Doctoral thesis

The gut-liver-axis in secondary sclerosing cholangitis after critical illness

submitted by

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1. Statutory Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Acknowledgements have been made in the text to all other material used. Throughout this thesis and in all related publications I followed the "Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz ".

Graz, 27th of April 2021

Andreas Blesl

2. Disclosures

Parts of this thesis have been published in:

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4. Table of contents

5. Abbreviations

AIH	autoimmune hepatitis
ALD	alcoholic liver disease
ANCOM	analysis of composition of microbiomes
ANOVA	analysis of variance
APC	argon plasma coagulation
ARDS	acute respiratory distress syndrome
BA	bile acids
BALB/c	albino, laboratory-bred strain of the house mouse
BMI	body mass index
BSH	bile salt hydrolases
CARD9	caspase recruitment domain-containing protein 9
CD	Crohn's disease
CIP	critically ill patients
CRP	C-reactive protein
CYP7A1	Cytochrome P450, Family 7, Subfamily A, Polypeptide 1
DAO	diaminoxidase
DNA	deoxyribonucleic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ERCP	endoscopic retrograde cholangiopancreatography
F/B ratio	Firmicutes/Bacteriodetes ratio
FDR	false discovery rate
FGF-19	Fibroblast growth factor 19
FMT	fecal microbiome transplantation
FXR	farnesoid X receptor

GGT	Gamma-Glutamyl Transferase
HC	healthy controls
HCO3	hydrogen carbonate
HCV	hepatitis C virus
HIV	human immunodeficiency virus
IBD	inflammatory bowel disease
ICU	intensive care unit
I-FABP	intestinal fatty-acid binding protein
INR	international normalized ratio
kPa	kilopascal
LBP	lipopolysaccharide-binding protein
LDA	linear discriminant analysis
LPS	lipopolysaccharides
MAMPs	microbial-associated molecular patterns
MELD	model of end-stage liver disease
MRCP	magnetic resonance cholangiopancreatography
NAFLD	non-alcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
NGM282	engineered fibroblast growth factor 19 analogue
NLRP3	NOD-, LRR- and pyrin domain-containing protein 3
NMMDS	non-metric multidimensional scaling
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
norUDCA	norUrsodeoxycholic acid
NSAIDS	non-steroidal anti-inflammatory drugs
OTU	operational taxonomic unit

PAMPs	pathogen-associated molecular patterns
PBC	primary biliary cholangitis
PCR	polymerase chain reaction
PHG	portal hypertensive gastropathy
PPAR	peroxisome proliferator-activated receptor
PPI	proton pump inhibitor
PSC	primary sclerosing cholangitis
RDA	redundancy analysis
RNA	ribonucleic acid
SC-CIP	secondary sclerosing cholangitis in critically ill patients
sCD14	soluble cluster of differentiation 14
TGF Beta	transforming growth factor beta
TGR5	Takeda G-protein-coupled receptor 5
TNF Alpha	tumor necrosis factor alpha
UC	Ulcerative Colitis
UDCA	ursodeoxycholic acid
VIF	variance inflation factor
VLP	virus-like particles

6. Abstract in German

Hintergrund: Die seltene cholestatische Lebererkrankung sekundär sklerosierende Cholangitis bei kritisch kranken Patienten (SC-CIP) tritt bei Patient*innen ohne vorherige Lebererkrankung nach einer Langzeitbehandlung auf einer Intensivstation mit invasiver Beatmung und hämodynamischer Unterstützung auf.

Ziele: Das Ziel der Arbeit war die Beschreibung der gesunden Darm-Leber-Achse und pathologische Störungen dieses Gleichgewichts bei chronischen cholestatischen Lebererkrankungen zu skizzieren. Wir planten die Zusammensetzung des Stuhlmikrobioms, die Darmpermeabilität, die bakterielle Translokation und die Gallensäureprofile aus dem Serum von SC-CIP-Patient*innen im Vergleich zu Patient*innen nach kritischer Erkrankung ohne Lebererkrankung, Patient*innen mit Zirrhose und gesunden Kontrollen im Rahmen einer Fallkontrollstudie zu untersuchen. Darüber hinaus haben wir uns zum Ziel gesetzt, die Häufigkeit und Eigenschaften von gastrointestinalen Blutungen bei SC-CIP im Rahmen einer retrospektiven Analyse zu bewerten.

Methoden: Das aktuelle Wissen über die Darm-Leber-Achse bei chronischen cholestatischen Lebererkrankungen wird in Form eines narrativen Reviews dargelegt. 16S-rDNA wurde aus dem Stuhl der vier Kohorten isoliert und unter Verwendung der Illumina-Technik sequenziert. Diaminoxidase, Zonulin, lösliches CD14 und Lipopolysaccharide Binding Protein wurden im Serum und Calprotectin im Stuhl bestimmt. Serumgallensäuren wurden durch Massenspektrometrie (HPLC-MS) analysiert. Für den retrospektiven Teil der Arbeit bezüglich gastrointestinaler Blutungen wurden Patient*innen mit diagnostizierter SC-CIP identifiziert und mit einer Kontrollgruppe von Patient*innen verglichen, bei denen sich in der Anamnese eine Intensivbehandlung nach einer Herzoperation fand, jedoch die Leber keinen Schaden nahm.

Ergebnisse: Im Rahmen des Reviews wurde dargestellt, dass die Darm-Leber-Achse bei chronisch cholestatischen Lebererkrankungen verändert ist und in die Pathogenese dieser Erkrankungen involviert sein dürfte. Achtzehn Patient*innen mit der Diagnose SC-CIP, 11 Kontrollen nach Intensivaufenthalt, 21 Patient*innen mit Leberzirrhose und 21 gesunde Kontrollen wurden in die Mikrobiomanalyse einbezogen. Das Mikrobiom von Patient*innen mit SC-CIP zeigte im Vergleich zu gesunden Kontrollen eine verringerte Alpha-Diversität und eine veränderte Beta-Diversität. Eine Verschiebung hin zu pathogenen Taxa und eine Oralisierung des fäkalen Mikrobioms wurde beobachtet. Eine beeinträchtigte

Darmpermeabilität, eine Erhöhung der Biomarker für bakterielle Translokation und ein verändertes Serumgallensäureprofil wurden zusätzlich in der SC-CIP Kohorte erkannt. Die Kontrollgruppe nach Intensivaufenthalt zeigte ebenso eine verringerte Diversität und eine veränderte taxonomische Zusammensetzung des Mikrobioms im Vergleich zu gesunden Kontrollen. Dreiundfünfzig Patient*innen mit SC-CIP und 19 Kontrollen wurden in die retrospektive Studie eingeschlossen. Die Häufigkeit gastrointestinaler Blutungen betrug 30% bei SC-CIP (16 Patient*innen) und 5% in der Kontrollgruppe (ein Patient) ($p = 0,03$). Bei Patient*innen mit SC-CIP wurden 13 Blutungen im oberen Gastrointestinaltrakt beobachtet. Der häufigste Grund für Blutungen waren gastroduodenale Ulzerationen.

Schlussfolgerung: Die Darm-Leber-Achse ist bei SC-CIP gestört. Der Lebererkrankung kann aber nicht alleine beschuldigt werden diese Veränderungen hervorzurufen, da die Mikrobiomzusammensetzung der Kontrollgruppe mit kritischer Erkrankung aber ohne Leberpathologie ebenfalls gestört zu sein scheint. Gastrointestinale Blutungen sind eine häufige Komplikation bei Patienten mit SC-CIP, es bleibt jedoch unklar, welcher Faktor für diese Häufung verantwortlich ist.

7. Abstract in Englisch

Background: The rare chronic cholestatic liver disease secondary sclerosing cholangitis in critically ill patients (SC-CIP) occurs in patients after long-term intensive care treatment with invasive ventilation and hemodynamic support without prior liver pathology.

Aims: We aimed to describe the healthy gut-liver axis and to outline pathological disruptions of this equilibrium in chronic cholestatic liver diseases. We planned to assess the stool microbiome composition, gut permeability, bacterial translocation, and serum bile acid profiles of SC-CIP patients compared to patients after critical illness without liver disease (CIP controls), patients with cirrhosis, and healthy controls within a case-control study. Furthermore, we aimed to evaluate the frequency and characteristics of gastrointestinal bleedings in SC-CIP with a retrospective analysis.

Methods: We conducted a narrative review outlining the actual knowledge about the gut-liver axis in chronic cholestatic liver diseases. 16S rDNA was isolated from stool of the four cohorts and sequenced using the Illumina technique. Diamine oxidase, zonulin, soluble CD14 and lipopolysaccharide-binding protein were determined in serum and calprotectin in stool. Serum bile acids were analyzed by high-performance liquid chromatography-mass spectrometry

(HPLC-MS). For the retrospective part concerning gastrointestinal bleedings, patients with diagnosed SC-CIP were identified and compared to a control group of patients with history of intensive care treatment after cardiac surgery, but without development of SC-CIP.

Results: With the conducted review, we could demonstrate that the gut-liver axis is altered in chronic cholestatic liver diseases and that observed alterations seem to play a role in the pathogenesis of these diseases. Eighteen SC-CIP patients, 11 CIP controls, 21 cirrhotic patients, and 21 healthy controls were included in the microbiome analysis. The microbiome of SC-CIP patients showed reduced alpha diversity and altered beta diversity compared to healthy controls. A shift towards pathogenic taxa and an oralization of the fecal microbiome was observed. Impaired gut permeability, elevation of biomarkers of bacterial translocation, and an altered serum bile acid profile were further recognized in SC-CIP. CIP controls also showed decreased diversity and a changed taxonomic composition of the microbiome compared to healthy controls. Fifty-three patients with SC-CIP and 19 controls were included in the retrospective study. Frequency of gastrointestinal bleeding was 30% in SC-CIP (16 patients) and 5% in the control group (1 patient) ($p=0.03$). In SC-CIP, 13 bleedings were reported in the upper gastrointestinal tract. Most common reasons for bleeding were gastroduodenal ulcers.

Conclusion: The gut–liver axis is altered in SC-CIP. The liver disease cannot solely be accused to induce these alterations, since there seems to be an additional lasting effect of the long-term intensive care treatment on microbiome composition. Gastrointestinal bleeding is a frequent complication in patients with SC-CIP, but it remains to be determined which factor is responsible for this susceptibility.

8. Introduction

8.1. Chronic cholestatic liver diseases

Cholestasis in liver diseases occurs due to defective biliary anatomy or impaired biliary flow (1). By its appearance, cholestasis can be divided into acute (choledocholithiasis, malignancy, and others) and chronic forms. The designation chronic cholestatic liver diseases implies inherit cholestatic diseases, the generic term secondary sclerosing cholangitis (SSC) subsuming diseases induced by ischemia, mechanical obstruction, autoimmune responses, infections, or liver damage due to toxic substances as well as primary sclerosing cholangitis (PSC) and primary biliary cholangitis (PBC) (2, 3).

Incidence of these chronic cholestatic diseases (SSC, PSC, and PBC) is rare (0.3 to 5.8 per 100.000 people per year) and the pathogenesis is still not completely explored yet, but points towards a multifactorial evolution of these diseases. Clinical symptoms can occur in all ages, with elevated serum liver parameters, jaundice, and pruritus being the most common. Sex distribution is not equal in all three disease types and without liver transplantation prognosis is still limited in many patients because causal treatment options are restricted for PBC and are lacking for SSC and PSC (2, 4, 5).

8.2. Secondary sclerosing cholangitis in critically ill patients (SC-CIP)

Secondary sclerosing cholangitis in critically ill patients (SC-CIP) is a sub-entity of SSC. It was first described in 2001 (6). The disease occurs in selected patients, primarily men, after long-term intensive care treatment with invasive ventilation and with the need for vasoactive drugs because of hemodynamic instability. Affected patients do not suffer from prior liver pathologies (7). Severe internal diseases as sepsis, myocardial infarction, pancreatitis or acute respiratory distress syndrome (ARDS) as well as burns, (poly)trauma, cardiac and thoracic surgery are reasons for intensive care treatment with an increased risk for the development of SC-CIP (3, 8, 9). Additionally, abdominal obesity boosts occurrence of the disease (10). Usually, it takes intensive care treatment between 30 to 40 days to develop SC-CIP, but an exception with a short intensive care unit (ICU) stay of nine days has been reported (7, 11). Cholestatic liver parameters typically start rising around day 5-7 of the ICU stay with the first often being the gamma-glutamyl transpeptidase (GGT) (9, 12). Elevation of bilirubin may take 20 days to occur. Interestingly, a decrease of these levels can emerge in disease course despite ongoing progression of the disease (13). Diagnosis can be established with display of pathologic features of the biliary tree by magnetic resonance cholangiopancreatography (MRCP) or endoscopic

retrograde cholangiopancreatography (ERCP) (12). Therapeutic efforts include antibiotic treatment of bacterial cholangitis and removal of biliary casts by ERCP situated in the intra- or extrahepatic biliary tree (14). But because of the lack of causal drug therapy liver transplantation remains the only curative treatment option (15). Prognosis of the disease is serious in many cases and transplant-free survival is stated to be around 40 months (9). If liver transplantation is carried out, outcomes are comparable to other indications for transplantation (16).

8.3. PSC and PBC

PSC induces inflammation of the intra- and extrahepatic biliary tree and therefore causes progressive liver damage leading to fibrosis and liver cirrhosis which may make liver transplantation necessary. PSC occurs more frequently in men (65-70%) and is often combined with inflammatory bowel diseases (IBD) (70-80%). The risk for hepatobiliary malignancies is increased as well as the risk for colorectal carcinoma in patients with concomitant IBD (17-20). As mentioned, medical treatment is not approved yet and mean liver transplantation-free survival is 14.5 years (21, 22). Without liver transplantation, prognosis is often limited and progression to cirrhosis can develop within weeks in the worst case (13, 14, 16). PBC causes progressive devastation of the intrahepatic biliary tree and is first diagnosed typically in women of middle age. The sex ratio is reported to be 10:1. Initial clinical symptoms can comprise pruritus, fatigue, and jaundice. For PBC, medical treatment options have been explored: ursodeoxycholic acid, bezafibrate, and obeticholic acid can be used in clinical routine to treat patients. Because of these increased conservative treatment options, liver transplantation can be avoided in many patients and only need to be carried out in 4% of affected patients (2, 4, 5, 23).

8.4. Etiologies of chronic cholestatic liver diseases

Up to now, etiologies of chronic cholestatic liver diseases have not been fully understood (2). The widely accepted hypothesis for the occurrence of SC-CIP implies ischemic injury of the biliary system during a critical illness caused by hypotension with consecutive formation of biliary casts (12). In contrast to hepatocytes, which receive perfusion from the hepatic arteries and the portal vein, blood circulation in the biliary epithelium is only ensured by branches of the hepatic arteries. Therefore, the biliary tree is more vulnerable to ischemic injury (9, 24). Recurrent biliary infections in these patients may further promote disease progression (12). Additionally, occurrence of the disease has been found to be favored by nucleotide-binding

oligomerization domain-containing protein 2 (NOD2) gene variants (25). In PSC, genetic and environmental factors have been investigated and it was successful to describe associations with the human leukocyte antigen system and other gene loci. As a limitation, a clear causal relationship could not be confirmed (26, 27). As disease recurrence after liver transplantation is observed in PSC patients, it seems plausible that extrahepatic drivers play a relevant role in the pathogenesis (18). The evolution of PBC is favored by environmental triggers like infections (Epstein-Barr virus, urinary tract and mycobacterial infections, Chlamydia, Helicobacter pylori) and smoking, as well as by genetic predisposition and autoimmune factors (4, 28-34).

Within the last years, more and more data about the role of the microbiome and the gut barrier in the pathophysiology of liver diseases including the cholestatic liver pathologies PSC and PBC have been generated. The underlying concept is called the gut-liver axis, which is thought to be a common pathogenetic principle and a therapeutic target worth investigating (35).

8.5. The gut-liver axis

8.5.1. Introduction

Interactions between the gut and the liver work in both directions, from the liver to the gut and from the gut to the liver (36-38) (Figure 1). The gut microbiome contains thousands of species with trillions of microbes ranging from bacteria to fungi, viruses, protozoa, and archaea. (39). In early life, composition of the gut microbiome is affected by kind of delivery (natural birth vs. caesarean section), breastfeeding, and genetics. Microbiome composition changes with age and is further influenced by food and drug intake, smoking, physical activity, and stress (40, 41). Disruption of the equilibrium of the gut microbiome leads to dysbiosis and further to augmented gut permeability, which prepares the ground for translocation of microbes and microbial products called microbial or pathogen-associated molecular patterns (MAMPs/PAMPs) (37, 42, 43). These patterns are released into the blood circulation and can be recognized by immune receptors of liver cells, which activate inflammation cascades leading to fibrosis and further to cirrhosis in liver diseases (43). On the other hand, the liver interacts with the gut through the biliary system and the systemic circulation by producing and releasing bile acids and inflammatory mediators (f.e. cytokines) (44). Primary bile acids are synthesized from cholesterol in the liver. They are then conjugated with taurine or glycine in hepatocytes and these conjugated bile acids are dispersed into the intestine where they are responsible for the absorption of lipids and fat-soluble vitamins. In the terminal ileum, the vast amount of bile acids is actively reabsorbed. The remaining 5% are metabolized to secondary bile acids by microbial metabolisms in the colon and are either passively reabsorbed or excreted. Conversion

from primary to secondary bile acids (deconjugation and dihydroxylation) is conducted by bile salt hydrolases (BSH) and 7α-dehydroxylase. These enzymes are expressed by gut microbes of all major phyla and by the genera *Bacteroides*, *Eubacterium, Clostridium, Escherichia,* and *Lactobacillus*. (43, 45-47). The function of bile acids is not limited to the uptake of lipids. They are further used as signaling metabolites and are able to interact with cellular receptors of the gut and with luminal microbes (46, 48, 49).

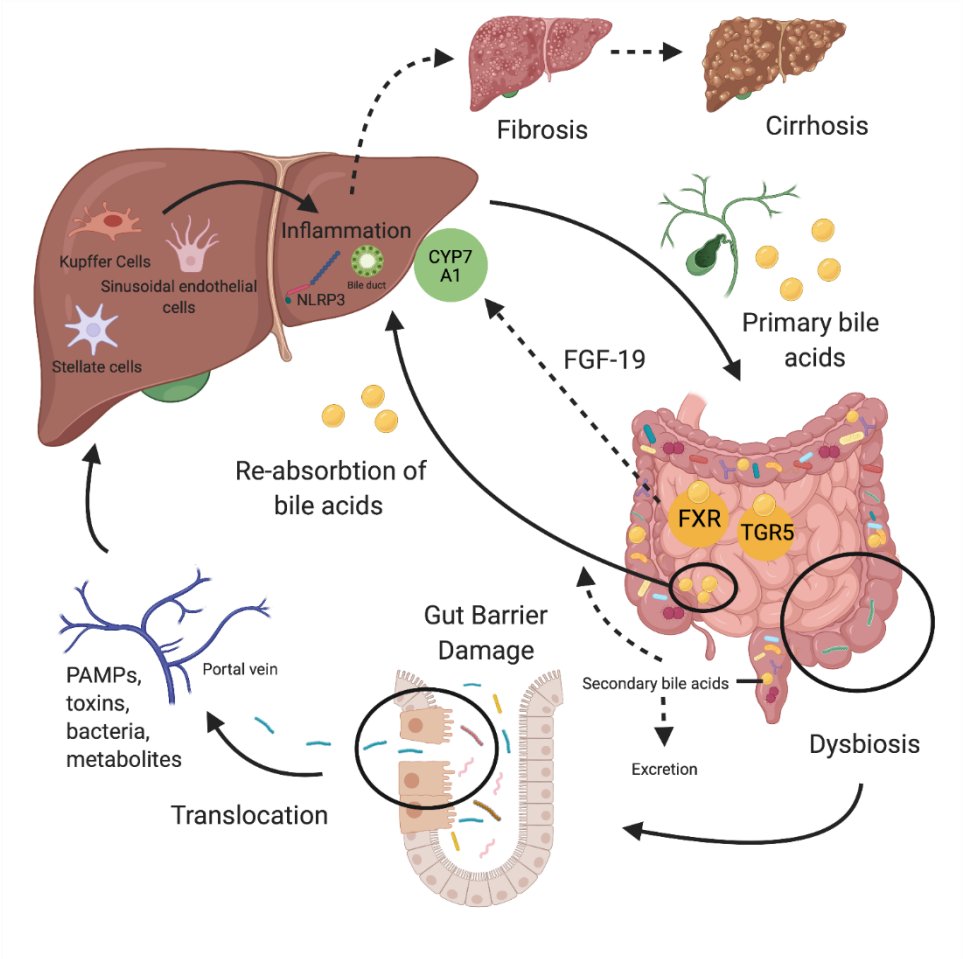

Figure 1. The gut-liver axis. Dysbiosis of the microbiome harms the gut barrier and leads to translocation of bacteria, metabolites, toxins, and pathogen-associated molecular patterns (PAMPs). They can reach the liver through the portal vein and induce inflammation cascades causing fibrosis and cirrhosis in the course of liver diseases. Bile acids are synthesized from cholesterol in the liver. Following conjugation, they are released into the biliary system and arrive in the gut as conjugated primary bile acids. In the intestine, bile acids enable fat digestion and they interact with cellular receptors and gut microbes. Farnesoid X receptor (FXR) and Takeda G-protein-coupled receptor 5 (TGR5) are the most important of these receptors. Binding of bile acids to FXR activates synthesis of fibroblast growth factor 19 (FGF-19) which acts as negative feedback mechanism for bile acid synthesis in hepatocytes. Most bile acids are reabsorbed in the terminal ileum, but a small number is released into

the colon. Gut microbes inhabiting the colon metabolize these bile acids to secondary bile acids, which are also reabsorbed or excreted. CYP7A1 = Cytochrome P450, Family 7, Subfamily A, Polypeptide 1; NLRP3 = NOD-, LRR- and pyrin domain-containing protein 3. Figure reproduced from (50) with permission of Nutrients. Figure created by Andreas Blesl with BioRender.com.

