Dissertation

Biomarkers of bacterial translocation and their prognostic value in patients with liver cirrhosis

submitted by

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Statuatory 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“.

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Rainer Florian eh

Disclosures

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Table of Contents

Abbreviations

A

ACLF ..... acute-on-chronic liver failure

AD ..... acute decompensation

AKI ..... acute kidney injury

apoA-I..... apolipoprotein A-I

AUC..... area under the curve

AUROC ..... area under receiver operating characteristic

B

BT..... bacterial translocation

C

CPS..... Child-Pugh score

CRP..... C-reactive protein

D

DIS ..... distension of intercellular spaces

DNA..... desoxyribonucleic acid

E

EASL ..... European Association for the Study of the Liver

EASL-CLIF .. European Association for the Study of the Liver - Chronic Liver Failure

F

FiO_2..... fraction of inspired oxygen

H

HCV ..... hepatitis C virus

HDL..... high-density lipoprotein

HDL-C..... high-density lipoprotein cholesterol

HE ..... hepatic encephalopathy

HR..... hazard ratio

HRS ..... hepatorenal syndrome

H&E..... hematoxylin & eosin

I

IDL ..... intermediate-density lipoprotein

IL-6..... interleukin 6

IL-8..... interleukin 8

INR..... international normalised ratio

IRB ..... Institutional Review Board

L

LDL ..... low-density lipoprotein

LEfSe ..... Linear discriminant analysis Effect Size

LPS ..... lipopolysaccharides

LTA ..... lipoteichoic acid

L/M ratio ..... lactulose/mannitol ratio

M

MAP ..... mean arterial pressure

MELD ..... model for end-stage liver disease

MLN..... mesenteric lymph node

MV ..... microvilli

O

OLT ..... orthotopic liver transplantation

OR..... odds ratio

OTU..... operational taxonomic unit

P

PAMPs ..... pathogen-associated molecular patterns

PaO_2..... partial pressure of arterial oxygen

PLT..... platelet count

PPI ..... proton pump inhibitor

PVT ..... portal vein thrombosis

R

RDA..... redundancy analysis

ROC ..... receiver operating characteristic

S

SBP..... spontaneous bacterial peritonitis

SC..... stable cirrhosis

sCD163..... soluble CD163

sMR..... soluble mannose receptor

SOFA ..... Sequential Organ Failure Assessment

SpO_2 ..... oxygen saturation measured by pulse oximetry

T

TEM ..... transmission electron microscopy

TJ..... tight junction

TLR ..... toll-like receptors

TNF-α ..... tumor necrosis factor α

U

UNOS..... United Network for Organ Sharing

V

VLDL..... very low-density lipoprotein

V/C ratio..... villus to crypt ratio

W

WBC..... white blood cell count

Abstract

Background: In patients with liver cirrhosis, prognosis prediction is of particular importance due to the variable disease course and the need to select and rank end-stage patients for liver transplantation, especially in the context of organ shortage. Most of the well-studied prognostic factors and scores are based on parameters of disease severity and do not take into account representatives of disease activity and progression. Yet, an important role of different components of the gut-liver axis & intestinal barrier on liver disease progression was recently suggested. Therefore, the aim of this scientific work was to investigate the prognostic value of several biomarkers related to bacterial translocation in patients with liver cirrhosis.

Methods: Patients in different stages of chronic liver disease (from compensated cirrhosis to acute-on-chronic liver failure) were investigated in terms of patient-specific parameters (e.g., etiology of liver disease, proton pump inhibitor intake), stool microbiome composition (i.e., 16S sequencing of stool samples), morphological alterations of the epithelial barrier (i.e., distal duodenal biopsies and analysis via light and transmission electron microscopy), markers of intestinal inflammation (i.e., fecal calprotectin levels), markers of intestinal permeability (i.e., fecal zonulin levels and the lactulose/mannitol test), markers of bacterial translocation (i.e., serum endotoxin levels), markers of hepatic & systemic inflammation (i.e., macrophage activation markers serum sCD163 & sMR as well as CRP, IL-6, IL-8 & TNF-α) and lipid metabolism parameters (i.e., HDL-C and apoA-I). Patients were monitored for occurrence of complications (including infection), transplantation and death.

Results: In total, 536 patients were collected in 2 cohorts, 256 patients with stable cirrhosis in a Grazer single-center and 280 patients with acute decompensation in a European multicenter cohort. In patients with liver cirrhosis, significant changes were observed at all levels of the intestinal barrier including distinctive alterations in gut microbial composition and intestinal epithelial structure, evidence of intestinal inflammation as well as increased intestinal permeability, bacterial translocation and systemic inflammation. Furthermore, levels of lipid metabolism markers HDL-C and apoA-I were significantly reduced. We found relevant associations between PPI use and changes in gut microbiome (in particular increased abundance of *Veillonella parvula* and *Streptococcus salivarius*) as well as other aspects of the intestinal

barrier, culminating in a higher complication and mortality risk in patients with PPI intake. The increased appearance of *V. parvula* in stool microbiome (*i.e.*, *V. parvula* dysbiosis) was linked to intestinal barrier dysfunction, the development of cirrhosis-associated complications, infections and mortality. Macrophage activation parameters sCD163 & sMR have proven their predictive value in liver cirrhosis patients, with better data for sCD163 in terms of survival prediction and for sMR in terms of cirrhosis-associated complication. Furthermore, we identified lipid metabolism parameters HDL-C and apoA-I as stable predictors of disease progression, complications, infections and survival, respectively.

Conclusion: We have proven the prognostic value of several biomarkers related to bacterial translocation in patients with liver cirrhosis, most notably *V. parvula* abundance, sCD163, sMR, HDL-C and apoA-I levels. Some of the solitary parameters demonstrated similar performance in comparison to established composite scores MELD & Child-Pugh, probably due to their qualities as representatives of disease progression.

Abstract in German

Hintergrund: Patient*innen mit Leberzirrhose zeigen unterschiedliche Krankheitsverläufe von langjähriger kompensierter Erkrankung hin zu rascher Progredienz ins Endstadium mit Auftreten von Zirrhose-assoziierten Komplikationen. Eine möglichst genaue Prognoseprädiktion ist bei dieser Erkrankung von besonderer Bedeutung, da Patient*innen mit hohem Risiko hinsichtlich Lebertransplantation evaluiert und auf der Warteliste priorisiert werden müssen. Die derzeit verwendeten prognostischen Scores basieren vorwiegend auf Parametern welche die Krankheitsschwere, jedoch nicht die Krankheits-Aktivität abbilden. In den letzten Jahren konnte ein Einfluss der Darm-Leber-Achse auf die Krankheits-Progression bei Patient*innen mit Leberzirrhose gezeigt werden. Das Ziel dieser wissenschaftlichen Arbeit war es, unterschiedliche Parameter der Darm-Leber-Achse sowie der bakteriellen Translokation hinsichtlich deren prognostischen Nutzen zu untersuchen.

Methoden: Es wurden Patient*innen in verschiedenen Stadien der chronischen Lebererkrankung (von kompensierter Zirrhose bis hin zum akut-auf-chronischen Leberversagen) eingeschlossen und folgende Parameter der Darm-Leber Achse untersucht: Patienten-spezifische Merkmale (z.B. Einnahme von Protonenpumpen-Hemmern), Zusammensetzung des Stuhl-Mikrobioms (16S-Sequenzierung von Stuhlproben), morphologische Veränderungen der Darmbarriere (Duodenal-Biopsien und Analyse mittels Licht- und Transmissionselektronenmikroskopie), intestinale Entzündung (Calprotectin-Werte im Stuhl), Marker der intestinalen Permeabilität (Zonulin-Spiegel im Stuhl & Laktulose/Mannitol-Test), Marker der bakteriellen Translokation (Serum-Endotoxin-Spiegel), hepatische & systemische Entzündung (Makrophagenaktivierungs-Marker sCD163 & sMR sowie CRP, IL-6, IL-8 & TNF-α) und Lipidstoffwechsel-Parameter (HDL-C und apoA-I). Die Patient*innen wurden hinsichtlich des Auftretens von Zirrhose-assoziierten Komplikationen (einschließlich Infektionen), Transplantation und Tod observiert.

Ergebnisse: Insgesamt wurden 536 Patient*innen aus zwei Kohorten eingeschlossen: 256 Patient*innen mit stabiler Leberzirrhose aus einer Grazer Kohorte sowie 280 Patient*innen mit akuter hepatischer Dekompensation aus einer europäischen multizentrischen Studie. Bei Patient*innen mit Leberzirrhose zeigten sich relevante Veränderungen auf allen Ebenen der intestinalen Barriere, vor allem

Veränderungen in der Zusammensetzung des Darmmikrobioms. Patient*innen mit Leberzirrhose zeigten Hinweise für eine erhöhte Darmdurchlässigkeit; Marker der bakteriellen Translokation sowie der intestinalen & systemische Entzündung waren erhöht. Zudem waren die Lipidstoffwechsel-Parameter HDL-C und apoA-I signifikant erniedrigt.

Es zeigten sich signifikante Assoziationen zwischen der Einnahme von Protonenpumpen-Hemmern und Veränderungen des Darmmikrobioms, insbesondere ein vermehrtes Auftreten von Veillonella parvula und Streptococcus salivarius im Stuhl. Patient*innen mit einer V. parvula-Dysbiose zeigten Hinweise für eine gestörte Darmbarriere sowie ein erhöhtes Risiko für die Entwicklung von Zirrhose-assoziierten Komplikationen, Infektion und Mortalität. Auch für die Makrophagenaktivierungs-Parameter sCD163 & sMR konnte ein prognostischer Nutzen nachgewiesen werden, wobei sCD163 bessere Daten hinsichtlich der Vorhersage des Überlebens und sMR für Zirrhose-assoziierte Komplikationen zeigte. HDL-C und apoA-I erwiesen sich als robuste Prädiktoren für die Vorhersage des Krankheitsverlauf, von Komplikationen, Infektionen und des Überlebens.

Fazit: Viele Parameter der Darm-Leber-Achse und der bakteriellen Translokation eignen sich als prognostische Marker bei Patient*innen mit Leberzirrhose. Insbesondere die Serumspiegel von sCD163, sMR, HDL-C und apoA-I zeigen einen ähnlichen prognostischen Nutzen im Verglich zu den etablierten Scores wie z.B. dem Child-Pugh oder MELD Score.

1. Introduction

1.1. A brief history of prognosis prediction

“It appears to me a most noble thing for the physician to practice prognosis: for by foreseeing and foretelling, by the side of the sick, the present, the past, and the future, and explaining as many things as patients leave untold, he may be more believed to know the circumstances of the sick; so that men will have the courage to entrust themselves to the physician” (1). This famous opening of the treatise *On Prognostics*, a section of the *Corpus Hippocraticum* written around 400 BC, demonstrates the essential role attested to prognosis prediction already at the time of ancient Greeks (2). By now, prognosis has become one of the cornerstones of clinical practice: Every diagnosis demands for a prediction of the likely course, and every drug prescription is based on the expectation of a distinct response. Since an accurate prognosis is usually dependent on multiple differently weighted parameters (*e.g.,* gender, age, comorbidities, the natural course of a disease, the effectiveness of available treatments…) and therefore, a complex scientific and statistical issue, prognosis prediction was largely based on experience rather than empirical findings up until the first half of the last century, when prognostic models were developed. One of the first prognostic scores was the *Pathologic Index Rating*, which was published in 1953 in Annals of Internal Medicine by Sidney Schnur and developed to predict the outcome of patients admitted to hospital with acute myocardial infarction (3). This model used a combination of different patient-specific parameters to categorize patients into different risk groups. As in this model, prognosis generally refers to life expectancy (*prognosis quod vitam*), but it can also address specific segments such as response rates and complication risks of distinct treatments (4). It is important to note that a prognosis is not a definite prediction, as the course of a disease or condition can vary widely from person to person and can be affected by many different factors. It is therefore not surprising that even in the late 20th century, studies highlighting the value of clinicians’ estimations compared to the calculation of prognostic scores were published. One of these studies was the SUPPORT trial (Study to Understand Prognoses and Preferences for Outcomes and Risk of Treatment) showing that life expectancy of seriously ill hospitalized patients was

predicted only slightly less precise by attending physicians compared with a prognostic model (5). However, with the improvement of statistical procedures and the gathering of large patient cohorts, prognosis prediction for individual patients improved. In the last decades, innumerable prognostic models have been developed for almost every medical condition, including cancer, infection, heart and liver disease. These models have helped medical professionals to make more accurate predictions about the likely outcome of a disease, guide treatment decisions and help patients and their relatives understand what to expect.

1.2. The liver and its pathologies

Cirrhosis and other chronic liver diseases are a major cause of morbidity and mortality with globally about two million deaths annually (6). In Austria, the estimated mortality rate for chronic liver disease stands at 19.4 per 100,000, surpassing the average rate observed across Europe (7). In industrialized nations, chronic alcohol use ranks among the leading causes for chronic liver disease, whereas in developing countries, viral infections are the predominant cause (8). Despite the range of potential causes for damage (see Table 1), the liver typically responds in a consistent manner: Persistent damage results in the replacement of destroyed parenchyma with connective tissue, leading to a distortion of the liver's normal architecture and the development of liver fibrosis and cirrhosis (9).

Alcohol-related liver disease	Autoimmune hepatitis
Viral hepatitis	Cirrhose cardiaque
Cholestatic liver diseases (primary biliary cholangitis, primary sclerosing cholangitis…)	Metabolic diseases (hemochromatosis, Wilson's disease, alpha-1 antitrypsin deficiency…)
Drug-induced liver damage	Other (including tropical diseases like schistosomiasis, liver fluke…)

Table 1. Common causes of chronic liver disease, adapted from (10)

1.3. Bacterial translocation in patients with liver disease

Patients with liver cirrhosis are more prone to experiencing severe infections that often manifest without a clear focus and significantly worsen patient's prognosis (11–13). Studies have demonstrated that patients suffering from chronic liver disease are

six times more likely to develop bacteremia compared to a non-cirrhotic control group, and among cirrhosis patients who acquire bacterial infections, mortality is increased about four-fold (14,15). A frequently occurring infectious complication in chronic liver disease is spontaneous bacterial peritonitis (SBP), which is, in the majority of cases, caused by microorganisms that typically inhabit the intestines (16). The risk of infection (and particularly of SBP) caused by enteric bacteria such as *Klebsiella pneumoniae* or *Enterococcus faecalis* is significantly diminished following intestinal decontamination with antibiotics (16). Based on these findings, a gut origin of these infections was suggested, with the possibility that bacteria could enter the body through an impaired intestinal barrier and translocate to mesenteric lymph nodes (MLNs), the portal venous & systemic circulation, and ascitic fluid (17). In order to assess the prevalence of *bacterial translocation* (BT), Cirera et al. analyzed MLNs of cirrhosis patients undergoing laparotomy either for hepatic resection or liver transplantation (16). Enteric bacteria were cultured from MLNs in approximately 10% of all patients, with a higher prevalence observed in individuals with Child's C (30.8%) compared to those with Child's A (3.4%) cirrhosis. Also, Llovet et al. demonstrated that bacterial strains retrieved from MLNs exhibited genetic similarity to strains causing SBP in rodents with chronic liver disease (18). Therefore, the translocation of bacteria to MLNs and their consecutive distribution was suggested one of the main steps leading to bacterial infection in patients with cirrhosis.

The function of the intestinal barrier and therefore, the prevention of BT, depends on the integrity and interaction of three important factors (17):

(1) The gut microbiome representing the ***ecologic barrier***

(2) Enteral cells & their cohesion via intercellular junctions forming the ***mechanical barrier***

(3) The clearance of translocating microorganisms and their constituents by the immune system, the ***immune barrier***

1.3.1. The ecologic barrier

The human gut contains > $1x10^{14}$ microorganisms, whose interaction with human cells provide many beneficial effects (19). This includes for example breakdown of nutrients, activation & preparation of the immune system and suppression of potential pathogens (20). However, depending on the composition of the intestinal microbiome, this relationship may also be harmful: variations in microbial compositions are involved in the development of a wide range of diseases, including autoimmune, metabolic, mental health and liver disorders. However, the composition of the intestinal microbiome is, vice versa, influenced by several factors including many of the above-mentioned diseases. Therefore, the cause-effect relationship between many disorders and microbiome composition is controversially debated (21). A microbial imbalance or maladaptation (i.e., dysbiosis) can persist, even when the leading trigger has been eliminated (e.g., discontinuation of an antibiotic therapy) (22).

In patients with liver cirrhosis, recent studies using DNA sequencing methods revealed systemic alterations of the stool microbiome including an increased abundance of Firmicutes, Proteobacteria & Fusobacteria and a decreased abundance of Bacteroidetes (23). Another key feature of intestinal dysbiosis in patients with liver disease is reduced microbial diversity. In fact, an adequate biodiversity is a prerequisite for a microbial community to suppress the colonization and growth of potential pathogens. In patients with liver disease, this impaired *colonization resistance* is indicated by high detection rates of microorganisms that are usually not represented in the gut microbiome (*non-autochthonous taxa*). For instance, *Veillonella* and *Streptococcus*, two genera that are usually found in the oral cavity, are increasingly identified in the stool of patients with liver disease compared to healthy controls (23). Higher rates of non-autochthonous taxa were shown to be associated with the detection of bacterial products in the systemic circulation (e.g., lipopolysaccharides) and a higher infection risk in these patients (24).

A similar phenomenon is observed in patients taking proton pump inhibitors (PPIs), even in patients without liver disease. This is considered to be a result not only of reduced gastric acid secretion and subsequently impaired pathogen clearance (25), but also of modification of the intestinal microbiome, leaving it vulnerable to pathogen

colonization and proliferation (26). Consistently, recent studies have found a link between long-term PPI use and increased risk of infection, especially of enteric origin (27–30).

1.3.2. The mechanical barrier

The majority of the gastrointestinal lumen is lined with a single layer of cells that create a three-dimensional arrangement of invaginations (crypts) and protuberances (villi). The epithelial cells forming this cell-layer, predominantly enterocytes, have to manage two rather divergent tasks: the uptake of required food components on the one hand, and the rejection of toxins, microorganisms & antigens on the other hand. To effectively carry out these functions, complex tissue organization and the creation of tightly regulated transportation routes are necessary (31).

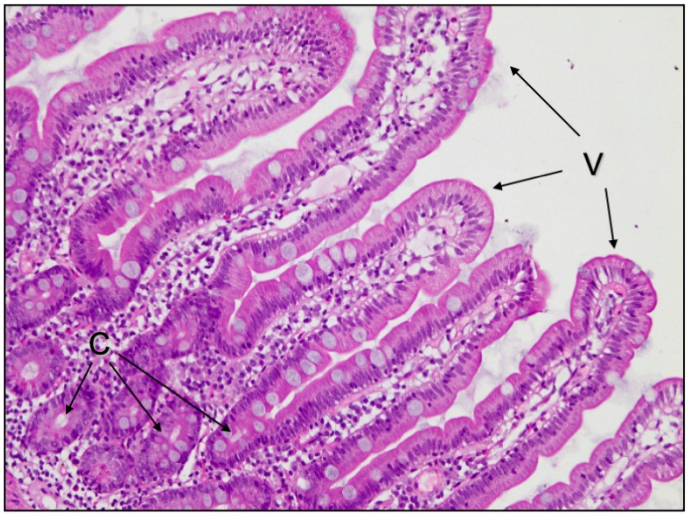

Figure 1. Regular Intestinal Mucosa: Villi (V) emerge as protrusions of the mucous membrane covered with epithelial cells. Crypts (C) are located in-between the villi as indentations of the epithelium into the lamina propria; H&E stain, 100x, duodenal biopsy sample taken from healthy control.

Given the structural arrangement of the mucosal layer, two different ways of nutrient uptake can be differentiated (32). Firstly, substances can be absorbed via the *transcellular pathway*, by crossing the lipid bilayer membrane of epithelial cells. Since water-soluble compounds are not capable of permeating eukaryotic cell membranes, enterocytes are equipped with transporters for these compounds (e.g., amino acids, sugars, electrolytes, fatty acids) (33). Alternatively, substances can pass the mucosal layer by utilizing the paracellular space between two adjacent enterocytes. This *paracellular pathway* is non-directional & passive and derives from varying concentrations of substances on either side of the epithelium (34). Furthermore, it is not controlled by membrane transporters or channels; instead, its extent is restricted

by junctional complexes that connect neighboring epithelial cells. An important role is attributed to the tight junctions (TJs), specialized cell-cell connections located near the apical surface of enterocytes. By their ability to respond to environmental influences, TJs form a modifiable barrier and therefore, are regarded as regulators of molecule permeation via the paracellular pathway (35).

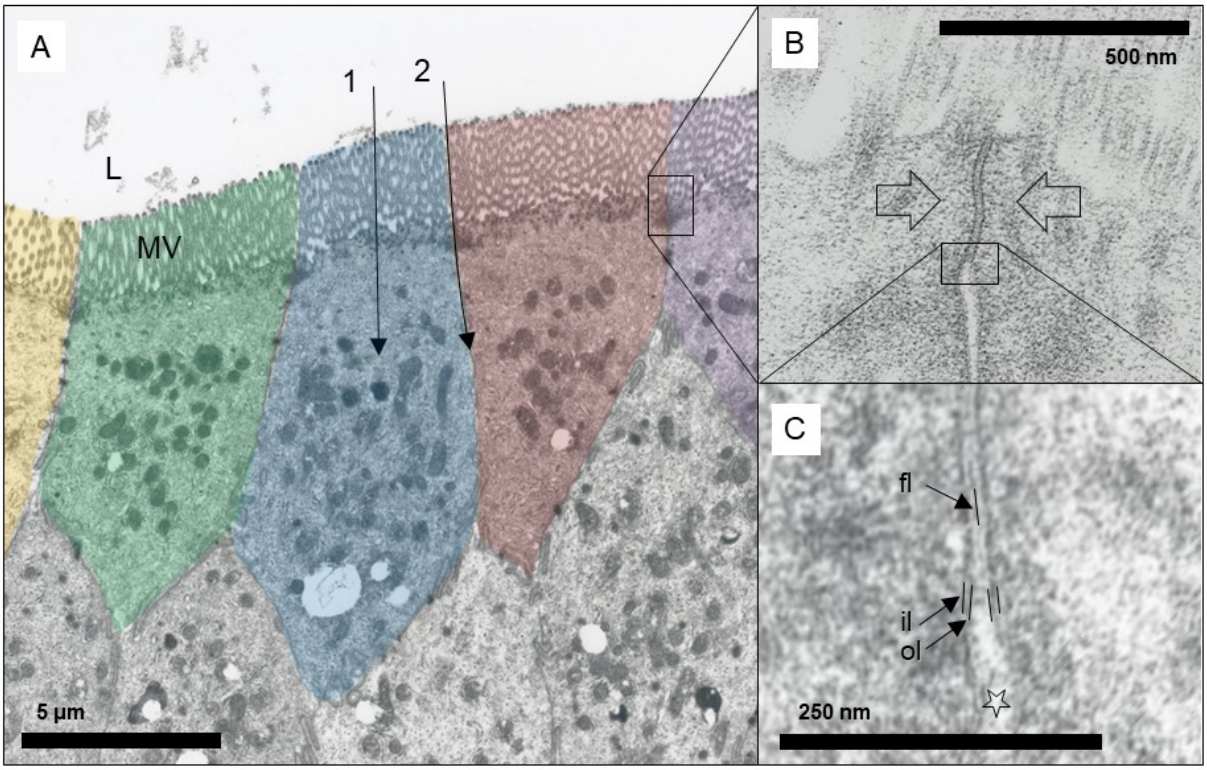

Figure 2. Electron micrograph of healthy epithelial cells and their junctional complexes. A: Transcellular (1) and paracellular pathway (2), the enterocytes are colored for better depiction. L, luminal side; MV, microvilli; B: The tight junction is located near the luminal surface of the enterocytes (in-between the two arrows) and is surrounded by a slight condensation of cytoplasmatic material. C: Higher magnification reveals approaching of the outer leaflets (ol) to form the fusion line (fl), here the intercellular space (open star) is obliterated. il = inner leaflet.

