (**Figure 1C**). Of note, baseline C4 levels were higher in females compared to males (179 [95 to 274] vs 55 [28 to 103] ng/mL , $P = .017$), but C4 levels of both sex groups of patients were significantly higher compared to healthy controls (data not shown). No clinical or biochemical explanation was found for the sex difference. None of the other laboratory parameters were different between males and females.

Figure 1. Dysregulated bile salt synthesis in IF patients with a temporary double enterostomy prior

to CR. (A) Baseline values of TBS, FGF19 and C4 of IF patients with a TDE ($n = 12$) compared with controls ($n = 12$). Note, C4 levels were available for 6 out of 12 healthy volunteers. (B) Correlation between FGF19 and C4 levels in IF patients with a TDE (blue circles) and controls (black circles). (C) Correlation between jejunal efflux adjusted by weight, and FGF19 or C4. Data is depicted as mean $\pm$ 95% CI. Differences were evaluated by Mann-Whitney U test. Correlations were evaluated with Spearman's (ρ) correlation coefficient. *P* values are depicted.

Chyme reinfusion restores the regulatory bile salt-FGF19 axis

Reinfusion of proximal small intestinal secretions into the distal small bowel allows enterohepatic recycling of bile salts to the liver and may accordingly reinstate the gut-liver axis of bile salt-FGF19 signaling. For this reason, we studied the effect of CR on the time courses of plasma TBS, FGF19 and C4. The individual responses are shown in **Supplementary Figures 2A & 2B**. Systemic TBS levels did not change significantly after introducing CR ($P = .112$) (**Figure 2A**). FGF19 levels increased after CR initiation ($P = .002$) with a transient peak after 7 days, and reaching a new steady-state level that was higher than at baseline, thereafter (**Figure 2A**). In line with elevated FGF19 levels, plasma C4 levels were reduced after start of CR ($P < .001$) (**Figure 2A**), indicating reduced bile salt synthesis during CR. The percentage change from baseline is shown in **Figure 2B**. Most pronounced responses of FGF19 and C4 were seen after 7 days of CR (**Figure 2B**). TBS levels were unaltered after 7 days of CR (1.9 [1.4 to 3.0] vs 1.2 [0.5 to 3.6] $\mu\text{mol/L}$, $P = .773$) (**Figure 2C**) FGF19 levels were higher for all but one patient after 7 days compared to baseline values (0.023 [0.012 to 0.061] vs 0.128 [0.077 to 0.208] ng/mL, $P = .003$) (**Figure 2C**). Conversely, and in line with FGF19 function, C4 levels were consistently lower after 7 days compared to baseline values (95 [38 to 196] vs [18 [11 to 26] ng/mL, $P < .001$) (**Figure 2C**).

Further, gene expressions of *FXR* and the FXR-regulated organic solute transporter subunits α/β (*OST α/β* , engaged in export of bile salts from the enterocyte to the venous capillaries) were upregulated after CR ($P < .05$ for all) (**Figure 2D**). Transcripts of the apical sodium-dependent bile salt transporter (*ASBT*), implicated in uptake of bile salts from the intestinal lumen were not altered after CR ($P = .612$) (**Figure 2D**). Expression of *DIET1*, a modulator of FGF19 secretion²⁶, was significantly increased after 21 days (**Figure 2D**). For reasons unknown, *FGF19* mRNA was not detected in the majority of specimens, irrespective of sampling prior to, or after 3 weeks of CR (data not shown).

Eleven patients (92%) had jejunal effluxes above 1200 g/24 hrs, and a single patient had an output of 678 g/24 hrs prior to CR. Inherent to the design of the device for reinfusing chyme, jejunal efflux could not be quantified during the course of CR. All patients developed stools or colonic stoma output after CR, with a single patient having persistently a high fecal output as a consequence of diarrhea. Nevertheless, intestinal output declined significantly during CR (2245 [1366 to 2913] vs 203 [125 to 375] g/24 hrs, $P < .001$) (**Figure 2E**).

Figure 2. CR restores homeostatic control of bile salt synthesis within 7 days. Twelve patients with a temporary double enterostomy on PN underwent CR for up to 7 weeks. Blood was sampled on the depicted time points before and after start of CR, and analyzed for (A) TBS, FGF19 and C4. (B) Relative change from baseline over time for TBS, FGF19 and C4. (C) Individual changes of TBS, FGF19 and C4 in the first week of CR. (D) Paired small intestinal biopsies were studied (n = 7 patients) at 3 days before CR and 21 days after CR and analyzed for mRNA expression of *FXR* and genes related to bile salt transport. (E) Proximal jejunal output at start of CR, and stomal or fecal output at discharge from the clinic/end of CR were assessed to address intestinal output changes. Data is depicted as mean $\pm$ 95% CI. Trends in time were evaluated by ANOVA with repeated measures. mRNA expression and intestinal output

differences were evaluated by Wilcoxon matched-pairs signed ranks sum test. *P* values are depicted. Asterisks indicate significance levels: *(*P* < .05), **(*P* < .01) and *** (*P* < .001).

Chyme reinfusion alters composition of circulating bile salts

Re-entry of chyme into the distal small bowel and colon restores interaction between the gut microbiota and host bile salts and results in microbial bile salt metabolism²⁷. At baseline, gut microbiota-derived secondary bile salts and deconjugated bile salt species were virtually absent (**Figure 3A & 3B**). The molar fractions of secondary and deconjugated bile salt species increased during CR treatment (*P* = .004 and *P* < .001, respectively) and reflect the restored contact between bile salts and bile salt-metabolizing gut microbes (**Figure 3A & 3B**). Moreover, the fractions of glycine-conjugated bile salts were elevated after CR (*P* = .025) (**Figure 3C**), also mirrored by lower ratio of taurine- to glycine-conjugated bile salts (*P* = .008) (**Supplementary Figure S3A**). Individual plasma bile salt species are reported in **Figure 3E**. Note, absolute plasma levels of a number of secondary bile salts ((glyco)deoxycholate, (glyco)lithocholate and ursodeoxycholate) (all *P* < .05) were significantly increased after 7 days of CR treatment (**Figure 3E**). An overview of absolute levels of all detected bile salt species at the respective time points, is provided in **Supplementary Table S4**. Absolute levels of the most abundant sulfated bile salt species (i.e. glycochenodeoxycholate-3-sulfate, GCDCA-3S), which are typically formed under conditions of cholestasis²⁸, were reduced after CR (*P* = .049) (**Figure 3D**), and showed a positive correlation with hepatobiliary injury markers ALP and GGT (ρ = +0.65, *P* = .026 and ρ = +0.50, *P* = .104, respectively) (**Supplementary Figure S3B**). Finally, GCDCA, GCA and TCA were strongly correlated to ALT (ρ = +0.66, *P* = .024, ρ = +0.83, *P* = .002 and ρ = +0.76, *P* = .006, respectively) (**Supplementary Figure S3C**).

Furthermore, we analyzed individual chyme bile salt species at baseline and after 7 days of CR treatment. Mole fractions of individual bile salt species in chyme did not show major shifts (**Figure 3F**). Note, the high fraction of unconjugated bile salt species (50.1%). Intriguingly, the total fraction of unconjugated bile salt species in chyme (i.e. CA, CDCA and DCA) was unexpectedly high, considering that >99% of biliary bile salts are conjugated²⁹. A possible explanation could be bacterial overgrowth in the proximal small intestine and attendant microbial bile salt deconjugation.

Re-introducing bile salts in the distal small bowel leads to FGF19 production. In support

of this notion, mole fractions of GCA ($\rho = +0.71$, $P = .014$) and GCDCA ($\rho = +0.71$, $P = .014$) were strongly associated with plasma FGF19 levels (**Figure 3G**), but significant associations with plasma C4 levels were not found (**Figure 3G**).

Figure 3. Alteration of serum bile salt composition after CR initiation. Time course of circulating bile salt composition during CR ($n = 12$). Graphs show time courses of (A) primary and secondary bile salts, (B) deconjugated and conjugated bile salts, (C) glycine- and taurine-conjugated bile salts and (D) the sulfated bile salt species GCDCA-3S. (E) Individual plasma bile salt species. (F) Chyme bile salt composition, fraction of individual bile salt species (%) of total bile salt composition. (G) Correlation between chyme GCA and GCDCA and plasma levels of FGF19 and C4. Data is depicted as mean $\pm$ 95% CI. Trends in time were evaluated by ANOVA with repeated measures. P values are depicted. Asterisks indicate significance levels: $^*(P < .05)$ and $^{**}(P < .01)$.