8.5.2. Gut permeability and bacterial translocation

8.5.2.1. Introduction

Enterocytes, which are kept together by junctional proteins, form the intestinal barrier (51). This barrier is maintained by commensal bacteria in the gut lumen producing short-chain fatty acids and thereby inhibiting pathogenic microbes as well as by a layer of mucins, antibacterial substances, and immunoglobulins (43, 51-54). The physiological function of the intestinal barrier is to regulate transportation of nutrients across tight junctions and to impede passing of microbes from the mucosa and therefore hinder them to enter the systemic circulation (43). Integrity of this barrier can be impaired by dysbiosis of the gut microbiome induced by infections, inflammation of different origins, western diet, antibiotic usage, or alcohol consumption (51, 55-61). Microbiome alterations also influence the mucus film. It was demonstrated that reduced abundance of *Akkermansia muciniphila* decreases the mucus layer (62-67). A disrupted gut barrier enables microbes and PAMPs to translocate into the systemic circulation and to get to the liver through the portal vein (43). In the liver, they induce inflammation through receptors on hepatic stellate cells, Kupffer cells, liver sinusoidal endothelial cells, and probably by activating the NLRP3 inflammasome (37, 68-71). Further mechanisms playing their part in the pathogenesis of liver diseases include translocation of short-chain fatty acids like butyrate, choline metabolites involved in accumulation of triglycerides in the liver, and ethanol and its metabolite acetaldehyde from the gut (43, 72-74). A certain amount of bacterial translocation is physiological in healthy humans and has an important role in building up host immunity. In liver diseases, especially in cirrhosis, bacterial translocation is augmented and is known to be a pathophysiological process of disease progression influenced by misguided reactions of the immune system to microbes and PAMPs, bacterial overgrowth, and lymphatic tissue defects (75-77).

Direct assessment of gut permeability and bacterial translocation in clinical conditions is difficult. Therefore, biomarkers present a decent alternative option to indirectly evaluate the function of the gut barrier. Assessment of the transfer of differently sized sugars across the gut epithelium by investigating lactulose, rhamnose, and mannitol urinary excretion after oral ingestion (lactulose:rhamnose and lactulose:mannitol ratio) is considered as the gold standard

to describe the amount of small intestinal permeability in context to the disposable absorptive space (78-80). As a limitation, the test is difficult to carry out for patients, pre-analytic errors can harm findings, and quantitative analytic methods of different sugars are not sufficiently available for usage in clinical routine (78). To screen for augmented intestinal permeability, the radioactive 51Cr-EDTA can be used by measuring its concentration in urine. However, the usage of radioactive tracers in clinical routine as well as in research settings is cumbersome (81). Therefore, biomarkers that can be assessed from stool or serum samples are of great interest. Enterocytes located in the mucosal layer of the jejunum and the colon express intestinal fatty-acid binding protein (I-FABP). As a consequence, the protein is released into the systemic circulation if epithelial cells get damaged and I-FABP plasma levels have been implemented as marker of gut permeability and cell death. Correlations of this protein with activity in celiac disease and ischemia have been suggested (81-85). Secretory immunoglobulin A (sIgA) can be found in the mucosal surface of the intestine and is responsible for gut homeostasis and composition of the gut microbiome, inhibits inflammation, and protects against pathogens and toxins (54, 86-88). By measuring its concentration and composition in stool the crosstalk between the immune system and the microbiome at the gut barrier can be outlined. Another biomarker for the assessment of gut permeability is Zonulin, a protein measured in stool and thought to be pre-haptoglobin 2. Because of the modulation of intracellular tight junctions, it impacts intestinal permeability (89, 90). It has been shown to be modifiable by interventions targeting the microbiome (91, 92). The enzyme diamine oxidase (DAO) can be determined in the intestine from the duodenum to the ileum and is responsible for oxidative deamination of histamine. Investigations showed the inverse correlation of serum concentration of the enzyme with gut permeability of the small intestine. Because it can be measured in serum it is easier to obtain and store compared with stool samples (93, 94). Additionally, tight junction proteins (f.e. claudin-3 in urine) and the amino acid citrulline, secreted by enterocytes of the small bowel, can be investigated (81). The PAMP lipopolysaccharide (LPS) consists of glycolipids from the outer membrane of gram-negative bacteria and presents a useful marker for bacterial translocation and endotoxemia. LPS-binding protein (LBP) correlates with LPS and is able to bind to receptor CD14, existing in soluble (sCD14) and in membrane-bound (mCD14) forms, which can induce inflammation. LBP and sCD14 levels are surrogate parameters for endotoxemia and can be measured practically with sandwich ELISA kits in plasma (22, 95, 96). At least, calprotectin, a protein located primarily in neutrophils, but additionally also in monocytes and macrophages, is released into the gut lumen during inflammation of the intestine and can be measured in feces (97).

8.5.2.2. Chronic cholestatic liver diseases

The fact that PSC patients often suffer from concomitant IBD and that colectomy prior to liver transplantation reduces PSC recurrence risk in the donor liver supports the hypothesis of the involvement of the gut-liver axis in disease emergence and progression (98, 99). The IBD-PSC phenotype seems to be a distinct IBD phenotype and often presents with an atypical mild right-sided colitis with rectal sparing and backwash ileitis (100-102). In both Crohn's disease and ulcerative colitis, increased intestinal permeability was linked to genetic variants of NOD2 (103-106). However, a reliable association of genetic variants predisposing to increased gut permeability and leading to the evolution of PSC could not be proven yet (107). A key question still remains insufficiently explored: are alterations of gut permeability a direct consequence of the inflammatory state or a primary impairment? Data acquired from animal models suggest that mucosal inflammation in IBD is a consequence of failure of the gut barrier and that therapeutical improvement of the gut barrier function inhibits inflammation (108-113). In mice, disruption of the barrier led to dysbiosis of the gut microbiome and caused colitis (114). The other way round, dysbiosis can also alter permeability and trigger liver injury. *Klebsiella pneumonia* was suggested to harm the epithelial barrier and induce bacterial translocation and liver inflammation in PSC patients. Hepatic T-helper 17 cells, which can cause an immunological reaction favoring liver injury, were associated with increased permeability (115). Furthermore, the translocation of *Lactobacillus gasseri* led to interleukin-17–mediated damage of the liver of mice (116). In humans, biomarkers released from T cells and related to interferon-gamma as well as LBP and sCD14 were augmented in PSC and were associated with reduced transplant-free survival. sCD14 was further increased in patients with hepatobiliary cancer. A relationship between gut barrier dysfunction, inflammation, and cancer development can therefore be discussed (22, 117). In biliary epithelial cells of liver specimens of PSC (and also PBC) patients abnormal high endotoxin accumulation has been reported (118). Additionally, augmented markers of structural gut damage were linked to worse outcome in PSC (119). Increased permeability in PSC could not be confirmed with measurement of excretion of lactulose/L-rhamnose in one study, probably caused by the small number of recruited patients and because of described technical barriers of this assay (120). In summary, data suggest that increased gut permeability and consequently bacterial translocation are key features in PSC disease evolution. Furthermore, biomarkers indicating gut permeability show correlation with clinical outcome parameters. Still, it is not finally clarified if gut barrier failure precedes or follows the emergence of the disease.

Bacterial translocation is also debated to be part of the pathogenesis of PBC (121). In BALB/c mice, long-term exposure to bacterial antigens led to occurrence of histological features of the liver comparable to PBC (122). In humans, increased permeability of the small intestine and the stomach was reported in PBC patients (105, 123, 124). In damaged bile ducts of liver samples and serum of PBC patients, lipoteichoic acid, a part of the cell wall of gram-positive bacteria, was elevated compared to a control group suffering from hepatitis C (125). Additionally, increased levels of LPS were described in PBC (126). In conclusion, described findings suggest impairment of the gut barrier in PBC patients, but the impact of this alteration in disease evolution still needs to be determined.

8.5.3. Bile acids

8.5.3.1. Introduction

A healthy gut-liver axis is dependent on a functioning enterohepatic circulation of bile acids. Accumulation of bile acids can induce oxidative stress and inflammation in the liver resulting in biliary fibrosis and cirrhosis (127). Primary bile acids located in the gut lumen can bind to farnesoid X receptor (FXR) in intestinal epithelial cells. FXR binding activates transcription of fibroblast growth factor 19 (FGF-19), which is then released into the circulation. FGF-19 further proceeds to the liver and lowers bile acid synthesis via CYP7A1 (43, 46, 127-129) and exercises impact on lipid and glucose metabolism (130-133). Takeda G-protein-coupled receptor 5 (TGR5) is the second crucial receptor in enterocytes for bile acid binding. It is involved in energy, glucose, and metabolic homeostasis and can activate anti-inflammatory immune responses (134-139). Direct and indirect effects of bile acids on the composition of the gut microbiome include the promotion of antimicrobial peptides by FXR binding and the damage of bacterial cell membranes. This impact is more pronounced in the small intestine and accounts for small bowel bacterial overgrowth in PBC, cirrhosis, and in mouse models following bile duct ligation (37, 43, 140-144). Contrary, certain species comprising *Escherichia coli* and *Helicobacter spp.* are resistant to the influence of bile acids (145-148), whereas diminution of secondary bile acids, which hold down growth of *Clostridioides difficile* spores, lowers colonization resistance (149). As already mentioned, primary bile acids are metabolized to secondary bile acids in the colon by microbes. In patients with advanced liver cirrhosis, increased primary bile acids and lower levels of secondary bile acids resulting in a lower secondary/primary bile acid ratio in feces have been associated with dysbiosis (150). Additionally, bile acids can impair the gut barrier through direct toxicity (129). Vice versa antibiotic intake, dietary habits and the gut microbiome influence the composition of the bile

acid pool and its size (140, 151). Microbes of the gut as *Ruminococcus gnavus N53* can even produce the beneficial bile acid ursodeoxycholic acid (UDCA), which is approved for the treatment of PBC and used with less evidence in SC-CIP and PSC (152). Microbiome transplantation in patients with *Clostridioides difficile* infection also restored bile acid pool by increasing secondary bile acids (153). On the other hand, the intake of vancomycin in obese patients resulted in a reduction of alpha diversity of the gut microbiome causing limitation of bile acid dehydroxylation (154).

8.5.3.2. Chronic cholestatic liver diseases

Bile acid composition in patients with early-stage PSC and healthy controls was shown to be similar (155), but patients suffering from PSC, SSC, and obstructive jaundice demonstrated reduced bile acid pool size in bile fluid (156-159). Mechanisms explaining this unexpected finding are not explored sufficiently yet. Decreased hepatocellular release of bile acids, leakage of the biliary system, and re-absorption of bile acids are discussed explanation attempts (157). Bile acid levels in plasma and the primary-to-secondary bile acid ratio were elevated in PSC patients compared to a healthy control cohort in a recently conducted, large study with inclusion of more than 500 PSC patients. A model including changes in bile acid composition demonstrated the ability to predicted hepatic decompensation, which was associated with an increase of most bile acids and an augmentation of conjugated forms (160). In addition to alterations of bile acid composition and pool size, also elevation of interleukin-8 levels was described in the bile of PSC patients, suggesting persistent inflammation in the course of disease progression (161).

Hydrophobic bile acids are toxic for many human cells. The reason why the biliary epithelium is not harmed by these salts remains under debate. One hypothesis implies protection by HCO3 biliary secretion entitled the HCO3 umbrella (162). In PBC patients, the HCO3 umbrella of bile ducts is defective, causing a decrease of the physiologically alkaline pH near the surface of hepatocytes and cholangiocytes (162, 163). Elevation of taurine conjugates of chenodeoxycholic acid was reported in the bile fluid of PBC sufferers. Behind this finding, the hypothesis that taurine conjugation protects cholangiocytes from harm caused by the failure of the HCO3 umbrella by reducing hepatotoxicity of hydrophobic bile acids has been created (164). Furthermore, modified serum and fecal bile acid composition in therapy naïve PBC patients compared to healthy controls and reduced conversion from primary to secondary and from conjugated to unconjugated bile acids were reported. Impeded microbial metabolism disrupting dehydroxylation was suggested as a possible explanation. Alterations were more

pronounced in patients with advanced-stage PBC (165). Patients suffering from pruritus presented increased total and glyco-conjugated primary bile acids in serum when compared to patients with absence of pruritus (166).

8.5.3.3. Bile acid microbiome

The bile is not a sterile fluid (167-169). The bile microbiome was first described in samples from patients undergoing resection of the gallbladder. Comparable to the gut, *Bacteriodetes, Firmicutes, Actinobacteria,* and *Proteobacteria* are the most prominent phyla (168). The microbial composition was reported to be dependent on age and sex (169).

PSC patients showed reduced alpha diversity in the bile fluid and expansion of pathobionts compared to healthy probands. The most prominent changes included the increase of the genera *Staphylococcus* and *Enterococcus* and the species *Enterococcus faecalis* and *Veillonella dispar* in PSC patients (156). *Enterococcus faecalis* can induce epithelial barrier alterations and inflammation (170). In mice, the increase of *Enterococcus spp* was associated with the exacerbation of alcoholic liver disease caused by proton pump inhibitor use (171). *Veillonella dispar* was reported to cause meningitis, endocarditis, and joint infections (172-176). *Veillonella spp* are elevated in Crohn's disease patients who have disease recurrence after surgical resection (177) and in people using proton pump inhibitors (91, 178). Furthermore, an association of bile microbiome composition with disease severity and complications is discussed. The abundance of *Streptococcus* could only be correlated with alpha diversity of the bile microbiome and disease severity in PSC patients with concomitant presence of dysplasia or cholangiocarcinoma. (155). Evidence of the alteration of the function of the bile microbiome was created by the demonstration of increased proinflammatory and invasive bacterial metabolic pathways of PSC patients (156).

For SC-CIP, microbial bile analysis with conventional culture methods during ERCP showed bacterial detection in 95% of samples. The distribution of monomicrobial and polymicrobial growth was nearly 50% each. Most numerous microbes found were gram-positive bacteria (50%) with *Enterococcus spp.* being dominant, gram-negative (30%), and candida (10%) (179). Sequencing data from bile of SC-CIP and PBC patients are presently not available. In conclusion, findings outlined in this chapter show changes in bile acid pool size and bile acid composition of the bile, feces, and blood as well as biliary bacterial overgrowth and dysbiosis of the bile microbiome in chronic cholestatic liver diseases. Because of the correlation of these

alterations with outcome parameters, an important role of these changes in the pathogenesis and progression of chronic cholestatic diseases can be hypothesized.

8.5.4. Gut microbiome

8.5.4.1. Introduction

The concept of dysbiosis of the gut microbiome, characterized by a change of microbial composition, participating in the evolution and progression of liver diseases is widely established. As already highlighted, dysbiosis impacts the gut barrier function and bile acid composition and therefore causes fibrosis and cirrhosis (101, 127). Additionally, there seems to be a role of dysbiosis in the carcinogenesis of hepatocellular cancer (180). Disruptions of the microbiome have been demonstrated for chronic cholestatic liver diseases, but also for nonalcoholic fatty liver disease (NAFLD), alcohol-induced liver disease (ALD), drug-induced liver injury, viral-induced liver diseases, and cirrhosis (19, 36, 101, 181). When interpreting description of changes of the gut microbiome in different diseases the same question always arises: Are these alterations part of the cause or the consequence of the disease? To approach this question, understanding the functions of the microbiome and the effects of these alterations is essential. In ALD, this has been proven quite convincingly: Patients with ALD develop slow bowel transit and bacterial overgrowth and show an elevation of *Proteobacteria* in favor of *Firmicutes* and *Bacteriodetes*. Increase of *Streptococci, Bifidobacteteria,* and *Enterobacteria* has additionally been demonstrated in severe alcoholic hepatitis (101, 182, 183). Studies investigating the occurrence of dysbiosis through alcohol use in animal models and humans without preexisting liver diseases support the thesis of the evolution of ALD being connected to alterations of the microbiome (184, 185). On the other hand, a single alcohol binge does not seem to be enough to induce dysbiosis (186). In patients with alcoholic hepatitis, correlation of the abundance of *Enterococcus faecalis,* which produces the exotoxin cytolysin, with disease severity and mortality could be demonstrated (187). Reproduction of these findings was not successful for patients with non-alcoholic steatohepatitis (188).

When interpreting descriptions of alterations of the gut microbiome from distinct studies it is crucial to keep in mind that the microbial composition differs between feces and mucosal biopsies, mainly due to the diverse abundance of the genus *Bacteroides* (189). Furthermore, different sampling methods (f.e. brushing, biopsy) can lead to different results. Biopsies from different locations of the gastrointestinal tract can also be the reason for heterogeneity (189-191). At last, the microbiome composition is different from person to person and is significantly affected by environmental factors like drug intake or diet (192, 193).

8.5.4.2. PSC

Determination of the gut microbiome and description of differences in microbial composition in PSC, PSC-IBD, IBD, and healthy controls has attracted quite some interest within the last couple of years. The gut microbiome in feces was recently examined in a large, bilateral PSC cohort. Aims did not only included description of compositional but also functional changes of the microbiome by using metagenomic shotgun sequencing. Healthy probands and IBD patients served as controls. The authors could demonstrate decreased microbial richness and impaired abundance of several species including a rise of *Clostridium* species, a trending elevation of *Veillonella* species, and a decrease of *Eubacterium spp*. and *Ruminococcus obeum* in PSC patients. Additionally, impaired vitamin B6 and branched-chain amino acids synthesis was reported. The reduction of plasma levels of these metabolites could be associated with decreased liver transplantation-free survival pointing out a relation between disease course and function of the microbiome (194). Investigation of the fecal and the salivary microbiome in PSC patients was performed in another recent study. PSC patients showed dysbiosis in fecal and salivary probes compared to healthy probands, independent of underlying IBD in PSC patients. Decreased alpha diversity, describing the mean species diversity of an ecosystem, and changes in beta diversity, describing spatial alterations in species composition, were reported in feces of PSC patients. Multiple alterations of the gut microbiome composition on species level could be shown in PSC patients compared to healthy controls. These modifications comprised a reduction of the relative abundance of *Bacteroides thetaiotaomicron* and *Faecalibacterium prausnitzii,* both known to be commensals. The potentially pathogenic species *Veillonella dispar, Streptococcus salivarius*, *Ruminococcus gnavus, Bacteroides fragilis,* a few *Clostridium species,* as well as the genera *Blautia, Lactobacillus,* and *Enterococcus* were elevated in PSC (195). In total, 11 peer-reviewed original full papers investigating the microbiome in PSC patients could be identified (194-204) (Table 1). Although results presented in these manuscripts differ, some commonalities for the fecal microbiome can be recapitulated: Compared to healthy controls, PSC patients have reduced alpha diversity and dysbiosis. Although reported alterations of microbiome composition vary at least partly from study to study, some common patterns can be summed up: increase of *Veillonella* and *Clostridium* species and the genera *Lactobacillus, Enterococcus* and *Streptococcus* in PSC as well as decreased abundance of *Coprococcus* and *Faecalibacterium*. Concomitant IBD does not seem to significantly influence microbiome composition in PSC patients. Concerning the mucosal microbiome, existing data is even less comparable. One study investigating mucosal biopsies showed differing microbial profiles and transcriptomes when comparing all included

groups (PSC, IBD, healthy controls). Additionally, PSC-IBD patients had upregulation of bile acid signaling pathways (196). Hardly any alterations between the mucosal microbiome in PSC and healthy controls were described in two other studies (203, 204).

Many other diseases apart from cholestatic liver diseases have reduced alpha diversity of the gut microbiome. This ranges from other liver diseases to IBD and metabolic disorders (193, 205, 206). Decreased alpha diversity gives pathobionts the possibility to overgrow commensals and cause the evolution of diseases. Gut infection with *Clostridioides difficile* is the classical disease induced by this mechanism (207). In a very small pilot study with inclusion of ten patients with PSC-IBD, ameliorated diversity after fecal microbiome transplantation and a drop in alkaline phosphatase levels of more than 50% in three patients could be shown (208). *Veillonella spp* do have a relevant pathogenic potential (209). *Veillonella* abundance can serve as biomarker indicating disease severity in autoimmune and alcoholic hepatitis as well as in cirrhosis (178, 209-213). The species *Veillonella parvula* can produce LPS and trigger cytokine release leading to hepatic inflammation in vitro and in humans (209, 214). Additionally, long-term PPI intake and mortality in cirrhotic patients could be correlated with its abundance (178). *Faecalibacterium prausnitzii,* a highly abundant, butyrate-producing commensal in human feces, leads to the induction of anti-inflammatory effects exerted through the release of salicylic acid and downregulation of cytokines involved in the emergence of inflammation (215). Its decrease has previously been reported in liver diseases (f.e. NAFLD), in gastrointestinal pathologies (IBS, IBD, and colon cancer), in metabolic diseases (obesity and diabetes mellitus type 2) as well as in psychiatric and neurologic diseases (215).

Altogether, dysbiosis of the intestinal microbiome in PSC has been shown and impact on disease course has been proposed. Reported findings support the important role of the gut-liver axis in the pathophysiology of this disease. However, additional scientific efforts should focus on the exploration of the distinction which factors (nutrition, drug intake, disease severity, comorbidities) induce changes in microbiome composition and which are just consequences of the disease.

Table 1. Overview of trials investigating the gut microbiome in PSC. Solely full papers are indicated. Since reported key findings of microbiome composition are an extract of findings, selection is affected by the personal opinion of the authors. Table reproduced and adapted from (50) with permission of Nutrients.

