

*Alla mia famiglia e
alla mia vita, Maria*

*“Se vostro figlio vuole fare lo scrittore o il poeta sconsigliatelo
fermamente. Se continua minacciatelo di diseredarlo. Oltre queste
prove, se resiste, cominciate a ringraziare Dio di avervi dato un figlio
ispirato, diverso dagli altri”*

Grazia Deledda

PREFACE

It is important to remember that this PhD work could not be accomplished without being surrounded by special persons. During these four intense years of PhD, I have grown so much as a person, both professionally and personally. I would therefore like to mention all the persons that contributed to this PhD thesis and supported me during this journey.

I would like to thank my promotor, Prof. Peter Stärkel. When I started the PhD, I could not imagine all the different aspects of the intestinal barrier I was going to investigate. You always supported me in all the crazy ideas I have had during the project, you believed in me, and I will always thank you for this. I have learned a lot from you on the clinical aspects and translational approaches in research. We discussed sometimes for hours on scientific aspects of the project, but not only this. I will always remember all the time we passed in our Friday's meetings in Manhattan discussing about everything. Your guidance has been fundamental for my growth, and I can promise you I will continue to contribute to the field with my curiosity and pure passion.

This work and personal growth could have never been accomplished without the mentorship and support of a fantastic person and scientist, Prof. Isabelle Leclercq. I always remember my first presentation in the lab when I was presenting my project. I prepared a very long presentation including any details of the possible experiments and alternative experimental plans I could have done during the thesis. After around two hours of presentation, when almost all people were literally dying, you stopped my presentation saying that it was too much. Indeed, you were right. When I came back home, I immediately realized you did it because I needed to improve not only my presentation skills but also the way of crystallizing the research questions. You continued to support me in the following months and thanks to your guidance I found my way of doing research. I will always thank you for your recommendations and guidance during these years.

Teamwork is fundamental in research, and I would have never achieved my goals without my colleagues, the fantastic GAEN team. I would like to thank Rita for having welcomed me in the lab, guiding me through my first experiments and for all the energy you transmitted to me. Thank you, Justine, for all the help in difficult moments and great time passed together during these years. Thank you, Maxime and Petit Max, for being always present and for the crazy fantastic days we passed together. Thank you, Arturo di Napoli, Dili, Camille, Sebastian, Nicolas, Yves, and Dora, for having supported me. I would also like to thank Boris, Simon, Nathacha for the help and support in conducting experiments. Thanks to the Master student's whom I have had the great opportunity to work with, Joyce and Joaquim, your support was essential for this work. I would also like to thank all my collaborators in the university and around the world that made this project possible.

I would like to thank my PhD jury, Prof. Sonia Brichard, Prof. Hubert Piessevaux, Prof. Philippe de Timary, Prof. Laure Bindels who followed me

during my project and helped me to crystallize my ideas. Special thanks to external jury members Prof. Bin Gao and Prof. Sebastian Müller for having accepted reviewing this PhD work. The private defense was really upgraded thanks to your vast knowledge and experience.

During this PhD thesis, in the GAEN lab, I also had the chance to meet the most special person in the world, Maria. You saved my life and I saved yours. We faced several difficult times, and we were able to overcome any single problem. I always told you "We win". Indeed, we won, and we achieved all this together. I am so lucky to have you by my side!

I would also like to thank all my friends that continuously supported me during my studies and during the PhD and that continue to believe in me.

Last, but not least, I would like to thank my family. You have been always present from the day zero, you supported me in all my decisions, and I am not sure I will ever be able to thank you enough for what you did, and you continue to do. This PhD work would not have been possible without you!

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Summary

Chronic alcohol consumption is one of the leading causes of chronic liver disease and liver-related deaths worldwide. Although more than 90–95% of people with excessive alcohol drinking develop fatty liver, only a minority (10–20%) of subjects with an alcohol use disorder (AUD) ultimately progress to more advanced stages of alcohol-associated liver disease (ALD) and its related complications. Even if some advances have been made in our understanding of the factors involved in the development of ALD, there are still no FDA-approved therapies for treating patients with ALD and abstinence is considered the most effective treatment option.

The intestine and the liver closely communicate via bidirectional links through the biliary tract, portal vein, and systemic circulation. The gut acts as a barrier to protect our body against invading pathogens and potentially harmful substances. This PhD work has focused on the characterization of the microbial and host changes in the intestinal barrier linked to alcohol abuse and progression of ALD at early disease stages in humans. We tested whether alterations in the duodenal mucosa-associated and fecal microbiota associated with host alterations of the gut barrier functions in AUD patients and whether these changes could be related to ALD progression. We analyzed mechanisms implicated in gut immune dysfunctions as well as systemic inflammatory responses linked to microbial translocation and liver disease severity.

Early progressive forms of ALD (e.g., steatohepatitis and fibrosis) represent silent asymptomatic stages, in which non-invasive detection of severe forms is crucial for the correct management of these patients. Keratin 18 is the major keratin in hepatocytes and upon hepatocellular damage and death is released in the systemic circulation in fragments called K18-M65. Since these fragments are released in the systemic circulation, we first evaluated whether circulating keratin 18 levels (K18-M65) could predict progressive forms of ALD at early pre-cirrhotic stages. Remarkably, we found high levels of K18-M65 in AUD patients with progressive ALD while the levels in the non-progressive ALD were similar to those in healthy controls. Circulating keratin 18 highly correlate with transaminases and predicted ALD independently from transaminases as well as other potential confounders (gender, age, body mass index (BMI)), controlled attenuation parameter (CAP) and liver stiffness. We demonstrated that K18-M65 is a reliable biomarker to detect early progressive ALD.

Therefore, we used K18-M65 in combination with clinical parameters in the next study aiming at investigating the relationship between changes in the intestinal barrier and ALD severity. We revealed that only a subset of AUD patients had increased intestinal paracellular and vascular permeability ("leaky gut") not related to changes of the duodenal microbiota or alterations of the intestinal epithelium but associated with compositional changes of the fecal microbiota. Leaky gut alone did not explain increased translocation of Gram+, Gram- and fungal microbes and/or their products in AUD patients. By contrast, duodenal dysbiosis with a dominance shift towards specific potential pathogenic bacteria, elevated translocation of microbes and/or their products, and reduced number of duodenal immune effector cells (macrophages and T cells) associated with ALD progression.

The diminution of duodenal T cells associated with elevated translocation of microbes and/or their products led us to hypothesize that this reduction in immune effector T cells is involved in defective immunosurveillance against microbes. We tested our hypothesis in the third study and found a specific reduction of mucosal CD8+ T resident memory (TRM) cells in AUD patients with progressive ALD. Transcriptomic analysis and *in vitro* studies revealed apoptosis in TRM lymphocytes driven by lysosomal membrane permeabilization and release of cathepsin B into the cytosol. Moreover, mitochondria uncoupling and altered lipid metabolism in TRM associated with programmed cell death. These changes were linked to changes in intestinal and systemic sphingolipids, which could possibly contribute to this phenomenon. As a consequence of TRM cell death, we revealed elevated translocation of microbes and/or their products, supporting that changes in TRM lymphocytes facilitate translocation of microbes and/or their products in progressive ALD.

Increased translocation of microbes and/or their products may provoke the activation of immune responses potentially contributing to ALD. The consequences of elevated translocation of microbes and/or their products in circulating monocytes activation were explored in the fourth study. Remarkably, the number of total blood monocytes increased in AUD patients compared to controls. Among the 3 monocyte subpopulations, intermediate and non-classical increased while classical monocytes decreased compared to controls. Monocytes from AUD patients had up-regulated IL1 β and IL8 levels together with TLR2 and down-stream AP-1 while fungal sensor CARD9 was down-regulated. Strikingly, IL1 β and IL8 were differentially secreted in

monocytes from AUD patients upon stimulation *in vitro* with the TLR2 ligand Peptidoglycan. In addition, systemic IL8 and monocytes changes correlated with elevated translocation of microbes and/or their products. We next investigated whether these two inflammatory mediators were elevated in the livers in patients with severe forms of ALD. Remarkably, hepatic IL1 β and IL8 increased with ALD severity together with liver macrophages activation and up-regulation of chemokines involved in monocytes attraction. Overall, these results point to a contribution of activated monocytes to systemic inflammation and ALD. Monocytes likely infiltrate the liver, transform into monocyte-derived macrophages and release IL1 β and IL8 in response to Peptidoglycan and TLR2 activation. In this fourth study we did not explore other liver immune responses implicated in early ALD progression.

Therefore, in the final study of this PhD thesis, the principal aim was to assess liver inflammatory and immune responses at early stages of ALD and to correlate them with liver damage and repair mechanisms and/or progression of fibrosis. We here demonstrated that a low proliferative capacity was accompanied by increased apoptosis and a deficient IL6/Stat3 signaling in hepatocytes during early ALD. Moreover, we revealed also predominantly in hepatocytes activation of TLR7/Interferon I axis which was strongly associated with the development of significant fibrosis. In parallel, activation of pro-inflammatory macrophages leads to release of IL1 β in the liver thus contributing to chronic inflammation. The source of TLR7 ligands (e.g., miRNA and/or viruses) may be derived from the gut and it will require future investigations.

All these data support the role of the different components of the intestinal barrier (e.g., microbiota and host factors) as contributors in the pathophysiology of ALD in humans. This PhD work has comprehensively investigated different factors involved in gut barrier dysfunctions and ALD. However, other mechanisms and cell types implicated in the gut-liver axis remain to be investigated, in particular the changes in the intestinal epithelium related to reduced immunosurveillance against microbes, the role of microbial and host metabolites in intestinal and hepatic dysfunctions during ALD, the contribution of other components of the immune system (e.g. the complement system, plasma cells and other cell types) in defective immunosurveillance of microbes and molecular mechanisms involved in invasion and dissemination of microbes and/or their products in the portal and systemic circulation.

Innovative technological developments will help us to identify promising biomarkers and finally understand the complexity of the different factors and pathways involved in gut barrier dysfunction during ALD. A deep understanding of the causal links between changes in the gut microbiome and intestinal barrier dysfunction at different stages of ALD will help us develop new diagnostic and prognostic tools and identify new therapeutic targets.

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LIST OF ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
ADH	Alcohol dehydrogenase
AH	Severe Alcohol-associated hepatitis
ALD	Alcohol-associated liver disease
ALDH	Aldehyde dehydrogenase
ALT	Alanine transaminase
AMPK	AMP-activated protein kinase
ASH	Alcohol-associated steatohepatitis
AST	Aspartate transaminase
AUD	Alcohol use disorder
AUDIT	Alcohol use disorder identification test
AUROC	Area under the receiving operating characteristic
AWS	Alcohol withdrawal syndrome
BMI	Body mass index
BMP	Bone morphogenetic protein
CAP	Controlled attenuation parameter
CARD	Caspase recruitment domain
CD	Cluster of differentiation
CRP	C-reactive protein
CVD	Cardiovascular disease
DALYs	Disability-adjusted life years
DAMPs	Danger-associated molecular patterns
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DSM	Diagnostic and statistical manual of mental disorders
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor

ELISA Enzyme-linked immune assay

FDA Food and Drug Administration

GALT Gut-associated lymphoid tissue

GAPs Goblet cell-associated antigen passages

GVB Gut vascular barrier

HSCs Hepatic stellate cells

IBD Inflammatory bowel disease

IBS Irritable bowel syndrome

IFABP Intestinal fatty acid binding protein

IFN Interferon

IL Interleukin

INR International normalized ratio

K18 Keratin 18

KLF Krüppel-like factor

LBP Lipopolysaccharide binding protein

LFTs Liver function tests

LPS Lipopolysaccharide

MAIT Mucosa-associated invariant T cells

MDB Mallory-Denk bodies

NAD⁺ Nicotinamide adenine dinucleotide – oxidized form

NADH Nicotinamide adenine dinucleotide – reduced form

NAFLD Non-alcoholic fatty liver disease

NASH Non-alcoholic steatohepatitis

NOD Nucleotide oligomerization domain receptors

OTU Operational taxonomic unit

PAMPs Pathogen-associated molecular patterns

PAS Periodic acid Schiff

PBMCs Peripheral blood mononuclear cells

PCR Polymerase chain reaction

PEG Polyethylene glycol

PGN Peptidoglycan

PGRPs Peptidoglycan recognition proteins

PPAR Peroxisome proliferator-activated receptor

PVF Perivenular fibrosis

RNA Ribonucleic acid

ROS Reactive oxygen species

SALVE Study of Alcohol-related liver disease in Europe

SM Sphingomyelin

SPDEF SAM Pointed Domain Containing ETS Transcription Factor

SREBP Sterol regulatory element-binding proteins

TCR T cell receptor

TLR Toll like receptor

TGF Transforming growth factor

TNF Tumor necrosis factor

TRM T resident memory lymphocytes

UCP Uncoupling protein

WHO World Health Organization

ZO Zonula occludens

INTRODUCTION

1 ALCOHOL USE DISORDER AND ALCOHOL-ASSOCIATED LIVER DISEASE

Alcohol is a psychoactive substance with toxic and dependence-producing properties^{*1}. Alcoholic drinks have been produced and consumed by humans for

thousands of years. The importance of these alcoholic beverages is evident in the multiplicity of customs and regulations that developed around their production and uses. They often became central in the most valued personal and social ceremonies, especially rites of passage, and were ubiquitous in such activities as births, initiations, marriages, compacts, feasts, conclaves, crowning, magic rites, medicine, worship, hospitality, war making, peacemaking, and funerals^{*2}. Beside this role of alcohol in the society, chronic alcohol consumption and alcohol dependence have represented and continue to represent one of the major cause of diseases and injury conditions.

The diagnosis of alcohol dependence is described in the Diagnostic and Statistical Manual of Mental Disorders (DSM). In May 2013, the American Psychiatric Association issued the fifth edition (DSM-5) in which the terms **alcohol abuse and alcohol dependence**, previously considered as two distinct disorders in the fourth edition, have been integrated into a **single disorder** called **alcohol use disorder (AUD)**. The DSM-V defines AUD as a **problematic pattern of alcohol use** leading to **clinically significant impairment or distress**, with **graded levels of severity** depending on the number of diagnostic criteria met. There are 11 diagnostic criteria and anyone meeting at least two of them during the last year qualifies for a diagnosis of AUD. Severity is established based on the number of criteria met, ranging from mild (2–3 criteria), to moderate (4–5 criteria) and severe (≥ 6 criteria)¹ (figure 1).

^{*1} Drug dependence is a chronic, progressive disease characterized by significant impairment that is directly associated with persistent and excessive use of a psychoactive substance.

^{*2} <https://www.britannica.com/topic/alcohol-consumption/Alcohol-and-society>

DSM-IV

In the past year, have you:

	Found that drinking—or being sick from drinking—often interfered with taking care of your home or family? Or caused job troubles? Or school problems?
Any 1 = ALCOHOL ABUSE	More than once gotten into situations while or after drinking that increased your chances of getting hurt (such as driving, swimming, using machinery, walking in a dangerous area, or having unsafe sex)?
	More than once gotten arrested, been held at a police station, or had other legal problems because of your drinking? **This is not included in DSM-5**
	Continued to drink even though it was causing trouble with your family or friends?
	Had to drink much more than you once did to get the effect you want? Or found that your usual number of drinks had much less effect than before?
	Found that when the effects of alcohol were wearing off, you had withdrawal symptoms, such as trouble sleeping, shakiness, restlessness, nausea, sweating, a racing heart, or a seizure? Or sensed things that were not there?
Any 3 = ALCOHOL DEPENDENCE	Had times when you ended up drinking more, or longer, than you intended?
NCE	More than once wanted to cut down or stop drinking, or tried to, but couldn't?
	Spent a lot of time drinking? Or being sick or getting over other aftereffects?
	Given up or cut back on activities that were important or interesting to you, or gave you pleasure, in order to drink?
	Continued to drink even though it was making you feel depressed or anxious or adding to another health problem? Or after having had a memory blackout?

DSM-5

In the past year, have you:

	Had times when you ended up drinking more, or longer, than you intended?
	More than once wanted to cut down or stop drinking, or tried to, but couldn't?
	Spent a lot of time drinking? Or being sick or getting over other aftereffects?
	Wanted a drink so badly you couldn't think of anything else? **This is new to DSM-5**
	Found that drinking—or being sick from drinking—often interfered with taking care of your home or family? Or caused job troubles? Or school problems?
	Continued to drink even though it was causing trouble with your family or friends?
	Given up or cut back on activities that were important or interesting to you, or gave you pleasure, in order to drink?
	More than once gotten into situations while or after drinking that increased your chances of getting hurt (such as driving, swimming, using machinery, walking in a dangerous area, or having unsafe sex)?
	Continued to drink even though it was making you feel depressed or anxious or adding to another health problem? Or after having had a memory blackout?
	Had to drink much more than you once did to get the effect you want? Or found that your usual number of drinks had much less effect than before?
	Found that when the effects of alcohol were wearing off, you had withdrawal symptoms, such as trouble sleeping, shakiness, restlessness, nausea, sweating, a racing heart, or a seizure? Or sensed things that were not there?
The presence of at least 2 of these symptoms indicates Alcohol Use Disorder (AUD) . The severity of the AUD is defined as: Mild: The presence of 2 to 3 symptoms Moderate: The presence of 4 to 5 symptoms Severe: The presence of 6 or more symptoms	

Figure 1. A comparison between DSM-IV and DSM-5, National Institute of Alcohol abuse and Alcoholism, 2013. DSM-IV described two distinct disorders, alcohol abuse and alcohol dependence, with specific criteria for each. Under DSM-IV, the diagnostic criteria for abuse and dependence were distinct: anyone meeting one or more of the “abuse” criteria within a 12-month period would receive the “abuse” diagnosis. Anyone with three or more of the “dependence” criteria during the same 12-month period would receive a “dependence” diagnosis. Under DSM-5, anyone meeting any 2 of the 11 criteria during the same 12-month period would receive a diagnosis of AUD. The severity of AUD—mild, moderate, or severe—is based on the number of criteria met. DSM-5 eliminates legal problems as a criterion and adds craving as a criterion for an AUD diagnosis. National Institute on Alcohol Abuse and Alcoholism, 2013.

Alcohol is consumed by more than half of the population in only three World health organization (WHO) regions – the European Region (59.9% of current drinkers), the Region of the Americas (54.1%) and the Western Pacific Region (53.8%). Differences in the levels of total alcohol consumption between regions of the world and between countries are the result of complex interactions between a wide range of factors. These include sociodemographic factors, prevalence rates of abstinence, level of economic development, religion and cultural norms, and the preferred alcoholic beverage types. For example, the WHO European Region is home to 13.7% of the world's population aged 15+ years but consumes more than a fifth (21.2%) of the total alcohol worldwide. Total alcohol per capita consumption has increased globally after a relatively stable phase between 2000 (5.7 litres of pure ethanol) and 2005 (5.5 litres). Since then, total per capita consumption rose from 5.5 litres in 2005 to 6.4 litres in 2010 and was still at the level of 6.4 litres in 2016 (figure 2)².

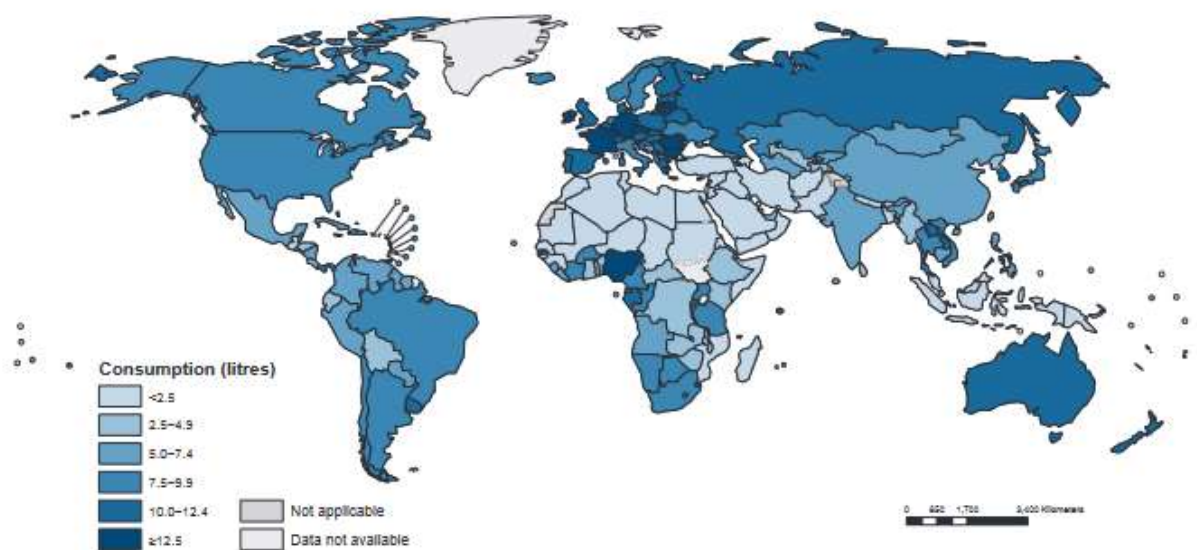


Figure 2. Total alcohol per capita consumption (APC) (15+ years; in litres of pure alcohol), WHO 2016.

Chronic alcohol consumption is one of the leading risk factors for population health worldwide. The harmful use of alcohol resulted in around 3 million deaths (5.3% of all deaths) globally in 2016. Alcohol led also to a large burden of disease and injury in 2016, causing 132.6 million DALYs^{*3} which represented 5.1% of all DALYs in that year. Among the 230 diseases to which alcohol is related, digestive diseases, unintentional injuries^{*4}, cardiovascular diseases (CVDs) and diabetes were the leading contributors to the estimated 3 million alcohol-attributable deaths in 2016, and were individually responsible for 21.3%, 20.9% and 19.0% of these deaths respectively². **Digestive diseases, especially liver cirrhosis, are becoming a more frequent cause of mortality and morbidity globally, especially in developed countries**³. Alcohol is causally related to an increase in the risk of both liver cirrhosis and pancreatitis⁴, causing an estimated 637000 digestive disease deaths and 23.3 million digestive disease DALYs in 2016. Within the burden of alcohol-associated digestive diseases, alcohol-associated liver cirrhosis caused 607000 deaths and 22.2 million DALYs, while alcohol-related pancreatitis resulted in 30000 deaths and 1.1 million DALYs².

1.1 Alcohol-associated liver disease: Disease spectrum and risk factors

Alcohol-associated liver disease (ALD) encompasses a spectrum of injury, ranging from fatty liver, also called as simple steatosis, to the more advanced stages, such as severe alcohol-associated hepatitis (AH) and decompensated cirrhosis^{*5}, which can ultimately lead to the development of hepatocellular carcinoma⁵ (figure 3). It is important to take into consideration that these stages are not necessarily distinct entities of the disease but rather multiple stages which may be present simultaneously in each individual.

Fatty liver develops in about 90% of individuals who drink more than 60 g/day of alcohol. Simple steatosis is usually asymptomatic and rapidly reversible upon abstinence. **Only a 20-30% of heavy drinkers progress into a form of liver injury called alcohol-associated steatohepatitis (ASH),** which is characterized by the

^{*3} Disability-adjusted life years (DALYs) represent the loss of the equivalent of one year of full health. DALYs for a disease or health condition are the sum of the years of life lost to due to premature mortality (YLLs) and the years lived with a disability (YLDs) due to prevalent cases of the disease or health condition in a population.

^{*4} Road injury, poisonings, falls, fire, heat and hot substances, drowning, exposure to mechanical forces and others

^{*5} Decompensated cirrhosis is defined as an acute deterioration in liver function in a patient with cirrhosis and is characterized by jaundice, ascites, hepatic encephalopathy, hepatorenal syndrome or variceal hemorrhage.

presence of steatosis and inflammation. Fibrosis is believed to start in the perivenular area. In certain patients the presence of fibrotic scars is accompanied by the disappearance of fatty liver. Progression of ALD culminates in the development of cirrhosis and its related complications. A subset of AUD patients develops severe alcohol-associated hepatitis, which has a substantially worse short-term prognosis. These advanced stages of ALD (AH and decompensated cirrhosis) are thought to be irreversible⁵. Therefore, a deeper **comprehension** of the molecular **mechanisms** implicated in ALD progression at **earlier disease stages** is crucial to reverse and/or ameliorate the liver disease.

The fact that only about 30% of heavy drinkers develop advanced ALD indicates that **other factors play an important role in the physiopathology**. Some factors that are correlated with disease progression have been identified, including sex, drinking patterns, genetics, obesity, and dietary factors^{5,6}. Those factors either increase susceptibility to liver damage or aggravate disease by acting synergistically with alcohol. The **amount of alcohol ingested** (independent of the form in which it is ingested) is the most important risk factor for the development of ALD. The risk of developing cirrhosis increases with the ingestion of >60-80 g/day of alcohol for 10 years or longer in men, and >20 g/day in women⁷. Another factor that has been identified is the **pattern of drinking**⁸. Drinking outside of mealtimes has been reported to increase the risk of ALD by 2.7-fold compared to those who consumed alcohol only at mealtimes. **Women have been found to be twice as sensitive to alcohol-associated hepatotoxicity** and may develop more severe ALD at lower doses and with shorter duration of alcohol consumption than men. This might be explained by differences in the relative amount of gastric alcohol dehydrogenase 7 (ADH7), a higher proportion of body fat in women, or changes in alcohol absorption with the menstrual cycle⁹. The presence and extent of protein calorie **malnutrition** play an important role in determining the outcome of patients with ALD. Mortality increases in direct proportion to the extent of malnutrition, approaching 80% in patients with severe malnutrition (i.e., less than 50% of normal)¹⁰. The interaction between obesity and alcohol, in particular for mild or moderate consumption, is controversial in humans¹¹. The incidence of obesity and the associated metabolic syndrome is increasing worldwide. A recent cohort study showed that patients with excessive alcohol consumption have increased prevalence of obesity and metabolic syndrome¹², which might increase the risk for liver disease progression. It has been

reported that moderate alcohol consumption increases the risk of development of hepatic steatosis in association with obesity. In contrast, moderate alcohol consumption reduces mortality in normal body mass index (BMI)^{*6} and overweight individuals. This beneficial effect was, however, lost in obese subjects. In addition to environmental factors, genetic factors predispose to ALD. **Polymorphisms^{*7} of genes involved in the metabolism of alcohol** (including alcohol dehydrogenase, acetaldehyde dehydrogenase and the cytochrome P450 system), **and in those which regulate endotoxin-mediated release of cytokines** have been associated with ALD¹³. Genomic-wide associations studies have identified some genetic risk loci for ALD progression, like for example a genetic variant in patatin-like phospholipase domain containing-3 (PNPLA3)¹⁴. However, the pathological interplay between environmental and genetic risk factors is incompletely understood.

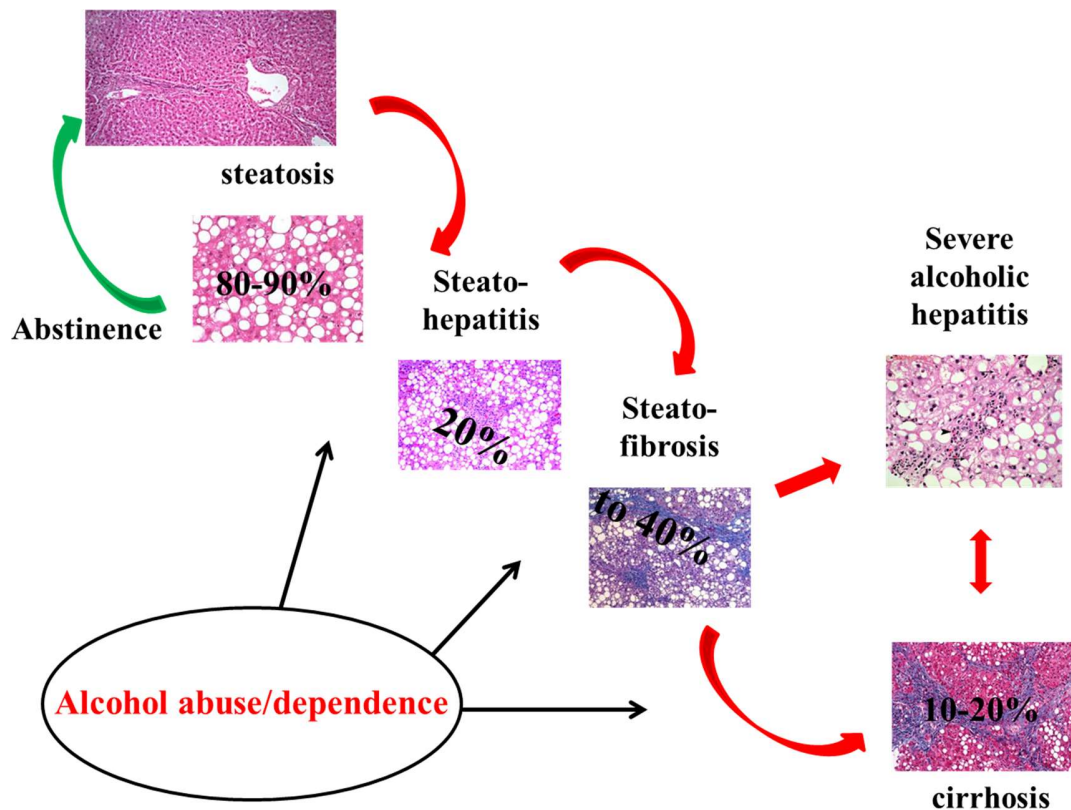


Figure 3. Spectrum of alcohol-associated liver disease.

^{*6} Body mass index (BMI) is a measure that relates body weight to height and is expressed in units of kg/m², resulting from mass in kilograms and height in meters. BMI is sometimes used to measure total body fat and whether a person is a healthy weight.

^{*7} Polymorphism involves one of two or more variants of a particular DNA sequence.

1.2 Diagnosis and management of alcohol-associated liver disease

The diagnosis of ALD is made based on a history of alcohol abuse, clinical evidence of liver disease, and supporting laboratory tests.

Detailed history on alcohol consumption to identify AUD is essential. As patients often underreport alcohol intake, questionnaires can be complemented by information from relatives (if appropriate) or by objective measures (e.g., physical signs of chronic alcohol use), and tests suggestive of alcohol abuse (i.e., elevated blood alcohol, γ -glutamyl transpeptidase or urinary ethyl glucuronide elevation). The primary screening tool to detect alcohol abuse and dependence is the **Alcohol Use Disorders Identification Test (AUDIT)** (figure 4). AUDIT is a 10-item questionnaire, which has been validated as a clinical tool for the accurate detection of alcohol consumption¹⁵ (figure 4). A biomarker in longstanding use, γ -glutamyl transferase (GGT), has been evaluated in several settings, including large population surveys. Unfortunately, low sensitivity and specificity limit the usefulness of elevated GGT to diagnose alcohol abuse the levels of which may fluctuate with extensive liver injury¹⁶. Carbohydrate-deficient transferrin combined with γ -glutamyl transferase has high sensitivity. However, the levels of carbohydrate-deficient transferrin may be confounded with increasing disease severity and active smoking¹⁶. The combination of GGT and Carbohydrate-deficient transferrin increases the specificity in the detection of alcohol consumption¹⁷. Newer biomarkers using metabolites of alcohol such as ethyl glucuronide can reveal alcohol use in the urine up to 3–4 days after the last alcohol drink. However, due to its high sensitivity, it can yield false-positive results with exposure to alcohol containing medications and hand sanitizers containing small amounts of ethanol. In addition, assessment of ethyl glucuronide in hair samples can detect alcohol use for a longer period of up to 1 month¹⁸.

The diagnosis of ALD is made by documentation of chronic alcohol consumption and evidence of liver disease. No single laboratory marker definitively establishes alcohol to be the etiology of liver disease. Furthermore, alcohol may be one of several factors causing liver injury, and the specific contributory role of alcohol alone may be difficult to evaluate in a patient with multifactorial liver disease. Different laboratory abnormalities, including elevated serum aminotransferases, have been reported in patients with alcohol-associated liver injury, and used to diagnose ALD. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT)

are typically elevated in patients with inflammation in the liver. However, even in the presence of fibrosis, serum transaminases can be at normal levels (< 40 IU/l) in certain patients. Therefore, an appropriate evaluation of liver fibrosis with non-invasive techniques is fundamental. As a high proportion of patients with histological features of ALD do not express any clinical symptoms or laboratory abnormalities, asymptomatic patients consuming a critical amount of alcohol should undergo appropriate screening investigations. Screening investigations should not only include liver function tests (LFTs), *i.e.*, gamma glutamyl transpeptidase, serum ALT and serum AST, but also **a test to detect liver fibrosis (e.g., transient elastography (Fibroscan®^{*8})) since advanced liver fibrosis may present with normal LFTs¹⁹.**

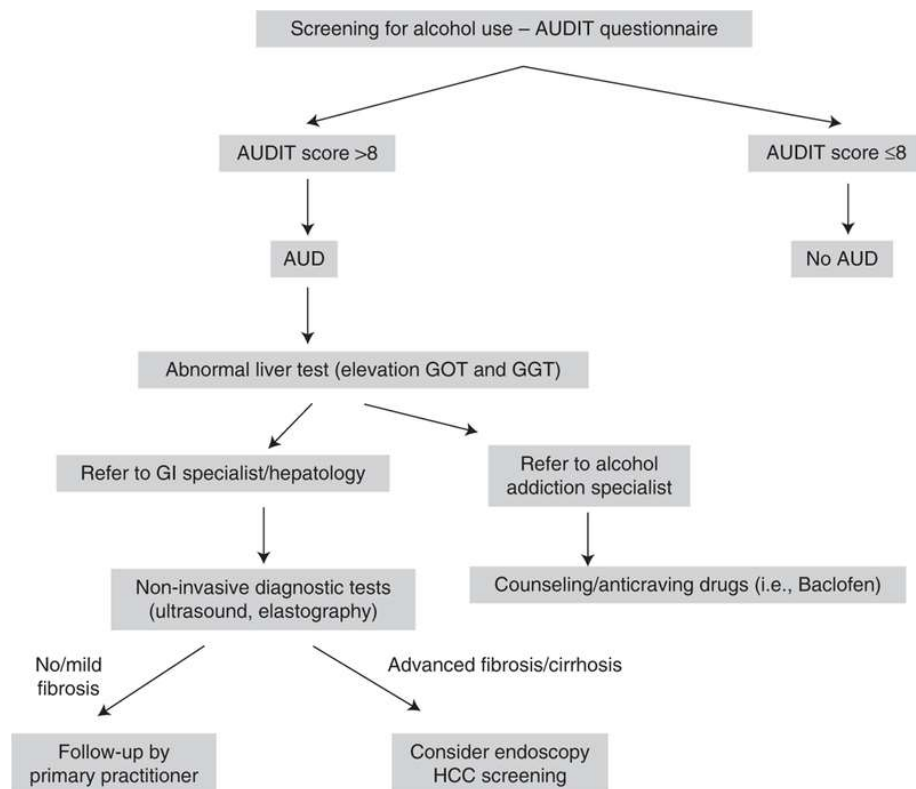


Figure 4. Diagnosis of alcohol use disorder (AUD) using AUDIT tool and on management of early alcohol-associated liver disease (ALD). Singal AK et al., *AM J Gastroenterol.*, 2018

^{*8} Fibroscan® device (Echosense, Paris, France) works by measuring shear wave velocity. The shear wave velocity can then be converted into liver elasticity, which is expressed in kilopascals (kPa), and represents an indirect measurement of fibrosis. In addition, it measures the so-called controlled attenuation parameter (CAP), the degree of ultrasound attenuation by hepatic fat. Results are measured in dB/m over a range of 100–400 dB/m.

In case of suspected advanced fibrosis or cirrhosis, serum albumin, prothrombin time or international normalized ratio (INR)^{*9}, serum bilirubin levels, as well as platelet and white blood cell counts should be determined to evaluate liver function and evidence of portal hypertension^{*10}. Whenever there is evidence of cirrhosis, upper gastrointestinal endoscopy should be performed to screen for esophageal varices, unless there is a low risk of having varices requiring treatment based on Baveno^{*11} criteria (platelets >150,000/μl of blood) and Fibroscan <20 kPa²⁰. A liver biopsy may be used to establish the definite diagnosis of ALD, to assess the exact stage and prognosis of liver disease and to exclude alternative or additional causes of liver injury. Usually, the liver biopsy is done percutaneously under ultrasound guidance or laparoscopically or via a transjugular approach. However, **a liver biopsy is an invasive procedure with significant morbidity**²¹. Therefore, a biopsy is not generally recommended for all patients with suspected ALD, but the risks should be carefully weighed against the clinical benefits and therapeutic consequences.

Patients with ALD are suffering from two different disorders, namely AUD and liver disease. Hence, the treatment should involve **integrated management targeting both the disorders**.

Alcohol abstinence represents a critical goal in patients with ALD since abstinence improves clinical outcomes at all stages of ALD. Many pharmacological agents have been used for treatment of AUD including disulfiram, acamprosate, gabapentin, naltrexone, topiramate, sertraline, and baclofen²². Of these, only baclofen, a γ-amino butyric acid-B receptor agonist has shown no major side effects in patients with ALD and cirrhosis²³. The other major approach to induce or to maintain alcohol abstinence in patients with ALD is behavioral interventions such as motivational enhancement therapy, cognitive behavioral therapy, motivational interviewing, supportive therapy, and psychoeducation²⁴. **Alcohol withdrawal syndrome (AWS) is a common condition affecting AUD patients who abruptly**

^{*9} International normalized ratio is a laboratory measurement of how long it takes blood to form a clot.

^{*10} Portal hypertension is the hemodynamic abnormality associated with the most severe complications of cirrhosis. Hepatic venous pressure gradient (HVPG) measurement is the gold-standard method to assess the presence of portal hypertension, which is defined as HVPG ≥10 mmHg.

^{*11} Baveno is a sequel of meetings where experts aim to develop definitions of key events in portal hypertension and variceal bleeding, to review the existing evidence on the natural history, the diagnosis, and the therapeutic modalities of portal hypertension, and to issue evidence-based recommendations for the conduct of clinical trials and the management of patients.

discontinue or markedly decrease alcohol consumption. Light or moderate AWS usually develops within 6–24 hours after the last drink and symptoms may include nausea/vomiting, hypertension, tachycardia, tremors, hyperreflexia, irritability, anxiety, and headache. Benzodiazepines are the most used drugs to treat AWS, and the main goal of this treatment is to prevent the life-threatening onset of major withdrawal signs such as seizures and delirium tremens²⁵.

Standardized and reproducible assessment of disease activity and fibrosis is a prerequisite for histology-based patient stratification, prognosis, and monitoring of treatment effects. By contrast of other chronic liver diseases such as Non-alcoholic fatty liver disease (NAFLD) and viral hepatitis, few proposals for specific grading and staging systems of ALD have been made and **a universally accepted system for the full clinical spectrum of ALD is currently lacking**. Recently, a group of European liver pathologists, members of the EASL^{*12}-endorsed consortium for the Study of Alcohol-related Liver disease in Europe (SALVE), comprising the SALVE Histopathology Group (SHG), convened to **design a morphological grading and staging system valid for the whole clinical spectrum of the disease**. A semiquantitative evaluation method was defined using numerical scores for macrovesicular steatosis (0-3), hepatocellular ballooning^{*13} (0-2), Mallory-Denk Bodies (MDB)^{*14} (0-2), and lobular neutrophils (0-2). The SALVE staging system details 7 SALVE fibrosis stages (SFS) comprising 4 pre-cirrhotic (SFS 0, 1, 2, and 3), and 3 cirrhotic stages (SFS 4A, 4B, and 4C) (figure 5)²⁶.

^{*12} European Association for the Study of Liver diseases

^{*13} Hepatocyte ballooning is a special form of liver cell degeneration associated with cell swelling and enlargement found particularly in steatohepatitis.

^{*14} Mallory-Denk bodies (MDB), are cytoplasmic hyaline inclusions of hepatocytes.

Steatosis (S) grade: Macrovesicular steatosis*; % parenchymal involvement

Score 0: <5%

Score 1: 5-33%

Score 2: 34-66%

Score 3: >66%

Activity (A) grade: Sum of scores for hepatocellular and lobular inflammation

Hepatocellular injury (ballooning (B) or Mallory-Denk bodies (MDB))**

Score 0: None-rare

Score 1: Few[§]

Score 2: Many^{§§}

Lobular neutrophils (LN)

Score 0: None-rare

Score 1: Few[§]

Score 2: Many^{§§} and/or satellitosis[¶]

Cholestasis type

Canalicular cholestasis (CC)

Score 0: None

Score 1: Present

Ductular cholestasis (DC)

Score 0: None

Score 1: Present

SALVE grade is described by itemization of each of the component scores:

S 0-3, A (B/MDB 0-2 + LN 0-2), CC 0-1, DC 0-1

SALVE, Consortium for the Study of Alcohol-related Liver disease in Europe.

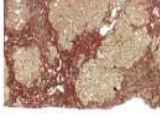
*Lipid vacuoles in the cytoplasm of hepatocytes larger than the hepatocellular nucleus.

**If scores for ballooning and Mallory-Denk bodies are unequal the higher score is applied.

§Feature is appreciated after a reasonable search and is present in few microscopic fields.

§§Feature is frequent and easy to find without searching and present in many microscopic fields.

¶Neutrophils surrounding ballooned hepatocytes.

SFS	Description	Morphological changes ^a	Examples	
0	No fibrosis	Fibrosis is absent		
1	Mild fibrosis	Periportal fibrosis only or PCF ^b in zone(s) 3 ± 2 <i>SFS 1P^c: PCF in zone(s) 3 ± 2 only</i>		
2	Moderate fibrosis	Periportal fibrosis and PCF in zone(s) 3 ± 2		
3	Severe fibrosis	≥1 complete septum ^d bridging portal tracts and/or central veins, ±PCF <i>SFS 3P^c: Perilobular PCF and/or complete septal PCF ± few dense septa ± venous lesions</i>		
4A	Cirrhosis thin septa	≥1 parenchymal nodule ^e , thin septa ^f , ±1 broad septum ^g , ±PCF <i>SFS 4AP^c: Severe PCF^b in >50% of parenchyma, indistinct parenchymal nodules^e</i>		
4B	Cirrhosis broad septa	Parenchymal nodules, >1 broad septum ^g , ±1 very broad septum ^h , ±PCF <i>SFS 4BP^c: Severe PCF in >50% of parenchyma</i>		
4C	Cirrhosis very broad septa	Parenchymal nodules, >1 very broad septum ^h , ±PCF <i>SFS 4CP^c: Severe PCF in >50% of parenchyma</i>		

PCF: pericellular fibrosis; SALVE, Consortium for the Study of Alcohol-related Liver disease in Europe; SFS, SALVE Fibrosis Stage.

^aDescription of the full range of topographical abnormal fibrosis including the degree of both dense septal and pericellular fibrosis.

^bPericellular fibrosis: Collagen fibers surrounding single or small groups of hepatocytes.

^cOPTIONAL, the presence of pericellular fibrosis as a dominant fibrosis type may be classified as "P" substage.

^dComplete septum: Fibrous band consisting mainly of collagen fibers resembling septa in viral hepatitis or septal PCF crossing biopsy diameter and linking portal tracts, portal tracts and central veins, or central veins.

^eParenchymal nodule without evidence of portal-central relations surrounded by dense septa.

^fThin septum: Dense septum, <50% of diameter of smallest parenchymal nodule.

^gBroad septum: Dense septum, ≥50% of the diameter of smallest parenchymal nodule but not thicker.

^hPCF evaluated at **LOW magnification** (20x or 40x total magnification).

ⁱParenchymal areas of indistinct nodular shape dissected by severe PCF.

^jVery broad septum: Dense septum, wider than the diameter of smallest parenchymal nodule.

Figure 5. SALVE grading of alcohol-associated liver disease based on histological features. Lackner C. et al., *Journal of Hepatology*, 2021.