Chyme reinfusion restores intestinal function and protects against liver injury

CR restored endocrine regulation of bile salt synthesis and allowed microbial metabolism of bile salts. Next, we studied whether CR recovered intestinal function and ameliorated liver injury. We first assessed whether CR could improve intestinal function. At baseline, 8 patients had plasma CIT concentrations $\leq 20 \mu\text{mol/L}$ (**Figure 4A**), indicating impaired intestinal function³⁰. Remaining 4 patients (2 on PN) with higher CIT levels had a combination of persistent high jejunal efflux (median 2170 mL) and long period of hospital admission (median 47 days) prior to CR, justifying CR treatment. CIT concentrations went up after CR initiation ($P = .004$) and levels considered normal³⁰ were attained after 5 weeks in all patients (**Figure 4A**). Serum

albumin levels increased after CR initiation ($P < .001$) and were in the normal range for 6 of 12 patients (50%) after 7 weeks (**Figure 4B**). Individual plasma CIT and albumin time courses are depicted in **Supplementary Figure S4**. Expression of the epithelium-specific genes villin-1 (*VIL1*, cytoskeletal component), sucrase-isomaltase (*SI*, carbohydrate digestive enzyme) and cubilin (*CUBN*, engaged in vitamin B12 absorption) were determined to study the impact of CR on enterocyte function. CR significantly increased *VIL1* ($P = .016$) and *SI* ($P = .031$) mRNA levels, whereas *CUBN* expression levels were not altered during CR ($P = .156$) (**Figure 4D**). Further, the mRNA level of ornithine carbamoyl transferase (*OCT*, involved in CIT synthesis) was increased after CR ($P = .016$), in line with increased plasma CIT levels after 21 days of CR (**Figure 4A & 4C**). We evaluated the local inflammatory response (small intestinal *IL6* mRNA level). *IL6* mRNA expression was decreased after CR ($P < .001$). In addition, transcript levels of pro-inflammatory cytokines including *TNF α* and *IL-1 β* (**Figure 4D**), *IL-8* and *IL-17* (**Supplementary Figure S5**) were decreased after three weeks of CR treatment (all $P < .05$). mRNA levels of the anti-inflammatory cytokine *IL-10* was also decreased after three weeks of CR treatment (**Figure 4D**).

Secondly, we studied the effect of CR on liver injury. Serum baseline elevations of ALP and GGT were significantly reduced after 49 days of CR ($P < .001$ and $P = .003$, respectively), approaching the respective normal ranges after 35 days (**Figure 4E**). Total bilirubin levels were within the normal range at baseline and were not affected by CR ($P = .408$) (**Figure 4E**). ALT and AST levels were above the normal range at baseline, although the time course displayed declining trends in time, these were not significant ($P = .150$ and $P = .189$, respectively) (**Supplementary Figure S6**).

Finally, we evaluated the systemic inflammatory response (CRP and pro-inflammatory cytokine levels). Serum CRP were at relatively low levels and decreased non-significantly during CR ($P = .052$) (**Figure 4F**). We also tested systemic levels of pro-inflammatory cytokines *IL6* and *TNF- α* , but found levels to be below the detection limit of the immune assays for 10 and 12 out of 12 patients, respectively (data not shown), highlighting the non-inflammatory state of the patients before and during CR.

Improvement of the nutritional status by chyme reinfusion

During CR, the oral feeding met the recommended dietary allowance for energy and protein intake. Three patients with residual malabsorption received additional enteral nutrition by “en Y” enteroclysis. Consequently, PN and/or IVS could be stopped in 9

out of 10 patients (90%) within a median of 3 [1 to 9] days after start of CR. Most patients ($n = 8$) had poor nutritional status before CR, but this improved during CR (Supplementary Table S5).

Figure 4. CR recovers intestinal absorptive function and ameliorates liver injury. Time course of markers for intestinal absorptive function and nutritional status during CR. (A) CIT values of individual patients at baseline (left panel) with values >20 $\mu\text{mol/L}$ in a dashed box and time course of CIT (right panel). (B) Time course of albumin (dashed lines represents normal value). (C) Relative change from baseline over time for CIT and albumin. (D) Paired mRNA expression of epithelium specific genes (*VIL-1*, *SI*, *CUBN*, and *OCT*) and local inflammation (*IL6*, *TNF-alpha*, *IL-1β*, *IL-8*, *IL-17* and *IL-10*) at baseline and 3 weeks after CR. (E) Time course of liver injury markers (ALP, GGT and total bilirubin) and (F) systemic inflammation (CRP). Data is depicted as mean $\pm$ 95% CI. Trends in time were evaluated by ANOVA with repeated measures. Gene expression differences were evaluated by Wilcoxon matched-pairs signed ranks sum test. P values are depicted.

The C4 response after 7 days dependent of the FGF19 response highly predicts

the course of CIT

Evaluation of plasma time courses of FGF19 and C4 showed that the reciprocal response in the first week was followed by a gradual increase of CIT levels and decrease of liver injury markers (ALP and GGT) (**Figure 1** and **Figure 4**). Therefore, we constructed a linear mixed model with random intercepts to evaluate the association between the change in FGF19 and C4 after 7 days of CR (Δ) and alterations in markers of intestinal function (CIT) and liver injury (ALP and GGT) from day 7 onwards. First, the reciprocal interaction between $\Delta_{\text{FGF19}} * \Delta_{\text{C4}}$ significantly predicted CIT values over time (estimate: -0.54 , 95% CI: $[-0.91 \text{ to } -0.18]$, $P = .007$). The interaction plot ($R^2 = +0.67$, $P = .007$) is depicted in **Figure 5** and demonstrates that the FGF19 response within the first 7 days, dependent on the C4 response, predicts CIT values over time. Finally, the Δ_{FGF19} (per 0.1 unit increase) predicts the course of ALP (estimate: -22.0 , 95% CI: $[-40.2 \text{ to } -3.8]$, $P = .022$) and tended to predict GGT (estimate: -26.8 , 95% CI: $[-58.3 \text{ to } 47.6]$, $P = .095$) (data not shown).

Figure 5. The C4 response, dependent on the FGF19 response, after 7 days predicts the plasma course of CIT over time. A linear mixed effects model was constructed to evaluate whether the response of FGF19 and C4 (difference between 7 days and baseline values = Δ) predicts the course of CIT over time. **(A)** Interaction plot depicting the association between the interaction of $\Delta_{\text{FGF19}} * \Delta_{\text{C4}}$ after 7 days and predicted CIT values over time (from 7 days onwards). The Δ_{FGF19} (ng/mL) is depicted on the x-axis and the Δ_{C4} (ng/mL) is shown at the corresponding dots. The y-axis shows the predicted CIT values ($\mu\text{mol/L}$). The regression line is shown with 95% confidence interval. R-squared and P values are depicted.

Discussion

CR re-establishes small intestinal continuity and hence functionally restores the enterohepatic circulation of bile salts. In this prospective study, the key finding is that CR rapidly reinstates the regulatory bile salt-FGF19 axis and further restores intestinal function, reverses liver injury and contributes to the improvement of the nutritional status in IF patients with a TDE. Our findings support a role for recovery of homeostatic control of bile salt synthesis by CR in patients with IF with a TDE. The pathophysiology and mechanistic findings were schematically summarized in **Figure 6**.

Figure 6. The pathophysiology and mechanistic findings in the present study.

To our knowledge the bile salt-FGF19 axis was not previously investigated in IF patients undergoing extracorporeal recovery of intestinal continuity. In our study, we observed that endocrine control of bile salt synthesis was recovered after 7 days of CR followed by a new equilibrium phase with higher FGF19 levels compared to the pre-CR period. This is in line with studies showing that administration of an FGF19 analogue strongly suppressed serum C4 levels in healthy human volunteers within 7 days and in patients with chronic liver disease after 4 or 12-weeks of treatment³¹⁻³³. The restored regulatory bile salt-FGF19 axis, evidenced by decline in C4 levels, was an anticipated effect of reinstating the enterohepatic circulation of bile salts. Furthermore, functional recovery of the enterohepatic circulation of bile salts was also reflected by larger abundance of microbiome-derived secondary and deconjugated bile salt species, indicating restored microbial metabolism of the gut¹⁶.

The initial response of FGF19 and C4 levels after 7 days of CR was followed by

gradually increasing CIT levels, indicating that restoration of the bile salt-FGF19 axis preceded recovery of intestinal function. Interestingly, the initial FGF19 response, dependent on C4, was highly associated with the later course of CIT over time. This would imply that restoring endocrine control of bile salt synthesis is an early step in functional recovery of the intestine. A recent study demonstrated involvement of FGF19 in preserving intestinal barrier integrity and protection against intestinal inflammation mediated by FXR³⁴.