Author	Publication Date	Cohort	Sample Origin	Methods	Key Findings of Microbiome Composition in PSC Patients in Feces or Mucosal Biopsies Compared to HC	Assessed Confounders of Microbiome Composition
Kummen et al. (194)	2020	57 PSC, 79PSC-IBD, 93 IBD, 158 HC	Feces	Metagenomic shotgun sequencing	↓ Diversity ↑ Clostridium ↓ Eubacterium spp. ↓ Ruminococcus obeum	Disease phenotype, UDCA intake, disease duration and severity
Lapidot et al. (195)	2020	17 PSC, 18 PSC-IBD, 30 HC	Feces, saliva	16S rRNA, Illumina MiSeq	↓ Diversity ↑ Streptococcus salivarius ↑ Veillonella dispar ↑ Ruminococcus gnavus ↑ Bacteroides fragilis ↑ Clostridium species ↑ Enterococcus ↑ Blautia ↑ Lactobacillus ↑ Enterobacteriaceae ↓ Bacteroides thetaiotaomicron ↓ Faecalibacterium prausnitzii	Disease phenotype, lifestyle, dietary habits, smoking
Quraishi et al. (196)	2020	10 PSC-IBD, 10 UC, 10 HC	Mucosal biopsies, sigmoid	16S rRNA, Illumina MiSeq	↑ Pseudomonas ↑ Streptoccocus ↑ Haemophilus parainfluenzae ↓ Lachnospiraceae	None
Lemoinne et al. (197)	2020	22 PSC, 27 PSC-IBD, 33 IBD, 30 HC	Feces	16S rRNA, Illumina MiSeq	↓ Diversity ↑ Veillonella ↓ Ruminococcus ↓ Faecalibacterium ↓ Lachnoclostridium ↓ Blautia	Disease phenotype, age, gender, smoking, drug intake, disease severity
Rühlemann et al. (198)	2019	62 PSC, 75 PSC-IBD, 118 UC, 133 HC	Feces	16S rRNA, Illumina MiSeq	↓ Diversity ↑ Veillonella ↑ Streptococcus ↑ Lactobacillus ↑ Enterococcus ↓ Coprococcus ↓ Holdemanella ↓ Desulfovibrio ↓ Faecalibacterium	Disease phenotype, UDCA, 5-ASA or azathioprine intake, colonic inflammation, diet

					↓ Clostridium IV	
Kummen et al. (199)	2017	30 PSC, 55 PSC-IBD, 36 UC, 263 HC	Feces	16S rRNA, Illumina MiSeq	↓ Diversity ↑ Veillonella ↓ Succinivibrio ↓ Desulfovibrio ↓ Coprococcus ↓ Phascolarctobacterium	Disease phenotype, age, gender, smoking status, BMI, drug intake, liver transplantation, disease duration and severity, concomitant autoimmune diseases
Bajer et al. (200)	2017	11 PSC, 32 PSC-IBD, 32 UC, 31 HC	Feces	16S rRNA, Illumina MiSeq	↑ Rothia ↑ Enterococcus ↑ Streptococcus ↑ Clostridium ↑ Veillonella ↑ Haemophilus ↓ Coprococcus catus ↓ Faecalibacterium prausnitzii ↓ Ruminococcus gnavus ↓ Prevotella copri ↓ Adlercreutzia equolifaciens	Disease phenotype
Sabino et al. (201)	2016	18 PSC, 48 PSC-IBD, 13 UC, 30 CD, 66 HC	Feces	16S rRNA, Illumina MiSeq	↓ Diversity ↑ Streptococcus ↑ Enterococcus ↑ Lactobacillus ↑ Fusobacterium ↑ Morganella ↓ Anaerostipes	Disease phenotype, gender, age, BMI, smoking status, UDCA intake, antibiotic intake, disease severity, liver transplantation
Torres et al. (202)	2016	1 PSC, 19 PSC-IBD, 13 UC, 2 CD, 9 HC	Mucosal biopsies, ileum, right and left Colon	16S rRNA, Illumina MiSeq	↑ Barnesiellaceae ↑ Blautia	Disease phenotype, disease severity
Kevans et al. (203)	2016	31 PSC-IBD, 56 UC	Mucosal biopsies, left colon	16S rRNA, Illumina MiSeq	HC not included in the study	Geographical origin
Rossen et al. (204)	2015	12 PSC-IBD, 11 UC, 9 HC	Mucosal biopsies, ileocoecum	16S rRNA, HITChip	↓ Diversity ↓ uncultured Clostridiales II	None

↓ = lower, ↑ = higher, PSC = primary sclerosing cholangitis, IBD = inflammatory bowel disease, UC = ulcerative colitis, CD = Crohn's disease, HC = healthy controls

8.5.4.3. PBC

To date, the gut microbiome of PBC patients has been studied in five trials (165, 216-219) (Table 2). Recently, in a study with 76 PBC patients of whom almost all included patients were treated with UDCA for more than a year at sample collection, lower alpha diversity compared to a healthy control group was reported. The primary reason for distinct beta diversity between the two groups was the increase of the genera *Lactobacillus, Streptococcus,* and *Bifidobacterium* in PBC. The genus *Enterococcus* was additionally elevated in PBC whereas the class *Clostridia* including the butyrate-producing genera *Anaerostipes* and *Faecalibacterium* was elevated in healthy controls. The study also demonstrated reduced abundance of *Faecalibacterium* in patients not responding to treatment with UDCA (216). A trial including 65 UDCA naïve PBC patients was conducted by Chen et al. In addition to outline changes in gut microbiome composition, the authors aimed to describe the interactions of the gut microbiome and bile acids as already highlighted above. PBC patients were shown, amongst others, to have an increase of the genera *Streptococcus, Klebsiella, Lactobacillus,* and *Veillonella*. Healthy controls on the other side had increased abundance of *Faecalibacterium* and *Bacteroides.* Strong association of UDCA levels with these genera could be demonstrated. Furthermore, positive correlation of enriched genera in PBC patients and negative correlation of genera augmented in healthy controls with secondary bile acids in serum were reported. This finding suggests dependence of secondary bile acid levels from microbiome composition due to conversion of primary to secondary bile acids performed by gut microbes (165, 220). Another group could further link abundance of *Veillonella* in the oral cavity of patients suffering from autoimmune liver diseases (AIH, PBC) to cytokine levels in saliva (217). For UDCA, the ability to restore the gut microbiome, at least partly, by decreasing the species *Streptococcus spp, Haemophilus spp,* and *Pseudomonas spp* and by increasing *Bacteroidetes spp, Oscillospira spp,* and *Sutterella spp* was reported (218). Together, mentioned findings indicate that alterations of the gut microbiome in PBC patients are similar to those in PSC and therefore suggest similar pathophysiological mechanisms concerning the gut-liver axis in those diseases.

Table 2. Summary of studies having explored the gut microbiome composition in PBC. Key findings of microbiome composition highlighted in this table were selected subjectively by the authors and present only a selection of findings. Table reproduced and adapted from (50) with permission of Nutrients.

Author	Publication Date	Cohort	Sample Origin	Methods	Key Findings of Microbiome Composition in PBC Patients in Feces Compared to HC	Assessed Confounders of Microbiome Composition
Furukawa et al. (216)	2020	76 PBC, 23 HC	Feces	16S rRNA, Illumina MiSeq	↓ Diversity ↑ Enterococcus ↑ Streptococcus ↑ Lactobacillus ↑ Bifidobacterium ↓ Clostridiales	UDCA treatment effect, PPI intake
Chen et al. (165)	2020	65 PBC, 109 HC	Feces	16S rRNA, Illumina MiSeq	↑ Lactobacillus ↑ Streptococcus ↑ Klebsiella ↑ Veillonella ↓ Faecalibacterium ↓ Bacteroides	UDCA intake
Abe et al. (217)	2018	39 PBC, 17 AIH, 15 HC	Feces, Saliva	16S rRNA, Terminal restriction fragment length polymorphism	↑ Lactobacillales ↓ Clostridium subcluster XIVa	None
Tang et al. (218)	2018	60 PBC, 80 HC	Feces	16S rRNA, Illumina MiSeq	↓ Diversity ↓ Bacteroidetes spp. ↓ Sutterella ↓ Oscillospira ↓ Faecalibacterium ↑ Haemophilus ↑ Veillonella ↑ Clostridium ↑ Lactobacillus ↑ Streptococcus ↑ Pseudomonas ↑ Klebsiella ↑ unknown genus of Enterobacteriaceae	UDCA intake, disease severity, gender, age, BMI
Lv et al. (219)	2016	42 PBC, 30 HC	Feces	16S rRNA, I llumina MiSeq	Multiple alterations mentioned, for example: ↑ Veillonella ↑ Streptococcus ↑ Klebsiella ↑ Enterobacteriaceae ↓ Lachnospiraceae	None

↓ = lower, ↑ = higher, PBC = primary biliary cirrhosis, HC = healthy controls, PPI = proton pump inhibitor, BMI = body mass index

8.5.5. Gut mycobiome

8.5.5.1. Introduction

On the contrary to bacteria, which represent 99% of all microbial genes found in the gut microbiome, fungi only account for 0.01–0.1% of all microbes (221). Data already available suggest that the composition of the gut mycobiome is very variable and fragile to environmental influences. Especially eating habits are said to play an important role as studies were able to link diet to mycobiome composition (222-224). Recently, also ethnicity, urbanization, and geographic location were identified as influencing factors (225). In total, 267 species were described in studies investigating the gut mycobiome but only 15 of these species were found in more than five studies (222, 226). Another limitation is that some reported fungi are not even able to inhabit the gut caused by environmental matters like body temperature. These fungi may more likely represent species ingested via diet or aerosols. The human gut seems to be a good environment for growth of *Malassezia* and *Candida (f.e. Candida albicans)* species. Other fungi like *Dipodascaceae* and *Cladosporium* (including *Aspergillus* and *Penicillium*) are classified as being able to inhabit the gut, although it is unlikely that they have their origin in the gut. Furthermore, there are species like *Saccharomyces cerevisiae* and many *Aspergillus* species that are capable of surviving the gut environment but are said to be more pronounced in plants, soil, and the air. Ultimately, *Penicillinum aff. commune* and *Debaryomyces hansenii* are not able to colonize the gut at all (222, 227, 228). The human core mycobiome seems to consist of *Saccharomyces, Malassezia, Candida,* and *Cladosporium* (221, 229, 230). Another relevant factor is that analytic methods used for investigating the mycobiome have some important limitations. Variations in DNA extraction techniques, the absence of uniform primer pairs, different sequencing methods and software, and the lack of an entire database for homogeneous taxonomic assignment makes it hard to compare different trials (231-233). It is reported that fungi have an essential role in keeping the homeostasis of the gut microbiome and for immunity (234, 235). In animal models, concomitant *Candida albicans* substitution during antibiotic intake was able to support bacterial recovery, but additionally, it changed gut microbiome composition sustainably (236). The usage of antifungal therapy in mice decreased fungal diversity and caused an overgrowth of bacterial pathobionts which resulted in augmented colonic inflammation (237). Another important finding, reported in rats, is that *Saccharomyces boulardii* can secret enzymes that have the capability to neutralize toxins of *Clostridioides*

difficile and *Escherichia coli* (238, 239). Equal to bacteria, fungi can also use PAMPs for communication with the immune system (234). Dectin-1 (CLEC7A), a receptor that detects β-glucans located in the cell wall of fungi and therefore protects the organism against fungal infections, was reported to be the key receptor for fungi in the gut (240, 241). Through this receptor, fungi are able to trigger the production of pro-inflammatory cytokines by caspase-associated recruitment domain-containing protein 9 (CARD9) and activate T helper 17 (Th17) immune responses (242). The blockade of Dectin-1 led to an amelioration of colitis in mice. On top, mice having a Dectin-1 deficiency showed resistance to dextran sodium sulfate and T cell-induced colitis (241). The role of the disruption of the fungal microbiome in the pathogenesis of disease has been determined for gastrointestinal disorders as IBD and colorectal cancer as well as for metabolic diseases (obesity, atherosclerosis) (235, 243-250). Interestingly, patients with diabetes mellitus type 1 have been reported to demonstrate elevated fungal diversity (251). Fungal dysbiosis could be associated with increased intestinal hypersensitivity in IBS patients, but the enhanced diagnostic and therapeutic value of investigating the mycobiome was doubted (252, 253). Alterations of the mycobiome characterized by *Candida* overgrowth and reduced overall diversity in ALD and by reduced diversity and changed fungal composition in NAFLD have been reported (234).

8.5.5.2. Chronic cholestatic liver diseases

The role of the gut mycobiome in chronic cholestatic liver diseases was solely investigated in PSC patients yet and results are partly heterogeneous. Lemoinne et al. recently presented their findings using ITS2 sequencing. Comparing PSC, PSC-IBD, IBD, and healthy probands no differences in alpha diversity could be observed. When combining PSC and PSC-IBD patients into one group, higher diversity in comparison to IBD could be shown. Besides, the fungi-to-bacteria diversity ratio was elevated in PSC patients. *Ascomycota* and *Basidiomycota* were to most abundant phyla reported in this investigation. Dysbiosis, characterized by the decrease of *Saccharomyces cerevisiae* and by the increase of the genus *Exophiala* (detected in just five patients), was described in PSC patients (197). An anti-inflammatory effect of *Saccharomyces cerevisiae,* a species related to the probiotic strain *Saccharomyces boulardii,* was proposed due to the induction of the production of interleukin-10 (246). This finding is supported by amelioration of colitis caused by an invasive *Escherichia coli* species through *Saccharomyces cerevisiae* application in mice (254). Another study group presented their mycobiome data of PSC patients in form of a letter (255). Results included unchanged alpha and beta diversity in PSC patients. Although composition of the mycobiome was comparable to previous findings,

Exophiala could not be detected in the gut of any of the included PSC patients in this cohort. Complementary, increase of *Sordariomycetes*, likely caused by the species *Trichocladium griseum*, and *Candida* was found in PSC patients (255). Regional differences are not a sufficient explanation for these divergent findings since both studies were conducted on mainland Europe. Concerning immunity, it was reported that *Candida albicans* cultivated from bile of PSC patients could trigger production of interleukin-17A, which leads to activation of Th17 cells. These cells are said to protect mucosal and epithelial surfaces from microbes but are also participating in the pathogenesis of IBD and further autoimmune diseases (256-261). Furthermore, biliary candida infections have been associated with an unfavorable disease course in PSC (262). Also in case reports, fungal infections have been linked to liver diseases: *Exophiala dermatitidis*-induced systemic Phaeohyphomycosis led to jaundice causing pruritus, abdominal pain, fever, and fatigue in a young woman. Findings on ERCP resembled the picture of sclerosing cholangitis (263). The same species was reported to have induced liver cirrhosis (264). To recap, alterations of the composition of the gut mycobiome seem to affect bacterial formation and may jointly be responsible for the occurrence of PSC. But by now, available knowledge is too limited and inconsistent and still needs to be generated for chronic cholestatic liver diseases other than PSC.

8.5.6. Gut virome

8.5.6.1. Introduction

Equal to fungi, viruses make up under 1% of all gut microbes. Absolute numbers reported are: 10^8 to 10^9 virus-like particles (VLP) per gram stool. Bacteriophages which function as opponents to bacteria and have the ability to modulate bacterial composition, are the most numerous viral representatives (221, 265-268). Also similar to fungi, the existence of a core gut phageome was proposed by investigating healthy humans (269). Eukaryotic-targeting viruses are the second component of the virome (221) and have lately been investigated in IBD (270, 271). It was reported that the gut is exposed to at least 16 families of animal RNA or DNA viruses directly after birth (221, 272, 273). Plant-pathogenic RNA viruses were reported to be most numerous in the healthy human gut (274). The inconsistent methodology of trials, the expensive use of shotgun metagenomics, the difficult to perform bioinformatics, and the absence of uniform marker genes make the description of a general healthy virome impossible to date. (221, 275, 276). Existing data hint at a diverse, staple, and very individual virome in humans (277). Dependence of the composition from age, ethnicity, location of living, grad of urbanization, and diet have been reported (278-280). Alterations of the fecal virome in IBD,

celiac disease, and colorectal cancer were caused by animal viruses in the gut and these diseases showed a higher phage diversity (281-284). Additionally, changes in the viral composition of the gut have been reported in graft-versus-host disease, HIV, diabetes mellitus type 2, and malnutrition (285-288). Expecting women suffering from diabetes mellitus type 1 presented a distinct virome when comparing them to pregnant women without diabetes (289). The vast amount of data of the intestinal virome was generated from fecal samples, but studies assessing mucosal biopsies have been conducted for IBD patients (271, 290, 291). Dysbiosis with higher abundance of *Caudovirales* bacteriophages was reported and the investigators managed to correlate dysbiosis with functional impairment of the intestine and inflammation (290). With application of FMT in patients suffering from *Clostridioides difficile* infection, a decline of *Caudovirales* bacteriophages of the recipient could be achieved. Simultaneously, overgrowth with donor *Caudovirales* was linked to healing (292). Donor stool with higher diversity of bacteriophages, but with a reduced abundance of these microbes was more effective in treating *Clostridioides difficile* infections (293). FMT usage in hematologic patients suffering from graft-versus-host disease ended up in higher abundance of *Caudovirales* phages (294). Same as for bacteria, FMT proved to be effective in inducing changes of the gut virome of ill patients towards donor composition (293, 295-297). Complementary to the influence of VLPs on the bacterial microbiome, the virome interacts with the immune system (221). In mice, the increase of bacteriophages was associated with disordered mucosal immunity and led to inflammation of the gut (298).

8.5.6.2. Liver diseases

With exception of a small group of PBC patients included as control group in one trial, the virome has not yet been investigated in chronic cholestatic liver diseases (299). Investigations have been performed for NAFLD, alcoholic hepatitis, and liver cirrhosis (299-301). In NAFLD, a total of 420 distinct viral species could be reported in feces of all included patients, but solely 19 of these species could be detected in at least 50% of all samples. Healthy individuals and 13 PBC patients suffering from a mild disease course served as controls. Decreased viral diversity and lower numbers of bacteriophages compared to other viruses were reported for NAFLD patients. These changes were further pronounced by underlying liver fibrosis. F2-F4 fibrosis could even be predicted with a model containing age of the patient, aspartate aminotransferase (AST) levels, and fecal viral diversity. Results with similar accuracy could not be achieved if using bacterial diversity (299). In patients with alcoholic hepatitis, VLPs were more divers and changes of the abundance of certain taxa (*Staphylococcus* phages*, Herpesviridae*) could be

associated with disease severity and mortality (300). On the contrary, the correlation between fecal bacteriophages, cirrhosis specifications, and clinical outcome after 90-days was limited (301). In addition to fecal investigations, the virome has also been studied in plasma. Patients after liver transplantation presented high abundance of *Anelloviruses*. These alterations could be associated with infections and nephrotoxicity (302).

8.5.7. Therapeutic interventions targeting the gut microbiome in liver diseases

Therapeutic strategies for the modulation of the gut microbiome comprise prebiotics, probiotics, antibiotics, and FMT (36) (Figure 2). For humans, prebiotics are indigestible types of carbs (mostly fiber), but beneficial gut bacteria can metabolize them. In a mouse model, they demonstrated efficacy in preventing alcohol-induced liver inflammation and steatosis. In humans, reduction of hepatic lipogenesis could be shown (303-305). Probiotics are live bacteria and have mainly been investigated for NAFLD, ALD, and cirrhosis. Restoration of gut microbiome composition, reduction of bacterial translocation, and re-establishment of the gut barrier are effects ascribed to probiotic usage (36, 306-311). Liver inflammation is inhibited by avoidance of TLR4 activation by LPS and by the reduction of proinflammatory cytokines (312, 313). In cirrhotic patients, oral use of rifaximin, an antibiotic that is sparsely absorbed into the systemic circulation, reduced endotoxemia and showed the ability to modulate the gut microbiome (314, 315). FMT was efficient in restoring the microbiome and lowering liver inflammation and damage in mice (304, 316). Furthermore, it decreased the severity of liver diseases, prolonged survival in ALD patients after one year (317), and positively influenced composition of the microbiome and reduced liver inflammation in NASH (318). In addition to mentioned treatment options, nutrition presents a powerful tool to influence microbiome composition. In mice, high intake of fat caused gut dysbiosis with decimation of butyrate-producing microbes and triggered LPS production through gram-negative bacteria which induced liver inflammation and the occurrence of NASH (75, 319, 320). In humans, the influence of dietary interventions and weight reduction on gut permeability in liver diseases remains conflicting (321, 322). Modulation of the gut-liver axis can additionally be achieved with usage of FXR agonists which showed the ability to treat bile acid dysmetabolism, ameliorate gut barrier function, reduce bacterial overgrowth, and improve liver inflammation, at least in rats (323). Newly investigated interventions targeting the gut-liver axis include bacteriophages which demonstrated efficacy in influencing microbiome composition in mice (324). Bacteriophages targeting *Enterococcus faecalis* inhibited levels of cytolysin, an exotoxin

of this species, in the liver and consequently hindered ethanol-induced liver disease (187). Modulation of the mycobiome with amphotericin B was successful in preventing alcohol-induced liver damage in mice (325).

In PSC, therapeutic usage of the antibiotics metronidazole and vancomycin led to reduction of alkaline phosphatase and bilirubin levels as well as the Mayo PSC Risk Score. If this biochemical improvement is caused by direct bacteriostatic effects of antibiotics or by immunomodulating effects of vancomycin targeting TNF-α and TGF-β pathways still needs to be investigated (326-329). Minocycline intake for a year reduced alkaline phosphatase levels and the Mayo PSC Risk Score. On the other hand, rifaximin has not shown efficacy in PSC (330, 331). A probiotic formulation containing four *Lactobacillus* and two *Bifidobacillus* strains was ineffective in a small cohort of PSC-IBD patients when applied over 3 months (332). As mentioned, Allegretti et al. investigated FMT for the treatment of PSC-IBD patients in a very small cohort and suggested restoration of the gut microbiome after FMT and reduction of markers of cholestasis (208). With exception of rifampicin for the treatment of pruritus in PBC, data are lacking for the use of pre- or probiotics, antibiotics, or FMT interventions in SC-CIP and PBC.

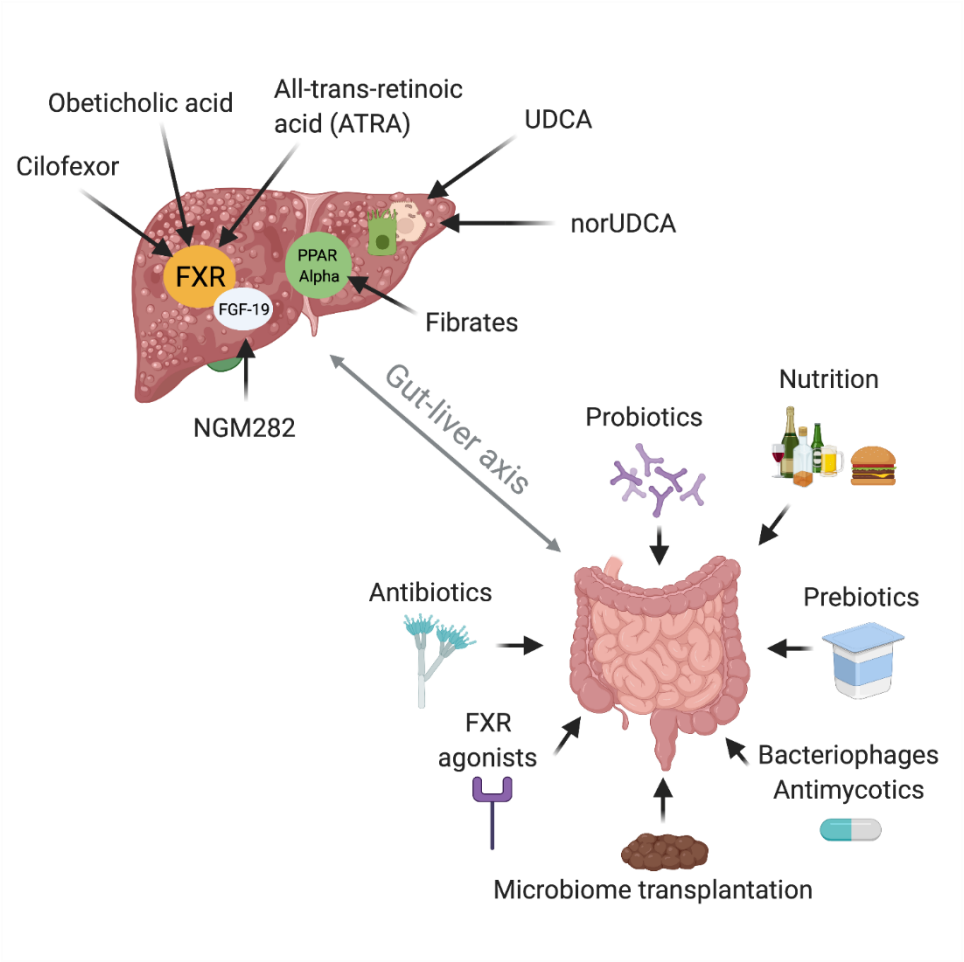

Figure 2. Therapeutic strategies. Actual available or currently investigated treatment options for modulation of the gut microbiome in liver diseases and investigated and approved drugs targeting bile acids in chronic cholestatic liver diseases. Known options to target microbiome composition and therefore the gut-liver axis are prebiotics, probiotics, antibiotics, FMT, and nutrition. Antimycotics and Bacteriophages are still in a very early stage of investigation. Same counts for FXR agonists concerning the ability to influence the gut microbiome. Approved medical options for treatment of PBC comprise UDCA, obeticholic acid, and fibrates. Approved drugs are currently not available for the treatment of SC-CIP and PSC. norUDCA, other PPAR ligands, all-trans-retinoic acid, cilofexor, and NGM282 are still under investigation. FXR = Farnesoid X receptor, FGF-19 = Fibroblast growth factor 19, PPAR = Peroxisome proliferator-activated receptor, NGM282 = an engineered fibroblast growth factor 19 analogue, UDCA = Ursodeoxycholic acid, norUDCA = norUrsodeoxycholic acid. Figure reproduced from (50) with permission of Nutrients. Figure created by Andreas Blesl with BioRender.com.