1.3 Physiopathology of alcohol-associated liver disease

1.3.1 Alcohol-associated fatty liver (simple steatosis)

Steatosis, the earliest response of the liver to alcohol abuse, is characterized by the **accumulation of fat (mainly triglycerides, phospholipids, and cholesterol esters) in hepatocytes**. Early studies indicated that alcohol consumption increases the ratio of reduced nicotinamide adenine dinucleotide/oxidized nicotinamide adenine dinucleotide in hepatocytes, which disrupts mitochondrial β -oxidation of fatty acids and results in steatosis²⁷. Alcohol intake has also been shown to **augment the supply of lipids to the liver from the small intestine**²⁸, and increasing **mobilization of fatty acids from adipose tissue and uptake of fatty acids by the liver**²⁷. Recent studies indicate that alcohol exposure, directly or indirectly, regulates lipid metabolism-associated transcription factors; this stimulates lipogenesis and inhibits fatty acid oxidation. Ethanol increases fatty acid synthesis in hepatocytes via up-regulation of sterol regulatory element-binding protein 1c (SREBP-1c), a transcription factor that promotes fatty acid synthesis via up-regulation of lipogenic genes²⁹. Alcohol consumption could directly increase transcription of SREBP-1c gene via its metabolite acetaldehyde or indirectly increase SREBP-1c expression by activating processes and factors that stimulate SREBP-1c expression, such as the endoplasmic reticulum response to **cell stress, adenosine and innate immune receptors which bind microbes and/or their products**³⁰. Moreover, alcohol consumption inhibits fatty acid oxidation in hepatocytes mainly via inactivation of the peroxisome proliferator-activated receptor (PPAR)- α , a nuclear hormone receptor that controls transcription of a range of genes involved in free fatty acid transport and oxidation. The ethanol metabolite acetaldehyde, but not ethanol itself, directly inhibits the transcriptional activation activity and DNA-binding ability of PPAR- α in hepatocytes^{31,32}. In addition to modulating fat metabolism-associated transcription factors, ethanol can also affect the activities of enzymes involved in fat metabolism by inhibiting AMPK^{*15}, which reduces fat metabolism and fatty liver. AMPK is a serine-threonine kinase that can phosphorylate and subsequently inactivate acetyl-CoA carboxylase (ACC), a rate-limiting enzyme for fatty acid synthesis. Inactivation of ACC also reduces levels of

*15 5' Adenosine monophosphate (AMP)-activated protein kinase, a central regulator of energy homeostasis, which coordinates metabolic pathways and thus balances nutrient supply with energy demand.

malonyl-CoA, a precursor in fatty acid synthesis and an inhibitor of carnitine palmitoyltransferase 1, a rate-limiting enzyme for fatty acid oxidation³¹ (figure 6).

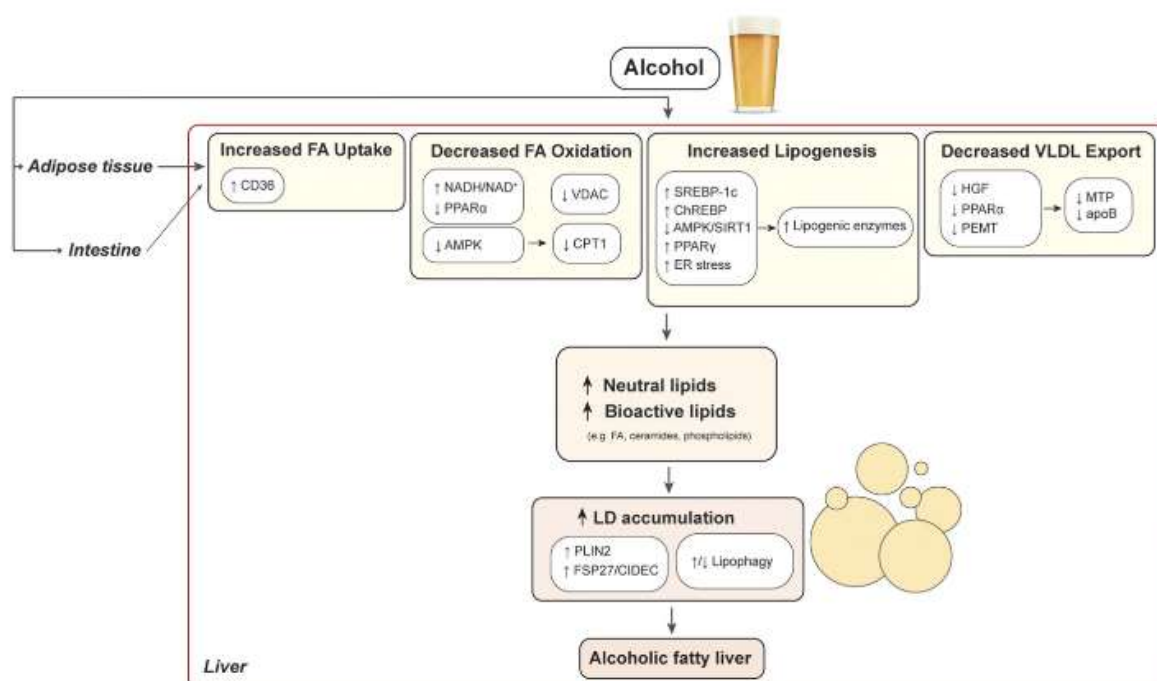


Figure 6. Potential mechanisms underlying the alcohol-associated fatty liver. Hepatic sterol regulatory element binding protein 1 is responsible for fatty acid synthesis and peroxisome proliferator activated receptor- α , - protein kinase (AMPK) and adiponectin are responsible for fatty acid oxidation. Alcohol also increases lipids supply from the small gut. Ethanol may influence the activity of PPAR- α , SREBP-1, and AMPK directly or through adiponectin and tumor necrosis factor- α . These effects activate the lipogenic pathways and inhibit fatty acid oxidation. Besides the fatty acid synthesis and oxidation, ethanol also alters lipid droplets (the storage form of triglycerides) metabolism in hepatocytes and very low-density lipoproteins secretion from liver. All these alterations contribute to alcohol-induced steatosis. Jeon S. and Carr R., *Journal of lipid research*, 2020.

1.3.2 Alcohol-associated steatohepatitis (ASH)

A subset of AUD patients progresses to a form of liver injury called steatohepatitis, which is characterized by the presence of steatosis and inflammation. This form of the disease is usually silent, it can be diagnosed by the concomitant presence of steatosis and elevated transaminases and by using a liver biopsy. In this early stage of the disease, fatty liver is associated with presence of hepatocellular injury (e.g.,

Mallory-Denk bodies, ballooning), inflammation (e.g., infiltration of inflammatory cells and elevated hepatic levels of immune mediators) and absence of significant fibrosis.

Multiple studies have highlighted the fact that multiple “hits” contribute to the development of ASH⁶.

Ethanol hepatotoxicity

Ethanol metabolism generates reactive oxygen species and causes lipid peroxidation, mitochondrial glutathione depletion, and S-adenosylmethionine depletion; all these products subsequently prime and sensitize hepatocytes to injury. Excess acetaldehyde binds covalently to microtubules, resulting in the intracellular retention of normally excreted proteins and the subsequent swelling of the hepatocytes. Acetaldehyde is a reactive compound; it is highly toxic to hepatocytes because it forms a variety of protein and DNA adducts that promote glutathione depletion, lipid peroxidation, and mitochondrial damage³³. Acetaldehyde is transformed into acetate by the enzyme called acetaldehyde dehydrogenase (ALDH). Although acetate has no direct hepatotoxicity, it is believed to regulate the inflammatory response in patients with AH via the up-regulation of proinflammatory cytokines in macrophages³⁴.

The fact that only a minority of heavy drinkers develop progressive form of ALD suggest that **ethanol hepatotoxicity might not be sufficient** to promote development of ALD, and other factors likely contribute to liver disease progression.

Hepatocellular injury

Alcohol, oxidative stress and immune responses can eventually lead to hepatocyte death. Damaged hepatocytes release endogenous DAMPs^{*16}, which are usually hidden from the extracellular environment. DAMPs activate cellular pattern recognition receptors, which results in sterile inflammation³⁵. Features include the production of proinflammatory cytokines, recruitment of immune cells to the site of injury, and assembly of a cytosolic protein complex machinery termed the

^{*16} Damage-associated molecular patterns are molecules within cells that are a component of the innate immune response released from damaged or dying cells

“inflammasome”^{*17}, which conveys DAMP signals into proinflammatory cytokines (e.g., interleukin IL-1 β)³⁶.

Emerging evidence suggests an important role of different forms of hepatocyte cell death (e.g., apoptosis^{*18}, necrosis^{*19}) in liver disease. During the early phases of cell death, enzymes called caspases are activated and cleave different substrates, including proteins of the cytoskeleton. **Keratin 18 is the principal cytoskeletal protein of the hepatocytes**, and it plays an important role in the maintenance of cellular integrity³⁷. The cleavage of keratin 18 at two distinct sites (Asp238 and Asp396) during cell death results in the release of two different peptides in the systemic circulation: **K18-M65**, an epitope exposed to fragmented K18 released from dying cells, thus considered as a marker of **hepatocyte damage**, and **K18-M30**, a neo-epitope mapped to positions 387 to 396 of K18, so called K18-Asp396 that is only revealed after caspase cleavage of the protein and is postulated as a selective biomarker^{*20} of **apoptosis**³⁷. The fact that these cleaved products of Cytokeratin 18 are **released in the systemic circulation** pave the way to their **possible use as a diagnostic tool in early “silent” stages of ALD to potentially discriminate patients with steatohepatitis and fibrosis**³⁸.

Impairment of liver regeneration

The liver is unique in its ability to regenerate in response to injury or loss of tissue. Liver regeneration from partial hepatectomy or acute injury has been extensively studied in animal models. Regeneration of the liver can be more correctly defined as compensatory hyperplasia where in the remaining liver tissue expands to meet the metabolic needs of the organism. Recovery of liver mass occurs mainly via proliferation of remaining adult hepatocytes, biliary epithelial cells, and endothelial cells. Under pathogenic conditions in which proliferation of hepatocytes is inhibited, liver progenitor cells (oval cells or ductular hepatocytes) proliferate and differentiate into hepatocytes or biliary epithelial cells³⁹. In both experimental animals and humans, increased rates of hepatocyte death occur at the earliest stages of alcoholic liver

*17 The inflammasome is a multiprotein intracellular complex that detects pathogenic microorganisms and sterile stressors, and that activates the highly pro-inflammatory cytokines interleukin IL-1 β and IL-18

*18 Apoptosis is the morphologic description of a form of programmed cell death.

*19 Necrosis is an uncontrolled type of cell death that happens before the end of the natural life span of the cell.

*20 A biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease.

disease⁴⁰. In severe alcohol-associated hepatitis, the degree of ductular reaction expansion correlates with disease progression and short-term survival⁴¹. It is thought that long-term alcohol consumption not only causes hepatocyte death but also inhibits hepatocyte proliferation in patients, thereby contributing to the pathogenesis of ALD. However, **data in humans about the role of liver cell proliferation during ALD are scarce**. In particular, **it is not known in which stages of ALD (ASH or liver fibrosis) impairment of liver regeneration might play a role in the pathophysiology of liver disease**.

Activation of innate and adaptive immune responses

A large body of evidence has suggested that activation of systemic and hepatic inflammatory processes play an important role in the development of ASH and progression to fibrosis, cirrhosis, and hepatocellular carcinoma. **Infiltration of innate and adaptive immune cells in the liver in response to hepatic injury and/or translocation of microbial components coming from the intestine** represent a key feature of ALD⁴².

The liver is a key, frontline immune organ. **Ideally positioned to detect pathogens entering the body via the gut, the liver appears designed to detect, capture, and clear bacteria, fungi, viruses, and macromolecules**⁴³. Containing the largest collection of phagocytic cells in the body, this organ is an important barrier between us and the outside world. Importantly, **as portal blood also transports many foreign but harmless molecules (e.g., food antigens), the liver's default immune status is anti-inflammatory or immunotolerant; however, under appropriate conditions, the liver can mount a rapid and robust immune response**. This balance between immunity and tolerance is essential to liver function. Dynamic interactions between the numerous populations of immune cells in the liver are key to maintaining this balance and overall tissue health⁴⁴.

The liver is a unique organ in that it is supplied by both arterial and venous blood. The anatomical organization of the liver allows these two blood supplies to mix and to bath the various structures and cells in the liver with their contents. Oxygen-rich arterial blood enters the liver via the hepatic artery; however, this is a minor source of the blood supplied to the liver. Most of the blood entering the liver is supplied by the portal vein. **Portal blood** is rich in both **nutrients and pathogen-derived molecules**⁴⁵.

When the gut barrier is disrupted, higher amounts of microbes and/or their products translocate to the liver, leading to the activation of the hepatic immune system through pattern recognition receptors like Toll-like receptors (TLRs). TLRs (ten members in humans and twelve members in mice) are a family of pattern-recognition receptors that play a critical role in the activation of innate immune system by **recognizing pathogen-associated molecular patterns (PAMPs) coming from the gut**^{*21} which subsequently leads to activation of antigen-specific adaptive immunity⁴⁶. Endogenous components derived from dying host cells, termed damage-associated molecular patterns (DAMPs), can also activate TLRs. TLRs are expressed widely in immune and non-immune cells in the liver⁴⁶.

It has been proposed that activation of the TLR4 signaling pathway by LPS plays a pivotal role in disease progression. Mice deficient in TLR4, CD14, and LPS-binding protein (LBP) show resistance to alcohol-induced liver injury⁴⁷. Other studies in mice show the pathophysiological importance of TLR4 in resident macrophages (e.g., Kupffer cells), monocyte-derived macrophages, and hepatic stellate cells⁴⁸. However, **data in AUD patients at different liver disease stage are scarce.**

Macrophages represent the first line of defense against infection and injury of the tissue. Kupffer cells, the largest population of tissue macrophages in the body (~80%), are liver resident macrophages that fulfill important roles of clearing pathogen-associated molecular patterns and translocated gut bacteria present in portal blood⁴⁹. **Circulating inflammatory monocytes are recruited to the liver during inflammation and fibrosis, main mechanisms possibly involved in ALD progression.** Once in the liver, monocytes transform into monocyte-derived macrophages and release many pro-inflammatory mediators (e.g., IL-1 β , TNF- α)

^{*21} The TLR ligands are generally categorized into three types: proteins, nucleic acids, and lipid-based elements. TLR4 recognizes LPS (also known as endotoxin), a Gram-negative bacterial cell wall component. The recognition process is enhanced by LPS-binding protein (LBP) that carries LPS to the CD14 molecule, where it then binds to the MD2–TLR4 complex. This complex forms a symmetrical TLR4 homodimer. TLR2 can form a heterodimer with either TLR1 to sense bacterial triacylated lipopeptides, or with TLR6 to recognize bacterial diacylated lipopeptides like MALP-2. Because of the sequence similarity with TLR1 or TLR6, TLR10 senses triacylated lipopeptides by heterodimerization with TLR2. In addition, the scavenger receptor CD36 enhances recognition capability of TLR2 to their ligands. Thus, the dimerization of these TLRs increases the specificity and sensitivity of the recognition for a variety of microbial components. TLR5, the receptor for bacterial flagellin, is expressed on dendritic cells (DCs) on the lamina propria to maintain homeostasis of intestinal immunity. TLR3 senses double-stranded RNA produced during viral replication in the endolysosome. Mouse TLR7 and human TLR7/8 recognize viral single stranded RNA. TLR9 acts as a receptor for unmethylated CpG islands in bacterial and viral DNA.

which have been shown to play a key role in ALD progression **in animal models**⁵⁰. Human studies generally focused on the role of monocytes and monocyte-derived macrophages in late-stages liver disease such as decompensated cirrhosis and severe AH^{51,52}. **Little is known about the role of monocytes and monocyte-derived macrophages in the development of systemic inflammation and alcohol-associated liver disease progression in response to microbial antigens in earlier stages of ALD.**

Infiltration of liver by parenchymal neutrophils is a prominent feature of AH⁵⁰. Up-regulation of IL-8, CXCL1 (Gro- α), and IL-17 in liver contributes to this infiltration and the severity of AH⁵³. However, a recent investigation found that alcohol-associated hepatitis patients with more neutrophils had a better outcome than those with less neutrophil infiltration, probably due to defective bacterial clearance and impaired liver regeneration⁵⁴.

Activation of adaptive immune responses may also favor the progression to ASH and to the more advanced stages of ALD. However, **data in early stages of ALD are lacking**. In advanced stages of ALD, presence of both CD4+ and CD8+ lymphocytes correlated significantly with regenerating nodules, intralobular inflammation, central sclerosis, and abnormalities of Kupffer cells⁵⁵. Another study by Liaskou *et al.* investigated whether these intrahepatic T cells in patients with advanced ALD reflected bystander activation or were a consequence of antigen- specific activation by using high-throughput T cell receptor (TCR) sequencing analysis. The authors identified the oligoclonal nature of these lymphocytes with signs of clonal expansions and the least T cell diversity⁵⁶. Although activated T cells are often detected in ALD and may contribute to disease pathogenesis, excessive alcohol drinking also caused **broad immunosuppression** including inhibition of T cells, thereby resulting in an increased risk of bacterial infection in patients with ALD⁵⁷. Human livers contain abundant mucosa-associated invariant T (MAIT) cells, representing 20%–50% of intrahepatic T cells. MAIT cells express invariant TCR that recognizes microbial riboflavin/vitamin B2 metabolites presented by the major histocompatibility complex class I-related protein 1, playing a key role in controlling bacterial infection⁵⁸. A marked reduction of MAIT cells has been observed in patients with advanced ALD, which may contribute to the **increased risk of bacterial infection** in these patients⁵⁹.

1.3.3 Liver fibrosis

In chronic liver disease, fibrosis develops as the result of an imbalance between the new deposition of extracellular matrix (ECM) and its resorption, representing the wound healing response of the liver to repeated injury. The accumulation of ECM in the fibrotic liver is quantitatively, qualitatively, and topographically abnormal. The end-result is the development of cirrhosis, a diffuse morphology that is characterized by excess fibrous tissue circumscribing parenchymal nodules, which consist of regenerating hepatocytes, and by alterations in hepatic vascular architecture⁶⁰. Collagen deposition around terminal hepatic veins has been described as one of the earliest fibrotic changes associated with chronic alcohol consumption, in a process called perivenular fibrosis (PVF). PVF seems to correlate with the amount and duration of alcohol consumption and may represent an important predictor of disease progression with development of fibrosis and cirrhosis⁶¹.

The onset of liver fibrosis is usually subtle and most of the related morbidity and mortality occurs after the development of cirrhosis. In most patients, progression to cirrhosis occurs after an interval of 10 to 20 years. Major clinical complications of cirrhosis include ascites, renal failure, hepatic encephalopathy, variceal bleeding, and hepatorenal syndrome. The disease can progress from compensated to decompensated cirrhosis, often associated with life-threatening complications⁷. Liver transplantation is usually the only effective therapy¹⁶.

Mechanistically, hepatocyte injury followed by inflammation and activation of the innate immune system leads to liver fibrosis mediated by hepatic stellate cell activation and extracellular matrix (ECM) secretion and deposition. Quiescent hepatic stellate cells (qHSCs) are in the space of Disse, a perisinusoidal space between hepatocytes and LSECs. qHSCs store vitamin A in lipid droplets, which represent the major vitamin A storage site for humans. Activated hepatic stellate cells (aHSCs/myofibroblasts) are the major source of ECM production in the liver fibrosis induced by alcohol consumption and contribute to liver inflammation by secreting cytokines⁶².

Hepatic stellate cell activation results from the inflammatory activity of liver immune cells, predominantly macrophages. Macrophage-derived TGF- β 1 activates hepatic stellate cells and is the most potent known fibrogenic agonist. Hepatic macrophages, including Kupffer cells and recruited monocyte-derived macrophages, also enhance

liver fibrosis by promoting the survival of aHSCs in a NF- κ B-dependent manner. Macrophage/hepatic stellate cell crosstalk is mediated by IL-1 β , and TNF- α . ROS produced upon ethanol metabolism stimulates the production of collagen I in aHSCs/myofibroblasts, acting as an intracellular signaling mediator for TGF- β 1-induced fibrosis⁶³. **In animal models, it has also been demonstrated that activation of innate receptors of microbial antigens, such as TLR4, can lead to hepatic macrophages activation with subsequent release of inflammatory and fibrogenic cytokines, production of ROS, activation of hepatic stellate cells (HSCs) and portal fibroblasts that transdifferentiate into collagen producing myofibroblast⁶⁴.** Kupffer cells and macrophages are targets for the TLR4 ligand LPS, and these cells express profibrogenic cytokines that activate hepatic stellate cells, which also express TLR4. Several lines of evidence indicate that LPS/TLR4 signaling provides a mechanistic link between inflammation and fibrosis. In response to LPS, hepatic stellate cells express chemokines (MCP-1, MIP-1 α) and adhesion molecules (ICAM-1, VCAM-1, and E-selectin) to recruit circulating monocytes which transform into monocyte-derived macrophages in the liver⁶⁴. **However, data in humans about the possible involvement of innate immunity and specific innate receptors in the development of alcohol-associated fibrosis are lacking.**

Taken all together, these investigations have shown that multiple factors (e.g., hepatocellular injury, impaired liver regeneration, **activation of hepatic immune responses by gut-derived molecules**) likely contribute to the progression of ALD from the “benign” form of the disease, namely simple steatosis (figure 7). Emerging body of evidence supports a **key role of the intestinal changes in the development of alcohol-associated liver disease**. However, a **deeper characterization of the alterations in the gut-liver axis at early disease stages (e.g., ASH and significant fibrosis) is currently lacking**. Understanding the molecular mechanisms of the changes happening in the gut at these ALD stages could provide us with the **possible identification of therapeutic targets** in a disease without any FDA-approved drugs. Therefore, this PhD work focuses on the characterization of the changes in the intestinal barrier possibly contributing to the progression of ALD in humans.

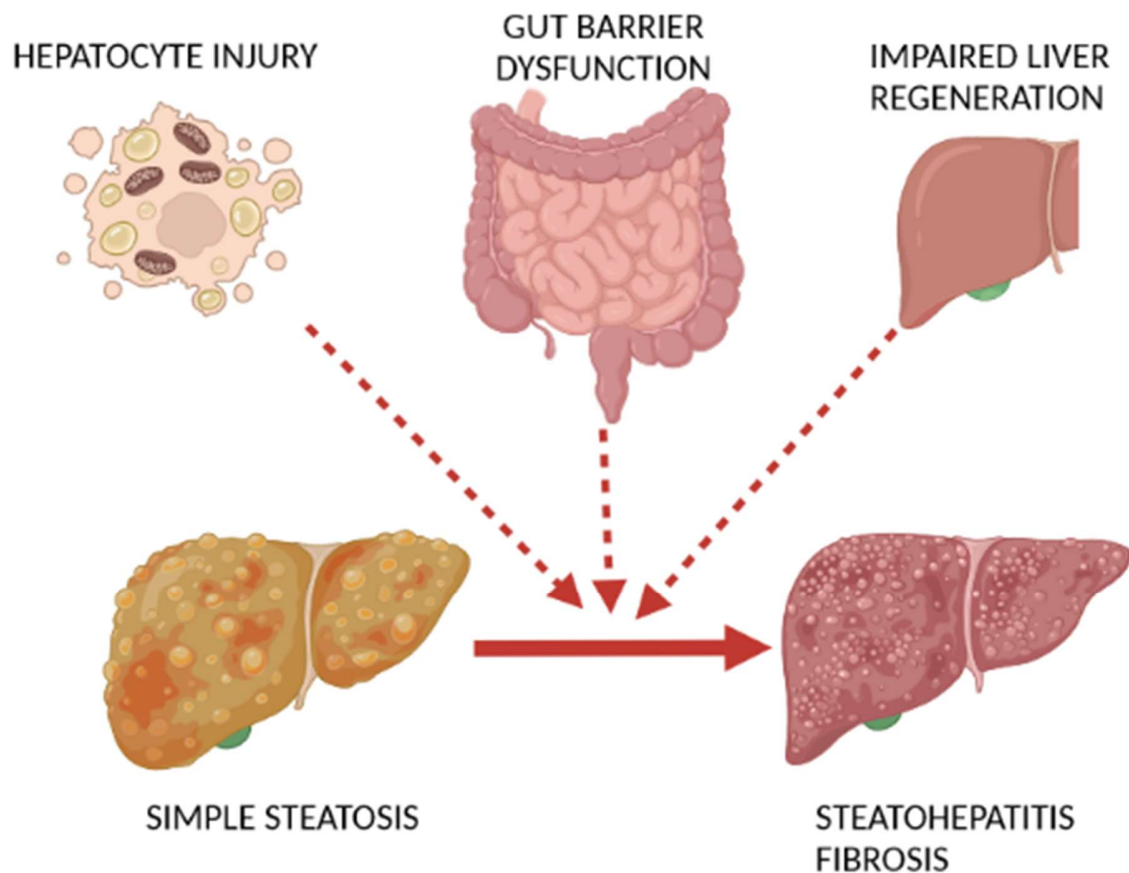


Figure 7. Schematic representation of the factors possibly involved in the progression from simple fatty liver to steatohepatitis and fibrosis.

1.4 Alcohol metabolism

To better understand the pathophysiology of alcohol-associated liver disease, it is important to take into consideration how ethanol is absorbed and metabolized in the human gastrointestinal tract. Ethanol is absorbed by simple diffusion through the mucosa of the gastrointestinal tract. The rate of diffusion of ethanol across the intestinal mucosa is determined by the concentration gradient between the intestinal lumen and the sub-epithelial capillaries, regional blood flow and the permeability of the mucosa in question. **High concentrations of ethanol, similar to those of ingested beverages, are reached only in the duodenum and jejunum.** Luminal alcohol concentrations in the ileum, cecum, and colon are similar to serum ethanol concentrations, suggesting that ethanol reaches the intestinal lumen from blood capillaries. Luminal ethanol can be metabolized by the host because ethanol

metabolizing enzymes are expressed in the intestinal wall. Alcohol dehydrogenase (ADH), the main enzyme involved in the first step of ethanol oxidation, has been found in all parts of the gut. Ethanol metabolism has been shown to occur in the small intestine but seems to be in small quantities compared with that documented in the stomach⁶⁵.

Exposure of the mucosal side of the small intestine to alcohol inhibits the active transport of numerous nutrients across the epithelial layer. An inhibition of absorption has been reported for monosaccharides, several L-amino acid residues and lipids (fatty acids, monoglycerides) as well as for some vitamins⁶⁵. **The pathophysiological mechanisms of the disturbed digestion and/or absorption of lipids and protein in AUD subjects have not been clarified.** An important contribution to this field came from a study on AUD without confounding disorders such as cirrhosis or pancreatic insufficiency. **When the duodenal and jejunal absorption of a nutrient solution containing a mixture of proteins, lipids, and carbohydrates (90% dextrin maltose) was determined using an intestinal perfusion technique, the duodenal absorption of all three nutrients was lower in AUD patients compared to age-matched controls, but jejunal absorption rates were not decreased⁶⁶.** The authors explained the different absorption rates in the duodenum versus the jejunum of the alcoholics as being due to the **more pronounced alcohol-induced mucosal alterations in the duodenum⁶⁶**. When duodenal biopsies from AUD patients were studied, some authors found normal histology by light-microscopy while others demonstrated significant histological alterations^{65,67}. The conflicting results may be explained by the fact that, in most subjects studied, the endoscopy was performed 3–14 days after admission. Considering the high regeneration rate of the small intestinal epithelium, many changes might have healed within the interval between starting abstinence and the endoscopy. **Standardization of the clinical setting in which AUD patients are recruited is therefore key to dissect molecular alterations in the duodenal mucosa linked to active chronic alcohol consumption.**

Ethanol's hepatotoxicity is mainly mediated through its conversion to acetaldehyde. There are 3 classic main hepatocyte pathways of ethanol metabolism that lead to the production of acetaldehyde: a) Alcohol dehydrogenase (ADH). This enzyme is found in the hepatic cytosol and is the major pathway of ethanol metabolism. Multiple isoenzymes can be found in the hepatic cytosol. Catalysis of this

reaction requires the conversion of nicotinamide adenine dinucleotide NAD⁺ to NADH. b) Catalase. Found in the hepatocyte peroxisomes, it can metabolize ethanol into acetaldehyde through the conversion of H₂O₂ to 2H₂O. This process catalyzes a small proportion of remaining ethanol. c) Cytochrome P450-2E1 (CYP2E1). This microsomal enzyme also contributes to ethanol conversion to acetaldehyde but is only significant in situations of excessive alcohol consumption. Oxidation of ethanol by this enzyme also leads to the production of reactive oxygen species (ROS)⁶⁸.

1.5 THE INTESTINAL BARRIER^{*22}

The intestinal tract is the largest interface in the body that is in contact with the external environment, fulfilling the function of nutrient absorption and allowing the survival of commensal symbiotic microbes while avoiding the growth and invasion of potential pathogens. The gut represents therefore a major line of defense for our body in which different epithelial, stromal and immune cell types work in concert to fight off pathogens and dampen their attachment and invasion to the tissues. Intestinal cells have co-evolved with trillions of a wide range of microbes, which are called the gut microbiota, in a beneficial and mutualistic relationship. The microbiota contributes to many physiological functions of the host barrier, while in return the host offers food and a correct habitat. In addition, the gut microbiota also contributes to the maintenance of the gut barrier by defending it against the invasion of pathogens.

1.5.1 Gut microbiota

The gut is the natural habitat for 100 trillion microorganisms (bacteria, archaea, fungi and viruses)⁶⁹. This community of microbe's genome—the microbiome— encodes 100 times more genes than the human genome⁷⁰. These microbes exert a remarkable **influence on the host during homeostasis and disease**. Indeed, the gut microbiota can modulate multiple physiological functions of the host, such as strengthening gut integrity and shaping the intestinal epithelium, harvesting energy⁷¹, protecting against pathogens⁷² and regulating immune response⁷³.

The **gut microbiome** represents our **first line of defense against potential pathogens**⁷⁴. Intestinal microbes compete with pathogens for nutrients and produce antimicrobial molecules called bacteriocins⁷⁵ which shape the composition of the gut microbiota. Commensal microbes further contribute to the development of the

^{*22} This section was adapted from Maccioni L et al., *Int J Mol Sci* 2021, Annex 1

mucosal immune system via direct interactions with host epithelial and immune cells⁷⁶. In addition, the gut microbiome is responsible for a number of vital metabolic and signaling functions for the host, such as biotransformation of bile acids⁷⁷, production of short-chain fatty acids⁷¹, synthesis of all B vitamins and vitamin K⁷⁸ as well as synthesis of essential amino acids⁷⁹. The gut microbiota communities are highly dynamic and colonize the different regions of the gastrointestinal tract according to the microenvironment. Some specific features are important for colonization, such as the source of available nutrients, the most adapted habitat (lumen or attached to the mucosa) for microbial growth and maintenance as well as the symbiosis status with the host⁸⁰ (Figure 8). The gastrointestinal tract is characterized by a **great regional diversity**, housing extensive gut microbes and providing different functions. Each region is geographically specialized in gene expression and function, regulating complex and diverse digestive, immunological, metabolic, and endocrinological processes. Whether it is the acidity in the stomach, the entry point of bile acids and pancreatic secretions, the digestive and absorptive surfaces of the small intestine, or water extraction and stool formation in the colon, these properties create critical environmental conditions that determine microbial community assemblage, fitness, and functions that mutually benefit host and microbes⁸¹ (Figure 8).

Most of the new techniques used to analyze bacterial composition are based on next-generation sequence analysis of the 16S ribosomal genes (16S rRNA also called 16S rDNA), which are highly conserved between bacterial species and are therefore used for bacterial identification. The identification is based on the similarity of their 16S rRNA gene sequences to previously analyzed sequences present in the Ribosomal Database Project⁸². Operational taxonomic units (OTUs) are used to categorize 16S rRNA gene sequences that differ by less than a fixed sequence dissimilarity threshold, most commonly 3%⁸³. Different measures can be used to assess the differences in the microbiota between groups of patients, for example in disease stages. The most widely used diversities are α - and β -diversity: the former considers the average diversity within a habitat type at a local scale while the latter indicates the differentiation between microbial communities from different environments⁸⁴. The vast majority of the studies analyze the gut microbiota composition in stool samples, which is easily sampled and rich in microbial diversity, density, and abundance⁸⁵. However, analysis of the microbial communities in the small intestine is usually not performed

given the difficulties to access the small gut especially for certain diseases. Studying **microbiota composition only in stool samples represents a limitation** if we consider the high heterogeneity and functionality of the microbial communities along the gut. Therefore, in this PhD thesis, gut microbiota profiling of the mucosa-associated microbiota in distal duodenal biopsies together with fecal microbiota analysis has been performed to evaluate whether local changes might be associated with dysfunctions of the intestinal barrier and/or progression of ALD.

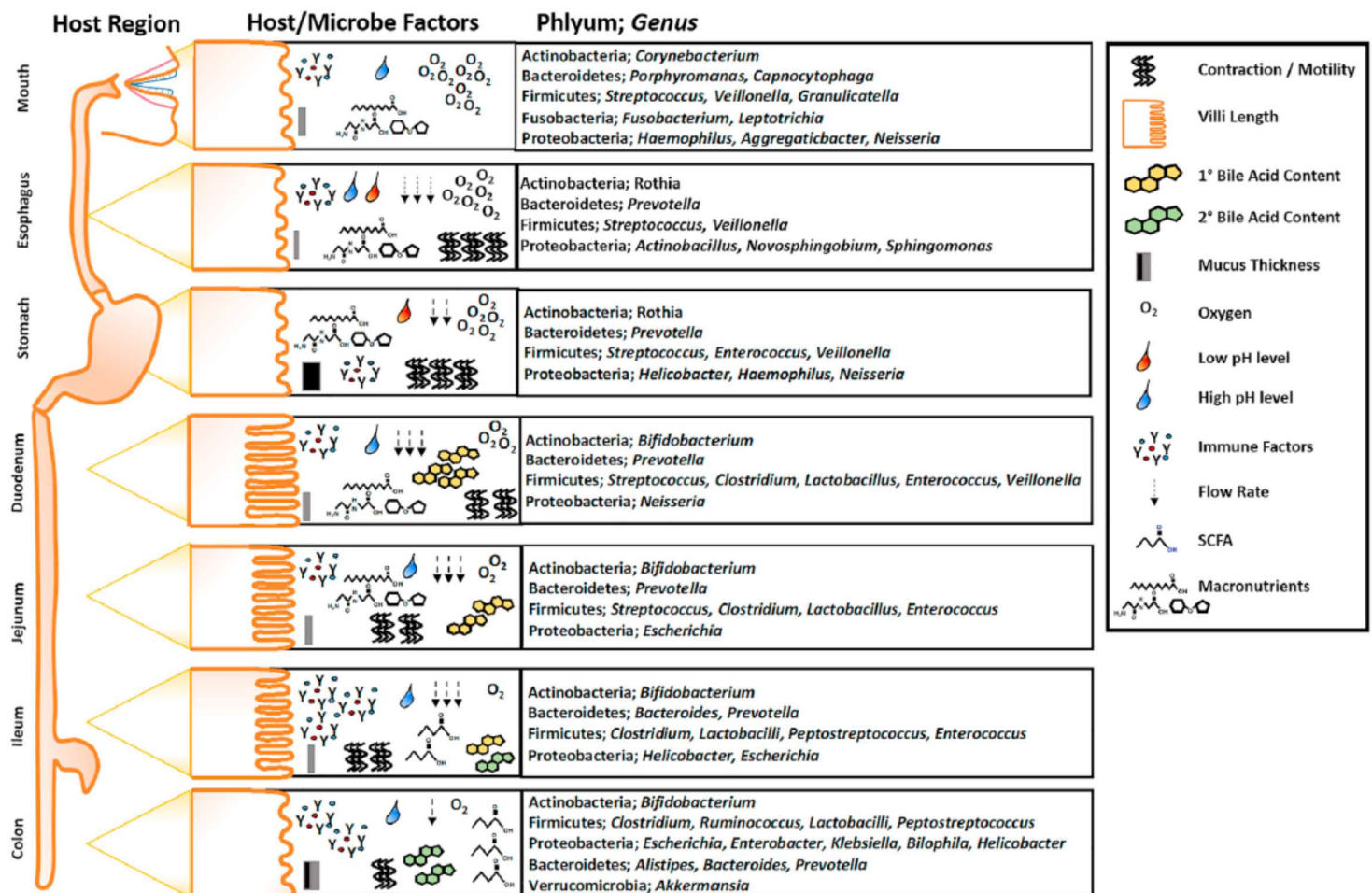


Figure 8. Representative Host and Microbial Factors and Community Membership along the gastrointestinal (GI) tract, Martinez-Guryn et al., *Cell & Host Microbe*, 2019.

1.5.2 The small intestinal epithelium

The principal functions of the intestinal barrier are nutrient absorption and defense against invading pathogens and potentially harmful substances. Mucosal cell types dynamically contribute to a **balance between protective immunity and prevention of excessive immune responses against nutrients and commensal microbes**. A first single-layer of cells, called the intestinal epithelium, facilitate absorption of nutrients while preventing microbial attachment and their translocation into the blood circulation⁸⁶.

The small intestinal epithelium is organized in villi and crypts of Lieberkühn. Adult stem cells reside at the base of the crypts and give rise to all absorptive and secretory cells making up the epithelial layer⁸⁷. Moving up the crypt, the transit amplifying (TA) cells, the progeny lineage of the stem cells, can be found, which are fast dividing cells differentiating into various epithelial cell types. The transit-amplifying cells divide 4-5 times in the crypts in a time frame of approximately 2 days before being integrated into the villus at which point they are fully differentiated⁸⁸. The differentiated cells reach the top of the villus in roughly 3 days where they undergo spontaneous apoptosis with the remains of the cell being dispersed into the lumen. The intestinal epithelium is completely renewed every 4 to 5 days in humans and **this constant renewal of the epithelium is of utmost importance for it to be able to perform correctly its functions**. To ensure the renewal of cells, multiple pathways such as the Wnt/ β -catenin, Notch, Epidermal growth factor (EGF) and bone morphogenesis protein (BMP) signalling pathways act together and crosstalk, thereby permitting a correct balance between proliferation in the crypts and apoptosis in the villi. Proliferation and differentiation are tightly linked and pathways known to be important in proliferation were also described to play a role in the differentiation process and vice versa⁸⁹. As for the proliferation **the differentiation process needs to be very well orchestrated to ensure that all types of cells are present in the correct proportions**.

Gut barrier functions are insured by different epithelial cell types (Figure 9). Secretory goblet cells produce mucus, a viscous fluid enriched in mucin glycoproteins that form a first physical barrier against luminal microbes. Commensal bacteria also degrade glycans to extract the energy content which then they share with the host in a mutualistic relationship⁹⁰. Moreover, goblet cells produce antimicrobial peptides, such

as trefoil factor⁹¹ and resistin-like molecule beta⁹², to dampen mucosal attachment of microbes. In addition, they are also able to present bacterial antigens to dendritic cells via the so called “goblet cells antigen-associated passages” (GAPs), thus educating the intestinal immune system⁹³. Paneth cells, which reside at the base of the crypts, contribute to gut barrier function by producing and releasing in the crypt lumen antimicrobial molecules such as antimicrobial peptides, the regenerating islet-derived 3 (Reg3) family of proteins, lysozyme and secretory phospholipase A2⁹⁴. Microfold cells, also known as M cells, are located in follicle-associated epithelium. They uptake antigens and microorganisms from the lumen and deliver to organized lymphoid tissues within the mucosa⁹⁵. Enteroendocrine cells (EECs) represent 1% of the epithelium and are key sensors of the intestinal luminal environment. In particular, EECs are able to sense microbial metabolites and can orchestrate immune responses⁹⁶. Absorptive enterocytes also contribute to defense mechanisms by regulating paracellular permeability with tight junction proteins and directly recognizing a variety of PAMPs with different pattern recognition receptors, including Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-containing proteins (NODs)⁹⁷. The surface of absorptive enterocytes is covered by a layer of microvilli, in which are present the enzymes that are needed to digest dietary component, as well as nutrient transporters. Microvilli increase the surface area available for digestion, which is already increased by the long villi in the proximal small intestine, where most digestion occurs. Furthermore, a dense glycocalyx consisting of extended and heavily glycosylated membrane mucins covers the surface of enterocytes reinforcing the protective properties of the intestinal mucus⁹⁸.

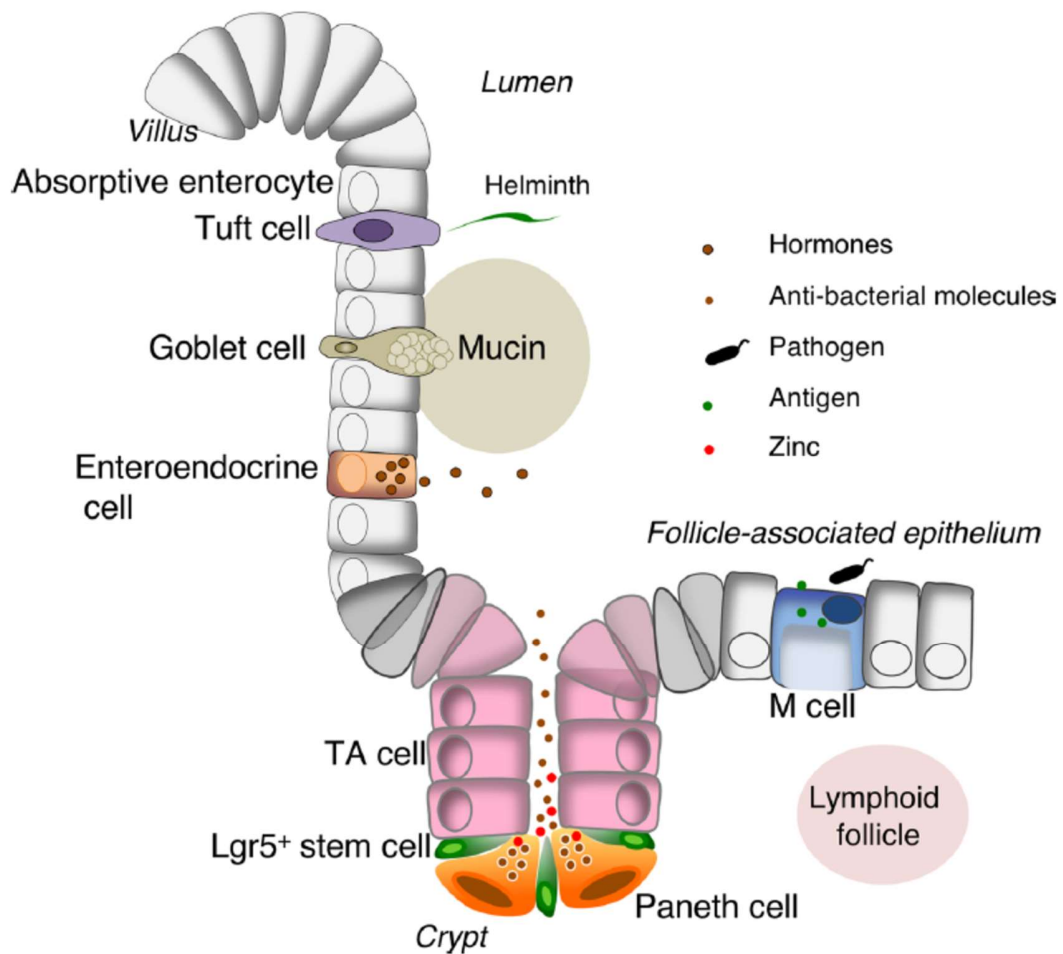


Figure 9. Locations and functions of intestinal epithelial cells in the small gut, Ohashi W et al., *Dig. Dis. Sci.*, 2019

1.5.3 The intestinal immune system

The gut contains the largest number of immune cells of any tissue in the human body. The mucosal immune system plays an important role in orchestrating intestinal defense mechanisms against potential pathogenic microorganisms by coordinating innate and adaptive responses. Immune cells strategically localize in different compartments of the gut mucosa according to the expression of different receptors and **capacity to sense directly or indirectly microbes or their products**⁹⁹.

Macrophages are in part found under the epithelium in the lamina propria where they engulf and kill invading microorganisms and subsequently expose their antigens to adaptive immune cells. Unlike phagocytes in other parts of the body, gut macrophages produce large amounts of regulatory cytokines such as IL-10 and contribute to **maintain a tolerant status in the intestine while avoiding excessive**

inflammatory reactions¹⁰⁰. The intestinal macrophages are continuously replaced by circulating monocytes. However, a recent report sheds light on the existence of long-lived tissue-resident gut macrophages that are not replaced by infiltrating monocytes¹⁰¹.