Our findings also demonstrated upregulated mRNA expression of the bile salt sensor FXR, the bile salt transporters *OSTα/β*, and epithelium specific genes *VIL-1*, *SI* and *OCT* in the distal small intestine after 21 days of CR. Interestingly, *FXR* expression was negatively related to the pro-inflammatory cytokine (*IL6*) and positively related to intestinal epithelial-specific genes (*VIL1*, *SI*, *CUBN* and *OCT*) (data not shown). Mechanistically, this may be attributed to normalized trans-epithelial bile salt flux facilitated by CR, hence intestinal FXR activation. Bile salts play a direct role in preserving the intestinal barrier function and protection against intestinal inflammation, effects mediated by FXR³⁵.

Remarkably, findings in the present study showed that jejunal output prior to CR was strongly related to systemic C4 levels. A plausible explanation could be that enhanced synthesis and biliary secretion of bile salts induces a choleric effect and eventually elevated jejunal output³⁶. Since the enterohepatic circulation of bile salts was recovered and C4 levels were normalized after CR, it is likely that reduced stomal or fecal output after CR treatment was, in part, due to restoration of the ileal brake⁶, a direct consequence of recovery of the bile salt-FGF19 axis. Indeed, patients with chronic primary bile acid diarrhea have high C4 levels³⁷. Note, a single patient remained to have a high fecal output (>2 liters per day) after 49 days of CR treatment. This patient had persistently the lowest FGF19 and highest C4 level during the course of CR. Intriguingly, this patient underwent a partial gastrectomy prior CR. High gastrin levels are observed after gastrectomy and are related to increased choleresis^{38, 39}.

The major strength of our study was long term follow-up with multiple sampling points, which allowed us to analyze the time course of homeostatic control of bile salt synthesis. This study has certain limitations. First, considering the complexity of IF, we designed a pre- and post-intervention study without any control group without CR treatment. Importantly, CR is recommended, whenever feasible, by ASPEN guidelines

and ESPEN recommendations. Withholding this effective treatment for the purpose of a controlled study, would have been unethical. In addition, patients already had a median pre-CR period of 4 weeks with unsuccessful treatment with PN or IVS. Secondly, ileal biopsies were taken 3 days before and 3 weeks after CR. However, changes in FGF19 and C4 levels were rapidly seen after 7 days of CR treatment. Thus, sequential biopsies taken at each corresponding time point of blood collection would be more informative about transcriptional changes during CR. Obviously, frequent biopsies is a huge burden for patients and not justified by clinical necessity. Nonetheless, alterations in circulating FGF19 and C4 levels correspond well to transcriptional changes^{40, 41}.

The findings of this study make it conceivable that the beneficial effects of CR are mediated in part through the restored bile salt-FGF19 signaling axis. Clinically, CR in surgical patients with a distal entrance of the small bowel should be considered in case IF develops, because CR might also benefit postoperative outcomes considering the persevering effects on intestinal function. In addition, in case patients need to undergo a temporary proximal enterostomy (e.g. during acute mesenteric ischemia), establishment of a double-barreled enterostomy or placement of a distal entrance of the small bowel should be considered. Finally, an important clinical implication of our study is that administration of FGF19 analogues could be considered in IF patients without an accessible distal small bowel.

In conclusion, CR improves intestinal and liver function in IF patients with a TDE. The beneficial effects of CR are partly mediated by activation of bile salt-FGF19 signaling leading to restored regulation of hepatic bile salt synthesis, and is associated with improved gut-liver health. CR needs to be considered as first-line treatment in IF patients with a TDE. Further, novel therapeutic approaches with FGF19 analogues might be promising in the management of IF and related complications in case a distal intestine is not accessible.

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Supplementary Table S1. Primer sequences.

Gene	Forward sequence	Reverse sequence
<i>36B4</i> †	CCTCGTGGAAGTGACATCGT	ATCTGCTTGGAGCCCACATT
<i>PSMB4</i> †	CTCGGCCAGATGGTGATTGA	AGGCTACACCAAGCATGTCC
<i>REEP5</i> †	ACTACATGCTGAAGTGTGGCT	TCCAGTCTGGTTTAGGTGCTC
<i>HPRT1</i> †	CCTGGCGTCGTGATTAGTGAT	GGGCTACAATGTGATGGCCT
<i>FXRiso</i>	ACAGAACAAGTGGCAGGTC	CTGAAGAAACCTTTACACCCCTC
<i>SLC10A2</i> *	TCGACTCTGGGAGCATCGTA	CCGCGATGGACCCAATTTTA
<i>SLC51A</i> *	ACCCTTTGCCCCATCAAGAG	GCACACGGCATAAACGAGG
<i>SLC51B</i> *	GGGTCTAAGGACCTCGTTGC	TTCTTTTCTGCTTGCCTGGATG
<i>DIET1</i>	AAACTGCACAACTGTGGGAGA	GCAGCTTCCTGTTGTGCTTG
<i>VIL1</i>	ATGAGCCATGCGCTGAACTT	GCATGGATGTGGCATCGAAC
<i>SI</i>	AGACACCTGCTGTTGATGAAAT	GAGAGTCATTCCACGGCCTC
<i>CUBN</i>	GCGTCCTTTGTTAGCCGATG	GGGTGGACATGACGCTGTAA
<i>OTC</i>	CACCTTCAGGCAGCTACTCC	GCAGCAACTTTAGCAGTCTTCA
<i>IL6</i>	CATCCTCGACGGCATCTCAG	TCACCAGGCAAGTCTCCTCA
<i>CXCL8</i> *	GCTCTGTGTGAAGGTGCAGTT	ACCCAGTTTTCTTGGGGTC
<i>IL1β</i>	GCTCTGTGTGAAGGTGCAGTT	ACCCAGTTTTCTTGGGGTC
<i>TNFα</i>	TCCCCAGGGACCTCTCTCTA	GAGGGTTTGCTACAACATGGG
<i>IL17</i>	AACCGATCCACCTCACCTTG	TCTCTTGCTGGATGGGGACA
<i>IL10</i>	ACATCAAGGCGCATGTGAAC	TAGAGTCGCCACCCTGATGT

†Housekeeping genes. *Synonyms: *SLC10A2*, *ASBT*; *SLC51A*, *OSTα*; *SLC51B*, *OSTβ*; *CXCL8*, *IL8*.

Supplementary Table S2. Individual patient data.

Patient	Sex	Age	BMI	Weight loss (%)	Etiology	Double enterostoma	Efferent anatomy	Small bowel resection	Colectomy	Home-setting CR	PN volume (mL/24u)	Additional enteroclysis Volume (mL/24u)
#1	Male	65	20,7	7	Ischemia	double- barrelled stoma	ileo-colo-rectal	jejunum	No	No	2500	-
#2	Female	77	15,7	21	Mechanical obstruction	double-barrelled stoma	ileo-colo-rectal	jejunum	No	No	-	-
#3	Male	83	20,4	14	ischemia	double-barrelled stoma	ileo-colo-rectal	jejunum	No	No	2500	-
#4	Female	37	23,8	18	Mechanical obstruction	double-barrelled stoma	ileo-colo-rectal	multiple	Sigmoid resection. Hartman Left colectomy	Yes	2500	-
#5	Female	72	27,9	0	Postoperative peritonitis	double-barrelled stoma	ileo-colo-stoma	No	No	No	3000	-
#6	Male	78	31,6	2	Postoperative peritonitis	double-barrelled stoma	ileo-colo-rectal	Jejunum	No	No	1500	500
#7	Male	81	22,6	5	Mechanical obstruction	loop stoma	ileo-colo-rectal	No	No	No	1000	500
#9	Male	71	19,5	16	Postoperative peritonitis	loop stoma	ileo-colo-rectal	No	No	No	2000	500
#10	Female	51	27,8	12	Postoperative peritonitis	double-barrelled stoma	ileo-colo-rectal	middle part of the small bowel	No	No	1250	-
#11	Female	64	17,0	10	Postoperative peritonitis	double-barrelled stoma	ileo-colo-stoma	No	No	No	1000	-
#12	Male	81	21,7	26	Ischemia	double-barrelled stoma	ileo-colo-stoma	jejunal	Left and transverse colectomy	No	-	-
#13	Male	52	25,2	4	Ischemia	double-barrelled stoma	ileo-colo-rectal	middle part of the small bowel	No	Yes	3000	-

Supplementary Table S3. Demographics of healthy controls versus intestinal failure patients.