8.6. The gut barrier and the gut microbiome in critically ill patients

It has been reported that 50% of all patients being treated at an intensive care unit (ICU) suffer enterocyte damage and more than 60% present GI symptoms (333). Stress, induced by ICU treatment, can lead to alterations of mucosal blood flow and gut motility as well as to increased

intestinal permeability (333, 334). It was suggested that gut barrier damage may occur as early as one hour after sepsis onset and that this state lasts for at least two days (335). The intestinal circulation can be harmed by catecholamine treatment. This may lead to enhanced dysfunction of the gut barrier and may therefore trigger infectious complications induced by increased bacterial translocation and may be part of the factors leading to multiple organ failure (333, 336). Augmented gut permeability indicated by elevation of I-FAB and decrease of citrulline could be correlated with worsened outcome and increased mortality in critically ill patients (337). These findings suggest, that disruption of the gut barrier is a complication of the critical illness. Concerning the evolution of SC-CIP, it can be hypothesized that gut barrier defects may be an essential factor in the pathophysiological cascade of the disease as suggested for PSC and PBC. But this alteration alone is not a sufficient explanation for the occurrence of SC-CIP since gut barrier failure seems to be much more common than the disease.

The gut microbiome composition was shown to be altered in critically ill patients (338). Predominance of pathogenic microbes, reduction in beneficial species and lower bacterial diversity have been reported (339). Caused by the lowering of *Firmicutes*, *Proteobacteria* remain the most abundant phylum in these patients (340). Influencing factors apart from features of the critical illness and the ICU stay itself include the intake of antibiotics, proton pump inhibitors, vasopressors, and opioids during ICU treatment (341). It could recently be demonstrated that different antibiotics have distinct impacts on microbiome composition (342). Modulation of the microbiome could be achieved with probiotic interventions in early sepsis but failed to demonstrated effects on length of hospital stay and mortality in patients treated on a respiratory care unit (343, 344).

8.7. Gastrointestinal bleedings in critically ill patients

Gastrointestinal (GI) bleeding is a frequent complication in patients treated on an ICU. It occurs with a frequency of 1.2 to 14% (345-348). Ulcers and erosions in the upper GI tract have been reported to be the most common causes of bleeding (346). Additionally, upper GI bleeding is a regularly observed complication in patients suffering from liver cirrhosis (349). Portal hypertension leading to esophageal and gastric varices represents the main bleeding cause in these patients (350). Despite the frequent observation of asymptomatic peptic ulcers in patients with cirrhosis, bleeding from these lesions is less frequent than variceal bleeding, although bleeding from ulcers may be favored by coagulopathy occurring due to hepatic malfunction in disease course (351-354). The third most common reason for upper GI bleeding in cirrhotic patients is portal hypertensive gastropathy (PHG). Dieulafoy's lesions, Mallory-Weiss

syndrome, erosive gastritis, esophagitis, and gastric vascular ectasia are complications with an increased but still lower potential bleeding risk than varices and ulcers (355, 356). Increased frequency of Dieulafoy's lesions has been communicated in patients with advanced liver diseases (357, 358). Common causes of lower GI bleeding in cirrhotic patients include hemorrhoids and portal hypertensive colo- and enteropathy (359). The frequency of GI bleedings in patients suffering from non-cirrhotic chronic liver diseases still needs to determined. Furthermore, no data can be found describing the occurrence of GI bleedings in neither non-cirrhotic, nor cirrhotic patients with SC-CIP.

8.8. Aims

In summary, it was outlined that the gut-liver axis, consisting of the gut microbiome, the gut barrier enabling bacterial translocation, and the bile acid circle, seems to play an important role in the evolution and progression of chronic cholestatic liver diseases (PSC, PBC). The gut-liver axis has not been investigated in SC-CIP patients yet. Furthermore, GI bleeding seems to be a common complication in ICU patients as well as in people suffering from liver diseases, at least in those with a higher grade of liver dysfunction. To date, the frequency and the reasons for GI bleeding in SC-CIP patients have not been investigated. Clinical observations in our daily routine in the endoscopy unit suggest that this complication occurs with a relevant frequency.

Consequently, this scientific work aimed to describe the gut microbiome, gut permeability including bacterial translocation and the composition and alterations of serum bile acid profiles for the first time in patients suffering from SC-CIP through the performance of a case-control study ("The gut-liver axis in SC-CIP"). Three control groups (patients after ICU treatment, but without signs of liver damage, cirrhotic patients, and healthy probands) were included in the study. In addition, the aim of this thesis was to report the frequency and reasons of GI bleedings in SC-CIP for the first time, while on ICU as well as in the long-term, by doing a retrospective analysis ("GI bleeding in SC-CIP").

9. Material and Methods

9.1. Case-Control Study "The gut-liver axis in SC-CIP" (360)

9.1.1. Study population

From December 2014 to May 2015 patients suffering from SC-CIP were recruited at two study centers (Medical University of Graz, Graz, Austria, and Saarland University Hospital, Homburg, Germany). Eligibility for the study was assessed by a physician in the outpatient

liver clinics of the study centers. Exclusion criteria comprised other reasons of cholestasis like choledocholithiasis, IgG4-associated cholangitis, toxic cholestasis, and other chronic cholestatic liver diseases (PSC, PBC). Diagnosis of SC-CIP was based on the medical history (ICU stay in the past, lack of liver pathologies prior to ICU treatment) and the previously reported typical cholangiographic criteria using ERCP or MRCP as diagnostic modalities. These criteria include irregularity of intrahepatic bile ducts with strictures and prestenotic dilations, bile duct rarefication, and the presence of biliary casts (7). From the included probands, no one had undergone biliary surgery or suffered from concomitant IBD. After obtaining informed consent from patients, blood and stool samples were collected for investigation at a single time point.

In the first control group (CIP controls) patients with history of a critical illness with the necessity for intensive care treatment, but without the occurrence of liver complications were included. Inclusion of these patients was done between July 2018 and January 2019 directly at the rehabilitation center Tobelbad near Graz with the help of treating physicians employed at the rehabilitation center. Patients in this cohort were required to have needed at least two days of invasive ventilation and hemodynamic support with vasoactive drugs during their ICU stay. Exclusion criteria comprised the presence of any liver or gut pathology, increased serum alkaline phosphatase levels as well as suspicious laboratory findings suggesting liver damage or cirrhosis. Abdominal sonography or elastography of the liver was not performed prior to study inclusion. Blood and stool probes were collected at a single time point after patients had signed the informed consent form.

Patients included in the second control group (cirrhosis) suffered from alcohol-induced cirrhosis. These patients were matched for age and sex from an earlier gathered study cohort by our study group. Recruitment of these patients was done at the liver outpatient clinic at the study center in Graz, Austria from July 2012 to September 2013. Patients were initially screened for an intervention study. Diagnosis of liver cirrhosis was proven with histopathological examinations of liver biopsy samples or was based on a combination of clinical, laboratory, and radiological features depending on availability. Alcohol withdrawal within two weeks before study inclusion was necessary for eligibility. Ongoing infections at screening, gastrointestinal bleeding within two weeks prior to inclusion, intake of immune-modulating drugs, renal failure (creatinine over 1.7 mg/dL), hepatic encephalopathy stage two or higher, other severe comorbidities, malignancy, and pregnancy presented exclusion criteria. Blood and stool samples were obtained before any intervention was carried out. In this study protocol also the

recruitment of healthy controls (healthy) without existing liver or gut pathologies was intended and was finally carried out at the study center in Graz, Austria from October to December 2014.

9.1.2. Liver stiffness and laboratory assessments

Liver stiffness of SC-CIP patients was evaluated with elastography on a Fibroscan 502 touch (Echosens, Paris, France). Because no cut-offs for the diagnosis of liver fibrosis and cirrhosis in SC-CIP have been reported to date, cut-offs for PSC were used. Cirrhosis was diagnosed with values exceeding 14.3 kPa. As cut-off for liver fibrosis, 8.8 kPa was chosen (361). Full blood count, liver enzymes, coagulation parameters and C-reactive protein were determined by routine laboratory procedures.

9.1.3. Microbiome analyses

Patients received detailed written instructions for adequate stool collection and timely transport to the study center. Alternatively, stool samples could directly be picked up by the study team. Stool samples were frozen at -80°C upon arrival at the study center until sequencing was performed. Total DNA was extracted from frozen stool samples using MagnaPure LC DNA Isolation Kit III (Bacteria, Fungi) (Roche, Mannheim, Germany) according to manufacturer's instructions including mechanic and enzymatic lysis (362). Hypervariable regions V1-V2 were amplified in a target-specific PCR using the primers 27F and R357 (27F-AGAGTTTGATCCTGGCTCAG; R357-CTGCTGCCTYCCGTA) and sequenced with the Illumina MiSeq technique (Illumina, Eindhoven, The Netherlands) (362). The resulting FASTQ files were used for data analysis. Sequencing was done in cooperation with the Core Facility for Molecular Biology at the Center for Medical Research in Graz.

9.1.4. Gut permeability markers

Enzyme-linked immunosorbent assays (ELISA) were utilized to determine calprotectin, zonulin, and serum diaminoxidase (DAO) levels (Immundiagnostic AG, Bensheim, Germany). Lipopolysaccharide-binding protein (LPB) and sCD14 levels were measured with a ready-to-use solid-phase sandwich ELISA (Hycult Biotechnology, Uden, The Netherlands).

9.1.5. Serum bile acids

Analysis of serum bile acids was performed as previously reported by Amplatz et al. (363) from deproteinized serum using a high-performance liquid chromatography (HPLC) equipped with

a Nucleoshell C18 reversed-phase column (Macherey-Nagel, Düren, Germany). This was followed by identification of bile acids with a Q Exactive hybrid quadrupole-orbitrap mass spectrometer (Thermo Fisher Scientific).

9.1.6. Statistical analyses

The R package "dada2" was utilized to pre-process sequencing data (364, 365). Raw forward and reverse sequences were trimmed to a length of 250 and 200 bases respectively and low-quality reads were removed from the analysis (maximum expected errors = 2). Dada2 core sample inference algorithm was used to infer true sequence variants for each sample individually. Paired reads were then merged and chimeras were removed. Low abundant features (i.e. less than ten copies found and present in less than three samples) were excluded from further analysis. Taxonomy was assigned using a naïve Bayesian classifier and SILVA v132 as a reference database. OTUs were blasted using the NCBI database (101). Mentioned species names in the manuscript had accordance of more than 97%. Lower accordance was graded as not further definable. After pre-processing, the average count per sample was 113.031 (standard deviation: 56.149; range: 19.479 to 235.167). In total, 7.912.177 sequencing reads with a fraction of non-zero values of 0.113 were obtained. Hellinger transformation was used for normalization of data. To remove contaminants, chloroplast and cyanobacteria filtering was carried out. Alpha diversity analysis was performed in Calypso 8.84 (http://cgenome.net/calypso/) using Chao1, Shannon Index and Richness on a rarefied feature table (sequencing depth 19479). To assess differences in alpha diversity between groups, analysis of variance (ANOVA) with Bonferroni correction was used. Beta diversity and factors influencing microbiome composition were determined with the use of Redundancy Analysis (RDA) based on Bray Curtis dissimilarity. Confounders with a $p<0.1$ were considered for a multivariate model using RDA+ in Calypso 8.84. Multicollinearity was defined as a variance inflation factor of two or higher. To avoid the potential bias introduced by collinear explanatory variables, parallel alternative models were constructed. Assessment of differentially abundant taxa was done with Analysis of Composition of Microbiomes (ANCOM). To select taxa associated with the four different groups, LDA-effect size (LEfSe) was used. Furthermore, network analysis was based on Spearman's rho associations between groups and statin intake compared to no statin intake by converting the pairwise correlations into dissimilarities to ordinate nodes in a two-dimensional PCoA plot. Bile acid multivariate analyses were done in PAST (Version 3.26) using non-metric multidimensional scaling (NMMDS) with Bray–Curtis dissimilarity. All other statistical analyses were done in SPSS Version 26 using Kolmogorov–

Smirnov-Test to assess normal distribution. To test for significance comparing two groups Student's T-Test and Man Whitney U Test were used. Additionally, the non-parametric Kruskal–Wallis one-way analysis of variance (one-way ANOVA) was utilized to compare the four groups to each other. Correction of multiple comparisons was done with usage of the Bonferroni correction. Correlations were calculated using Pearson correlation coefficient. All tests were carried out on a 5% significance level.

9.1.7. Ethical Considerations

The different study parts have been approved by the research ethics committee of the Medical University of Graz (26-569 ex 13/14 and 23-096 ex 10/11), Ärztekammer des Saarlands (177/15), and the AUVA (35/2017). Registration numbers on clinicaltrials.gov are NCT02545309 and NCT01607528. The study was performed in accordance with the ethical standards laid down in the Declaration of Helsinki and its amendments.

9.2. Retrospective Study "GI bleeding in SC-CIP" (366)

9.2.1. Study population

For this study, patients with the established diagnosis of SC-CIP were identified from the medical records of the Medical University of Graz, Graz, Austria. The identification phase lasted from May 2020 to November 2020. Patients were followed retrospectively from the time point of the provoking incident leading to ICU treatment and subsequently to the disease SC-CIP. The observation period ended with the death of the proband or alternatively in November 2020 if still alive. Diagnosis of SC-CIP was established by clinical suspicion and by typical cholangiographic findings on ERCP or MRCP, identically to the criteria already defined in the method section of the study "The gut-liver axis in SC-CIP" (irregular intrahepatic bile ducts with strictures and prestenotic dilations, bile duct rarefication, and biliary casts). Alternatively, diagnosis could also be established with liver biopsy. Exclusion criteria included cholestasis of other origins (choledocholithiasis, PSC, PBC, IgG4-associated cholangitis, acute cholestatic liver failure of unknown origin, and toxic cholestasis).

Patients with the necessity of prolonged intensive care treatment after cardiac surgery served as control group for the SC-CIP group. Because of their underlying cardiovascular diseases, the complicated surgical interventions performed, and the long-term intensive care treatment those patients were considered to be at risk for development of SC-CIP. These patients were initially

recruited prospectively for another study. Patients who did not develop SC-CIP were included in the present study as control group and were monitored retrospectively for the occurrence of GI bleedings.

9.2.2. Definitions

Bleedings were noted when any sign of GI hemorrhage occurred during the observation period. This comprised obvious GI bleeding including distinct hematemesis, hematochezia, and melena as well as active or suspended bleeding upon endoscopy and obvious bleeding on computer tomography angiography or direct angiography. Furthermore, if assessment of occult GI bleeding was performed and was positive for occult blood, this was also classified as GI hemorrhage.

9.2.3. Primary Endpoint

The occurrence of GI bleedings in SC-CIP patients compared to the control group within the observation period was chosen as primary endpoint.

9.2.4. Secondary Endpoints

Secondary endpoints comprised the description of bleeding characteristics (bleeding source, techniques to achieve hemostasis), the outcome of patients (number of administered blood units, mortality) and the evaluation of the vascular anatomy of SC-CIP patients upon cross-sectional imagine techniques (computer tomography, magnetic resonance tomography) to check for alterations of vascular anatomy potentially increasing the bleeding risk.

9.2.5. Data collection

Patients suffering from SC-CIP were identified using a keyword search in the medical documentation system of the study center. SC-CIP, secondary sclerosing cholangitis, and sclerosing cholangitis were used as keywords. Patients with PSC were concomitantly identified with this search but were not included in the present study. Only patients with a definite diagnosis of SC-CIP were included in the analysis. If the diagnosis was unsure but possible and another reason for cholestasis appeared to be more likely, patients were also not included. From the included patients the medical records were scanned for the occurrence of GI bleedings and if bleedings had occurred, bleeding characteristics and outcomes were documented. This work was solely done by the author of this thesis and the first author of the manuscript. Patients of

the control group were identified directly through an ongoing prospective study (currently on hold due to the Covid-19 pandemic) which intended to examine the prevalence of SC-CIP in patients undergoing cardiac surgery with the subsequent need for intensive care treatment. Patients with the need for mechanical ventilation +/- extracorporeal membrane oxygenation for five consecutive days or more were included in the present study if they did not develop SC-CIP. Gathering of additional information and evaluation for bleeding events was done in retrospect by the author of this thesis.

9.2.6. Radiological evaluation

Assessment of vascular anatomy and the presence of vascular stenosis in SC-CIP patients was done by a radiologist of the study center using available cross-sectional imaging (contrast-enhanced and unenhanced computer tomography, contrast-enhanced and unenhanced magnetic resonance tomography, computer tomography angiography, magnetic resonance angiography). The images which were chosen for interpretation were the ones available closest to the initial incident leading to ICU treatment. All images were performed due to necessary reasons for ensuring adequate medical care, none of them was performed for study purposes.

9.2.7. Statistical analysis

Statistical analysis was done using SPSS Version 25. Kolmogorov-Smirnov-Test was used to test for normal distribution. Descriptive statistics were utilized for description of variables; Chi quadrat, Student's T-Test, and Mann-Whitney U Test to check for significance. All tests were performed on a 5% significance level.

9.2.8. Ethical Considerations

Data were obtained from two studies, both approved by the research ethics committees of the Medical University of Graz (26-569 ex 13/14 and 30-342 ex 17/18) and registered at clinicaltrials.gov (NCT02545309 and NCT03566797). The studies were performed in accordance with the ethical standards laid down in the Declaration of Helsinki and its amendments.

10. Results

10.1. Case-Control Study "The gut-liver axis in SC-CIP" (360)

10.1.1. Patient characteristics

Eighteen patients suffering from SC-CIP, 11 patients with history of long-term ICU treatment without subsequently occurring liver disease (CIP controls), 21 patients with the diagnosis of alcohol-induced liver cirrhosis (cirrhosis), and 21 healthy controls (healthy) were included in the analysis (SC-CIP: age 59 ± 13 (mean ± SD) years, 13 men, BMI: 27.2 ± 5.3 kg/m^2; CIP controls: age 54 ± 15 years, 8 men, BMI: 25.5 ± 3.0 kg/m^2; cirrhosis: age 58 ± 9 years, 16 men, BMI 27.3 ± 5.0 kg/m^2; healthy: age 58 ± 7 years, 9 men, BMI: 25.3 ± 3.1 kg/m^2). Age, sex and BMI did not differ between the four groups. In Table 3 patient characteristics are outlined.

Table 3. Baseline characteristics for all four examined groups including age, sex, body mass index, most common drugs taken at sample acquisition, and selected laboratory markers. Table reproduced and adapted from (360) with permission of Nutrients.

Characteristics	SC-CIP (n=18)	CIP controls (n=11)	Cirrhosis (n=21)	Healthy (n=21)	p-value
Age (years); mean (±SD)	59 ± 12.7 yr	54 ± 15 yr	58 ± 9 yr	58 ± 6.9 yr	0.827
Sex (male/female)	13/5	8/3	16/5	9/12	0.204
Body Mass Index; mean (±SD)	27.2 ± 5.3	25.5 ± 3	27.3 ± 5	25.3 ± 3.1	0.350
Proton pump inhibitors (yes/no)	6/12	2/9	13/8	2/19	0.002
Antihypertensives (yes/no)	8/10	1/10	4/17	10/11	0.114
NSAIDs (yes/no)	0/18	3/8	5/16	2/19	0.088
Platelet function inhibitors (yes/no)	4/14	5/6	0/21	1/20	0.002
Statins (yes/no)	3/15	2/9	1/20	1/20	0.388
Thyroid medication (yes/no)	4/14	5/6	1/20	4/17	0.054
Beta blockers (yes/no)	6/12	2/9	14/7	5/16	0.004
AP (U/l); mean (±SD)	287.4 ± 262.2	89.8 ± 20.9	111.5 ± 47.2	61.8 ± 14.8	<0.001
GGT (U/l); mean (±SD)	518.1 ± 371.2	34.6 ± 15.3	213.6 ± 182.1	21.2 ± 10.5	<0.001
Bilirubin (mg/dl); mean (±SD)	2.0 ± 3.2	0.3 ± 0.2	1.2 ± 0.6	0.4 ± 0.8	0.02
Creatinine (mg/dl); mean (±SD)	1.0 ± 0.4	0.6 ± 0.2	0.9 ± 0.3	0.9 ± 0.2	0.2
CRP (mg/l); mean (±SD)	12.7 ± 13.6	6.4 ± 7.2	3.7 ± 3,7	2.1 ± 2.2	<0.001
INR; mean (±SD)	1.2 ± 0.4	1.0 ± 0.09	1.2 ± 0.3	1.0 ± 0.04	0.001

NSAIDs = non-steroidal anti-inflammatory drugs, AP = alkaline phosphatase, GGT = gamma-glutamyl-transferase, CRP = C-reactive protein, INR = international normalized ratio

Initial diagnosis in SC-CIP was made between 2006 and 2013. Patients received ICU treatment for different reasons comprising internal medicine pathologies like myocardial infarction and acute respiratory distress syndrome (ARDS) as well as polytrauma and burns. Seven patients suffered from liver cirrhosis at time of study inclusion (liver stiffness: 37.6 ± 19.4 (mean ± SD) kiloPascal (kPa); MELD score: 13 ± 5). Liver fibrosis (liver stiffness 9.6 ± 0.5 (mean ± SD) kPa) was recognized in four patients. All included patients had normal liver function prior to ICU treatment. One patient reported fully recovered drug-induced liver injury caused by tuberculostatic agents in the past. Duration of ICU treatment was 42 ± 20 (mean ± SD) days in this cohort. Invasive ventilation was required for 28 ± 18 days and hemodynamic support with catecholamines was administered for 12 ± 8 days. Five patients needed hemodialysis during their ICU stay. The interval between ICU admittance and diagnosis of SC-CIP was 311 ± 442 (mean ± SD) days (range 23 – 1523 days). Details are given in Table 4. None of the included patients died within the follow-up period of 457 ± 394 (mean ± SD) days. One patient had to undergo orthotopic liver transplantation. One patient was still receiving hemodialysis at study inclusion due to chronic kidney insufficiency. Study inclusion and sample acquisition were performed almost three and a half years after ICU treatment (41 ± 20 (mean ± SD) months). At time of inclusion, 12 out of 18 patients received treatment with ursodeoxycholic acid (UDCA).