In transmission electron microscopy, TJs are observed as regions with narrowed intercellular spaces positioned near the luminal border of enterocytes (see Figure 2). At higher magnifications, it becomes apparent that in the TJ region, the outer leaflets of neighboring cell membranes come closer and fuse to create a joint line known as the fusion line. The intercellular space seems eliminated within these areas. Therefore, the typical three-layered structure of the cell membrane (comprising two electron-dense lipid layers with a less electron-dense intermediate layer) is replaced by a five-layered configuration. This new structure consists of the two inner leaflets of the cell membranes (electron-dense), the fusion line (electron-dense) and two less electron-dense intermediate layers.

On a molecular basis, TJs are mainly composed of two different components: (1) *Adhesive transmembrane proteins* forming the core of TJs, protruding into the intercellular space & creating the paracellular barrier, and (2) *peripheral proteins* that connect transmembrane proteins with bunches of the perijunctional cytoskeleton in order to anchor the TJs within the cells (36). Although more than 40 different proteins forming TJs are currently known, the claudin family of transmembrane proteins have been identified as the most important: they define TJ selectivity via the formation of modifiable strands that establish connections with claudin proteins on neighboring cells (37). Evidence suggests that the selection and combination of diverse claudin subtypes influences paracellular selectivity (including size, ionic charge preference and electrical resistance): Certain claudins lead to an increased porosity of the paracellular barrier (e.g., claudin-2) allowing small substances to pass, while others reduce paracellular permeability by 'tightening' the intercellular gap (e.g., claudin-1). Currently, more than 20 different claudin subtypes are known in eukaryotes (38) and therefore, the potential combinations for TJ assembly and likely variations in TJ function appear to be extensive (33,39).

Intestinal permeability can be assessed by various methods including the lactulose/mannitol test (i.e., the calculation of the urinary excretion ratio of the two non-metabolized sugars lactulose & mannitol after oral administration) or the measurement of zonulin blood or stool levels (a protein that influences on tight junction function) (40). In patients with chronic liver disease, these tests indicate a disruption of the intestinal barrier: Permeability has been shown to increase, especially in patients with alcohol use (41), ascites (11) or severe septic complications (42), and increases with the stage of cirrhosis (43–46). Increased intestinal permeability is accompanied by evidence of bacterial products like lipopolysaccharides (LPS, or endotoxin) in the systemic circulation. In fact, a low level of endotoxin leakage to the portal vein has been observed also in healthy individuals and can be cleared by the phagocytic activity of the reticuloendothelial system (47). In patients with liver disease, an excessive amount of these bacterial products is found in the systemic circulation indicating a disrupted mechanical barrier (48).

1.3.3. The immune barrier

Even in healthy individuals, BT appears to occur regularly without causing infection or any other adverse consequences, provided that the body's defense mechanisms are functional. Every aspect of the immune system, including cell-mediated (T-cells, macrophages etc.), mucosal (secretory immunoglobulins) and humoral (plasma immunoglobulins) immunity play a crucial role in preventing BT (49). In patients with liver cirrhosis, various defense mechanisms are compromised, encompassing both humoral and cellular elements: Research indicates a diminished phagocytic capacity of reticuloendothelial cells, along with a decreased opsonic activity of serum and ascitic fluid. This reduction is especially noticed in patients with SBP and attributed to an impaired hepatic synthesis of complement factors (13). Again, these impairments have been shown to be associated with infection and mortality risk in patients with cirrhosis: the dysfunction of neutrophils, indicated by a decreased phagocytotic & killing capacity and an increased oxidative resting burst, is associated with an increased susceptibility to infections and occurrence of complications in patients with chronic liver disease (50,51).

1.4. Implications of bacterial translocation

The intactness of the intestinal epithelial barrier is essential not just in fending off viable microorganisms, but also in repelling numerous other potentially harmful substances for the body. Hence, the scope of possible effects caused by a compromised intestinal function extends beyond mere infection. In chronic liver disease, it is hypothesized that hepatic inflammation and fibrosis progression is maintained by pathogen-associated molecular patterns (PAMPs, including LPS) that originate from the intestine and increasingly reach the liver via the portal system (52). Within the liver, these PAMPs are recognized by toll-like-receptors (TLRs) on immune cells that subsequently release pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α) and reactive oxygen species (13,53). Ultimately, liver inflammation triggered by these cytokines leads to fibrosis progression and cirrhosis development (54,55).

TLRs are usually expressed on sentinel cells such as dendritic cells and macrophages. Resident macrophages in the hepatic sinusoids (known as Kupffer

cells) serve as the first contact point for gut-derived PAMPs. In fact, macrophage activation can be assessed by measuring serum levels of solube (s)CD163 and soluble mannose receptor (sMR); these macrophage activation parameters are elevated in patients with liver disease and/or portal hypertension (54,56–58). CD163, the receptor for hemoglobin-haptoglobin complexes, is found on macrophages and monocytes and released into the bloodstream as soluble CD163 after shedding from these cells (59). sCD163 levels are associated with prognosis in patients with alcoholic hepatitis (60), liver cirrhosis (58,61,62) and acute-on-chronic liver failure (63). sMR, the shedding product of the mannose receptor, is predominantly found on macrophages and dendritic cells and has been shown to be elevated especially in patients with liver disease and in patients with critical illness (54,55).

BT not only affects the liver and other down-streamed organs but also the gut itself: Weakening of the intestinal barrier results in microorganisms and toxic substances coming into contact with the patient's immune system within the gut wall and thus, results in inflammatory changes of the intestinal lining. (17). Furthermore, liver function (or dysfunction) affects the integrity of different components of the intestinal barrier. For instance, it has been shown that the composition of the intestinal microbiome dynamically changes after liver surgery and that gut-derived metabolites play important roles in liver regeneration (64). The concept of the 'gut-liver axis' comprises these manifold associations between the intestinal microbiome, the intestinal barrier and the liver.

1.4.1. The role of lipoproteins in innate immunity

Lipoproteins are complex compounds consisting of a lipid core (comprising triglycerides and cholesterol) and a shell of proteins & phospholipids. The five lipoprotein classes [i.e., high-density lipoproteins (HDLs), low-density lipoproteins (LDLs), intermediate-density lipoproteins (IDLs), very low-density lipoproteins (VLDLs) and chylomicrons] can be separated by their density and are primarily responsible for the transport of lipids in human blood (65). Since the liver plays a crucial role in lipid metabolism and in assembling lipoproteins, patients with impaired liver function often experience low cholesterol levels (66). Chronic liver disease is associated with a decrease in blood levels of high-density lipoprotein cholesterol

(HDL-C), LDL (67) and apolipoprotein (apo) A-I, the main protein component of HDL particles (68). It has been shown that lipoprotein levels correlate inversely with severity of liver disease (69,70). However, alterations in lipoprotein metabolism have also proven prognostic relevance in patients with liver cirrhosis: Low levels of HDL-C and apoA-I have been shown to associate with death or need for liver transplantation in these patients (71). This can be explained by the fact that lipoproteins also play important roles for the innate immune system with a multitude of functions (see Figure 3).

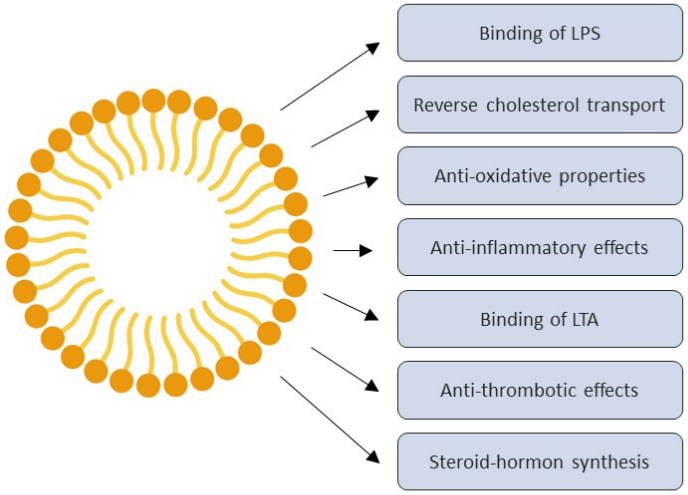

Figure 3. Functions of HDL particles. LPS; lipopolysaccharide, a constituent of gram-negative cell walls; LTA, lipoteichoic acid, a component of gram-positive bacteria.

With regard to bacterial translocation, worth mentioning is the capacity of HDL to bind and neutralize bacterial LPS (72,73). Therefore, sufficient and functional HDL particles seem crucial for endotoxin clearance, inflammation suppression and generally, disease progression and prognosis in patients with liver disease.

1.5. Prognosis prediction in patients with chronic liver disease

The natural history of liver cirrhosis is highly variably encompassing 1-year mortality rates between 1 and 57% (74). This is because liver cirrhosis itself extends through different stages, a usually asymptomatic compensated stage followed by decompensation that is defined by the occurrence of ascites, hepatic encephalopathy (HE) or variceal hemorrhage (75). Patients with compensated cirrhosis usually have a satisfactory quality of life and may remain in this stage for many years. The progression depends on multiple factors such as etiology of liver disease or the possibility of stopping / slowing the underlying damaging process. Clinical outcomes

and survival rates in different stages are highly variable with a mean life-expectancy of 10-13 years in compensated patients and roughly 2 years in patients with decompensated cirrhosis, respectively (76). Cohort studies indicate that approximately 5-7% of patients transition from a compensated to a decompensated stage each year (74). Ascites is the most frequent first decompensating event to occur, therefore it is considered a landmark sign of decompensation.

In patients with liver cirrhosis adequate prognosis prediction is of particular importance due to the availability of a highly effective but limited treatment option, i.e., liver transplantation. The need for adequate prognostic models and scoring systems is evident in order to manage patients in terms of indication and optimal timing of liver transplantation in the context of organ shortage. Different prognostic models have been developed including systems comprising clinical variables (e.g., Child-Pugh score), only numerical data (e.g., MELD score) and those that use primarily clinical features (e.g., D'Amico clinical stages of cirrhosis).

1.5.1. Prognostic Markers and Systems

A large number of non-invasive and invasive markers and systems have been investigated for prognosis prediction in patients with chronic liver disease.

Child-Pugh score: One of the first prognostic scores was proposed by Child and Turcotte in 1964 to predict surgery risk in patients undergoing porto-caval shunting for variceal bleeding (77). Patients with liver cirrhosis were divided into three categories according to their 'functional hepatic reserve': Child-Turcotte A, B and C, corresponding to minimally, moderately or severely impaired functional hepatic reserve. Their score was based on five variables, three clinical (i.e., nutritional status, ascites & encephalopathy) and two laboratory parameters (bilirubin & albumin) that were selected based on the authors' clinical experience. It is therefore noteworthy that a slightly modified score, the Child-Pugh score (CPS) proposed by Pugh et al. in 1973, is even today one of the most widely used and best-studied prognostic score in patients with liver cirrhosis (78). Pugh modified the score by removing nutritional status and adding a coagulation parameter. He also assigned 1, 2 or 3 points to each of the variables allowing the calculation of the Child-Pugh score that finally ranges from 5-15 points, with a score of 5 and 6 corresponding to Child-Pugh class A, a

score of 7-9 corresponding to class B and a score of 10-15 to Child-Pugh class C (see Figure 4).

Clinical and Biochemical Measurements	Points scored for Increasing Abnormality		
	1	2	3
Encephalopathy (grade)*	None	1 and 2	3 and 4
Ascites	Absent	Slight	Moderate
Bilirubin (mg. per 100 ml.)	1–2	2–3	>3
Albumin (g. per 100 ml.)	3·5	2·8–3·5	<2·8
Prothrombin time (sec. prolonged)	1–4	4–6	>6
For primary biliary cirrhosis:—			
Bilirubin (mg. per 100 ml.)	1–4	4–10	>10

* According to grading of Trey, Burns, and Saunders (1966).

Figure 4. The Child-Pugh Score as it was published in the original paper by Pugh et al. (78).

MELD score: In 2000, Malinchoc introduced a novel sore to predict the survival of patients undergoing transjugular intrahepatic portosystemic shunt (TIPS) intervention, the model for end-stage liver disease (MELD) score (79). The primary version of this score included etiology of liver disease (alcoholic & cholestatic versus other) and three laboratory variables (bilirubin, creatinine and INR). The parameters were selected by multivariable analysis using Cox regression analysis (80) and the natural logarithm of bilirubin, creatinine and INR were entered to reduce the effect of outliers. It soon became evident that the MELD score was particularly useful in the setting of advanced or decompensated liver cirrhosis, presumably because TIPS was performed in patients with recurrent variceal hemorrhage or refractory ascites. Therefore, the United Network for Organ Sharing (UNOS) adapted the score in 2002 (etiology was removed & a maximum threshold for creatinine was established at 4 mg/dl to prevent a very high score in patients with renal insufficiency) and approved the MELD score as primary allocation tool for patients awaiting liver transplantation in the United States (81,82). The current MELD score is calculated using the following equation:

$$MELD\ score = 9.6 * ln(creatinine) + 3.8 * ln(bilirubin) + 11.2 * ln(INR) + 6.4$$

Since its first introduction in 2000, several modifications of the MELD score have been published including the MELD-lactate (adding lactate), MELD-Na (adding sodium), MELD-Plus (adding different variables such as white blood count & age)

and the MELD 3.0 scores (adding albumin and sex) (83). Among these, the MELD-Na score has demonstrated to significantly improve prediction of waitlist mortality and therefore, is nowadays widely used to rank priority of patients awaiting liver transplantation (84,85).

Clinical staging models: Although Child-Pugh and MELD scores have proven their relevance in determining mortality risk in patients with liver cirrhosis, their main limitation lies in assessing the severity of liver disease at a single time point regardless of the course and progression of each patient through different stages. One of the easiest and most common ways to stage patients with liver cirrhosis is according to the absence or presence of clinical complications into compensated and decompensated liver cirrhosis (75). However, patients in either of the two stages demonstrate high variability in terms of complication and mortality risk. As a result, multilevel clinical staging systems have been developed such as D'Amicos clinical stages of cirrhosis, differentiating between four stages: stages 1 and 2 reflecting compensated cirrhosis (without clinical complications or only non-bleeding esophageal varices), stages 3 and 4 representing decompensated disease (patients with ascites or bleeding esophageal varices, respectively) (74). During the last decades, several other important drivers of prognosis (*e.g.,* systemic inflammation, extrahepatic organ function) have been recognized and additional stages have been introduced (*e.g.,* acute-on-chronic liver failure representing patients with high short-term mortality). An attempt to provide a novel staging system including these new insights was ventured by Jalan et al. (86) in 2021 (see Figure 5).

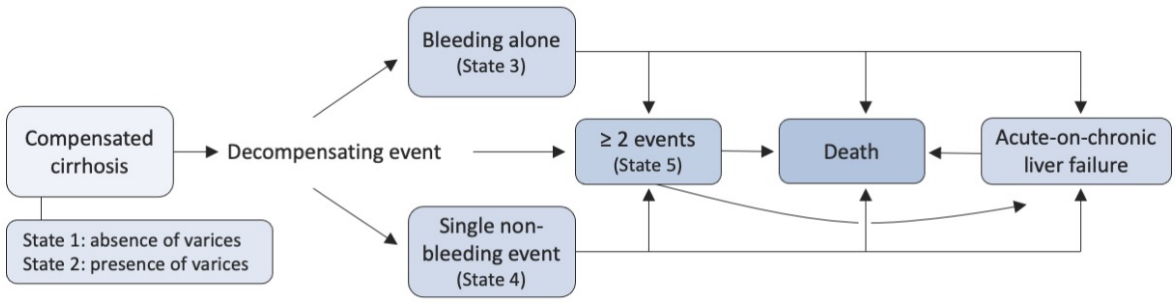

Figure 5. Clinical trajectory of cirrhosis across different clinical states. States 1 and 2 are defined by the absence or presence of varices, respectively. In state 3, variceal bleeding occurs, state 4 encompasses any other non-bleeding event, and state 5 includes the occurrence of 2 or more events. Acute-on-chronic liver failure may occur at any state and worsens prognosis significantly. Mortality risk rises with increasing disease states. Adapted from (86).

Acute decompensation (AD) and acute-on-chronic liver failure (ACLF): The term acute-on-chronic liver failure (ACLF) was initially coined by Jalan and Williams in the year 2002 (87) and emerged from the recognition of a clinical syndrome associated with a very high short-term mortality in patients with acutely decompensated liver disease. ACLF is characterized by three key features: it is often precipitated by pro-inflammatory events (*e.g.,* bacterial infection or alcoholic hepatitis), characterized by intense systemic inflammation and often associated with one or more extrahepatic organ failures (88). Several definition and diagnostic criteria of ACLF exist, but usually address the role of initiating events and the occurrence of different organ failures. There is a consensus that ACLF represents not only decompensation of liver cirrhosis, but a distinct syndrome that can develop on the basis of acute decompensation (AD). A large proportion of patients admitted to hospital develop ACLF during their first episode of AD of liver cirrhosis (about 40%) (89). Therefore, the occurrence of AD is an essential clinical feature for the definition and diagnosis of ACLF.

The most widely accepted definition of ACLF was proposed by the EASL-CLIF (European Association for the Study of the Liver – Chronic Liver Failure) consortium in 2013 and identifies organ failures with the use of a modified Sequential Organ Failure Assessment (SOFA) score. The score is based on liver, kidney, circulatory, respiratory and brain function as well as coagulation parameters and permits stratification of patients in subgroups with different mortality risks (see Table 2).

Organ System	*1 Point*	*2 Points*	*3 Points*
Liver	Bilirubin <6 mg/dl	Bilirubin 6.0–11.9 mg/dl	Bilirubin ≥ 12 mg/dl
Kidney	Creatinine <1.5 mg/dl Creatinine 1.5–1.9 mg/dl	Creatinine 2.0–3.4 mg/dl	Creatinine ≥ 3.5 mg/dl or RRT
Circulation	MAP ≥ 70 mmHg	MAP < 70 mmHg	Vasopressor requirement
Respiration	PaO_2/FiO_2 > 300 SpO_2/FiO_2 > 357	PaO_2/FiO_2 201 - 300 SpO_2/FiO_2 215 - 357	PaO_2/FiO_2 ≤ 200 SpO_2/FiO_2 ≤ 214 or mechanical ventilation

Brain (West Haven Criteria)	Grade 0	Grade 1 - 2	Grade 3 - 4
Coagulation	INR < 2.0	INR 2.0 – 2.4	INR ≥ 2.5

Table 2. The Sequential Organ Failure (SOFA) score proposed by the EASL-CLIF consortium. For each organ system, points are collected ranging from 1 (normal function) to 3 points (severe dysfunction). The light blue cells indicate organ dysfunction, the dark blue cells indicate organ failure. FiO_2 fraction of inspired oxygen, MAP mean arterial pressure, INR international normalized ratio, PaO_2 arterial oxygen partial pressure, RRT renal-replacement therapy, SpO_2 oxygen saturation (pulse oximetry).

According to the number and selection of organ failures at diagnosis, patients are stratified into four prognostic stages: Patients without an organ failure or a single, non-kidney organ failure without kidney dysfunction or brain dysfunction do not have ACLF and therefore, have a comparatively good prognosis (28-day-mortality 4.4 – 6.3%). Patients with only kidney failure or a single organ failure with kidney or brain dysfunction are classified as ACLF-1 (28-day-mortality 18.6 – 27.8%), patients with two organ failures as ACLF-2 (32.0%) and patients with 3 or more organ failures as ACLF-3 (68.0-88.9%) (88).

The EASL-CLIF consortium has also proposed and validated a prognostic score for patients with AD without ACLF, the CLIF-C AD score (90):

$$\textit{CLIF-C AD Score} = 10 * [0.03 * \textit{age} + 0.66 * \ln(\textit{creatinine}) + 1.71 * \ln(\textit{INR}) + 0.88 * \ln(\textit{WBC}) - 0.05 * \textit{Sodium} + 8]$$

The CLIF-C AD score was shown to be more accurate in the prediction of outcomes in these patients when compared to the MELD or MELD-Na score (90).

1.6. Aims

As it was outlined, prognosis prediction is of particular importance in patients with liver cirrhosis due to the variable disease course and the need to select and rank end-stage patients for liver transplantation, especially in the context of organ shortage. While most of the well-studied prognostic factors and scores are based on parameters of disease severity (*e.g.,* bilirubin, presence of ascites), they do not take into account markers or representatives of disease progression. Yet, an important role of different components of the gut-liver axis & intestinal barrier on liver disease

progression was recently suggested. Chronic liver disease behaves like a chronic inflammatory condition where the immune system is constantly stimulated by microorganisms and bacterial products of intestinal origin. In liver disease, impairments have been described in every subsection of the intestinal barrier, including alterations in gut microbiome composition, increased permeability of the epithelium and malfunctions of the innate and adaptive immune systems.

Therefore, the aim of this scientific work was to investigate the prognostic value of several biomarkers related to bacterial translocation in patients with liver cirrhosis.

2. Materials & Methods

For this study, patients in different stages of chronic liver disease (from compensated cirrhosis to ACLF) were investigated in terms of patient-specific parameters (e.g., etiology of liver disease, PPI intake), stool microbiome composition (i.e., 16S sequencing of stool samples), morphological alterations of the epithelial barrier (i.e., distal duodenal biopsies and analysis via light and transmission electron microscopy), markers of intestinal inflammation (i.e., fecal calprotectin levels) & permeability (i.e., fecal zonulin levels and the lactulose/mannitol test), markers of bacterial translocation (i.e., serum endotoxin levels), markers of systemic inflammation (i.e., macrophage activation markers serum sCD163 & sMR as well as CRP, IL-6, IL-8 & TNF-α) and lipid metabolism parameters (i.e., HDL-C and apoA-I). An overview of investigated parameters is given in Figure 6. Patients were monitored for occurrence of complications (including infection), transplantation or death. The prognostic value of biomarkers related to bacterial translocation was compared to established composite scores including Child-Pugh and MELD scores.

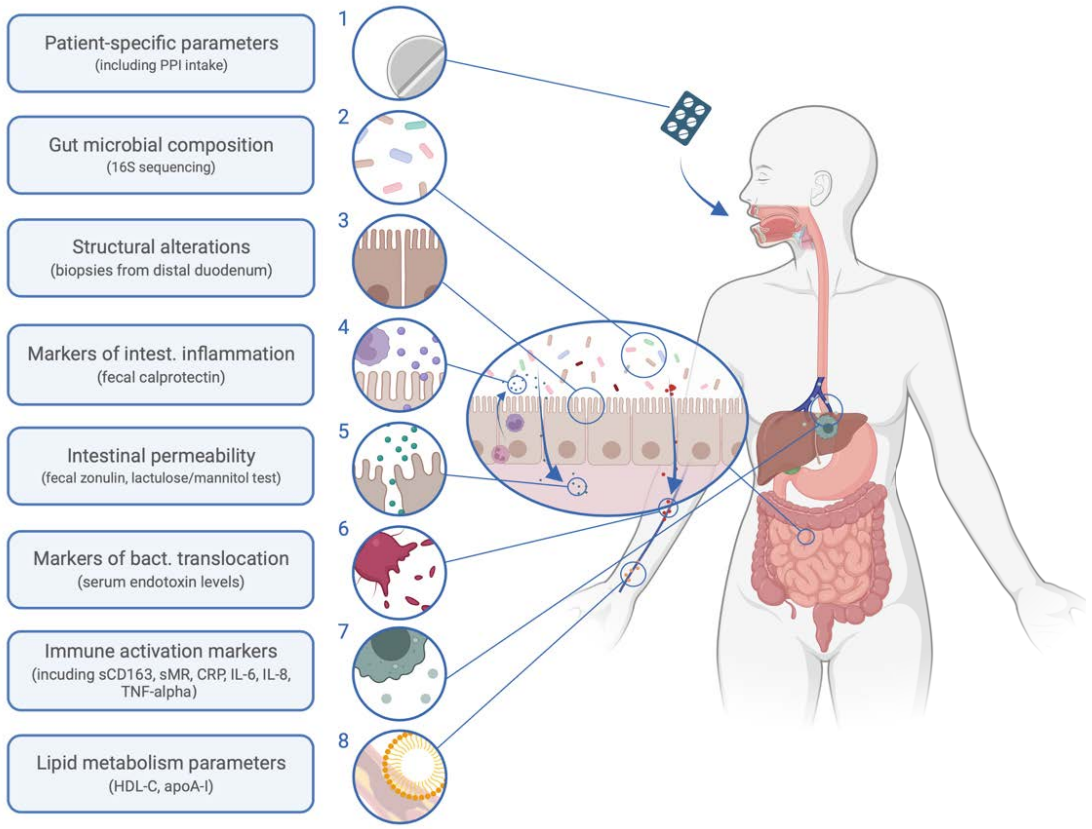

Figure 6. Determinants and biomarkers of bacterial translocation, as assessed in this thesis. PPI, proton pump inhibitor; sMR soluble mannose receptor; CRP, C-reactive protein; IL, interleukin; TNF, tumor necrosis factor; HDL, high density lipoprotein; apoA-I, apolipoprotein A-I. This figure was created with biorender.com.