Variable (n = 12)	Healthy controls	IF-patients	<i>P</i>
Age (years)	54.7 ± 19.7	67.7 ± 14.5	.079
Sex (male vs female)	8 vs 4	7 vs 5	.178
BMI (kg/m ²)	24.1 ± 3.6	22.8 ± 4.7	.455

Data are expressed as mean ± standard deviation (SD). *P* values in bold represent significant statistical differences. BMI indicates body mass index; Data of healthy controls are adapted from Plummer M.P., *Crit Care Med.* 2016 Sep;44(9):e790-6.

Supplementary Table S4. Overview of plasma bile salt species before and during chyme reinfusion.

Bile salt species (nmol/L)	Baseline			7 days			21 days			35 days			49 days		
CA	31	13	79	83	18	110	70	24	106	44	27	114	76	38	152
DCA	12	6	13	57	6	94	29	8	92	48	6	130	35	12	109
CDCA	46	9	180	109	29	305	138	44	219	114	50	237	160	105	424
LCA	4	3	5	7	4	17	8	4	17	10	3	20	6	4	20
UDCA	0	0	2	6	2	19	6	1	61	6	3	17	7	3	8
GCA	892	716	1625	86	23	363	74	33	242	34	8	417	84	23	427
GDCA	9	5	10	30	5	77	14	5	65	25	7	50	21	7	40
GCDCA	885	404	1446	240	161	829	245	208	401	250	142	527	302	111	935
GLCA	0	0	0	5	0	14	0	0	10	5	0	29	3	0	11
GUDCA	0	0	0	0	0	8	0	0	34	0	0	0	0	0	0
TCA	298	195	556	23	0	174	5	0	81	8	0	74	7	0	38
TDCA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TCDCa	47	16	135	6	0	48	2	0	21	0	0	17	6	0	19

Data are presented as median + [IQR]. 1st column: median, 2nd column: 25th percentile and 3rd column 75th percentile.

Supplementary Table S5. Evolution of nutritional status before and during CR.

Variable (n = 12)	Before CR	During CR	<i>P</i>
Energy intake, kcal/kg/day	25.9 [20.9 to 28.4]	30.5 [27.9 to 36.8]	.123
Protein intake, g/kg/day	1.0 [0.9 to 1.2]	1.4 [1.0 to 1.5]	.069
Weight loss, %	10.9 [4.5 to 16.4]	9.9 [5.4 to 14.1]	.161
BMI, kg/m ²	22.2 [20.2 to 25.8]	22.7 [20.3 to 25.4]	.754
Serum albumin, g/L	24.3 [20.1 to 29.3]	32.3 [29.3 to 35.8]	.002
Serum albumin < 30 g/L, n (%)	9 (75)	4 (33)	.012
NRI	71.6 [65.3 to 80.3]	84.7 [80.9 to 88.9]	.017
NRI < 83.5, n (%)	11 (92)	4 (33)	
83.5 ≤ NRI < 97.5, n (%)	1 (8)	8 (67)	
97.5 ≤ NRI ≤ 97.5, n (%)	0 (0)	0 (0)	.053

Data are median [IQR]. *P* values in bold represent significant statistical differences. BMI indicates body mass index; NRI, nutritional risk index.

Study design

Supplementary Figure S1. Study design. Overview of study design.

S2-A

S2-B

Supplementary Figure S2A-B. Individual plasma FGF19, C4 and TBS time courses.

Individual plasma time courses of (A) FGF19, C4 and (B) TBS in all twelve patients. In (A) the interrupted line depicts FGF19 and the black line C4 levels.

Supplementary Figure S3. Ratio of taurine- to glycine-conjugated bile salts and association of GCDCA-3S with liver injury markers. Time course of (A) the ratio of taurine- to glycine-conjugated bile salts and (B) correlation between GCDCA-3S and ALP and GGT (area under the curve). (C) Correlation between GCDCA-3S, GCA, TCA and ALT levels (area under the curve). Trends in time were evaluated by ANOVA with repeated measures. Correlations were evaluated with Spearman's (ρ) correlation coefficient. P values are depicted.

Supplementary Figure S4. Individual plasma CIT and albumin plasma time courses. Individual plasma time courses of CIT and albumin of all patients. The interrupted line depicts CIT and the black line albumin levels.

Supplementary Figure S5. mRNA expression of *IL-8* and *IL-17*. mRNA expression of pro-inflammatory cytokines *IL-8* and *IL-17*. *P* values are depicted.

Supplementary Figure S6. Liver injury markers plasma time courses. Plasma time courses of ALT and AST. Trends in time were evaluated by ANOVA with repeated measures. *P* values are depicted.

Chapter 7

General discussion

A disrupted enterohepatic circulation (EHC) of bile salts leads to unfavorable outcomes in surgical patients ^{1,2}. Patients with hyperbilirubinemia due to biliary obstruction have a high rate of severe complications after partial liver resection ³. In addition, due to the loss of bile salts (i.e. liver and/or gallbladder tissue) and diminished bile salt signaling, patients undergoing partial hepatectomy are at risk of liver failure and death, especially in case of a small future liver remnant (FLR) ⁴. Furthermore, patients with an interrupted EHC due to a temporary jejunal double enterostomy can develop intestinal failure (IF) and intestinal failure associated liver disfunction (IFALD) because of loss of succus entericus ⁵. To improve clinical outcomes, various treatments targeting the circulation and signaling function of bile salts are employed/tested in surgical patients or experimental models, including preoperative biliary drainage (BD) ⁶, bile salt receptor activation ^{7,8}, and chyme reinfusion ⁵.

The central aim of the current thesis is to investigate the role of restored or activation of the enterohepatic bile salt signaling axis, as well as consequences of cholestasis on liver regeneration and intestinal failure (secondary to a temporary double enterostomy). This translated into five main specific aims: 1) to assess the effect and optimal route of preoperative biliary drainage in patients with resectable pCCA (**Chapter 2**); 2) to explore precision-cut liver slices (PCLS) as a model for studying factors affecting human liver regeneration *ex vivo* (**Chapter 3**); 3) to investigate the influence of cholestasis on portal vein embolization (PVE)-induced FLR hypertrophy in patients (**Chapter 4**); 4) to study the mechanism of obeticholic acid (OCA)-augmented hypertrophy in experimental PVE (**Chapter 5**); and 5) to study the contribution of the bile salt-fibroblast growth factor 19 (FGF19) axis in intestinal failure patients receiving chyme reinfusion (**Chapter 6**).

Preoperative biliary drainage for resectable perihilar cholangiocarcinoma

Preoperative BD is used to decompress the biliary tree and improve FLR function before liver resection. However, the value of preoperative BD in resectable pCCA is under debate. Additionally, the best drainage route (percutaneous transhepatic biliary drainage, PTBD or endoscopic biliary drainage, EBD) still needs to be determined. In **Chapter 2**, we conducted a systematic review of systematic reviews (SRs) to assess the effect of preoperative BD versus no preoperative BD, and the superiority of EBD versus PTBD, on perioperative and long-term outcomes in patients with resectable

pCCA. The results indicate that preoperative BD might be beneficial in strictly selected patients. In addition, EBD might be associated with higher short-term drainage-related morbidity (e.g. cholangitis and pancreatitis), but more favorable long-term survival. It should be emphasized that all but one of the original studies included in the respective SRs had a retrospective design, which could not avoid confounding factors in the study period. Furthermore, most included SRs had a high risk of bias according the ROBIS assessment tool. Notably, there is considerable variability in the inclusion criteria of original studies and the numbers of participants and events abstracted across the SRs. These differences could be caused by variable periods of study inclusion, inclusion criteria, and data extraction errors. Errors in the data extraction procedure are an important source of bias in SRs ⁹. The influence of risk of bias on pooled results could lead to under- or overestimation of the actual intervention effects, resulting in a limitation of the validity of drawn conclusions ¹⁰.

The one randomized controlled trial (RCT) conducted thus far, demonstrated higher mortality in the PTBD compared to the EBD group ¹¹. PTBD increased perioperative mortality in patients with resectable pCCA, with 3 of 11 fatalities occurring before surgery ¹¹. This study was prematurely stopped because of higher mortality in the PTBD group. A possible reason is that externally draining bile in the PTBD procedure results in an impaired contribution of bile salt signaling to the regenerative response and processes. Future studies to compare PTBD with reinfusion of bile fluid, to EBD in patients with pCCA are recommended.