Patients recruited for the first control group (CIP controls) had a history of intensive care treatment, but in contrast to SC-CIP, they and did not develop any liver diseases. Causes for the critical illness comprised mainly traumas in this group, with injuries of the spine being most numerous. Time of ICU treatment was 19 ± 12 (mean ± SD) days and time of invasive ventilation was 14 ± 9 days. Both, ICU treatment duration and ventilation time, were significantly shorter compared to SC-CIP. All patients needed hemodynamic support with application of vasoactive drugs, but it was not possible to assess length of this treatment exactly in retrospect. One single patient required hemodialysis during the ICU stay. The interval between ICU admission and inclusion in the study was 12 ± 10 (mean ± SD) months (range 95 – 907 days) and therefore shorter than this interval in SC-CIP patients. Patients in the cirrhosis group had all received the diagnosis of alcohol-associated liver cirrhosis with Child-Pugh class A (5 or 6 points) and a MELD score of 11.0 ± 2.6 (mean ± SD). Healthy controls did not report gut or liver pathologies and additionally did not suffer from other relevant distinct diseases like cardiovascular pathologies.

Table 4. ICU treatment and disease characteristics of SC-CIP and CIP controls. Table reproduced and adapted from (360) with permission of Nutrients.

Characteristics	SC-CIP (n=18)	CIP-controls (n=11)	p-value
Cirrhosis/Fibrosis/None	7/4/7	0/0/11	0.004
ICU treatment (days); mean (±SD)	42 ± 20	19 ± 12	0.001
Ventilation time (days); mean (±SD)	28 ± 18	14 ± 9	0.036
Catecholamine treatment (days); mean (±SD)	12 ± 8	n.a.	n.a.
Interval ICU admission – SC-CIP diagnosis (days); mean (±SD)	311 ± 441	n.a.	n.a.
Hemodialysis (yes/no)	5/13	1/10	0.228
Treatment with UDCA (yes/no)	12/6	0/11	<0.001
Death (yes/no)	0/18	0/11	n.a.
Liver transplantation (yes/no)	1/17	0/11	0.426

ICU = intensive care unit, UDCA = ursodeoxycholic acid

10.1.2. Microbiome analysis

10.1.2.1. Alpha diversity

Chao 1 and Richness Index were significantly reduced in SC-CIP, CIP controls and cirrhosis patients compared to healthy controls ($p<0.001$ for all three groups). No significant difference could be observed when comparing SC-CIP, CIP controls and cirrhosis among themselves. Shannon Index did only suggest decreased diversity in CIP controls and cirrhosis ($p<0.05$) (Figure 3).

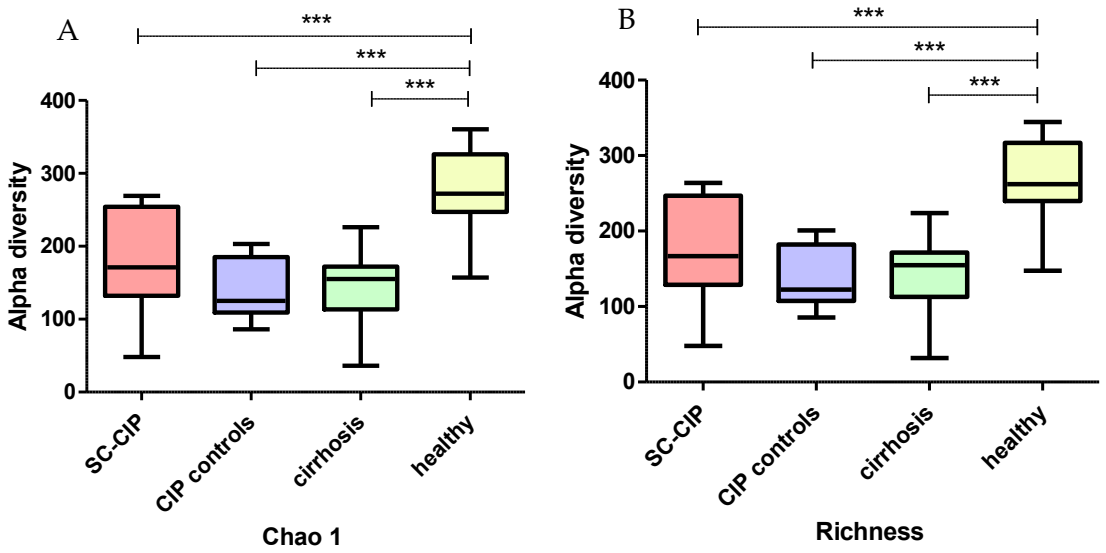

Figure 3. Alpha diversity. Boxplots indicating levels of mean alpha diversity of SC-CIP (red), CIP controls (violet), cirrhosis (green) and healthy (yellow) using (A) Chao 1 and (B) Richness Index.

Significant findings are shown with stars. *** $p \leq 0.001$. For statistical comparisons, analysis of variance (ANOVA) with Bonferroni correction was used. Figure reproduced from (360) with permission of Nutrients. Figure created by Andreas Blesl.

10.1.2.2. Beta diversity and multivariate analysis

RDA analysis indicated significant differences in beta diversity for all comparisons between the four groups on genus level ($p<0.001$) (Figure 4). To check for additional influencing factors of microbiome composition on OTU level to group allocation, the influence of sex, age, BMI, cirrhosis, CRP, creatinine, INR, thrombocytes, bilirubin, serum and stool parameters of gut permeability, intestinal inflammation, bacterial translocation, bile acid composition and common drugs (PPI, antihypertensives, NSAIDs, statins, beta blockers, thyroid medication, and antiplatelet agents) was examined. The initially conducted univariate RDA analysis revealed cirrhosis, PPI intake, beta blockers, statins, antiplatelet agents, BMI, CRP, INR, calprotectin, zonulin, DAO, LBP, and sCD14 as possible explanatory variables for microbiome composition with a p-value <0.1. When assessing collinearity with group allocation, cirrhosis and sCD14 had a Variance Inflation Factor (VIF) greater than two and low tolerance levels (VIF: cirrhosis: 3.08, sCD14: 2.56). Consequently, two distinct alternative models were created: the first excluding cirrhosis and sCD14 and including group allocation as explanatory variable, and the second model including cirrhosis and sCD14 but excluding group allocation instead. Multicollinearity could be excluded for cirrhosis. The first model (Model 1) identified group allocation ($p=0.001$) and statin use ($p=0.006$) as independent predictors of microbiome composition. The second model (Model 2) identified cirrhosis ($p=0.003$), statins ($p=0.001$), antiplatelet agents ($p=0.001$), the BMI ($p=0.001$), and calprotectin ($p=0.022$) as independent predictors (Figure 5, Table 5).

We further performed a network analysis to visualize the effects of statin intake and group allocation (Figure 6). Similarities of SC-CIP and cirrhosis in the genera *Lactobacillus* and *Blautia* could be visualized by overlap of colors. Patients with intake of statins were indicated to be closely related to healthy controls.

Next, SC-CIP and CIP control were compared to each other to examine factors favoring the occurrence of SC-CIP in critically ill patients. Variables included in the analysis contained additionally to the above-mentioned factors time of invasive ventilation, length of ICU treatment, hemodialysis, and catecholamine administration. Probably caused by the limited sample size in both compared cohorts, only sCD14, bilirubin, and BMI remained as potential

explanatory variables on univariate RDA analysis. These variables showed a Variance Inflation Factor (VIF) smaller than two and high tolerance levels, but any of these could be identified as significant explanatory variable in the multivariate model (Table 6).

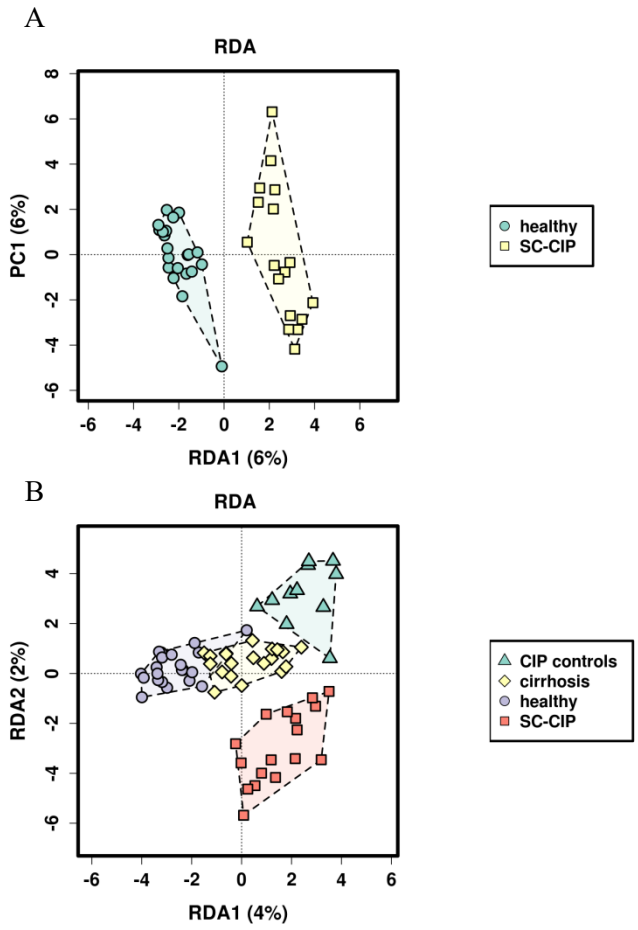

Figure 4. Beta diversity. Redundancy (RDA) analysis based on BrayCurtis dissimilarity on genus level. Each point on the diagram indicates one study proband. (A) SC-CIP (yellow) compared to healthy (green) (p=0.001), and (B) SC-CIP (red), CIP controls (green), cirrhosis (yellow) and healthy (violet) compared among themselves (p=0.001). Findings indicate that all groups showed differences in beta diversity when compared to each other. PC = principal component. Figure reproduced from (360) with permission of Nutrients. Figure created by Andreas Blesl.

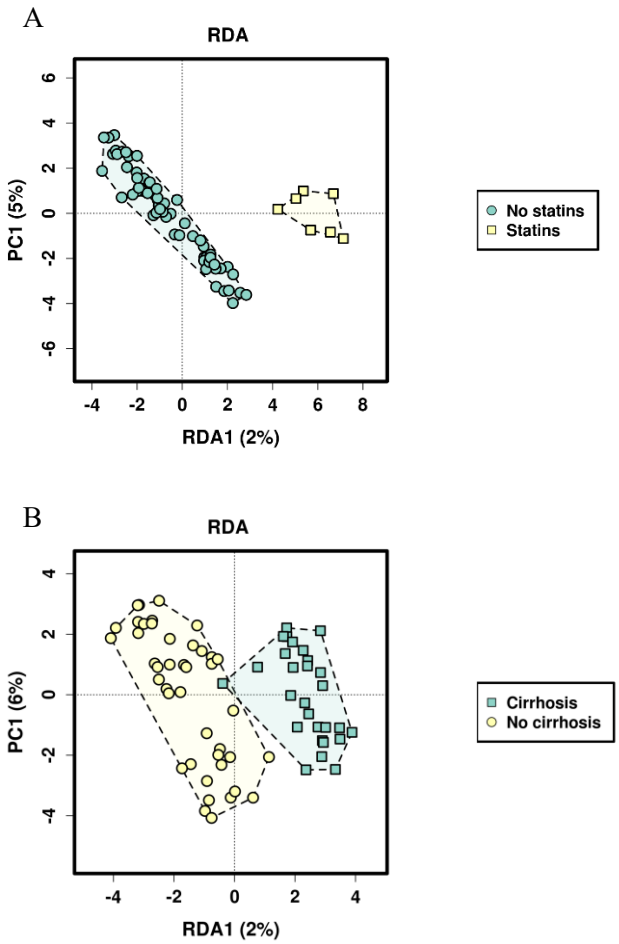

Figure 5. Redundancy analysis. Plots for statin intake and cirrhosis. Each point on the diagram indicates one study proband. (A) People not taking statins are shown in green, people taking statins are shown in orange (p=0.004). (B) Patients with cirrhosis are drawn green, controls not suffering from cirrhosis in yellow (p=0.004). In both graphs, clustering of the distinct groups can be observed. This points out that cirrhosis and statin intake are potential explanatory variables for microbiome composition. Figure reproduced from (360) with permission of Nutrients. Figure created by Andreas Blesl.

Table 5. Results of the two multivariate models (RDA+) used to examine microbiome-influencing factors. Group allocation, statin intake, intake of antiplatelet agents, BMI, calprotectin, and cirrhosis remained possible explanatory variables, with only statin intake being significant in both models. In the first column of each model, the variance is shown, in column two results of the F-test are demonstrated and in column three the p-value is indicated. Table reproduced and adapted from (360) with permission of Nutrients.

	Model 1			**Model 2**		
	Variance	**F**	***p***	**Variance**	**F**	***p***
Group	82.62	1.90	0.001			
Proton pump inhibitors	15.39	1.06	0.220	16.76	1.06	0.194
Statins	18.85	1.30	0.007	24.38	1.54	0.001
Beta Blockers	15.13	1.05	0.250	16.61	1.05	0.245
Antiplatelet agents	15.03	1.04	0.304	24.09	1.52	0.001
Body mass index	14.58	1.01	0.496	24.21	1.53	0.001
CRP	13.51	0.93	0.751	15.11	0.95	0.673
INR	15.29	1.06	0.225	16.43	1.04	0.314
Calprotectin	16.10	1.11	0.102	19.54	1.23	0.022
DAO	14.01	0.97	0.600	15.85	1.00	0.464
LBP	14.03	0.97	0.623	15.05	0.95	0.678
Zonulin	13.00	0.90	0.846	15.34	0.97	0.620
Cirrhosis				22.46	1.42	0.003
sCD14				13.73	0.87	0.924

CRP = C-reactive protein, INR = international normalized ratio, DAO = diaminoxidase, LBP = lipopolysaccharide-binding protein; sCD14 = soluble cluster of differentiation 14

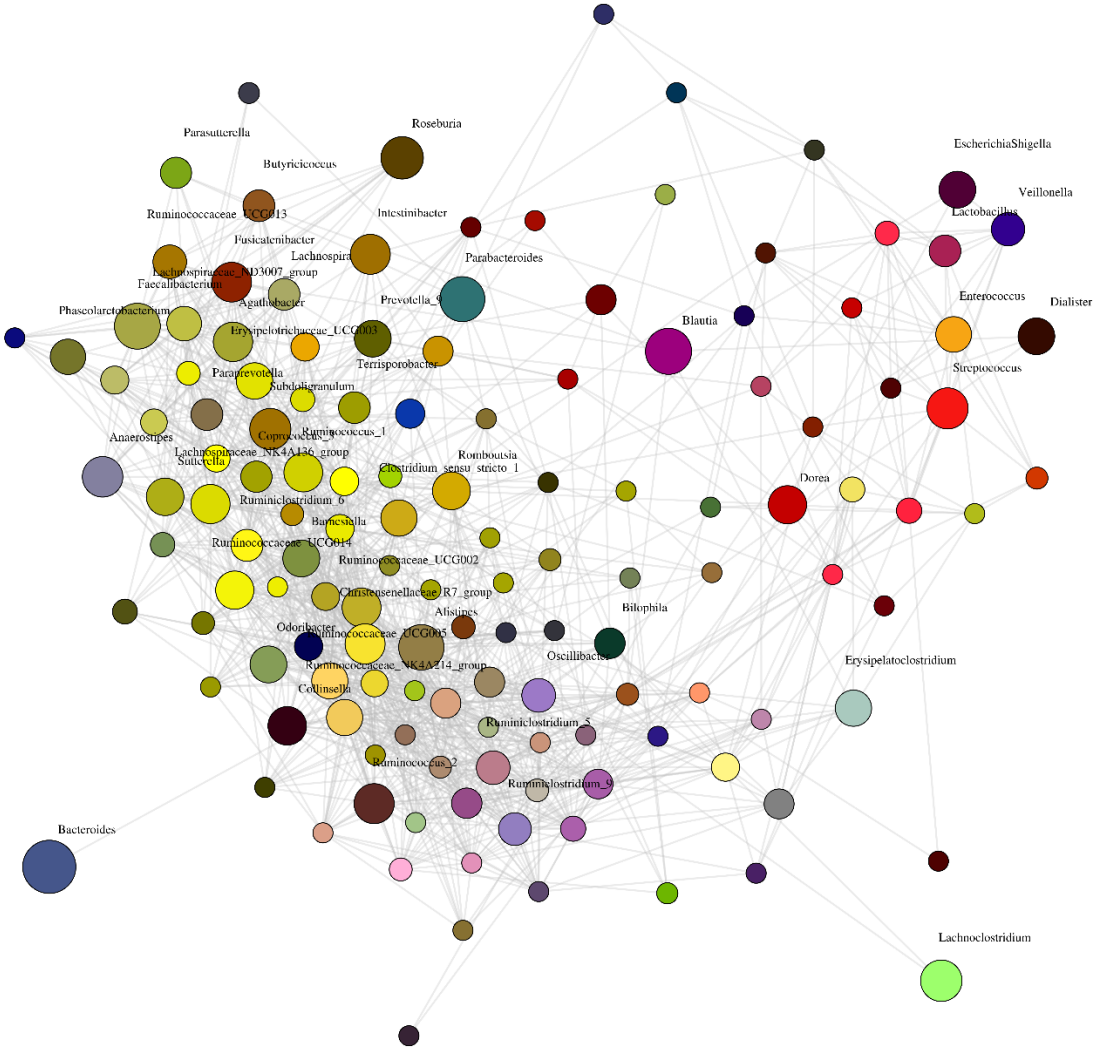

Figure 6. Network analysis. Principal coordinates analysis (PCoA) using Spearman‘s rho correlation. Taxa are indicated as dots, taxa abundance as dot size and edges show positive and negative associations. Green: statin intake, red: SC-CIP, purple: CIP controls, blue: cirrhosis, yellow: healthy. Similarities of microbiome composition are indicated with overlap of colors. Figure reproduced from (360) with permission of Nutrients. Figure created by Andreas Blesl.

Table 6. Possible explanatory variables for differences in microbiome composition of SC-CIP and CIP controls. BMI, bilirubin, sCD14 dropped out in the multivariate analysis with RDA+ and only group allocation remained as potential explanatory variable. In column one, the variance is shown, in column two results of the F-test are indicated and in column three the p-value is described. Table reproduced and adapted from (360) with permission of Nutrients.

	Variance	**F**	***p***
Group	57.99	1.40	0.001
Body mass index	29.89	0.72	0.844
Bilirubin	58.02	1.40	0.094
sCD14	32.57	0.79	0.947

sCD14 = soluble cluster of differentiation 14

10.1.2.3. Taxonomy

With usage of Analysis of Composition of Microbiomes (ANCOM) taxonomic differences from species to phylum levels between the included groups were demonstrated. First, healthy controls were compared to the three disease groups (SC-CIP, CIP controls, cirrhosis). In SC-CIP, the species *Streptococcus parasanguinis* and *Rothia dentocariosa,* both inhabitants of the oral cavity*,* the facultative pathogen *Enterococcus faecium*, the probiotic strain *Streptococcus thermophilus,* and *Sellimonas intestinalis* were elevated compared to healthy. On the other hand, the butyrate-producing *Anaerostipes hadrus* was more abundant in healthy controls. In CIP controls, the rare pathogenic species *Erysipelatoclostridium ramosum* was elevated compared to healthy controls whereas the potentially beneficial, butyrate-producing *Faecalibacterium prausnitzii* was less abundant. In cirrhosis, a potential butyrate-producing, undefined *Coprococcus* and a *Romboutsia* species were determined to have lower levels compared to healthy. Additional to alterations on species level, multiple differences on genus, family, order, class, and phylum level became evident and are indicated in Tables 7, 8, 9. In SC-CIP the well-known and good characterized genera *Streptococcus*, *Enterococcus*, *Lactobacillus*, *Bifidobacterium*, *Rothia*, and *Lactococcus* were elevated compared to healthy, whereas *Anaerostipes* and *Coprococcus* were less abundant.

Table 7. Taxonomic differences observed with Analysis of Composition of Microbiomes (ANCOM) between SC-CIP and healthy on all taxonomic levels. Data expressed as arithmetic mean. Table reproduced and adapted from (360) with permission of Nutrients.