Dendritic cells (DCs) orchestrate mucosal immunity by acquiring antigens in the epithelium through goblet or microfold epithelial cells or by directly capturing and sampling luminal antigens. They then present antigens to T cells and B cells in the mucosa or in organized secondary lymphoid structures known as Peyer's Patches, as well as in mesenteric lymph nodes¹⁰². Tolerogenic dendritic cells promote differentiation of regulatory T cells (Treg)¹⁰³, which in turn are able to induce tolerogenic DCs via IL-10, TGF- β and other surface molecules¹⁰⁴. DCs in gut-associated lymphoid tissue (GALT) and mesenteric lymph nodes further promote differentiation of B cells into immunoglobulin A (IgA)-producing plasma cells and memory B cells. Dimers of immunoglobulin A are transported through intestinal epithelial cells and secreted into the lumen where it binds commensal and pathogenic microbes, without initiating a pro-inflammatory response¹⁰⁵, and therefore contributes to mucosal defense.

The intestine also represents the home of the highest number of T lymphocytes in the human body. T cell numbers are several fold-higher in the duodenum and jejunum compared to the ileum, decreasing even further in the colon⁹⁹. The majority of mucosal T cells are locally activated and resident populations of CD8+ and CD4+ T lymphocytes called **T resident memory cells (TRM)** play an important role in the maintenance of **gut microbial immune surveillance**. These lymphocytes are long-lived cells present in both epithelium and lamina propria and allow the fast initiation of targeted immune responses against invading pathobionts¹⁰⁶. The different actors of the small intestinal barrier are schematically represented in Figure 10.

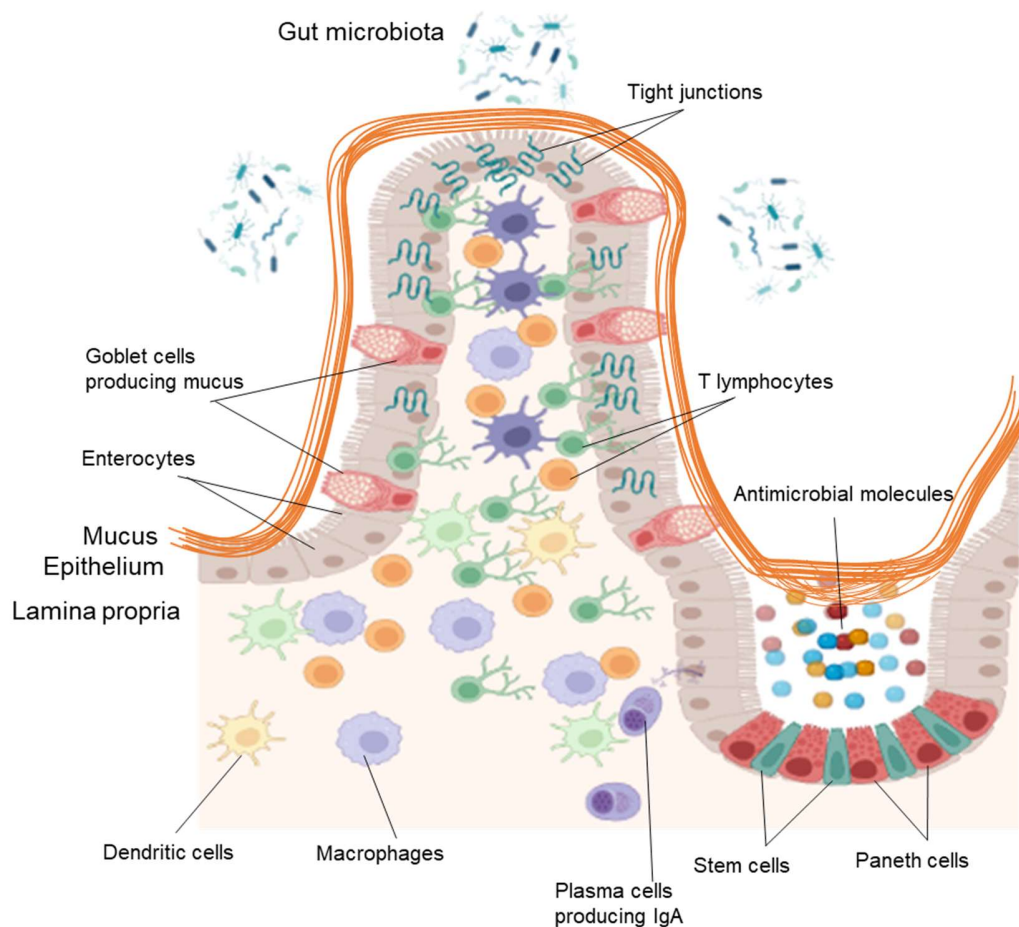


Figure 10. The small intestinal barrier in healthy conditions. The intestinal barrier is composed of three main layers: the mucus, the epithelium and the lamina propria. The epithelium which is overlaid by a thin and discontinuous mucus layer together with enterocytes and specialized cells constitute a first line of defense. Immune cells in the mucosa further fine-tune defense mechanisms against pathogens. Macrophages underlying the epithelium regulate immune response by phagocytosis of microbes and production of large amounts of anti-inflammatory cytokines, thus preventing excessive immune responses. Dendritic cells capture, process and present microbial antigens to different adaptive immune cells. T lymphocytes rapidly act against pathogens by killing infected cells, producing cytokines and coordinating immune responses. Maccioni L et al., *Int J Mol Sci*, 2021 (Annex 1).

Defects in the functions ensured by the different cell types of the intestinal barrier are associated, in chronic intestinal diseases and liver diseases, with microbial overgrowth, attachment and invasion of microbes into distant tissues via the portal

and/or systemic circulation with subsequent activation of immune responses¹⁰⁷. The **increased passage/translocation of metabolites and/or products coming from the gut microbiota**, a process called **microbial translocation**, appear to be linked to dysregulation of the gut barrier functions in different diseases of the gastrointestinal tract, such as inflammatory bowel disease (IBD), celiac disease, irritable bowel syndrome (IBS)¹⁰⁸. This process can occur via different routes (e.g., paracellular or transcellular) and mechanisms, which remain largely unknown.

Since the gut cannot be regarded as a simple and static barrier, different techniques are used to evaluate its different functions. The research question as well as the study design and accessibility to biological samples drive the choice for the techniques used to assess intestinal barrier function. Therefore, **a combination of complementary techniques** has been used in this PhD thesis to gain a better insight into the functionality of the gut barrier in AUD patients and its relation to ALD progression at early pre-cirrhotic stages.

1.6 METHODS TO EVALUATE INTESTINAL BARRIER FUNCTION

1.6.1 Intestinal permeability

The first line of defense of the gut barrier is represented by epithelium in which **epithelial cell membranes and tight junction** proteins limit the **selective passage** of relatively small molecules ($>300 \text{ Da}^{*23}$) **in the paracellular space**. These tight junction proteins include integral and transmembrane proteins, such as claudins, occludin, junctional adhesion molecules and zonula occludens-1 (ZO-1)¹⁰⁹.

Histological evaluation is important to assess the integrity of the epithelial barrier as well as the morphology of the small intestine (e.g., villi and crypts length). **Intestinal epithelial damage** can be further evaluated non-invasively by measuring **serum levels of intestinal fatty acid binding protein (I-FABP)**, released by enterocytes in the blood circulation upon damage¹¹⁰. Electron microscopy led to the discovery of tight junctions. Moreover, immunofluorescence of different tight junction proteins can be used to visualize their structure, which is closely related to their functions. Protein and mRNA transcript levels of tight junction molecules can be assessed with western blotting and quantitative PCR^{*24}, respectively. However, **tight junction** proteins are

*²³ Dalton (Da) is a unit of mass that is equal to one twelfth of the mass of a free carbon-12 atom at rest.

*²⁴ Polymerase Chain Reaction (PCR) is a laboratory technique used to amplify DNA sequences. The method involves using short DNA sequences called primers to select the portion of the genome to be amplified.

mainly regulated at the structural level and therefore evaluation of their structural features by immunohistochemistry/**immunofluorescence is likely the most appropriated method**¹⁰⁹.

In addition, paracellular intestinal permeability can be evaluated under *in vivo* conditions via the enteral administration of non-digestible markers. This method is based on the assessment of the flow from the gut lumen to extraintestinal space such as blood, urine or specific organs. Non-digestible markers used for this purpose in humans are the radioactive probe ⁵¹Cr-EDTA, the lactulose/mannitol ratio and polyethylene glycol-400 (PEG-400)¹¹¹. Each test to evaluate intestinal permeability and barrier function has its own advantages and disadvantages. Among them, **⁵¹Cr-EDTA represents the gold standard to evaluate paracellular intestinal permeability** for two main reasons: first, it is not degraded by microbes and second, but not in order of importance, measuring the urinary excretion at different time points allows the evaluation of intestinal permeability along the whole intestinal tract¹¹².

The intestinal epithelium does not represent the sole layer that controls the selective passage of molecules. Within the lamina propria the **capillary endothelium** partly restricts the passage of **relatively big molecules present in the blood stream such as albumin**, which has a molecular weight of 66.5 kDa. This additional barrier is called **gut vascular barrier (GVB)**, formed by endothelial cells held together by tight junctions and adherens junctions and sustained by enteric glial cells and pericytes, which form the gut vascular unit¹¹³ (Figure 11). The passage of molecules from the intestinal blood stream to the lumen and the related vascular permeability can be evaluated by measuring albumin in the stools. Assessment of fecal albumin for the measurement of gut vascular permeability can result in false negative results when albumin synthesis by the hepatocytes is decreased (e.g., advanced stages of liver disease) or in albumin losing diseases (e.g. kidney disease)¹¹¹. So far, no studies in humans have evaluated the possible use of fecal albumin in conditions where albumin concentrations in the circulation are in normal ranges. The liver of AUD patients at early stages of ALD presents a preserved synthetic function and in principle also a normal kidney function in pre-cirrhotic stages of ALD. Therefore, we have evaluated in this PhD study intestinal vascular permeability by measuring fecal albumin together

with immunofluorescence analysis in distal duodenal biopsies of plasmalemma vesicle-associated protein-1 (PV-1), a marker of endothelial cells permeability¹¹³.

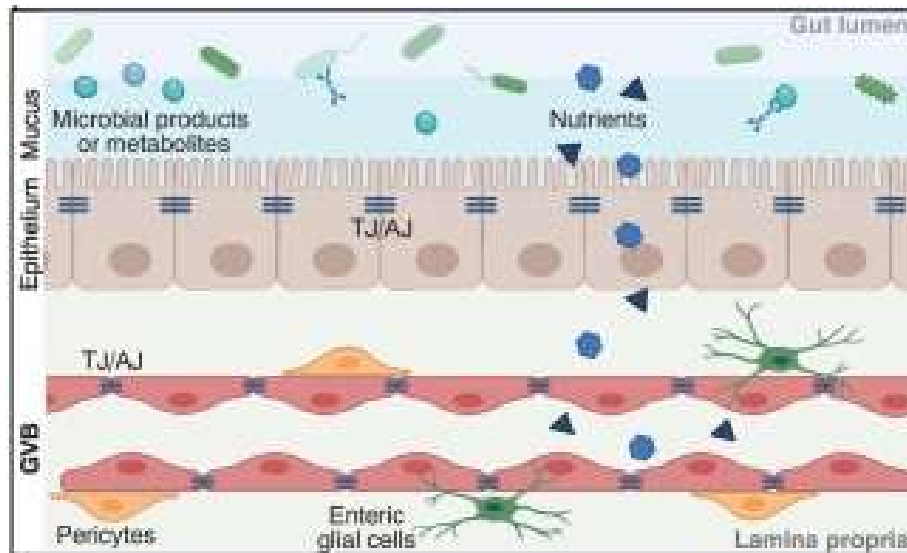


Figure 11. Gut epithelial and vascular barrier in health conditions, Brescia P. and Rescigno M., *Trends in Molecular Medicine*, 2021.

1.6.2 Microbial translocation

The passage of viable microbes and/or their products into the blood circulation is highly controlled and restricted by the intestinal barrier. The mechanisms underlying microbial translocation are largely unknown and likely involve defective responses of multiple cell types in the gut barrier. Animal models of ALD and data obtained in advanced stages of liver disease have highlighted the importance of translocation of microbes in the portal and systemic circulation^{114,115}. Different methods have been developed to assess the circulating levels of gut-derived and translocated PAMPs. The Gram-negative product Lipopolysaccharide (LPS) can be measured by the Limulus assay or by other ELISA assays. Peptidoglycan, part of the bacterial cell wall, can also be measured by different ELISA kits¹¹⁶. **The direct measurement of microbial products needs highly sterile conditions and can easily lead to false positive results. To overcome this problem, serum levels of so-called surrogate markers of microbial translocation are often measured.** Markers of microbial translocation are proteins released by multiple cell types in response to microbial antigens, and bind microbes or some of their parts^{117,118}. In addition, serum levels of antibodies directed against certain bacteria or fungi can be measured as an indirect evaluation of microbial translocation and response to microbes.

Translocation of viable microbes appear to play an important role in advanced stages of liver disease since a high percentage of AUD patients with decompensated cirrhosis and severe AH have increased incidence of infections, which are a cause of morbidity and mortality¹¹⁹. Aerobic and anaerobic culture techniques are used to detect the presence of viable microbes in both circulation and tissues¹¹⁴. However, only a minority of microbes can be cultured by traditional culture techniques. To elucidate the role of different non-cultivable microbes in the gut microbiota, metagenomics^{*25} approaches have been developed to analyze their genome in relation to healthy and diseases conditions¹²⁰.

1.6.3 Intestinal immune responses

Mucosal immunity plays a pivotal role in protecting the intestine from invasion of pathobionts while preventing excessive inflammatory responses. The comprehensive study of gut immune responses in human pathology represents a significant challenge given the difficulty to access tissues in non-advanced stages of diseases. In contrast to intestinal permeability, epithelial damage and microbial translocation, which can be assessed non-invasively with the use of different markers, a complete evaluation of the functions of gut immune cells can be achieved only by accessing the tissues and by applying different complementary techniques. It is fundamental that clinical settings are standardized to evaluate gut immune cells in biopsies obtained from patients in the same “active” status to avoid biases. The highly-standardized clinical setting of the alcohol withdrawal unit (described in the general design of the research, pages 54-55) allows the characterization of duodenal immune cells from actively drinking and abstinent AUD patients at different ALD stages.

The gut harbors numerous immune cell types, which can be identified and evaluated by **immunohistochemistry/immunofluorescence** staining as well as by **flow cytometry analysis**. Effector molecules, such as cytokines and antimicrobials, and chemoattractants (e.g., chemokines) can be assessed by **qPCR and Western blotting**. To get a better insight into the functionality of the immune cells, innovative bulk RNA and single-cell **RNA sequencing technologies** have been developed to study transcriptional signatures in isolated gut immunocytes linked to different diseases¹²¹. Finally, since transcriptional signatures do not provide a complete

^{*25} Metagenomics is the study of the genomic information extracted directly from clinical or environmental samples.

overview of the functions of the immune cells, different ***in vitro* approaches** can be used to evaluate different mechanisms (cell death, metabolism, cytokine's release etc.) in isolated FACS^{*26}-sorted intestinal immune cells.

1.7 GUT BARRIER DYSFUNCTION IN CHRONIC ALCOHOL CONSUMPTION

Chronic alcohol consumption in rodents and humans is associated with small **intestinal bacterial overgrowth**¹²², alterations in the composition of the intestinal micro- and mycobiota, also called **gut dysbiosis**¹²³, and increased **microbial translocation (translocation of microbes and/or their products)**¹¹⁴. In addition, both experimental and clinical evidence show modifications at multiple levels of the gut barrier, including the epithelium and immune system, which might in turn shape the microbial changes, allow microbes to translocate to the portal circulation, and eventually participate in alcohol-associated liver disease progression. We here focus on the **dysfunctional defense mechanisms** in the different compartments and cell types that finally contribute to the overall failure of the gut barrier.

1.7.1 Alcohol-associated changes in the gut microbiome

Most of the studies have investigated the changes in the microbiota composition looking specifically at bacteria. Chronic alcohol consumption can lead to intestinal bacterial overgrowth¹²², compositional changes in the bacterial microbiota¹²⁴, and elevated systemic levels of bacterial-derived products¹¹⁴. Several studies have assessed the changes in the intestinal microbiome in ALD¹²⁵. A comparison between these studies is not always possible since there are significant differences in design, data collection methodology and ALD stage. Recent advances in microbial sequencing technologies have allowed us to identify specific patterns of microbial alterations in patients with different stages of ALD. Culturing stool samples from AUD patients revealed decreased numbers of *Bifidobacterium*, *Lactobacillus*, and *Enterococcus* compared with healthy controls. In 2012, Mutlu *et al.* characterized the gut microbiota composition in alcohol use disorder patients using non-cultured, next-generation sequencing technologies and showed that only a subgroup of AUD subjects had altered colonic microbiota composition¹²⁶. Indeed, a third of subjects were defined as having a different microbiota mainly consisting of lower abundance of Bacteroidetes and higher ones of Proteobacteria in the colonic mucosa. Two other

^{*26} Fluorescence-activated cell sorting (FACS) is a technique to purify specific cell populations based on phenotypes/markers detected by flow cytometry.

studies of our own group confirmed the presence of intestinal dysbiosis in stool samples of AUD patients¹²⁷. We further demonstrated that alcoholics exhibit reduced numbers of the beneficial bacteria including *Lactobacillus* spp. and *Bifidobacterium* spp. Intriguingly, alcohol abstinence alone resulted in a restoration of suppressed levels of *Bifidobacterium* spp. and *Lactobacillus* spp¹²⁷.

Recent reports indicate alcohol-associated overgrowth of gut fungal populations¹²⁸, with reduced fungal diversity and a proportional increase in *Candida albicans* (*C. albicans*) and *Malassezia restricta* (*M. restricta*) in patients with alcohol-use disorder¹²⁹. Regarding the enteric viruses, only one study by Jiang et al. has evaluated the fecal virome of patients with ALD at different disease stage (e.g., severe alcohol-associated hepatitis, alcohol-use disorder) in comparison to healthy controls¹³⁰. Patients with severe alcohol-associated hepatitis were characterized by a marked increase in mammalian viruses, such as Parvoviridae and Herpesviridae¹³⁰. In addition, many bacteriophages were more abundant in patients with severe alcohol-associated hepatitis, including *Staphylococcus*, *Escherichia*, Enterobacteria, and *Enterococcus* phages¹³⁰.

Gut dysbiosis is likely one factor that contributes to increased microbial translocation, either directly or in an indirect manner. Overrepresented pathogenic microbes can attach and invade the host by yet not completely elucidated mechanisms. Moreover, loss of commensal microbes involved in different metabolic functions (e.g., transformation of bile acids, synthesis of vitamins, short-chain fatty acids and amino acids) can negatively affect the intestinal epithelium and immune system¹³¹.

1.7.2 Alcohol-associated changes in the intestinal epithelium

Several independent studies have shown alcohol-induced disruption of tight junction proteins (e.g., zonula occludens 1 (ZO-1), occludin, and claudins) and subsequent increased paracellular permeability, also called **leaky gut, primarily confined to the proximal small intestine**¹³². The exact molecular mechanisms of increased intestinal permeability remain however largely unknown. Studies conducted in animal models and using intestinal cell lines such as CaCo-2 have shown the potential involvement of inflammatory pathways in the dysregulation of tight junction proteins^{133,134}. However, no studies have identified pathways and mechanisms related to proximal small gut permeability in AUD patients at early pre-cirrhotic stages, in which additional

factors characterizing liver decompensation (e.g., portal hypertension) are not involved in the process¹³².

Both animal models and subjects with an alcohol-use disorder are characterized by increased production of mucus in the proximal small intestine¹³⁵. Mucin-2 deficiency in mice leads to amelioration of alcohol-induced liver disease. The protective effect was demonstrated to be dependent of changes in the composition of the microbiota and increased expression of Reg3 antimicrobial molecules resulted in reduced bacterial overload and translocation in mice¹³⁵. Moreover, glycosylation of the mucins in the intestine is impaired since Fut2, an enzyme involved in α 1–2-fucosylation at the cell surface of enterocytes and mucus¹³⁶, is downregulated in AUD patients¹³⁷. Membrane and secreted α 1–2-linked fucose can be cleaved by bacterial fucosidase, and the liberated L-fucose is utilized by certain bacteria¹³⁸. Fut2 deficiency could therefore induce overgrowth of potential pathogens in the mucosa. Deficiency of Fut2 exacerbates chronic ethanol-induced liver injury, steatosis, and inflammation in mice¹³⁷, supporting its protective role against alcohol-induced liver disease.

Chronic alcohol consumption reduces gene and protein expression of Reg3 γ and Reg3 β in murine small intestine and duodenal Reg3 γ in AUD patients, indicating impaired function of specialized Paneth cells¹²⁴. Deficiency in Reg3 lectins in mice enhances bacterial translocation into the liver with subsequent activation of the immune responses¹²⁴. Furthermore, Reg3 knockout mice chronically exposed to ethanol had worsened liver injury linked to increased bacterial translocation¹²². Interestingly, diminution of the antimicrobial molecules Reg3 are associated with increased number of mucosa-associated bacteria¹²².

The maintenance of a correct balance between proliferation and differentiation programs is crucial for functions of the gut barrier, such as correct absorption of nutrients and coordination of defense mechanisms against invading microbes. Mice chronically fed ethanol are characterized by damage of adult stem cells via dysregulation of Wnt/ β -Catenin signaling and reduced expression of stem cell markers such as Lgr5 and Bmi1¹³⁹. In contrast, animal models of chronic and binge alcohol consumption show crypt hyperplasia (increased crypt length) with enhanced proliferation through the Wnt/ β -Catenin and Erk1/2 signaling pathways¹⁴⁰. Currently, **human data about the intestinal epithelium of AUD patients are scarce** and not related to active drinking status. Epithelial alterations could influence both defective

defense mechanisms as well as malabsorption of nutrients, a well-known characteristic of patients with an alcohol use disorder¹⁴¹.

1.7.3 Alcohol-associated alteration in the gut immune system

Chronic alcohol consumption is associated with **impairment of both innate and adaptive immune responses**. How these alterations affect attachment and invasion of microbes in the gut mucosa in the different stages of ALD is still incompletely understood.

Macrophages in the lamina propria that produce TNF- α are increased in the proximal small intestine of mice chronically fed ethanol as well as in AUD patients¹⁴². Which subsets of macrophages produce this inflammatory cytokine remain unknown as well as its significance for defense mechanisms. Moreover, no studies so far have investigated the tolerogenic properties of intestinal macrophages in AUD patients.

Dendritic cells, together with macrophages, orchestrate immune responses in the gut barrier through presentation of the processed antigens to adaptive immune cells. Few studies have investigated the impact of alcohol abuse on dendritic cells. Acute alcohol intake alters the function and cytokine production of human monocyte-derived DCs in the blood circulation¹⁴³. Moreover, chronic alcohol consumption is associated with changes in the distribution, immunophenotype, and secretion of inflammatory mediators¹⁴⁴. Rhesus macaques chronically exposed to ethanol show a reduced number of bone marrow and circulating pools of myeloid dendritic cells, with suppression of a costimulatory molecule such as CD83, which may attenuate the ability of DCs to promote T cell expansion¹⁴⁵. These data support the detrimental effect of alcohol abuse on the function of dendritic cells. However, no study has characterized the effect of chronic alcohol consumption on intestinal DCs in mice and humans related to or suffering from ALD.

The number of intestinal IgA-secreting plasma cells is reduced in experimental models of ethanol-induced liver disease in mice¹⁴⁶. In humans, studies aimed at investigating IgA during ALD are scarce and contradictory. In one study, patients with decompensated cirrhosis had reduced intestinal secretion of IgA compared to compensated alcohol-associated cirrhosis¹⁴⁷. By contrast, two other studies reported similar levels of IgA secretion¹⁴⁸ and IgA-secreting plasma cells¹⁴⁹. Characterization of IgA levels with different methods as well as characterization of IgA-plasma cells at different stages of ALD and the possible impact of a period of abstinence before

admission may partially explain these conflicting observations. Future studies are needed to elucidate the role of IgA in the development of early and late stages of alcohol-associated liver disease.

Intestinal T cells may play an important role in gut barrier dysfunction during ALD. However, **the contribution of changes in intestinal T lymphocytes in defective immunosurveillance of microbes remains largely unknown, especially at early ALD stages.** Impaired adaptive immunity linked to defective microbial immunosurveillance is a characteristic of more advanced liver disease, such as alcoholic hepatitis and cirrhosis. Reduced mucosa-associated invariant T (MAIT) cells, which play an important role against bacterial infections, have been observed in advanced ALD along with compromised antibacterial and cytotoxic responses ⁵⁹.

1.8 HOW CAN GUT BARRIER DYSFUNCTION CONTRIBUTE TO ALD PROGRESSION?

The intestine and liver communicate via bidirectional links through the biliary tract, portal vein, and systemic circulation. The liver synthesizes and transports primary bile acids and antimicrobial molecules to the intestinal lumen through the bile duct. In the gut, the host and microbes metabolize endogenous (bile acids and amino acids) and exogenous substrates (dietary and environmental factors) into products that are then transported to the liver through the portal vein. Furthermore, the systemic circulation connects the gut-liver axis by transporting liver metabolites into the intestine ¹⁵⁰. Many of those products or metabolites (e.g., bile acids, fatty acids, ethanol metabolites) might act as signaling molecules at the local and distant sites where they activate various signaling processes (e.g., cell growth and differentiation, host metabolism, and immune responses) involved in regulation of the intestinal barrier ¹⁵¹. Changes in the levels and in the structure of these signaling molecules, for example, a different proportion of secondary and primary bile acids, can negatively affect the processes (e.g., intestinal permeability, epithelial differentiation, absorption of nutrients and immunity) essential for defense mechanisms in the gut. As a result, breakdown of gut barrier function allows microbes and/or their products to get into the liver and systemic circulation, where they are recognized by different pattern recognition receptors in various cell types with subsequent activation of immune responses (Figure 12). **According to the stage of ALD, the cell types, and signaling pathways involved, activation of a pro-inflammatory response against microbial antigens might be beneficial or detrimental for liver disease progression** ¹⁵². We here discuss some

recent findings that support the link between gut barrier dysfunction, systemic inflammation, and impaired hepatic immunity in the pathogenesis of early and advanced stages of ALD.

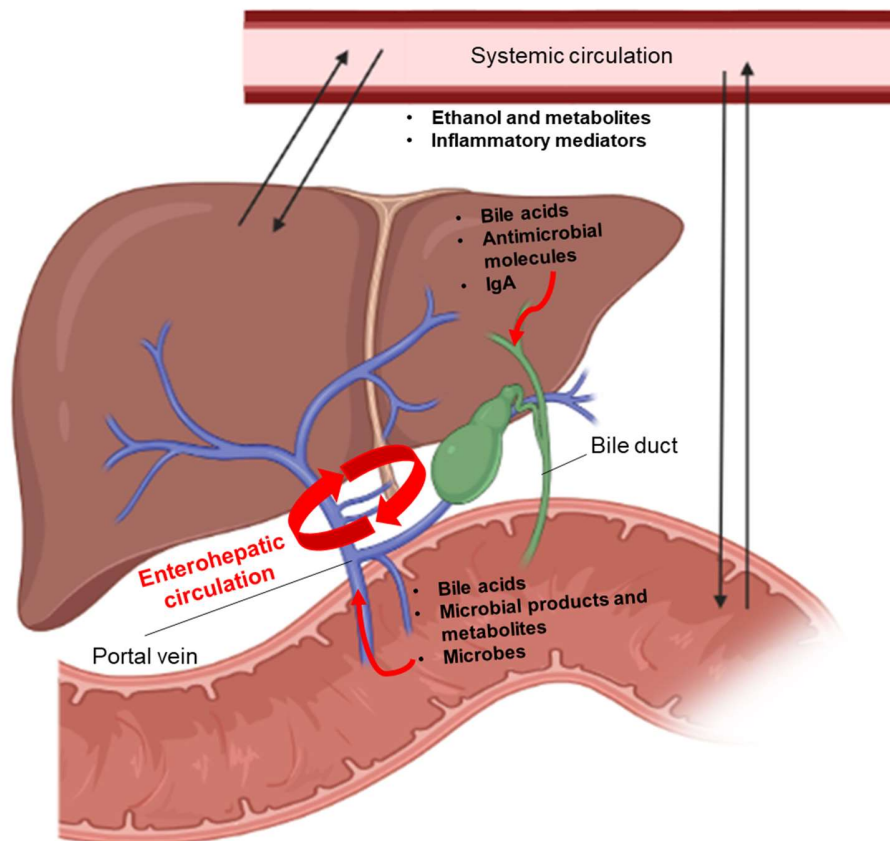


Figure 12. Bidirectional communication between the gut and the liver during Alcohol-associated liver disease. The liver produces primary bile acids, antimicrobial molecules, and IgA that are released in the intestine through the bile duct. In the intestine, these molecules contribute to shape the microbiota. In addition, primary bile acids are converted into secondary bile acids by the gut microbiota. Bile acids, which are reabsorbed in the terminal ileum, microbial products and metabolites, as well as viable microbes are transported to the liver through the portal vein. Once in the liver, they are implicated in triggering immune and inflammatory responses that might lead to liver disease. Moreover, the gut–liver axis is connected via the systemic circulation where ethanol, ethanol-derived metabolites, as well as other inflammatory mediators (cytokines, metabolites, etc.) can reach the two organs, thus influencing their functions. Maccioni L. et al., *Int J Mol Sci*, 2021 (Annex 1).

1.8.1 Systemic inflammatory responses

Increased systemic translocation of bacterial products, such as lipopolysaccharides (LPS) and peptidoglycans (PGN), have been shown to be associated with elevated plasma levels of inflammatory cytokines (IL1 β , IL8, IL18, TNF- α , and IL6) in AUD patients¹⁵³. Mechanistic analyses have shown that in AUD patients, **LPS and to a**

greater extent PGN can contribute to the activation of peripheral blood mononuclear cells (PBMCs). PBMCs are likely contributing to PGN-triggered release of **IL1 β , IL8, and IL18** into the blood ¹⁵³. In contrast, TNF- α and IL6 were not elevated in PBMCs, suggesting that their increased plasma levels might originate from other sources ¹⁵⁴. Inflammatory chemokine monocyte chemoattractant protein 1 (MCP-1) levels in the plasma are increased in actively drinking subjects compared to the controls ¹⁵⁵. This may promote **monocyte recruitment and infiltration** into the liver where they transform into inflammatory monocyte-derived macrophages. This process has been observed in mice chronically fed ethanol ¹⁵⁶ and it is believed to play an important role in the early pathogenesis of ALD ⁵⁰. Future studies are needed to clarify the pathways linked to this process. In contrast to the early stages of alcohol-associated liver disease, severe alcohol-associated hepatitis is characterized by massive hepatic infiltration of neutrophils coming from the circulation ⁵⁰. However, a recent investigation found that alcohol-associated hepatitis patients with more neutrophils had a better outcome than those with less neutrophil infiltration ⁵⁴, probably due to defective bacterial clearance and impaired liver regeneration.

1.8.2 Impairment of hepatic immune responses against microbes

Increasing evidence supports the role of unbalanced immunity in the different stages of ALD. When the gut barrier is disrupted, higher amounts of microbes and/or their products translocate to the liver ¹³², leading to the activation of the hepatic immune system through pattern recognition receptors like Toll-like receptors (TLRs) ⁶⁴. Animal models of ALD have shown that activation of the TLR4 signaling pathway plays an important role in ethanol- induced liver injury ⁴⁷. Other studies in mice show the pathophysiological importance of TLR4 in resident macrophages (e.g., Kupffer cells), monocyte-derived macrophages, and hepatic stellate cells ⁴⁸. The concept of TLR4 activation with subsequent TNF- α release has conducted to translational approaches such as the anti-TNF- α therapies for patients with severe alcoholic hepatitis. The disappointing outcome of these clinical trials ¹⁵⁷ highlight two main facts in the field of alcohol-associated liver disease: firstly, **data obtained in animal models of ALD cannot be necessarily extrapolated to humans** ¹⁵⁸; secondly, but not in order of importance, **inflammatory responses can be beneficial or detrimental depending on the cell type involved and stage of liver disease** (Figure 13).

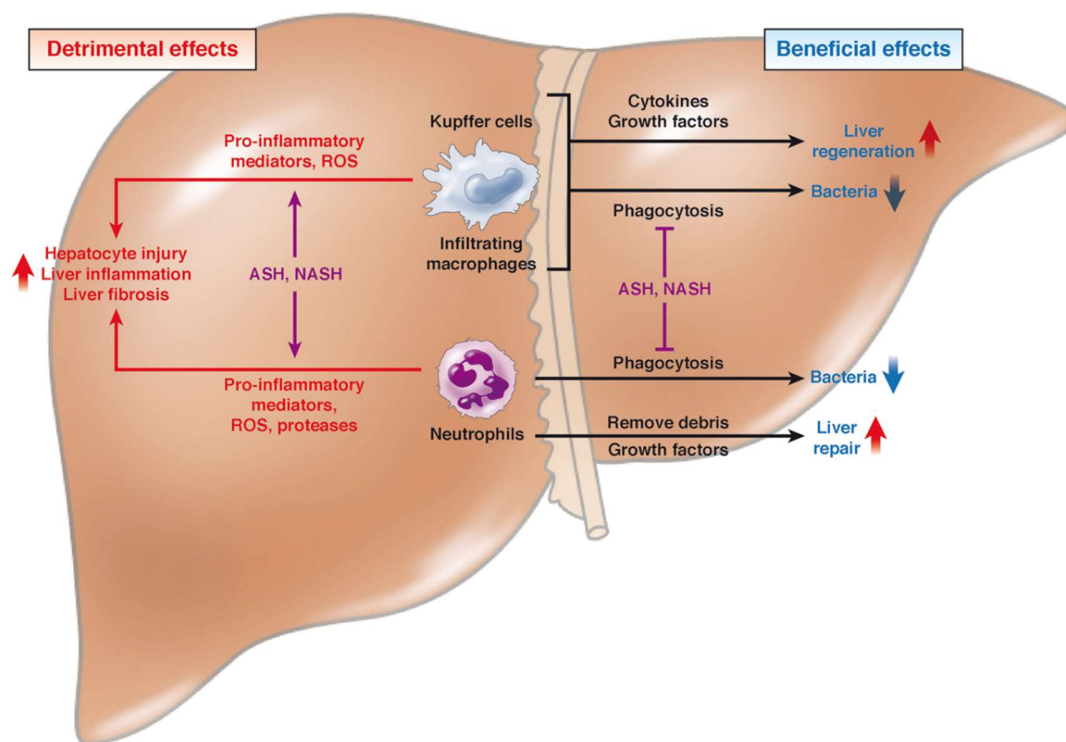


Figure 13. Detrimental and beneficial effects of innate immune cells in ALD and NAFLD, Gao B. and Tsukamoto H., *Gastroenterology*, 2016.

1.8.3 Limitations of animal models of alcohol-associated liver disease

To study the mechanisms implicated in ALD progression, mice and rats are the most widely used models. Murine models of chronic alcohol exposure have increased our knowledge about the potential role of the gut-liver axis in ALD evolution¹³². Changes of the gut microbiota composition^{159,124}, increased paracellular permeability^{160,161} and subsequent translocation of microbial products to the liver activate immune responses are implicated in ethanol-induced liver disease progression¹²⁵. Manipulations designed to interfere with this process, all improved liver disease in animals^{142,122,162,163,135}. However, these data cannot necessarily be extrapolated to human pathology for several reasons. Animals have a natural aversion to alcohol^{158,164}, a 5 times faster ethanol metabolism¹⁶⁵, and profound differences in their immune system¹⁶⁶ and their microbiota¹⁶⁷ compared to humans. Animals do only develop mild forms of ALD (e.g., simple steatosis with no or little inflammation) upon chronic alcohol feeding and do not resume the liver-damage pattern observed in humans¹⁶⁸.

Therefore, human studies conducted in **highly-standardized clinical settings** and aiming at investigating the role of the **gut-liver axis** are needed to **decipher molecular mechanisms implicated in the progression of ALD**. These mechanisms could be potentially targeted to ameliorate gut barrier function in alcohol use disorder patients with ALD and possibly prevent progression of liver disease. In contrast to other studies considering mainly advanced stages of liver disease, the aim of this PhD work was to primarily investigate potential mechanisms operating in humans at earlier stages of ALD where interventions would be the most meaningful.

AIMS AND STUDY DESIGN OF THE RESEARCH

AIMS

Changes in the intestinal barrier may trigger and promote the progression of alcohol-associated liver disease in humans. Several studies conducted principally in animal models have highlighted that increased gut permeability or “leaky gut” promotes translocation of microbes and/or their products, activation of intestinal and liver inflammatory responses, ultimately leading to ALD progression. Currently, human data able to bridge findings in animal models to human pathology in ALD are extremely scarce. The intestinal barrier represents the most complex barrier of the human body in which multiple cell types and factors contribute to the overall gut function. We are currently lacking a comprehensive characterization of gut barrier dysfunctions (microbial and host-related changes) which may drive progression of liver disease at early disease stages in humans.

Therefore, the aim of this PhD work is to evaluate intestinal barrier alterations in AUD patients and to investigate mechanisms linking gut barrier dysfunctions and activation of systemic and liver inflammatory responses involved in ALD progression at early pre-cirrhotic stages. We also investigated which factors may underlie the potential gut-liver interactions.

To follow the general aim, several hypotheses were generated step-by-step during the courses of the studies of this thesis. The following specific aims were addressed:

1. Only a minority of AUD patients develop progressive forms of ALD. The development of steatohepatitis and fibrosis in early pre-cirrhotic stages is usually silent. Hepatocytes cell damage is thought to play a major role in the progression of ALD. In the first chapter of this thesis, we aimed at **evaluating the diagnostic power of circulating keratin 18 levels**, a marker of liver cell damage, **for the detection of progressive forms of ALD**. We used a combination of non-invasive techniques to classify AUD patients into progressive and non-progressive ALD and we tested the diagnostic efficiency of K18-M65 in discriminating the two forms of the disease.
2. Alterations in the gut-liver axis (e.g., increased intestinal permeability, microbial translocation, changes in the gut microbiota and intestinal immune responses) may contribute to the progression of ALD. The second chapter of this thesis focused on the **characterization of the relationship between intestinal permeability, the duodenal and fecal microbiome, translocation of microbes and/or their**

products, and gut immunity in AUD patients and how these changes are associated with liver disease. To test this, we analyzed epithelial and vascular permeability, microbial translocation markers and the composition of the microbiota attached to the duodenal mucosa and in the stools of actively drinking AUD patients. We finally correlated the observed changes with the pattern of liver disease.

3. Little is known about the gut immune system in intestinal barrier dysfunction in at early stages of ALD in humans. Since elevated microbial translocation was associated with diminution of T cells, we aimed at characterizing in the third chapter of this thesis the **molecular changes in the duodenal T cell compartment** and to assess the **relationship between duodenal T lymphocytes and systemic translocation of microbes and/or their products** in AUD patients. We finally correlate the observed changes with the pattern and severity of liver disease.

4. Increased systemic translocation of bacterial products, such as Lipopolysaccharides (LPS) and Peptidoglycans (PGN) have been shown to be associated with **elevated plasma levels of inflammatory cytokines** (IL1 β , IL8, IL18, TNF- α and IL6) in AUD patients. Mechanistic analyses have shown that in AUD patients LPS and to a higher extent PGN can contribute to the activation of peripheral blood mononuclear cells (PBMCs). PBMCs are likely contributing to PGN-triggered release of IL1 β , IL8 and IL18 into the blood of AUD. Among PBMCs, **monocytes** represent the cell type with the highest potential to secrete these **cytokines**. Therefore, in the fourth chapter of this thesis we explored the **links between monocytes, microbial translocation, systemic inflammation, and ALD**.

5. Multiple factors (e.g., hepatocellular injury, impaired liver regeneration, activation of hepatic immune responses by gut-derived molecules) likely contribute to the progression of ALD from the “benign” form of the disease, namely simple steatosis. However, data in humans are scarce especially for early stages of ALD where rigorously standardized conditions for patient selection and sampling are required. The aim of the fifth chapter of this thesis was to assess **liver inflammatory and immune responses at early stages of ALD in humans and to correlate them with liver damage and repair mechanisms and/or progression of fibrosis**.

STUDY DESIGN OF THE RESEARCH

The studies reported in the thesis were all conducted to a similar design which is directly dependent on the alcohol-withdrawal program followed by AUD patients in our hospital Cliniques Universitaires Saint Luc, Brussels. This unit was created in 2003 in a collaborative project between the department of Hepato-gastroenterology and Psychiatry to allow **management of alcohol use disorder and favor/facilitate the possibility of developing a prolonged abstinence**. The alcohol withdrawal program is highly standardized. AUD patients arrived on Monday morning at the hospital, and they received vitamins B and most of them benzodiazepines for which the dose is adapted according to withdrawal symptoms. All AUD patients recruited for the study consumed alcohol until their admission in the unit or the day before. On the day of admission, Fibroscan® (Echosense, Paris, France) combined with the controlled attenuation parameter (CAP) were performed and a fasting blood sample (serum, plasma) was drawn. A 24 h urine collection for ^{51}Cr -EDTA evaluation was obtained on the second day and a gastroscopy with distal duodenal biopsies on the same day. Stools samples were collected from the first bowel movement after admission. In addition, patients underwent an abdominal Doppler ultrasound on the second day as part of the routine work-up in the unit. Liver biopsies were taken on Wednesday in patients with suspicion of liver disease. The rehabilitation program consisted in 2 weeks at the hospital separated by one week where they go back home (Figure 1). AUD patients who completed the withdrawal program were tested twice, at the beginning of the detoxification program (T1) as explained above, and at the end of the third week, after 17 days of abstinence (T2). The patients who relapsed during the week at home were excluded from the study at T2.

Additional inclusion criteria were first admission to the alcohol withdrawal unit and absence of previously diagnosed significant alcoholic liver disease.

Patients with diabetes were eligible for the study if they were on stable treatment (metformin, oral anti-diabetics with or without insulin) and a glycosylated hemoglobin level < 7% at admission.

Patients were excluded from the analysis if they had one of the following conditions:

- Inflammatory bowel disease or other chronic inflammatory or autoimmune diseases

- History of cancer within 5 years prior to admission
- Known liver disease of any other etiology
- Clinical signs of liver decompensation
- Clinically significant cardio-vascular, pulmonary, or renal co-morbidities
- Clinical and biochemical signs of active infection ($t^{\circ} > 37,5^{\circ}\text{C}$, viral syndrome, high C-reactive protein (CRP), and high White Blood cell count)
- Administration of antibiotics, probiotics, glucocorticoids, and nonsteroidal anti-inflammatory drugs during the two months preceding enrollment.

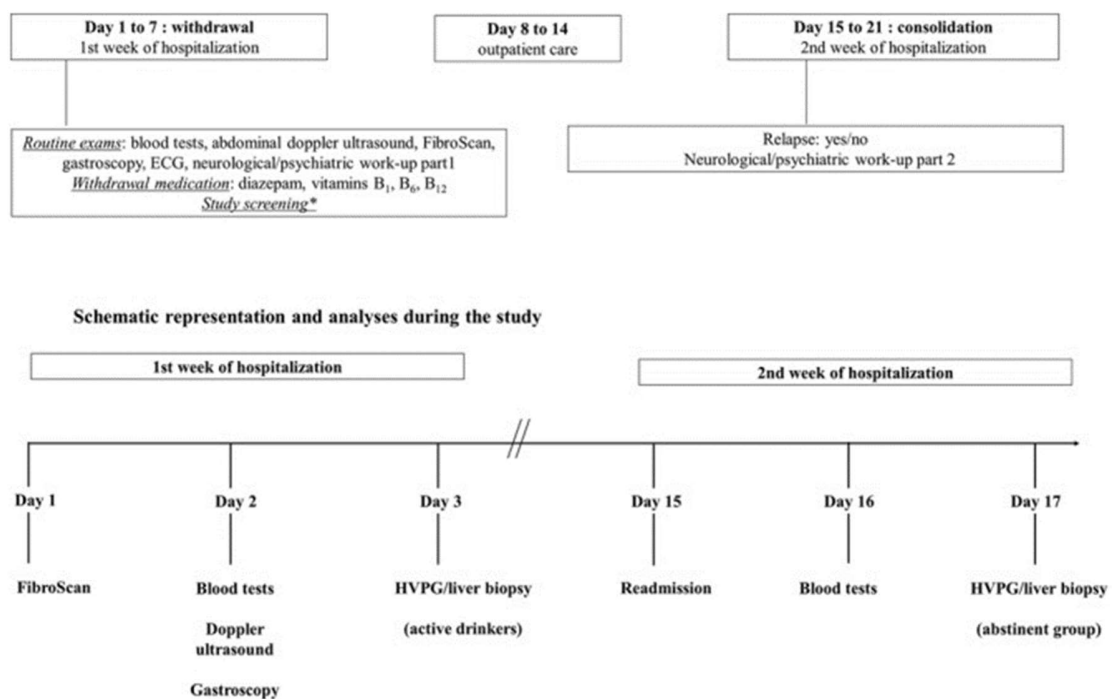


Fig.1 Standardized working scheme of the alcohol withdrawal unit.