In addition, further studies should pay attention to the differences of internal or external biliary drainage on perioperative and postoperative outcomes in resectable pCCA. Because internal drainage elicits a different physiological response compared to external drainage that abrogates the digestive, signaling and antimicrobial roles of bile salts. Abated activation of ileal farnesoid X receptor (FXR) can result in the loss of negative feedback control of hepatic bile salt synthesis and bile salt overload with attendant hepatotoxicity when export to the biliary network is hampered ¹. In the setting of external drainage, it can be considered to give bile back orally or via a naso-gastric tube, to allow beneficial antimicrobial and signaling actions of bile salts. ¹²⁻¹⁴.

PCLS as an *in vitro* model to study human liver regeneration

Liver regeneration requires the temporally orchestrated proliferation of hepatocytes as well as all the other cell types that support liver function, such as vascular endothelial cells, innate immune cells, and bile duct-lining cholangiocytes^{15, 16}. Paracrine signaling via soluble factors (e.g. angiocrine factors) is pivotal in this process, and restoration of the livers' functional architecture¹⁷. As an *ex vivo* model, PCLS contains various parenchymal and non-parenchymal cells, imitating liver original environment¹⁸. PCLS may be a good platform to study the cell-cell interactions in liver regeneration, and test strategies to augment liver growth.

In **Chapter 3**, we used FGF19 and bile salt nuclear receptors agonists to test the suitability of human PCLS (hPCLS) as an *in vitro* model to study liver regeneration. The responsiveness of hPCLS to FGF19 could not be verified at the level of ERK1/2, a kinase that is activated after binding of FGF19 to its receptor FGFR4. ERK1/2 activation (phosphorylated form) was already maximal without addition of FGF19. This likely results from external triggers during harvesting or processing of tissue for hPCLS preparation. Unfortunately, the expression of FGF19-regulated genes could not be used as a read-out of FGF19 activity, as *CYP7A1* became undetectable after >19 hrs of culturing and *PCK1* was drastically reduced. Since we could not verify FGF19 responsiveness in hPCLS, we cannot draw a conclusion on the role of FGF19 in driving hepatocellular proliferation in humans.

Constitutive androstane receptor (CAR) activation by CITCO (6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime) was reported to induce hepatocyte proliferation in PCLS, including slices from the liver of one patient¹⁹. Despite carefully replicating experimental conditions, however, we could not reproduce the proliferative effect of CITCO in hPCLS derived from three distinct patients. Because induction of CAR target gene *CYP2E1* by CITCO was not observed in hPCLS, we cannot draw a conclusion on CAR action.

In its current form, the hPCLS model is not suitable for studying the role of FGF19 on liver regeneration. Further measures including systematic evaluation of patient factors (background liver disease, prior chemotherapy, ischemic time, etc), and culturing conditions (e.g. optimized recovery time, strategies to prevent dedifferentiation etc.) should be considered in optimizing the hPCLS as model to study -and therapeutically modulate- liver regeneration.

Bile salt homeostasis on PVE-induced liver regeneration

The FXR agonist OCA accelerated liver regeneration following PVE, reflected by increased hepatic volume and proliferation in the non-embolized lobe in a standardized rabbit model ⁷. However, the underlying mechanism has not been defined yet. In **Chapter 4** we aim to reveal the mechanisms underlying accelerated FLR growth in OCA-treated rabbit undergoing PVE. We observed earlier normalization of serum total bile salt levels after PVE in OCA-treated animals. In the non-embolized lobe, bile salts levels were lower upon OCA treatment, which may be caused by reduced bile salt synthesis combined with enhanced basolateral export of bile salts. Serum bile salt composition profile showed no major shift by OCA treatment. However, the absolute levels of lithocholic acid (LCA, a cytotoxic bile salt) and hyodeoxycholic acid (HDCA) were lower in OCA-treated animals, and their levels were negatively correlated with FLR hypertrophy. Furthermore, transcript profiling by RNA sequencing and subsequent pathway analysis of differentially expressed genes, provided support for an important role of bile salt homeostasis to accelerate FLR hypertrophy. Additional candidates for mediating growth-stimulating effects of OCA include the secreted factors bone morphogenetic protein 3 (Bmp3) and Indian hedgehog (Ihh). This study indicates that improved bile salt homeostasis underlies the increased hypertrophy of the non-embolized liver lobe in OCA-treated animals.

In the clinical setting, patients often have damaged liver parenchyma and function before the PVE procedure, such as cholestasis, chemotherapy-associated liver injury (CALI) and liver fibrosis. Impaired liver function is associated with subsequent attenuated liver regeneration after PVE ²⁰. Treatment with OCA may have potential to accelerate FLR volume gain in patients before liver resection. This can be beneficial to shorten the interval between PVE and liver resection, thus decreasing the risk of tumor progression (and degree of CALI) during this interval. OCA has been shown to improve hepatic fibrosis and reduce blood cholestatic markers in patients with chronic liver diseases in recent clinical trials ^{21, 22}. A large body of safety data in humans is available for OCA from phase II-IV trials ²¹⁻²³ and thus application of OCA in surgical patients for inducing liver regeneration is promising. However, an essential additional preclinical step would be the demonstration of lack of effects of OCA on tumor progression in the embolized segments. Once optimized, hPCLS derived from tumoral liver tissue may be usable to study this aspect in a human model system.

The role of bile salt homeostasis on liver growth in the rabbit model of PVE led us to investigate the influence of cholestasis on FLR hypertrophy in patients undergoing PVE. Thus, in **Chapter 5**, we retrospectively analyzed data from 50 patients (31 colorectal liver metastases (CRLM), 19 pCCA) who underwent PVE before major liver resection in Maastricht University Medical Center (Maastricht, The Netherlands) or the Uniklinik RWTH Aachen (Aachen, Germany). We found that FLR volumes increased after PVE in both patients with CRLM (all non-cholestatic) and pCCA (majority still cholestatic despite being drained). The degree of hypertrophy and kinetic growth rate, two measures reflecting FLR hypertrophy induced by PVE, were not different between patients with pCCA and CRLM. Patients with pCCA are routinely drained prior to resection, and also here the majority of these patients were drained prior to PVE. After drainage, pCCA patients with or without hyperbilirubinemia had comparable indices of FLR hypertrophy. Furthermore, neither serum bilirubin levels before drainage, nor before PVE were correlated with indices of FLR hypertrophy.

Because blood samples were not available in this retrospective cohort, we could not determine serum levels of bile salt homeostasis-related molecules, such as total and individual bile salts, FGF19, and C4 (a marker of synthesis of bile salt). As mentioned in **Chapter 4**, bile salts play an important role in liver regeneration via activation of the nuclear receptor FXR in the ileum and the liver. Future prospective studies with longitudinal collection of blood samples are recommended to further assess the association of the bile salt-FGF19 axis with FLR hypertrophy in patients undergoing PVE.

Through analyzing the impact of uni- vs. bilateral biliary drainage on FLR hypertrophy, we found that unilateral drainage of FLR induced higher DH than bilateral drainage. This finding suggests that unilateral drainage might be superior when performing initial biliary drainage. In addition, elevated inflammatory response (increased C-reactive protein levels) was associated with impaired liver growth in patients with pCCA. Serum C-reactive protein alterations may be promising to be used as a surrogate marker of PVE-induced FLR hypertrophy in patients with pCCA. Due to limited number of patients in our study, however, studies with larger number of participants are required to substantiate this notion.

Chyme reinfusion and bile salt-FGF19 axis

Enterocutaneous fistulae or surgically created (small intestinal) enterostomies disrupt the enterohepatic circulation and can result in (functional) intestinal failure ⁵. Several retrospective studies showed that chyme reinfusion (CR) can improve nutritional status, intestinal absorptive function, and reverse liver injury, in patients with acute intestinal failure (IF) ^{5, 24, 25}. In **Chapter 6** we describe a prospective study with a pre- and post-intervention design to investigate a potential contribution of the bile salt-FGF19 axis to the beneficial actions of CR in intestinal failure patients with a temporary double enterostomy (RESCUE study).