SC-CIP vs. Healthy		
Phylum	**SC-CIP**	**Healthy**
Actinobacteria	3.19	0.62
Bacteriodetes	28.94	43.27
Patescibacteria	0.039	0.0046
Tenericutes	0	0.17
Class		
Alphaproteobacteria	0.033	0.57
Bacilli	8.99	0.4
Order		
Betaproteobacteriales	0.92	1.38
Izimaplasmatales	0	0.16
Lactobacillales	8.95	0.4
Micrococcales	0.096	0.015
Rhodospirillales	0.033	0.57
Family		
Carnobacteriaceae	0.023	0.0043
Christensenellaceae	0.5	3.01
Enterococcaceae	3.17	0.022
Micrococcaceae	0.097	0.015
Streptococcaceae	4.34	0.35
Genus		
Actinomyces	0.17	0.019
Anaerostipes	0.17	1.43
Atopobium	0.042	0.0019
Bifidobacterium	0.021	0.0022
Coprococcus_2	0.033	0.67
Enterococcus	3.3	0.023
Lachnospira	0.13	0.27
Lactobacillus	1.43	0.025
Lactococcus	0.14	0.013
Rothia	0.1	0.016
Ruminiclostridium	0.047	0.87
Ruminococcaceae_UCG005	0.14	0.78
Ruminococcaceae_UCG014	0.87	2.9
Sellimonas	0.29	0.024
Solobacterium	0.023	0.0024
Streptococcus	4.46	0.37
Sutterella	0.29	1.05
OTU		

Streptococcus thermophilus	0.69	0.033
Species		
Streptococcus parasanguinis	0.33	0.042
Rothia dentocariosa	0.029	0
Sellimonas intestinalis	0.074	0.0024
Enterococcus faecium	0.37	0
Anaerostipes hadrus	0.072	0.87

OTU = operational taxonomic unit

Table 8. Taxonomic differences found with Analysis of Composition of Microbiomes (ANCOM) between CIP controls and healthy on all taxonomic levels. Data expressed as arithmetic mean. Table reproduced and adapted from (360) with permission of Nutrients.

CIP controls vs. Healthy		
Phylum	**CIP controls**	**Healthy**
Firmicutes	49.45	53.21
Fusobacteria	0.03	0.0019
Proteobacteria	1.48	2.67
Synergistetes	0	0.00087
Class		
Bacteriodia	47	43.43
Clostridia	41.04	47.83
Gammaproteobacteria	1.63	1.8
Order		
Betaproteobacteriales	0.87	1.38
Micrococcales	0.041	0.015
Family		
Micrococcaceae	0.041	0.015
Neisseriaceae	0.032	0.00083
Genus		
Agathobacter	0.51	1.94
Coprococcus_2	0.12	0.57
Eisenbergiella	0.32	0.015
Faecalibacterium	1.47	5.9
Neisseria	0.056	0.0009
Sellimonas	0.44	0.024
Species		
Erysipelatoclostridium ramosum	0.65	0.012
Faecalibacterium prausnitzii	1.28	5.91

Table 9. Taxonomic differences observed with Analysis of Composition of Microbiomes (ANCOM) between cirrhosis and healthy on all taxonomic levels. Data expressed as arithmetic mean. Table reproduced and adapted from (360) with permission of Nutrients.

Cirrhosis vs. Healthy		
Phylum	**Cirrhosis**	**Healthy**
Synergistetes	0.0013	0.00017
Class		
Clostridia	36	47.83
Erysipelotrichia	1.3	2.61
Oxyphotobacteria	0.0032	0.00064
Synergistia	0.0013	0.00088
Order		
Bacteroidales	45.78	43.41
Betaproteobacteriales	1.24	1.38
Clostridiales	36.02	47.84
Corynebacteriales	0.00031	0.00029
Erysipelotrichales	1.3	2.61
Family		
Christensenellaceae	0.72	3.01
Clostridiaceae_1	0.13	0.9
Peptostreptococcaceae	0.82	2.83
Genus		
Coprococcus_2	0.025	0.67
Romboutsia	0.22	1.13
Veillonella	0.4	0.033
OTU		
Uncultured Bacterium	0.09	0.28
Uncultured Bacterium	0.00075	0.2
Species		
Coprococcus_2_NA	0.023	0.5
Romboutsia_NA	0.18	1.01

OTU = operational taxonomic unit

Second, variations between the three disease groups (SC-CIP, CIP controls, cirrhosis) were evaluated using the same procedure. *Prevotella melaninogenica,* a species inhabiting the oral cavity which is capable to cause opportunistic infections, and a not further classified *Neisseria* species showed decreased abundance in SC-CIP compared to CIP controls. *Anaerostipes hadrus* was more abundant in cirrhosis when compared to SC-CIP. Further taxonomic differences on genus, family, order, class, and phylum level are outlined in Tables 10, 11, 12.

Table 10. Taxonomic differences observed with Analysis of Composition of Microbiomes (ANCOM) between SC-CIP and CIP controls on all taxonomic levels. Data expressed as arithmetic mean. Table reproduced and adapted from (360) with permission of Nutrients.

SC-CIP vs. CIP controls		
Phylum	**SC-CIP**	**CIP controls**
Firmicutes	64.1	49.45
Patescibacteria	0.039	0.00053
Tenericutes	0	0.047
Class		
Bacilli	8.99	3.45
Bacteroidia	28.91	47
Mollicutes	0	0.047
Saccharimonadia	0.039	0.00053
Family		
Neisseriaceae	0	0.032
Genus		
Anaerotruncus	0.01	0.42
Bifidobacterium	0.021	0.0052
Coprobacillus	0.044	0.26
Fusicatenibacter	1.65	0.51
Intestinimonas	0.019	0.14
Neisseria	0	0.036
Oscillibacter	0.13	0.77
Prevotella_7	0	0.038
Solobacterium	0.023	0.0023
OTU		
Uncultured Bacterium	0.042	0.71
Species		
Neisseria_NA	0	0.032
Prevotella_7_melaninogenica	0	0.03

OTU = operational taxonomic unit

Table 11. Taxonomic differences observed with Analysis of Composition of Microbiomes (ANCOM) between SC-CIP and cirrhosis on all taxonomic levels. Data expressed as arithmetic mean. Table reproduced and adapted from (360) with permission of Nutrients.

SC-CIP vs. Cirrhosis		
Phylum	**SC-CIP**	**Cirrhosis**
Actinobacteria	3.19	1.21
Bacteroidetes	28.94	45.86
Firmicutes	64.1	46.83
Patescibacteria	0.039	0.0002
Tenericutes	0	0.018
Class		
Alphaproteobacteria	0.033	1.69
Mollicutes	0	0.018
Order		
Rhodospirillales	0.033	1.69
Family		
Atopobiaceae	0.57	0.0074
Bifidobacteriaceae	0.018	0
Eggerthellaceae	0.59	0.069
Erysipelotrichaceae	3.51	1.34
Lachnospiraceae	25.49	21.57
Peptostreptococcaceae	5.95	0.82
Genus		
Actinomyces	0.17	0.048
Anaerostipes	0.17	2.44
Bifidobacterium	0.021	0
Dorea	1.52	0.43
Lachnospira	0.13	0.29
Solobacterium	0.023	0.0028
Species		
Anaerostipes hadrus	0.072	2.04

Table 12. Taxonomic differences observed with Analysis of Composition of Microbiomes (ANCOM) between CIP controls and cirrhosis on all taxonomic levels. Data expressed as arithmetic mean. Table reproduced and adapted from (360) with permission of Nutrients.

CIP controls vs. Cirrhosis		
Phylum	**CIP controls**	**Cirrhosis**
Firmicutes	49.45	46.83
Proteobacteria	1.48	6.06
Synergistetes	0	0.0013
Class		
Alphaproteobacteria	0.3	1.69
Clostridia	41.04	36
Erysipelotrichia	2.55	1.3
Gammaproteobacteria	1	3.99
Synergistia	0	0.0013
Order		
Bacteriodales	47	45.78
Clostridiales	41.04	36.02
Corynebacteriales	0	0.00031
Erysipelotrichiales	2.55	1.3
Propionibacteriales	0.00068	0.0074
Rhodospirillales	0.3	1.69
Synergistales	0	0.0013
Family		
Eggerthellaceae	0.51	0.069
Neisseriaceae	0.032	0.00073
OTU		
Unclutured Bacterium	0.0026	0.25
Unclutured Bacterium	0.11	0.012
Unclutured Bacterium	0.61	0

OTU = operational taxonomic unit

10.1.2.4. Microbiome composition

Supervised machine learning algorithms as a feature selection method on genus level (LefSE) were used to further explore microbiome composition. Confirming the results obtained with ANCOM, *Lactobacillus* and *Streptococcus,* both counted as oral commensal bacteria, and *Enterococcus,* as well as *Romboutsia* and the butyrate-producing *Butyricicoccus,* were associated with SC-CIP. Associations with CIP controls could be demonstrated for the cellulosome-producing microbe *Ruminiclostridium,* the favorable genus *Odoribacter*, the genus *Merdibacter***,** involved in maintenance of metabolic homeostasis, as well as for *Eisenbergiella*,

Coprobacillus, Erysipelatoclostridium, Oscillibacter, and *Anaerotruncus.* For cirrhosis, *Rothia*, an oral inhabitant, *Alistipes,* suggested playing a role in colorectal cancer evolution and the short-chain fatty acid-producing *Anaerostipes* and *Lachnospira*, as well as *Tyzzerella* showed associations. At last, for healthy controls association with *Prevotella*, a genus situated mainly in the oral cavity and reported to be involved in development of abscesses, the butyrate-producing *Coprococcus*, *Clostridium senso stricto*, and the very abundant *Ruminococcus* could be demonstrated (Figure 7).

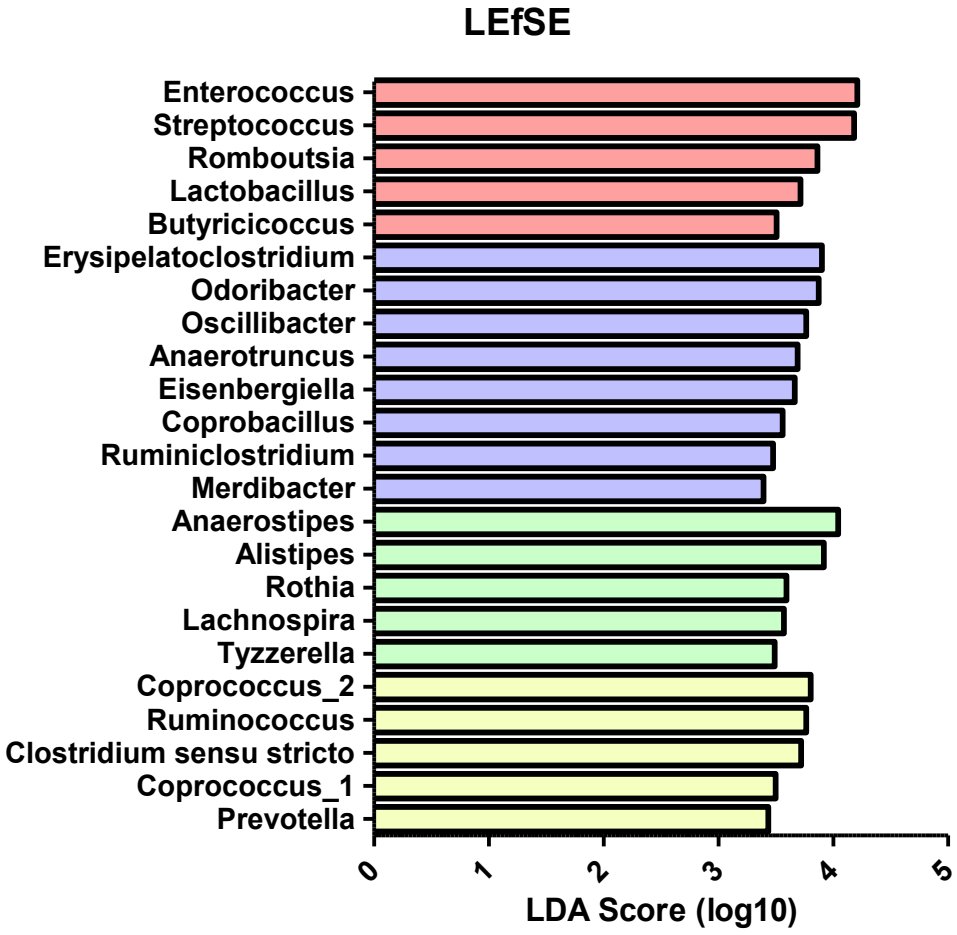

Figure 7. LEfse analysis. Linear discriminant analysis-effect size (LEfse) results for SC-CIP (red), CIP controls (violet), cirrhosis (green), and healthy (yellow). The highlighted genera are associated with microbiome composition of the respective group. The length of the bar indicates the logarithmic linear discriminant analysis (LDA) score. Figure reproduced from (360) with permission of Nutrients. Figure created by Andreas Blesl.

10.1.3. Gut permeability and bacterial translocation

Serum levels of the gut permeability marker diaminoxidase (DAO) were elevated in the three disease groups compared to healthy (SC-CIP – healthy: p=0.005; CIP controls – healthy: p=0.013, cirrhosis – healthy: p=0.001). Differences concerning zonulin and calprotectin in feces could not be observed, but CRP was higher in SC-CIP and CIP controls compared to healthy and in SC-CIP compared to cirrhosis (SC-CIP - healthy: p<0.001; CIP controls - healthy: p=0.016; SC-CIP - cirrhosis: p=0.04). LBP levels, used as marker for bacterial translocation, were not distinct in all comparisons, but soluble CD14 (sCD14) showed increased levels in SC-CIP and cirrhosis compared to healthy (p<0.001) and in cirrhosis compared to CIP controls (p=0.01) (Figure 8). Furthermore, positive correlations of markers of gut permeability, bacterial translocation, and inflammation with liver damage and stiffness and nutritional status could be outlined (Table 13).

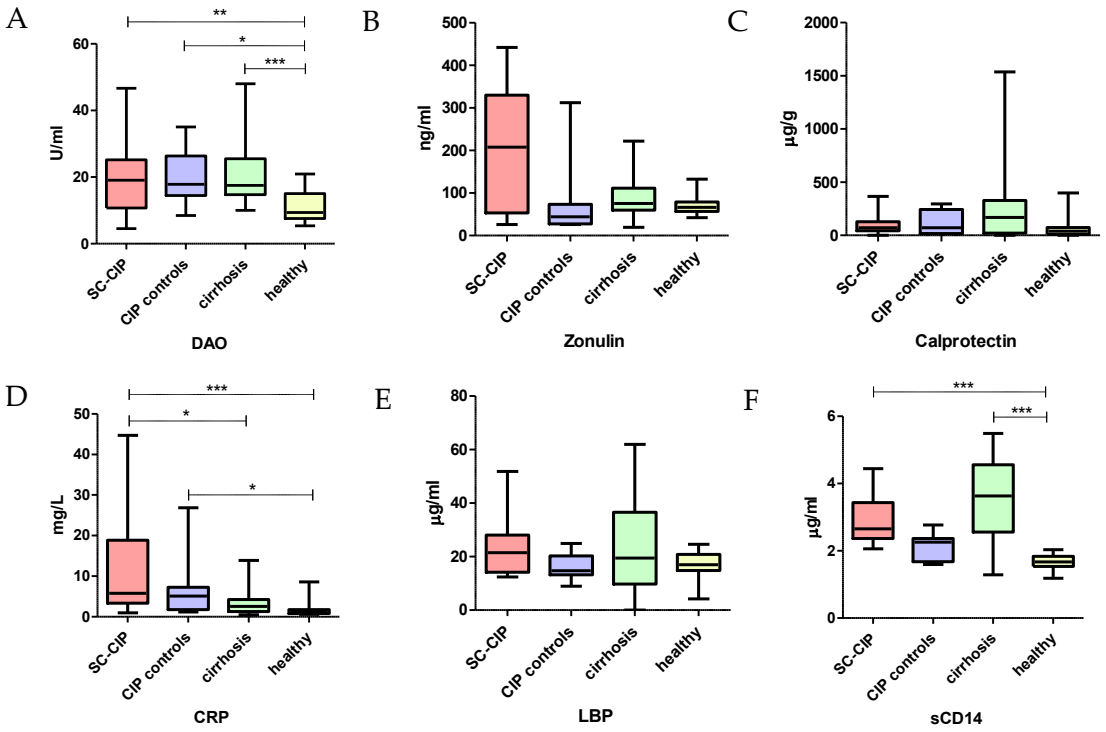

Figure 8. Gut permeability and bacterial translocation. Boxplots indicating markers of gut permeability, bacterial translocation, and intestinal and systemic inflammation. (A) diaminoxidase (B) zonulin (C) calprotectin (D) C-reactive protein (E) lipopolysaccharide-binding protein (F) soluble cluster of differentiation 14. SC-CIP (red), CIP controls (violet), cirrhosis (green), healthy (yellow). Significances are demonstrated with stars within the diagrams. * p≤0.05; ** p≤0.01; *** p≤0.001. For statistical analysis, analysis of variance (ANOVA) with Bonferroni correction was used; DAO = diaminoxidase, CRP = C-reactive protein, LBP = lipopolysaccharide-binding protein, sCD14 = soluble cluster of

differentiation 14. Figure reproduced from (360) with permission of Nutrients. Figure created by Andreas Blesl.

Table 13. Correlations of biomarkers of gut permeability, bacterial translocation, intestinal and systemic inflammation with age, body mass index, liver and kidney function pictured on a heatmap. Spearman`s correlation coefficient is indicated in the table. Significant results ($p<0.05$) are framed. The greener a field is drawn, the better is the positive correlation, the redder a field is drawn the more pronounced is the negative correlation. Table reproduced and adapted from (360) with permission of Nutrients.

	Zonulin	DAO	sCD14	LBP	CRP	Calprotectin
Creatinine	-0.042	-0.074	-0.052	0.147	0.047	0.019
Bilirubin	0.037	0.226	0.432	0.27	0.554	0.029
INR	0.107	0.286	0.339	-0.013	-0.08	0.092
Stiffness	-0.207	0.629	0.268	0.512	0.52	0.457
Age	-0.023	0.026	0.015	0.14	0.229	0.05
Body mass index	-0.03	0.32	0.226	0.073	0.235	-0.081

DAO = diaminoxidase, sCD14 = soluble cluster of differentiation 14, LBP = lipopolysaccharide-binding protein, CRP = C-reactive protein, INR = international normalized ratio

10.1.4. Serum bile acids

Total serum bile acids were higher in SC-CIP and cirrhosis compared to healthy ($p<0.001$) and in cirrhosis compared to CIP controls ($p=0.007$). Caused most likely by UDCA intake in 12 out of 18 patients in SC-CIP, UDCA was increased in SC-CIP compared to healthy ($p=0.02$), but not when compared to the other two groups. Therefore, UDCA was not excluded in the following evaluation. Primary and secondary bile acids were increased in cirrhosis compared to healthy ($p=0.05$; $p=0.01$) and total conjugated and unconjugated bile acids in SC-CIP and cirrhosis compared to healthy (total conjugated both groups: $p<0.001$, total unconjugated both groups: $p=0.02$). Total conjugated bile acids were higher in cirrhosis than in CIP controls ($p=0.007$). On individual bile acid level, the primary bile acid cholic acid was unchanged between the groups, whereas chenodeoxycholic acid was augmented in cirrhosis compared to healthy ($p=0.02$). The secondary bile acid deoxycholic acid was increased in cirrhosis compared to healthy ($p=0.05$). Lithocholic acid demonstrated high levels in SC-CIP and cirrhosis and levels close to zero in CIP controls and healthy (Figure 9). With multivariate NMMDS analysis, changed bile acid profiles were found in healthy compared to SC-CIP, CIP control, and cirrhosis. The analysis was performed with and without inclusion of UDCA ($R=0.26$, $p<0.001$;

SC-CIP - healthy: p<0.001; cirrhosis - healthy: p<0.001; CIP controls - healthy: p=0.006). Differences between SC-CIP and cirrhosis could additionally be demonstrated, independent of inclusion of UDCA in the analysis (p=0.03) (Figure 10). Several positive correlations of bile acid levels with markers of bacterial translocation, gut permeability and liver damage could be highlighted (Table 14).

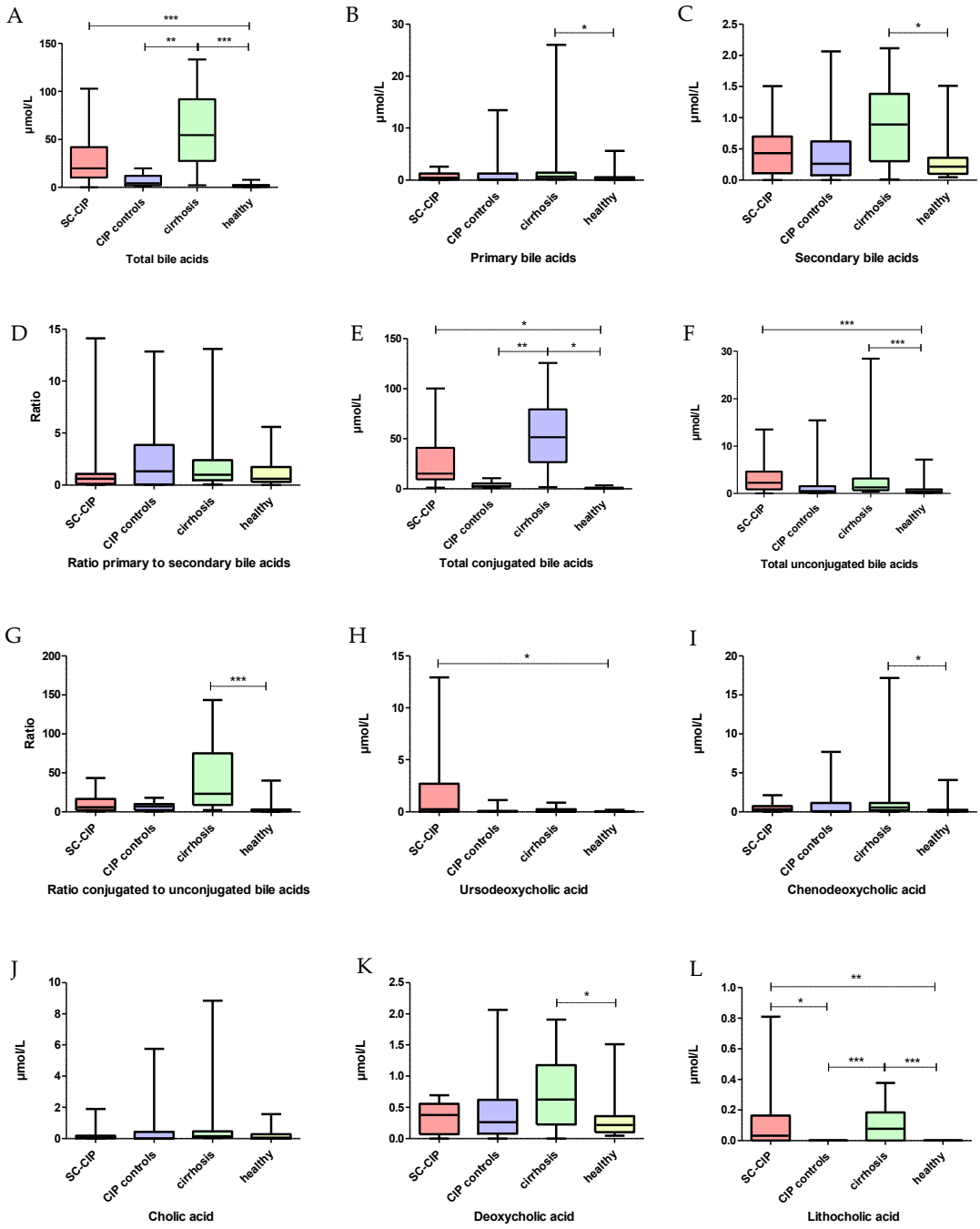

Figure 9. Serum bile acids. Boxplots for comparison of bile acid levels. (A) total bile acids (B) primary bile acids (C) secondary bile acids (D) ratio primary to secondary bile acids (E) total conjugated bile

acids (F) total unconjugated bile acids (G) ratio conjugated to unconjugated bile acids (H) ursodeoxycholic acid (I) chenodeoxycholic acid (J) cholic acid (K) desoxycholic acid (L) lithocholic acid. SC-CIP (red), CIP controls (violet), cirrhosis (green), healthy (yellow). Significances are indicated with stars within the diagrams. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. For statistical analysis, analysis of variance (ANOVA) with Bonferroni correction was used. Figure reproduced from (360) with permission of Nutrients. Figure created by Andreas Blesl.