2.1. Patients

In total, 536 patients with liver cirrhosis from 2 different cohorts were included:

The first cohort consists of 256 consecutive patients with *stable cirrhosis*, recruited between 2011 and 2016 at the Medical University of Graz either from the liver outpatient clinic or the gastrointestinal ward. These patients were collected from three different studies: (I) An intervention study assessing the efficacy of a 6-month probiotic intervention (see Horvath et al. (91), n=101 patients), (II) an observational study evaluating the prevalence of pulmonary hypertension and hepatopulmonary syndrome (see Douschan et al. (92), n=136 patients) and (III) a small pilot study investigating duodenal structural alterations in patients with liver cirrhosis (for further information, see (93), n=19 patients).

The second cohort comprises 280 patients with *decompensated cirrhosis* with or without ACLF who were included in the multicenter CANONIC study between February and September 2011 [for further details, see Moreau et al. (94)]. The CANONIC study was conducted in 12 European studies.

In all patients, the diagnosis of cirrhosis was either based on histology or a combination of imaging, clinical and biochemical signs of liver disease. Patients with presence or history of hepatocellular carcinoma or solid organ transplantation were excluded.

Patient characteristics, blood & stool samples were obtained, and the lactulose/mannitol test & upper gastrointestinal endoscopy were performed at baseline according to the different study protocols. Patients admitted to the hospital were assessed for the presence of AD or ACLF as defined by Moreau et al (94). In patients with stable and compensated cirrhosis at baseline, data on the occurrence of complications were collected. Complications of liver cirrhosis were categorized as follows: development of new-onset ascites or HE in a patient without ascites / HE at baseline, development of SBP, hepatorenal syndrome, new-onset jaundice (clinically detectable or bilirubin >3 mg/dl), occurrence of portal vein thrombosis, upper gastrointestinal bleeding due to portal hypertension (bleeding from esophageal varices or portal hypertensive gastropathy), or death from liver disease. Additionally,

the occurrence of severe infection requiring antibiotic treatment was recorded in patients with stable cirrhosis at baseline.

Patients with liver chronic liver disease were compared to 40 age- and sex-matched healthy controls who did not meet the following exclusion criteria: history of liver disease, renal disease, cardiovascular disease, pregnancy, obesity, dyslipidemia, diabetes or signs of inflammation.

All studies have been approved by the local Institutional Review Boards (IRBs) in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient unless the requirement had been waived by the local IRB.

2.2. Laboratory assays

Gut microbiome composition was analyzed from fecal spot samples; DNA was isolated using MagnaPure LC DNA Isolation Kit III (Roche, Mannheim, Germany). The V1-2 hypervariable region was amplified and sequenced using the MiSeq Reagent V3 kit.

Inflammation and gut permeability were studied by fecal calprotectin, fecal zonulin, sCD163, sMR, CRP, TNF-α, IL-6 and IL-8 using nuclear magnetic resonance or ELISA according to manufacturer's instructions (95). Serum endotoxin (LPS) was measured in a cell-based detection assay with an adapted HEK-Blue LPS Detection Kit (Invivogen, San Diego, CA, USA) as previously described (91). The differential sugar absorption test was performed with 10g lactulose & 5g mannitol and analyzed by nuclear magnetic resonance spectroscopy as previously described (91). Levels of total cholesterol were measured enzymatically (Diasys, Holzheim, Germany). The levels of total HDL-C were determined using homogeneous assays from Denka Seiken Co., Ltd. (Tokyo, Japan) (96). ApoA-I was determined by immunoturbidimetry as previously described (97). Analyses were performed on an Olympus AU680 analyzer (Beckman Coulter, Brea, USA).

2.3. Structural analysis of duodenal biopsies

For examination using the light microscope, duodenal biopsy specimens were fixed in formalin, embedded in paraffin, cut into 3 µm thick sections and stained with

hematoxylin-eosin. The expression of claudin-1 was investigated using immunohistochemistry according to manufacturer's instructions. To detect apoptotic cells, sections were stained with antibodies against caspase-3.

For examination using the electron microscope, semi-thin sections were initially stained with toluidine blue. After fixation in 2.0% glutaraldehyde in 0.1M phosphate buffer (pH 7.2), specimens were post-fixed in 1.0% osmium tetroxide, dehydrated in ethanol and embedded in resin for sectioning. Two ultrathin sections (approx. 70–80 nm) were placed on 200 mesh copper grids, one section per grid. The mesh copper grids were contrast-stained with lead citrate and uranyl acetate for examination via transmission electron microscopy (TEM). Images obtained from TEM were analyzed using ImageJ.

2.4. Statistical analysis

Statistical analyses were performed using SPSS 28 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 9 (GraphPad Software, San Diego, USA). Continuous variables were reported by medians (Q1-Q3), count data by absolute frequencies and percentages. Comparisons between groups were done for categorical variables with the chi-square or Fisher's exact test and for continuous variables with the Mann-Whitney U or Kruskal-Wallis test. Spearman's rank test was used to determine correlations between biomarkers of bacterial translocation and other variables of interest.

Stool microbiome analyses were performed using QIIME (98) on a local Galaxy instance (https://galaxy.medunigraz.at/). The Chao1 index was used to quantify alpha-diversity. Taxonomic classification was conducted using SILVA database (99); when necessary, sequences were blasted in NCBI database to refine classification (100). Operational taxonomic units (OTUs) found only in one individual and low abundant OTUs (minimum fraction count of 0.05%) were removed from analysis. Benjamini-Hochberg procedure was used to account for multiple testing. The web-based program Calypso 7.14 (http://cgenome.net/calypso/) was utilized for redundancy analysis (RDA) and LEfSe (Linear discriminant analysis Effect Size) calculations.

Mortality rates were compared by Kaplan-Meier curves and log-rank tests. Discriminative power was assessed with the area under receiver operating characteristic curve (AUC) and corresponding 95%CI. Diagnostic performance was measured with the Youden index. Univariate Cox regression analysis was used to assess mortality and infection rates, binary logistic regression analysis to assess 1-year complication rates. Hazard ratios (HRs) estimated from Cox models and odds ratios (ORs) estimated from logistic regression were reported as relative risks with corresponding 95%CIs. A *p*-value <0.05 was considered to be statistically significant.

3. Results

3.1. Overview and patient characteristics

In total, 536 patients were enrolled in two cohorts, 256 patients with stable cirrhosis (SC) in the single-center cohort from Graz and 280 decompensated patients with or without ACLF in the European multicenter cohort. An overview of available parameters in different cohorts and follow-up is given in Figure 7.

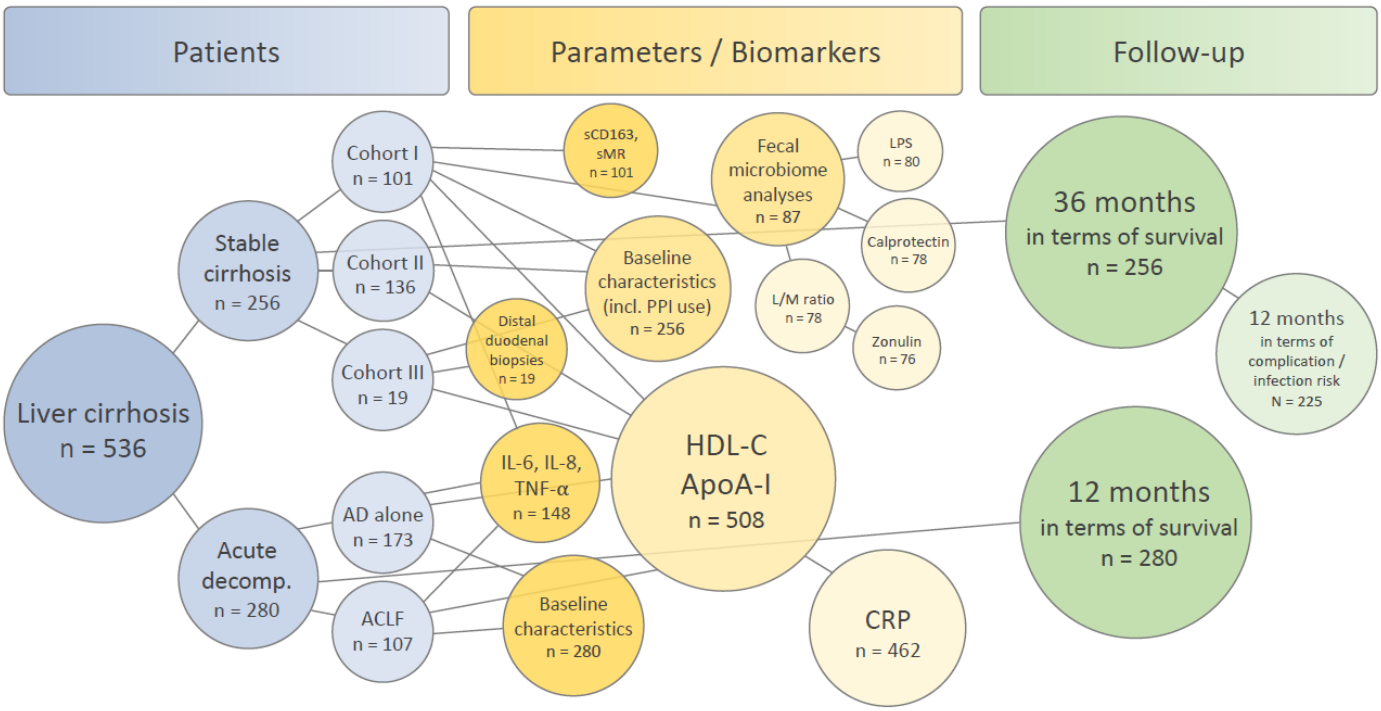

Figure 7. Overview of patient cohorts, available parameters and follow-up. IL, interleukin; sMR soluble mannose receptor; TNF, tumor necrosis factor; PPI, proton pump inhibitor; L/M ratio, lactulose/mannitol ratio; HDL-C, high density lipoprotein cholesterol; LPS, lipopolysaccharide; CRP, C-reactive protein.

Among 256 patients with SC, 148 (57.8%) were categorized as Child-Pugh class A, 84 (32.8%) as Child-Pugh class B and 24 patients (9.4%) as Child-Pugh C. Among 280 patients with AD from the European cohort, the diagnosis of ACLF was made in 107 patients (38.2%). Further information regarding characteristics of patients and controls is found in Table 3.

	Controls	Stable cirrhosis	Acute Decompensation	
			No ACLF	ACLF
N	*40*	*256*	*173*	*107*
Age [years]	57 (48, 64)	57 (51, 63)	58 (51, 67)	55 (48, 64)
Gender (% male)	60%	73%	64%	64%
Etiology of cirrhosis				
Alcohol		57%	53%	57%
HCV		16%	31%	28%
Other		27%	16%	15%
Child-Pugh score		6 (5, 8)	9 (8, 11)	11 (10, 13)
MELD		12 (9, 16)	16 (12, 20)	27 (22, 33)
CLIF-C AD score		n/a	52 (46, 57)	n/a
CLIF-C ACLF score		n/a	n/a	49 (43, 56)
WBC [10^9/l]	6.0 (5.1, 6.7)	5.1 (3.9, 6.5)	5.7 (4.1, 8.2)	8.4 (5.3, 12.3)
PLT [10^9/l]	249 (218, 270)	111 (80, 150)	97 (59, 141)	67 (42, 114)
Bilirubin [mg/dl]	0.5 (0.4, 0.7)	1.4 (0.8, 2.8)	2.8 (1.4, 5.6)	7.4 (2.3, 18.7)
Creatinine [mg/dl]	0.8 (0.7, 0.9)	0.8 (0.7, 1.0)	0.9 (0.7, 1.2)	1.9 (0.9, 3.2)
INR	1.01 (0.98, 1.04)	1.28 (1.16, 1.48)	1.46 (1.27, 1.75)	1.88 (1.40, 2.52)
Sodium [mmol/l]	140 (139, 141)	139 (136, 141)	136 (133, 139)	134 (128, 137)
Albumin [g/dl]	4.4 (4.3, 4.6)	4.0 (3.3, 4.4)	2.9 (2.6, 3.2)	3.0 (2.3, 3.4)

Table 3. Characteristics of Study participants. AD, acute decompensation; ACLF, acute-on-chronic liver failure; WBC, white blood cell count; PLT, platelet count; INR, international normalized ratio. Data are shown as median (Q1, Q3). n/a, not applicable.

3.2. Markers of bacterial translocation - associations with liver disease severity

3.2.1. PPI intake

PPI intake was assessed in patients with SC. At baseline, about half of patients (n=120, 47%) were taking proton pump inhibitors (data was missing in n=5). PPI intake was evenly distributed in patients with compensated (67 of 144 Child-Pugh A patients, 47%) and advanced liver cirrhosis (53 of 107 Child-Pugh B and C patients, 50%).

3.2.2. Alterations in gut microbial composition of cirrhosis patients

Microbiome data was available in 87 patients with liver cirrhosis and 21 healthy controls (for baseline data in these patients, see Table 9). We observed that alpha-diversity (measured as Chao1-index) was significantly reduced in patients with liver cirrhosis compared to controls (p<0.001). We did not observe specific clustering of

the groups in the PCoA (principal coordinates analysis) plots. Accordingly, we did not find significant differences between groups based on Bray-Curtis dissimilarity or weighted UniFrac. However, RDA found cirrhosis to significantly influence on microbiome composition (p=0.001), as shown in Figure 8.

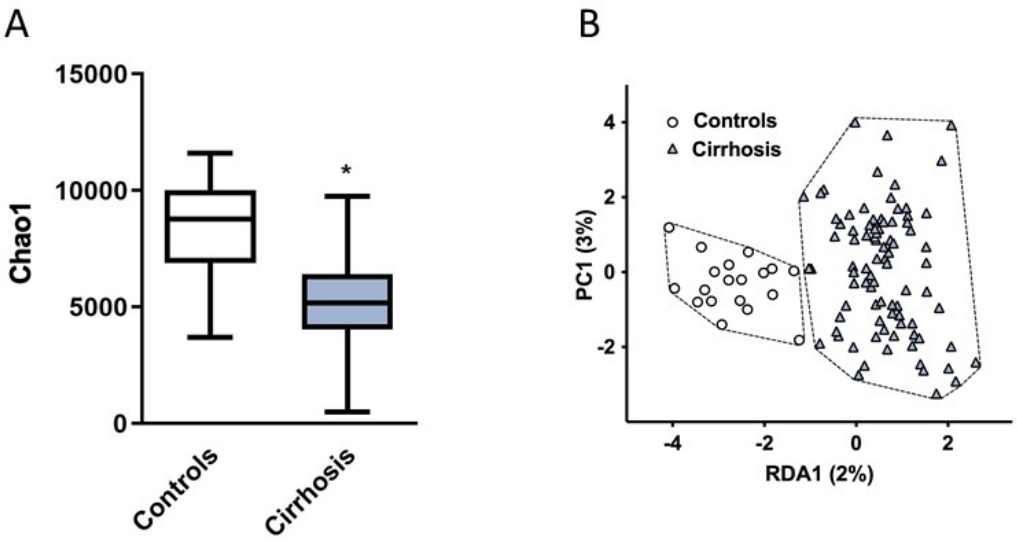

Figure 8. The impact of liver cirrhosis on gut microbial composition. A: Alpha diversity based on Chao1 index of cirrhosis patients (n=87) compared to healthy controls (n=19). B: Redundancy analysis of OTU abundance data and the presence of cirrhosis; *p<0.001 vs. controls.

To identify discriminatory genera between healthy individuals and patients with cirrhosis, LEfSe was employed. It revealed an association of the genera *Bacteroides, Blautia, Enterococcus, Streptococcus, Veillonella and Lactobacillus* with cirrhosis, while beneficial genera including *Ruminococcus* and *Prevotella* were attributed to healthy subjects. On family level, Bacteroidaceae, Streptococcaceae, Clostridiaceae 1 and Lactobacillaceae were associated with cirrhosis and differentially abundant between groups. Families Ruminococcaceae, Christensenellaceae, Peptostreptococcaceae and Erysipelotrichaceae were associated with healthy subjects. On order level, significant differences between groups were found in Clostridiales, Erysipelotrichales and NB1-n (health-related) as well as in Lactobacillales (cirrhosis-associated). The classes Erysipelotrichia and Mollicutes (controls) and Bacilli (cirrhosis) showed significant differences between patients and controls. On phylum level, Firmicutes and Tenericutes were associated with a healthy microbiome and Bacteroidetes with cirrhosis; all three were significantly different between our groups.

In patients with liver cirrhosis, severity and etiology of disease had an influence on microbiome composition (p=0.013 and p=0.065, respectively), assessed by redundancy analysis (see Figure 9).

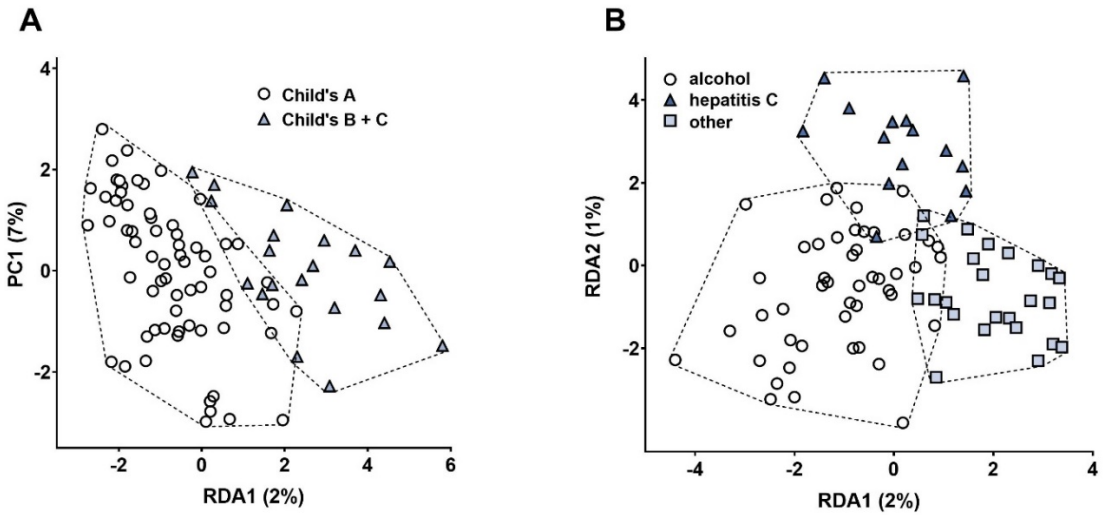

Figure 9. The impact of disease severity and etiology on gut microbial composition. Redundancy analysis of OTU abundance data. A: Severity of liver disease (Child-Pugh grade), B: Etiology of cirrhosis.

3.2.3. Structural alterations of the epithelial barrier

19 patients with liver cirrhosis at an early (Child-Pugh class A, n=12) or advanced (Child-Pugh classes B or C, n=6) disease stage underwent upper gastrointestinal endoscopy with distal duodenal biopsies. Alterations observed in light and transmission electron microscopy were compared to findings in 10 healthy controls (for baseline characteristics, see Table 4).

	Controls	*Cirrhosis*
N	10	19
Age [years]	48 (42; 62)	57 (53; 64)
Gender (% male)	10%	68%
Etiology of cirrhosis		
Alcohol	n/a	13 (69%)
HCV	n/a	1 (5%)
Other	n/a	5 (26%)
Child-Pugh class		
A	n/a	12 (63%)
B and C	n/a	7 (37%)
MELD Score	n/a	10 (9; 14)
PPI use (%)	50%	63%

Table 4. Baseline characteristics of controls and cirrhotic patients undergoing upper gastrointestinal endoscopy. HCV, hepatitis C virus; PPI, proton pump inhibitor; Data are shown as median (Q1, Q3). n/a, not applicable.

Light microscopy:

In healthy individuals, the usual villus-to-crypt ratio (V/C ratio) in the distal duodenum (i.e., the ratio between the length of the villi and the depth of the crypts) stands at ≥3:1. In the control group, only one person exhibited a decreased V/C ratio (10%), whereas in the setting of cirrhosis, 8 patients showed shortened villi (42%). Patients with advanced cirrhosis were at greater risk of having a disturbed V/C ratio.

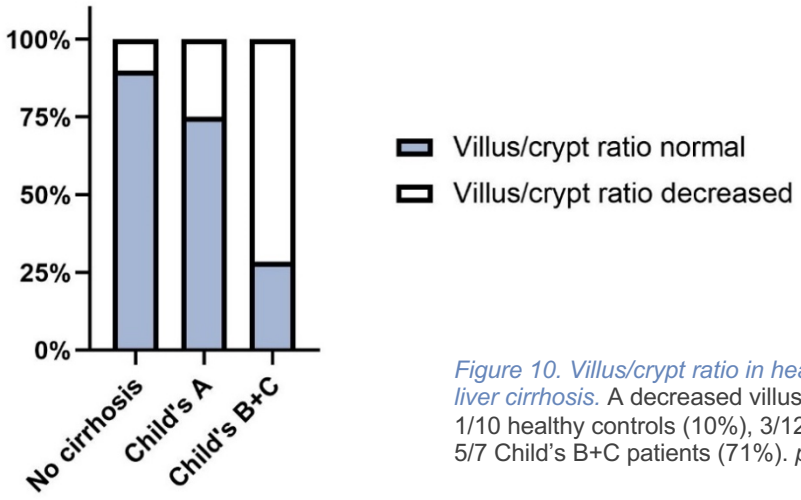

Figure 10. Villus/crypt ratio in healthy controls and patients with liver cirrhosis. A decreased villus/crypt ratio was observed in 1/10 healthy controls (10%), 3/12 Child's A patients (25%) and 5/7 Child's B+C patients (71%). *p*=0.017.

In addition to a decrease in size, duodenal villi also exhibited widening; this phenomenon was particularly noticeable in patients with advanced liver cirrhosis.

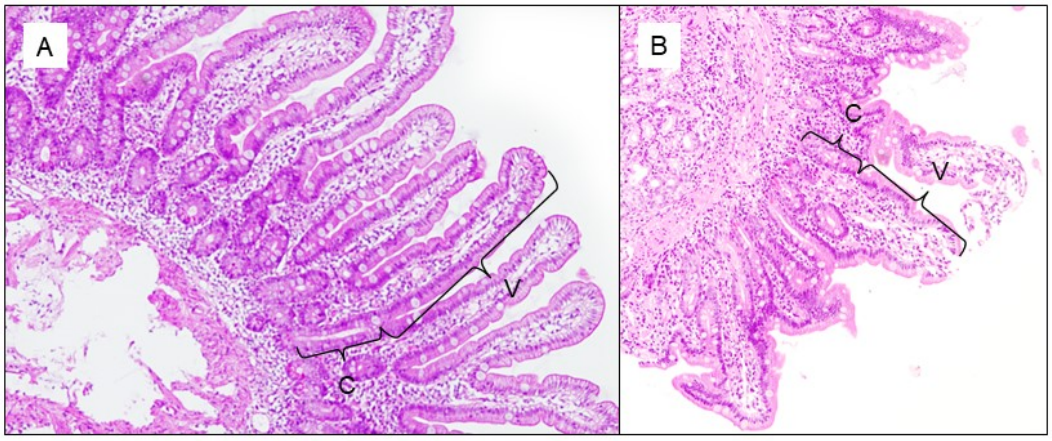

Figure 11. Duodenal mucosa in healthy control (A) and patient with liver cirrhosis (B). In the setting of cirrhosis, villi appear flattened and broadened (patient with Child-Pugh C cirrhosis, 13 points), leading to a decreased villous/crypt ratio. V, villi; C, crypt; H&E stain, 40x.