RESULTS

CHAPTER 1: Circulating cytokeratin 18 detects progressive forms of alcohol-associated liver disease in early pre-cirrhotic stages

The diagnosis of severe forms of alcohol-associated liver disease (ALD) often requires a liver biopsy. However, this invasive technique is not readily accessible and carries the risk of potential life-threatening complications (e.g., bleeding). In routine clinical practice, the diagnosis of ALD is based on the performance of non-invasive techniques such as imaging for the detection of fibrosis and measurement of serum transaminases levels for liver cell damage. These techniques are not always able to discriminate progressive forms of ALD that precedes the development of liver decompensation, namely steatohepatitis and fibrosis, therefore, biomarkers capable of differentiating these silent forms of ALD from benign fatty liver are needed.

In our studies, progressive ALD is a combination of clinical markers the patients are presenting at admission to the unit. Minimal or non-progressive ALD is defined as no liver abnormalities or maximum simple steatosis and progressive ALD as clinical features of steatohepatitis (steatosis plus increased transaminases) or fibrosis, based on Fibroscan® evaluation. Since this evaluation represents just the status at admission without any longitudinal follow-up, our definition does not imply that liver disease in these patients will inevitably progress to more advanced stages in the future.

Since hepatocytes damage leads to the release in the blood circulation of keratin 18 fragments called K18-M65, we aimed at evaluating in this first study the diagnostic accuracy of circulating keratin 18 levels to detect progressive forms of ALD at early pre-cirrhotic stages. We tested the ability of K18-M65 to predict non-invasive measurements of progressive ALD as well as the relationship between this biomarker and liver inflammatory signatures linked to ALD physiopathology.

We here demonstrated the **high diagnostic value of keratin 18 fragment M65 (K18-M65)** for the early detection of progressive forms of ALD, namely steatohepatitis and fibrosis. Elevated K18-M65 levels are primarily markers of hepatocytes damage and can be used to detect “silent” severe forms of liver disease in early ALD.

The results of this first chapter have been submitted

Circulating cytokeratin 18 detects progressive forms of alcohol-associated liver disease in early pre-cirrhotic stages

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Acknowledgements: This study was supported in part by NIH grants R01 AA24726, R37 AA020703, U01 AA026939, U01 AA026939-04S1, by Award Number BX004594 from the Biomedical Laboratory Research & Development Service of the VA Office of Research and Development, and a Biocodex Microbiota Foundation Grant (to B.S.) and services provided by NIH centers P30 DK120515 and P50 AA011999, by grants from Fond National de Recherche Scientifique Belgium (J.0146.17 and T.0217.18) and Action de Recherche Concertée (ARC), Université Catholique de Louvain, Belgium to P.S.

Conflicts of interest: B.S. has been consulting for Ferring Research Institute, HOST Therabiomics, Intercept Pharmaceuticals, Mabwell Therapeutics, Patara Pharmaceuticals and Takeda. B.S.'s institution UC San Diego has received research support from Axial Biotherapeutics, BiomX, CymaBay Therapeutics, NGM Biopharmaceuticals, Prodigy Biotech and Synlogic Operating Company. B.S. is founder of Nterica Bio. UC San Diego has filed several patents with B.S. as inventor related to this work. P.S. received grant support from Gilead Sciences Belgium.

Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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ABSTRACT

Background Progression of alcohol-associated liver disease (ALD) in early pre-cirrhotic stages is a silent process. Circulating cytokeratin 18 levels (K18-M65) predict severe events and mortality in more advanced stages of ALD, but data in early stages are scarce. We aimed at evaluating the diagnostic accuracy of K18-M65 to detect progressive forms of early ALD.

Methods We prospectively included two cohorts of actively drinking patients (training (n=162) and validation (n=78)) with alcohol use disorder (AUD) who follow a rehabilitation program and matched healthy controls (n=21). Clinical and laboratory data (liver stiffness, controlled attenuation parameter (CAP), AST/ALT) served to distinguish AUD patients with simple steatosis (non-progressive) and steatohepatitis/fibrosis (progressive ALD). We measured serum K18-M65 levels and assessed its ability to predict progressive ALD.

Results High levels of K18-M65 characterized AUD patients with progressive ALD while the levels in the non-progressive ALD were similar to those in healthy controls (AUROC=0.8704; $p<0.0001$), with an optimal cut-off of 265.9 U/l. Circulating cytokeratin 18 highly correlate with transaminases and predicted independently from transaminases as well as other potential confounders CAP and liver stiffness. In addition, K18-M65 did not differentiate between fibrosis and steatohepatitis and were related to hepatocellular damage, as confirmed by the positive correlations with histological signs of hepatocellular damage and liver inflammation (all $p<0.05$). The K18-M65 cut-off identified was able to detect progressive ALD in a second validation cohort with high accuracy (sensitivity and specificity of 86.67% and 96.67%, respectively).

Conclusions Serum K18-M65 strongly predicts the progression of ALD at early pre-cirrhotic stages.

INTRODUCTION

Chronic alcohol consumption is one of the leading risk factors for population health worldwide. Alcohol-associated liver disease (ALD) encompasses a spectrum of injury, ranging from benign fatty liver, also called steatosis, to advanced stages including cirrhosis and eventually the development of hepatocellular carcinoma¹. Fatty liver develops in about 90% of individuals who drink more than 60 g/day of alcohol. Simple steatosis is usually asymptomatic and rapidly reversible upon abstinence. A subset of AUD patients progresses to steatohepatitis (ASH), which is characterized by the presence of steatosis and inflammation. Being clinically silent, this form of the disease can be suspected by the concomitant presence of steatosis and elevated transaminases and formally confirmed by a liver biopsies². In this early stage of the disease, fatty liver is associated with presence of hepatocellular injury (e.g., Mallory-Denk bodies, ballooning), inflammation (e.g., infiltration of inflammatory cells, elevated hepatic levels of immune mediators) without significant fibrosis. Some patients will progress to fibrosis, which develops as

the result of an imbalance between the new deposition of extracellular matrix (ECM) and its resorption, representing the wound healing response of the liver to repeated injury. The onset of liver fibrosis is usually subtle and most of the related morbidity and mortality occurs after the development of cirrhosis³.

Alcohol excess in conjunction with elevated serum aminotransferases (Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT)) are used to diagnose ALD. However, serum transaminases can be normal in certain patients even if fibrosis is present. Therefore, an appropriate evaluation of liver fibrosis with non-invasive techniques is fundamental⁴ for example with transient elastography (Fibroscan®)⁵.

Emerging evidence suggests an important role of different forms of hepatocyte cell death (e.g., apoptosis, necrosis) in the progression of ALD. Keratin 18 (K18) is the principal cytoskeletal protein of the hepatocytes, and it plays an important role in the maintenance of cellular integrity⁶. The cleavage of keratin 18 at two distinct sites (Asp238 and Asp396) during cell death results in the release of two different peptides in the systemic circulation: K18-M65, an epitope exposed to fragmented K18 released from dying cells, thus considered as a marker of hepatocyte damage, and K18-M30, a neo-epitope mapped to positions 387 to 396 of K18, so called K18-Asp396. K18-M30 is only revealed after caspase cleavage of the protein and is postulated as a selective biomarker of apoptosis⁶. The fact that these cleaved products of keratin 18 are released in the systemic circulation pave the way for their possible use as a diagnostic tool in early "silent" stages of ALD to potentially discriminate patients with steatohepatitis and fibrosis⁷.

We recently showed increased serum K18-M65 levels in AUD patients compared to healthy controls. The highest levels of K18-M65 were found in AUD patients with progressive forms of ALD (ASH and fibrosis). In another study, K18-M65 levels were strongly correlated with histological signs of liver cell damage while a weak correlation was found with liver fibrosis⁸. Hence, K18-M65 appears to be a promising biomarker for the non-invasive evaluation of progression of ALD⁹.

To better evaluate the diagnostic power of K18-M65 in the detection of progressive forms of ALD at early pre-cirrhotic stages, we here analyzed serum K18-M65 levels in a big cohort of actively drinking AUD patients undergoing a highly standardized rehabilitation program. We identified the best cut-off and we used it to validate in a second validation cohort of AUD patients. We finally correlated K18-M65 levels with histological signs of liver disease as well as with liver inflammatory markers using liver biopsies.

METHODS

Patients

Alcohol use disorder (AUD) patients (Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition criteria) admitted for elective alcohol withdrawal from October 2014 until October 2019 to dedicative alcohol withdrawal unit followed a highly standardized and controlled 3-week detoxification and rehabilitation program (Fig.1). They were compared to healthy volunteers matched for gender, age and BMI (social drinkers consuming <20g of alcohol/day). All patients reported long-term (>1year) alcohol consumption (>60g/day) and were actively drinking until the day of admission. Additional inclusion and exclusion criteria are available in Supplementary data. AUD patients of the validation cohort were admitted for elective alcohol withdrawal from October 2019 until December 2020. All clinical and baseline biochemical data were collected prospectively for all patients.

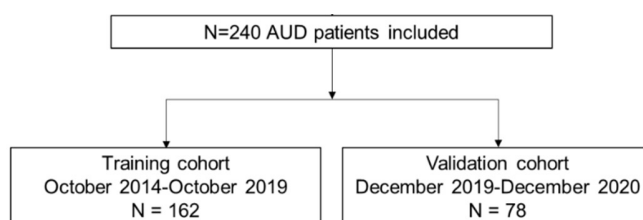


Fig.1 Study design for the training and validation cohorts of alcohol use disorder (AUD) patients.

Examinations and sample collections (Supplementary Fig.1)

On the day of admission, Fibroscan® (Echosense, Paris, France) combined with the controlled attenuation parameter (CAP) was performed and a fasting blood sample (serum, plasma) was drawn. In addition, patients underwent an abdominal Doppler ultrasound on the third day as part of the routine work-up in the unit. A transjugular liver biopsy was routinely proposed to all patients with suspicion of significant fibrosis $\geq F2$ on FibroScan (≥ 7.6 kPa, or doppler ultrasound with a dysmorphic liver or splenomegaly or alterations of the portal vein flow).

Ethical aspects

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institution's human research and ethical committee (B403201422657). Written informed consent was obtained from all patients and healthy volunteers.

Determination of serum biomarkers of liver cell damage

Serum keratin 18 (K18) was used to assess liver cell damage (K18-M65 ELISA kit; TECOmedical AG, Sissach, Switzerland). All assays were performed in duplicate following the manufacturer's instructions. Routine biochemical markers including AST, ALT, GGT, alkaline phosphatase, bilirubin, INR, albumin and creatinine were determined by the clinical biochemistry laboratory of the hospital.

Histological evaluation of ALD

Liver sections were stained with hematoxylin and eosin and Masson's trichrome blue (fibrosis). Histological grading of ALD was assessed by an experienced pathologist as previously described¹⁰ using semi-quantitative scores for steatosis, activity (hepatocellular injury and lobular neutrophils), cholestasis and fibrosis. Briefly, a semiquantitative evaluation method was applied using numerical scores for macrovesicular steatosis (0-3), hepatocellular ballooning (0-2), Mallory-Denk Bodies (MDB) (0-2), and lobular neutrophils (0-2). The SALVE staging system details 7 SALVE fibrosis stages (SFS) comprising 4 pre-cirrhotic (SFS 0, 1, 2, and 3), and 3 cirrhotic stages (SFS 4A, 4B, and 4C).

Reverse-Transcription and quantitative Real-Time Polymerase Chain Reaction

Liver tissue messenger RNA (mRNA) was assessed by quantitative real-time polymerase chain reaction (PCR) and quantified as described¹¹. Primer sequences are listed in supplementary material.

Statistics

Data were analyzed using Graph Pad Prism 9 (GraphPad Software, San Diego, CA, USA) and presented as mean $\pm$ standard error of the mean (SEM) unless otherwise indicated. Categorical clinical data between groups were compared by Chi-squared test. Normality was assessed by the Kolmogorov-Smirnov test followed by t-tests for normally distributed data, or the Wilcoxon test for nonnormally distributed data. Data were compared by one-way ANOVA for multiple groups, followed by Bonferroni's post hoc test for pairwise comparisons. Pearson's or Spearman's

correlation tests were used for correlations between data sets. A p value <0.05 was considered as statistically significant. Area under the receiver-operating characteristics curves (AUROC) were constructed using the method by Hanley and McNeil. Youden's statistics was performed for the determination of the threshold values. Multiple linear and logistic regressions were used to determine the independent factors associated with levels of biomarkers. Biomarker levels were Z score normalized before multivariate regression analyses.

RESULTS

Patients' characteristics

The study population of the training cohort consists of 162, middle-aged, predominantly male AUD patients and 21 healthy controls matched for age, gender, and BMI. Demographic and biochemical data are provided in Table 1. The characteristics of the validation cohort (n=78) were similar to the training cohort and are shown in supplementary table 1. All patients reported alcohol consumption until the evening prior to their admission.

Keratin 18 detects progressive forms of ALD

During damage of hepatocytes, a cleavage product of keratin 18 called K18-M65 is released into the circulation and could serve as a biomarker to distinguish progressive forms of alcohol-associated liver disease (e.g., steatohepatitis and fibrosis) from the more benign forms of ALD (e.g., simple fatty liver and no liver disease). We therefore wanted to investigate whether serum levels of K18-M65 could detect with high performance progressive forms of ALD. The pattern of liver disease in AUD patients was clinically classified as depicted in Table 2 and 3 (training and validation cohort, respectively) into non-progressive and progressive ALD. This classification was based on the presence/absence of elevated transaminases, presence of steatosis (CAP $\geq$ 250 dB/m) and the presence of significant fibrosis (liver stiffness $\geq$ 7.6 kPa). The non-progressive ALD group was defined as normal transaminases, with or without presence of steatosis and absence of fibrosis. The progressive ALD group was defined as steatohepatitis (increased levels of either AST or ALT and presence of steatosis) or fibrosis with or without steatohepatitis (Supplementary table 2 and 3 for the training and validation cohort, respectively). All the patients with steatohepatitis or fibrosis had a preserved synthetic liver function and showed no clinical signs of liver decompensation. Doppler ultrasound ruled out any significant vascular or biliary problem in the liver.

Table 1. Baseline demographic and biochemical data of the training population

<i>Demographics</i>				
	Healthy volunteers (n = 21)		AUD patients (n = 162)	p value
Gender (female/male)	7 (33%) / 14 (67%)		47 (29%) / 115 (71%)	.6829
	Mean ± SEM			
Age (years)	45.3 ± 9.5		48.2 ± 8.8	.2722
Height (m)	1.75 ± 0.07		1.73 ± 0.08	.2834
Weight (kg)	75.8 ± 10.6		74.1 ± 10.5	.6068
BMI	24.5 ± 3.1		24.6 ± 3.0	.8940
<i>Biochemistry</i>				
	Mean ± SEM (normal range)			
AST (IU/l)	17.8 ± 3.5		76.8 ± 56 (<40)	.0240
ALT (IU/l)	10.9 ± 2.9		57.6 ± 37.3 (<40)	.0039
γ-GT (IU/l)	22.4 ± 8.6		233.6 ± 223.6 (<40)	.0572
ALP (IU/l)	52 ± 11		80 ± 24 (30-120)	.0196
Bilirubin (mg/dl)	0.21 ± 0.13		0.6 ± 0.3 (0.3-1.2)	.0040
Albumin (g/l)	44 ± 0.8		45.97 ± 4.05 (35-52)	.3428

Abbreviations: BMI, body mass index; AST, Aspartate transaminase; ALT, Alanine transaminase; γ-GT, gamma-glutamyltransferase; ALP, alkaline phosphatase

Table 2. Characteristics of the patient's subgroups in the training AUD cohort based on clinical evaluation of ALD (Progressive vs Non-progressive)

<i>Demographics</i>				
	Non-progressive ALD (n = 67)		Progressive ALD (n = 95)	p value
Gender (female/male)	22 (33%) / 45 (67%)		25 (26%) / 70 (74%)	.3678
	Mean ± SEM			
Age (years)	47.8 ± 9.3		48.5 ± 8.4	.6996
Height (m)	1.72 ± 0.06		1.73 ± 9.5	.7805
Weight (kg)	72.2 ± 9.3		75.5 ± 11.2	.1346
BMI	24.2 ± 2.6		24.9 ± 3.3	.2295
<i>Biochemistry</i>				
	Mean ± SEM (normal range)			
AST (IU/l)	26.2 ± 6.6		113 ± 65.9 (<40)	<.0001
ALT (IU/l)	23.3 ± 7.8		82.1 ± 39.2 (<40)	<.0001
γ-GT (IU/l)	53 ± 30.8		360.5 ± 278 (<40)	<.0001
ALP (IU/l)	64.7 ± 14.3		90.8 ± 28.8 (30-120)	<.0001
Bilirubin (mg/dl)	0.48 ± 0.18		0.8 ± 0.4 (0.3-1.2)	<.0001
Albumin (g/l)	46.9 ± 2.8		45.8 ± 4.3 (35-52)	.2149
CAP (dB/m)	256 ± 46		316 ± 31 (<250)	<.0001
LS (kPa)	4.75 ± 0.95		11.4 ± 5.6 (<7.6)	<.0001

Table 3. Characteristics of the patient's subgroups in the validation AUD cohort based on clinical evaluation of ALD (Progressive vs Non-progressive)

<i>Demographics</i>			
	Non-progressive ALD (n = 33)	Progressive ALD (n = 45)	p value
Gender (female/male)	14 (42%) / 19 (58%)	11 (25%) / 34 (75%)	.0927
	Mean $\pm$ SEM		
Age (years)	49.9 $\pm$ 9.8	49.5 $\pm$ 7.7	.9350
Height (m)	1.73 $\pm$ 0.08	1.74 $\pm$ 0.07	.8580
Weight (kg)	72.1 $\pm$ 12.4	76.7 $\pm$ 11.4	.1798
BMI	24.8 $\pm$ 4.2	25.2 $\pm$ 3.3	.7175
<i>Biochemistry</i>			
	Mean $\pm$ SEM (normal range)		
AST (IU/l)	28.9 $\pm$ 7.1	103.6 $\pm$ 49.4 (<40)	<.0001
ALT (IU/l)	26.9 $\pm$ 8.7	85.7 $\pm$ 46.9 (<40)	<.0001
γ -GT (IU/l)	80.5 $\pm$ 51.3	350.8 $\pm$ 264.3 (<40)	<.0001
ALP (IU/l)	76.9 $\pm$ 18.2	94.6 $\pm$ 28.8 (30-120)	.0143
Bilirubin (mg/dl)	0.51 $\pm$ 0.23	0.8 $\pm$ 0.5 (0.3-1.2)	.0255
Albumin (g/l)	44.9 $\pm$ 2.2	45.7 $\pm$ 2.7 (35-52)	.6826
CAP (dB/m)	255 $\pm$ 40	311 $\pm$ 35 (<250)	<.0001
LS (kPa)	5.1 $\pm$ 1	12.2 $\pm$ 8.5 (<7.6)	.0026

High levels of K18-M65 characterized AUD patients with progressive ALD while the levels in the non-progressive ALD were similar to those in healthy controls (Fig.2A). Remarkably, K18-M65 levels did not separate AUD patients with steatohepatitis compared to fibrosis (Supplementary Fig.2), supporting that K18-M65 is a marker related to liver cell damage regardless of fibrosis. Receiver operating curve (ROC) analysis indicated that serum K18-M65 levels allowed to distinguish with high accuracy progressive ALD from the non-progressive forms of liver disease (AUROC=0.8704; 95%CI: 0.8143-0.9264; $p < 0.0001$) (Fig.2B). Youden's statistics identified an optimal cut-off of 265.9 U/l with the best specificity and sensitivity profile (79.1% and 85.26%, respectively) for separating both groups.

We next evaluated whether the K18-M65 cut-off identified in the training cohort was able to detect progressive ALD in our validation cohort. Similar high K18-M65 levels were again found in the progressive ALD group (Supplementary Fig.3), again independently on presence of fibrosis (Supplementary Fig.4). Sensitivity and specificity in this validation cohort were 86.67% and 96.67%, respectively. The cut-off of 265.9 U/l for K18-M65 predicted progressive ALD with high accuracy as indicated by a very high positive likelihood ratio (28.6) and low negative likelihood ratio (0.14) (Table 4).

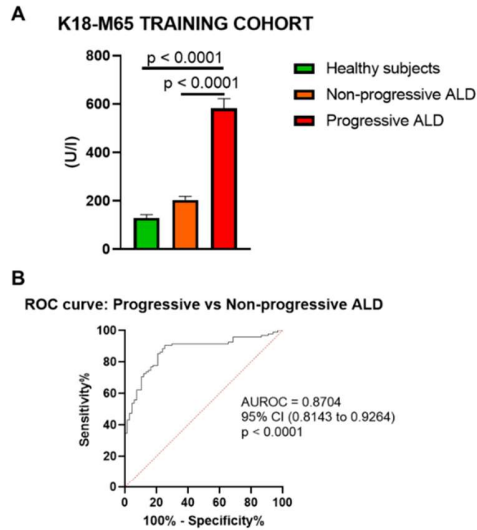


Fig.2 Serum cytokeratin 18 (K18-M65) detects progressive forms of alcohol-associated liver disease (ALD). High serum K18-M65 levels characterized progressive ALD (n=95) compared to non-progressive ALD (n=67) and healthy controls (n=21) (A). Circulating cytokeratin 18 levels differentiate severe forms of alcoholic liver disease from the non-progressive forms of alcoholic liver disease, as shown by Receiver Operating Characteristic (ROC) analysis (B) (AUROC= 0.8704; 95% CI: 0.8143-0.9264; $p < 0.0001$).

Table 4. Statistical analysis of serum CK18-M65 levels as a biomarker of progressive ALD in the validation AUD cohort

Statistics	Value	95% CI
Positive Likelihood Ratio	28.6	4.14 to 197.73
Negative Likelihood Ratio	0.14	0.07 to 0.29
Sensitivity (%)	86.67	73.21 to 94.95
Specificity (%)	96.97	84.24% to 99.92%

Cytokeratin 18 highly correlates with liver cell damage

Since our classification was based on biochemical and imaging data on liver cell damage, steatosis, and fibrosis, we next evaluated the relation between K18-M65 levels and these parameters in the training cohort. Serum K18-M65 levels highly correlated with transaminases ($r = 0.6796$, $r = 0.6628$ and $r = 0.5989$ for AST, ALT, and γ -GT, respectively; all $p < 0.0001$). A weaker but significant correlation was found between K18-M65 and imaging data on steatosis (CAP) and liver stiffness (Table 5). Although both transaminases correlated with K18-M65, the

AST/ALT ratio did not ($r=0.0397$; $p = 0.5415$). Similar correlations were seen in the validation cohort, with cytokeratin 18 correlating higher with AST and ALT levels compared to CAP and liver stiffness (Supplementary Table 4), confirming that K18-M65 levels are primarily related to hepatocytes damage.

Table 5. Spearman's correlations between CK18-M65 levels and biochemical and imaging data in the training AUD cohort

	CK18-M65
AST	****0.6796
ALT	****0.6628
γ -GT	****0.5989
ALP	****0.3087
CAP	****0.4533
LS	****0.4467

**** = $p < 0.0001$

Cytokeratin 18 is an independent predictor of severe forms of ALD

We next evaluated whether K18-M65 levels were an independent predictor of progressive ALD. To evaluate this, since the diagnostic power of cytokeratin 18 and correlations were confirmed in the two cohorts, we pooled the training and validation cohort. We then assessed the predictive independence of K18-M65 from possible confounding factors known to be associated with a higher risk of more severe liver disease (e.g., age, gender, BMI) as well as the independence of K18-M65 from transaminases levels and liver stiffness. We found that K18-M65, AST, ALT and liver stiffness independently predicts progressive forms of ALD, with the highest odd ratio found for cytokeratin 18 (Odd ratio 25.81 with 95% CI 3.166 to 336.1; $p < 0.0001$) (Table 6).

As transaminases levels were used in our classification together with imaging data, we then asked if circulating cytokeratin 18 could bring some information more than AST, ALT levels related to steatosis and fibrosis. By using multivariate regression analysis, we therefore tested whether cytokeratin 18 and/or AST, ALT levels could predict CAP and liver stiffness. Interestingly, K18-M65 but not AST/ALT predicted CAP while both cytokeratin 18 and transaminases predicted independently liver stiffness (Supplementary table 5 and 6), indicating that K18-M65 can bring some information more on the pathological status on the liver independently on transaminases. Remarkably, ALT levels negatively predicted liver stiffness by contrast of AST.

Table 6. Multivariate logistic regression analysis to test independent prediction of progressive ALD by using CK18-M65 levels

Variable	Odds ratios	95% CI	p value
Age	0.9521	0.8681-1.032	0.2527
BMI	1.019	0.8356-1.252	0.8486
Gender	1.807	0.2392-16.74	0.5736
K18-M65	25.81	3.166-336.1	0.0055
AST	1.108	1.048-1.197	0.0019
ALT	1.092	1.007-1.204	0.0474
LS	3.832	2.229-9.054	0.0001

Relationship between histological signs of ALD, inflammation, and cytokeratin 18

We finally wanted to evaluate whether serum K18-M65 levels correlated with histological signs of ALD and/or liver gene expression of inflammatory mediators. To do this, we first assessed histological severity of ALD in 27 liver biopsies obtained from AUD patients of the two cohorts and we correlated these scores with serum K18-M65 levels. We found a positive correlation between K18 and hepatocytes ballooning ($p < 0.05$) but not with fibrosis scores.

In addition, we evaluated K18-M65 in relation to mRNA levels of inflammatory mediators involved in the progression of ALD (cytokines and pro-fibrogenic factors). Interestingly, K18-M65 significantly correlated with the inflammatory mediator IL8 ($p < 0.05$) but not with factors implicated in fibrosis (e.g., Col1 α 1, α -SMA and Timp1) (Table 7).

Overall, these results further indicate the relation of K18-M65 with hepatocytes damage and inflammation, regardless of fibrosis.

Table 7. Spearman's correlations between CK18-M65 levels and histological signs of liver disease as well as liver inflammatory parameters

	CK18-M65
IL8	*0.3896
Col1a1	0.0271
a-SMA	-0.2131
Timp1	-0.0412

*= p<0.05

DISCUSSION

We here showed the high diagnostic value of cytokeratin 18 fragment M65 (K18-M65) for the early detection of progressive forms of alcohol-associated liver disease, namely steatohepatitis and fibrosis. High K18-M65 levels are primarily markers of hepatocytes damage and could be used to detect "silent" severe forms of liver disease in early pre-cirrhotic stages of ALD.

The early and non-invasive diagnosis of ALD is crucial to allow a correct control of disease progression. This study demonstrated that circulating levels of K18-M65 can be useful in the non-invasive diagnosis of progressive ALD. By contrast of other studies aiming at investigating the value of K18-M65 in more advanced stages of ALD (e.g., decompensated cirrhosis and severe alcoholic hepatitis)^{12,13,14}, our research primarily focuses on the detection of liver disease at early stages of ALD. We found high serum levels of K18-M65 in AUD classified, according to transaminases, liver stiffness and CAP measurements, as patients having progressive forms of ALD. Interestingly, circulating cytokeratin 18 levels were similar in AUD with benign forms of the disease (e.g., simple steatosis and no liver disease) compared to healthy controls, showing no or minimal liver cell damage at these stages of ALD. High K18-M65 levels did not differ significantly in AUD with steatohepatitis compared to fibrosis and did not correlate with liver gene expression of pro-fibrogenic factors. This result confirms a previous report showing a weak correlation between cytokeratin 18 and fibrosis evaluation⁸. By contrast, circulating cytokeratin levels highly correlated with serum transaminases, histological signs of hepatocytes damage and liver gene expression of the inflammatory mediator IL8. This point to a link between K18-M65 release into the blood circulation, liver cell damage and hepatic inflammation.

Another major finding of this study is that this biomarker was able to predict progressive ALD in a second validation cohort with high accuracy. We identified the best cut-off (265.9 U/l) with high sensitivity and specificity and validated it in the second big cohort of AUD patients with a high

performance. Moreover, the predictive value of circulating K18-M65 was independent from possible confounding factors, such as age, gender and BMI. A main strength of this investigation is the unique patient cohorts of a high number of heavily, actively drinking AUD patients in whom collections of the blood and imaging techniques have been performed in a strict, highly standardized clinical program. Other studies have reported the diagnostic value of K18-M65 in discriminating progressive forms of fatty liver disease with different etiology, namely patients with benign non-alcoholic fatty liver disease (NAFLD) from non-alcoholic steatohepatitis (NASH)^{15,16,17}. Our study extends current data on the use of circulating cytokeratin 18 levels also to early stages of ALD. The high diagnostic power of our integrated approach might prove useful for monitoring liver disease progression and/or regression in AUD patients.

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Supporting material/data

Cytokeratin 18 correlates with severity of alcohol-associated liver disease in patients with an alcohol use disorder

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Supplementary material

- Patients:
 - *Inclusion-exclusion criteria*
- Methods
 - *Fibroscan® measurements*
 - *Transjugular liver biopsy and tissue sampling*
 - *Primers Quantitative Real-Time Polymerase Chain Reaction*
- Results
- Supplementary figures

Patients, methods

Inclusion-exclusion criteria

Additional inclusion criteria were first admission to the alcohol withdrawal unit and absence of previously diagnosed significant alcohol-associated liver disease.

Patients with diabetes were eligible for the study if they were on stable treatment (metformin, oral anti-diabetics with or without insulin) and a glycosylated hemoglobin level < 7% at admission.

Patients were excluded from the analysis if they had one of the following conditions:

- Inflammatory bowel disease or other chronic inflammatory or autoimmune diseases
- History of cancer within 5 years prior to admission
- Known liver disease of any other etiology
- Clinically significant cardio-vascular, pulmonary or renal co-morbidities
- Clinical and biochemical signs of active infection ($t^{\circ} > 37,5^{\circ}\text{C}$, viral syndrome, high CRP and high White Blood cell count)
- Administration of antibiotics, probiotics, glucocorticoids and nonsteroidal anti-inflammatory drugs during the two months preceding enrollment.

Fibroscan® measurements

Transient elastography measurements were performed using the Fibroscan® device (Echosens, Paris, France) by an experienced examiner blinded to the patient's data. Fibroscan® was considered as valid if at least 10 validated measurements were obtained with an interquartile range of less than 30%. The final result expressed in kPa was the median of all valid measurements obtained. We used the cut-offs for alcoholic patients proposed by Nguyen-Khac et al. (Aliment Pharmacol Ther. 2008; 28:1188-98), i.e. 7.6, 11, 19.5 kPa for F2, F3 and F4, respectively.

Controlled attenuation parameter (CAP) was obtained during the same procedure. We used a median cut-off of 250 dB/m to diagnose steatosis as suggested by Kalas T et al. (J Hepatol 2017; 66:1022–1030). An interquartile range of 50 dB/m was deemed acceptable to validate the result.

Transjugular liver biopsy and tissue sampling

Liver biopsy was performed using a transjugular liver access and biopsy needle (16G) set (Cook, Bjaeverskov, Denmark). After administration of local anaesthesia to the neck, a catheter was introduced into the right jugular vein and guided under fluoroscopy into the right main hepatic vein. Separate tissue samples were taken and routinely fixed in formalin, snap-frozen in liquid nitrogen, and preserved in RNAlater solution (Invitrogen, Carlsbad, CA). After a complete routine histopathological examination of the biopsy samples by an experienced liver pathologist, surplus tissue was recovered for further research purposes.

Biopsy samples of ≥15 mm in length, including a minimum of 6 portal tracts, were considered suitable or when the pathologist could establish the degree of fibrosis with certainty on a smaller sample.

Quantitative Real-Time Polymerase Chain Reaction

Primers were as follows:

IL8: forward (F)-5'- CTAGGACAAGAGCCAGGAAGAAAC-3' and reverse (R)-5'- CGGCCAGCTTGGAAGTCA-3';

Col1a1: (F)-5'- GCAAGACAGTGATTGAATACAAAACC-3' and (R)-5'- ACGTCGAAGCCGAATTCCT-3';

α-SMA: (F)-5'- CAAGTGATCACCATCGGAAATG-3' and (R)-5'- GACTCCATCCCGATGAAGGA-3';

Timp1: (F)-5'- GCTGACATCCGGTTCGTCTAC-3'; (R)-5'- GTTGTGGGACCTGTGGAAGTATC-3';

Rpl-19: (F)-5'-CAAGCGGATTCTCATGGAACA-3'; (R)-5'-TGGTCAGCCAGGAGCTTC T-3'.

Supplementary table 1. Baseline demographic and biochemical data of the validation AUD cohort

<i>Demographics</i>	
	AUD patients (n = 78)
Gender (female/male)	25 (32%) / 53 (68%)
Age (years)	49.6 ± 8.6
Height (m)	1.74 ± 0.08
Weight (kg)	74.8 ± 12.1
BMI	25.1 ± 3.7
AST (IU/l)	72.0 ± 46.1
ALT (IU/l)	60.8 ± 40.3
γ-GT (IU/l)	236.4 ± 204.5
ALP (IU/l)	87.1 ± 23.8
Bilirubin (mg/dl)	0.68 ± 0.37
Albumin (g/l)	45.6 ± 2.5

Supplementary Table 2. Characteristics of the patient's subgroups in the training AUD cohort based on clinical evaluation of ALD

	Non-progressive liver disease (n=67)		Progressive liver disease (n=95)	
	No liver disease (n=30)	Simple steatosis (n=37)	Steatohepatitis (n=35)	Fibrosis (n=60)
	AST and ALT < 40 IU/L	AST and ALT < 40 IU/L	AST and/or ALT > 40 IU/L	With or without AST/ALT > 40 IU/L
<i>Clinical parameters</i>	CAP < 250 dB/m	CAP > 250 dB/m	CAP > 250 dB/m	With or without CAP > 250 dB/m
	kPa < 7.6	kPa < 7.6	kPa < 7.6	kPa > 7.6
<i>Clinical data</i>				
AST (IU/l; N<40)	23.1 ± 4.45	28.7 ± 7.38	89.5 ± 34.14	126.31 ± 80.78
ALT (IU/l; N<40)	19.83 ± 5.11	26.1 ± 8.9	80.24 ± 26.92	83.15 ± 46.22
γ-GT (IU/l; N<40)	43.8 ± 24.2	60.11 ± 34.72	249.32 ± 173.96	423 ± 328
ALP (IU/l; N<130)	61.38 ± 11.15	67.42 ± 16.06	74.32 ± 15.72	100.15 ± 34.23
Bilirubin (mg/dl; N<1.2)	0.46 ± 0.18	0.49 ± 0.17	0.63 ± 0.29	0.89 ± 0.47
Albumin (g/l; N>35)	47 ± 2.9	46.8 ± 2.9	47 ± 3.6	44.4 ± 5.5
CAP (dB/m)	207 ± 24	295 ± 31	317 ± 26	314 ± 37
(LS) kPa	4.97 ± 1.08	4.58 ± 0.86	5.92 ± 1.14	14.6 ± 7.1

Supplementary Table 3. Characteristics of the patient's subgroups in the validation AUD cohort based on clinical evaluation of ALD

	Non-progressive liver disease (n=33)		Progressive liver disease (n=45)	
	No liver disease (n=15)	Simple steatosis (n=18)	Steatohepatitis (n=26)	Fibrosis (n=19)
	AST and ALT < 40 IU/L	AST and ALT < 40 IU/L	AST and/or ALT > 40 IU/L	With or without AST/ALT > 40 IU/L
<i>Clinical parameters</i>	CAP < 250 dB/m kPa < 7.6	CAP > 250 dB/m kPa < 7.6	CAP > 250 dB/m kPa < 7.6	With or without CAP > 250 dB/m kPa > 7.6
<i>Clinical data</i>				
AST (IU/l; N<40)	24.3 ± 5.7	32.9 ± 7.1	102.7 ± 48.9	104.8 ± 49.6
ALT (IU/l; N<40)	20.4 ± 6.1	32.3 ± 9.6	96.6 ± 47.7	70.8 ± 41.6
γ-GT (IU/l; N<40)	61.3 ± 43.2	96.6 ± 56.5	194.7 ± 100.3	564.2 ± 388.9
ALP (IU/l; N<130)	76.9 ± 20.2	77.0 ± 16.6	84.0 ± 18.8	109.1 ± 35.9
Bilirubin (mg/dl; N<1.2)	0.48 ± 0.21	0.52 ± 0.24	0.66 ± 0.27	1.04 ± 0.74
Albumin (g/l; N>35)	46 ± 2	45 ± 2.2	46.9 ± 2.1	44.1 ± 3.5
CAP (dB/m)	215 ± 28	284 ± 28	312.9 ± 21.6	309 ± 54
(LS) kPa	4.5 ± 1.1	5.5 ± 0.8	5.8 ± 0.9	20.9 ± 14.1

Supplementary table 4. Spearman's correlations between CK18-M65 levels and biochemical and imaging data in the validation AUD cohort

	CK18-M65
AST	****0.7846
ALT	****0.7169
γ-GT	****0.6364
ALP	0.0966
CAP	****0.5419
LS	****0.4513

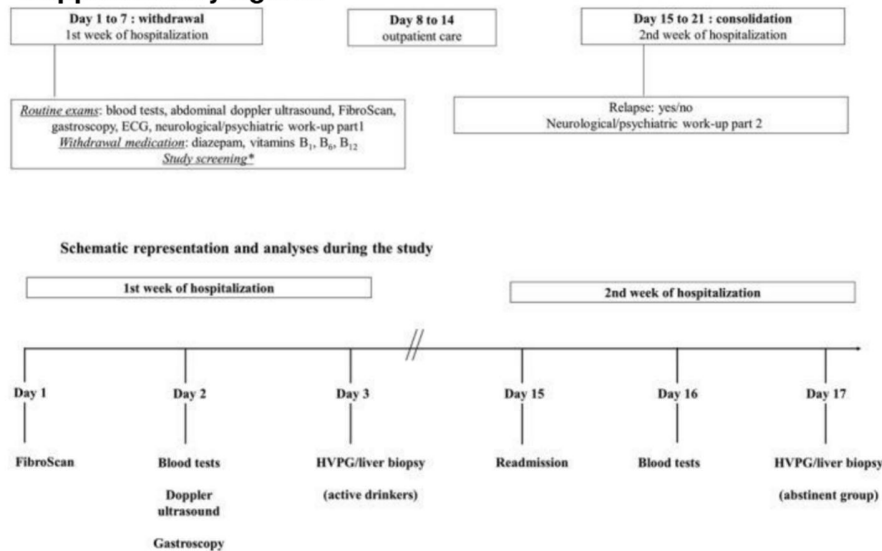
Supplementary table 5. Multivariate linear regression analysis for the prediction of controlled attenuation parameters (CAP) values

	Estimate	p value
Age	0.7999	0.0178
AST	8.776	0.32
ALT	9.087	0.2363
BMI	5.884	<0.0001
Gender	-3.866	0.6145
M65	10.34	0.0142
Liver stiffness	-0.6149	0.1228

Supplementary table 6. Multivariate linear regression analysis for the prediction of liver stiffness values

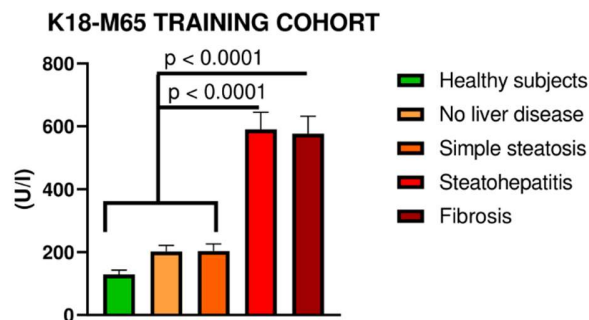
	Estimate	p value
Age	0.1315	0.0326
AST	10.08	<0.0001
ALT	-7.43	<0,0001
BMI	0.8172	<0,0001
Gender	-0.5446	0.697
M65	1.599	0.0379
CAP	-0.02041	0.1228

Supplementary figures



Supplementary Figure 1. Standardized working scheme of the alcohol withdrawal unit.

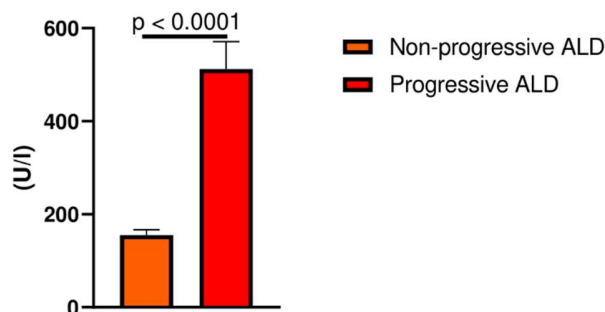
Alcohol use disorder (AUD) patients are undergoing a highly standardized clinical work-up with fixed sampling of biological specimens.



Supplementary Figure 2. Serum cytokeratin 18 (K18-M65) according to different stages of liver disease in the training cohort.

Elevated K18-M65 was only found in progressive ALD (steatohepatitis and fibrosis) independently from the development of fibrosis.

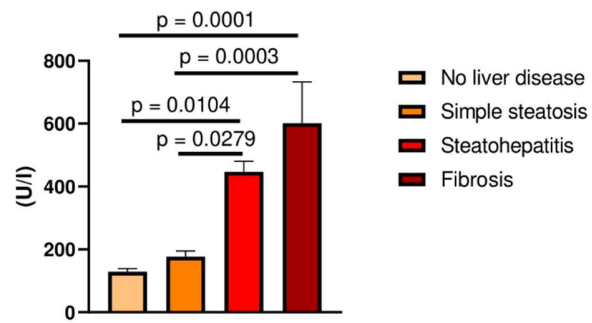
K18-M65 VALIDATION COHORT



Supplementary Figure 3. Evaluation of serum K18-M65 levels in the validation cohort.

High levels of K18-M65 were found in patients with progressive alcohol-associated liver disease (ALD) (n=45) compared to non-progressive ALD (n=33).

K18-M65 VALIDATION COHORT



Supplementary Figure 4. Serum cytokeratin 18 (K18-M65) according to different stages of liver disease in the validation cohort.

Elevated K18-M65 was only found in progressive ALD (steatohepatitis and fibrosis) independently from the development of fibrosis.

CHAPTER 2: Intestinal permeability, microbial translocation, changes in duodenal and fecal microbiota and their associations with alcoholic liver disease in humans

Data obtained in animals chronically exposed to ethanol have highlighted a role of the gut-liver axis in the development of alcohol-associated liver disease. Changes in the composition of the gut microbiota, increased intestinal permeability, subsequent translocation of microbes and/or their products from the gut lumen to the liver could lead to the activation of gut and hepatic immune responses implicated in disease progression. The first aim of this study was to evaluate the relationship between leaky gut, the duodenal and fecal microbiome, gut immune responses and microbial translocation in actively drinking AUD patients. The second aim was to assess whether these changes are associated with liver disease. Finally, we evaluated whether a short period of abstinence was able to restore alcohol-related changes in the gut and liver cell damage.

We showed that **only a subgroup of AUD patients had increased intestinal permeability linked to changes in the fecal microbiota**. We further demonstrated that **alterations in the duodenal mucosa-associated microbiota together with elevated microbial translocation are associated with liver disease progression**. Intriguingly, the **increase** of surrogate markers for Gram+, Gram-, and fungal **microbial translocation** in the blood of AUD patients **occurred independently from leaky gut but was associated with a diminution of duodenal effector immune cells**, such as macrophages and T cells.