Table 14. Correlations of serum bile acid profiles with markers of gut permeability, bacterial translocation, intestinal and systemic inflammation and liver function parameters. Spearman`s correlation coefficient is indicated in the table. Significant results ($p<0.05$) are framed. The greener a field is drawn the better is the positive correlation, the redder a field is drawn the more pronounced is the negative correlation. Table reproduced and adapted from (360) with permission of Nutrients.

	T BA	P/S BA	U/C BA	P BA	S BA	TC BA	TU BA
sCD14	0.529	0.108	0.562	0.109	0.196	0.517	0.093
Zonulin	-0.011	-0.149	-0.041	-0.059	0.031	0.14	0.111
DAO	0.367	0.287	0.217	0.309	0.123	0.331	0.283
LBP	0.1	-0.08	0.117	-0.107	0.032	0.106	-0.125
CRP	0.143	-0.008	0.131	-0.049	-0.13	0.133	-0.033
Calprotectin	0.028	-0.076	0.293	-0.016	0.035	0.011	-0.034
Creatinine	-0.062	-0.221	-0.051	-0.204	0.095	-0.066	-0.171
Bilirubin	0.268	0.088	0.391	-0.014	-0.067	0.294	-0.049
INR	0.353	0.109	0.308	0.102	0.34	0.303	0.098
Stiffness	0.194	0.193	0.64	0.293	-0.33	0.356	-0.305

T BA = total bile acids, P/S BA = Primary/secondary bile acids, U/C BA = unconjugated/conjugated bile acids, P BA = primary bile acids, S BA = secondary bile acids, TC BA = total conjugated bile acids, TU BA = total unconjugated bile acids, sCD14 = soluble cluster of differentiation 14, DAO = diaminoxidase, LBP = lipopolysaccharide-binding protein, CRP = C-reactive protein, INR = international normalized ratio

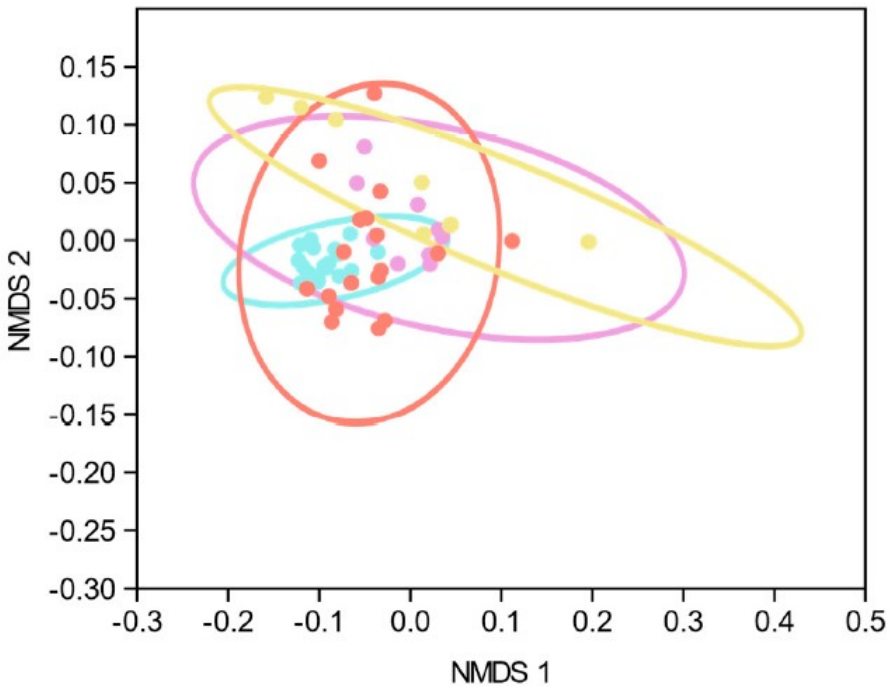

Figure 10. Serum bile acid composition. Principal coordinates analysis (PCoA) plot for serum bile acid composition using non-metric multidimensional scaling (Bray–Curtis) for analysis. Orange: SC-CIP, violet: CIP controls, turquoise: cirrhosis, yellow: healthy. Each point on the diagram presents one study proband. Distinct bile acid profiles could be demonstrated for healthy compared to SC-CIP, CIP control and cirrhosis and between SC-CIP and cirrhosis. R=0.26, p<0.001; SC-CIP–healthy: p<0.001; CIP controls–healthy: p=0.006, cirrhosis–healthy: p<0.001; NMDS = Non-metric multidimensional scaling. Figure reproduced from (360) with permission of Nutrients. Figure created by Andreas Blesl.

10.2. Retrospective Study "GI bleeding in SC-CIP" (366)

10.2.1. Patient characteristics

Fifty-three patients suffering from SC-CIP and 19 controls with history of long-term ICU treatment due to cardiac surgery but without the diagnosis SC-CIP were included in the analysis. Baseline characteristics are outlined in Tables 15 and 16. Age at study inclusion was 57 ± 14 (mean ± SD) years in SC-CIP and 73 ± 10 years in the control group (p<0.001). One-quarter of patients in both groups were female (SC-CIP: 14 (26%), controls: 4 (21%)). Length of ICU stay was not distinct between the two groups (p=0.1). Causes for intensive care treatment in SC-CIP comprised internal medicine emergencies such as myocardial infarction

and ARDS, but also polytrauma and burns. Diagnosis of SC-CIP was established 12 ± 22 (mean ± SD) months after ICU admission. Patients included in the control group were hospitalized because of acute myocardial infarction, aortic dissection, or valvular heart disease with the necessity for cardiac surgery. Within the observation period, nine SC-CIP (17%) needed orthotopic liver transplantation and 16 patients (30%) died, mainly due to complications linked to the initial incident causing the ICU stay. In the control group, six deaths (32%) were observed. Mean follow-up was nearly five years in SC-CIP and one and a half years in the control group. In total, 3053 patient months (254 years) in SC-CIP and 296 patient months (25 years) in the control group were overviewed.

Table 15. Baseline characteristics of patients suffering from SC-CIP included in the analysis. Table reproduced and adapted from (366) with permission of the Journal of Clinical Medicine.

	SC-CIP (n= 53)
Age at study inclusion (years); mean (±SD)	57 (±14)
Female; n (%)	14 (26)
Deaths; n (%)	16 (30)
Age at death (years); mean (±SD)	64 (±11)
Length of ICU stay (days); mean (±SD)	40 (±22)
Platelets at diagnosis (10^9/l); mean (±SD)	280800 (±132000)
Bilirubin at diagnosis (mg/dl); mean (±SD)	5.8 (±7.4)
Alkaline phosphatase at diagnosis (U/l); mean (±SD)	701 (±499)
GGT at diagnosis (U/l); mean (±SD)	1022 (±726)
Prothrombin time at diagnosis (%); mean (±SD)	84 (±27)
Albumin at diagnosis (g/dl); mean (±SD)	3.6 (±0.8)
Creatinine at diagnosis (mg/dl); mean (±SD)	1.4 (±1.1)
Liver transplantation; n (%)	9 (17)
GI bleeding; n (%)	16 (30)
Follow-up (months); mean (±SD)	58 (±47)

SC-CIP = secondary sclerosing cholangitis in critically ill patients, SD = standard deviation, GI = gastrointestinal, GGT = gamma-glutamyltransferase

Table 16. Baseline characteristics of patients included in the control group. All patients were treated on an intensive care unit because of carried-out cardiac surgery. Table reproduced and adapted from (366) with permission of the Journal of Clinical Medicine.

	Controls (n= 19)
Age at study inclusion (years); mean (±SD)	73 (±10)
Female; n (%)	4 (21)
Deaths; n (%)	6 (32)
Age at death (years); mean (±SD)	60 (±16)
Length of ICU stay (days); mean (±SD)	32 (±27)
GI bleeding; n (%)	1 (5)
Follow-up (months); mean (±SD)	16 (±11)

SD = standard deviation, ICU = intensive care unit, GI = gastrointestinal

10.2.2. Frequency of GI bleeding

GI bleeding was observed in 16 patients (30%) of the SC-CIP cohort and one patient (5%) of the control group (p=0.03). Bleeding occurred 13 ± 19 (mean ± SD) months after ICU admission in SC-CIP and 10 patients developed bleeding within the first year after ICU admission (data not available for two patients). Three bleedings occurred during the ICU stay (Table 17). Cirrhosis was present in only three SC-CIP patients (19%) at onset of bleeding, five patients (31%) had splenomegaly and four (25%) received oral anticoagulation. Platelets were significantly (p=0.01), GGT (p=0.1) and alkaline phosphatase (p=0.4) numerically decreased at bleeding than at time of diagnosis, whereas bilirubin (p=0.1) was numerically elevated at bleeding. The majority of patients presented with normal prothrombin time at bleeding. Bleedings were grave in SC-CIP, with hemoglobin levels of 8.0 ± 1.7 (mean ± SD) g/dl at the time of bleeding recognition.

Table 17. Characteristics of patients with SC-CIP and GI bleeding. Table reproduced and adapted from (366) with permission of the Journal of Clinical Medicine.

	SC-CIP (n = 16)
Interval ICU-GI bleeding (months); mean (±SD)	13 (±19)
Interval SC-CIP diagnosis-GI bleeding (months); mean (±SD)	11 (±23)
Bleeding during ICU stay; n (%)	3 (19)
Cirrhosis at GI bleeding; n (%)	3 (19)
Splenomegaly at GI bleeding; n (%)	5 (31)
Signs of portal hypertension upon endoscopy; n (%)	2 (13)
Anticoagulation at GI bleeding; n (%)	4 (25)
Hemoglobin at GI bleeding (g/dl); mean (±SD)	8.0 (±1.7)
Platelets at GI bleeding (10^9/l); mean (±SD)	173000 (±116000)
Bilirubin at GI bleeding (mg/dl); mean (±SD)	8.0 (±7.0)
Alkaline phosphatase at GI bleeding (U/l); mean (±SD)	614 (±559)
GGT at GI bleeding (U/l); mean (±SD)	671 (±494)
Prothrombin time at GI bleeding (%); mean (±SD)	70 (±31)
Albumin at GI bleeding (g/dl); mean (±SD)	2.6 (±0.6)
Creatinine at GI bleeding (mg/dl); mean (±SD)	1.4 (±0.7)

SC-CIP = secondary sclerosing cholangitis in critically ill patients, ICU = intensive care unit, GI = gastrointestinal, SD = standard deviation, GGT = gamma-glutamyltransferase

10.2.3. Characteristics of GI bleedings in SC-CIP

In 13 patients with SC-CIP the bleeding location was identified in the upper GI tract. The main bleeding causes were ulcers located in all parts of the upper GI tract (see bleeding characteristics Table 18). Interestingly, ulcers in the stomach emerged at atypical locations for peptic ulcers (see examples Figure 11) and visible vessels without ulceration were detected (Dieulafoy's lesions). In a single patient the bleeding source could not be elucidated. Bleeding originated from the colon in only two patients (ulcers in the rectum of unknown origin in the first patient, atypical visible vessel without ulceration in the ascending colon in the second patient). Eight patients underwent gastric biopsies, helicobacter pylori could not be found in any of them. To achieve hemostasis, submucosal diluted adrenalin, hemostatic gel, hemoclips, argon plasma coagulation (APC), and hemostatic powder were applied. One patient received coiling via angiography because endoscopic intervention failed. Supportive medical treatment with PPI was administered in all patients. Dosing widely differed between patients and ranged from 40mg once daily to 80mg three times a day or continuous application with an infusion pump.

80% of patients received blood units, six needed more than five of them. One death occurred due to GI bleeding in a patient with liver cirrhosis caused by SC-CIP.

10.2.4. Characteristics of GI bleeding in the control group

Only one hint for a GI bleeding episode was observed in the control group. This was a positive test for occult blood in stool in a single patient lacking additional bleeding signs. Suspected bleeding terminated spontaneously, endoscopic evaluation was omitted.

Table 18. Characteristics of GI bleedings in SC-CIP. Patients with GI bleedings were numbered from one to sixteen. Patient 13 died due to GI bleeding. Bleeding was not lethal in the other 15 patients. ICU – bleeding indicates the time from admission to ICU to the occurrence of gastrointestinal bleeding in months. Hemoglobin levels were recorded at time of recognition of gastrointestinal bleeding. Table reproduced and adapted from (366) with permission of the Journal of Clinical Medicine.

Patient	ICU - bleeding	Hemoglobin (g/dl)	Location of bleeding	Bleeding source	Therapy	Blood units
1	69	7.3	Duodenum	Ulcer	PPI	3
2	1	8.6	Esophagus, Stomach	Hemorrhagic mucosa	PPI	No
3	31	8.6	Stomach	Ulcer	PPI	1
4	18	4.9	Unkown	Unknown	PPI	>5
5	2	7.5	Rectum	Ulcers	PPI, HG	Unknown
6	2	11.0	Duodenum	Angiodysplasia, trauma of DHC stent	PPI, APC	No
7	2	5.3	Stomach	Ulcer, Visible vessel	PPI, A, HC	2
8	1	11.2	Esophagus, Stomach	Ulcers	PPI	>5
9	unknown	unknown	Esophagus, Stomach	Ulcers	PPI	No
10	33	7.3	Duodenum	Bleeding from papilla after EPT	PPI, A	3
11	8	8.8	Esophagus	Coagulum ora serrata, no visible lession	PPI, A	3
12	2	8.8	Duodenum	Ulcers	PPI, Coiling (IR)	>5
13	unknown	7.6	Stomach	Ulcer	PPI, HC	>5
14	9	8.1	Colon ascendens	Visible vessel without ulceration	PPI, HC	>5
15	0	8.1	Duodenum	Visible vessel without ulceration	PPI, A, HC, HP	>5
16	9	6.6	Duodenum	Ulcer	PPI, HC	4

ICU = intensive care unit, PPI = proton pump inhibitor, A=adrenalin, HC = hemoclip, HG = hemostatic gel, APC= argon plasma coagulation, HP = hemostatic powder, IR = interventional radiology

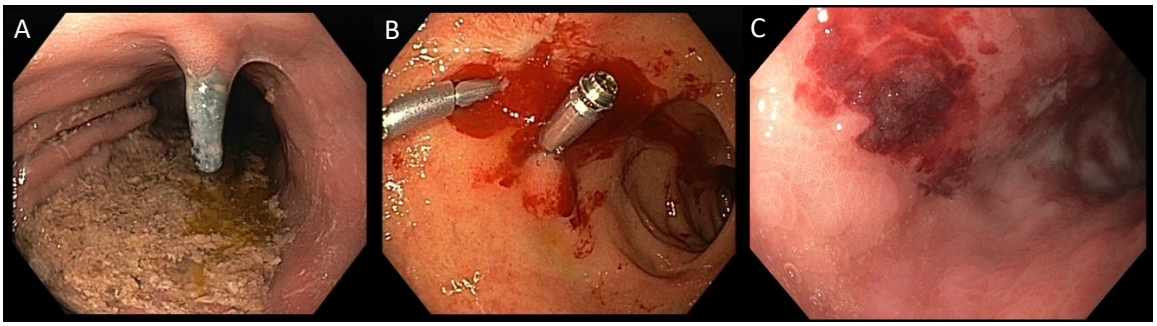

Figure 11. Bleeding sources of three patients included in the study. (A) Patient 7. Second look endoscopy after suspension of bleeding. Endoscopy revealed a hemoclip in the body of the stomach located at the small curvature. The bleeding originated from a visible vessel from an ulcer at an atypical location. (B) Patient 16. Endoscopy demonstrated active bleeding from an ulcer located in the duodenal bulb. The bleeding was categorized as Forrest 1b. Hemostasis could finally be ensured with the use of hemoclips. (C) Patient 9. Endoscopy revealed several ulcers in the stomach with one of them demonstrating bleeding signs (coagulum). The bleeding was rated as Forrest 2b. As no active bleeding was observed, the endoscopist decided not to intervene. Instead, medical therapy with proton pump inhibitors was initiated. Figure reproduced from (366) with permission of the Journal of Clinical Medicine. Figure created by Andreas Blesl.

10.2.5. Radiological evaluation

Altered vascular anatomy or suspected vascular stenosis were observed with similar frequency in SC-CIP patients with and without GI bleedings (SC-CIP with bleeding: altered anatomy: n=5 (31%), stenosis: n=1 (6%); SC-CIP without bleeding: altered anatomy n=8 (24%), stenosis: n=5 (15%) (data of 3 patients missing)) (Table 19). Variants most commonly observed comprise modified origins of hepatic arteries. Additionally, stenosis of the coeliac trunk, the common hepatic artery, and the superior mesenteric artery were recognized. In one case innate compression of the coeliac trunk by the diaphragm was suspected, in the other patients stenosis seemed to be caused by arteriosclerosis. A specific risk pattern increasing the risk for GI bleeding could not be characterized.

Table 19. Description of alterations of the vascular anatomy of SC-CIP highlighting standard variants and vascular stenosis. The cohort was divided depending on the occurrence of GI bleeding. Table reproduced and adapted from (366) with permission of the Journal of Clinical Medicine.

	SC-CIP without GI bleeding (n=37)	**SC-CIP with GI bleeding (n=16)**
Imaging not available	n = 3	n = 0
Normal vascular anatomy/no stenosis	n = 21	n = 10
RHA arising from superior mesenteric artery	n = 3	n = 2
LHA arising from left gastric artery	n = 2	n = 1
LHA and RHA arising from coeliac trunk	n = 1	n = 0
LHA and RHA arising from coeliac trunk, GD artery arising from LHA	n = 1	n = 1
Common hepatic artery arising from the aorta	n = 0	n = 1
Accessory renal artery	n = 1	n = 0
Stenosis of the coeliac trunk	n = 3	n = 1
Stenosis of the common hepatic artery	n = 1	n = 0
Stenosis of the superior mesenteric artery	n = 1	n = 0

RHA = right hepatic artery, LHA = left hepatic artery, GD = gastroduodenal

11. Discussion

SC-CIP is a chronic cholestatic liver disease that is induced by long-term intensive care treatment in patients without prior existing liver diseases (50). Since the disease is relatively rare and has been characterized for the first time only 20 years ago, relevant gaps of knowledge about the pathophysiology and the heterogeneous course of the disease remain (6, 12). What is already known about this disease is that it is triggered by severe illnesses, basically independent of the type of the disease, but occurrence is mainly described after cardiac surgery, burns, polytrauma as well as in patients with myocardial infarction and acute respiratory distress syndrome (ARDS), recently also described in a Covid-19 patient (14, 367, 368). Additional to a severe illness, the patient has to undergo invasive ventilation and hemodynamic support with vasoactive drugs to acquire the disease (50). Interestingly, all patients have a healthy liver before ICU treatment, the disease does not seem to appear in prior harmed organs (14). Even though a lot of patients on an ICU fulfill these requirements and seem to be potentially at risk for the disease, only a very small number of them develop it. SC-CIP favors men in a ratio between 2:1 and 9:1 (14), but reasons for this predominance remain unclear. The same is observed in PSC and females also seem to have a milder disease course of this disease (17, 369). On the other hand, mortality rates are higher in critically ill patients with male sex under

the age of fifty compared to women (370). Consequently, it remains to be determined if SC-CIP is absolutely more common in men, or if men experience the more severe illnesses leading to SC-CIP. Immunological effects of sex hormones, pregnancy, genetics, differences in environmental factors and dietary habits, and sex-related differences in microbiome composition have been discussed as reasons for the unequal incidence of liver diseases in the two sexes (371). Furthermore, it seems quite ensured that the pathophysiology of SC-CIP comprises ischemic damage of the biliary tree which consequently leads to biliary infections and casts blocking the bile flow (360). It was suggested that there might be a genetic background, namely NOD2 variants, favoring the evolution of the disease (25). In the existing literature it is indicated that the disease often takes an unfavorable course with rapid formation of liver cirrhosis, subsequently leading to liver transplantation because an established conservative treatment is not available (9, 14). But as also demonstrated with our SC-CIP cohorts, a relevant proportion of patients also suffer from milder disease courses with slow or even no progression to cirrhosis (360).

Despite the increased knowledge about the disease, relevant key questions with important implications for clinical care have not been successfully addressed so far: Which patients acquire the disease and because of which reason? Is the trigger a preexisting condition like body weight, sex, or genetics? Does the disease occur in context with drug intake or preexisting medical conditions? Are the main drivers the severity of the underlying illness and the length of the ICU stay? Are other, not so obvious, pathophysiological drivers working in the background? Is the gut-liver axis involved in disease evolution as described for other liver pathologies? If yes, are alterations just a consequence of the disease or involved in disease evolution? How can we identify patients with an increased risk for an unfavorable course? What are the complications patients have to suffer during disease course and can we prevent them? Probably the answers to these questions would prepare the ground for the development of prevention strategies, targeted treatments, and improved outcome.

To contribute to the solution of these important questions, we have conducted the present studies. We were able to highlight that patients suffering from SC-CIP have a distinct gut microbiome composition, increase gut permeability and bacterial translocation, and an altered serum bile acid profile compared to healthy controls. Besides, also the control group (CIP controls) including patients after ICU treatment without the occurrence of the liver disease presented changes of the gut microbiome, elevated gut permeability, and a distinct serum bile acid profile compared to healthy controls. Additionally, we could demonstrate that SC-CIP

patients seem to be vulnerable to GI bleeding during disease course, mainly of the upper GI tract, with partly atypical bleeding locations.