Furthermore, three phenomena especially at the tips of the villi were evident: (I) a separation of the enterocytes with distension of the intercellular spaces (DIS), (II) a detachment of enterocytes from the subjacent layer (the lamina propria), and (III) a complete loss of enterocytes (denudation) in some villi.

DIS was observed particularly in the lower (basal) portion of epithelial cells, whereas at the luminal part, enterocytes remained closely adhered to each other (see Figure 12). To quantify these changes, villi with DIS were counted and set in relation to the total number of villi observed in every slide. In patients with liver cirrhosis, there was a trend towards an increased occurrence of DIS (40.9% vs. 60.0% in healthy controls and liver disease patients, respectively; p=0.055).

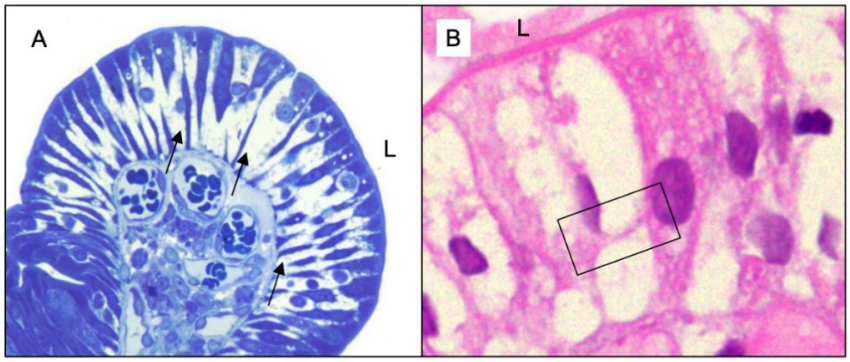

Figure 12. Villi with enterocytes separated by a distended intercellular space. A: Especially in the lower portion of enterocytes, they seem dispersed. Arrows indicate the expanded intercellular spaces. Toluidine blue stain, 200x; B: The open box marks a strand between two dispersed enterocytes, where cell-cell connections attempt to maintain contact, H&E stain, 400x; L, intestinal lumen.

Furthermore, the appearance of subepithelial blebs (due to a detachment of the epithelial layer from the lamina propria) and denuded villi (probably due to subsequent shedding of enterocytes) was investigated in both study groups. Again, villi with presence of either of these two phenomena were counted and related to the total number of villi in every slide. In Table 5, an overview of the relative frequencies in healthy controls and liver cirrhosis patients can be found:

	Controls	*Cirrhosis*	*p-value*
N	10	19	
Subepithelial dilatation [% of villi]	8.2 (5; 13)	21.7 (11; 29)	0.021
Denudation [% of villi]	18.2 (12; 21)	32 (18; 45)	0.013

Table 5. Comparison of Villi with subepithelial dilatation and denudation.

In patients with liver cirrhosis, both subepithelial dilatation and denuded villi were counted more frequently than in healthy controls (see Figure 13).

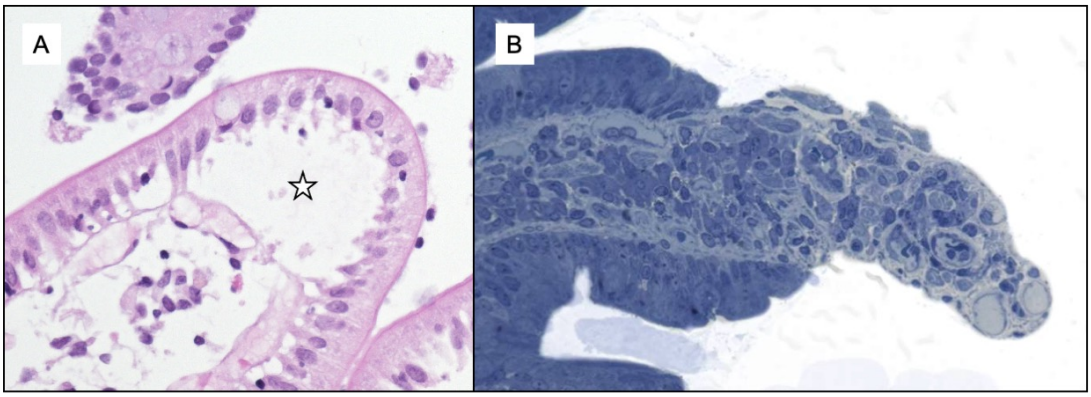

Figure 13. Dilatation of subepithelial spaces and denudation of villi in patients with cirrhosis. A: The epithelial layer is separated from the basement membrane forming a subepithelial bleb (open star), H&E stain, 400x. B: Denuded villus after loss of epithelium, toluidine blue stain, 200x.

Immunohistochemistry:

Expression of **claudin-1** was investigated in distal duodenal biopsy samples. In both crypts and villi, expression of claudin-1 was significantly decreased in patients with liver cirrhosis (Figure 14).

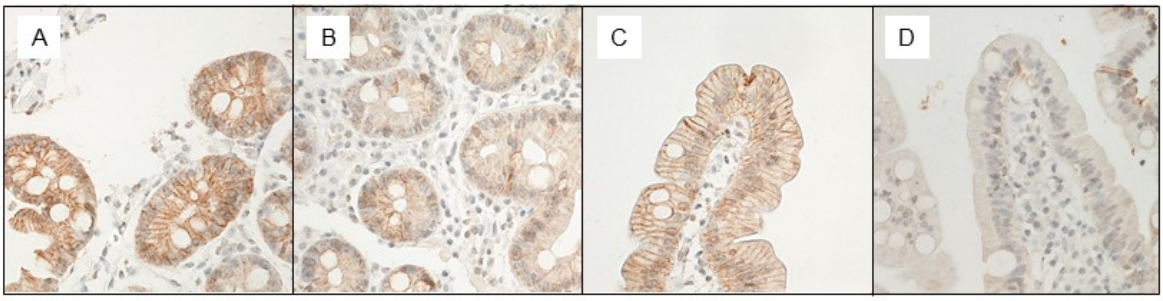

Figure 14. Claudin-1 expression in crypts (AB) and villi (CD) of healthy controls (AD) and patients with liver cirrhosis (BD). A: Crypt of a healthy control with marked claudin-1 expression. B: Crypt of a patient with liver cirrhosis (Child's A, 5 points) with less marked claudin-1 expression. C: Villi of a healthy control with marked claudin-1 expression. D: Villi of a patient with liver cirrhosis (Child's B, 8 points) with less marked claudin-1 expression.

Claudin-1 expression gradually decreased with increasing severity of liver disease, as shown in Figure 15.

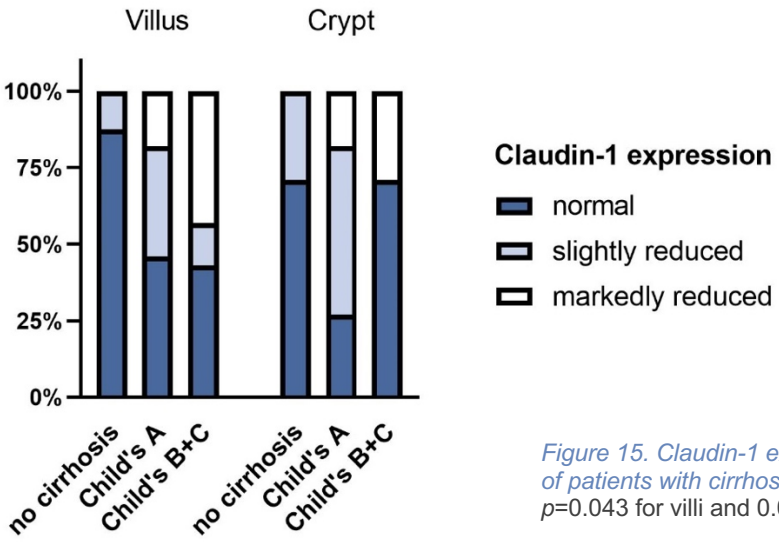

Figure 15. Claudin-1 expression in villi and crypts of patients with cirrhosis and healthy controls.
p=0.043 for villi and 0.040 for crypts.

After labeling with **caspase-3 antibodies**, apoptotic cells were detected in most patients (93%) and controls (70%) at the tips of the villi. However, in patients with liver cirrhosis, significantly more villi carried apoptotic cells when compared to healthy controls (% of villi with apoptotic cells in liver cirrhosis vs healthy controls: 15.4% vs. 6.5%, *p*=0.004).

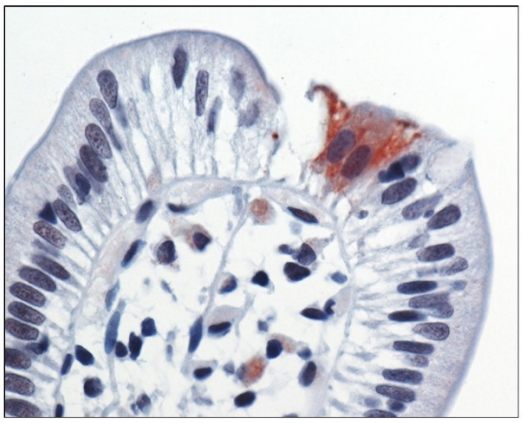

Figure 16. Apoptotic cells located at the tip of a villus. Anticaspase-3 stain (brown-colored cells), 400x.

Transmission-electron microscopy:

Similar to observations under the light microscope, expanded intercellular spaces, especially in the basal parts of enterocytes with preserved cell-junctions at the luminal borders were observed under TEM in patients with liver cirrhosis. This phenomenon was particularly prominent at the tips of the villi.

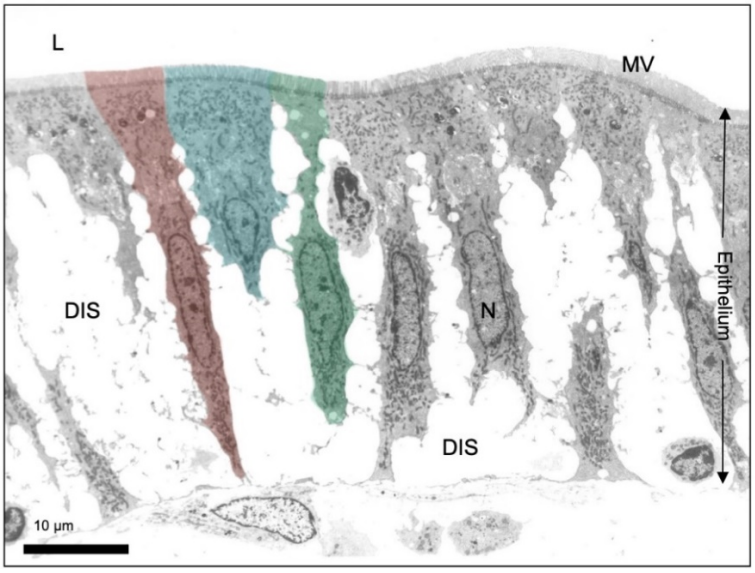

Figure 17. Columnar epithelium of a patient with liver cirrhosis (Child-Pugh score 8 points). The epithelial cells are separated by dilated extracellular spaces (DIS), especially at the lower portion of cells. Some enterocytes are colored for better depiction. L, luminal side; MV, microvilli; N, nucleus of an enterocyte.

Furthermore, alterations in tight-junction ultrastructure were assessed. In every study participant, at least 15 TJ complexes were analyzed.

At lower magnifications, TJs from patients with chronic liver disease demonstrated similar ultrastructural characteristics compared to healthy controls (see Figure 18).

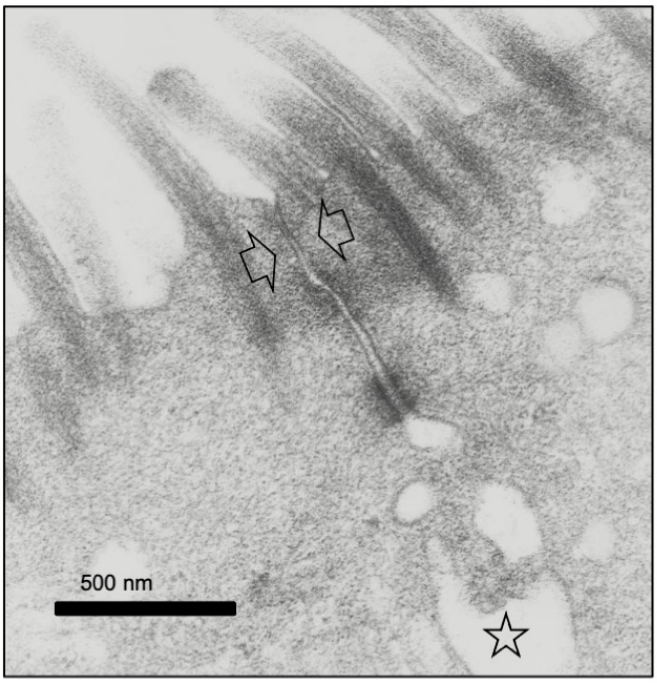

Figure 18. TJ complex of a patient with liver cirrhosis (Child-Pugh score 8 points). The open star depicts a distended intercellular space; bar line: 500nm.

Subsequently, the distance between the two inner leaflets of the enterocytes' cell membranes in the area of TJs (TJ-gap width) was measured in cirrhosis patients and healthy controls as shown in Figure 19.

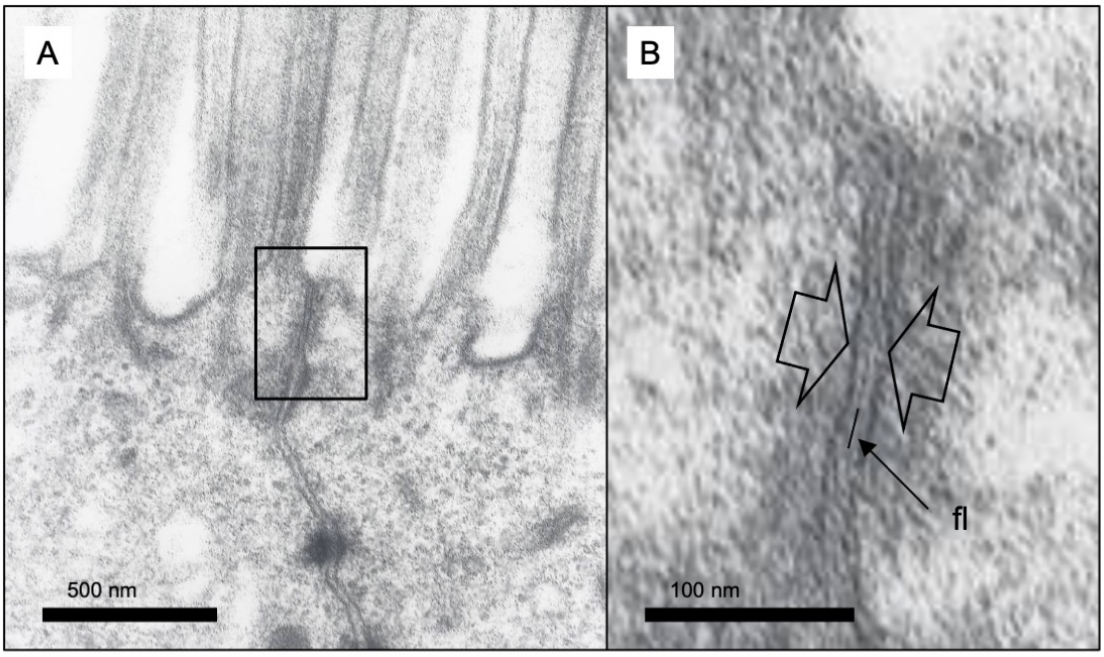

Figure 19. TJ complex from a healthy control. A: The tight junctions are located at the luminal end of the enterocytes' lateral walls (square), bar line: 500nm. B: In Figure B, the TJ complex with in the square of figure A is magnified. The TJ-gap width was defined as the distance between the two inner leaflets of the cell membranes (arrows), fl, fusion line; bar line: 100nm.

There were no differences between mean TJ gap width of healthy controls and of cirrhosis patients when areas without DIS were measured (10.3 nm and 10.2 nm, respectively, p=0.809). However, TJ gap width in areas with DIS was significantly increased compared to healthy controls (10.3 nm and 11.6 nm, respectively, p<0.001).

Occasionally, spreading of the fused outer layers of the cell membrane within the TJs (possibly corresponding to strand breaks) were observed. This phenomenon was depicted in 2 patients with liver cirrhosis (see Figure 20) and not in healthy controls.

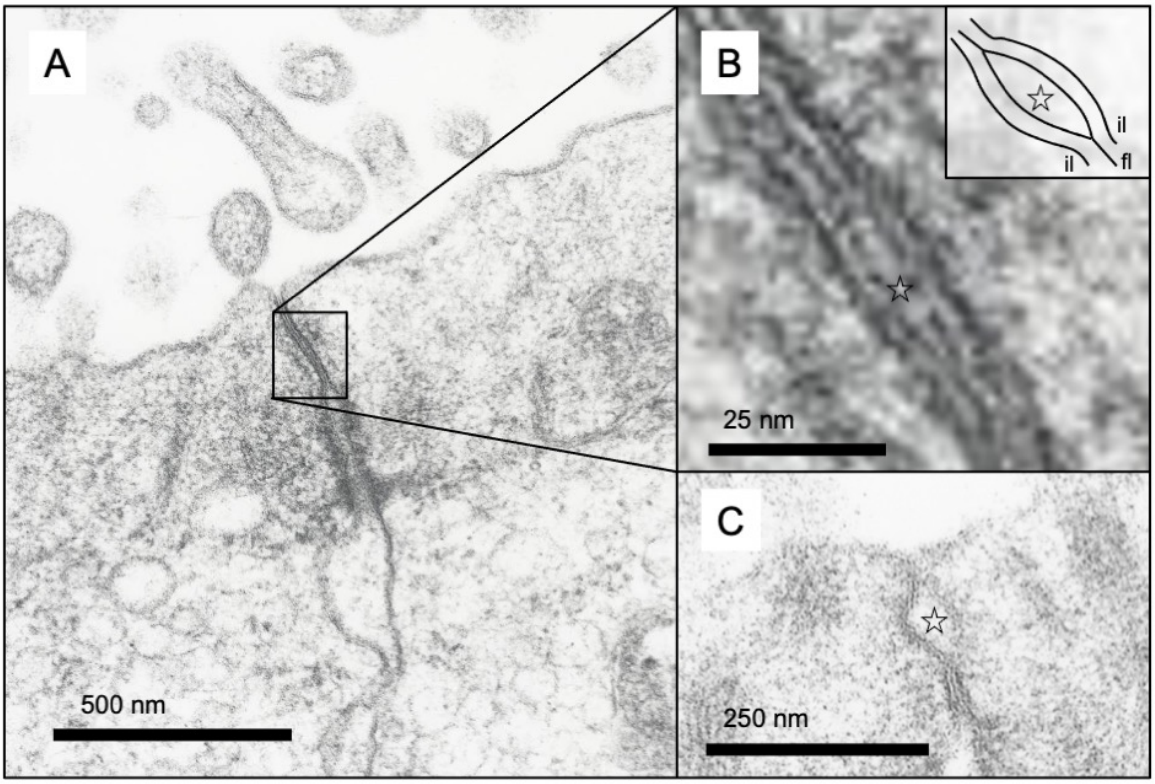

Figure 20. Strand breaks of TJ complexes in patients with liver cirrhosis. A: TJ of a patient with Child's B liver cirrhosis. B Higher magnification of the TJ complex shows a strand break of the fusion line. C: Electron micrograph of a TJ complex in a patient with Child's A cirrhosis showing spreading of the fusion line. il, inner leaflet; fl, fusion line.

Furthermore, the mean microvilli (MV) length of enterocytes was measured in at least 10 cells and compared between patients with liver cirrhosis and healthy controls. In the group of patients with liver disease, the brush border appeared less tall and additionally less densely packed. Figure 21 shows representative cases of MV in patients with liver cirrhosis and healthy individuals.

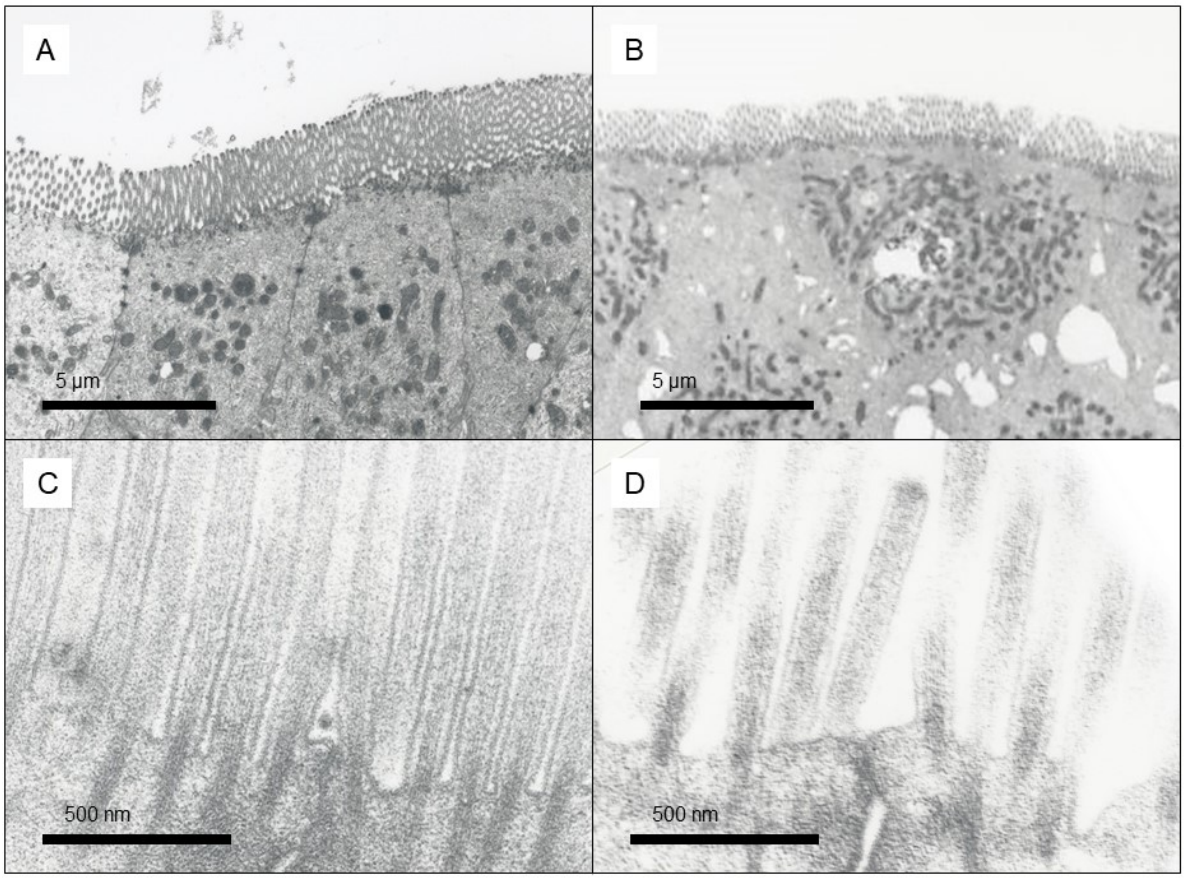

Figure 21. Microvilli in control and cirrhosis patients. A, C: TEM of the brush border in a healthy control. The microvilli stand close to each other and attain a normal size. B, D: Microvilli in a representative patient with liver cirrhosis; they are shorter and less densely packed.

Statistical analysis indicated that microvilli were significantly shorter in patients with liver cirrhosis compared to healthy individuals (1270nm in cirrhosis patients vs. 1570nm in healthy controls, p=0.008).

In isolated instances, we found evidence of necrosis in cells at the villous tips (including swelling of cytoplasm & nuclei and disruption of plasma membrane & organelles) in patients with liver cirrhosis (see Figure 22).