The results of this second chapter have been published in *Gut Microbes* 2020, Issue 1, Volume 12

Intestinal permeability, microbial translocation, changes in duodenal and fecal microbiota, and their associations with alcoholic liver disease progression in humans

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To cite this article: Luca Maccioni , Bei Gao , Sophie Leclercq , Boris Pirlot , Yves Horsmans , Philippe De Timary , Isabelle Leclercq , Derrick Fouts , Bernd Schnabl & Peter Stärkel (2020): Intestinal permeability, microbial translocation, changes in duodenal and fecal microbiota, and their associations with alcoholic liver disease progression in humans, Gut Microbes

To link to this article: <https://doi.org/10.1080/19490976.2020.1782157>



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Published online: 26 Jun 2020.



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Intestinal permeability, microbial translocation, changes in duodenal and fecal microbiota, and their associations with alcoholic liver disease progression in humans

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ABSTRACT

Background: Animal data suggest a role of the gut-liver axis in progression of alcoholic liver disease (ALD), but human data are scarce especially for early disease stages.

Methods: We included patients with alcohol use disorder (AUD) who follow a rehabilitation program and matched healthy controls. We determined intestinal epithelial and vascular permeability (IP) (using urinary excretion of ⁵¹Cr-EDTA, fecal albumin content, and immunohistochemistry in distal duodenal biopsies), epithelial damage (histology, serum iFABP, and intestinal gene expression), and microbial translocation (Gram – and Gram + serum markers by ELISA). Duodenal mucosa-associated microbiota and fecal microbiota were analyzed by 16 S rRNA sequencing. ALD was staged by Fibroscan® (liver stiffness, controlled attenuation parameter) in combination with serum AST, ALT, and CK18-M65.

Results: Only a subset of AUD patients had increased ⁵¹Cr-EDTA and fecal albumin together with disrupted tight junctions and vasculature expression of plasmalemma Vesicle-Associated Protein-1. The so-defined increased intestinal permeability was not related to changes of the duodenal microbiota or alterations of the intestinal epithelium but associated with compositional changes of the fecal microbiota. Leaky gut alone did not explain increased microbial translocation in AUD patients. By contrast, duodenal dysbiosis with a dominance shift toward specific potential pathogenic bacteria genera (*Streptococcus*, *Shuttleworthia*, *Rothia*), increased IP and elevated markers of microbial translocation characterized AUD patients with progressive ALD (steato-hepatitis, steato-fibrosis).

Conclusion: Progressive ALD already at early disease stages is associated with duodenal mucosa-associated dysbiosis and elevated microbial translocation. Surprisingly, such modifications were not linked with increased IP. Rather, increased IP appears related to fecal microbiota dysbiosis.

ARTICLE HISTORY

Received 21 March 2020
Revised 20 May 2020
Accepted 3 June 2020

KEYWORDS

Alcohol; liver disease; dysbiosis; microbiota; gut barrier; microbial translocation; CK-18; alcohol use disorder; alcohol abstinence

Introduction

Alcoholic liver disease (ALD) is one of the leading causes of chronic liver disease worldwide. Although around 90% of patients with alcohol use disorders (AUD) develop steatosis, only a minority progress to the more severe forms of liver disease and its related complications. Currently, the main mechanisms involved in disease progression are not completely understood¹.

Animal models of chronic alcohol exposure highlight the potential role of the gut-liver axis in ALD evolution.² Changes of the gut microbiota

composition,^{3,4} disruption of the gut barrier function,^{5,6} and subsequent translocation of microbial products to the liver could activate immune responses implicated in disease progression.⁷ Manipulations designed to interfere with this process, all improved liver disease in animals.^{8–12} However, these data cannot necessarily be extrapolated to human pathology for several reasons. Animals have a natural aversion to alcohol,^{13,14} a 5 times faster ethanol metabolism,¹⁵ and profound differences in their immune system¹⁶ and

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their microbiota¹⁷ compared to humans. Animals do only develop mild forms of ALD upon chronic alcohol feeding and do not resume the liver-damage pattern observed in humans.¹⁸ Human studies generally focused on the role of the gut-liver axis in severe alcoholic hepatitis and decompensated cirrhosis.^{19–23} Little is known about the mechanisms operating at earlier stages of ALD and only two studies reported increased intestinal permeability (IP) in association with alterations of the fecal microbiota in less than 50% of AUD patients.^{24,25} Liver disease was not assessed in these reports.

The aim of our present study is to assess the relationship between IP, the duodenal and fecal microbiome, and microbial translocation in AUD patients and how these changes are associated with liver disease. To test this, we analyzed epithelial and vascular permeability, microbial translocation markers, and the composition of the microbiota attached to the duodenal mucosa and in the stools of actively drinking AUD patients. We finally correlated the observed changes with the pattern of liver disease.

Methods

Patients

Alcohol use disorder (AUD) patients (Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition criteria) admitted for elective alcohol withdrawal from April 2017 until January 2019 to dedicated alcohol withdrawal unit followed a highly standardized and controlled 3-week detoxification and rehabilitation program (Figure 1). They were compared to healthy volunteers matched for gender, age, and BMI (social drinkers consuming <20 g of alcohol/day) in a one (controls) to four (AUD patients) ratio. All patients reported long-term (>1 year) alcohol consumption (>60 g/day) and were actively drinking until the day of admission. Additional inclusion and exclusion criteria are available in Supplementary data. All clinical and baseline biochemical data were collected prospectively for all patients, and due to methodological and logistical reasons, the different investigations analysis were performed only in representative cohorts of patients, as indicated in the figure legends.

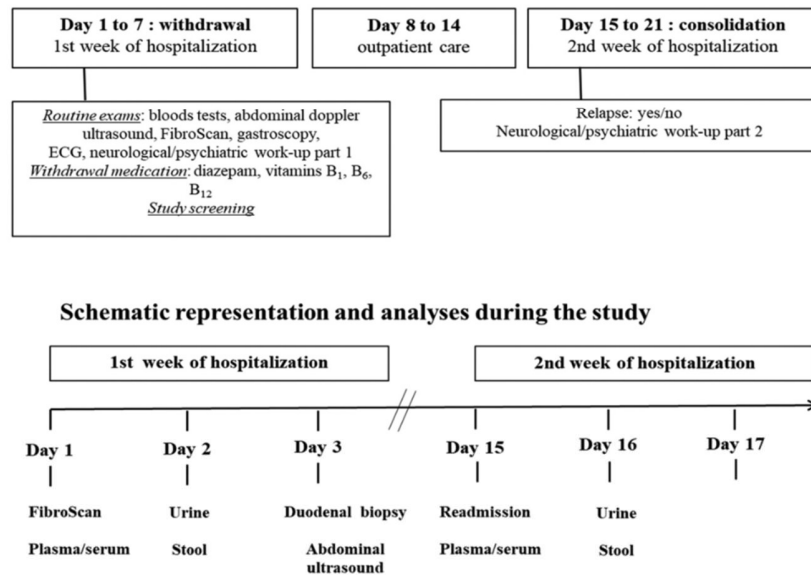


Figure 1. Standardized working scheme of the alcohol withdrawal unit. Alcohol use disorder (AUD) patients are undergoing a highly standardized clinical work-up with fixed sampling of biological specimens.

Examinations and sample collections (Figure 1)

On the day of admission, Fibroscan® (Echosense, Paris, France) combined with the controlled attenuation parameter (CAP) were performed and a fasting blood sample (serum, plasma) was drawn. A 24 h urine collection for ⁵¹Cr-EDTA determination (see below) was obtained on the second day and a gastroscopy with distal duodenal biopsies on the following day (details in supplementary material). Stools samples were collected from the first bowel movement after admission.

In addition, patients underwent an abdominal Doppler ultrasound on the third day as part of the routine work-up in the unit.

Ethical aspects

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institution's human research and ethical committee (B403201422657). Written informed consent was obtained from all patients and healthy volunteers. We followed the STROBE criteria for reporting cohort studies.

Measurement of intestinal permeability (IP) and intestinal epithelial cell damage

IP was assessed by measuring the urinary excretion of the radioactive probe ⁵¹Cr-EDTA, as described previously²⁴ and the fecal albumin content using a commercial ELISA kit (Human Albumin ELISA Kit, Immundiagnostik AG, Bensheim, Germany) (details in supplementary material).

Intestinal epithelial cell damage was measured by ELISA using serum intestinal fatty acid-binding protein (i-FABP) as a surrogate marker following the manufacturer's instructions (Human i-FABP ELISA kit, HycultBiotech, Uden, Holland).

Histology and morphometric indices

Duodenal sections were stained with Hematoxylin and Eosin and examined by an experienced gastrointestinal pathologist as a part of the standardized routine procedure of the alcohol withdrawal unit. Slides were then digitalized using a SCN400 slide

scanner (Leica Biosystems, Wetzlar, Germany) at X20 magnification and subjected to morphometric analysis. The major axis length of several villi was measured (details in supplementary material).

Immunohistochemistry

Formalin fixed duodenal sections were routinely stained and analyzed by immunohistochemistry using standard methods followed by quantification (details in supplementary material).

Immunofluorescence

Five µm cryosections were stained with the primary and secondary antibodies followed by quantification (details in supplementary material).

16 S rRNA sequencing and data analysis

DNA extraction and 16 S rRNA library were constructed as described previously.²⁶ 16 S sequence reads were processed using MOTHUR-base 16 S analysis workflow to determine the operational taxonomic units as described previously.^{11,27,28} Phyloseq package was used for the α-diversity (observed OTUs, Chao 1, Shannon, and Simpson) and β-diversity (weighted and unweighted Unifrac) analysis.²⁹ Linear discriminant analysis (LDA) effect size (LEfSe) was used to compare the difference between different groups.³⁰

Determination of serum biomarkers of liver cell damage and microbial translocation

In addition to AST and ALT, serum cytokeratin 18 (CK18) was used to assess liver cell damage (CK18-M65 ELISA kit; TECOmedical AG, Sissach, Switzerland). Microbial translocation was determined using soluble CD14, Lipopolysaccharide Binding Protein (Human CD14 Quantikine ELISA kit sCD14 and Human LBP duoset ELISA, Biotechne Ltd., Abingdon, United Kingdom) and Peptidoglycan Recognition Proteins (Human PGRPs ELISA kit, Thermofisher, Merelbeke, Belgium), respectively. All assays were performed in duplicate following the manufacturer's instructions.

Reverse-transcription and quantitative real-time polymerase chain reaction

Duodenal tissue messenger RNA (mRNA) was assessed by quantitative real-time polymerase chain reaction (PCR) and quantified as described.³¹ Primer sequences are listed in supplementary material.

Statistics

Data were analyzed using Graph Pad Prism 7 (GraphPad Software, San Diego, CA, USA) and presented as mean $\pm$ standard error of the mean (SEM) unless otherwise indicated. Normality was assessed by the Kolmogorov–Smirnov test followed by t-tests for normally distributed data, or the Wilcoxon test for nonnormally distributed data. Data were compared by one-way ANOVA for multiple groups, followed by Bonferroni's post hoc test for pairwise comparisons. Wilcoxon or paired t-tests compared data of patients before and after abstinence. Pearson's or Spearman's correlation tests were used for correlations between data sets. A *p* value $< .05$ was considered as statistically significant. Area under the receiver-operating characteristics curves (AUROC) were constructed using the method by Hanley and McNeil. Youden's statistics was performed for the determination of the threshold values. MaAsLin2 analysis (<https://bitbucket.org/biobakery/maaslin2/src/default/>) was used to determine the association between microbiota and serum surrogate markers of microbial translocation.

Results

Study population

The study population consists of a cohort of 106, middle-aged, predominantly male AUD patients and 24 volunteers. Demographic and biochemical data are provided in Table 1. All patients reported alcohol consumption until the evening prior to their admission. Twenty-seven percent (29/106) had detectable blood alcohol concentrations on the following day at admission with a median level of 0.5 g/L (range 0.1–2.5 g/L).

Table 1. Baseline demographic and biochemical data of the study population.

Demographics			
	Healthy volunteers (n = 24)	AUD patients (n = 106)	p value
Gender (female/ male)	10 (41.6%)/14 (58.4%)	28 (26.4%)/78 (73.6%)	.1380
	Mean $\pm$ SEM		
Age (years)	42 $\pm$ 11	46 $\pm$ 9.2	.1537
Height (m)	1.74 $\pm$ 0.08	1.75 $\pm$ 0.07	.9583
Weight (kg)	71.4 $\pm$ 9.4	75 $\pm$ 11.3	.2598
BMI	23.4 $\pm$ 2.9	24.6 $\pm$ 3.2	.2012
Biochemistry			
	Mean $\pm$ SEM (normal range)		
AST (IU/L)	17.8 $\pm$ 3.5	64 $\pm$ 44.4 (<40)	.0274
ALT (IU/L)	10.9 $\pm$ 2.9	53.5 $\pm$ 34.6 (<40)	.0052
γ -GT (IU/L)	22.4 $\pm$ 8.6	211.4 $\pm$ 217.6 (<40)	.1745
ALP (IU/L)	52 $\pm$ 11	79 $\pm$ 24 (30–120)	.0578
Bilirubin (mg/dL)	0.21 $\pm$ 0.13	0.55 $\pm$ 0.24 (0.3–1.2)	.0106
Albumin (g/L)	44 $\pm$ 0.8	47 $\pm$ 4 (35–52)	.0717
Creatinine	0.97 $\pm$ 0.13	0.8 $\pm$ 0.1 (<1.2)	.0021

Abbreviations: BMI, body mass index; AST, Aspartate transaminase; ALT, Alanine transaminase; γ -GT, gamma-glutamyltransferase; ALP, alkaline phosphatase

Clinical classification of patients according to severity of liver disease

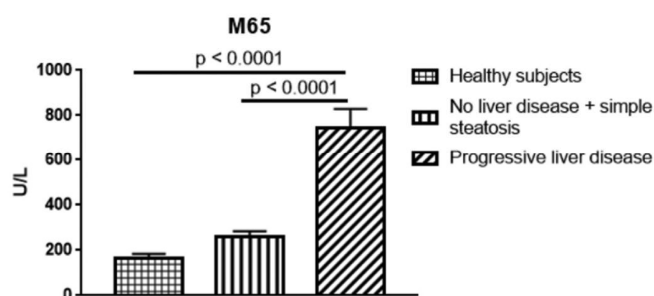
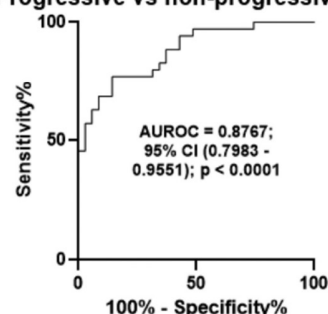
To allow a more detailed analysis related to clinical parameters we assessed the pattern of liver disease in AUD patients who were clinically classified as depicted in Table 2 into non-progressive (no liver disease/simple steatosis) and progressive liver disease (steato-hepatitis/steato-fibrosis). All the patients with steato-hepatitis or steato-fibrosis had a preserved synthetic liver function and showed no clinical signs of liver decompensation. Doppler ultrasound ruled out any significant vascular or biliary problem in the liver.

Serum cytokeratin 18 levels (CK18) further refine the clinical classification

Cytokeratin 18 is released upon cell damage (necrosis, apoptosis) and has been considered as a liver-specific cell damage marker.³² Serum CK18-M65 increased significantly in AUD patients compared to healthy volunteers. When looking at the different clinical subgroups, high CK-M65 levels were found in AUD patients with progressive liver disease but not in those with non-progressive forms of the disease (Figure 2). Receiver operating curve (ROC) analysis showed that serum CK18-M65 levels allowed to distinguish with high accuracy progressive ALD from the non-progressive forms of liver disease

Table 2. Characteristics of the patient's subgroups based on clinical evaluation of ALD.

	Non-progressive liver disease (n = 53)		Progressive liver disease (n = 45)	
	No liver disease (n = 24)	Simple steatosis (n = 29)	Steato-hepatitis (n = 29)	Steato-fibrosis (n = 16)
<i>Clinical parameters</i>	AST/ALT < 40 IU/L	AST/ALT < 40 IU/L	AST/ALT > 40 IU/L	AST/ALT > 40 IU/L
	CAP < 250 dB/m kPa < 7.6	CAP > 250 dB/m kPa < 7.6	CAP > 250 dB/m kPa < 7.6	CAP > 250 dB/m kPa > 7.6
<i>Clinical data</i>				
AST (IU/L; N < 40)	22.16 ± 4.68	28.93 ± 5.73	89.78 ± 34.24	127.125 ± 89.05
ALT (IU/L; N < 40)	19.21 ± 5.17	27.76 ± 9.13	84.57 ± 30.37	88.44 ± 55.99
γ-GT (IU/L; N < 40)	35.3 ± 18.48	68.48 ± 41.32	217.25 ± 151.62	521 ± 372.87
ALP (IU/L; N < 130)	61.74 ± 10.88	68.82 ± 15.25	76.25 ± 14.82	111.69 ± 35.77
Bilirubin (mg/dL; N < 1.2)	0.46 ± 0.18	0.48 ± 0.18	0.51 ± 0.14	0.76 ± 0.44
Albumin (g/L; N > 35)	47.46 ± 3.36	47.64 ± 2.95	47.99 ± 3.68	45.67 ± 6.25
CAP (dB/m)	202 ± 22	297 ± 29	319 ± 24	296 ± 45
kPa	4.85 ± 0.81	4.79 ± 0.95	5.53 ± 0.92	20.69 ± 12.06

**Progressive vs non-progressive ALD**

Cutoff	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio	J
> 401.9	77.14	60.98% to 87.93%	82.86	67.32% to 91.90%	4.5	1.59
> 416.2	77.14	60.98% to 87.93%	85.71	70.62% to 93.74%	5.4	1.6185
> 425.2	74.29	57.93% to 85.84%	85.71	70.62% to 93.74%	5.2	1.59

Figure 2. Serum cytokeratin 18 (CK18-M65) as a surrogate marker of liver damage. High serum CK18-M65 levels characterized progressive ALD (upper) and differentiate severe forms of alcoholic liver disease from the non-progressive forms of alcoholic liver disease, as shown by Receiver Operating Characteristic (ROC) analysis (middle) (AUROC = 0.8767; 95% CI: 0.7983–0.9551; $p < .0001$). Below, the cutoff identified using the Youden's statistics (416.2 U/L) was characterized by high sensitivity and specificity (77.14% and 85.71%, respectively).

(AUROC = 0.8767; 95%CI: 0.7983–0.9551; $p < .0001$). We then identified the cutoff using the Youden's statistics (416 U/L) with the best specificity and sensitivity profile (85.7% and 77.1%, respectively). Interestingly, CK18-M65 levels did not separate patients with steato-hepatitis from those with steato-fibrosis within the progressive ALD group (Supplementary Figure 1). Thus, CK18-M65 was associated with liver damage regardless of fibrosis.

Overall, serum CK18-M65 seems to be a reliable diagnostic tool to identify progressive ALD with a high positive likelihood ratio (Table 3). Consequently, we used CK to adjust the definition of the group with no liver disease or steatosis (CK18 < 416 U/L, G1 group) and the group with steato-hepatitis or steato-fibrosis (CK > 416 U/L, G2 group).

Assessment of intestinal permeability (IP), morphology and microbial translocation in AUD patients

Intestinal paracellular and capillary leakiness³³ can be measured by the urinary excretion of the radioactive probe ⁵¹Cr-EDTA and fecal albumin content, respectively. Compared to controls, ⁵¹Cr-EDTA and fecal albumin content supported increased IP in actively drinking AUD (Figure 3a). The two markers were however not correlated ($r = 0.006$, $p = .9625$) meaning that they likely do not measure the same parameters of IP.³³ In addition, immunofluorescence showed up-regulated duodenal expression of plasmalemma Vesicle-Associated Protein 1 (PV-1), a marker of disruption of the Gut-Vascular Barrier³⁴ in AUD patients compared to controls (Figure 4c).

As morphological changes and/or enterocyte damage might be causes of gut barrier dysfunction, we studied mucosal morphology on duodenal biopsies and measured serum i-FABP levels as a marker

of intestinal epithelial cell damage. Compared to healthy volunteers, AUD patients were characterized by shorter villi and down-regulation of Villin gene expression (Figure 3b, c). Serum i-FABP did not significantly change between AUD patients and controls (Figure 3c).

Soluble CD14 (sCD14), lipopolysaccharide binding protein (LBP) and Peptidoglycan-recognition Proteins (PGRPs) have been used as serum surrogate markers of microbial translocation (MT) for Gram- and Gram+ bacteria, respectively.^{35–37} All three markers were significantly higher in AUD patients compared to healthy controls (Figure 3d). Chronic alcohol consumption has also been associated with an altered mycobiome and translocation of fungal products.²⁰ We thus assessed serum antibodies directed against *Saccharomyces cerevisiae* (ASCA) as a potential surrogate of fungal translocation. Serum ASCA IgA and IgG were low or undetectable in controls whereas high levels were observed in AUD patients (Supplementary Figure 4).

Only a subset of AUD patients shows increased IP together with disruption of tight junctions and vasculature expression of PV-1

When looking at the data more closely, about two-thirds of AUD patients had intestinal permeability measurements close to those of controls while 36–40% showed high IP based on fecal albumin and ⁵¹Cr-EDTA, respectively (Figure 4a). This separation of subjects into two categories was calculated according to a deviance criterion at a threshold of 1.65 SDs of the mean of the control group. High IP was principally related to the proximal small bowel as suggested by the small bowel component (duodenum, jejunum) of the ⁵¹Cr-EDTA measurements (Supplementary Figure 2) without significant modifications of the distal gut component (ileum, colon) (data not shown).

In accordance with ⁵¹Cr-EDTA data, Zonula Occludens 1 (ZO-1), a tight junction protein that regulates paracellular permeability, was disrupted in AUD subjects with high IP but not in those with normal IP (Figure 4b). By contrast, patients with high fecal albumin content showed a higher staining index of PV-1 in endothelial cells of the duodenal mucosa (Figure 4c).

Table 3. Statistical analysis of serum CK18-M65 levels as a biomarker of progressive ALD.

Statistics	Value	95% CI
Positive Likelihood Ratio	5.56	2.43 to 12.74
Negative Likelihood Ratio	0.24	0.12 to 0.47
Positive Predictive Value (%)	84.37	70.20 to 92.52
Negative Predictive Value (%)	81.08	68.60 to 89.37
Accuracy (%)	82.61	71.59 to 90.68

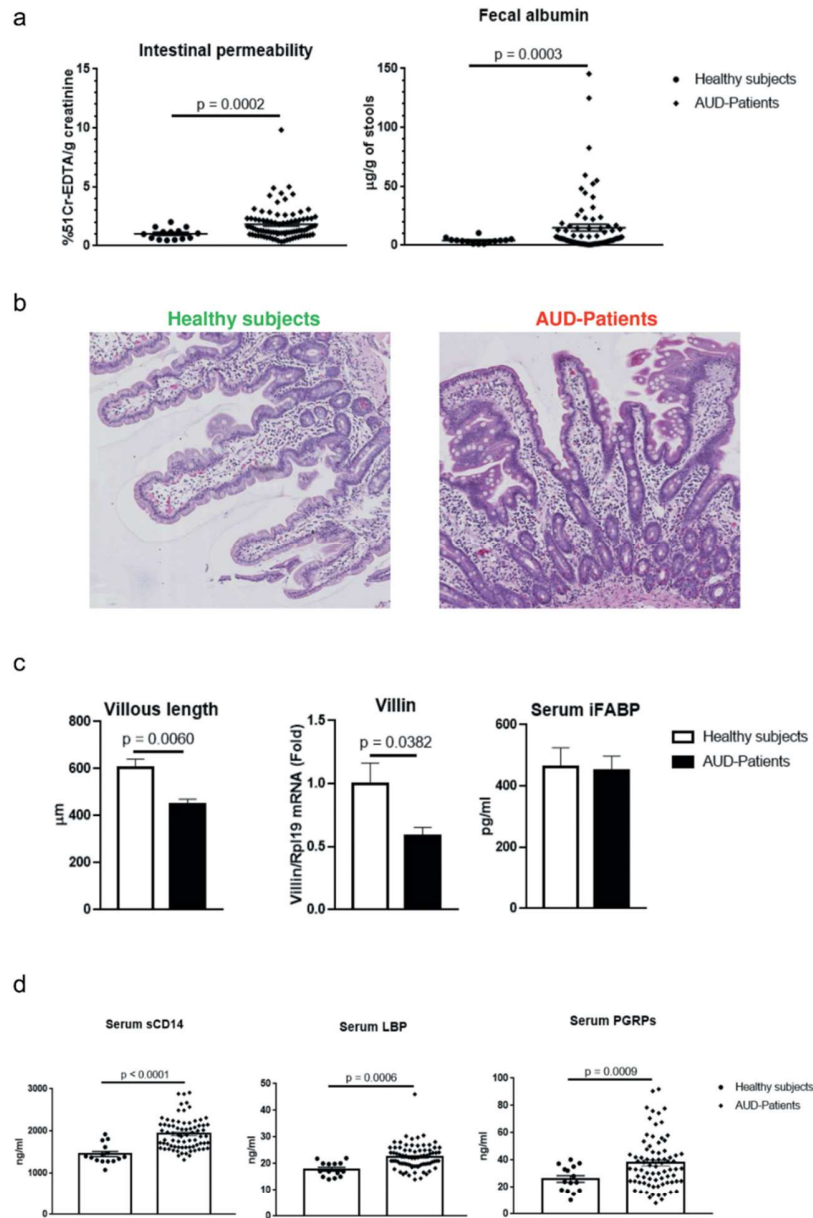


Figure 3. Assessment of intestinal permeability (IP), morphological features of the duodenal mucosa, enterocyte damage and microbial translocation. (a) IP measured *in vivo* by ⁵¹Cr-EDTA (n = 86) urinary excretion (left) and fecal albumin content (n = 78) (right) increased significantly in the overall alcohol use disorder (AUD) population compared to controls (n = 14). (b) Representative hematoxylin and eosin staining of duodenal biopsies from healthy volunteers and AUD patients. (c) Morphometric analysis showing statistically significant reduction of villi's length in AUD patients (n = 11) compared to controls (n = 5) associated with down-regulation of Villin gene expression by qPCR (middle) in AUD patients (n = 43) compared to controls (n = 9) whereas serum levels of the enterocyte damage marker (intestinal fatty acid binding protein, i-FABP) (right) did not differ between AUD patients (n = 77) and healthy subjects (n = 11). (d) Gram-negative soluble CD14 (sCD14) and Lipopolysaccharide Binding Protein (LBP) as well as the Gram-positive translocation marker Peptidoglycan Recognition Proteins (PGRPs) increased significantly in AUD patients (n = 75) compared to controls (n = 15).

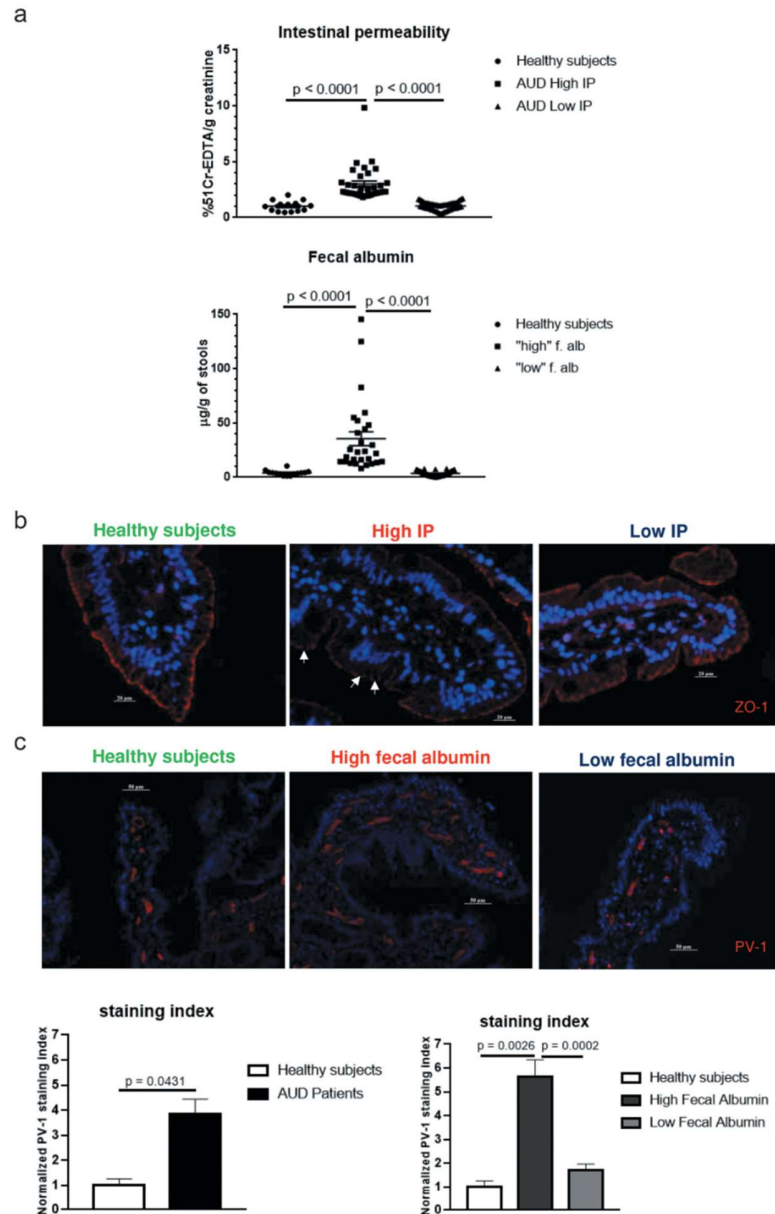


Figure 4. Assessment of intestinal permeability (IP) in relation to histological features. (a) More detailed analysis revealed that only one-third of AUD patients showed significantly increased IP ($n = 34$ and $n = 28$ for $^{51}\text{Cr-EDTA}$ and fecal albumin, respectively) compared to controls. This separation of subjects was calculated according to a deviance criterion at a threshold of 1.65 SDs of the mean of the control group. (b) Representative immunofluorescence of ZO-1 duodenal tissues from healthy volunteers, and AUD patients with high IP and low IP showing disrupted tight junctions (indicated by arrows) in AUD patients only with high IP. (c) Representative immunofluorescence staining of PV-1 in duodenal tissues of healthy volunteers and AUD patients with high and low fecal albumin, respectively. Increased expression PV-1 in vessels of AUD patients was found principally in association with high fecal albumin and confirmed by quantitative analysis (staining index).

Morphological changes or enterocyte damage do not associate with intestinal permeability

Strikingly, morphological changes were not more prominent in AUD patients with high IP (Supplementary Figure 3) suggesting that they do not play a major role in determining intestinal permeability. Increased IP was not associated with enterocyte damage since even after splitting them according to permeability measurements no differences in serum i-FABP were found compared to controls (Supplementary Figure 3).

Microbial translocation is not exclusively dependent on increased IP

Serum surrogate markers of microbial translocation (PGRPs, sCD14, and LBP) were significantly higher in AUD patients compared to healthy controls. However, the levels were similar in the patients with increased and normal gut permeability whether based on ⁵¹Cr-EDTA or fecal albumin measurements (Figure 5a–c). As with bacterial translocation markers, an increase in ASCA levels in AUD patients occurred independently from high IP and high fecal albumin (Supplementary Figure 4).

These observations suggest that, in AUD patients, systemic microbial translocation might occur even if IP is normal.

Reduced inflammatory cell infiltration and absence of a pro-inflammatory response characterized the duodenal mucosa of AUD patients

Since intestinal inflammation could contribute to intestinal cell damage and favor microbial translocation, we quantified duodenal mucosal infiltration by immune cells and assessed mucosal expression of major gut-specific pro-inflammatory cytokines. Overall, CD45-positive immune cells and more specifically CD68-positive macrophages and CD3-positive T cells were reduced in distal duodenal biopsies in AUD patients (Figure 6a–c). Although Interleukin-1beta (IL1β) mRNA was increased, other gut-specific pro-inflammatory cytokines, interleukin 17 and 22 as well as interferon-gamma (IFN-γ) remained unchanged in AUD patients compared to healthy controls (Figure 6d).

These results rule out mucosal inflammation as a significant driver of morphological changes and microbial translocation in AUD patients. Rather

a reduction of immune effector cells in the small gut might contribute to decreased immune surveillance and facilitate microbial translocation.

Assessment of the duodenal mucosa-associated microbiota and fecal microbiota in relation to IP

Changes in the duodenal mucosa-associated microbiota in AUD patients do not determine increased intestinal permeability

We have previously observed an increased number of duodenal mucosa-associated bacteria in AUD patients.⁹ We here analyzed the composition of the microbiota in duodenal biopsies of a representative group of actively drinking AUD patients. Albeit not-significant, duodenal-associated microbiota of AUD patients showed an increased number of operational taxonomic units (OTUs) compared to healthy volunteers (Observed species and Chao1) reflecting a higher richness. Intriguingly, this increased number of bacteria was accompanied by a reduced Simpson index in AUD patients. This observation suggests that the different OTUs are not evenly distributed and indicates that some bacterial taxa are more dominant than others (Figure 7a). Although the PCoA of β-diversity indexes (weighted and unweighted Unifrac) did not allow a clear separation between AUD patients and healthy controls (Figure 7a), we found that 10 bacterial genera were different between AUD and controls. As reflected by the LDA score, the relative abundance of *Nubsella*, *Rothia*, and *Streptococcus* was higher in AUD patients while the relative abundance of *Mycobacterium*, *Alcaligenes*, *Lachnoclostridium*, *Ralstonia*, *Rarobacter*, *Ethanoligenens*, and *Dolosigranulum* was higher in healthy subjects (Figure 7b).

We next assessed whether mucosa-associated microbiota changes were related to increased IP assessed by ⁵¹Cr-EDTA measurements. Despite the observed differences between AUD patients and healthy subjects, neither α-diversity indexes nor β-diversity did differ between AUD patients with high IP when compared to those with normal IP (Figure 7c, Supplementary Figure 5).

These observations suggest that changes in the duodenal microbiota do not necessarily associate with increased intestinal permeability.

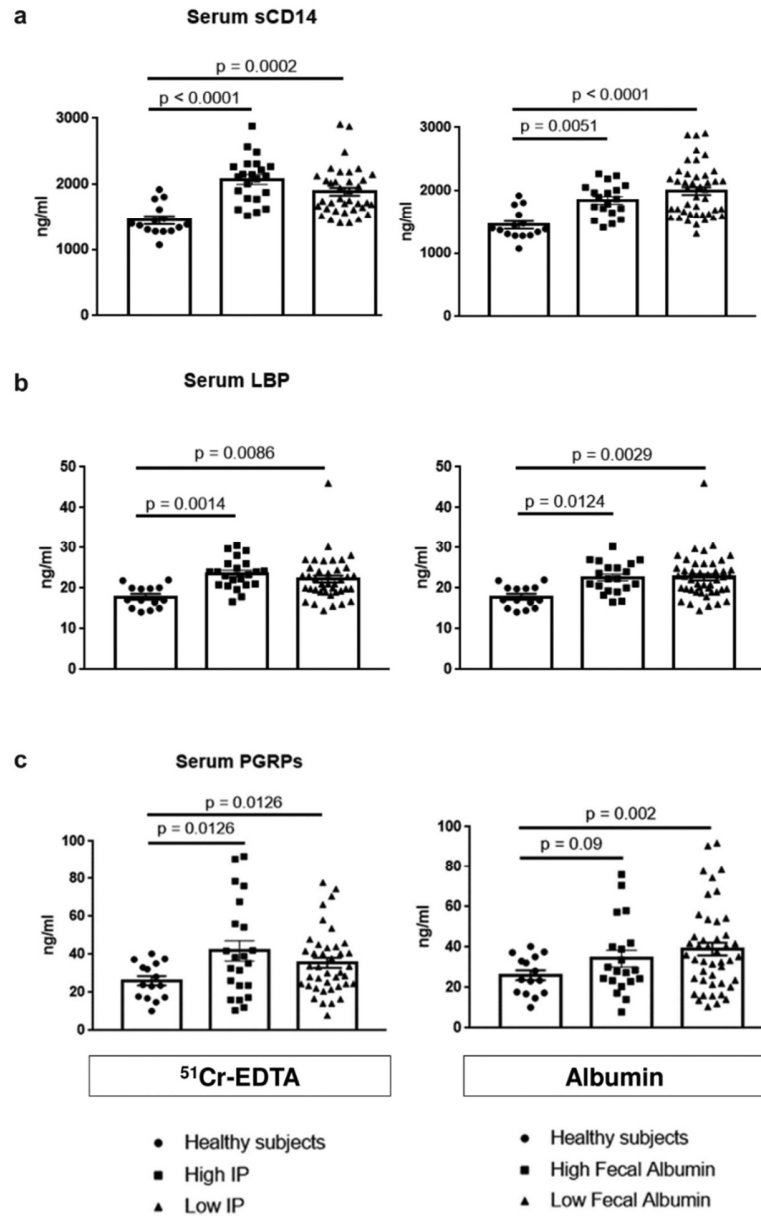


Figure 5. Assessment of serum microbial translocation markers in relation to intestinal permeability (IP). Gram-negative (a, b) soluble CD14 (sCD14) and Lipopolysaccharide Binding Protein (LBP) as well as the Gram-positive translocation marker (c) Peptidoglycan Recognition Proteins (PGRPs) increased significantly in AUD patients with high and low IP compared to controls while levels did not differ between high and low IP, measured by ⁵¹Cr-EDTA urinary excretion (left graphs) and fecal albumin content (right graphs).

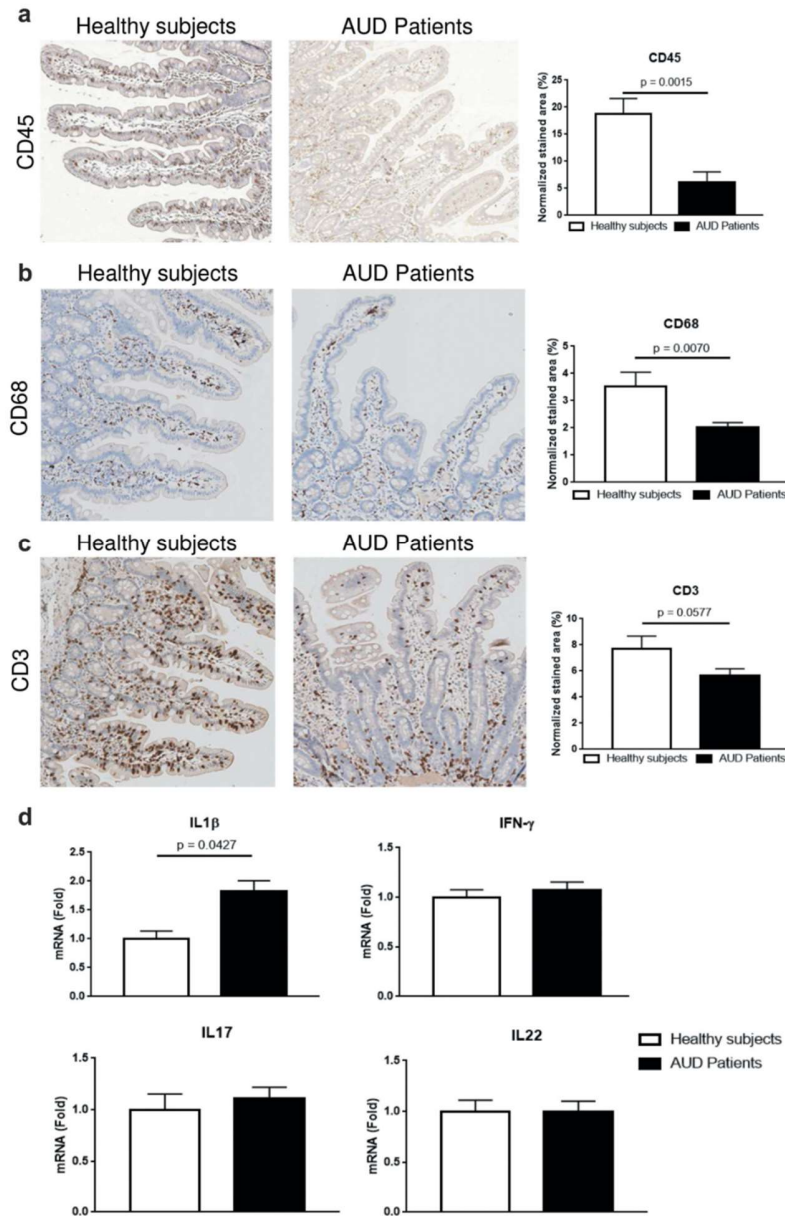


Figure 6. Evaluation of immune cells and inflammatory markers in the duodenum of alcohol use disorder (AUD) patients. Immunohistochemistry analysis of the hematopoietic cell marker CD45 (a) revealed a decrease of immune cells in the duodenal mucosa of AUD patients ($n = 11$) compared to controls ($n = 6$). Analysis of the macrophages marker CD68 (b) and the T cell marker CD3 (c) revealed a reduction of both cell types in the duodenal mucosa of alcohol use disorder (AUD) patients ($n = 15$) compared to healthy controls ($n = 8$). Duodenal gene expression (d) of Interleukin-1beta (IL1 β) increased in AUD patients ($n = 59$) compared to controls ($n = 13$) while mRNA levels of Interleukin-17(IL17), Interleukin-22 (IL22) and Interferon-gamma (IFN- γ) were similar between AUD patients and healthy volunteers.

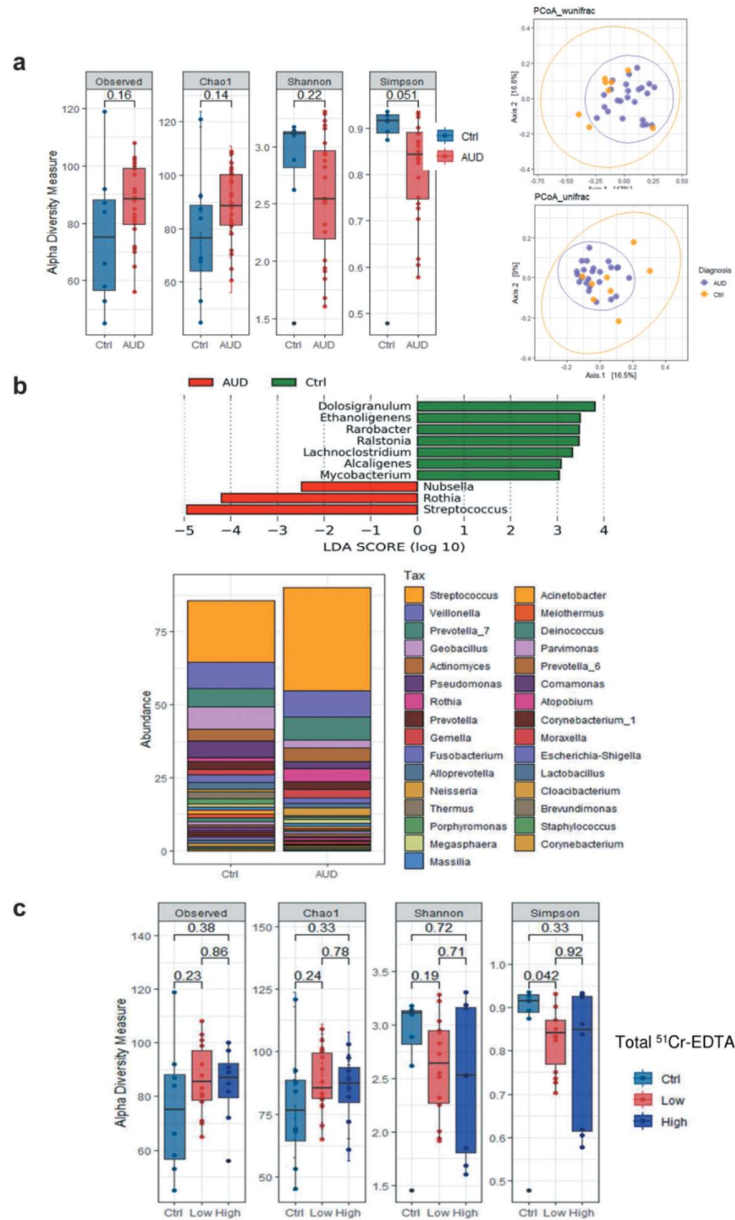


Figure 7. Evaluation of duodenal mucosa-associated microbiota in relation to intestinal permeability (IP). (a) Microbial α diversity (left), showed an increased number of operational taxonomic units (OTUs) (Observed and Chao1), in AUD patients ($n = 22$) compared to healthy volunteers ($n = 8$) indicating increased richness. This increased number of bacteria was accompanied by reduced evenness (Simpson index), with a shift regarding the dominance of certain genera, compared to controls. By contrast, β diversity (right) did not differ between AUD and controls. (b) Different genera overrepresented in the distal duodenum of AUD patients and healthy subjects, as assessed by linear discriminating analysis (LDA) (above) of AUD patients compared to controls at the genus level. Below, the relative abundance of the different genera in the two groups. Only genera with detection in at least 2% are shown. (c) No differences in terms of α diversity were found when AUD patients were split into high ($n = 8$) and low IP ($n = 14$), according to $^{51}\text{Cr-EDTA}$ measurements.