Disturbance of the bile salt-FGF19 axis in these patients was evident at baseline (prior to CR), as judged from low serum FGF19 and marked elevation of serum C4. CR induced a prompt increase in plasma FGF19 and decrease in C4 levels, indicating restored regulation of bile salt synthesis via endocrine FGF19 action. Fractions of secondary and deconjugated bile salts were increased after CR, reflecting restored contact between host bile salts and the colonic flora, allowing microbial bile salt metabolism. Furthermore, citrulline and albumin levels were gradually rising after CR, while abnormal serum liver tests normalized after CR, indicating restored intestinal function, improved nutritional status and amelioration of liver injury. CR increased ileal gene transcripts related to enterocyte number, and carbohydrate and bile salt handling. Finally, the reciprocal FGF19/C4 response after 7 days, predicted the plasma citrulline time course, indicating beneficial effects of restored hepatic synthesis of bile salt on improved intestinal function.

Remarkably, our study showed that jejunal output prior to CR was strongly related to systemic C4 levels. A plausible explanation could be that enhanced synthesis and biliary secretion of bile salts induces a choleretic effect and eventually elevated jejunal output ²⁶. In support of such notion, patients with chronic primary bile salt diarrhea have high C4 levels ²⁷.

The findings of this study make it conceivable that the beneficial effects of CR are mediated in part through a restored bile salt-FGF19 signaling axis. In clinics, CR need to be considered in surgical patients with a distal entrance of the small bowel in case IF develops. If the effluent part of the small intestine cannot be accessed for CR, systemic FXR/FGF19-based therapy could be considered. Engineered FGF19 variants were already shown to be effective in lowering bile salt synthesis in patients with cholestatic or metabolic liver disease ^{28, 29}.

Future perspective

Due to a relatively low incidence of pCCA, long-running RCTs with large sample sizes are lacking to provide strong evidence for the use (or not) of preoperative BD. Therefore, international multicenter cooperation is urgently needed to settle this medical question. The main issue in the current clinical practice is that uniform indications for preoperative BD are lacking in pCCA. This can be considered from at least three perspectives. Firstly, a serum bilirubin threshold needs to be determined for BD. Additionally, cholangitis needs to be considered because of its association with increased mortality in resectable pCCA⁴. Furthermore, small FLR can be an indication for preoperative BD. Because preoperative BD did not increase major morbidity after liver resection in jaundiced patients with small FLR³⁰.

In initial explorations of suitability of hPCLS as a model system to study liver regeneration, we observed rapid loss of *CYP7A1* mRNA expression to undetectable levels after 19 h of incubation. As *CYP7A1* expression is used as a readout for FGF19 signaling, optimization of culturing conditions such that a differentiated hepatocyte phenotype is maintained, is necessary to draw conclusions on FGF19 action on liver growth. In addition, RNA sequencing, phosphoproteome analysis, and metabolite profiling by mass spectrometry, can be used to find yet undefined targets of FGF19 action in hPCLS.

We retrospectively analyzed the influence of cholestasis on FLR hypertrophy after PVE through comparing patients with CRLM (all non-cholestatic) and pCCA (predominantly cholestatic)., Direct study of molecules that pertain to bile salt homeostasis in plasma and/or non-embolized liver tissue of clinical patients before and after PVE, can provide deeper insights into factors influencing PVE-induced liver hypertrophy. Bile salts levels at 5 h after PVE have been reported to be positively correlated with the increase in FRL volume in patients with CRLM³¹. However, the downstream pathways mediating FLR hypertrophy remain ill-defined. In addition, the impact of local inflammation on FLR growth should be investigated in patients and/or an optimized hPCLS model. Besides PVE, simultaneous embolization of portal and hepatic veins (PVE/HVE) is a new approach to induce FLR growth. Notably, PVE/HVE achieved better FLR hypertrophy and resectability than PVE alone³². It will be interesting to explore the potential role of bile salt signaling in PVE/HVE induced liver growth.

In the RESCUE study, we observed improved intestinal and liver function by the enteral

nutrition technique of chyme reinfusion, resulting in restored continuity of the EHC in intestinal failure patients with abnormal liver tests. As recommended by the European Society for Clinical Nutrition and Metabolism (ESPEN), oral or enteral nutrition (including distal enteral tube feeding) should be established in patients with intestinal failure-associated liver disease ³³. In addition, our study suggests that when patients need to undergo a temporary proximal enterostomy (e.g. during acute mesenteric ischemia), establishment of a double-barreled enterostomy or placement of a distal entrance of the small bowel should be considered in management of patients. Further, a plausible suggestion of our study is that (intramuscular) administration of FGF19 analogues could be beneficial to improve gut-liver function in intestinal failure patients when there is no accessible distal small bowel.

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Appendix I

Summary

Bile salts not only aid in absorption of dietary lipids, but also act as signaling molecules controlling negative feedback regulation of synthesis of bile salts, lipogenesis, and gluconeogenesis in the liver. The bile salt-fibroblast growth factor 19 (FGF19) signaling axis depends on an intact enterohepatic circulation (EHC) of bile salts, and is important for gut-liver health including promoting liver regeneration and improving intestinal failure (IF) and intestinal failure-associated liver disease (IFALD).

In **Chapter 1** we provided a general introduction to this thesis. We first introduced the physiology of the EHC of bile salts, the impact of biliary obstruction (e.g. perihilar cholangiocarcinoma, pCCA) on the EHC, and the interventional strategies (i.e. preoperative biliary drainage, BD) to decompress the biliary obstruction. Furthermore, we discussed the interrupted EHC due to loss of the 'source' of bile salts, such as partial hepatectomy, and the potential role of bile salt signaling on portal vein embolization (PVE)-induced liver hypertrophy. Moreover, we introduced disturbance of the EHC because of outflow of succus entericus in the stoma bag in patients with a temporary jejunal double enterostomy, and beneficial effects of chyme reinfusion (CR) in improving IF and IFALD. Finally, the aim and outline of this thesis were summarized.

In **Chapter 2** we gave a systematic review of systematic reviews to assess the effect and route of preoperative BD in patients with resectable perihilar cholangiocarcinoma (pCCA). Through searching database, eleven systematic reviews (SRs) with meta-analysis including 5950 patients were identified. All but one original studies in the SRs were retrospective. Ten of eleven SRs had high risk of bias. For preoperative BD *versus* no preoperative BD, all SRs showed no statistical differences in postoperative mortality. Preoperative BD was associated with increased postoperative major morbidity in 'simple criteria' patients receiving BD only based on the presence of jaundice. For endoscopic biliary drainage (EBD) *versus* percutaneous transhepatic biliary drainage (PTBD), three of four SRs showed that the postoperative mortality was not significantly different between the two drainage modes. EBD was associated with higher drainage-related overall morbidity, cholangitis and pancreatitis rates in three of four, three of five, and four of four SRs, respectively. PTBD was associated with higher seeding metastasis rates and worse postoperative overall survival. This study does not end the debate on the significance and preferred route of preoperative BD. The preoperative BD might need to be performed in strictly selected patients in terms of cholangitis, bilirubin levels and future liver remnant volume to avoid increased postoperative major morbidity. EBD might be associated with higher short-term

drainage-related morbidity but more favorable long-term oncological outcomes.

In **Chapter 3** we explored the suitability of human precision-cut liver slices (hPCLS) as an *in vitro* model to study human liver regeneration. hPCLS were derived from tumor-distal liver tissue from patients who underwent partial liver resection for colorectal liver metastases (CRLM). hPCLS were incubated with lipopolysaccharide (LPS), recombinant FGF19, and nuclear bile salt receptor agonists (obeticholic acid [OCA], farnesoid X receptor [FXR] agonist; 6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime [CITCO], constitutive androstane receptor [CAR] agonist; rifampicin, pregnane X receptor [PXR] agonist). 5C and Geltrex were used to assess their effectiveness in maintaining expression of genes related to a differentiated hepatocyte phenotype. mRNA expression of IL6, FXR/FGF19 pathway-related genes and proliferation-related genes were assessed. We found that FGF19 did not induce *IL6* mRNA expression in hPCLS. In addition, marked phosphorylation of extracellular signal-related kinase (ERK)1/2 was already observed in untreated liver slices. FGF19 had no additional effect on levels of phosphorylated ERK1/2. Expression of FGF19 pathway-related genes (*CYP7A1*, *FGFR4*, *KLB*, *PCK1*) rapidly declined upon hPCLS culturing. The use of 5C and Geltrex did not maintain expression of these genes during culturing of hPCLS. Furthermore, nuclear receptors agonists OCA and rifampicin did not induce cell proliferation in hPCLS. The efficacy of FGF19 and CAR treatment could not be ascertained in these exploratory experiments. Hence, questions regarding the involvement of the FGF19/IL6 axis in hepatocellular proliferation, and augmentation of liver growth by CAR agonism could not be answered with the current method of hPCLS source, preparation and culturing.