In the study "The gut-liver axis in SC-CIP" we investigated the composition of the gut microbiome in SC-CIP patients compared to three control groups. When dealing with alterations of the microbiome, the primary investigation always comprises the examination of alpha diversity, which describes the mean species diversity of an ecosystem. We found that alpha diversity was reduced in SC-CIP, CIP controls, and cirrhosis. This finding is not very surprising for the two groups with liver diseases, since same disruptions have been described for distinct liver pathologies including PSC, PBC, and cirrhosis (181, 194, 195, 199, 216, 372). Decreased diversity has also been reported for inflammatory bowel disease, obesity, diabetes, antibiotic intake, and in critically ill patients (205, 206, 342, 373-377). The fact that CIP controls also demonstrated a reduction in diversity was less expected because patients included in this cohort have started their ICU treatments in the mean one year before study inclusion. This highlights that there seems to be a potential long-lasting effect of the intensive care treatment itself on microbial diversity. On the contrary, in another cohort, fast recovery of dysbiosis after cardiac surgery with intensive care treatment was suggested, but patients included in this analysis had a mean ICU treatment length of just one day (378).

With the use of RDA analysis for the examination of beta diversity, distinct clustering of SC-CIP and CIP controls compared to healthy controls could be shown. Similar alterations in beta diversity have been highlighted for ICU patients within 48 hours after admission and in patients presenting with early sepsis without organ failure (338, 343). So far, it was enigmatic whether these changes persist in the long-term or if they are just temporary and self-limiting. Our data hint that altered beta diversity persists in SC-CIP because three years have passed between ICU admission and study inclusion in our cohort. As also CIP controls presented altered beta diversity in our study, it can be argued that changes have remained, at least partly, from ICU treatment and therefore changes found in SC-CIP may not solely be driven by the liver disease. Of course, also co-factors like diet and drug intake may play a significant role not to neglect, and may present confounders when comparing the different groups to each other. For example, in CIP controls not only the ICU treatment may have long-term impact on microbiome composition, but also the changed habits after the ICU stay. This comprises increased need for drugs like pain killers, changed mobility due to permanent physical disabilities, changed eating habits due to long-term rehabilitation in specialized institutions and the increased use of antibiotics caused by the elevated risk of hospital-acquired infections. One important limiting

factor of our study is the lack of longitudinal data from the included patients which makes it impossible to address these confounders more detailed.

Exploring influencing factors of microbiome composition other than solely group assignment, we could demonstrate that statins have an independent impact on microbiome composition in both applied multivariate models. In mice, statins also caused changes of the microbiome which improved metabolic disorders like hyperglycemia (379). For humans, it was recently reported that statin intake is negatively associated with obesity-induced dysbiosis (380). Statin therapy led to an increase of the anti-inflammatory species *Faecalibacterium prausnitzii* and *Akkermansia muciniphila* (381, 382). Additionally, cirrhosis, intake of antiplatelet agents, the body mass index, and calprotectin levels could be reported as further potential independent microbiome-modulating factors. To find cirrhosis in these factors is not surprising as it is established that cirrhosis causes dysbiosis and microbiome alterations. Microbiome signatures have been investigated as biomarkers for diagnosis and disease progression (181, 372, 383). As an extension to these findings, recent scientific aims focus on the impact of dysbiosis on the evolution of hepatocellular carcinoma and the potential to use microbiome alterations as biomarkers for early detection of malignancy (384-388). Also, the connection of obesity, nutritional status, and diet with microbiome composition has been broadly reported. Alterations of the gut microbiome are said to change energy homeostasis in the host and can induce energy storage leading to weight gain (389-392). Calprotectin is a marker for gut inflammation and gut inflammation leads to dysbiosis (374). It is unsure if the finding of antiplatelet agents being microbiome-modulating factors is directly associated with effects of the drugs on microbiome composition or if the underlying cardiovascular disease impacts the microbiome (393). We could not find NSAIDs to be associated with microbiome composition in the present study, which is in contrast to previous literature (394). Same as for characteristics of the ICU stay which did not seem to contribute to microbiome composition, this may be caused by the small sample size. Other factors include the low number of patients with NSAIDs intake and the long timespan from ICU treatment to sample acquisition which probably impeded findings for ICU characteristics.

Changes on all taxonomic levels could be reported for SC-CIP and cirrhosis compared to healthy. This is even more remarkable for SC-CIP because these patients were stable outpatients presenting a rather mild disease course and solely seven patients were suffering from cirrhosis, all without signs of decompensation. Further, the extend of differences in CIP controls compared to healthy was striking, because these patients were, as already mentioned, included

in the study in the mean one year after ICU admission and due to the lack of a liver disease, one would assume that microbiome composition might have restored. Firmicutes and Bacteriodetes, followed by Proteobacteria and Actinobacteria were the dominant phyla observed in all four groups. The Firmicutes/Bacteriodetes ratio was increased in SC-CIP compared to all other groups. Recently, associations of this ratio with irritable bowel syndrome, obesity, NAFLD, and cirrhosis as well as with ICU mortality have been demonstrated. However, its usefulness as a biomarker for critically ill patients has been doubted since the Firmicutes/Bacteriodetes ratio may be normal if both phyla are equally altered (372, 395, 396).

When looking in detail at microbiome composition in SC-CIP compared to healthy controls, a shift towards pathogenic taxa, highlighted by increased abundance of the genera *Streptococcus* and *Enterococcus,* was observed. Same findings have previously been reported in cirrhosis, acute on chronic liver failure, and NAFLD, and are discussed to influence the clinical phenotype and the prognosis of these diseases (397). It has been further observed that these alterations may augment the risk for *Clostridioides difficile* infections (398). In addition to the mentioned genera, *Lactobacillus*, *Romboutsia*, and *Butyricicoccus* were associated with SC-CIP. Especially the associations with *Lactobacillus, Streptococcus,* and *Enterococcus* overlap with findings in the cholestatic liver diseases PBC and PSC (195, 216). The increase of *Butyricicoccus* in SC-CIP highlights the fact that not only pathobionts, but also butyrate-producing bacteria with potential beneficial, anti-inflammatory effects, can be augmented in liver diseases. On the other hand, decrease of this genus has been reported for IBD (399-401). Furthermore, it has been shown in a mouse model that the butyrate-producing strain *Anaerostipes hadrus,* in our cohort decreased in SC-CIP*,* is able to disturb gut microbiome composition (402). These findings indicate very well the complex interplay of the gut microbiome and the variable effects microbes can exert depending on the setting.
Additional to these findings, increased abundance of microbes in feces normally inhabiting primarily the mouth and the upper GI tract could be demonstrated in SC-CIP patients of our cohort. *Rothia dentocariosa*, a bacterium inhabiting the mouth with potential pathogenic potential (403), and *Streptococcus parasanguinis*, generally associated with a healthy oral microflora (404), were elevated in fecal samples of SC-CIP. *Enterococcus faecium*, a partially antibiotic-resistant strain, was increased in SC-CIP and has been shown to be so too in long-term ICU patients (375, 405).

Critically ill patients being treated at an ICU may need long-term treatment in very serious cases including invasive ventilation with tracheotomy for sometimes week and long-lasting

hemodynamic support with vasoactive drugs and invasive procedures. Additionally, various medications are administered comprising antibiotics, antiviral and antifungal therapies as well as enteric or parenteral nutrition. It has been already demonstrated, that these interventions in combination with the underlying critical illness affect the composition of the microbiome on distinct body sites. These alterations may favor hospital-acquired infections, sepsis leading to multiorgan failure, disruptions in energy homeostasis, and cachexia with muscle wasting (375, 378, 406-409). The decreased bowel transit time in ICU patients caused by electrolyte and metabolic disturbances and by the administration of sedatives and opiates may be one of the plausible causes leading to microbiome alterations (410). For this reasons, therapeutic interventions such as probiotics, symbiotics and fecal microbiome transplantation, aiming to modulate the gut microbiome and therefore reducing the occurrence of enteritis and ventilator-associated pneumonia in ICU patients, have been investigated (411-414). To date, data about long-term outcome of ICU patients concerning microbiome alterations are lacking. In our study, we have included a control group (CIP controls) consisting of critically ill patients who did not develop SC-CIP. As inclusion of these patients was done in average one year after admission to ICU, this cohort does not only serve as control group for SC-CIP but also sheds light on long-term microbiome alterations of ICU treatment but with the already mentioned confounding factors not to be forgotten. Compared to healthy controls, these patients still demonstrated alterations of the microbial composition on all taxonomic levels. On species level, the decrease of the beneficial, butyrate-producing species *Faecalibacterium prausnitzii* which is crucial for the function of enterocytes and was shown to be depleted in various intestinal disorders and in cholestatic liver diseases as well as the elevation of *Erysipelatoclostridium ramosum,* a species reported to be involved in the evolution of the metabolic syndrome, could be reported (50, 415-417). Together these findings suggest that, independent of the presence of a liver disease, dysbiosis can be observed in SC-CIP and CIP controls. As a logical consequence, the question occurs, whether certain microbial alterations could induce the evolution of the liver disease. Indeed, we were able to highlight that the microbiomes of these two cohorts present great differences comprising shifts of highly abundant classes like *Bacteroidia* and *Bacilli* as well as changes on species level with lower abundance of an unnamed *Neisseria* species and *Prevotella melaninogenica* in SC-CIP. Of course, direct associations of certain microbes with SC-CIP evolution cannot be concluded with our findings, therefore the most powerful tool would be the conduction of a prospective trial including patients developing SC-CIP and collecting longitudinal data of each patient.

Dysbiosis of the gut microbiome in patients suffering from alcohol-induced liver cirrhosis has previously been reported. Findings included the elevation of *Bifidobacteria*, *Streptococci*, *Veillonella,* and *Enterobacteria* and the reduction of *Faecalibacterium* in cirrhosis (416, 418, 419). The abundance of *Streptococcus* was recently reported to be able to predict severity of liver injury in these patients (420).In our cohort with cirrhosis caused by alcohol consumption, we could find decreased diversity in comparison to healthy controls, and on genus level, increased abundance of *Veillonella* and decreased abundance of *Romboutsia* and *Coprococcus*. The commensal and potential pathogenic genus *Veillonella*, known to be able to cause severe diseases, was reported to be associated with hepatic encephalopathy in cirrhosis, but may also be an effect of PPI treatment (173, 176, 178, 213). Furthermore, it could recently be identified as bile acid-sensitive and could be associated with response to a FGF-19 analog in patients with NASH (421).

It was outlined in detail in the introduction concerning the gut-liver axis that changes of the gut microbiome can lead to a leaky gut and endotoxemia. This finding was observed in gastrointestinal diseases as well as in liver diseases (422). Whether increased intestinal permeability induces the development of liver diseases or whether it is a consequence is not fully clarified yet. Concerning IBD, it could be the first step in the pathophysiology of the disease (108). With our present study, we were able to describe this pathophysiological link between the microbiome and the gut barrier for the first time in SC-CIP as we could highlight alterations in both systems. SC-CIP demonstrated elevated biomarkers of gut permeability (DAO), bacterial translocation (sCD14), and systemic inflammation (CRP) compared to healthy controls, although clinical signs of inflammation could not be observed in any included patient. sCD14 levels have been considered to be a surrogate marker for endotoxemia and were also reported to be augmented in PSC. Additionally, sCD14 showed association with limited transplant-free survival (22, 423). With data acquired through our investigations, we can hypothesize that increased gut permeability may not be solely driven by the liver disease, but may also be a consequence of the ICU treatment since also CIP controls show increase of DAO highlighting increased permeability. With distinct markers for permeability (I-FABP, citrulline), increased gut permeability in critically ill patients has already been reported and was linked to mortality (337). The reported positive correlations of inflammation and gut permeability markers with parameters of liver function in our study suggest relation of gut permeability with the severity of the liver disease. Comparable findings have earlier been

reported by our study group with usage of the same biomarkers for gut permeability and bacterial translocation in cirrhotic patients (424).

As final important part of the gut-liver axis, bile acids, enabling the liver to communicate with the gut, have to be discussed. The enterohepatic circulation has already been outlined in the introduction, highlighting the production of primary bile acids from cholesterol in hepatocytes, the secretion of conjugated bile acids into the intestine, the function of bile acids in fat digestion and immunology, and the reuptake of bile acids in the terminal ileum or the proceeding into the colon with metabolism to secondary bile acids through gut microbes (50). Therefore, disruption of the microbiome by inflammation, diet, or antibiotic intake induces also changes in the bile acid profile (425). Data on bile acid composition and pool size are rare for chronic cholestatic liver diseases. Within this study, we have investigated bile acids in serum. Not surprisingly for a cholestatic disease, total serum bile acids were found to be elevated in SC-CIP. This finding is in accordance with earlier investigations in PSC, PBC, and in NAFLD (426, 427). Besides, we could demonstrate altered bile acid profiles in SC-CIP and cirrhosis compared to healthy controls. Conjugation of bile acids in the liver aims to ameliorate hydrophilicity and to protect the epithelium against toxic effects of bile acids (50). Same as described for PSC and PBC (426), elevation of conjugated bile acids could be reported by ourselves in SC-CIP. It was reported from investigations on mice, that taurine-conjugated bile acids of gall bladder aspirates were linked to overgrowth of pathobionts and the evolution of colitis. Hence, a connection between bile acid and gut microbiome composition can be suggested. Furthermore, germ-free or antibiotic-treated rodents also showed the same elevation of taurine conjugates in plasma as well as in the liver which gives way for the interpretation that the observed increase of conjugated bile acids in our cohort may have a connection to the reduction of microbiome diversity and antibiotic use in SC-CIP (428). The elevation of taurine conjugated bile acids has also been observed in the bile fluid of PBC, as already mentioned (164, 429).

In the retrospective study “GI bleeding in SC-CIP” we explored the frequency of GI bleeding in SC-CIP patients compared to a control group with history of long-term intensive care treatment after cardiac surgery without the emergence of SC-CIP. We were able to demonstrate a high rate of GI bleedings in SC-CIP (30%) compared to the control group (5%). Currently, we are the only ones having explored this topic in SC-CIP patients.

The occurrence of GI bleeding in critically ill patients is not uncommon and is mainly caused by gastroduodenal ulcers (346, 348, 430). This is the reason why the usage of stress ulcer

prophylaxis was implemented in clinical routine for critically ill patients, although the benefit on mortality remains a controverse discussion point (431-433). Respiratory and renal failure, liver diseases, coagulopathy, and male sex have been described as risk factors for GI bleeding (346, 430). At first sight, it seems logical that the main driver for GI bleeding in SC-CIP patients may be the complicated long-term ICU treatment these patients had to undergo. On the one hand, only three SC-CIP patients suffered bleeding while on ICU in our cohort and on the other hand, the reported total rate of 30% of patients experiencing bleeding is high since the highest reported number of GI bleedings in critically ill patients to date is only half of this percentage and this study was conducted even before the PPI era (348). As a limitation, our study is not completely comparable, as we have counted GI bleedings not only during the ICU stay but also during follow-up of, in the mean, nearly five years. Interval of ICU admission to onset of bleeding was on average more than a year in SC-CIP but we could report that seven out of the 16 patients suffered bleeding within two months after ICU admission (interval of two probands unknown). On the other hand, bleedings also occurred long after the ICU stay. To overcome this problem of limited comparability, we have included a control group in our analysis. In this control group, only one occult GI bleeding occurred and terminated spontaneously without intervention. At this point, it is necessary to mention another possible bias: the shorter mean follow-up of only one and a half years in this cohort, but still, the difference between the two groups seems convincing. For SC-CIP it remains unclear whether the disease itself favors GI bleeding or whether SC-CIP patients recruited for our study present a negative selection of especially ill patients. What makes the second hypothesis less likely is the fact that also patients included in the control group were extremely ill and suffered, for example, from aortic dissection with the need for complicated emergency surgery and long-term ICU treatment. Additionally, patients in the control group were older than SC-CIP patients and one would assume that these patients are more susceptible to complications like GI bleedings.

It is a fact that patients suffering from cirrhosis are at increased risk for GI bleeding, but the risk for patients with liver fibrosis yet needs to be determined (349). The main cause for upper GI bleeding in cirrhotic patients is bleeding due to portal hypertension from esophageal and gastric varices as well as from portal hypertensive gastropathy (430, 434). But since in our SC-CIP cohort less than 20% had cirrhosis at time of bleeding and signs of portal hypertension (splenomegaly, thrombopenia) were rare, it seems unlikely that portal hypertension was the only or the main driver for the high rate of bleedings in SC-CIP. This is supported by the fact that none of the observed bleedings in SC-CIP originated from varices.

Peptic ulcers are the main cause of nonvariceal upper GI bleeding (30 to 60%) and account for 20 to 30% of upper GI tract bleedings in liver cirrhosis (349, 435, 436). Increased mortality due to bleeding from peptic ulcers has been described for patients suffering from cirrhotic and non-cirrhotic liver diseases. Cirrhotic patients also seem to have an increased risk for recurrent in-hospital bleeding as well as for long-term re-bleedings (352, 357, 435, 437, 438). Similar to these described findings, we could observe bleeding from ulcers as the main bleeding source (over 55% of all bleedings) in SC-CIP patients. Previously reported risk factors for peptic ulcer bleeding include intake of non-steroidal anti-inflammatory drugs (NSAIDs), age over 60 years as well as co-administration of aspirin, antiplatelet agents, or steroids (439-442). As a consequence of these risk factors, one would assume that the GI bleeding rate should be higher in the control group than in SC-CIP since this cohort was older and therapy with antiplatelets and anticoagulants is frequently necessary in patients suffering from cardiovascular diseases. We were not able to adequately assess the intake of NSAIDs in both cohorts, but in clinical routine NSAIDs are not frequently prescribed for patients with advanced liver diseases.

Despite that the pathophysiology of SC-CIP is not fully understood yet, ischemic damage of the biliary tree seems to be a confirmed factor, maybe the leading one, involved in the evolution of this disease and has been observed in liver biopsies (14, 443). The vulnerability of the biliary system to hypotension during intensive care treatment is caused by the singular vascular supply from the hepatic artery (444-446). It was communicated that in a single patient sclerosing arteriopathy with intima thickening was observed upon histology after liver explanation (24). Vascular supply of the liver is very variable and only about 80% of humans have vascular anatomy of the hepatic artery reported to be the standard variant (447, 448). It is currently unknown if alterations of the vascular supply make patients more vulnerable to the development of SC-CIP. Furthermore, it remains enigmatic if altered vascular anatomy may also change the perfusion of the stomach and the duodenum and therefore may increase the risk for upper GI bleeding. In our presented study, only about 60% of patients included in the SC-CIP cohort had normal vascular anatomy, but we were not able to find specific alterations which we could link to an increased bleeding risk. Further, we were not able to describe collateral circulation caused by portal hypertension in any SC-CIP patient. As an important limitation, the radiological evaluation is dependent on the quality and applied protocols of cross-sectional imaging techniques, hence anatomy of smaller arteries, vascular malformations und exact stenosis-quantification could not be reported.

Both studies presented in this work have relevant limitations. Concerning “The gut-liver axis in SC-CIP” it seems important to mention that the cross-sectional study design is not suited to establish causal relationships. As SC-CIP is a very rare disease, a downside of this study is the rather small sample size. To overcome this problem, we included SC-CIP patients from two study centers, but patient numbers increased only marginal. Also, recruitment for the CIP cohort was challenging as recruitment of patients with history of intensive care treatment but at study inclusion discharged from hospital, the longer the better, was difficult. Furthermore, there are possible confounders concerning patient characteristics (very variable drug intake, recruitment in different settings (outpatients vs. patients in a rehabilitation center)), differences in mobility between SC-CIP and CIP controls (injuries of the spine leading to permanent disabilities in CIP controls), inclusion of cirrhotic and non-cirrhotic patients in SC-CIP) in this study, which have to be kept in mind when interpreting the findings. The lack of longitudinal data with collection of stool samples at just one timepoint makes the analysis susceptible to daily fluctuations of microbiome composition and does not allow to draw conclusions on longitudinal effects. In addition, 16S sequencing is inferior to whole metagenome sequencing techniques concerning microbial resolution. This may cause limited species identification. For this reason, only sequences that exactly matched the reference genome were reported on species level.

Limitations of the study “GI bleeding in SC-CIP” comprise the retrospective study design which implicates variable data quality. Therefore, it seems possible that GI bleedings may have been underreported since bleeding episodes could only be assessed when documented in the medical information system used for this study. If occurring in hospitals not connected to this system, bleedings could have been missed, but this accounts for both groups. Another limitation is the smaller number of patients included in the control group and the fact that there are differences in some baseline characteristics between the two groups. Additionally, follow-up was shorter in the control group than in SC-CIP (one and a half years vs. five years). But this should not reduce the strength of our findings too much since the majority of bleedings in SC-CIP occurred within the observation period of the control group.

In conclusion, we were able to demonstrate that SC-CIP is associated with dysbiosis of the fecal gut microbiome implicating structural and functional alterations in microbiome composition. An oralization of the fecal microbiome as well as increased abundance of potential pathogenic microbes were detected. Further, augmented gut permeability leading to bacterial translocation and inflammation and an altered serum bile acid profile could be reported. With inclusion of the chosen control groups, we were able to demonstrate that alterations observed in SC-CIP do

not seem to be caused solely by the liver disease, as also long-term microbiome changes in patients after ICU treatment without liver diseases were found. In addition to these findings about the gut-liver axis, we generated data suggesting GI bleed to be a frequent complication of SC-CIP patients, presenting with partially atypical bleeding locations. The association with portal hypertension or long-term ICU treatment may not be an acceptable explanation. We therefore hypothesized that alterations of vascular supply may trigger bleeding risk, but we were not able to confirm this hypothesis with our findings.

For the future, larger, prospective, and longitudinal studies investigating the disease SC-CIP need to be conducted to shed light on the pathophysiology and on disease course to be able to identify patients at risk for the development and the progression of this disease. In a second step, it is crucial to find appropriate treatment strategies for chronic cholestatic liver diseases which do not exist for SC-CIP and PSC yet. Concerning the gut-liver axis, descriptive investigations of microbiome composition are available for most liver diseases, now also for SC-CIP, but the understanding of microbiome functions and interactions needs to be improved. We need to focus on the description of the order of the occurrence of alterations to be able to better distinguish between trigger and consequence. Further, investigating the gut mycobiome and the virome in chronic cholestatic liver diseases seems to be a promising target for future investigations.

12. References

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