Reduced richness and evenness of the fecal microbiota in AUD patients is associated with increased intestinal permeability

We assessed whether changes of the fecal microbiota were associated with leaky gut in our cohort of AUD patients. We compared the fecal microbiota of AUD patients and healthy controls. Microbial α -diversity indexes were lower in AUD patients, reflecting reduced richness (observed species, Chao1) and evenness (Shannon). This also indicates that the number of OTUs was lower and less evenly distributed in the fecal samples of AUD patients compared to controls. (Figure 8a). Furthermore, the PCoA of UniFrac indexes (β -diversity) allows a separation between the AUD patients and healthy subjects suggesting that bacterial profiles are different between these two groups (Figure 8b). Linear discriminating analysis (LDA) revealed a higher abundance of five genera such as *Gemella*, *Actinomyces*, *Desulfovibrio*, *Subdoligranulum*, and *Akkermansia* in AUD patients compared to healthy subjects (Supplementary Figure 6).

Compared to controls, reduced richness, and evenness of the fecal microbiota were principally found in AUD patients with high but not in normal IP (Figure 8c). By contrast, bacterial composition (β -diversity) of the stool microflora were similar in high and low IP (Figure 8d, Supplementary Figure 7).

These observations suggest that compositional changes of the fecal microbiota could be associated with increased intestinal permeability.

Relationship between progressive ALD, intestinal permeability, microbial translocation, and microbiota

Elevated translocation of microbial products has been linked in ALD models to the progression of liver disease.^{38,39} After refining of the clinical subgroups using CK18-M65 biomarker, we assessed whether IP, microbial translocation markers, duodenal, and fecal microbiota changes were associated with progressive forms of liver disease.

Increased IP and Gram-negative translocation markers are associated with progressive forms of liver disease

⁵¹Cr-EDTA increased significantly with progressive ALD compared to controls but no differences were found between non-progressive ALD and healthy

volunteers. By contrast, fecal albumin increased independently of ALD severity (Figure 9a). Serum microbial Gram-negative markers sCD14 and LBP were upregulated in AUD patients with the highest levels found in progressive ALD (Figure 9b). Both markers also significantly correlated with CK18-M65 ($r = 0.39$ and 0.378 ; $p < .001$, respectively).

By contrast, PGRPs rose independently of the stage of liver disease in AUD patients (Figure 9b) as did serum ASCA IgA and IgG (Supplementary Figure 8).

Increased richness and predominance shift in the duodenal mucosa-associated microbiota, but not in the fecal microbiota, in patients with progressive ALD

AUD patients with progressive ALD showed increased richness (observed species, Chao1) as well as reduced evenness with a dominance shift toward some bacterial taxa (Simpson) of their mucosa-associated microbiota whereas no significant changes were found in AUD with non-progressive liver disease compared to controls (Figure 10a). While the overall bacterial profiles (β -diversity) did not differ between the groups (Supplementary Figure 9a), we found that six OTUs were different between progressive ALD and controls. LDA scores indicated that *Streptococcus*, *Shuttleworthia*, and *Rothia* were overrepresented in AUD patients with progressive ALD while *Mycobacterium*, *Alcaligenes*, and *Deinococcus* were specific of healthy volunteers (Figure 10b).

We finally assessed whether dysbiosis of the fecal microbiota was also associated with progressive ALD. Interestingly, and in contrast to the duodenal microbiota, no differences in α - and β -diversity indexes and composition of the fecal microbiota were observed when the cohort was split into AUD patients with or without progressive liver disease compared to controls (Figure 10c, Supplementary Figure 9b).

Thus, increased richness with a dominance shift toward specific bacteria of the duodenal mucosa-associated microbiota and not of the fecal microbiota, are associated with progressive ALD.

Microbial translocation markers correlate with duodenal microbiota changes in progressive ALD

We next assessed the association between microbiota and two representative serum surrogate markers of

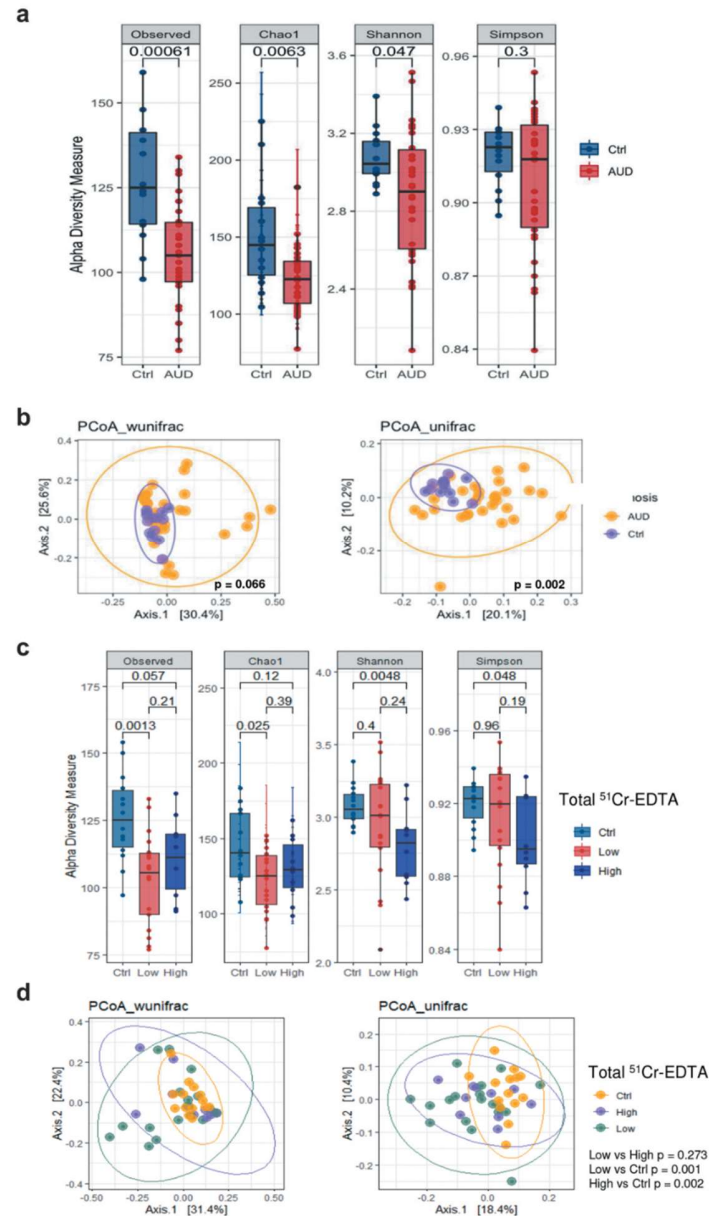


Figure 8. Fecal microbiota assessment in AUD patients in relation to intestinal permeability (IP). (a) Microbial α diversity showed reduced number of operational taxonomic units (OTUs) (Observed and Chao1), in AUD patients ($n = 30$) compared to healthy volunteers ($n = 13$) indicating reduced richness. Intriguingly, this diminished number of bacteria was accompanied by decreased evenness (Shannon index), reflecting the loss of rare species, compared to controls. (b) Comparison of principal coordinate analysis (PCoA) by using weighted and unweighted UniFrac distance showed that overall fecal microbiota composition (β diversity) was different between AUD patients and controls. Each dot represents one sample and the distance between the samples represents the difference in community composition of the samples. (c) Reduced evenness (Shannon and Simpson) were found only in AUD patients with high IP compared to controls. (d) Fecal microflora composition (β diversity) did not change between AUD patients with high and low IP.

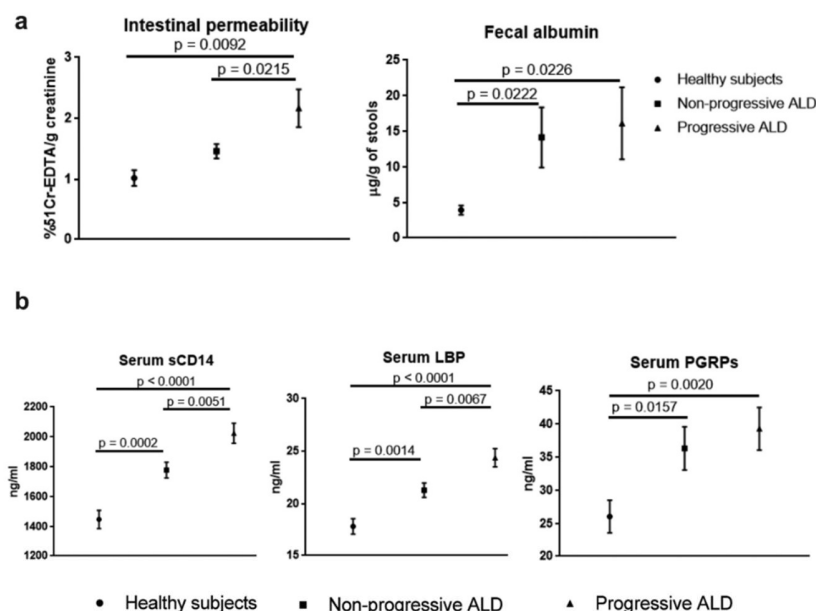


Figure 9. Relationship between intestinal permeability (IP), microbial translocation and stage of alcoholic liver disease (ALD). (a) IP assessed by ^{51}Cr -EDTA (left) increased significantly with progressive ALD while levels in non-progressive ALD remained close to controls. High fecal albumin (right) was independent from liver disease severity and already significantly increased in non-progressive ALD compared to controls. (b) Gram-negative serum microbial translocation markers soluble CD14 (sCD14) and Lipopolysaccharide Binding Protein (LBP) levels gradually increased with the highest levels observed in progressive ALD, while the Gram-positive translocation marker Peptidoglycan Recognition Proteins (PGRPs) increased independently from the stage of ALD compared to controls.

microbial translocation (sCD14 and PGRPs). In patients with non-progressive ALD, six duodenal and four fecal microbial species correlated almost exclusively with the translocation marker sCD14.

By contrast, the number of duodenal microbial species that correlated with translocation markers further increased to 12 in progressive ALD, 5 species correlating with sCD14 and 7 with PGRPs. Additionally, five species in the fecal samples showed a correlation principally with PGRPs. All individual correlations were statistically significant (p -values < 0.05). However, after adjusting for multiple comparisons, the q -values were greater than 0.5 (Table 4).

A short time of abstinence restores normal intestinal permeability and fecal microbiota but does not normalize microbial translocation and liver damage

In the absence of effective pharmacological therapy, abstinence constitutes the foundation of ALD

treatment. We, therefore, assessed whether a short period of abstinence, often encountered in AUD patients, was able to restore alcohol-related changes in the gut and liver cell damage. We retested AUD patients who remained abstinent at the end of 3-wk detoxification program (T2). IP of AUD patients with both high ^{51}Cr -EDTA urinary excretion and high fecal albumin content returned to values observed in the control group and remained low in those with already normal levels at admission (T1) (Figure 11a). In parallel, serum sCD14 levels also significantly decreased upon abstinence. By contrast, neither LBP nor PGRPs were modified by alcohol cessation (Figure 11b) suggesting the persistence of some microbial translocation. In addition, serum CK18-M65 decreased after abstinence (Figure 11c) but remained significantly higher than control levels, indicating persisting but attenuated liver damage.

These observations indirectly confirm that increased IP is not absolutely required for microbial

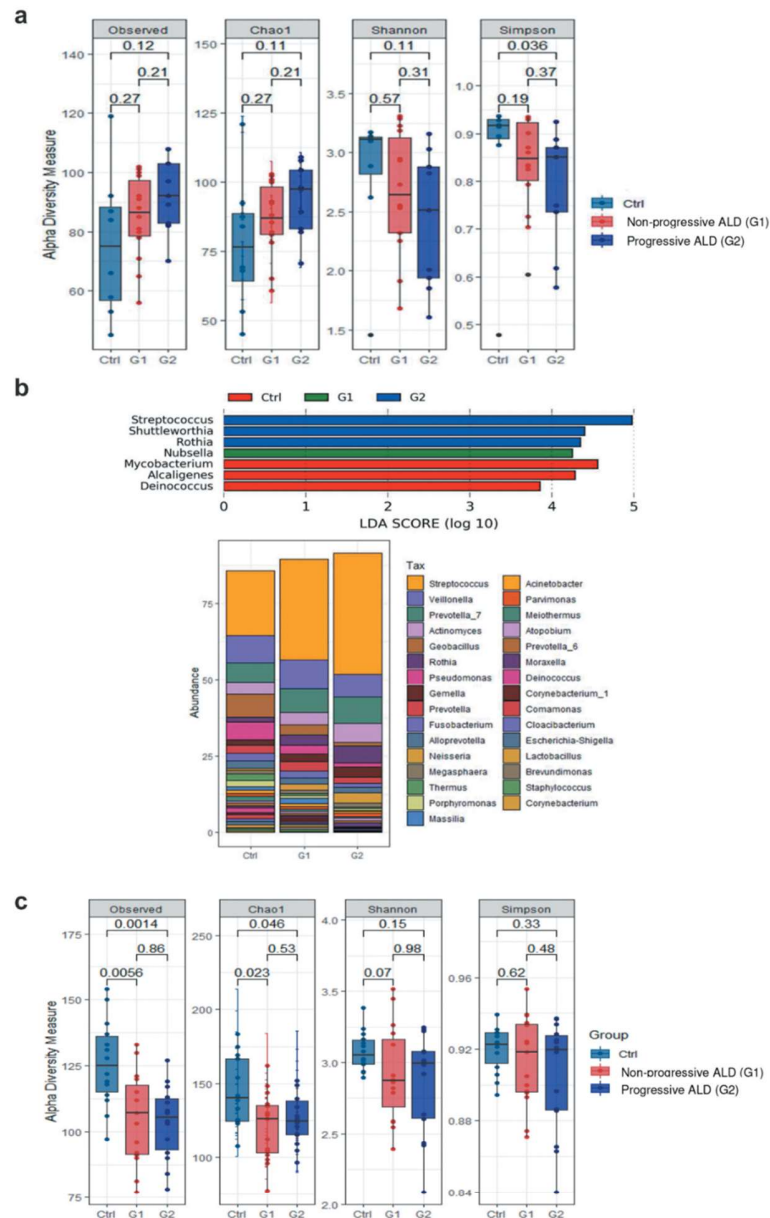


Figure 10. Analysis of the duodenal mucosa-associated microbiota and fecal microbiota in relation to alcoholic liver disease (ALD) severity. (a) Microbial diversity (α diversity), plotted for healthy controls (Ctrl), AUD patients with non-progressive ALD (G1) and progressive ALD (G2), showed that G2 is characterized by increased richness as well as a reduced evenness with a shift in bacterial dominance (Simpson index) of their mucosa-associated microbiota compared to controls. (b) Different genera overrepresented in the distal duodenum of AUD patients in relation to ALD severity and healthy subjects (Ctrl). Results of linear discriminant analysis (LDA) of AUD patients (G1 and G2) compared to controls at the genus level. Below, relative abundance of the different genera in the three groups. Only genera with detection in at least 2% are shown. (c) By contrast to the duodenal mucosa-associated microbiota, fecal microbial α diversity did not differ in AUD patients when they were split according to the severity of liver disease.

Table 4. Correlation between microbiota species in duodenum and stools and serum surrogate markers of microbial translocation sCD14 and PGRPs at different stages of alcoholic liver disease (ALD).

Microbe	Gram	Marker	Coef	stderr	N	p-val	q-val
Non-progressive ALD							
<i>Microbiota: duodenal</i>							
Gemella	Positive	PGRPs	-0.0014923	0.00044804	14	0.01037279	0.83197791
Bacillus	Positive	sCD14	-6.97E-06	2.53E-06	14	0.02498809	0.83197791
Corynebacterium	Positive	sCD14	-1.71E-05	6.28E-06	14	0.02598272	0.83197791
Novosphingobium	Negative	sCD14	-2.09E-06	6.95E-07	14	0.01674825	0.83197791
Cloacibacterium	Negative	sCD14	-2.76E-05	1.07E-05	14	0.03262759	0.83197791
Sphingobacterium	Negative	sCD14	-2.24E-06	8.89E-07	14	0.03591045	0.83197791
<i>Microbiota: fecal</i>							
Unclassified_Erysipelotrichaceae	Positive	sCD14	-1.36E-05	5.86E-06	14	0.04246871	0.90377608
Hespellia	Positive	sCD14	8.51E-06	3.70E-06	14	0.04430796	0.90377608
Dialister	Negative	sCD14	5.85E-05	2.61E-05	14	0.04868563	0.90377608
Clostridium_XIVb	Negative	sCD14	5.32E-06	2.38E-06	14	0.04903367	0.90377608
Progressive ALD							
<i>Microbiota: duodenal</i>							
Rikenellaceae_RC9_gut_group	Negative	PGRPs	6.06E-05	1.55E-05	9	0.011346	0.57354547
Bergeyella	Negative	PGRPs	0.00011534	3.19E-05	9	0.01527949	0.57354547
Anaeroglobus	Negative	PGRPs	0.00010158	3.09E-05	9	0.02190414	0.57354547
Fretibacterium	Negative	PGRPs	6.84E-05	2.13E-05	9	0.02375486	0.57354547
Capnocytophaga	Negative	PGRPs	0.00061948	0.00022807	9	0.04196674	0.57354547
X.Eubacterium_nodatum_group	Positive	PGRPs	-5.78E-05	2.06E-05	9	0.03806405	0.57354547
Abiotrophia	Positive	PGRPs	0.00036362	0.0001331	9	0.04118104	0.57354547
Bergeyella	Negative	sCD14	2.50E-06	7.41E-07	9	0.01968319	0.57354547
Rikenellaceae_RC9_gut_group	Negative	sCD14	1.20E-06	3.60E-07	9	0.02062727	0.57354547
Roseateles	Negative	sCD14	-2.98E-06	9.85E-07	9	0.02921792	0.57354547
Aeromonas	Negative	sCD14	-2.65E-06	9.03E-07	9	0.03260309	0.57354547
Enterococcus	Positive	sCD14	7.42E-06	2.59E-06	9	0.0351744	0.57354547
<i>Microbiota: fecal</i>							
Unclassified_Rikenellaceae	Negative	sCD14	1.60E-06	5.63E-07	11	0.02496945	0.89385681
Gemella	Positive	PGRPs	6.63E-05	2.65E-05	11	0.04072833	0.89385681
Acidaminococcus	Negative	PGRPs	-0.000892	0.00035918	11	0.04200311	0.89385681
Roseburia	Positive	PGRPs	-0.0050578	0.0020531	11	0.04324419	0.89385681
Unclassified_Erysipelotrichaceae	Positive	PGRPs	0.00154982	0.00065127	11	0.04890272	0.89385681

translocation to occur but sustain a potential association of bacterial translocation with liver disease.

The microbial composition in the stool was also assessed at the end of 3 week-detoxification in AUD patients subdivided according to both permeability and ALD stage. Interestingly, we found an increased evenness (Shannon and Simpson) only in AUD patients with high IP (Figure 11d) while the number of observed species remains the same. By contrast, α -diversity indexes did not change in AUD patients with normal intestinal permeability (Figure 11d). Regarding ALD stage, we found a minor change in α -diversity indexes (richness and evenness) only in AUD patients with non-progressive ALD but not in progressive ALD (Figure 11e). Overall, microbial profile (β -diversity) did not change upon abstinence (Supplementary Figures 10,11).

These results support and reinforce the concept of a possible link between fecal microbiota dysbiosis and leaky gut but not with ALD progression in humans.

Discussion

Our work addresses the link between the gut and the liver at early stages of ALD in a unique human cohort. We demonstrate for the first time that alterations in the duodenal mucosa-associated microbiota together with elevated translocation of either microbial products and/or microbes themselves are associated with liver disease progression in a large human cohort of AUD patients. Intriguingly, the increase of surrogate markers for Gram+, Gram-, and fungal microbial translocation in the blood of AUD patients compared to controls occurred independently from both paracellular and vascular IP, measured by ^{51}Cr -EDTA and fecal albumin, respectively. Therefore, one might ask the question as to whether increased IP is absolutely required for microbes to cross the gut barrier. Microbial translocation might involve different mechanisms that do not necessarily depend on changes in paracellular and/or vascular gut leakiness. On the other hand, increased IP alone might

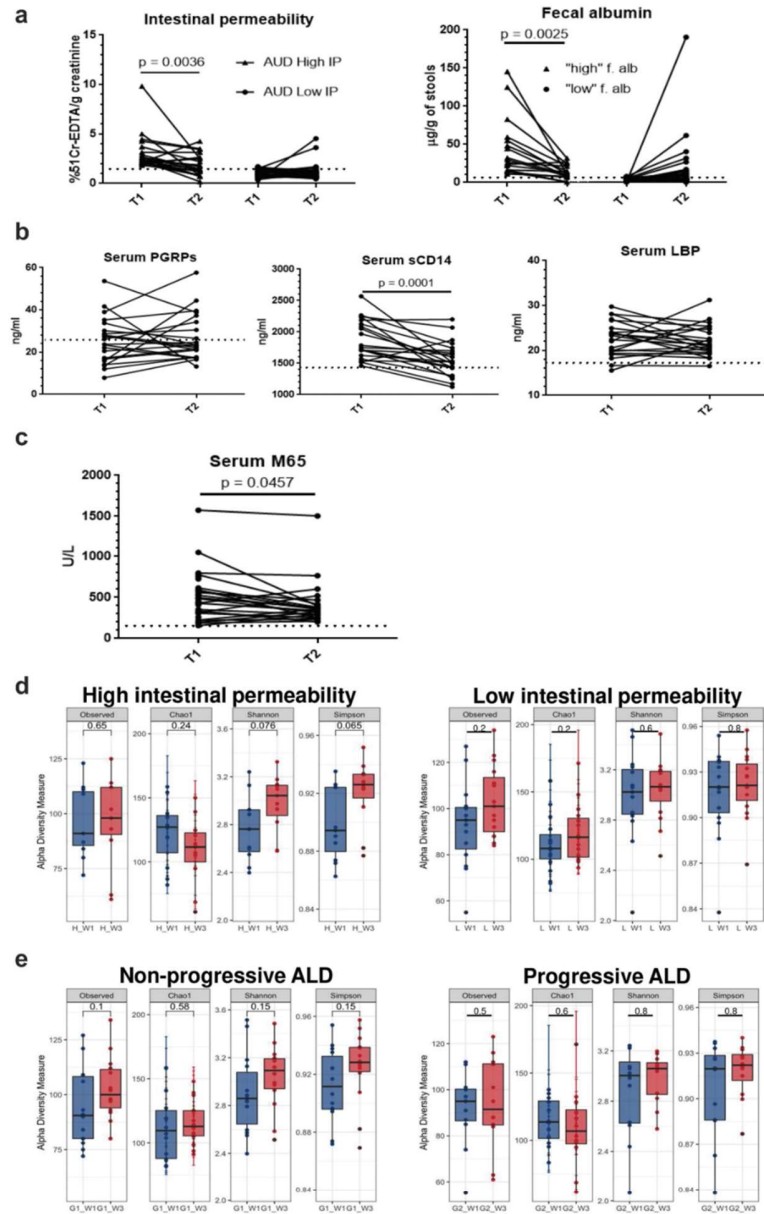


Figure 11. Impact of short time of abstinence on intestinal permeability (IP), microbial translocation, liver damage and microbiota composition. (a) In AUD patients with both high ^{51}Cr -EDTA urinary excretion and high fecal albumin content, levels returned to those observed in the control group (indicated with the dotted line) whereas no significant changes were found in AUD subjects with normal/low IP at T1. (b) Among microbial translocation markers, only serum soluble CD14 (sCD14) levels (middle) decreased upon abstinence but not LBP and PGRPs. (c) Levels of serum cytokeratin 18 (CK18-M65), a marker of liver cell damage, decreased significantly after abstinence but remained higher compared to controls (indicated with the dotted line). (d) Evenness (Shannon and Simpson) but not richness (observed species and Chao1) of the fecal microbiota increased in AUD patients with high intestinal permeability (left) after abstinence but not in those with initially normal intestinal permeability (right). (e) Observed species and evenness (Shannon and Simpson) were only slightly modified upon abstinence in AUD patients with non-progressive ALD but not in those with progressive ALD.

also not be sufficient to cause microbial translocation unless additional mechanisms in the complex interactions between host and microbes in the gut fail.

Inappropriate immune responses and/or transcytosis in the gut, as shown in several situations^{26,40} but not investigated in our study, might be one of those mechanisms also implicated in ALD. We found a decreased number of immune cells, such as macrophages and T cells, in the duodenal mucosa of AUD patients as well as little, if any, upregulation of gut-specific pro-inflammatory cytokines. This argues against a major inflammatory component as a driver for morphological changes and microbial translocation. Rather, one might speculate that this unexpected but intriguing decrease in immune effector cells leads to reduced immunosurveillance that may allow microbes to cross the gut barrier and reach the blood circulation. Indeed, an appropriate immune defense in the intestine should ward off the translocation and control invasion of bacteria and fungi into the tissues.⁴¹

Goblet cells also contribute to mucosal immunosurveillance. They may form the so-called goblet cell-associated antigen passages (GAPs) to deliver microbial antigens to antigen-presenting cells within the lamina propria.⁴⁰ It is tempting to speculate that under pathological conditions, microbes might also use this route in order to invade the intestine and finally enter the blood circulation. Thus, irrespectively of IP, the relative inefficiency of innate as well as adaptive components orchestrating the mucosal defense against microbial invaders, may explain, at least in part, our observation.

Gram-microbial translocation was associated with progressive ALD and correlated with liver cell damage marker CK18-M65. In addition, reduced richness with dominance shift toward potential pathogenic bacteria such as *Streptococcus*, *Shuttleworthia*, and *Rothia* characterized the duodenal-associated microbiota of AUD patients with progressive ALD. Intriguingly, all these genera belong to the Gram+ instead of Gram-bacteria. Thus, elevated sCD14 levels in the serum may not entirely reflect Gram-translocation because it recognizes ligands at the cell surface of both Gram- and Gram+ bacteria and it has been associated with Gram+ sepsis and mortality.^{42,43} Several studies reported a higher abundance in stool or saliva of *Streptococcus* in association with

cirrhosis severity, decompensation, and encephalopathy.^{22,44-46} Interestingly, both *Shuttleworthia* and *Rothia* are part of the normal oral microflora^{47,48} and are overrepresented in the distal duodenum of AUD patients with progressive ALD compared to controls. These findings suggest a possible extension of the oral microflora further down into the duodenum as a potential determinant in ALD progression, as already speculated in advanced stages of liver disease.⁴⁹ Proton pump inhibitor (PPIs) therapy⁵⁰ cannot explain the overrepresentation of oral microflora components in the duodenum of AUD patients since only a minority (<20%; data not shown) took PPIs on admission. Notably, *Rothia* spp. are generally considered organisms of low virulence in immunocompetent hosts but they have emerged as pathogens which can cause significant infections, for example, in patients with severe forms of liver disease.⁵¹ This predominance shift was accompanied by the diminution of important genera such as *Mycobacterium*, *Alcaligenes*, and *Lachnospirillum* characterizing the mucosa of healthy volunteers. Interestingly, *Alcaligenes* spp. have been shown to be important for the development, maturation, and maintenance of an appropriate gut immune system⁵² and its loss could be deleterious for immune surveillance in the intestine of AUD patients. Thus, our results revealed that specific opportunistic pathogens become dominant in the patients' small bowel mucosa who develop progressive ALD. Furthermore, the fact that intestinal permeability restores after short-term abstinence in opposite to microbial translocation (in particular Gram+) and liver damage indicates that both phenomena might indeed be linked. Our study confirms in humans, some observations made in mice of elevated translocation of microflora products related to disease severity.^{38,39}

Neither intestinal inflammation, morphological changes in the duodenum nor enterocyte damage provided a convincing explanation for IP changes. The reduction in villi length found in AUD patients might be caused by alterations of the differentiation program in the small intestinal epithelium and could influence absorption of various important nutrients.⁵³ Further studies are needed to investigate these processes and whether they result from toxicity related to alcohol and its metabolites and/

or are caused by alterations of the intestinal microbiome/metabolome. We also reveal important conceptual differences between the duodenal mucosa-associated and the fecal microbiota in relation to IP and ALD progression. We found that only a subgroup of AUD patients is characterized by increased IP associated with dysbiosis of the fecal microbiota but not with changes in the microflora attached to the duodenal mucosa. The present results confirm our previous observation in a different cohort of AUD patients with no or mild liver disease where fecal microbiota changes were associated with high IP.²⁴ Interestingly, metabolic analysis of fecal samples showed alterations of the metabolic profile linked to gut barrier dysfunction.^{24,54} ⁵¹Cr-EDTA measurements suggest that the permeability of the colon is preserved in AUD patients with fecal dysbiosis (not shown) which raises the question of how those changes indirectly interfere with events that occur in the small bowel. One might speculate that specific microbial metabolites which could reach the circulation could be linked to increased IP and appropriate metabolomics analysis together with a mechanistic approach is needed in order to elucidate the mechanisms involved in this intriguing observation. By contrast, fecal dysbiosis was not associated with ALD progression. However, we cannot exclude a potential link given its association with increased IP which could act as a facilitator of microbial translocation.

An intrinsic limitation of this study is that data are mainly based on associations/correlations, which do not formally prove a cause-and-effect relationship. However, given the difficulties in accessing tissues at early-stage disease, we believe our study adds significant insights into the role of the gut-liver axis in the early pathogenesis of ALD. Certainly, the main strength of this investigation is the unique patient cohort of a high number of heavily, actively drinking AUD patients in whom distal duodenal biopsies with collections of the blood, stools, and urine have been performed in a strict, highly standardized clinical program. We used point of care noninvasive techniques (Fibroscan®, controlled attenuation parameter (CAP), AST, and ALT levels) to diagnose and stage liver disease in combination with serum CK18-M65 levels. This allowed the identification

of liver damage with high accuracy even at early stages of ALD. A recent report underlines a benign disease course in nonalcoholic fatty liver disease in patients with normal transaminases.⁵⁵ Other studies suggest an added value of CK-18, a component of the cytoskeleton of hepatocytes which is released in the blood upon cell damage⁵⁶ in nonalcoholic steatohepatitis and viral disease.^{57–59} Our study extends current data on CK-18 also to ALD. The good diagnostic power of our integrated approach might prove useful for monitoring liver disease progression and/or regression in AUD patients.

Our observations may be relevant for clinical practice in the future since they suggest that many unfavorable factors are already linking the gut and the liver at early stages in human ALD. Short-term abstinence does not fully abolish microbial translocation, which can potentially lead to disease progression. Understanding the mechanisms underlying dysbiosis and translocation of microbes and/or their products into the blood circulation would provide us with new possible therapeutic targets at the frontlines of the complex host-microbes interactions during early human ALD.

Acknowledgments

This study was supported in part by NIH grants R01 AA24726 (to D.E.F., B.S., P.S.), U01 AA026939 (to D.E.F., B.S.) and services provided by P30 DK120515 and P50 AA011999, by grants from Fond National de Recherche Scientifique Belgium (J.0146.17 and T.0217.18) and Action de Recherche Concertée (ARC), Université Catholique de Louvain, Belgium to P.S. We thank the IREC imaging platform for their support in analyzing the results.

Disclosure of Potential Conflicts of Interest

B.S. has been consulting for Ferring Research Institute, Intercept Pharmaceuticals, HOST Therabiomics and Patara Pharmaceuticals. B.S.'s institution UC San Diego has received grant support from BiomX, NGM Biopharmaceuticals, CymaBay Therapeutics, and Synlogic Operating Company. P.S. received grant support from Gilead Sciences Belgium.

Funding

This work was supported by the Fonds national de la Recherche Scientifique - FNRS [J.0146.17]; Fonds national de la Recherche Scientifique - FNRS [T.0217.18]; National

Institutes of Health [U01 AA026939]; National Institutes of Health [R01 AA24726]; Université Catholique de Louvain [ARC 2018].

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Intestinal permeability, microbial translocation, changes in duodenal and fecal microbiota and their associations with alcoholic liver disease progression in humans

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Supplementary material/data

Content:

Supplementary methods

- Patients:
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Patients, methods

Inclusion-exclusion criteria

Additional inclusion criteria were first admission to the alcohol withdrawal unit and absence of previously diagnosed significant alcoholic liver disease.

Patients with diabetes were eligible for the study if they were on stable treatment (metformin, oral anti-diabetics with or without insulin) and a glycosylated hemoglobin level < 7% at admission.

Patients were excluded from the analysis if they had one of the following conditions:

- Inflammatory bowel disease or other chronic inflammatory or autoimmune diseases
- History of cancer within 5 years prior to admission
- Known liver disease of any other etiology
- Clinically significant cardio-vascular, pulmonary or renal co-morbidities
- Clinical and biochemical signs of active infection ($t^{\circ} > 37,5^{\circ}\text{C}$, viral syndrome, high CRP and high White Blood cell count)
- Administration of antibiotics, probiotics, glucocorticoids and nonsteroidal anti-inflammatory drugs during the two months preceding enrollment.

Fibroscan® measurements

Transient elastography measurements were performed using the Fibroscan® device (Echosens, Paris, France) by an experienced examiner blinded to the patient's data. Fibroscan® was considered as valid if at least 10 validated measurements were obtained with an interquartile range of less than 30%. The final result expressed in kPa was the median of all valid measurements obtained. We used the cut-offs for alcoholic patients proposed by Nguyen-Khac et al. (Aliment Pharmacol Ther. 2008; 28:1188-98), i.e. 7.8, 11, 19.5 kPa for F2, F3 and F4, respectively

Upper gastro-intestinal endoscopy and biopsy specimens

The standardized protocol of the alcohol withdrawal unit foresees a routine upper gastro-intestinal endoscopy within 72 hours of admission by an experienced endoscopist. Patients were slightly sedated with midazolam and 20mg of buthylhyosine was administered intravenously prior to the procedure in order to reduce intestinal peristaltic contractions. The endoscope was rapidly pushed under visual control into the distal duodenum. Care was taken to keep the transit time through the esophagus and the stomach as short as possible. Biopsies were taken from the distal duodenum with a single-use biopsy forceps (Boston Scientific, Galway, Ireland). During the first forceps passage, two duodenal biopsies were obtained for DNA extraction and 16S rRNA sequencing. Specimens were displayed on a sterile glass plate and rinsed repeatedly with sterile physiological solution to flush away non-adherent mucus and microbes, transferred into a sterile tube and immediately frozen in liquid nitrogen. Subsequent passages were performed to obtain additional biopsies which were preserved in RNA later (Invitrogen™, Thermo Fisher Scientific, Merelbeke, Belgium) or fixed in formalin.

Measurement of intestinal permeability

Intestinal permeability was assessed by measuring both the urinary excretion of the radioactive probe ^{51}Cr -EDTA and the fecal albumin content by ELISA.

Briefly, after an overnight fast and emptying of the bladder, patients drank a Nutridrink (200 mL, 150 kcal/100 mL) (Nutricia) containing 50 μCi (1.85 MBq) ^{51}Cr -EDTA. Two urine

collections were obtained: one from 0h to 4h representing the proximal intestine (duodenum, jejunum) and one from 4h to 24h representing the distal gut (ileum, colon). Radioactivity was measured in urine collections with a gamma counter (Cobra5003; Canberra Packard). Urinary excretion was expressed as the percentage of the ingested dose (ID) normalized to creatinine concentration (% ID/g creatinine). Permeability of the different parts was calculated from the respective urine collections. Total intestinal permeability was derived from the entire 24h urine collection.

In order to assess the fecal albumin content, proteins were extracted from at least 3 different parts of the stools of each patient using the extraction buffer provided in the kit (Human Albumin ELISA Kit, Immundiagnostik AG, Bensheim, Germany). Every single measurement was conducted in duplicate, and the data represent the mean of the different measurements performed.

Immunofluorescence

Intestinal samples were fixed in 10% formalin for 2 hours, washed and dehydrated in 30% sucrose for at least 4 hours and the included in OCT compound (Sakura, Kampenhout, Belgium). Five μ m cryosections were rehydrated, blocked with phosphate buffer saline (PBS) 1x, 10% bovine serum albumin (BSA), and 3% milk. Sections were stained with the primary antibodies (Polyclonal rabbit IgG anti- ZO-1 (61-7300, Thermofisher, Merelbeke, Belgium) 1/100 and anti-human PLVAP (clone174/2, Abcam, Cambridge, United Kingdom) 1/50 overnight at 4 °C) followed by incubation with a secondary antibody conjugated with the fluorochrome Alexa Fluor 594 donkey anti-rabbit at 1/1000. Before imaging, nuclei were counterstained with 4', 6-diamidin-2-phenylindole (DAPI). Fluorescence multiplex immunostained slides were digitalized using a Panoramic 250 FlashIII scanner (3DHistech, Budapest, Hungary).

PLVAP staining was quantified using the image analysis tool Author version 2017.2 (Visiopharm, Hørsholm, Denmark) and results were expressed as staining index (mean fluorescence intensity * stained area) on the duodenal biopsies.

Histology and morphometric indices

Since upper gastro-intestinal endoscopy is part of the standardized routine program of the alcohol withdrawal unit, biopsy specimens were routinely sent to the pathology department for examination by an experienced gastro-intestinal pathologist who generated a standard pathology report. After exclusion of any gastro-intestinal pathology susceptible to interfere with additional analyses of the specimens (e.g. celiac disease, Whipple's disease, collagenous sprue, inflammatory bowel disease, etc.), the duodenal sections were stained with Hematoxylin and Eosin and digitalized using a SCN400 slide scanner (Leica Biosystems, Wetzlar, Germany) at X20 magnification. Scanned slides were subjected to morphometric analysis with the image analysis tool Author version 2017.2 (Visiopharm, Hørsholm, Denmark). On each slide, villi were manually delineated and the major axis length of a simplified ellipse automatically drawn around the villus was measured. Results were expressed as the mean major axis length of at least 3 villi per slide.

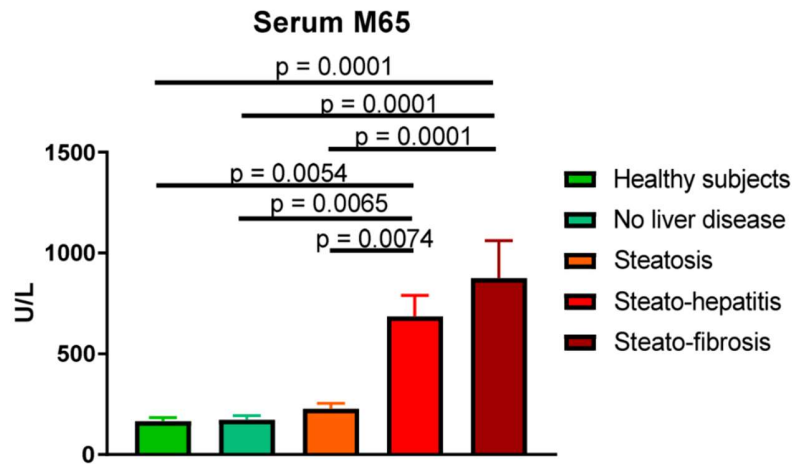
Immunohistochemistry

Duodenal sections were stained overnight at 4°C with antibodies against CD45 (Mouse monoclonal anti-CD45 LCA, Clone 2B11+PD7/26) 1/30, CD68 (Mouse monoclonal anti-CD68, Clone PG-M1) 1/100, and CD3 (Mouse monoclonal anti-CD3, Clone L26) 1/200, and digitalized using a SCN400 slide scanner (Leica Biosystems, Wetzlar, Germany) at X20 magnification. Scanned slides were subjected to quantification analysis with the image analysis tool Author version 2017.2 (Visiopharm, Hørsholm, Denmark). On each slide, villi were first manually delineated and then automatically delineated along their perimeter. Stained pixels were detected at high resolution (x20) using a thresholding classification method. Thresholds were adjusted on representative stained vs not stained regions. The same

parameters were kept constant for all slides. Results were expressed as the mean stained area in percentage of at least 3 villi per slide normalized to the total surface area of the villi taken in consideration.

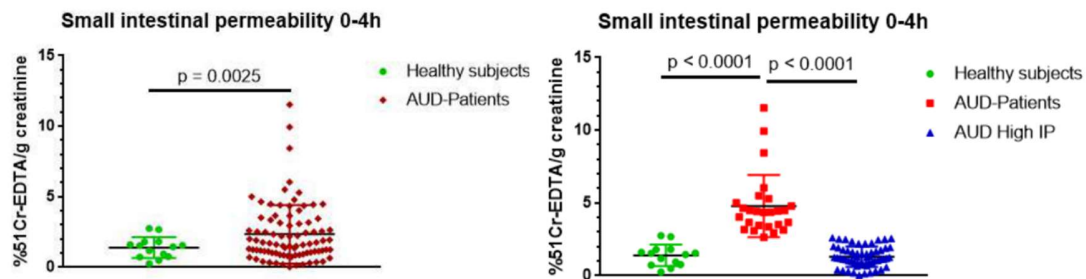
Reverse-Transcription and quantitative Real-Time Polymerase Chain Reaction

Primers were as follows: Villin: forward (F)-5'-CTGCTACATCATCCTGGCTATC-3' and reverse (R)-5'-GTCATCCATCTGTGTGGTGTAG-3'; IL1 β : (F)-5'-GCACGATGCACCTGTACGAT-3'; (R)-5'-AGACATCACCAAGCTTTTTTGCT-3'; IFN- γ : (F)-5'-GCATCCAAAAGAGTGTGGAG-3'; (R)-5'-GCAGGCAGGACAACCATTAC-3'; IL17: (F)-5'-TTAGACCCCATCAAGGGTTAAAT-3'; (R)-5'-TGACCTTCTCTTCCCTCTCTCTT-3'; IL22: (F)-5'-AAAGCTGGTAGGAAAATGAGTCC-3'; (R)-5'-ACCTCTATCCTTTCCACCTTGAG-3'; Rpl-19: (F)-5'-CAAGCGGATTCTCATGGAACA-3'; (R)-5'-TGGTCAGCCAGGAGCTTC T-3'.