In **Chapter 4** we investigated the mechanisms by which obeticholic acid (OCA) accelerates liver hypertrophy following PVE in a rabbit model. Specimen were analyzed from a previous experiment in which rabbits were treated by oral gavage with OCA (10 mg/kg, n=11) or vehicle (control group, n=11) prior to, and during the course of, PVE. Serum total bile salts (TBS), bile salt composition, C4 (marker of bile salt synthesis), and liver synthetic function were determined before, after 3 hrs, and at day 1, day 3, and day 7 after PVE. Ileal and hepatic genes expression were analyzed through targeted and untargeted approaches at day three after PVE, when the growth-enhancing effect of OCA was maximal. For correlation analyses, volumes of (non)embolized liver segments, and %volume gain, were previously determined by CT scan. We previously reported that PVE induced a larger volume gain of the non-

embolized liver lobe in OCA-treated animals. Effects of OCA were largest at 3 days after PVE (volume gain: 56.1% vs. 26.1% in OCA- and vehicle-treated animals, respectively). OCA treatment led to earlier normalization of serum TBS levels and lower bile salt content of the non-embolized liver lobe after PVE. OCA decreased mRNA expression of *Cyp7a1* (involved in bile salt synthesis) and promoted expression of *Slc51a/b* (involved in bile salt export) in the non-embolized liver lobe, likely contributing to the observed improved serum and liver bile salt homeostasis. Levels of lithocholic acid (LCA), a hepatotoxic bile salt, were lower in the OCA-treated animals compared to the control animals at day 1 and day 3 ($P=0.002$ and $P=0.031$, respectively). LCA levels were negatively related with caudal (i.e. non-embolized) liver volume (CLV) increase and the numbers of Ki-67 positive hepatocytes in this lobe ($\rho = -0.726$, $P < 0.001$; $\rho = -0.718$, $P = 0.009$, respectively). Both targeted and untargeted transcript analysis revealed associations with genes engaged in bile salt homeostasis and liver growth, adding to the notion that OCA-mediated improvement of bile salt homeostasis was in part responsible for enhanced PVE-induced liver growth. Additional factors candidates for mediating growth-stimulating effects of OCA include the secreted factors Bmp3 and Ihh. Our findings indicate that OCA improved bile salt homeostasis, which partly contributed to augmented hypertrophy of the non-embolized liver lobe in rabbits. OCA may be promising to increase the efficacy of PVE in patients with small future liver remnant planned for (extended) partial liver resection.

In **Chapter 5** we assessed the influence of cholestasis on PVE-induced future liver remnant (FLR) hypertrophy. Patients were enrolled with pCCA or CRLM, who underwent PVE before a (extended) right hemihepatectomy on 2016-2019. Volume of segments II and III were considered as FLR and assessed on pre- and post-embolization CT scans. Serum bilirubin above 50 $\mu\text{mol/L}$ was used as a clinical marker of cholestasis. The degree of hypertrophy (DH) as percentual increase, and kinetic growth rate (KGR) as percentage/week, were used to assess PVE-induced hypertrophy. A total of 50 patients (31 CRLM, 19 pCCA) were included. The DH and KGR were similar in patients with pCCA and CRLM. Neither bilirubin levels before biliary drainage (pCCA patients) nor before PVE (entire cohort) were correlated with DH or KGR. For patients with pCCA, unilateral drainage in FLR induced a higher DH than bilateral drainage (6.7 [4.9-7.9] versus 2.7 [1.5-4.2] %, $p = 0.012$). After biliary drainage, ten patients with pCCA remained hyperbilirubinemic, but had comparable DH and KGR to patients with bilirubin levels below 50 $\mu\text{mol/L}$ (DH: 5.6 [3.0-7.5] versus

5.7 [2.4-7.0] %, respectively, $p=0.806$; KGR: 1.7 [1.0-2.4] versus 1.9 [0.8-2.4] %/week, respectively, $p=1.000$). C-reactive protein levels before PVE were negatively correlated with DH and KGR in patients with pCCA (DH: $p=-0.539$, $p=0.038$; KGR: $p=-0.532$, $p=0.041$). Our findings indicate that there was no influence of cholestasis on hypertrophy of the FLR in patients undergoing PVE. Unilateral drainage in FLR was associated with higher liver hypertrophy than bilateral drainage. Elevated inflammation response appears to be associated with impaired liver growth in patients with pCCA.

Automated chyme reinfusion in intestinal failure patients with a temporary double enterostomy (TDE) restores intestinal function and protects against IFALD, but the mechanisms are incompletely understood. In **Chapter 6** we aimed to investigate whether beneficial effects of CR relate to functional recovery of enterohepatic signaling via the bile salt-FGF19 axis. Blood samples were collected from 12 patients, 3 days before, at start, and 1, 3, 5 and 7 weeks after CR initiation. Plasma FGF19, TBS, C4, citrulline (CIT), bile salt composition, liver tests and nutritional risk indices were determined. Paired small bowel biopsies prior to CR and after 21 days were taken and genes related to bile salt homeostasis and enterocyte function were assessed. CR induced an increase in plasma FGF19 and decreased C4 levels, indicating restored regulation of bile salt synthesis via endocrine FGF19 action. TBS remained unaltered during CR. Intestinal FXR was upregulated after 21 days of CR. Molar fractions of secondary and deconjugated bile salts were increased after CR, reflecting restored microbial metabolism of host bile salts. Furthermore, CIT and albumin levels were gradually rising after CR, while abnormal serum liver tests normalized after CR, indicating restored intestinal function, improved nutritional status and amelioration of IFALD. CR increased gene transcripts related to enterocyte number, carbohydrate handling and bile salt homeostasis. Taken together, beneficial effects of CR are partly mediated by recovery of the bile salt-FGF19 axis and subsequent homeostatic regulation of bile salt synthesis, contributing to treat/prevent IFALD.

In **Chapter 7** we provided a general discussion in the context of an important role of enterohepatic bile salt signaling in liver regeneration and intestinal failure, along with a perspective view on future studies and potential implications.

Appendix II

Impact

Relevance

Social and economic relevance

Enterohepatic circulation (EHC) of bile salt plays an important role in gut-liver function¹. However, surgical patients, especially patients with hepatobiliary malignancies, often suffer from a disrupted EHC, which has detrimental effects on gut-liver health². For instance, patients with perihilar cholangiocarcinoma (pCCA) have an obstructed EHC, due to malignant obstruction of the bile duct. These patients can develop obstructive jaundice and cholestatic liver injury³. Furthermore, for patients undergoing partial hepatectomy, especially when accompanied by concomitant removal of the gallbladder during extended resection procedures, the EHC can be temporarily disrupted due to removal of the 'source' of bile salts. Major liver resection can lead to post-hepatectomy liver failure (PHLF) and death, especially in patients with pCCA^{4, 55, 6744}. Likewise, patients with insufficient future liver remnant (FLR) volume suffer unfavorable high risk of PHLF and mortality after partial liver resection⁵. To solve this problem, the nuclear bile salt receptor farnesoid X receptor (FXR) agonist obeticholic acid (OCA) has been shown to accelerate hypertrophy of FLR in experimental portal vein embolization (PVE) model⁶. Pharmacological treatment with OCA has promise to induce augmented FLR hypertrophy in patients scheduled for extended partial liver resection, contributing to decreased incidence of PHLF. From economic perspective, it can avoid more medical costs through decreasing postoperative morbidity.

In addition, intestinal failure (IF) is another common health issue in digestive surgery. IF patients with a temporary double enterostomy (TDE) in the jejunum have a disrupted EHC due to outflow of succus entericus in the stoma bag⁷. These patients require parenteral nutrition (PN) for weeks or even months⁸. However, PN is associated with various complications, such as infectious, mechanical and metabolic complications⁹. An important consequence of IF can be the development of intestinal failure-associated liver disease (IFALD) that is characterized by cholestasis, hepatic steatosis and fibrosis. These complications can result in hospital readmissions and increased healthcare-related costs⁷. Additionally, the patients' quality of life is seriously affected by IF. Chyme (intestinal or fistula secretions) reinfusion (CR), an extracorporeal enteral nutrition technique, contributed to improved nutritional status, better intestinal absorptive function, recovery from IFALD, reduced intestinal secretions and shorter period to weaning of PN.