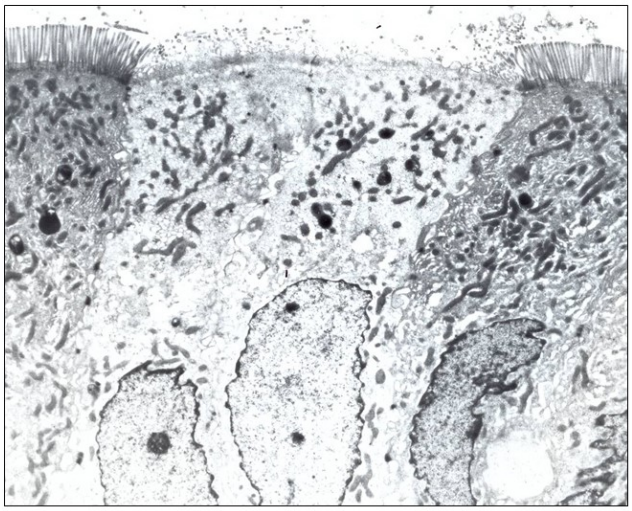

Figure 22. Cells with features of necrosis covering the villous tip in a patient with liver cirrhosis. The two cells in the middle show nuclear swelling, rarified & distorted microvilli and disruption of the plasma membrane.

3.2.4. Intestinal inflammation

Stool calprotectin as a marker of gut inflammation was measured in 77 patients with liver cirrhosis (n=59 Child-Pugh A patients; n=18 Child-Pugh B & C patients) and 23 healthy controls. In patients with liver cirrhosis, calprotectin was significantly elevated when compared to controls (115 vs. 40µg/mg, p<0.001) and increased with disease severity (Child-Pugh A: 95µg/mg, Child-Pugh B: 150µg/mg and Child-Pugh C: 180µg/mg, p=0.014, see Figure 23).

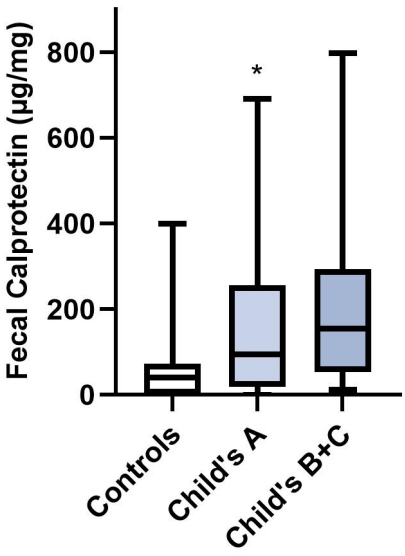

Figure 23. Levels of fecal calprotectin according to disease severity.
* p<0.01 vs. controls.

3.2.5. Intestinal permeability

For evaluation of intestinal permeability, fecal zonulin levels were measured and the lactulose/mannitol test was performed. These parameters were available in 78 patients with liver cirrhosis (n=60 Child-Pugh A patients & n=18 Child-Pugh B & C patients) and 25 healthy controls. There were no significant differences in zonulin levels measured in healthy controls and patients with liver cirrhosis (74ng/ml vs. 78ng/ml, *p*=0.437); however, there was a trend towards an increase in patients with advanced cirrhosis (Child-Pugh B+C: 90ng/ml, p=0.079).

Similar results were observed in terms of the lactulose/mannitol test: there were no significant differences between healthy individuals and patients with liver disease, but a significant increase in patients with advanced cirrhosis (lactulose/mannitol ratio in controls: <0.001, Child-Pugh A: 0.006, Child-Pugh B+C: 0.133, see Figure 24).

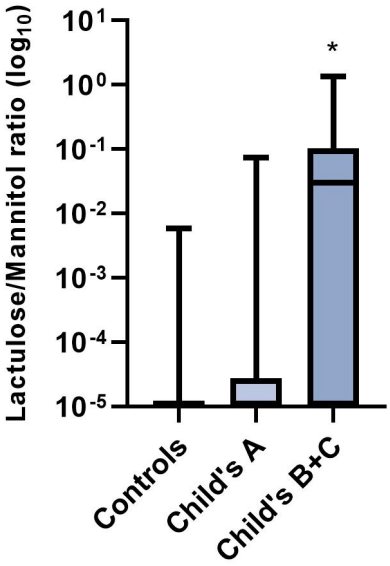

Figure 24. The lactulose/mannitol ratio according to disease severity.
* *p*<0.001 vs. Child's A.

3.2.6. Markers of bacterial translocation

LPS as a marker of bacterial translocation was measured in 80 patients with liver cirrhosis (n=61 Child-Pugh A patients & n=19 Child-Pugh B & C patients) and 31 healthy controls. In patients with liver cirrhosis, serum LPS levels were significantly elevated when compared to healthy controls (7.3 vs. 0.3 EU/ml, *p*<0.001). There was no difference between patients with compensated and advanced disease or in patients with or without PPI intake (*p*=0.923 and 0.996, respectively).

3.2.7. Markers of systemic inflammation

Macrophage activation markers sCD163 and sMR:

Macrophage activation markers sCD163 and sMR were measured in 101 patients with liver cirrhosis and 31 healthy controls. 72 patients had compensated cirrhosis (Child-Pugh class A), 29 patients had advanced disease (25 Child-Pugh class B and 4 Child-Pugh class C). For characteristics of patients and controls, see Table 6.

	Controls	*Cirrhosis*
N	31	101
Age [years]	57 (51; 63)	58 (47; 64)
Gender (% male)	72%	71%
Etiology of cirrhosis		
Alcohol	n/a	54 (53%)
HCV	n/a	21 (21%)
Other	n/a	26 (26%)
Child-Pugh class		
A	n/a	72 (71%)
B and C	n/a	29 (29%)
MELD Score	n/a	11 (8; 14)

Table 6. Baseline characteristics of controls and cirrhotic patients for assessment of macrophage activation parameters. HCV, hepatitis C virus; PPI, proton pump inhibitor; Data are shown as median (Q1, Q3). n/a, not applicable.

The median levels of sCD163 and sMR were significantly higher in patients with liver cirrhosis and also increased with the severity of liver disease (see Figure 25). Additionally, significantly higher levels of both markers could be measured in patients with ascites at baseline compared to those who did not suffer from ascites (ascites/no ascites = 7.95/4.43 mg/L and 61/39 µg/dL, respectively).

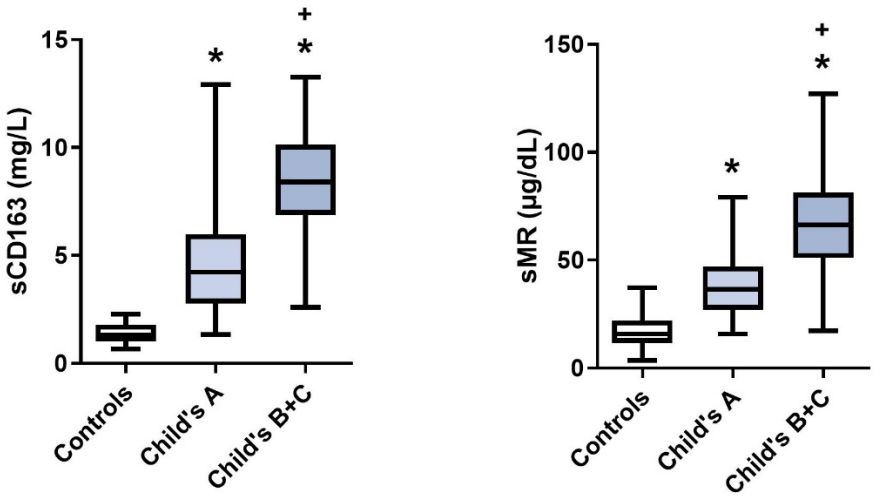

Figure 25. Macrophage activation markers sCD163 and sMR levels in healthy controls and cirrhosis patients classified according to disease severity. * $p<0.001$ vs. controls. $^{+}$ $p<0.001$ vs. Child's A. Adapted from (101).

Other markers of systemic inflammation:

Markers of systemic inflammation (CRP, TNF-α, IL-6, IL-8) were available also in the European multicenter cohort (in total n=252 patients with stable cirrhosis, 169 patients with AD and 108 ACLF patients). All markers were significantly elevated in patients with liver cirrhosis (see Table 7) and gradually increased with disease severity (see Figure 26).

	Controls	*Cirrhosis*	*p-value*
CRP [mg/L]	2.5 (1.0; 4.7)	6.3 (2.5; 22.3)	**0.003**
IL-6 [pg/mL]	0 (0; 1)	9 (0; 43)	**<0.001**
IL-8 [pg/mL]	0.9 (0; 5)	71.3 (30; 170)	**<0.001**
TNF-α [pg/mL]	0 (0; 1)	5 (0; 25)	**<0.001**

Table 7. Markers of systemic inflammation in healthy controls and cirrhosis patients. Data are shown as median (Q1, Q3).

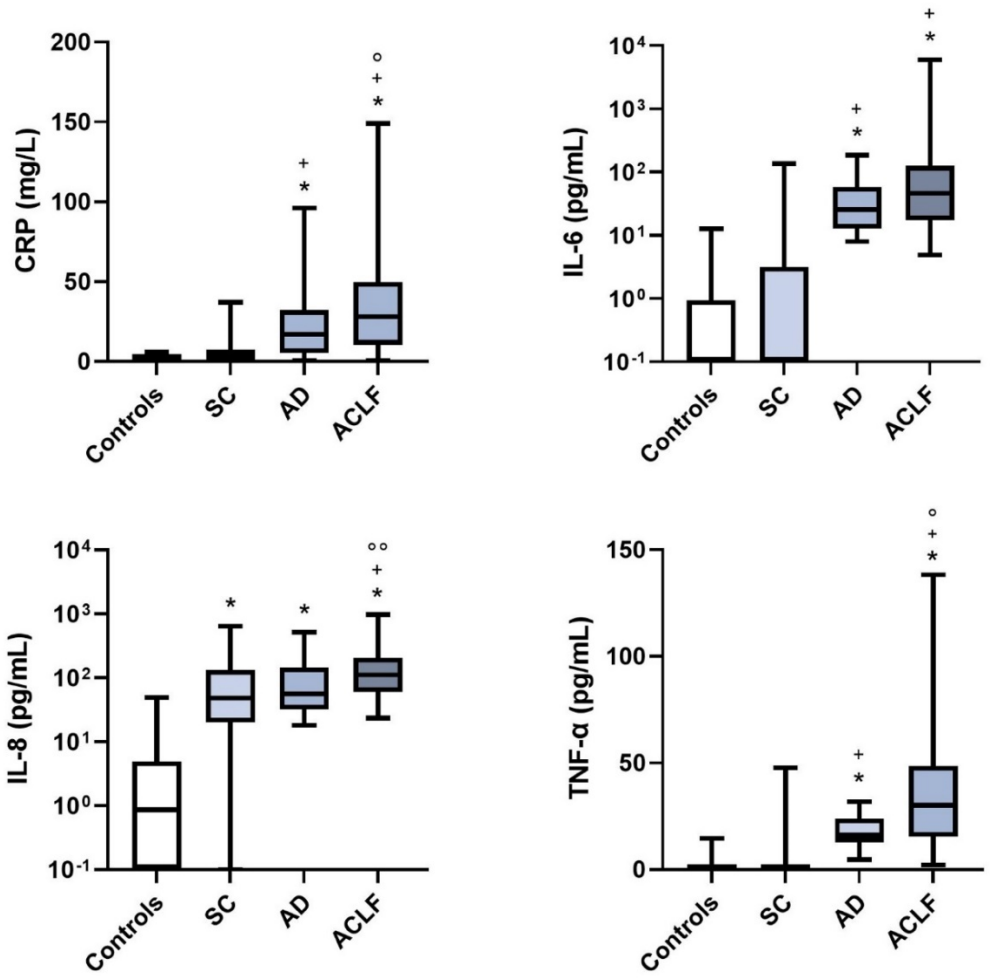

Figure 26. Markers of systemic inflammation CRP, TNF-α, IL-6 and IL-8 according to disease severity. SC, stable cirrhosis; ACLF, acute-on-chronic liver failure; AD, acute decompensation; IL, interleukin. * p<0.001 vs. control; $^{+}$ p<0.001 vs. SC; ° p<0.01, °° p<0.05 vs. AD. Adapted from (102).

3.2.8. Lipid metabolism parameters in liver disease

Due to the known effects of cholestasis on lipid metabolism and lipoprotein values, patients with cholestatic liver diseases were excluded from these analyses (103). Data on lipid metabolism parameters were available in 228 patients with SC in the Austrian single-center and 280 patients with AD in the European multicenter cohort. In patients with liver cirrhosis, the median levels of total cholesterol and HDL-related biomarkers (including HDL-C and apoA-I) were significantly reduced when compared to healthy controls. Furthermore, serum levels gradually declined with the severity of liver disease (Table 8, Figure 27).

	Controls	*SC*	*AD*	*ACLF*
N	40	228	173	107
Cholesterol [mg/dL]	231 (199; 285)	149 (120; 181)	95 (73; 126)	71 (42; 100)
HDL-C [mg/dL]	65 (52; 83)	47 (36; 60)	22 (11; 30)	11 (5; 20)
ApoA-I [mg/dL]	178 (152; 210)	122 (90; 149)	69 (43; 89)	33 (18; 67)

Table 8. HDL-related biomarkers according to disease severity. AD, acute decompensation; ACLF, acute-on-chronic liver failure; HDL-C, high-density lipoprotein cholesterol; apoA-I, apolipoprotein A1. Data are shown as median (Q1; Q3).

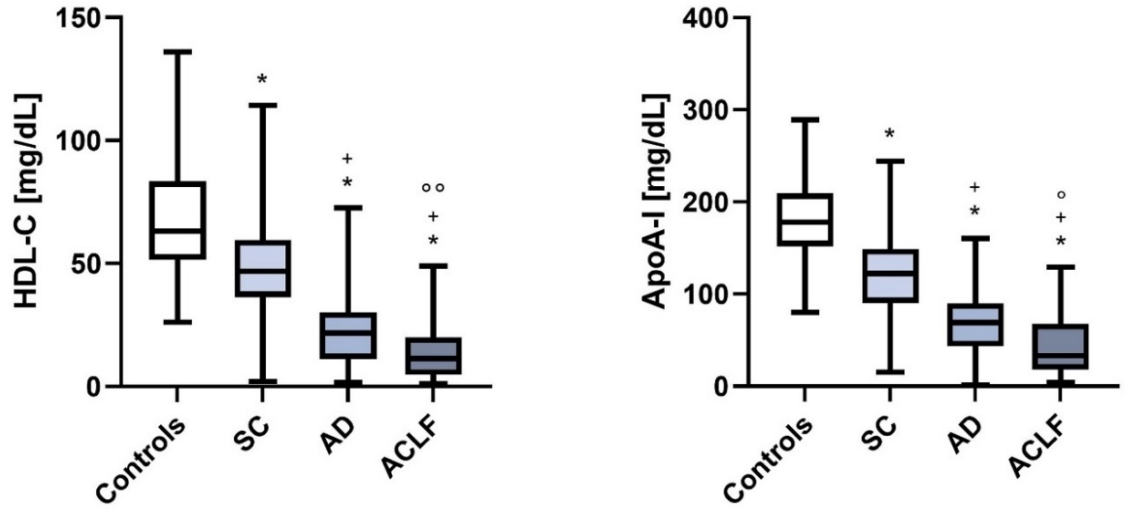

Figure 27. HDL-related biomarkers and their associations with liver disease severity. HDL-C (A) and ApoA-I (B) blood levels in healthy controls (n=40) and patients with SC (n=228), AD (n=173) and ACLF (n=107). HDL-C, high-density lipoprotein cholesterol; ApoA-I, apolipoprotein A1; SC, stable cirrhosis; AD, acute decompensation; ACLF, acute-on-chronic liver failure. * $p<0.001$, ** $p<0.05$ vs. control; $^{+}$ $p<0.001$ vs. SC; ° $p<0.001$, °° $p<0.05$ vs. AD. Adapted from (102).

3.3. Markers of bacterial translocation – interrelations and influences

3.3.1. The impact of PPI intake on gut microbial composition

In our cohort with available stool samples, 48 of 87 cirrhosis patients (55%) used PPIs; 23 of those patients (48% of PPI users) had documented indications for PPI use [gastritis or duodenitis (n=13), gastroesophageal reflux disease (n=7), use of non-steroidal anti-inflammatory drugs (NSAIDs, n=2), ulcers (n=1)]. All of the 48 patients had taken PPIs for more than 1 month, 75% of them for more than one year. The percentage of PPI users did not differ significantly between patients with stable (i.e., Child-Pugh class A) and advanced (i.e., Child-Pugh classes B and C) liver

disease, whereas PPI use without clear indication was more frequent in advanced liver disease (39% versus 80% of PPI-users, for stable and advanced liver disease, respectively; p=0.013).

	Controls	*Cirrhosis*	
		No PPI	PPI
N	19	39	48
Age [years]	60 (53; 64)	56 (51; 61)	59 (52; 64)
Gender (% male)	47%	62%	79%
Etiology of cirrhosis			
Alcohol	n/a	20 (51%)	25 (52%)
HCV	n/a	9 (23%)	10 (21%)
Other	n/a	10 (26%)	13 (27%)
Child-Pugh class			
A	n/a	34 (87%)	33 (69%)
B and C	n/a	5 (13%)	15 (31%)
MELD Score	n/a	9 (7; 13)	11 (9; 15)

Table 9. Baseline characteristics of controls and cirrhotic patients according to PPI use. HCV, hepatitis C virus; PPI, proton pump inhibitor; Data are shown as median (Q1, Q3). n/a, not applicable.

In patients with liver disease, intake of PPI showed no effect on microbial diversity; however, significant changes were observed in terms of microbiome composition (p=0.001, see Figure 28).

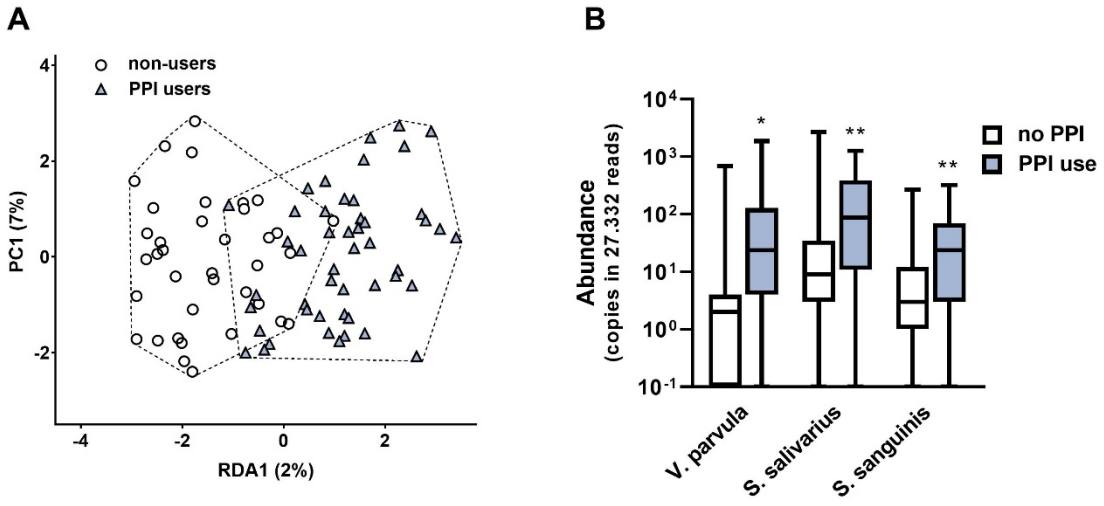

Figure 28. Alterations in the stool microbiome of cirrhosis patients in terms of PPI use / non-use. A: RDA-analysis based on Bray-Curtis dissimilarity matrix. B: Abundances (i.e., copies in 27.332 sequencing reads) of *Veillonella parvula*, *Streptococcis salivarius* and *Streptococcus sanguinis* in patients with liver cirrhosis according to PPI use / non-use. * p<0.001, ** p<0.05 vs. no PPI use. Adapted from (95).

In particular, significantly higher abundances of three species usually located in the oral cavity (i.e., *Veillonella parvula, Streptococcus salivarius* and *Streptococcus parasanguinis*) and lower abundances of *Subdoligranulum variabile* & a *Lachnospiraceae* species were found in stool of patients with PPI compared to patients without PPI intake. Of these 5 species, *V. parvula* abundance showed the best prediction for PPI use or non-use using receiver operating characteristics (AUROC=0.766; $p<0.001$), as listed in Table 10.

Discriminator	*AUROC*	*95% CI*	*p-value*
Veillonella parvula	0.733	0.66-0.87	**<0.001**
Streptococcus salivarius	0.728	0.62-0.84	**<0.001**
Streptococcus parasanguinis	0.711	0.60-0.82	**0.001**
Lachnospiraceae sp.	0.704	0.59-0.82	**0.001**
Subdoligranulum variabile	0.656	0.54-0.77	**0.013**

Table 10. Discriminatory power of differentially abundant OTUs to distinguish between PPI-user and non-user ranked according to AUROC. CI, confidence interval.

3.3.2. The impact of PPI intake & gut dysbiosis on markers of bacterial translocation

Besides impact on gut microbial composition, PPI intake also affected different parameters of bacterial translocation: Stool calprotectin levels were significantly elevated in patients with PPI intake (no PPI intake: 32µg/mg, PPI intake: 231µg/mg, $p<0.001$), same as stool zonulin levels as a marker of intestinal permeability (no PPI intake: 71ng/mL, PPI intake: 84ng/mL, $p=0.035$). Levels of macrophage activation marker sMR was significantly elevated in patients taking PPI (no PPI intake: 0.37µg/dL, PPI intake: 0.46 µg/dL, $p=0.029$). Furthermore, lipid metabolism parameters HDL-C & apoA-I were significantly lower in patients using PPI compared to non-users (HDL-C: no PPI intake: 51 mg/dL, PPI intake: 44 mg/dL, $p=0.026$; apoA-I: no PPI intake 131 mg/dL, PPI intake: 111 mg/dL, $p=0.026$).

However, there were no significant differences between patients with or without PPI use in the lactulose/mannitol ratio (L/M ratio), serum LPS levels, sCD163, and markers of systemic inflammation (see Table 11).

	PPI use	*No PPI use*	*p-value*
Fecal calprotectin [µg/mg]	231 (115; 348)	32 (0; 69)	**<0.001**
Fecal zonulin [ng/mL]	84 (60; 108)	71 (58; 85)	**0.035**
Lactulose/mannitol ratio [log_{10}]	-1.27 (-1.7; -0.9)	-1.37 (-1.6; -1.2)	0.221
LPS [EU/mL]	3.1 (0; 11)	2.4 (0; 7)	0.742
sCD163 [mg/L]	6.0 (3.5; 8.9)	4.5 (3.3; 6.9)	0.138
sMR [µg/dL]	0.46 (0.3; 0.6)	0.37 (0.3; 0.5)	**0.029**
CRP [mg/L]	3.2 (1; 9)	2.9 (1; 8)	0.294
IL-6 [pg/mL]	9.0 (0; 26)	3.7 (0; 15)	0.809
IL-8 [pg/mL]	53 (22; 174)	30 (14; 113)	0.084
TNF-α [pg/mL]	1.9 (0; 5)	1.3 (0; 4)	0.946
HDL-C [mg/dL]	44 (34; 56)	51 (39; 64)	**0.026**
ApoA-I [mg/dL]	111 (81; 146)	131 (102; 150)	**0.026**

Table 11. Associations between PPI use and different markers of bacterial translocation. Data are shown as median (Q1, Q3). LPS, lipopolysaccharide; ApoA-I, apolipoprotein A1; HDL-C, high-density lipoprotein cholesterol.