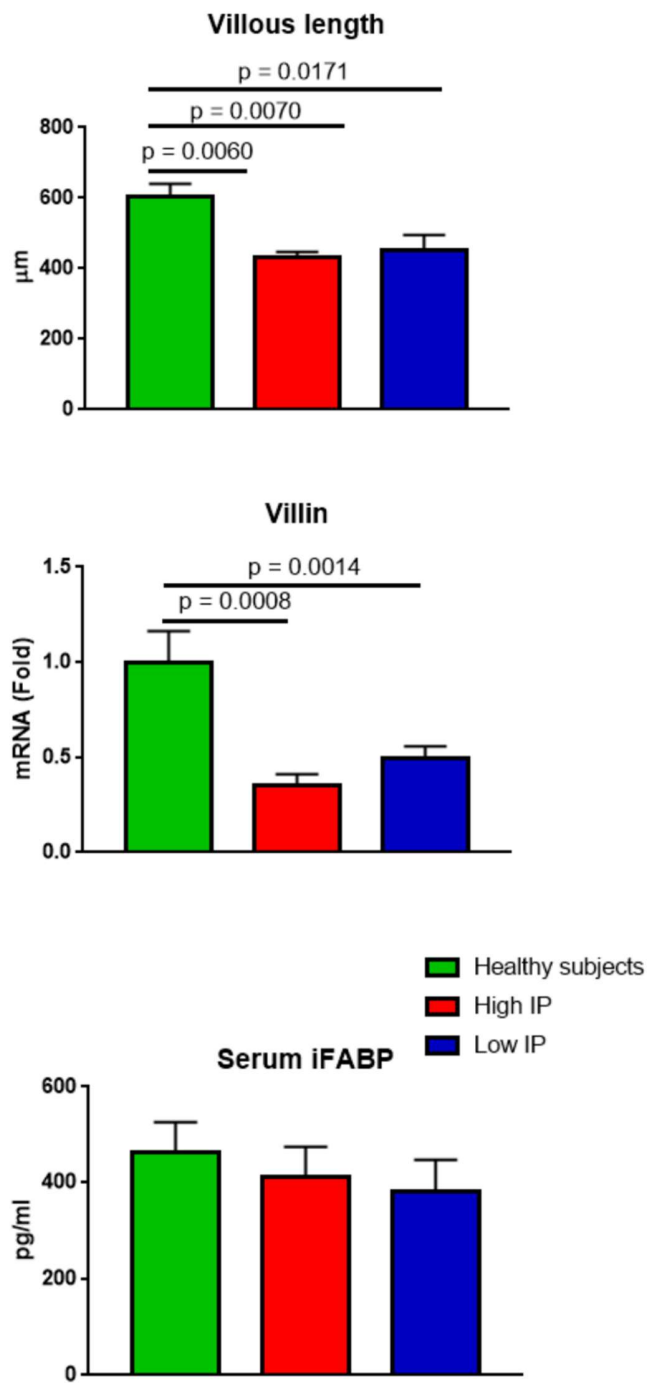
Supplementary Figure 1. Serum cytokeratin 18 (CK18-M65) according to different stages of liver disease.

Elevated CK18-M65 was only found in progressive ALD (steato-hepatitis and steato-fibrosis) independently from the development of fibrosis.



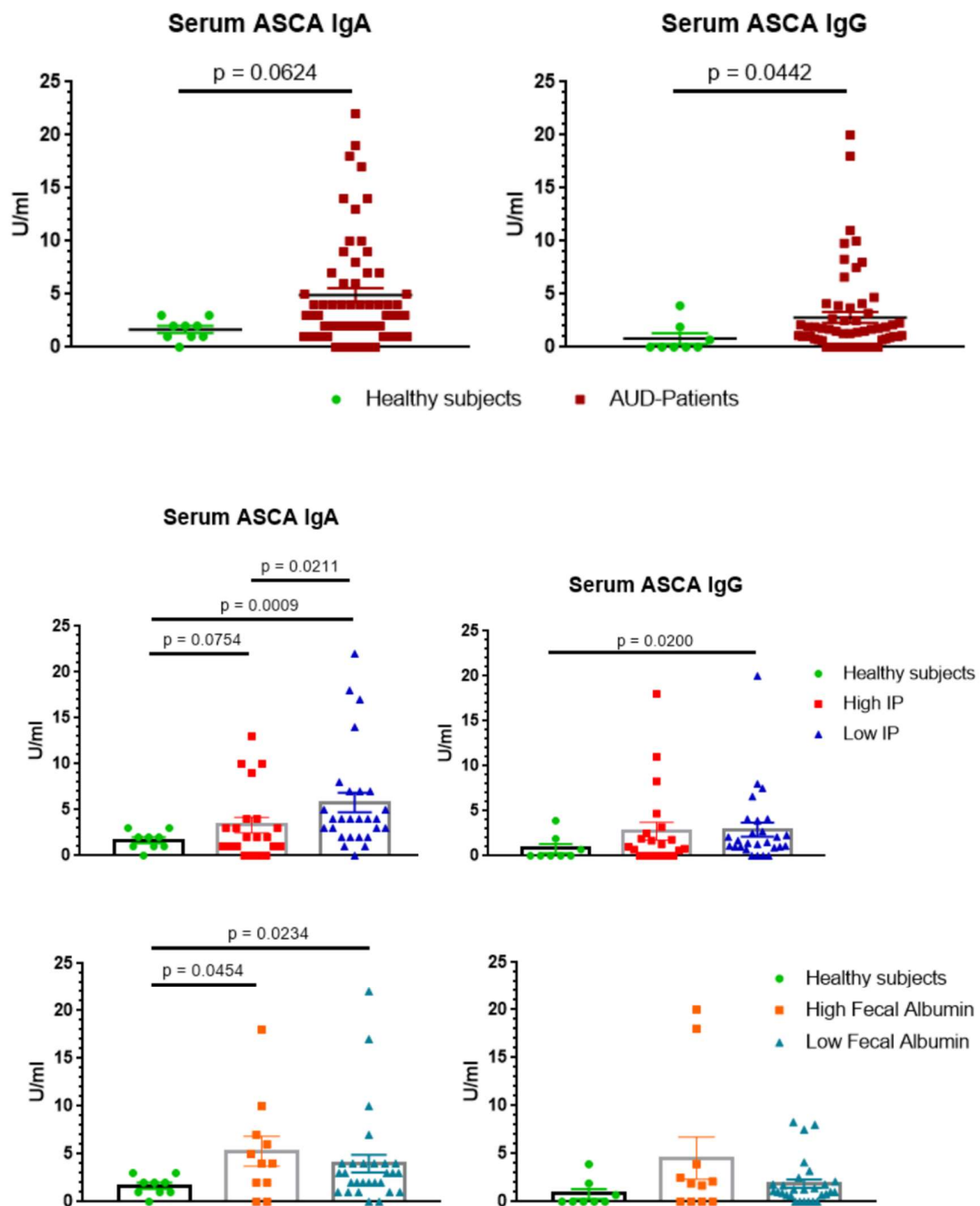
Supplementary Figure 2. Intestinal permeability (IP) in AUD patients in the small gut.

IP, based on ^{51}Cr -EDTA urinary excretion during the first four hours, representing the proximal small bowel fraction (duodenum, jejunum), increased significantly in AUD patients ($n=86$) compared to controls ($n=14$) (left). More detailed analysis revealed that only one-third of AUD patients showed significantly increased IP ($n=34$) compared to controls (right). This separation of subjects was calculated according to a deviance criterion at a threshold of 1.65 SDs of the mean of the control group.



Supplementary Fig.3 Villi's length, Villin gene expression and enterocyte damage according to intestinal permeability (IP).

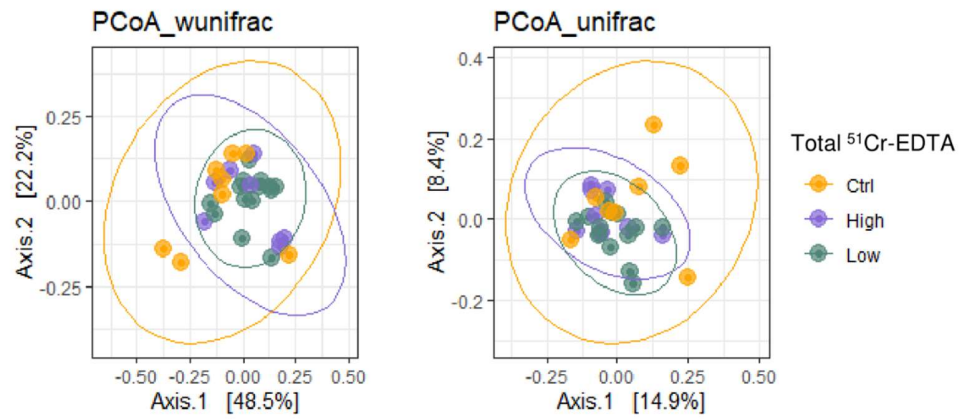
All the investigated parameters were similar when patients were split according to increased IP, measured by ^{51}Cr -EDTA urinary excretion.



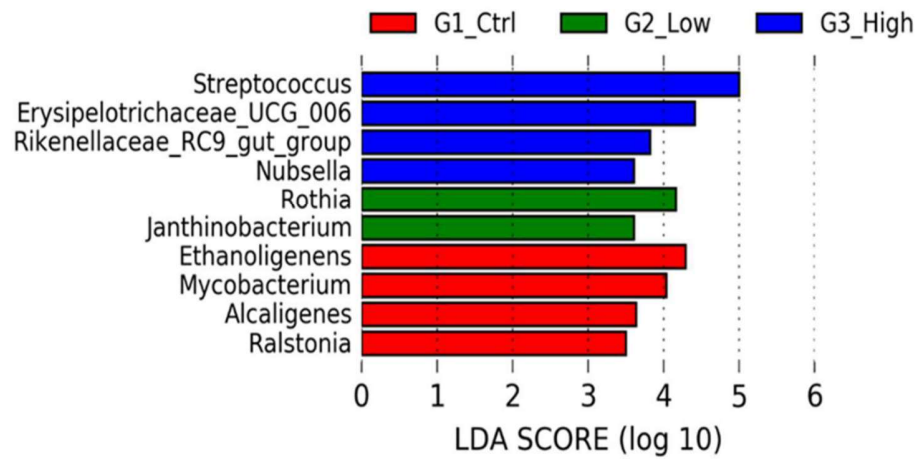
Supplementary Fig.4 Evaluation of serum ASCA levels in AUD patients in association with intestinal permeability (IP).

Serum ASCA levels increased significantly in AUD patients (n=60) compared to controls (n=9) (above) but independently of increased IP, measured by ^{51}Cr -EDTA urinary excretion (middle graphs) and fecal albumin content (lower graphs).

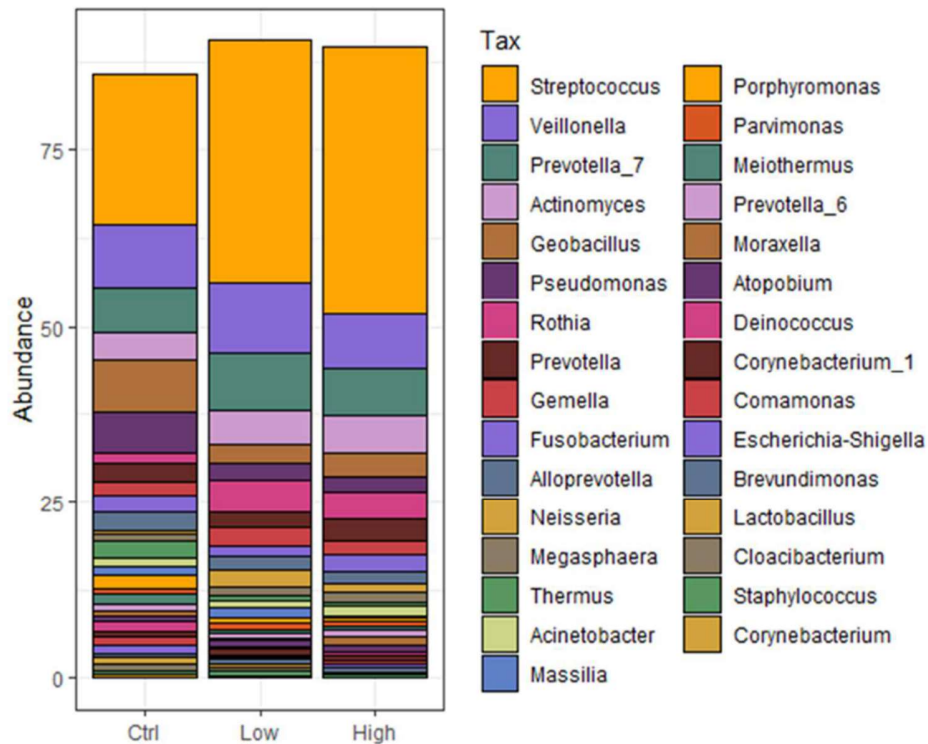
A



B



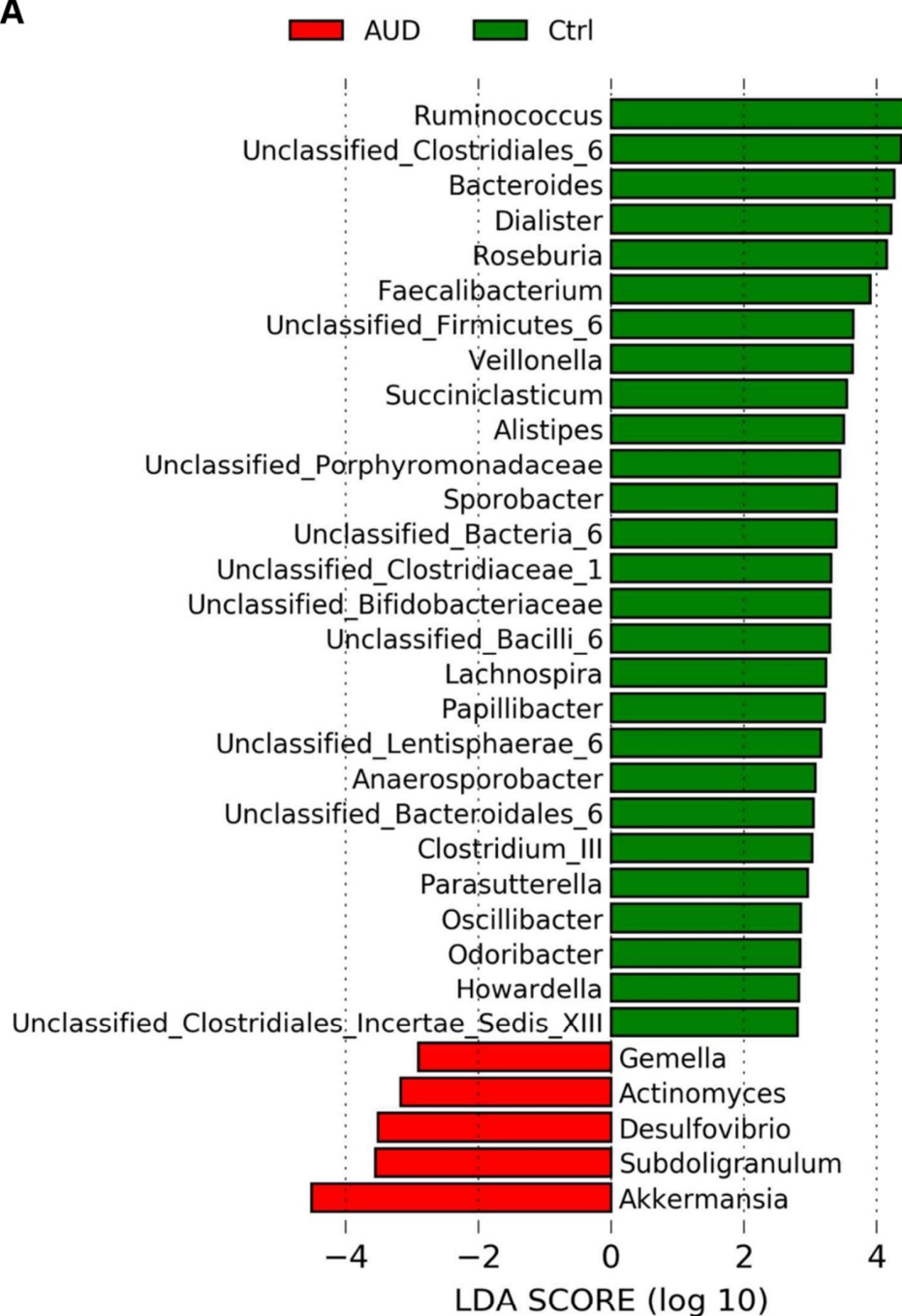
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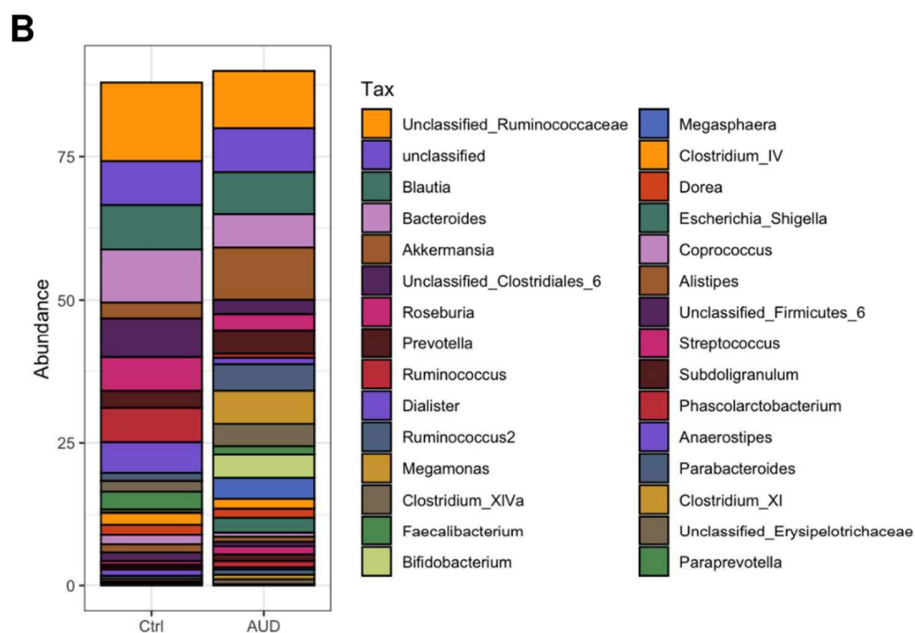


Supplementary Fig.5 Microflora composition of the duodenal mucosa-associated microbiota in association with intestinal permeability (IP).

(A) Microflora composition (β diversity) analysis of the duodenal microbiota showed no differences between AUD patients with high and low IP and healthy volunteers. (B) Results of linear discriminating analysis (LDA) effect size of AUD patients (high and low IP) compared to controls at the genus level. Different genera were overrepresented in the distal duodenum of AUD patients in relation to IP and healthy subjects (Ctrl). (C) Relative abundance of the different genera in the three groups. Only genera with detection in at least 2% are shown.

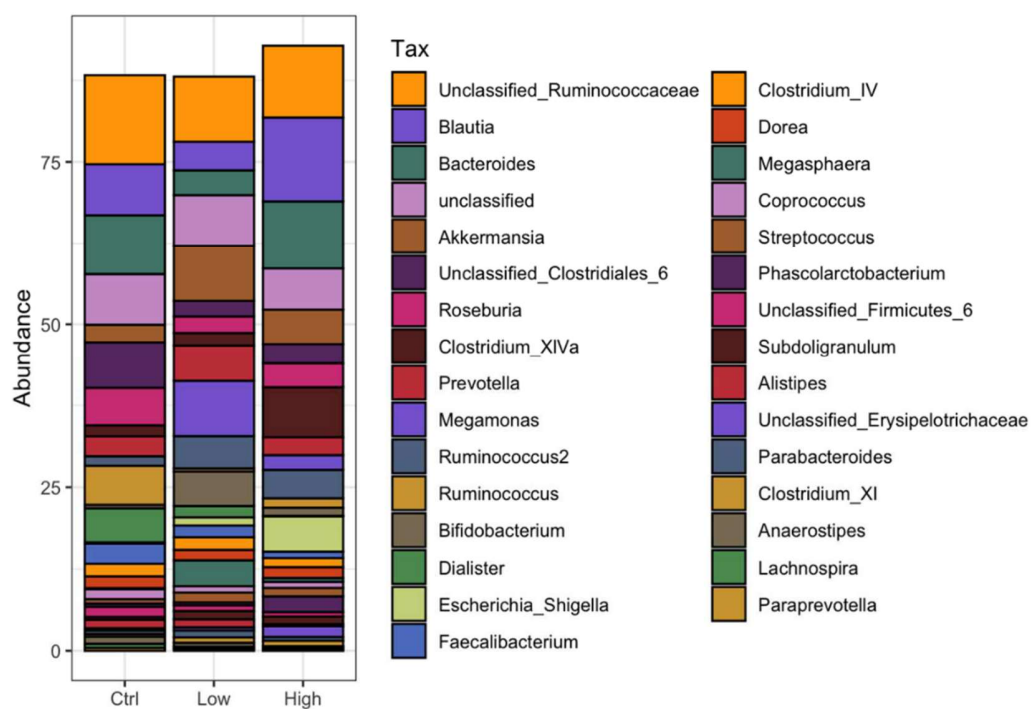
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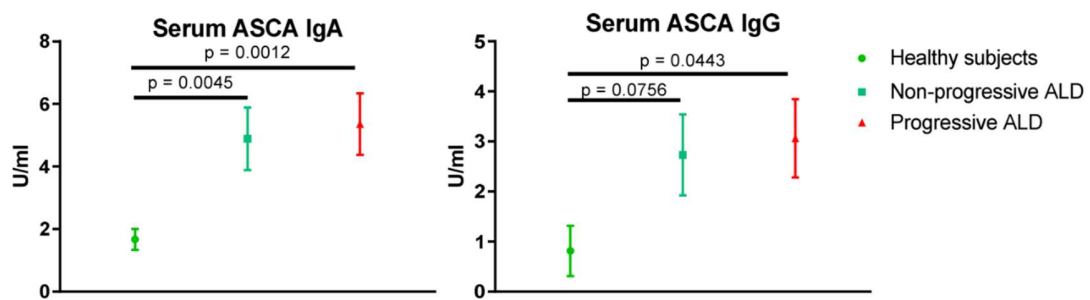


Supplementary Fig.6 Linear discriminating analysis (LDA) and relative abundance of bacterial genera in the stool of AUD patients and healthy volunteers.

(A) Higher abundance of *Gemella*, *Actinomyces*, *Desulfovibrio*, *Subdoligranulum* and *Akkermansia* characterized the stools of AUD patients compared to controls. (B) Relative abundance of the different genera in the two groups. Only genera with detection in at least 2% are shown.

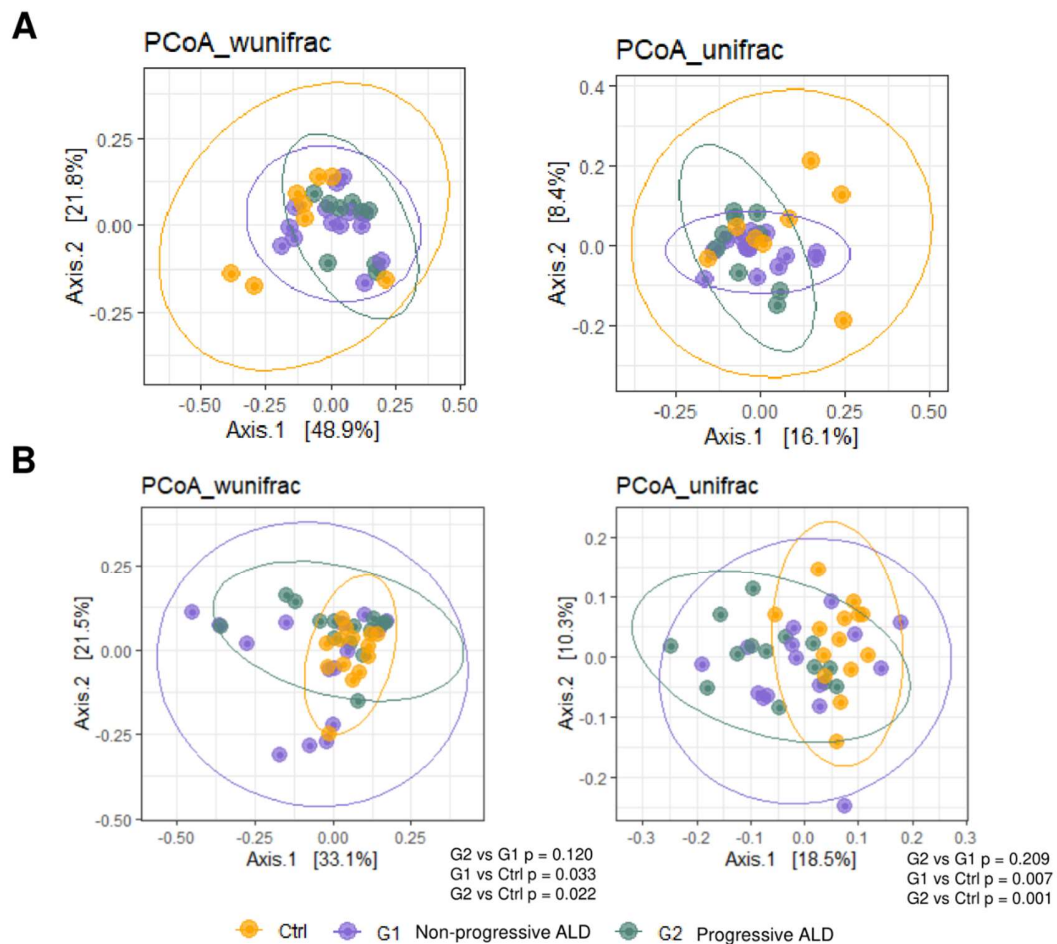


Supplementary Fig.7 Relative abundance of the different genera in AUD patients with high and low intestinal permeability (IP) and controls.



Supplementary Fig.8 Changes of serum ASCA levels in AUD patients in association with severity of liver disease.

Serum ASCA IgA and IgG increased independently from liver disease severity and were already significantly upregulated in non-progressive ALD compared to controls.

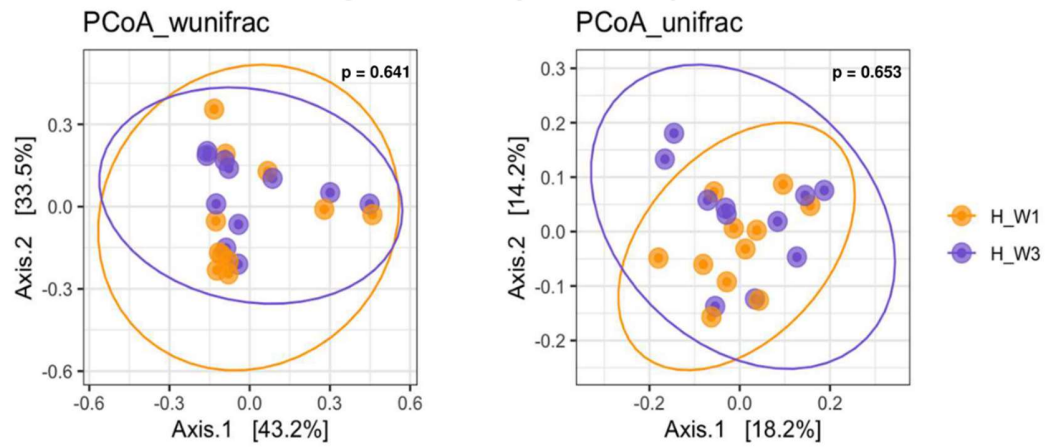


Supplementary Fig.9 Microflora composition in both duodenum and stools according to stage of liver disease.

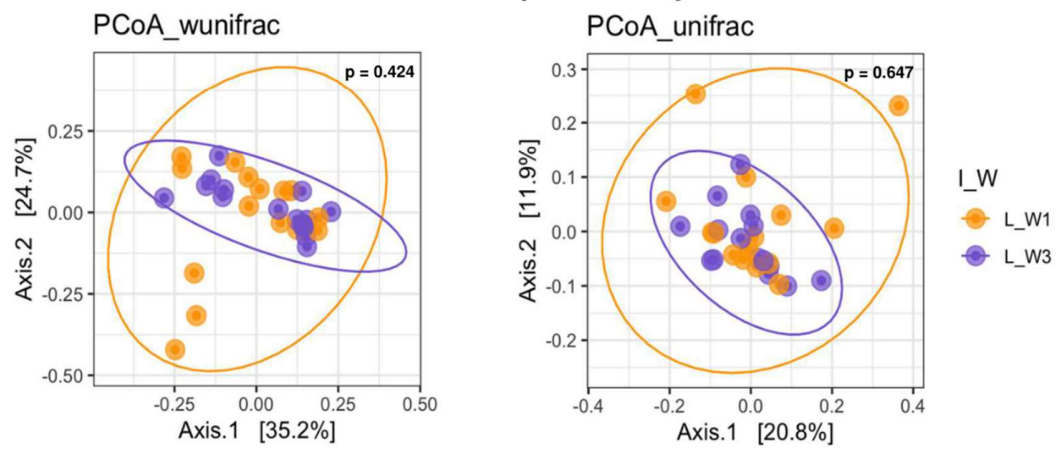
Microbial composition (β diversity) of the duodenal mucosa-associated (**A**) and fecal (**B**) microbiota did not change in relation to ALD severity in AUD patients.

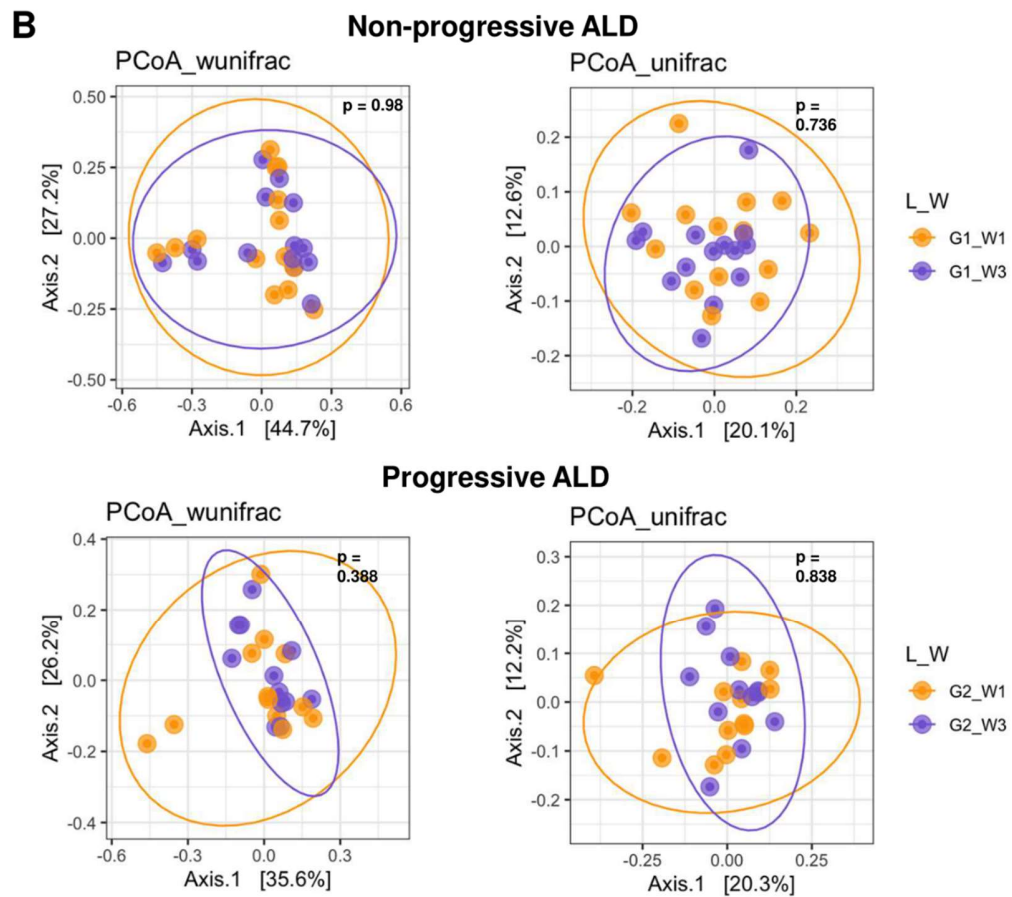
A

High intestinal permeability

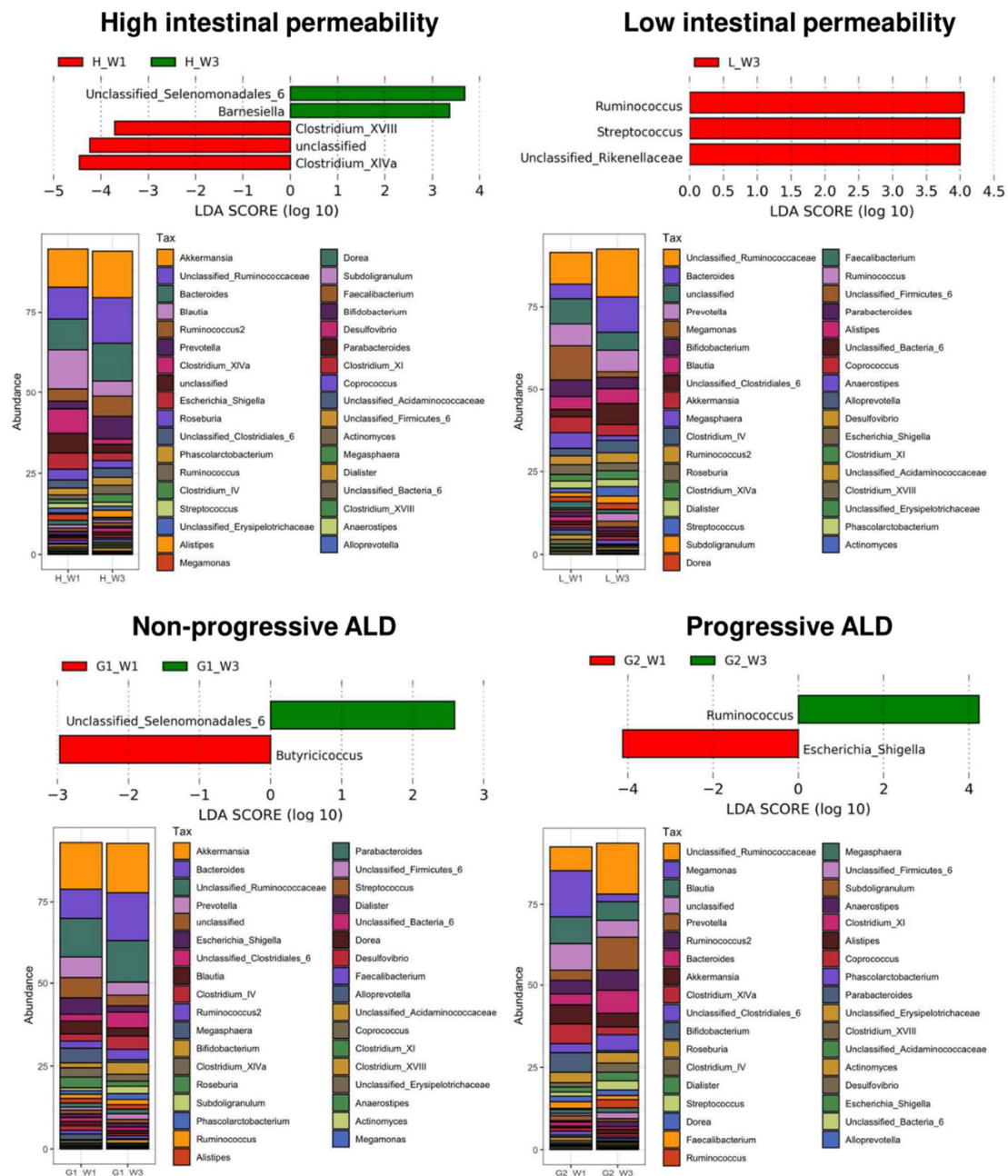


Low intestinal permeability





Supplementary Fig.10 Microbial profiles in the stools of AUD patients after abstinence.
Microbial composition (β diversity) of the fecal microbiota did not change after abstinence in AUD patients subdivided according to both intestinal permeability (A) and ALD stage (B).



Supplementary Fig.11 Linear discriminating analysis (LDA) and relative abundance of bacterial genera in the stool of AUD patients, subdivided according to both intestinal permeability and ALD stage, after abstinence.

Complementary discussion and conclusion

Elevated systemic translocation of microbes and/or their products occurred independently from increased intestinal permeability but was associated with reduced number of effector immune cells in the duodenum of AUD patients. In addition, alterations in the duodenal mucosa-associated microbiota together with elevated microbial translocation were linked to ALD progression. By contrast, changes in the fecal microbiota did not associate with liver disease severity but were related to leaky gut. These results support that mechanisms implicated in increased intestinal permeability and translocation of microbes and/or their products across the gut barrier could occur independently, and that alterations in the proximal small gut are likely directly linked to ALD physiopathology in early pre-cirrhotic stages. The fact that diminution of local sentinels in the duodenum involved in immunosurveillance against microbes, such as macrophages and T lymphocytes, associated with proximal small gut microbiota changes and translocation of microbes and/or their products indicate the potential contribution of this phenomenon in the progression of ALD.

In conclusion, we have characterized in chapter 2 the elements that link the gut and the liver during early ALD. Diminution of duodenal immune cells, changes in the duodenal microbiota and elevated translocation of microbes and/or their products appear to be linked to disease progression.

The perspectives of chapter 2 are the following:

- Evaluation of factors potentially involved in the impairment of mucosal immunosurveillance against microbes and disease progression.
- Characterization of molecular mechanism implicated in host-related changes in the intestine of AUD patients.
- Identification of immune cells and inflammatory pathways connecting translocation of microbes and/or their products and ALD progression.

CHAPTER 3: Duodenal CD8+ T resident memory cell apoptosis links gut barrier dysfunction, microbial translocation and alcohol-associated liver disease in humans

We have revealed in chapter 2 that bacterial and fungal systemic translocation and/or their products in AUD patients can occur independently from paracellular and vascular permeability. These results suggest that other compartments of the intestinal barrier also need to fail to allow microbes and/or their products to reach the blood circulation. Another key component for the maintenance of the gut barrier is the intestinal-associated immune system. Intestinal mucosal T cells represent key sentinels that protect against microbial attachment, invasion, and translocation. Since we showed that a reduced number of T cells characterized the mucosa of AUD patients in association with elevated microbial translocation and/or their products (chapter 2), the principal aim of this study was to evaluate the alterations in the duodenal T cell compartment and to assess the relationship between duodenal T lymphocytes and systemic microbial translocation in AUD patients. We then correlate the observed changes with the pattern and severity of liver disease.

Gut T lymphocytes rely for their generation and long-term survival on the consumption of exogenous lipids, which could impact their functions. In an ancillary study, we found that lipid profiles might differentiate progressive ALD from simple steatosis (annex 2). We therefore hypothesized that these lipids may interfere with some populations of intestinal T lymphocytes which was examined as a secondary aim in this study.

We revealed that apoptosis of duodenal CD8+ T resident memory cells (TRM) related to functional changes in lysosomes, mitochondria and lipid metabolism support an impaired gut adaptive immunity in AUD patients with progressive ALD. Interestingly, increased **sphingomyelin (d18:0/14:0) plasma levels correlated with apoptosis** and stimulation with the sphingomyelin cleavage product dihydroceramide **dose-dependently reduced viability of TRM**, pointing to a potential involvement of these sphingolipids in cell death.

The results of this third chapter have been submitted

Duodenal CD8⁺ T resident memory cell apoptosis links gut barrier dysfunction, microbial translocation and alcohol-associated liver disease in humans

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All authors read and approved the final manuscript.

Acknowledgements: This study was supported in part by NIH grants R01 AA24726, R37 AA020703, U01 AA026939, U01 AA026939-04S1, by Award Number BX004594 from the Biomedical Laboratory Research & Development Service of the VA Office of Research and Development, and a Biocodex Microbiota Foundation Grant (to B.S.) and services provided by NIH centers P30 DK120515 and P50 AA011999, by grants from Fond National de Recherche Scientifique Belgium (J.0146.17 and T.0217.18) and Action de Recherche Concertée (ARC), Université Catholique de Louvain, Belgium to P.S. We thank the IREC CytoFlux and imaging platforms for their support in analyzing the results.

Conflicts of interest: B.S. has been consulting for Ferring Research Institute, HOST Therabiomics, Intercept Pharmaceuticals, Mabwell Therapeutics, Patara Pharmaceuticals and Takeda. B.S.'s institution UC San Diego has received research support from Axial Biotherapeutics, BiomX, CymaBay Therapeutics, NGM Biopharmaceuticals, Prodigy Biotech and Synlogic Operating Company. B.S. is founder of Nterica Bio. UC San Diego has filed several patents with B.S. as inventor related to this work. P.S. received grant support from Gilead Sciences Belgium.

Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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ABSTRACT

Background Human data evaluating alterations in intestinal T cells, that play a key role in gut barrier function, in early stages of alcohol-associated liver disease (ALD) are lacking. We explored the links between intestinal T cells, microbial translocation, and ALD progression.

Methods Alcohol use disorder (AUD) patients following a rehabilitation program were compared to non-alcoholic fatty liver disease (NAFLD) subjects and healthy controls. Clinical and laboratory data (liver stiffness, controlled attenuation parameter, AST/ALT, CK18-M65) served to distinguish AUD patients with simple steatosis (non-progressive) and steato-hepatitis/fibrosis (progressive ALD). Serum microbial translocation markers were measured by ELISA. Plasma levels of sphingomyelins were analysed by targeted LC-MS. T lymphocytes in duodenal biopsies were characterized by immunohistochemistry, flow cytometry and RNA sequencing on FACS-sorted cells. Mechanisms for T cell alterations (apoptosis, lysosomal and mitochondrial dysfunctions) were assessed *in vitro* by various methods.

Results Only progressive ALD patients showed reduced numbers of duodenal CD8+ T resident memory (TRM) cells compared to NAFLD and controls, with a shift to the senescent phenotype. TRM transcriptomic analysis, *in vitro* analyses and pharmacological inhibition of Cathepsin B confirmed TRM apoptosis driven by lysosomal membrane permeabilization together with Cathepsin B release into the cytosol. Mitochondria uncoupling, altered lipid metabolism and increased sphingomyelin (d18:0/14:0) plasma levels correlated with apoptosis. Stimulation with the sphingomyelin cleavage product dihydroceramide dose-dependently reduced viability of TRM. Duodenal TRM phenotypic changes, apoptosis and transcriptomic alterations correlated with increased levels of microbial translocation markers. A short-term abstinence did not reverse TRM cell death in AUD.

Conclusions Apoptosis of duodenal CD8+ TRM related to functional changes in lysosomes, mitochondria and lipid metabolism support an impaired gut adaptive immunity in AUD patients with progressive ALD.

Keywords Gut immunity; Intestinal barrier; Lysosomes; Alcohol use disorder

INTRODUCTION

Although most patients with alcohol use disorders (AUD) develop steatosis, only a minority progress to the more severe forms of alcohol-associated liver disease (ALD). The mechanisms involved in disease progression are not completely understood but emerging evidence from animal models of chronic alcohol exposure suggests a role of the gut-liver axis implicating intestinal barrier dysfunction^{1,2} in liver disease progression. Increased intestinal permeability, gut microbiota changes and elevated microbial translocation have been linked to ALD development^{3,4}. However, the bacterial and fungal microbial translocation in AUD patients can occur independently from paracellular and vascular intestinal permeability⁵. These results suggest that other compartments of the intestinal barrier also need to fail to allow microbes to reach the blood circulation. Another key component for the maintenance of the gut barrier is the intestinal-associated immune system⁶. Little is known about the gut immune system in intestinal barrier dysfunction in at early stages of ALD in humans and studies generally focused on the overall role of gut immunity in decompensated cirrhosis⁷. We recently found a reduced number of T cells in the duodenal mucosa of AUD patients linked to elevated microbial translocation⁵.

Intestinal mucosal T cells act as local gate keepers to protect against microbial invasion⁸ thanks to their T cell receptor (TCR) repertoire⁹. Intestinal T resident memory (TRM) lymphocytes are the main resident population retained at mucosal sites due to CD103 and high CD69 expression.¹⁰ They

are long-lived cells¹¹ that originate from killer cell lectin-like receptor G1 (KLRG1) negative precursors, in opposite to KLRG1 + T cells that are short-lived and constantly replenished from the systemic circulation^{12,13}.

TRM are central in host defense in mucosal tissue allowing fast and targeted adaptive immune responses to invading pathobionts¹⁰ and their role is increasingly investigated in the development of chronic diseases especially in the intestine⁸.

We aimed at characterizing the alterations in the duodenal T cell compartment and to assess the relationship between duodenal T lymphocytes and systemic microbial translocation in AUD patients. We finally correlate the observed changes with the pattern and severity of liver disease.

METHODS

Patients

AUD patients (Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition criteria) admitted for elective alcohol withdrawal to a dedicated alcohol withdrawal unit were prospectively recruited from a highly standardized and controlled 3-week detoxification and rehabilitation program (Supplementary Fig.1). All patients reported long-term (>1 year) alcohol consumption (>60g/day) and were actively drinking until the day of admission. Additional inclusion and exclusion criteria are available in Supplementary data. Clinical and baseline biochemical data as well as transient elastography with controlled attenuation parameter (CAP) (Fibroscan®) were collected at admission. The presence of steatosis was further confirmed by doppler ultrasound. They were compared to healthy controls matched for gender, age and BMI (social drinkers consuming <20g of alcohol/day), to subjects with non-alcoholic fatty liver disease (NAFLD) in whom duodenal biopsies were obtained as a routine during work-up for bariatric surgery (details in supplementary material) as well as to abstinent AUD patients who completed the detoxification program.