Scientific relevance

In this thesis, we conducted several studies to investigate the role of enterohepatic bile salt signaling in compensatory liver growth and intestinal failure. Firstly, we systematically assessed the effect and route of preoperative biliary drainage (BD) in patients with resectable pCCA. Due to high risk of bias in the current available evidences, we propose future randomized prospective studies to evaluate the value of preoperative BD in resectable pCCA. Furthermore, we explored the suitability of human precision-cut liver slices (hPCLS) as an *in vitro* model to study liver regeneration. Further optimization of the procedure and/or culture conditions, and consideration of patient factors are required before (un)suitability of hPCLS as a model system can be judged. In addition, we revealed that OCA improves bile salt homeostasis after PVE in rabbits, likely through inhibiting hepatic synthesis and promoting export of bile salts. This underlies in part the accelerated hypertrophy of the non-embolized liver lobe. It is promising to further evaluate the effect of OCA in surgical patients with small FLR scheduled for (extended) hepatectomy. A large body of safety data with OCA is available from phase III and ongoing phase IV trials in patients with chronic cholestatic and metabolic liver diseases¹⁰⁻¹². Finally, through analyzing bile salt-FGF19 signaling in IF patients treated with chyme reinfusion, we revealed that chyme reinfusion restored EHC of bile salt and bile salt-FGF19 signaling, which partly contributed to improved gut-liver function in this patient population.

Target groups

The studies performed in this thesis provide novel knowledge and perspectives for the scientific community. We demonstrated that OCA improved bile salt homeostasis, and this in part contributes to accelerated hypertrophy of the non-embolized liver lobe after PVE in rabbits. These observations can inspire scientists and clinicians to explore the use of pharmacological activation of FXR to accelerate PVE-induced liver growth in human studies.

Meanwhile, insights from the studies described here can also benefit clinical doctors. Given the importance of the EHC of bile salts in daily practice, nutritionists can consider chyme reinfusion as first-line treatment in IF patients with a TDE. This can also improve/prevent some PN-related complications. In addition, because internal biliary

drainage can restore EHC of bile salt, physicians can consider this potential benefit when choosing a drainage method in patients with biliary obstruction.

The ultimate aim of medical research is to improve the health of humans. Our findings may contribute to improve outcomes of surgical patients, including patients planned for (extended) liver partial resection, and IF patients with TDE.

Innovation and Implementation

In this thesis, we investigated the link between enterohepatic bile salt signaling and liver regeneration and intestinal failure. For the first time, a systematic review of systematic reviews was conducted to assess the effect and route of preoperative BD in patients with resectable pCCA and indicated a high risk of bias in current available systematic reviews. Additionally, although our previous study ⁶ showed that OCA promoted liver regeneration following PVE in a rabbit model, the mechanism was not clarified. Our study indicated that improved bile salt homeostasis contributed to accelerated liver growth induced by PVE in OCA-treated animals. Furthermore, for IF patients with TDE, the RESCUE study revealed that restored enterohepatic bile salt-FGF19 signaling underlies in part the beneficial effects of chyme reinfusion on gut-liver function, thus reversing IFALD.

The effect of preoperative BD in resectable pCCA is under debate. Further studies with large sample sizes should focus on the indications for BD, subsequently determine what kind of patients with pCCA can benefit from preoperative BD. Additionally, the effectiveness of OCA for promoting liver growth has been investigated in the rabbit model of PVE. Future research to study effect of OCA on tumor growth in embolized lobes in pre-clinical setting are recommended. Moreover, in the nutritional units, chyme reinfusion (Fig. 1) should be considered as preferred treatment for IF patients with TDE to avoid potential PN-associated morbidity and improve intestinal rehabilitation. A portable roller pump would allow patients to be ambulant during the pre-operative setting ¹³.

Figure 1. Chyme reinfusion technique with the automated roller pumps. Continuous chyme reinfusion was performed in a closed system of extra-corporal circulation of chyme using the Enteromate II® system (Labodial, Clayes-sous-Bois, France). The left pump works permanently and aspirates the jejunal effluent from the stoma bag toward a plastic container. When the minimal volume of about 10 ml in the container is exceeded, the second pump starts and the contents are infused into the downstream small intestine. This illustration is a modification from a picture of Picot *et al.* (2017) ⁷.

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Appendix III

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Appendix IV

Curriculum Vitae

Xinwei Chang was born on the November 28th, 1988 in Henan, China. In September of 2009, he started his study on Clinical Medicine at Nanchang University and received his bachelor's degree in July of 2014. Later on, in September of 2017, he started his master's study on Surgery at Zhengzhou University. Her master thesis was entitled: "TERT promoter mutation in hepatocellular carcinoma and renal pelvic carcinoma". Under the supervision of Prof. Xiuxian Ma, he obtained his master's degree in July of 2017.

In September of 2017, he was awarded a national grant from the China Scholarship Council and started his PhD study at the Department of Surgery of Maastricht University. His research was supervised by Prof. Steven Olde Damink and Dr. Frank Schaap. During his PhD study, he focused on the role of bile salt-FGF19 signaling in promoting liver regeneration and improving intestinal failure. The results of his PhD studies are presented in this thesis. He was the member of European Association for the Study of the Liver and is also a member of Dutch Society of Hepatology. In 2019, he received a seeding grant from Division 2 of School of Nutrition and Translational Research in Metabolism in Maastricht University. In 2020, he was awarded a young investigator bursary in the International Liver Congress. After his PhD graduation in 2021, he will move back to China and start his clinical training in surgery.

Appendix V

List of publications

List of publications

1. Koelfat KVK*, Picot D*, **Chang X***, Desille M, van Eijk HM, van Kuijk SMJ, Lenicek M, Layec S, Carsin M, Dussaulx L, Seynhaeve E, Trivin F, Lacaze L, Thibault R**, Schaap FG**, Olde Damink SWM**. Chyme reinfusion restores the regulatory bile salt-FGF19 axis in intestinal failure patients. *Hepatology* 2021, doi: 10.1002/hep.32017. (*Authors share co-first authorship, **Authors share co-senior authorship)
2. **Chang X**, Schaap FG, Shen H, Dejong CHC, Groot Koerkamp B, van der Leij C, Neumann UP, Kleijnen J, Olde Damink SWM. The effect and route of preoperative biliary drainage in patients with resectable perihilar cholangiocarcinoma. (submitted)
3. **Chang X**, van Himbeeck C, Olthof PB, Jin H, Soons Z, van Eijk HM, van Goolen R, Jansen PL, van Gulik T, Heger M, Olde Damink SWM, Schaap FG. Obeticholic acid accelerates portal vein embolization-induced liver hypertrophy by improving bile salt homeostasis in rabbits. (submitted)
4. **Chang X***, Korenblik R*, Olij B, Knapen B, van der Leij C, Brandts L, Heise D, den Dulk M, Neumann UP, Schaap FG, van Dam R, Olde Damink SWM. No influence of cholestasis on portal vein embolization-induced hypertrophy of the future liver remnant. (*Authors share co-first authorship) (submitted)
5. Shen H, van der Kleij R, van der Boog PJM, **Chang X**, Wang W, Song X, Li Z, Lou X, Chavannes N. Prevalence of reduced kidney function, kidney function decline and related risk factors among a primary care population in China: A repeated cross-sectional study. (submitted)
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10. **Chang X**, Ma X, Zhu R, Li J. Imaging evaluation of residual cancer after radiofrequency ablation of hepatocellular carcinoma. *World Chinese Journal of Digestology* 2016, 24(10): 1539-1544.
11. **Chang X**, Ma X, Shen H, Li J, Qiao Z, Wan J. Clinical analysis of 12 patients with sarcomatoid carcinoma of the gallbladder. *World Chinese Journal of Digestology* 2016, 24(13): 2063-2069.

Oral presentations

1. May, 2021: Dutch Translational Metabolism Conference-Seminar 4 (online). Chyme reinfusion restores the regulatory bile salt-FGF19 axis in intestinal failure patients
2. November, 2020: NUTRIM Symposium (online). The effect of chyme reinfusion (CR) on the bile salt/fibroblast growth factor 19 (FGF19) signaling in intestinal failure patients with a temporary jejunal double enterostomy: RESCUE study
3. August, 2020: The International Liver Congress (online). The effect of chyme reinfusion on the bile salt/fibroblast growth factor 19 signaling in intestinal failure patients with a temporary jejunal double enterostomy
4. November, 2019: 16th International Medical Postgraduate Conference (Hradec Králové, Czech Republic). The effect of chyme reinfusion on the bile salt/FGF19 signaling axis in intestinal failure patients with a temporary jejunal double enterostomy

Poster presentations

- August, 2020: The International Liver Congress (online). Preoperative biliary drainage in perihilar cholangiocarcinoma: an overview of systematic reviews