Since *V. parvula* abundance showed the best prediction for PPI use / non-use (see Table 10), we assessed markers of bacterial translocation in patients with high and low *V. parvula* count. The cut-off between low and high abundance (3.5 copies of *V. parvula* in 16703 sequences) was calculated by Youden index based on the intake of PPI and corresponded to the 45th percentile. We found that high abundance of *V. parvula* was associated with increased calprotectin levels in stool (p<0.001), an increased lactulose-mannitol ratio (p=0.029), and significantly increased zonulin levels in stool (p=0.047). High abundance of *V. parvula* was also associated with increased levels of CRP (p=0.044) and hepatic macrophage activation markers sCD163 (p=0.004) and sMR (p=0.013). Furthermore, lipid metabolism parameters HDL-C and ApoA-I were significantly lower in patients with *V. parvula* dysbiosis (p=0.045 and 0.008, respectively).

There were no significant differences in levels of LPS, IL-6, IL-8 and TNF-α (p=0.374, 0.651, 0.097 and 0.051, respectively) in patients with/without *V. parvula* dysbiosis.

An overview of associations between different markers of bacterial translocation is given in Table 12.

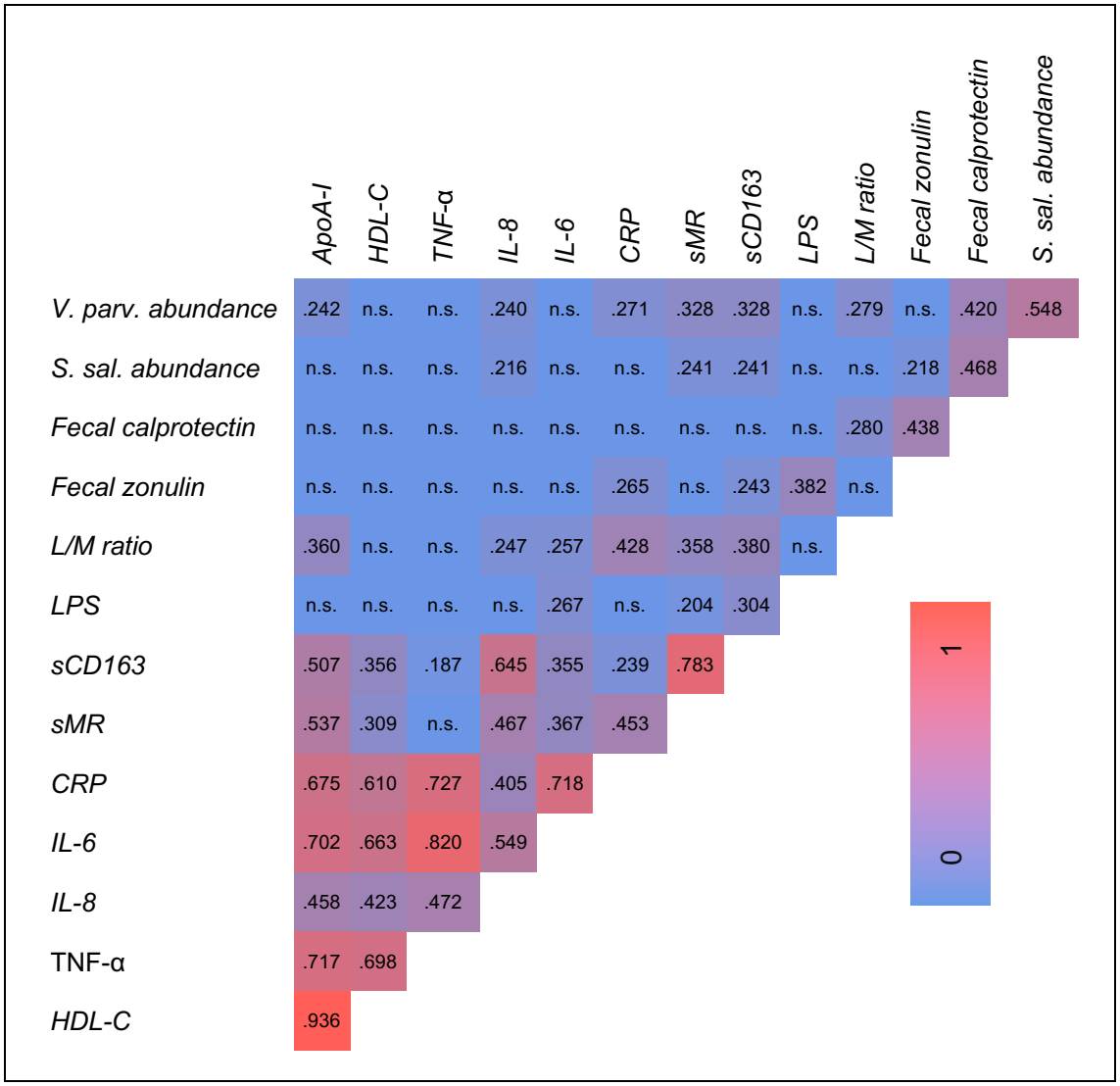

Table 12. Associations between different markers of bacterial translocations, evaluated with Spearman correlation. Given values are Spearman's correlation coefficients r_s. *V. parv.* abundance, *Veillonella parvula* abundance; *S. sal.* abundance, *Streptococcus salivarius* abundance; L/M ratio, lactulose/mannitol ratio; LPS, lipopolysaccharide; sMR, soluble mannose receptor, IL, interleukin; TNF, tumor necrosis factor; HDL-C, high density lipoprotein cholesterol; apo, apolipoprotein.

3.3.3. Structural alterations and associations with other markers of BT

In our cohort with available distal duodenal biopsies, some structural alterations were more frequently seen in patients taking PPI: A decreased villus/crypt ratio and an increased incidence of distended intercellular spaces were associated with PPI use (see Table 13). A decreased V/C ratio was further associated with a higher

lactulose/mannitol ratio and higher CRP levels. However, there was no association between V/C ratio and HDL levels (see Table 13), but HDL values significantly correlated with MV height (r_s=0.629, p=0.012), and MV height showed a trend towards an inverse association with L/M ratio (r_s=-0.412, *p*=0.090).

	PPI use *n = 12*	*No PPI use* *n = 7*	*p-value*
Villus/crypt ratio n (%)	7 (58%)	1 (14%)	**0.043**
Distension of intercellular spaces [% of villi]	65 (48; 79)	39 (30; 49)	**0.009**
Denudation of villi [% of villi]	38.5 (22; 59)	29.3 (14; 38)	0.236
TJ gap width [nm]	11.4 (10.2; 12.0)	10.8 (10.4; 11.8)	0.592

	V/C ratio decreased *n = 8*	*V/C ratio normal* *n = 11*	*p-value*
Lactulose/mannitol ratio [ratio]	0.022 (0.0; 0.1)	0.008 (0.0; 0.0)	**0.032**
CRP [mg/L]	4.8 (3; 14)	2.7 (1; 3)	**0.020**
HDL-C [mg/dL]	46 (19; 61)	49 (39; 58)	0.740
MV height [nm]	1335 (1056; 1630)	1460 (1240; 1645)	0.696

Table 13. Structural alterations and associations to other markers of bacterial translocation. Data are shown as count (%) or median (Q1, Q3), as appropriate. TJ, tight junction; CRP, C-reactive protein; MV, microvilli.

3.4. Prediction of cirrhosis-associated complications

In patients with SC at baseline (n=256), the prognostic utility of BT-related biomarkers regarding the occurrence of cirrhosis-associated complications within 12 months was investigated. Due to missing data or insufficient follow-up time, 31 patients had to be excluded from analysis (5 patients withdrew consent, 4 patients were transplanted within 1 year without prior development of cirrhosis complications, 22 patients had insufficient data in terms of complications). Overall, cirrhosis-associated complications occurred in 84 of 225 patients (37%); typically patients who developed complications experienced more than just a single complication (see Table 14).

Complication	*Count (%)*
All complications	84 (37%)
Ascites	45 (20%)
Hepatic encephalopathy	33 (15%)
SBP	6 (3%)
AKI-HRS	6 (3%)
Jaundice	17 (8%)
Bleeding	13 (6%)
PVT	3 (1%)
Death	23 (10%)

Table 14. Occurrence of cirrhosis-associated complications in patients with stable cirrhosis (n=225). SBP, spontaneous bacterial peritonitis; AKI, akute kidney injury; HRS, hepatorenal syndrome; PVT, portal vein thrombosis.

Furthermore, bacterial infection requiring antibiosis occurred in 55 patients (24%) within 12 months. The most frequent infections were respiratory tract infections (n=11, 20%), followed by urinary tract infections (including one patient with renal abscess, n=9, 16%), spontaneous bacterial peritonitis (n=6, 11%) and skin/wound infections (n=6, 11%). In each of 3 patients (5%), bacteremia caused by Staphylococcus aureus or invasive gastroenteritis (including one patient with *Clostridium difficile* enteritis) was the reason for the initiation of antibiosis, respectively. An abscess of the parotid gland, listeriosis, dental infection, sinusitis, cholangitis, cholecystitis and knee prosthesis infection were observed in 1 patient each. In 10 cases (18%), the cause of infection could not be clearly determined and antibiosis was started on the basis of symptoms and/or laboratory alterations.

3.4.1. The predictive value of PPI intake and V. parvula dysbiosis in terms of complication/infection risk

We tested whether PPI intake or *V. parvula* dysbiosis was associated with occurrence of cirrhosis-associated complications and/or infections. Univariate logistic regression revealed that patients with PPI use were more likely to develop complications within 12 months compared to non-users (OR=7.1; p=0.004). The most

frequent complication in PPI users was development of ascites (OR=17.3; *p*=0.008). Furthermore, we found that high abundance of *V. parvula* (cut-off = 125 copies of *V. parvula* out of 16,703 sequences calculated by Youden index for the occurrence of complications) predicted development of complications within one year (OR=6.9; *p*=0.002), in particular ascites (OR=6.3; *p*=0.008) and hepatic encephalopathy (OR=9.5; *p*=0.005). In addition, patients with a high *V. parvula* count had an increased infection risk (OR=3.0, *p*=0.004). There was only a trend between PPI intake and prediction of infection within 12 months (OR=1.8, p=0.066).

The predictive value of PPI intake and *V. parvula* abundance according to disease severity is shown in Table 15.

		Cirrhosis-associated complications		Infections	
		OR	*p-value*	*OR*	*p-value*
Child's A	PPI use	6.9	0.082	1.5	0.353
	V. parvula dysbiosis	1.4	0.778	11.0	**0.030**
Child's B + C	PPI use	2.8	**0.048**	2.4	0.063
	V. parvula dysbiosis	1.6	0.066	0.6	0.287

Table 15. Prediction of cirrhosis-associated complications and infections according to disease severity.

3.4.2. The predictive value of macrophage activation parameters in terms of complication/infection risk

Levels of sCD163 and sMR were significantly higher in patients who subsequently developed cirrhosis-associated complications than in those who did not (see Figure 29).

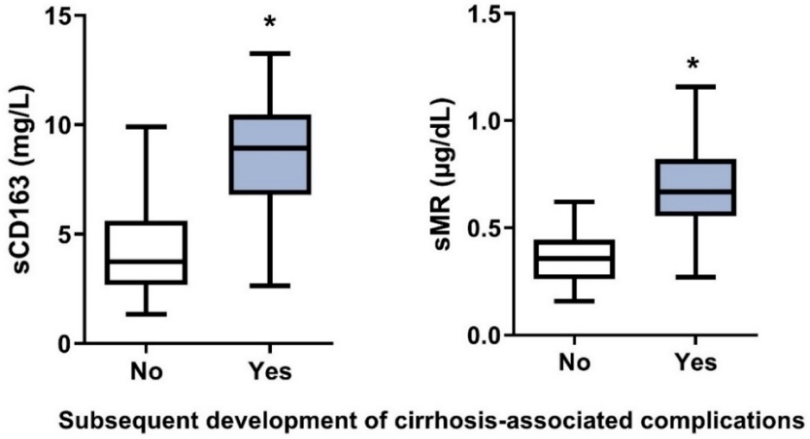

Figure 29. Levels of sCD163 and sMR in liver disease patients with and without subsequent complications. * $p<0.001$ vs. no complication. Adapted from (101).

Separate analyses of individual complications showed similar results: levels of macrophage activation parameters were significantly higher in patients who subsequently suffered from ascites, HE or jaundice (see Table 16). However, there were no differences observed in patients who developed infections requiring antibiotic therapy and those who remained free from relevant infections within 12 months.

		sCD163 *(mg/L)*	*p*-value	sMR *(µg/dL)*	*p*-value
All cirrhosis-associated complications	yes	8.92 (6.8; 10.5)	**<0.001**	66.8 (55; 82)	**<0.001**
	no	3.8 (2.7; 5.6)		35.7 (26; 45)	
Ascites	yes	8.41 (5.8; 10.0)	**<0.001**	67.3 (52; 81)	**<0.001**
	no	3.88 (2.7; 6.2)		35.7 (27; 46)	
Hepatic encephalopathy	yes	10.23 (8.0; 11.9)	**<0.001**	78.9 (57; 92)	**<0.001**
	no	4.29 (2.7; 6.7)		37.7 (27; 50)	
Jaundice	yes	9.46 (7.1; 10.5)	**0.002**	61.2 (50; 70)	**0.005**
	no	3.97 (2.7; 6.4)		36.2 (27; 46)	
Infection	yes	4.73 (3.6; 6.9)	0.665	40.6 (26; 49)	0.207
	no	5.61 (3.2; 8.3)		44.7 (32; 61)	

Table 16. Markers of macrophage activation in cirrhosis patients with subsequent development of complications/infection. Data are shown as median (Q1, Q3).

Univariate logistic regression analysis revealed both macrophage activation markers sCD163 and sMR together with Child-Pugh score, MELD, etiology and CRP, but not gender, age, fecal calprotectin, zonulin, L/M ratio, LPS, CRP, IL-6, IL-8 or TNF-α as predictors of 1-year complication rate.

Variable	OR (95% CI)	*p*-value	R^2
sCD163	1.67 (1.4-2.1)	**<0.001**	0.45
sMR	1.12 (1.1-1.2)	**<0.001**	0.56
Child-Pugh score	3.47 (2.1-5.8)	**<0.001**	0.48
MELD	1.35 (1.2-1.6)	**<0.001**	0.30
Etiology			0.16
alcohol	3.51 (1.0-13.4)	**0.047**	
hepatitis C	8.61 (1.9-40.0)	**0.006**	
CRP	1.14 (1.0-1.3)	**0.009**	0.12
Age	1.00 (0.9-1.0)	0.910	0.00
Gender	1.59 (0.6-4.5)	0.381	0.01
Fecal calprotectin	1.00 (1.0-1.0)	0.090	0.06
Fecal zonulin	1.01 (1.0-1.03)	0.144	0.04
Lactulose/mannitol ratio	2.17 (0.1-34.0)	0.583	0.01
LPS	1.00 (0.9-1.0)	0.869	0.00
IL-6	1.02 (1.0-1-0)	0.166	0.04
IL-8	1.00 (1.0-1.0)	0.358	0.00
TNF-α	0.85 (0.6-1.2)	0.358	0.04

Table 17. Univariate logistic regression analysis of clinical and biochemical factors associated with occurrence of complications; sMR, soluble mannose receptor; CRP, C-reactive protein; LPS, lipopolysaccharide; IL, interleukin: TNF, tumor necrosis factor.

The most favorable Nagelkerke's R square (as goodness-of-fit measure) was obtained using sMR. AUROC for diagnostic accuracy of sMR for 1-year complication rate was significantly higher compared to AUROC of MELD (p=0.034). The prognostic benefit of the Child-Pugh score was comparable to sCD163 and sMR (AUROC [95% CI] sCD163: 0.87 [0.78-0.93], sMR: 0.89 [0.81-0.95], CPS 0.83 [0.74-0.90], MELD 0.78 [0.68-0.86]). AUROCs for 1-year complication rates are depicted in Figure 30.

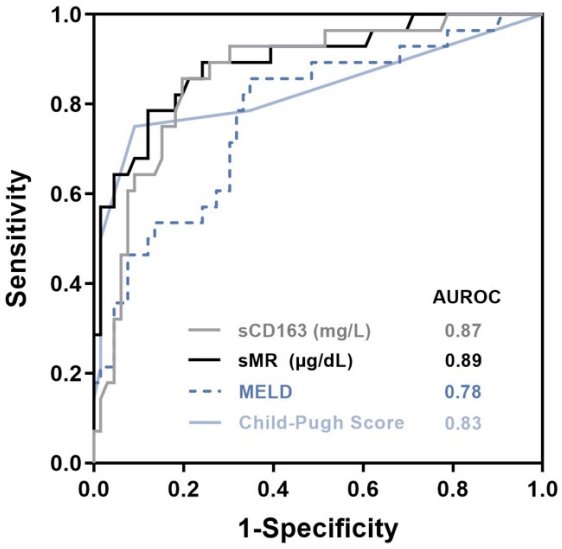

Figure 30. ROC curves illustrating the prognostic value of sCD163, sMR, MELD and Child-Pugh score as predictors of 1-year complication rate. Adapted from (101).

3.4.3. The predictive value of lipid metabolism parameters in terms of complication/infection risk

Serum levels of HDL-C and apoA-I were significantly decreased in patients with subsequent development of cirrhosis-associated complications and infections requiring antibiotic therapy (see Figure 31).

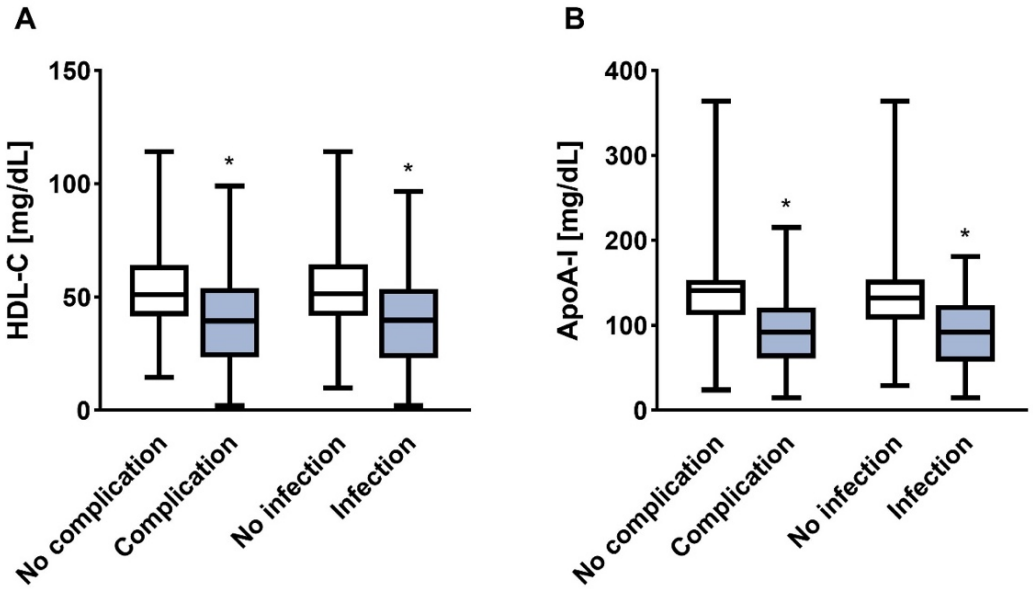

Figure 31. Levels of lipid metabolism parameters in cirrhosis patients with and without subsequent complications or infections. * p<0.001 vs. no complication / no infection.

Consistently, these parameters were associated with development of complications / infections in univariate logistic regression analysis (HDL-C for prediction of complications: OR=0.96, $p<0.001$; HDL-C for prediction of infection: OR=0.92, $p<0.002$; apoA-I for prediction of complications: OR=0.97, $p<0.001$; apoA-I for prediction of infection: OR=0.96, $p<0.001$).

For multivariate analysis, only 1 lipid-related biomarker and MELD score were included in order to avoid multicollinearity. Multivariable logistic regression revealed both HDL-C and apoA-I as independent predictors of cirrhosis-associated complications (see Table 18).

	OR (95% CI)	*p*-value	R^2
Univariate			
HDL-C	0.96 (0.9-1.0)	**<0.001**	0.16
ApoA-I	0.97 (0.9-1.0)	**<0.001**	0.32
MELD	1.30 (1.2-1.4)	**<0.001**	0.33
Add to MELD			
HDL-C	0.98 (0.9-1.0)	0.061	
MELD	1.26 (1.2-1.4)	**<0.001**	
Combined			0.35
ApoA-I	0.98 (1.0-1.0)	**0.001**	
MELD	1.19 (1.1-1.3)	**<0.001**	
Combined			0.39

Table 18. Independent factors to predict development of complications in stable cirrhosis. 12-month complication risk.

On ROC analysis, both HDL-C and apoA-I yielded high diagnostic accuracy for development of cirrhosis-associated complications and infections within 12 months (see Figure 32).

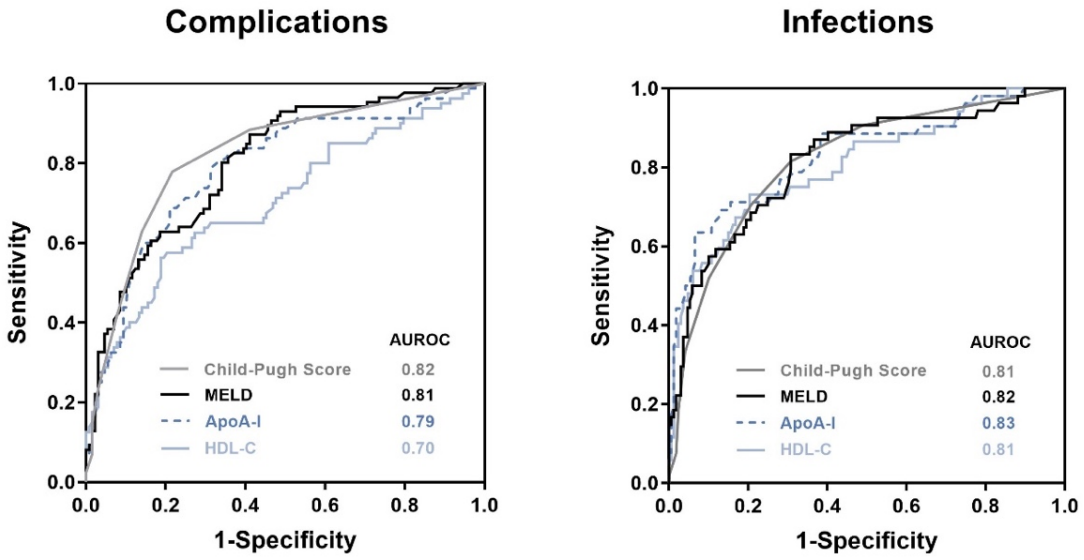

Figure 32. HDL-related biomarkers predict complications and infection. ROC curves illustrating the prognostic value of HDL-C, apoA-I, MELD and Child-Pugh score in terms of complication and infection risk (within 12 months). ApoA-I, apolipoprotein A1; HDL-C, high-density lipoprotein cholesterol; MELD, model for end-stage liver disease.

Using the Youden index, optimal cut-offs for prediction of 12-month infection risk could be calculated (<24.5 mg/dL for HDL-C and <72 mg/dL for apoA-I). On Kaplan-Meyer analysis, baseline HDL-C and apoA-I values below these cut-offs were associated with a markedly increased risk of subsequent infection, comparable to a MELD score of ≥22 points.

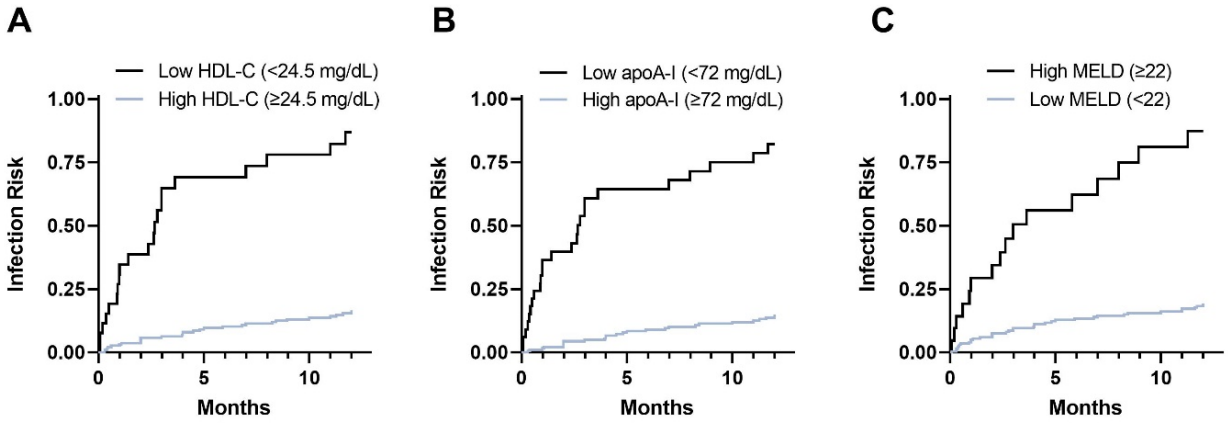

Figure 33. Kaplan-Meier plots depicting infection risk in patients with stable cirrhosis. Patients with (A) HDL-C levels <24.5 mg/dl (p<0.001 by log-rank test), (B) apoA-I levels <72 mg/dl (p<0.001 by log-rank test) or (C) a MELD score ≥22 (p<0.001 by log-rank test) experience significantly higher infection risks. ApoA-I, apolipoprotein A1; HDL-C, high-density lipoprotein cholesterol; MELD, MELD, model for end-stage liver disease.

3.5. Mortality risk after occurrence of complications

In stable cirrhosis patients who developed cirrhosis-associated complications within 12 months, mortality risk was significantly increased compared to patients who remained stable: Of 208 patients with an available follow-up time of 36 months, 79 patients (38%) developed complication(s) within 12 months, of whom 29 patients (37%) died. In contrast, 11% of stable patients (who remained stable, 14 out of 129 patients) died within 36 months (HR for patients who developed complications: 4.14, $p<0.001$). Furthermore, mortality risk was also increased in patients who developed an infection requiring antibiotic therapy (including 6 patients with SBP): 45 patients (22%) needed antibiosis due to bacterial infection, 16 of whom (36%) died within 36 months. Of 163 patients without subsequent infection, 19 patients (12%) died (HR for patients who develop infection: 3.90, $p<0.001$).

Complication	*HR (95% CI)*	*p-value*
All cirrhosis-associated complications	4.1 (2.2-7.9)	**<0.001**
Ascites	1.32 (0.8-2.3)	0.324
Hepatic encephalopathy	1.86 (1.1-3.4)	**0.043**
Bleeding	3.85 (1.7-8.9)	**0.002**
Infection	3.90 (2.5-6.2)	**<0.001**

Table 19. 3-year mortality risk after development of cirrhosis-associated complications and/or infection. Cox regression analysis.

Cox regression analysis including infections and the most frequent complications (i.e., ascites, HE, bleeding) revealed occurrence of infection the 2nd most hazardous complication after bleeding in patients with liver cirrhosis (see Table 19 & Figure 34).

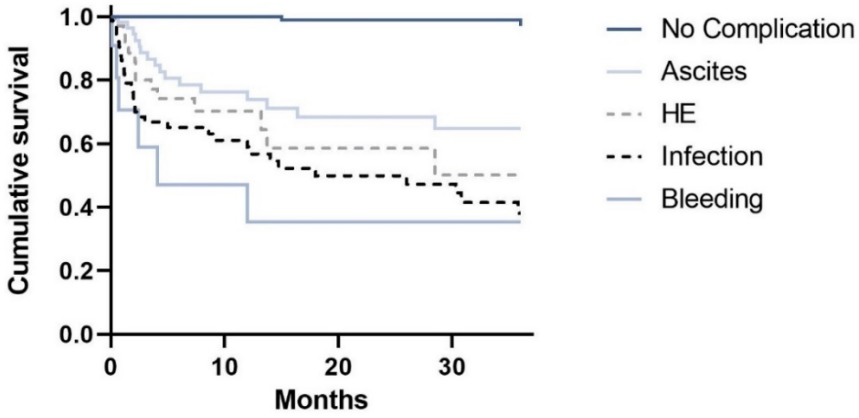

Figure 34. Kaplan-Meier plots of 3-year survival probability in patients with development of complications or infection. $p<0.001$ by log-rank test; HE, hepatic encephalopathy.

3.6. Survival prediction

Of 256 stable cirrhosis patients in the Austrian cohort, 51 patients (20%) died and 38 patients (15%) were transplanted within a median observation period of 24 months (range 0 - 71 months). 44% of patients in the European multicenter cohort (112 of 280 patients) died within 12 months and 40 patients (14%) were transplanted. Mortality and liver transplantation rates in different cohorts are given in Table 20.

	Stable cirrhosis	*Acute Decompensation*	
		No ACLF	*ACLF*
N	*256*	*173*	*107*
36-month mortality	14%	n/a	n/a
12-month mortality	7%	27%	56%
90-day mortality	4%	15%	42%
36-month OLT rate	15%	n/a	n/a
12-month OLT rate	9%	13%	17%
90-day OLT rate	3%	6%	10%

Table 20. Mortality and liver transplantation rates in different cohorts. OLT, orthotopic liver transplantation.

3.6.1. The predictive value of PPI intake and V. parvula dysbiosis in terms of survival

The predictive value of PPI intake and *V. parvula* dysbiosis was investigated in patients with stable cirrhosis with available stool samples (n=87). We found that 3-year mortality risk of patients with PPI use was elevated 5.1 times in comparison to non-users (HR 5.1, 95% CI 1.2-23.0, *p*=0.032) and remained significantly elevated after stratification for Child-Pugh score (HR 4.5, 95% CI 1.0-21.6, *p*=0.048). Furthermore, patients with *V. parvula* dysbiosis (cut-off = 90 copies of *V. parvula* out of 16,703 sequences calculated by Youden index for mortality risk) had a 4.3 times higher mortality risk (HR 4.3, 95% CI 1.5-12.5, *p*=0.008) compared to patients with a low *Veillonella* count. Interestingly, survival in patients with PPI intake without *V. parvula* dysbiosis (n=32 of 48 PPI users, 67%) was comparable to patients without PPI intake, only in patients with both PPI use and *V. parvula* dysbiosis (n=16 of 48 PPI users, 33%) mortality risk was significantly increased (see Figure 35)

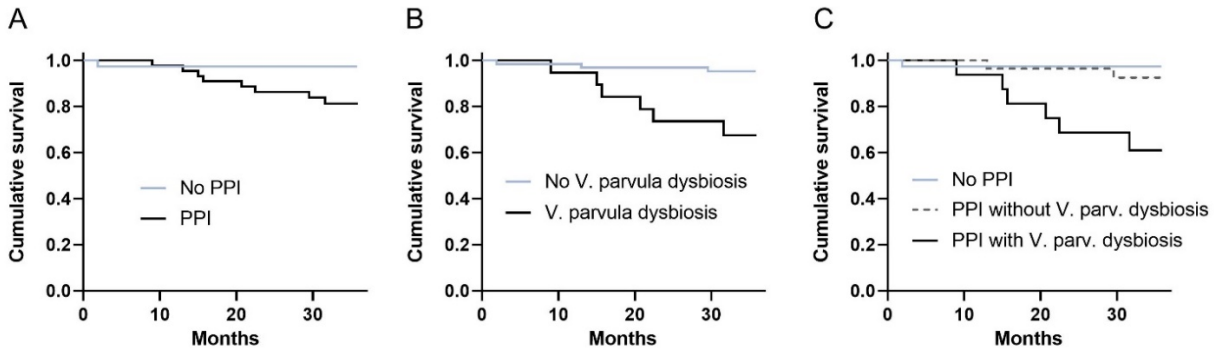

Figure 35. Kaplan-Meier curve for 4-year survival according to PPI intake and V. parvula dysbiosis. A: Mortality risk was significantly increased in (A) patients with PPI use (*p*=0.028 by log-rank test), (B) patients with *V. parvula* dysbiosis (cut-off = 90 copies of 16,703 sequences, *p*<0.001 by log-rank test) and (C) patients with PPI use & concomitant *V. parvula* dysbiosis, but not in patients with PPI use without *V. parvula* dysbiosis (*p*<0.001 by log-rank test comparing patients with PPI intake and *V. parvula* dysbiosis vs. patients without PPI use). Adapted from (95).

Additionally, CP and MELD scores were predictors of 3-year mortality in patients with liver disease on univariate analysis (see Table 21). *V. parvula* dysbiosis, but not PPI intake, remained an independent predictive variable when added to MELD or Child-Pugh score:

	HR (95% CI)	*p*-value
Univariate		
PPI use	5.1 (1.2-23.0)	**0.032**
V. parvula dysbiosis	4.3 (1.5-12.5)	**0.008**
MELD	1.2 (1.1-1.4)	**0.007**
Child-Pugh score	1.9 (1.4-2.7)	**<0.001**
Add to MELD		
PPI use	5.1 (0.6-43.3)	0.131
MELD	1.2 (1.0-1.3)	**0.039**
V. parvula dysbiosis	4.9 (1.1-23.2)	**0.042**
MELD	1.1 (0.9-1.3)	0.171
Add to Child-Pugh score		
PPI use	4.2 (0.5-36.7)	0.191
Child-Pugh score	1.7 (1.2-2.5)	**0.003**
V. parvula dysbiosis	3.7 (1.0-17.2)	**0.047**
Child-Pugh score	1.6 (1.1-2.4)	**0.012**

Table 21. Predictive value of PPI use and V. parvula dysbiosis in terms of 3-year mortality according to multivariate Cox regression analysis; HR, hazard ratio; CI, confidence interval.

3.6.2. Predictive value of markers of intestinal inflammation, permeability, bacterial translocation and immune activation

Univariate Cox-regression analysis revealed CP & MELD scores, L/M ratio, macrophage activation markers sCD163 & sMR, CRP and IL-6, but not age, gender, etiology, fecal calprotectin & zonulin levels, serum LPS, IL-8 and TNF-α as predictors of 3-year mortality:

Markers	HR (95% CI)	*p*-value
Patient-specific		
age	1.02 (1.0-1.1)	0.370
gender (male)	2.77 (0.8-9.4)	0.102
Cirrhosis-associated		
etiology (alcohol)	1.04 (0.4-2.9)	0.936
etiology (HCV)	0.91 (0.3-3.3)	0.889
Child-Pugh Score	1.60 (1.3-2.1)	**<0.001**
MELD	1.16 (1.1-1.3)	**0.003**
Intestinal inflammation		
fecal calprotectin	1.00 (1.0-1.0)	0.813
Intestinal permeability		
fecal zonulin	0.99 (0.9-1.0)	0.499
lactulose/mannitol ratio	4.68 (1.2-18.4)	**0.027**
Bacterial translocation		
serum LPS	1.02 (1.0-1.1)	0.475
Macrophage activation		
sCD163	1.32 (1.1-1.5)	**<0.001**
sMR	1.02 (1.0-1.1)	**0.004**
Systemic immune activation		
CRP	1.14 (1.1-1.2)	**0.003**
interleukin-6	1.00 (0.9-1.0)	0.570
interleukin-8	1.00 (1.0-1.0)	**0.031**
TNF-α	0.71 (0.2-2.1)	0.534

Table 22. Prognostic factors regarding 3-year survival in stable cirrhosis patients. Univariate Cox proportional hazards analysis of clinical and biochemical factors with assumed prognostic relevance. HR, hazard ratio; CI, confidence interval; HCV, hepatitis C virus; LPS, lipopolysaccharide; sMR, soluble mannose receptor, TNF, tumor necrosis factor.

Survival curves for macrophage activation markers, CRP & IL-8 are depicted in Figure 36. Optimal cut-offs for survival prediction were calculated using the Youden index (5.9 mg/L for sCD163, 45.5 µg/dL for sMR, 2.85 mg/L for CRP and 42.34 pg/mL for IL-8).

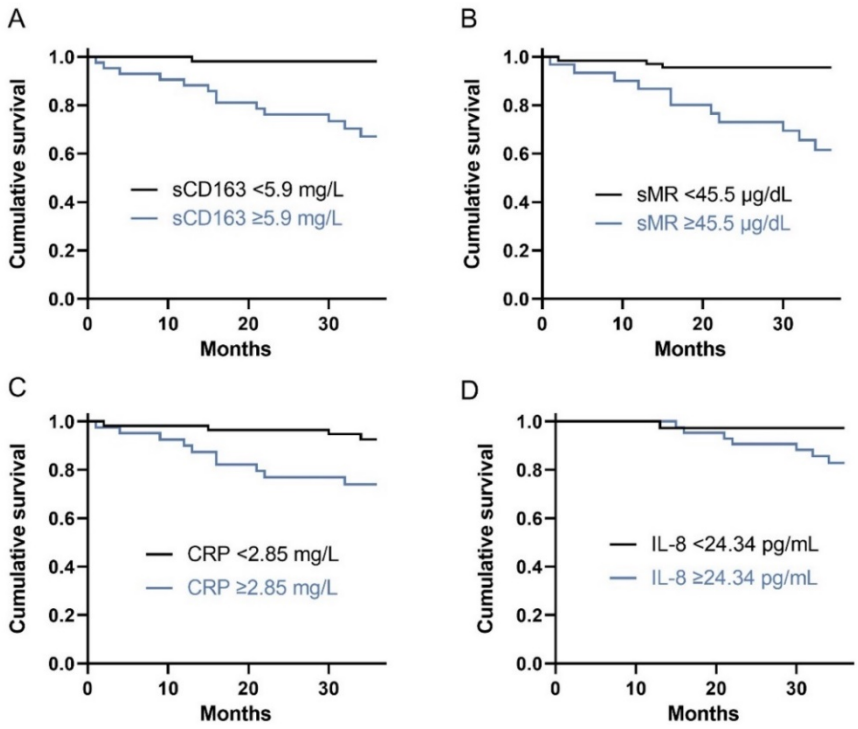

Figure 36. Kaplan-Meier curves for 3-year survival according to macrophage activation parameters, CRP & IL-8. A: sCD163, *p*<0.001 by log-rank test; B: sMR, *p*<0.001 by log-rank test; C: CRP, *p*=0.009 by log-rank test; D: IL-8, p=0.054. Adapted from (101).

On ROC analysis, sCD163 and sMR showed high diagnostic accuracy and were comparable to composite scores Child-Pugh and MELD in terms of 3-year survival [AUROC (95% CI), sCD163: 0.75 (0.6-0.9), sMR: 0.72 (0.6-0.8), Child-Pugh score 0.70 (0.6-0.8), MELD 0.67 (0.5-0.8)]. No relevant differences were observed regarding the prognostic significance of the different parameters by using methodology of Hanley & McNeil (see Table 23).

Parameters	*Difference between areas (95% CI)*	*z statistic*	*p-value*
Child-Pugh score & MELD	0.035 (-0.07-0.14)	0.660	0.509
Child-Pugh score & sCD163	0.047 (-0.08-0.17)	0.740	0.460
Child-Pugh score & sMR	0.019 (-0.09-0.14)	0.315	0.753
MELD & sCD163	0.082 (-0.05-0.22)	1.172	0.241
MELD & sMR	0.054 (-0.08-0.19)	0.796	0.426
sCD163 & sMR	0.028 (-0.06-0.12)	0.623	0.533

Table 23. Pairwise comparison of ROC curves regarding survival prediction. AUROCs were compared using methodology of Hanley & McNeil.

Using multivariate Cox regression analysis with parameters significant on univariate analysis, macrophage activation parameters sCD163 & sMR (but not lactulose/mannitol ratio, CRP and IL-8) remained independent predictive parameters when added to MELD. When added to Child-Pugh score, only sCD163 remained significant (see Table 24).

	HR (95% CI)	*p*-value		*HR (95% CI)*	*p-value*
Add to MELD			***Add to Child-Pugh score***		
sCD163	1.40 (1.1-1.8)	**0.011**	sCD163	1.37 (1.1-1.8)	**0.013**
MELD	1.07 (0.9-1.2)	0.404	CP score	1.28 (0.9-1.9)	0.227
sMR	13.2 (1.1-164)	**0.045**	sMR	4.04 (0.3-64)	0.324
MELD	1.13 (0.9-1.3)	0.157	CP score	1.70 (1.1-2.7)	**0.021**
L/M ratio	25.8 (0-4389)	0.215	L/M ratio	6.82 (0-1404)	0.480
MELD	1.20 (1.0-1.4)	**0.037**	CP score	1.89 (1.2-2.9)	**0.003**
CRP	0.95 (0.8-1.1)	0.517	CRP	0.92 (0.8-1.1)	0.328
MELD	1.22 (1.1-1.4)	**0.004**	CP score	2.05 (1.5-2.9)	**<0.001**
IL-8	1.00 (1.0-1.0)	0.833	IL-8	1.00 (1.0-1.0)	0.547
MELD	1.22 (1.0-1.4)	0.013	CP score	2.03 (1.4-3.0)	**<0.001**

Table 24. Predictive value of macrophage activation parameters sCD163 & sMR, lactulose mannitol ratio, CRP and IL-8 in terms of 3-year mortality according to multivariate Cox regression analysis. HR, hazard ratio; CI, confidence interval, sMR, soluble mannose receptor; MELD, model for end-stage liver disease; IL, interleukin, CRP, C-reactive protein, L/M ratio, lactulose/mannitol ratio; CP score, Child-Pugh score.

3.6.3. The predictive value of HDL & apoA-I

The impact of HDL-C and apoA-I on mortality risk was evaluated in both patients with stable cirrhosis (n = 228 after exclusion of cholestatic liver diseases) and AD (n = 280) using Cox regression analysis. On univariate analysis, both HDL-related biomarkers showed high prognostic value for outcomes of interest, i.e., 90-day and 12-month mortality. In order to avoid multicollinearity, only one lipid-related biomarker (either HDL-C or ApoA-I) and one composite score (either MELD or Child-Pugh score) were included in multivariate analyses. Both HDL-C and apoA-I remained independent prognostic variables for 90-day and 12-month mortality on multivariable analysis when added to MELD or Child-Pugh score (see Table 25).

	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
	90-day mortality		**12-month mortality**	
Univariate				
HDL-C	0.90 (0.9-0.9)	**<0.001**	0.93 (0.9-0.9)	**<0.001**
ApoA-I	0.96 (0.9-1.0)	**<0.001**	0.97 (0.9-1.0)	**<0.001**
Add to MELD				
HDL-C	0.93 (0.9-1.0)	**<0.001**	0.95 (0.9-1.0)	**<0.001**
MELD	1.09 (1.0-1.1)	**<0.001**	1.09 (1.1-1.1)	**<0.001**
ApoA-I	0.98 (0.9-1.0)	**<0.001**	0.98 (1.0-1.0)	**<0.001**
MELD	1.09 (1.0-1.1)	**<0.001**	1.09 (1.1-1.1)	**<0.001**
Add to Child-Pugh score				
HDL-C	0.92 (0.9-1.0)	**<0.001**	0.95 (0.9-1.0)	**<0.001**
Child-Pugh score	1.29 (1.1-1.5)	**<0.001**	1.28 (1.1-1.4)	**<0.001**
ApoA-I	0.97 (0.9-1.0)	**<0.001**	0.98 (1.0-1.0)	**<0.001**
Child-Pugh score	1.27 (1.1-1.5)	**<0.001**	1.25 (1.1-1.4)	**<0.001**

Table 25. Independent predictors of 90-day and 12-month mortality in n=508 patients with liver cirrhosis. Cox regression analysis; CI, confidence interval; HR, hazard ratio; HDL-C, high-density lipoprotein cholesterol; ApoA-I, apolipoprotein A1; MELD, model for end-stage liver disease.

On ROC analysis, HDL-C as well as apoA-I showed high diagnostic accuracy in terms of 12-month mortality for patients with stable cirrhosis, and 90-day mortality for patients with acute decompensation, respectively (see Figure 37).

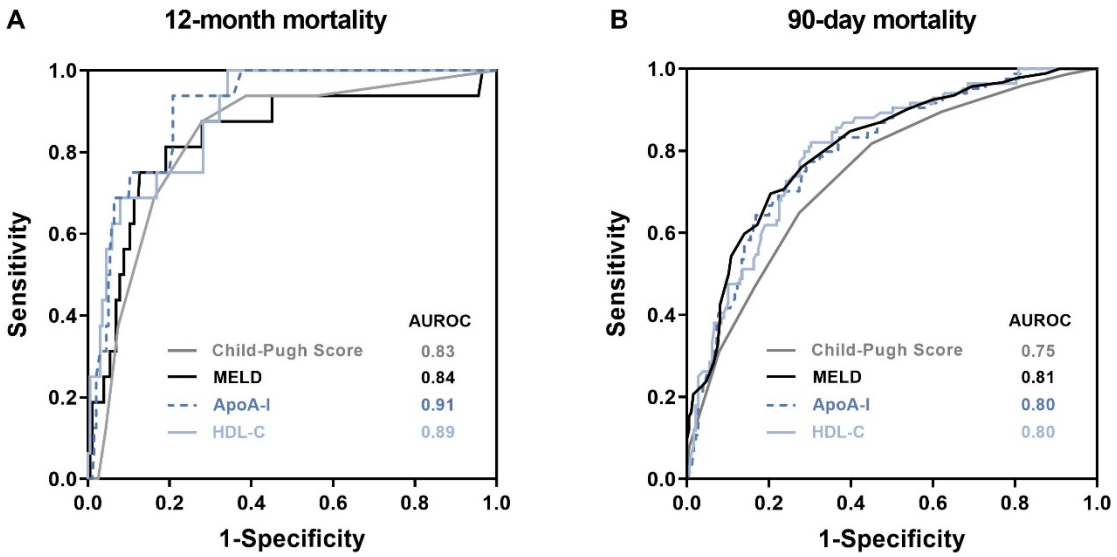

Figure 37. HDL-related parameters as predictors of mortality. ROC curves illustrating the prognostic value of HDL-C, apoA-I, MELD and Child-Pugh score in terms of 12-months mortality risk (A, stable cirrhosis patients) and 90-day mortality risk (B, patients with AD with/without ACLF). ApoA-I, apolipoprotein A1; HDL-C, high-density lipoprotein cholesterol; AD, acute decompensation; MELD, model for end-stage liver disease; ACLF, acute-on-chronic liver failure. Adapted from (102).

In the European cohort with acutely decompensated patients (n = 280), prognostic relevance of HDL-related biomarkers was further compared with the CLIF-C AD score (for patients with AD without ACLF) and with the CLIF-C ACLF score (for patients fulfilling ACLF-criteria). Again, both parameters HDL-C and apoA-I performed equally well compared to the respective composite scores. The highest diagnostic accuracies were found with HDL-C, apoA-I and MELD (see Table 26).

	AD + ACLF (n = 280)	AD (n = 173)	ACLF (n = 107)
	AUROC (95% CI)		
HDL-C	0.80 (0.7-0.9)	0.84 (0.8-0.9)	0.77 (0.7-0.9)
ApoA-I	0.80 (0.7-0.9)	0.84 (0.7-0.9)	0.75 (0.7-0.9)
MELD	0.81 (0.8-0.9)	0.82 (0.7-0.9)	0.74 (0.6-0.8)
Child-Pugh Score	0.75 (0.7-0.8)	0.77 (0.7-0.9)	0.67 (0.6-0.8)
CLIF-C AD Score		0.74 (0.6-0.8)	
CLIF-C ACLF Score			0.75 (0.7-0.9)

Table 26. Performance of HDL-related biomarkers and composite prognostic scores in terms of 90-day mortality. AD, acute decompensation; ACLF, acute-on-chronic liver failure; ApoA-I, apolipoprotein A1; CLIF-C, Chronic Liver Failure Consortium; HDL-C, high-density lipoprotein cholesterol; MELD, model for end-stage liver disease.

Optimal cut-offs for prediction of 90-day mortality were calculated using the Youden index and yielded <17 mg/dl for HDL-C and <50 mg/dl for apoA-I. Patients with baseline HDL-C or apoA-I values below these thresholds had a significantly reduced 90-day survival (Figure 38 A+B), comparable to that of patients with a baseline MELD of >22 points or a Child-Pugh Score > 9 (Figure 38 C+D).

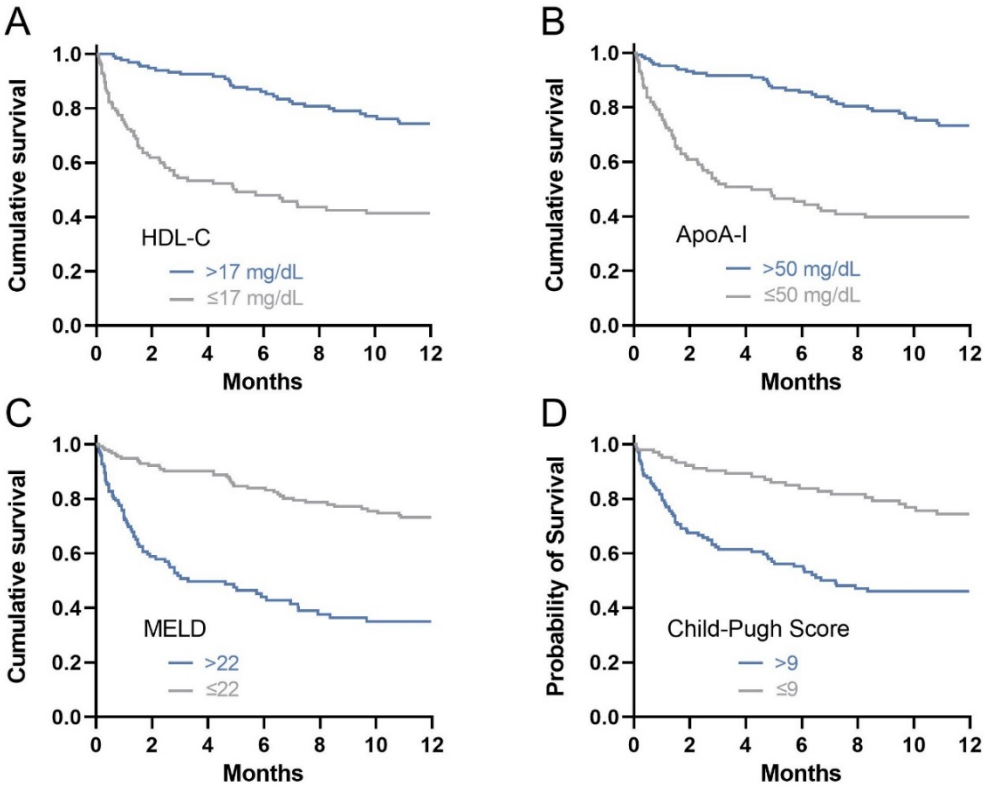

Figure 38. Survival plots of patients with acutely decompensated liver cirrhosis. Mortality was significantly increased in patients with (A) HDL-C <17 mg/dl (p <0.001 by log-rank test), (B) apoA-I <50 mg/dl (p <0.001 by log-rank test), (C) MELD >22 (p <0.001 by log-rank test) and (D) Child-Pugh score >9 (p<0.001 by log-rank test). ApoA-I, apolipoprotein A1; HDL-C, high-density lipoprotein cholesterol; MELD, model for end-stage liver disease. Adapted from (102).

4. Discussion

In patients with chronic liver disease, significant changes can be observed at all levels of the intestinal barrier, the ecological, mechanical and immune level. In our cirrhosis patients, we found distinctive alterations in stool microbial composition and intestinal epithelial structure, evidence of intestinal inflammation as well as increased intestinal permeability, bacterial translocation and systemic inflammation. Furthermore, levels of lipid metabolism markers HDL-C and apoA-I were significantly reduced. Many parameters demonstrated their value as predictors of cirrhosis-associated complications and mortality. We found significant associations between PPI use and changes in gut microbiome (in particular increased abundance of *V. parvula* and *S. salivarius*) as well as other aspects of the intestinal barrier, culminating in a higher complication and mortality risk in patients with PPI intake.

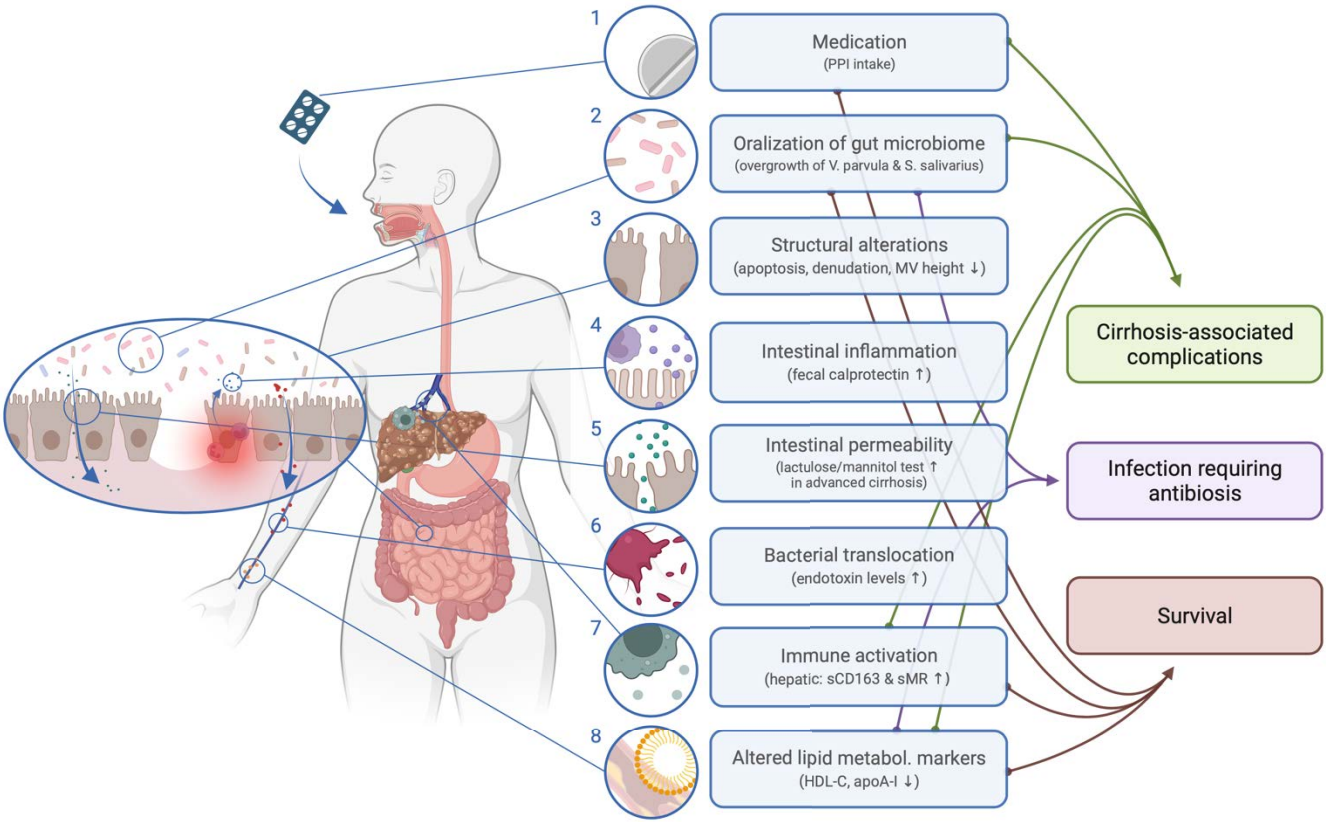

Figure 39. Alterations in biomarkers associated with bacterial translocation and their predictive value in terms of cirrhosis-associated complications, infection and survival. In patients with liver cirrhosis, we found imprudent PPI use, alterations in gut microbiome and small intestinal structure, evidence of intestinal inflammation, increased intestinal permeability, bacterial translocation and immune activation. Furthermore, lipid metabolism markers were significantly altered. In patients with PPI use, we found overgrowth of *V. parvula* and S. salivarius, significantly elevated fecal calprotectin, fecal zonulin, serum sMR and lower HDL-C & apoA-I levels. Arrows on the right side indicate predictive values of investigated biomarkers. PPI, proton pump inhibitor; ApoA-I, apolipoprotein A1; HDL-C, high-density lipoprotein cholesterol. This figure was created with biorender.com.

The increased appearance of *V. parvula* in stool microbiome (*i.e.*, *V. parvula* dysbiosis) was linked to barrier dysfunction, the development of cirrhosis-associated complications, infections and mortality. Macrophage activation parameters sCD163 & sMR have proven their predictive value in liver cirrhosis patients, with better data for sCD163 in terms of survival prediction and for sMR in terms of cirrhosis-associated complication. Furthermore, we identified lipid metabolism parameters HDL-C and apoA-I as robust predictors of disease progression, cirrhosis-associated complications and survival, respectively, in the setting of stable cirrhosis and also AD. An overview of our results is given in Figure 39.

4.1. Impact and prognostic value of PPI intake and V. parvula dysbiosis

We were able to demonstrate that PPI use in patients with liver cirrhosis is linked to an increased abundance of bacteria in stool that typically inhabit the oral cavity (*i.e., Veillonella parvula* and *Streptococcus salivarius*). This *'oralization of the gut microbiome'* was strongly associated with gut inflammation, damage to the intestinal barrier and high levels of systemic inflammatory markers. Patients with PPI intake had a significantly elevated risk for cirrhosis-associated complications and showed higher mortality rates. The same associations were found in terms of *V. parvula* abundance; furthermore, *V. parvula* dysbiosis was associated with an increased risk of infection requiring antibiotic therapy within 12 months.

Recent observations have highlighted the potential implications of PPI use on the gut microbiome, encompassing an elevated presence of bacteria that are typically detected in the oral cavity (104–107). Qin et al. have suggested this oralization also to be found in patients with liver cirrhosis in general (23); however, in their study they did not address the influence of PPI intake. Oralization is likely attributed to a reduced 'gastric clearance' of bacteria due to suppression of acid secretion (25,108). In our work, abundances of *V. parvula* and *S. salivarius* in cirrhosis patients without PPI use did not differ from healthy individuals, but were only increased when PPIs were administered.

Veillonella parvula is a non-motile, anaerobic coccus. Although it is commonly referred to as a commensal, it has been identified as an infectious agent in several

case reports (109–115). It is the most common *Veillonella* species found in subgingival plaques (116) and it often forms biofilms together with different *Streptococcus* species, including *S. salivarius* (117,118). Both species have been shown to induce pro-inflammatory reactions in dendritic cells (including TNF-α & CXCL8 production), which is even more pronounced when induced in co-cultures (119). CXCL8 is a chemoattractant for neutrophil granulocytes and promotes the production of calprotectin (120,121). This aligns with the high calprotectin concentrations we detected in our patients with PPI use and *V. parvula* dysbiosis. TNF-α mediated inflammation has been proven to disrupt tight junction function and thereby, to increase intestinal permeability and bacterial translocation (122). In our patients with high *Veillonella* abundance fecal zonulin levels and L/M ratios as markers of intestinal permeability were significantly elevated and thus, align with these observations.

Increased intestinal permeability and subsequent abacterial translocation are suspected to drive liver disease progression in patients with chronic liver disease. The connection between gut permeability and PPI intake has been illustrated in a recent study by Llorente et al.: In alcohol-fed mice, they showed associations of PPI intake with increased detection of pathogens in the intestines, their translocation to the portal vein and, ultimately, hepatic inflammation and liver disease progression (123). Consistently, we found elevated serum markers of macrophage activation and systemic inflammation in cirrhosis patient with PPI use. Furthermore, PPI-associated *V. parvula* dysbiosis and concomitant inflammation predicted the occurrence of complications and survival in cirrhosis, possibly as a result of dysbiosis-driven hepatic inflammation.

4.2. Structural alterations and associations with other markers of BT

Our work revealed several structural changes in distal duodenal mucosa of patients with liver cirrhosis at both the light and electron microscopic level. Light-microscopic findings encompassed a decreased V/C ratio (especially in patients with advanced cirrhosis), alterations at the villous tips (dilatation of the subepithelial space, denudation of villi, a trend towards increased occurrence of DIS, more apoptotic cells) and a reduced claudin-1 staining. At the electron microscopic level, TJs appeared morphologically preserved, except for an increased TJ-gap width in

epithelial areas where DIS was evident. In patients with liver cirrhosis, the height of the microvilli was significantly reduced compared to healthy controls. Noteworthy, in 2 patients with liver cirrhosis spreading of the fused outer layers of cell membranes (like blebs within the TJs) were observed, possibly corresponding to TJ strand breaks.

Cirrhosis patients with PPI use demonstrated a significantly reduced V/C ratio and an increased occurrence of DIS when compared to patients without PPI use. A decreased V/C ratio was associated with increased intestinal permeability indicated by an elevated L/M ratio; however, the high L/M ratio in our patients with advanced liver cirrhosis was predominantly based on an impaired mannitol excretion. Mannitol uptake primarily depends on total absorptive surface area rather than on integrity of the epithelial barrier and consistently, a reduced V/C ratio was observed particularly in our patients with Child-Pugh B & C cirrhosis (43).

Previous studies on electron microscopic characteristics of intestinal mucosa (and especially of TJ structure) in patients with chronic liver disease are scarce and have yielded contradictory results. The first study investigating duodenal biopsies of cirrhosis patients was published in 2002 by Such et al. (124) and indicated extended intercellular spaces in the basal regions of epithelial cells on light microscopy (according to DIS in our patients), but did not find any alterations in TJ structure on electron microscopy. In contrast, Bhonchal et al. documented an increased TJ gap width; though, in this study duodenal biopsy specimens from individuals with a history of chronic alcohol consumption were examined and these patients additionally suffered from direct toxic effects of alcohol on gut mucosa (125). In 2007, a research group from China investigated electron-microscopic characteristics of duodenal mucosa in cirrhosis patients and described a widening of TJ gaps when compared to healthy controls (126). In all three studies, a consistent observation was the presence of distorted and shortened microvilli. In our cirrhosis patients, records of TJ-gap widths in epithelial areas without DIS showed similar values compared to those in healthy controls. However, widened TJ gaps were noted in epithelial areas where DIS was evident. Although a statistically significant difference was measured, the clinical relevance seems debatable considering the minor difference of 1.3 nm.

In fact, regulation of TJ permeability is a complex process comprising a multitude of influences, and affecting different solutes according to size and charge. There has also been some evidence suggesting that small and large solutes cross the TJ barrier by means of two different pathways (36): Substances smaller than 4 Å are suspected to use the **pore pathway**, which is mainly regulated by TJ protein composition. In our patients with liver cirrhosis, we have shown a reduced TJ staining with claudin-1 antibodies on immunohistochemistry in crypts and villi. Since claudin-1 has been shown to 'tighten' the intercellular space, a reduced expression of claudin-1 might result in an increased paracellular permeability for small solutes. These findings are in concordance with previous studies showing a reduced expression of claudin-1 in duodenal biopsies of liver cirrhosis patients and associations with markers of bacterial translocation (127). Permeation of solutes larger than 4 Å (like substances used in the lactulose/mannitol test, *e.g.,* lactulose = 9.5 Å) is suspected to take place by means of the **leak pathway** via temporary breaks in the usually continuous TJ complex. Interestingly, we found TJs with spreading of the fused outer layers of cell membranes in 2 patients with liver cirrhosis, possibly corresponding to TJ strand breaks (see Figure 20). The leak pathway has been shown to react sensitive to any form of cellular injury and is probably mediated by an enhanced production of nitric oxide (NO) (36,127). In patients with liver cirrhosis NO levels are increased in the splanchnic area due to portal hypertension and its effect on endothelial cells (46). In our patients, we found evidence of congestion (including fluid accumulation in-between and underneath enterocytes) as well as cellular injury (including an increased number of apoptotic cells, features of cell necrosis on electron microscopy & denudation of villi probably as a result of cell shedding). Cellular injury is possibly caused by a deficiency in oxygen supply, since alterations primarily manifested in the region of the villi tips, where oxygen saturation is physiologically lower than laterally on the villi or within the crypts (17). In patients suffering from portal hypertension, a reduced oxygen tension within the gastrointestinal mucosa has been demonstrated using endoscopic reflectance spectrophotometry (128). Additionally, similar histological changes were observed in studies investigating the effects of ischemia and reperfusion on the small intestine of rats (129).

4.3. Prognostic value of macrophage activation parameters

Macrophage activation parameters sCD163 & sMR demonstrated their value as predictors of cirrhosis-associated complications and survival in a cohort of largey compensated cirrhosis patients; sCD163 especially for 3-year survival and sMR for occurrence of complications. Serum levels of sMR were elevated in patients with PPI use, both markers sCD163 & sMR in patients with V. parvula dysbiosis. Additionally, both variables were associated with markers of gut permeability (L/M ratio), bacterial translocation (LPS) and immune activation (CRP, IL-6, IL-8) suggesting potential associations between damage to the intestinal wall, activation of macrophages and inflammation.

Hepatic macrophages constitute over 80% of the entire macrophage population (130) and play a pivotal role in the onset and progression of liver inflammation and fibrosis (131,132). Increased serum levels of sCD163 & sMR reflect activity of liver macrophages (57,60) and show a strong association with disease severity and portal hypertension in patients with cirrhosis (58,63). In our patients, we noted a progressive increase in both values that correlated well with disease severity. Furthermore, sCD163 and sMR levels were significantly elevated in patients with ascites at the time of measurement. These findings underline the significance of macrophage activation in the development of portal hypertension, likely through the release of vasoactive compounds and activation of stellate cells (133). As fibrosis advances and portal hypertension progresses, the likelihood of cirrhosis-related complications increases. Hence, recent studies have demonstrated that sCD163 serves as a reliable indicator for prediction of disease progression (58) and the occurrence of variceal bleeding in cirrhosis (61). Both markers have proven their value in predicting survival of cirrhosis patients in different studies (58,61,63), regardless of etiology (134–136). In our patients, both sCD163 & sMR demonstrated similar prognostic utility in terms of long-term survival compared to established composite scores (Child-Pugh & MELD). sCD163 remained an independent, significant predictor of 3-year survival when added to MELD or Child-Pugh score.

Circulating levels of sMR were identified primarily as predictors of cirrhosis-associated complications. For instance, Laursen et al. (137) investigated the levels of sMR in patients awaiting TIPS implantation and found the highest sMR values in

patients suffering from acute variceal bleeding. In fact, the mannose receptor is found not only on macrophages but also on some dendritic as well as endothelial cells (138) that might be affected and stimulated in patients with portal hypertension. Thus, sMR might emerge as a somewhat distinct biomarker compared to sCD163, particularly for prediction of complications related to portal hypertension.

In patients with liver cirrhosis, macrophages appear to be constitutively activated, as we have also measured elevated levels of sCD163 & sMR in patients with compensated Child's A cirrhosis. This activation is suspected to be sustained by gut-derived PAMPs (like LPS) (60,139–141), which can be measured in large quantity in the portal blood of cirrhosis patients (16,142). Our findings are consistent with this hypothesis since both macrophage activation parameters correlated well with the lactulose/mannitol ratio (as marker of intestinal permeability), LPS (as marker of bacterial translocation) and several markers of systemic inflammation (IL-6, IL-8, CRP).

4.4. Prognostic value of HDL-related biomarkers

In this work, we established lipid-metabolism markers HDL-C and apoA-I as reliable predictors of complications and mortality in patients with compensated as well as decompensated liver disease. The predictive value of HDL-C and apoA-I, when considered individually and as easily accessible indicators, closely paralleled the prognostic capabilities of composite scores such as MELD, CLIF-C AD and CLIF-C ACLF.

Recent studies have shown significant changes in HDL maturation, metabolism and function in patients with liver cirrhosis (143). Our investigations revealed gradually decreasing serum levels of HDL-C and apoA-I (the main protein component of HDL particles) with increasing severity of liver disease, regardless of the underlying etiology. Additionally, we were able to demonstrate that these alterations can yield prognostic values: HDL-related biomarkers HDL-C and apoA-I were able to predict the development of cirrhosis-associated complications (including infection) and survival. Both markers are technically easy to measure in standard falaboratories and, as individual parameters, demonstrated satisfying prognostic accuracies for 90-day and also 12-month mortality. When HDL-C values at baseline drop below 17

mg/dl and those of apoA-I below 50 mg/dl, patients face a notably poor prognosis with an approximate 50% chance of survival at 90 days. Our findings align with a recent study published by Poynard et al., who examined 581 patients with alcohol-associated liver disease and showed significantly lower 12-month survival rates for patients with low levels of apoA-I (70).

In liver cirrhosis, bacterial infections are frequently encountered and contribute significantly to mortality rates (14). Infections in these patients are facilitated by various restrictions in immune function that accumulate with the progression of liver disease (144,145). Individuals with cirrhosis face a risk of sepsis that is over twice as high as that of a comparative population without liver disease, and their prognosis remains dismal despite advances in intensive medical care (146–148). Given the immunomodulatory functions of HDL particles, an involvement in the development of immune dysfunction seems reasonable: HDL controls cholesterol levels within cell membranes by promoting the extraction of cholesterol and other lipids, thereby reducing inflammatory receptor signaling (149,150). In animal models, HDL particles were able to bind LPS and therefore, to reduce cytokine production and inflammatory responses (151,152). This is consistent with a recent study by Trieb et al. that demonstrated a significantly impaired ability of HDL to suppress the LPS-induced activation of NF-κB (a pro-inflammatory transcription factor) and subsequent IL-6 and TNF-α production in patients with liver cirrhosis (143).

In line with these findings, we demonstrated a significant inverse association of HDL-C and apoA-I with hepatic (sCD163 & sMR) and systemic inflammatory markers (CRP, IL-6, IL-8 &TNF-α). Studies investigating possible beneficial effects of restoring HDL functions are already circulating: A recent ex vivo study demonstrated that the addition of reconstituted HDL was able to attenuate LPS-induced inflammatory processes in patients with advanced liver disease (153). Notably, a comparable therapeutic approach with infusions of reconstituted apoA-I (CSL112) has already been tested in patients with acute myocardial infarction and was confirmed in a recent phase II study (154). Considering the poor prognosis of patients with low HDL-C or apoA-I levels in our work, a therapeutic attempt to restore HDL and apoA-I functions in liver cirrhosis seems reasonable.

4.5. Strengths and limitations

The strengths of our work encompass the high number of patients (n=536) covering the whole spectrum of chronic liver disease (from stable cirrhosis to ACLF), and the notable observation period (36 months in the majority of cases). However, to create the large overall cohort, several smaller study collectives with different agendas and target parameters were merged and therefore, many translocation-associated parameters were not available in all patients (see Figure 7). Notably, distal duodenal biopsy samples were only available in 19 patients with liver cirrhosis since this sub-part was primarily designed to reveal structural differences between patients with liver cirrhosis and healthy controls. Hence, associations with markers of bacterial translocation or prognostic value of structural alterations could not be thoroughly examined or failed due to underpowering. Also, stool samples were not included in the study design of this sub-study, despite the fact that the correlation of microbiome changes to structural alterations would have been very intriguing.

Another limitation of this work pertains to the observational design. Our results do not prove causality and may be subject to unmeasured confounders. Since PPI use in our studies was not randomized but rather reflected real-life prescription habit, duration of PPI therapy varied between one month and several years. Fortunately, the percentage of PPI users did not differ significantly between patients with early (i.e., Child-Pugh class A) and advanced (i.e., Child-Pugh classes B and C) cirrhosis.

4.6. Conclusion

In conclusion, we have proven the prognostic value of several biomarkers related to bacterial translocation in patients with liver cirrhosis, most notably *V. parvula* abundance, sCD163, sMR, HDL-C and apoA-I levels. Some of the solitary parameters demonstrated similar performance in comparison to established composite scores MELD & Child-Pugh, probably due to their qualities as representatives of disease progression. We have shown that PPI intake in patients with liver cirrhosis is associated with distinctive alterations in the intestinal microbiome, especially with increased abundances of predominantly oral OTUs including *V. parvula* & *S. salivarius*. We have shown that this oralization is linked to intestinal inflammation, impairment of the gut barrier and higher levels of hepatic and

systemic inflammatory markers. Furthermore, we detected associations with altered lipid metabolism parameters that, considering the manifold functions of lipoproteins, might explain the higher infection, complication and mortality risk in these patients.

Finally, our data suggest different targets for therapeutic approaches in patients with liver cirrhosis, including the intestinal microbiome (to reduce PPI-associated side effects, oralization and inflammation), macrophages (to disrupt hepatic inflammation and fibrosis progression) and lipoproteins (to restore immune-associated functions of lipoprotein particles and probably reduce infection risk).

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