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institution's human research and ethical committee (B403201422657; 2014/15OCT/514). Written informed consent was obtained from all patients and healthy controls.

Clinical classification of AUD patients according to severity of liver disease

AUD patients were classified into non-progressive (no liver disease/simple steatosis) and progressive ALD (steato-hepatitis/steato-fibrosis) based on clinical parameters as depicted in supplementary table 2. Serum Cytokeratin 18 levels (CK18-M65 ELISA kit; TECOmedical AG, Sissach, Switzerland), a liver specific cell damage marker¹⁴ were used to adjust the clinical classification of the two groups (internally validated cut-off 270 U/l).

Analysis of duodenal immune cells

Duodenal T cells were isolated, characterized and quantified using various antibodies by flow cytometry with a BD FACSCanto™ II equipped with BD FACSDiva™ software (see supplementary material).

Assessment of cell death, lysosomal membrane permeabilization and mitochondrial ROS production

Apoptosis was assessed using the eBioscience™ Annexin V Apoptosis Detection Kit APC and further confirmed in cryosections with the terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) assay using an *in-situ* cell death detection kit (Roche, Vilvoorde, Belgium). Lysosomal membrane permeabilization and mitochondrial ROS production were measured with LysoTracker™ Deep Red and MitoSOX™ Red Mitochondrial Superoxide Indicator, respectively (all

ThermoFisher, Merelbeke, Belgium). All assays were performed following the manufacturer's instructions.

RNA sequencing

RNA sequencing from sorted duodenal TRM cells was isolated using QIAGEN RNeasy Plus Mini Kit and sequenced using the HiSeq4000 instrument. Count-based differential expression analysis was done with R-based Bioconductor package DESeq2. GSEA was performed with clusterProfiler (v.3.14.3) Bioconductor package and gene sets from Molecular Signature Database (msigdb v.7.1.1). p-values were adjusted for multiple testing (see supplementary material).

LC-MS/MS analysis of sphingolipids

Plasma lipids were analyzed by LC-MS/MS by using an Agilent 1290 HPLC system coupled with a high-resolution mass spectrometer system (Agilent 6550 ion funnel MS-qTOF) (see supplementary material).

Cell culture

Cell viability and apoptosis were evaluated in sorted TRM upon stimulation with different inhibitors and sphingolipids by using WST-1 and Caspase 3/7 assay, respectively (see supplementary material).

Statistics

Data were analyzed using Graph Pad Prism 9 (GraphPad Software, San Diego, CA, USA) and presented as mean $\pm$ standard error of the mean (SEM) unless otherwise indicated. Normality was assessed by the Kolmogorov-Smirnov test followed by t-tests or the Wilcoxon test as appropriate. Data were compared by one-way ANOVA for multiple groups, followed by Bonferroni's post hoc test for pairwise comparisons. Pearson's or Spearman's correlation tests were used for correlations between data sets. A p value <0.05 was considered as statistically significant. Flow cytometry data were analyzed using FlowJo (TreeStar).

RESULTS

Study population

The study population consists of a cohort of 105, middle-aged, predominantly male actively drinking AUD patients, 32 healthy controls, 10 NAFLD patients and 23 abstinent AUD (Table 1, Supplementary Table 1). Active drinkers reported alcohol consumption until the evening prior to admission. Thirty-four percent (36/105) had detectable blood alcohol concentrations at admission (median 0.4 g/l; range 0.1–2.8 g/l). Fifty patients were classified as having non-progressive and 50 patients as having progressive ALD (Supplementary table 2). All the AUD and NAFLD had a preserved synthetic liver function and no clinical signs of liver decompensation. Doppler ultrasound ruled out significant vascular or biliary problems. For methodological reasons and limited amounts of tissue available, the different analyses were performed in representative cohorts of patients.

Assessment of the different subsets of gut T lymphocytes

Reduced duodenal CD8+ T lymphocytes characterizes AUD patients

In actively drinking AUD patients the number of CD8+ T lymphocytes was lower than in healthy controls and in NAFLD patients (Fig.1A, B) which was further confirmed by flow cytometry quantification (Fig.1C). Reduction of CD8+ T cells occurred in both compartments, the epithelium and the lamina propria (Supplementary Fig.2). Interestingly, abstinent AUD patients had similar numbers of CD8+ T cells compared to actively drinking patients indicating that a short-term abstinence is not able to reverse this phenomenon. As Compared to healthy controls, AUD patients were characterized by similar number of mucosal CD4+ T cells (Supplementary Fig.3).

Diminished proportion of CD8+ KLRG1low CD103+ T resident memory cells in AUD patients

Mucosal CD8+ CD103+ T cells with low KLRG1 are considered as long-lived T resident memory cells while CD8+ KLRG1+ are short-lived lymphocytes^{15,16}. We found a significant reduction in the proportion of CD8+ KLRG1low T cells in AUD patients (Fig.1D). In addition, CD8+KLRG1low cells expressed CD103, a marker of intestinal T resident memory lymphocytes in humans¹³, thus confirming a specific diminished proportion of TRM lymphocytes in AUD patients (Fig.1E). The proportion of TRM expressing CD69+ allowing retention and local activation of the cells in the tissue was similar in AUD patients and controls (Fig.1E). This indicates that the reduction of TRMs in AUD cannot be explained by a loss of their ability to be retained in the tissue.

Table 1. Baseline demographic and biochemical data of the study population

Demographics

	Healthy controls (n = 32)	AUD patients (n = 105)	NAFLD (n=10)
Gender (female/male)	14 (44 %) / 18 (56 %)	31 (30 %) / 74 (70 %)	6 (60%) / 4 (40%)
	Mean $\pm$ SEM		
Age (years)	44.9 $\pm$ 9	48.2 $\pm$ 9	39.3 $\pm$ 12
Height (m)	1.73 $\pm$ 0.09	1.73 $\pm$ 0.06	1.68 $\pm$ 10
Weight (kg)	73.3 $\pm$ 12	73.1 $\pm$ 10.4	134 $\pm$ 19.5
BMI	24.4 $\pm$ 2.9	24.2 $\pm$ 3.2	45 $\pm$ 3

Biological and imaging data

	Mean $\pm$ SEM (normal range)		
AST (IU/l)	17.8 $\pm$ 3.5	72.5 $\pm$ 51.1 (<40)	57.1 $\pm$ 24.7
ALT (IU/l)	10.9 $\pm$ 2.9	58.6 $\pm$ 39.7 (<40)	94.4 $\pm$ 49.4
γ -GT (IU/l)	22.4 $\pm$ 8.6	260.9 $\pm$ 269.5 (<40)	68.9 $\pm$ 24.8
Bilirubin (mg/dl)	0.21 $\pm$ 0.13	0.63 $\pm$ 0.32 (0.3-1.2)	0.74 $\pm$ 0.24
Albumin (g/l)	44 $\pm$ 0.8	46.2 $\pm$ 3.1 (35-52)	45.6 $\pm$ 2.1
CAP (dB/m)	ND	281 $\pm$ 50	350 $\pm$ 36
kPa	ND	8.6 $\pm$ 5.3	12.5 $\pm$ 3.5
CK18-M65 (U/l)	173.6 $\pm$ 43.3	436.4 $\pm$ 321	765.9 $\pm$ 175.9
SAF score	ND	ND	S2A3F2

Abbreviations: BMI, body mass index; AST, Aspartate transaminase; ALT, Alanine transaminase; γ -GT, gamma-glutamyltransferase; ALP, alkaline phosphatase

Figure 1

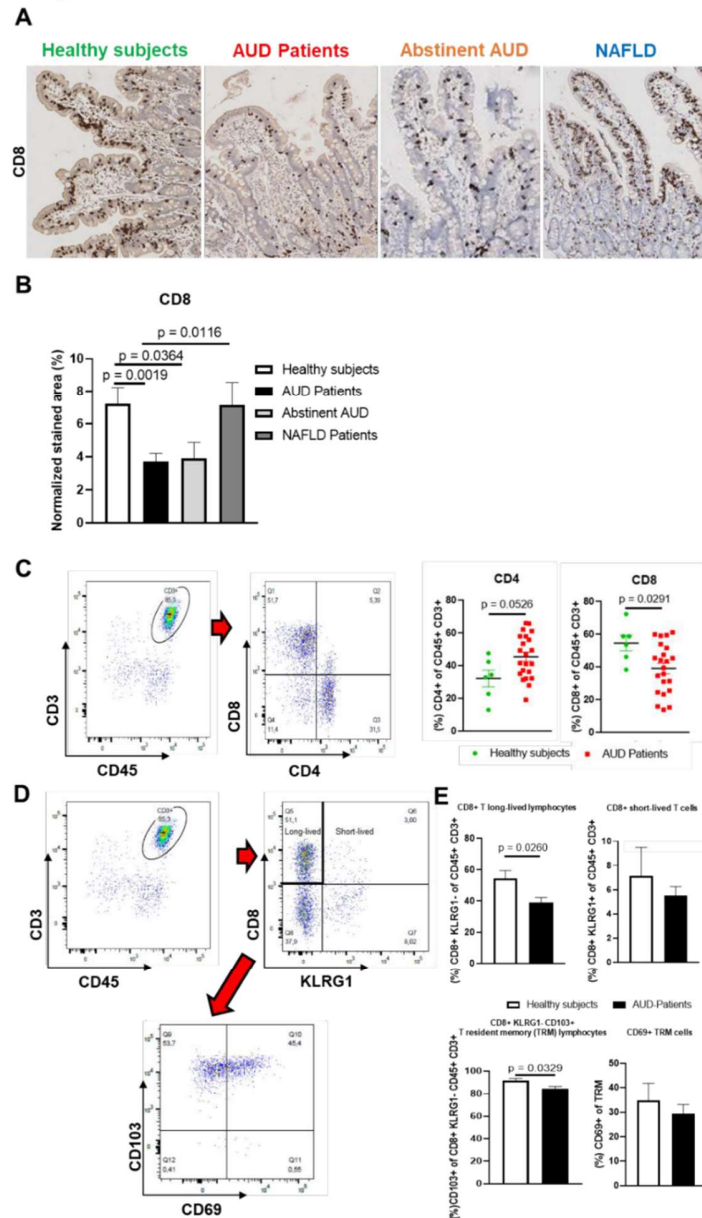


Fig.1 Evaluation of T lymphocytes subsets in the duodenum of alcohol use disorder (AUD) patients.

(A) Representative immunohistochemistry staining of duodenal CD8 + T lymphocytes showing a significant reduction of CD8+ in the duodenum of AUD patients (n=15) and abstinent AUD (n=7) compared to controls (n=9) and NAFLD patients (n=10) (B). (C) Flow cytometry analysis confirmed the significant reduction in the CD8+ T cells pool. (D) Gating strategy for CD8+ KLRG1low CD103+ long-lived T resident memory cells and CD8+KLRG1high short-lived T lymphocytes. (E) Significant reduction in the proportion of T resident memory cells in the duodenum of AUD patients compared to controls. Similar proportions of CD69+ TRM were found in AUD compared to controls.

Relationship between reduction of duodenal T resident memory cells (TRMs) and ALD

Specific transcriptomic alterations are present in patients with progressive ALD

Intestinal CD8⁺ TRM cells receive multiple signals from their tissue microenvironment that integrate into a transcriptional program driving differentiation and maintenance^{17,18}. We analyzed transcriptional profiles by RNA sequencing of TRMs isolated from AUD patients at different disease stages and healthy controls. Using principal-component analysis (PCA) (Fig.2A, Supplementary Fig.4), we found a main divergence between transcriptomic profiles of TRMs in patients with progressive ALD according to the second principal component (PC2) while those in the non-progressive ALD group clustered together with controls. Fifty-two and 41 genes were significantly up-regulated in TRM of patients with progressive ALD when compared with healthy controls or AUD patients with non-progressive ALD, respectively (Fig.2B, Supplementary Fig.5).

Applying gene set enrichment analysis (GSEA) to search for enriched pathways, we only found significantly up-regulated gene sets in TRM of patients with progressive ALD (Supplementary Fig.6). Among those gene sets, eighteen gene ontologies related to apoptosis were significantly enriched (Fig.2C), indicating that programmed cell death could be involved in TRMs reduction in patients with progressive ALD. Apoptosis transcriptional signature was further confirmed by KEGG analysis (Supplementary Fig.7). Several significantly enriched genes were related to lysosomes (e.g., CTSB, CTSH, LMNA) and point to the intrinsic apoptotic pathway while genes associated with the extrinsic pathway of apoptosis were not induced (Supplementary Fig.8).

Shift to the senescent phenotype in TRM in progressive ALD

Since terminally differentiation of T cells has been associated with cell death¹⁹, we evaluated the proportion of effector memory (CD45RA-CD197-) and terminally-differentiated effector T lymphocytes or TEMRA (CD45RA+CD197-). Proportions of TEMRA did not change significantly between AUD patients and controls (Supplementary Fig.9A). We next assessed whether TRM in AUD patients were characterized by differential expression of markers associated with immune dysfunction that could drive cell death. Proportions of TRM expressing programmed cell death 1 (PD1), a marker of immune exhaustion²⁰ highly expressed in TRM lymphocytes²¹, were reduced while CD57⁺ TRM increased indicating T cell senescence²² (Supplementary Fig.9B). Intriguingly this possible shift to the senescent phenotype was only observed in patients with progressive ALD (Fig.2D).

Figure 2

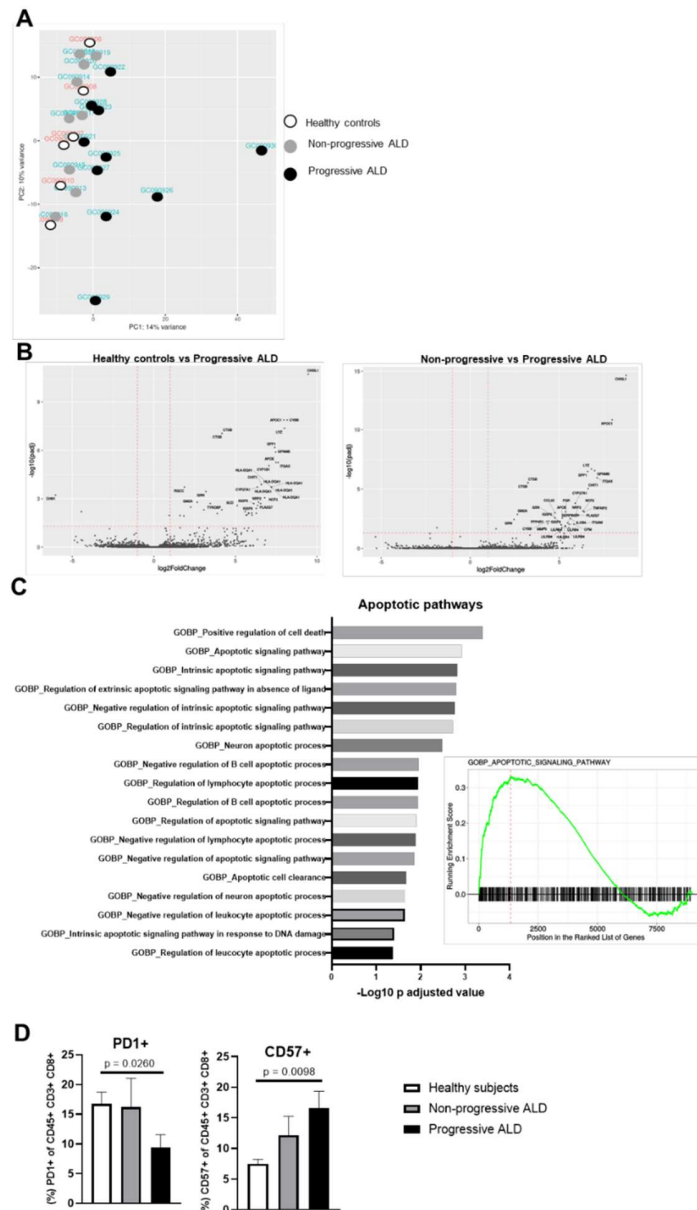


Fig.2 Transcriptomic analysis of duodenal T resident memory lymphocytes (TRM) from alcohol use disorder (AUD) patients and healthy controls. Principal component analysis of TRM from AUD patients (n=19) compared to controls (n=6) indicated different transcriptomic profiles (principal component 2; PC2) in the sub-group of patients with progressive alcoholic liver disease (ALD). (B) Significant enrichment of different genes in TRM of patients with progressive ALD compared to healthy volunteers (left) and non-progressive ALD (right). (C) Gene set enrichment analysis (GSEA) highlighted significant up-regulation of apoptosis gene sets in TRM only in progressive ALD. (D) Diminution of PD1+ and increased CD57+ TRM occur only in progressive ALD.

Assessment of mechanisms implicated in alterations of duodenal CD8+ TRM

Increased apoptosis of CD8+ T cells in patients with progressive forms of liver disease

Annexin V (ANX5) in a combination with a cell-membrane impermeable dye (e.g. DAPI) allows to distinguish late-apoptotic cells (ANX5+DAPI+) from “alive” cells (ANX5-DAPI-)²³. In accordance with RNAseq data, we found a significantly increased proportion of duodenal ANX5+ DAPI+ in parallel to a reduction of “alive” CD8+ T cells in AUD patients compared to controls (Fig.3A,B). The number of CD8 late apoptotic T cells inversely correlated with the overall proportion of CD8+ T lymphocytes (Fig.3C). We confirmed the increased number of apoptotic CD8+ T lymphocytes in actively drinking that persisted in abstinent AUD patients using a TUNEL assay, compared to no or little apoptosis in healthy controls and NAFLD patients (Fig.3D). Intriguingly, the highest levels of apoptotic duodenal CD8+ TRM were seen in patients with progressive ALD (Fig.3E), whereas the levels were similar in non-progressive ALD versus controls. Interestingly, proportions of CD4+ live and late-apoptotic T cells were similar in AUD patients and healthy controls (Supplementary Fig.10). Since increased proliferation could compensate the loss of cells due to apoptosis, we evaluated proliferation of CD8+ T cells using the proliferation marker Ki67 by immunofluorescence. Proliferation of CD8+ T lymphocytes was low with no difference between AUD patients and controls (Supplementary Fig.11).

Figure 3

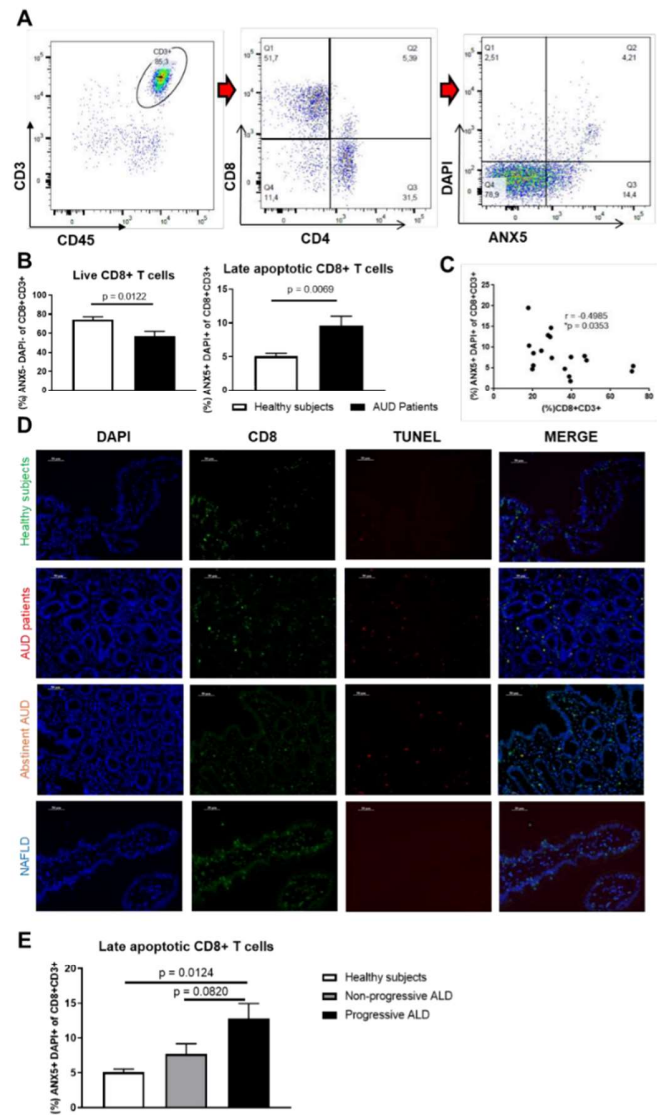


Fig.3 Assessment of cell death in mucosal CD8+ T lymphocytes.

(A, B) Analysis assessing cell death stage by Annexin V in combination with DAPI revealed a reduction of ANX5-DAPI-CD8+ T lymphocytes ("alive") and a significant increase in the proportion of ANX5+DAPI+ CD8+ T cells ("late-apoptotic"). (C) Significant inverse correlation between proportion of CD8+ T cells and proportion of "late-apoptotic" CD8+ T lymphocytes linking low CD8+ cell numbers to apoptosis. (D) Representative immunofluorescence staining of terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL), showing apoptotic cells CD8+ T cells preferentially in the duodenum of actively drinking and abstinent AUD patients compared to few apoptotic CD8+ T cells in healthy controls and NAFLD. (E) Increased proportion of late-apoptotic CD8+ T cells in patients with progressive ALD as compared to the other groups.

Lysosomal membrane permeabilization contributes to gut T resident memory cell death

RNA sequencing in mucosal TRM also indicated an enrichment of gene sets related to lysosomes (Fig.4A). Since lysosomal membrane permeabilization could contribute to programmed cell death²⁴, we assessed lysosomal integrity and alterations of lysosomal function using LysoTracker®. LysoTracker revealed a significant reduction of mean fluorescence intensity in duodenal CD8+ T resident memory lymphocytes from patients with progressive ALD as compared with non-progressive ALD (Fig.4B), indicating an increase in pH of lysosomes, a hallmark of lysosomal membrane permeabilization.

To further confirm lysosomal implication, we inhibited lysosomal protease Cathepsin B (CTSB), the highest expressed gene on RNAseq in TRM of patients with progressive ALD. Inhibition of Cathepsin B with its specific inhibitor CA-074²⁵, alone or in combination with ethanol was able to reduce caspase 3/7 activity compared to the cells with no inhibitor (Fig.4C) thus indicating that lysosomes via Cathepsin B contribute to mucosal TRM apoptosis.

Mitochondria uncoupling in CD8+ TRM lymphocytes associates with progressive ALD

Memory T lymphocytes are characterized by a high spare respiratory capacity, the basis of mitochondrial health, survival and rapid antigen recall response to enable durable immunity^{26,27}. We observed an enrichment of pathways related to oxidative phosphorylation and ATP metabolic processes in TRM from patients with progressive ALD (Supplementary Fig.12) indicating metabolic alterations in mitochondria.

Up-regulation of these gene sets can lead to increased mitochondrial ROS production, essential for T cell differentiation and clonal expansion²⁷. Using the MitoSOX assay to measure mitochondrial ROS production, we Surprisingly found reduced mitochondrial ROS production in TRM cells from patients with progressive ALD compared with with non-progressive liver disease (Fig.4D). Mitochondrial uncoupling protein 2 (UCP2) has the ability to moderate ROS production²⁸. We therefore assessed whether UCP2 could be implicated in reduced mitochondrial ROS using the UCP2 inhibitor Genipin. UCP2 inhibition significantly increased ROS production (Fig.4E), indicating that UCP2 possibly contributes to reduced mitochondrial ROS production.

Figure 4

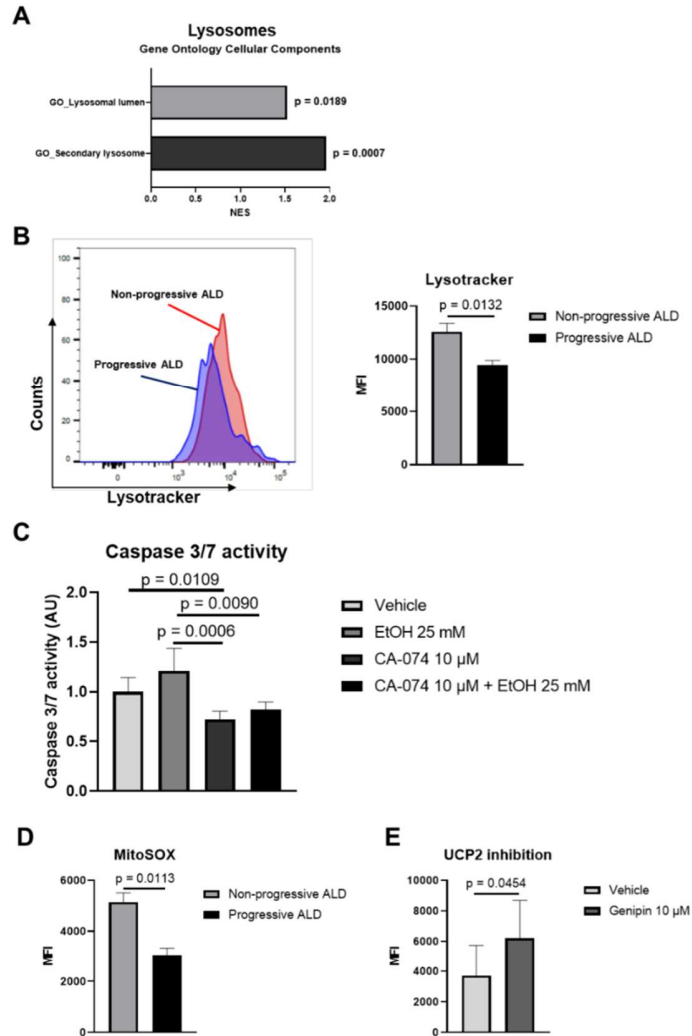


Fig.4 Evaluation of lysosomal function in T resident memory lymphocytes (TRM)

(A) Significant up-regulation of gene sets related to lysosomes. (B) Lysotracker mean fluorescence intensity (MFI) on flow cytometry (left) significantly decreased in TRM lymphocytes from patients with progressive alcoholic liver disease (ALD) compared to non-progressive ALD (right, n=4 /group). (C) Inhibition of Cathepsin B with CA-074 reduced caspase 3/7 activity in TRM lymphocytes(n=6/group). (D) MitoSOX revealed reduced mitochondrial ROS production in gut CD8+ T cells from patients with progressive ALD as compared to non-progressive ALD (n=3/group) which was rescued by (E) UCP2 inhibition using Genipin.

Up-regulation of gene sets linked to lipid metabolism correlate with specific sphingolipids

RNA sequencing data also showed that specific lipid transcriptomic signatures were enriched in TRM from patients with progressive ALD (Fig.5A, Supplementary Fig.13), with APOE, APOC1, SCD, LPL, IRS2, CYP1B1, CYP27A1 being the most significantly up-regulated genes together with transcripts of genes mediating transport and metabolism of long-chain fatty acids (see supplementary material). Lipid metabolism and especially sphingolipids might be linked to lysosomal dysfunction for example in human lysosomal storage disorders ²⁹.

Interestingly, we have previously shown that saturated sphingomyelins (e.g. SM (d32:0), SM (d36:0)) are associated with progression of ALD (Gao B. et al., paper under revision). We therefore determined plasma levels of these lipids using targeted high-resolution LC-MS/MS technology in positive ion mode. The chromatograms corresponding to the exact masses of these two sphingomyelins revealed two peaks (Fig.5B), indicating the presence of different isomers in the plasma. Fragmentation of these compounds in tandem mass spectrometric analyses was consistent with SM (d20:0/12:0) and SM (d18:0/14:0) corresponding to peak 1 and peak 2 of SM (d32:0), respectively, and SM (d18:0/18:0) and SM (d20:0/16:0) corresponding to peak 1 and peak 2 of SM (d36:0), respectively (Supplementary Fig.14A, B).

SM (d18:0/14:0) and SM (d20:0/16:0) were increased in the plasma of AUD patients compared to controls (Supplementary Fig.15) and, intriguingly, were specific of patients with progressive ALD (Fig.5C). Plasma levels of both sphingomyelins correlated with liver parameters used to classify the patients according to liver disease stage (supplementary material), supporting a potential contribution of these lipid species to ALD progression.

Correlation of lipid levels with transcriptomic profiles showed significant positive correlations with top-ranked genes of lipid metabolism/lysosomes (supplementary material), supporting a potential interaction between these lipids and duodenal TRMs. In addition, SM (d18:0/14:0) inversely correlated with the proportion of duodenal TRM lymphocytes (Fig. 5D) pointing to a potential toxicity of this sphingomyelin. To test this hypothesis, we assessed *in vitro* cell viability of sorted CD8⁺ TRM cells upon stimulation with C14 dihydroceramide, the cleavage product of sphingomyelin SM (d18:0/14:0) and found a reduced viability of CD8⁺ TRM lymphocytes in a dose-dependent manner (Fig.5E).

These results indicate that saturated sphingomyelins and derived dihydroceramides could contribute to intestinal CD8⁺ TRM lymphocytes death in patients with progressive ALD.

Altered duodenal sphingolipid metabolism in AUD patients

Absorption of dietary sphingomyelins requires hydrolysis to sphingosine by intestinal alkaline sphingomyelinase and a neutral ceramidase, encoded by the ENPP7 and ASAH2 genes, respectively³⁰. Assessment of these two enzymes in duodenal biopsies of AUD by qPCR revealed down-regulation of both ENPP7 and ASAH2 transcripts (Supplementary fig.16A). Therefore, increased plasma levels of these specific sphingomyelins are unlikely related to up-regulation of enzymes required for the uptake of dietary sphingomyelins, but might result from synthesis in the tissue itself. Ceramide synthase 6 (CERS6) is responsible for the synthesis of dihydroceramides with C14- and C16- N-fatty acids³¹. Duodenal CERS6 mRNA was up-regulated in AUD patients compared to healthy controls (Supplementary Fig.16B), suggesting that elevated levels of circulating sphingomyelins possibly derive from increased production in the proximal small gut.

Figure 5

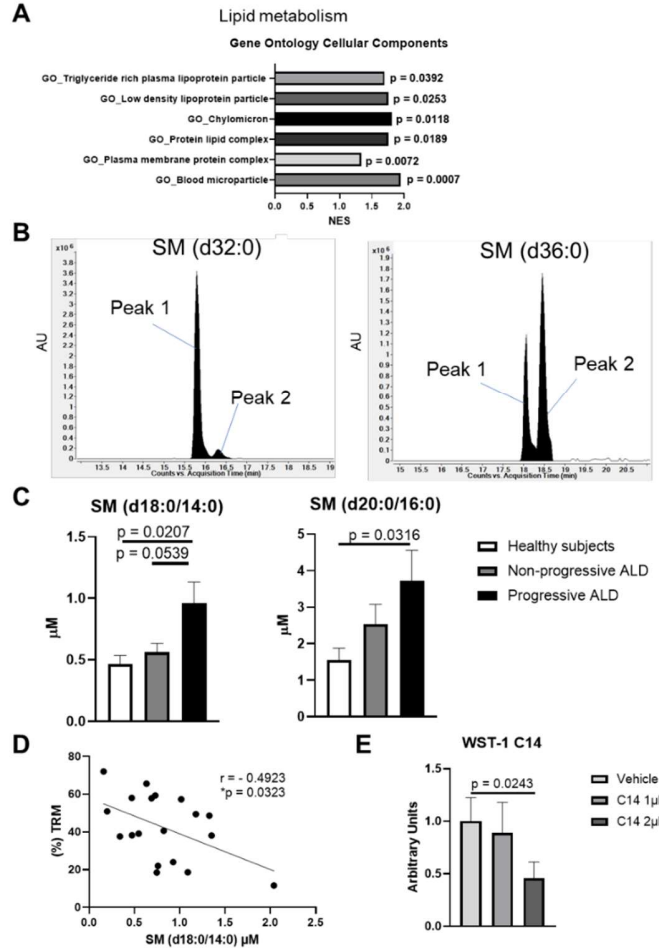


Fig.5 Lipids linking transcriptomic alterations in T resident memory lymphocytes (TRM) and cell viability in patients with progressive alcoholic liver disease (ALD).

(A) Significant enrichment of gene sets related to lipid metabolism in TRM of patients with progressive ALD. (B) Representative chromatogram showing the two peaks characterizing the two isomers of sphingomyelins (SM) (d32:0) and (d36:0). (C) Targeted analysis by LC-MS/MS showed increased plasma levels of SM (d20:0/16:0) and SM (d18:0/14:0) in patients with progressive ALD (n=9/group). (D) Low percentages of duodenal TRM cells correlated with higher plasma levels of SM (d18:0/14:0). (E) Reduced viability of CD8+ TRM lymphocytes after stimulation with C14:0 ceramide at increasing concentrations assessed by the WST-1 assay (n=3/group).

Relationship between intestinal CD8+ TRM lymphocytes and reduced immunosurveillance

Enrichment of gene sets associated with immunity and antimicrobial responses

Intestinal CD8+ TRM lymphocytes act as local gatekeepers to protect against microbial attachment and invasion. Consequently, we looked for alterations in the transcriptomic profile related to antimicrobial effects in TRMs. We found enrichment of gene sets mediating response to microbes, leukocyte activation and migration in TRM of patients with progressive ALD (Fig.6A). Focusing on responses against microbes, the most up-regulated gene was the anti-fungal mediator CHI3L1, followed by CHIT1 and the antimicrobial molecule LYZ (see supplementary material). Serum CHI3L1 levels increased progressively in ALD but did not restore upon abstinence. In comparison, levels were lower in NAFLD patients, which were similar to those in the non-progressive ALD. CHI3L1 levels correlated with CHI3L1 transcripts in TRM ($r=0.4770$; $p=0.0184$), pointing to a potential release of this anti-fungal factor by TRM.

Among cytokines, the most up-regulated gene was anti-inflammatory IL1 Receptor Antagonist (IL1RN), which was confirmed by quantitative PCR (Supplementary Fig.17A) in the mucosa of AUD patients compared to controls.

These results suggest that TRM cells might play a regulatory role in response to microbes in the duodenum of AUD patients with a decrease in numbers adversely affecting microbial control.

Elevated microbial translocation inversely correlated with alterations in CD8 TRM pool

We assessed whether increased serum levels of microbial translocation markers, characteristic of patients with progressive ALD⁵, correlate with changes in mucosal lymphocytes. Serum microbial Gram- marker soluble CD14 (sCD14) was specifically elevated in the progressive ALD group (Fig. 6C) while serum Gram+ marker Peptidoglycan Recognition Proteins (PGRPs) levels increased in actively drinking AUD, irrespectively of ALD stage, compared to controls (Supplementary Fig.17B). We observed a significant inverse correlation between number of CD8+ T cells and sCD14 (Fig.6D). sCD14 levels further correlated with the shift to the senescent TRM phenotype (PD1low, CD57high) in patients with progressive ALD ($r=-0.4328$ and $r=0.5271$, $p<0.05$, for PD1+ and CD57+ TRM, respectively).

The proportion of TRMs determined by flow cytometry also inversely correlated with the PGRPs levels ($r=-0.5159$; $p=0.0340$).

Taken together, our results support that alterations in duodenal CD8+ TRM likely contribute to reduced immune surveillance and facilitate microbial translocation. These changes strongly associated with progressive ALD. Reduction in the TRM pool did not reverse upon short-term abstinence and was not observed in NAFLD.

Figure 6

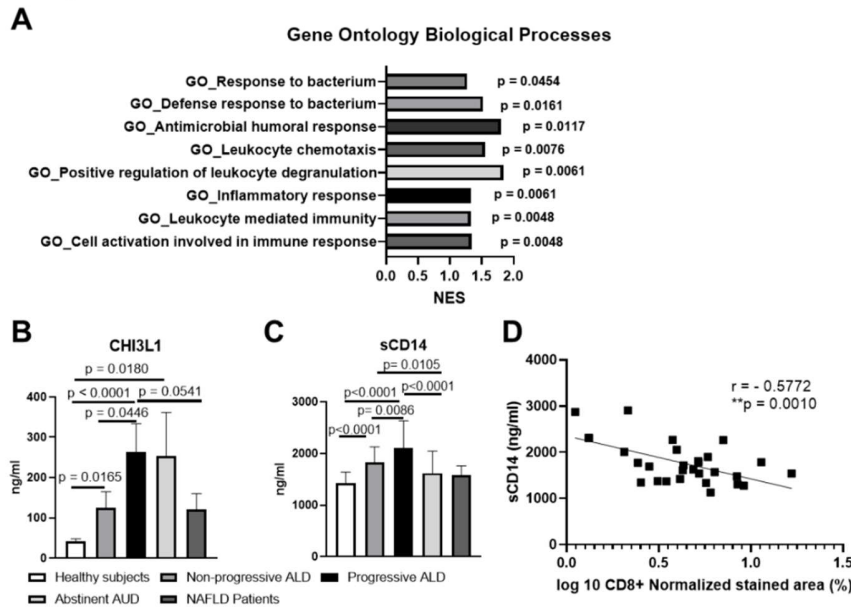


Fig.6 Evaluation of pathways related to inflammatory and antimicrobial responses in T resident memory (TRM) cells from patients with progressive alcoholic liver disease (ALD)

(A) Up-regulation of gene sets linked to inflammatory and anti-microbial responses in TRM cells from alcohol use disorder (AUD) patients with progressive ALD. (B) Serum levels of CHI3L1 increased progressively in progressive ALD, did not change upon abstinence and were higher compared to NAFLD subjects. (C) Serum levels of sCD14 microbial translocation marker increased significantly primarily in actively drinking patients with progressive ALD. (D) Inverse correlation between number of mucosal CD8+ T cells and serum levels of microbial translocation marker sCD14.

DISCUSSION

We addressed alterations in the duodenal adaptive immunity at early stages of ALD. We reveal for the first time that a specific reduction together with functional changes of duodenal CD8+ T resident memory lymphocytes in progressive ALD are correlated with elevated microbial translocation and liver disease progression in a large cohort of patients. TRM cells undergo apoptosis at least partially related to lysosome dysfunction and altered lipid metabolism.

The generation and long-term survival of pathogen-specific TRM lymphocytes is fundamental for local immunosurveillance and relies on consumption of exogenous free fatty acids by these cells¹⁸. This metabolic substrate preference distinguishes subpopulations of T cells in mucosal barriers from their circulating counterparts¹⁸. In our study, expression of gene sets related to lipid metabolism were up-regulated in TRM and correlated with elevated plasma levels of several sphingomyelins, specifically in patients with progressive ALD. These circulating bioactive lipids and/or their metabolic products could alter intestinal TRM, thus leading finally to cell death. *Ex vivo* stimulation of TRM from AUD patients with these lipid species revealed a reduced viability in a dose-dependent manner, which is consistent with a causal role of these specific sphingolipids in TRM cell death. sphingomyelin SM (d18:0/14:0) levels correlated with up-regulated lysosomal genes such as CTSB and lysosomal membrane permeabilization was increased on TRM cells of patients with progressive

ALD. Inhibition of Cathepsin B, the most significant lysosomal gene revealed by RNA Sequencing, reduces apoptosis thus improving survival of TRM. A link between sphingolipids, lysosomes and disease in humans has already been described, for example, in lysosomal storage disorders²⁹. Our result open the intriguing possibility that in AUD patients lysosome stability is an important regulator of the maintenance of memory CD8+ T cells³² in the duodenal mucosa.

Lipids were measured in the systemic circulation raising the question of their source. Sphingomyelins are virtually expressed in all the tissues but are especially abundant in the central nervous system³³. Dietary sphingomyelins need to be hydrolyzed by intestinal alkaline sphingomyelinase and neutral ceramidase to sphingosine, which is absorbed by enterocytes, converted to palmitic acid and integrated into chylomicron triglycerides³⁰. Both enzymes were down-regulated in the mucosa of AUD patients indicating that increased levels of sphingomyelins likely do not derive from the diet. Alternatively, sphingomyelins can be synthesized by the intestine in pathological conditions involving Ceramide synthetase 6³¹. The latter was up-regulated in the duodenum of AUD patients, supporting a possible role for local production of specific sphingolipids in the gut. Determining the level of those lipids directly in the gut mucosa could help to clarify this issue. On top of lysosomal alterations, we revealed reduced mitochondrial ROS production in TRM of patients with progressive liver disease linked to uncoupling by the uncoupling protein 2 (UCP2). Lower levels of mitochondrial ROS in intestinal CD8+ T lymphocytes, a condition known as a "reductive stress"³⁴, might impact their survival. Under normal conditions, the maintenance of correct ROS levels in mitochondria is important to initiate protective signaling cascades related to cell proliferation, differentiation, immune responses and survival^{34,35}. Further studies should allow to gain more insight into the complex interaction between redox homeostasis, metabolic status, cell death and immune responses in human intestinal CD8+ TRM.

We also revealed that TRM cells likely play an important role in alcohol-induced gut barrier dysfunction in patients with progressive ALD. Those cells up-regulated gene sets associated with leukocyte activation and response to microbes. Notably, this enrichment was not linked to pro-inflammatory responses but to regulatory mediators such as IL1RN and antimicrobial molecules such as CHI3L1, CHIT1 and LYZ. However, this apparently good response is counteracted by increased cell death. As a consequence, the lower the proportion of TRM, the higher the levels of Gram- and Gram+ translocation markers in the blood. In addition, enhanced shift to the senescence phenotype also correlated with sCD14 levels. As a net result, microbial immunosurveillance seems to be profoundly disturbed, allowing invading microbes from a severely dysbiotic microbiota in patients with progressive ALD to reach the circulation. It is tempting to speculate that reduced TRM also translates into decreased diversity and recognition of specific pathogens in the intestine which might contribute to liver disease progression. It would be interesting to study the TCR repertoire to assess their heterogeneity and to identify whether they are directed against specific bacterial and/or fungal species.

An intrinsic limitation of this study is that data are mainly based on associations/correlations, which do not formally prove a cause-and-effect relationship. We can also not exclude that other effector immune cells, such as macrophages and dendritic cells, might be defective in response to microbes. We have recently also shown a reduced number of macrophages in the duodenal mucosa of AUD patients⁵. Given the difficulties in accessing tissues at early-stage disease, we believe our study adds significant insights into the role of the impairment of gut adaptive immunity in the early pathogenesis of ALD. A reduced number of duodenal CD8+ T cells was not found in presence of metabolic steatohepatitis (NAFLD group) indicating that these changes are a hallmark of alcohol consumption and ALD. In addition, microbial translocation markers were not elevated in the NAFLD as they were in progressive ALD supporting that changes in microbial composition, leaky gut and ongoing liver disease in NAFLD are not sufficient to alter CD8 TRM cells. Alterations of gut adaptive immunity in the proximal small intestine occur early at pre-cirrhotic stages and appear to be linked

to alcohol abuse. However, ethanol did not directly induce TRM cell death *in vitro* indicating that alcohol alone might not be sufficient to drive this process.

In conclusion, we reveal an intriguing diminution of CD8⁺ T resident memory lymphocytes specifically in the duodenal mucosa of patients with progressive ALD and point to a contribution of lysosomal and mitochondrial alterations to their cell death. These changes correlate with functional alterations of the duodenal mucosa barrier, especially in AUD patients with progressive liver disease. Our findings support a beneficial role of CD8⁺ TRM in the human intestine and contribute to our understanding of these lymphocytes in ALD. Other studies have highlighted the protective properties of CD8⁺ T resident memory cells in several infectious and inflammatory diseases^{36,37}. Our observations may be relevant for clinical practice in the future since they suggest that impairment of the small intestinal adaptive immune response is linking the gut and the liver already at early stages in human ALD. Therefore, strategies aiming at reinforcing the intestinal immune system could prove beneficial in early human ALD.

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Supporting material/data

Duodenal CD8+ T resident memory cell apoptosis links gut barrier dysfunction, microbial translocation and alcohol-associated liver disease in humans

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Patients, methods

Inclusion-exclusion criteria

Additional inclusion criteria were first admission to the alcohol withdrawal unit and absence of previously diagnosed significant alcohol-associated liver disease.

Patients with diabetes were eligible for the study if they were on stable treatment (metformin, oral anti-diabetics with or without insulin) and a glycosylated hemoglobin level < 7% at admission.

Patients were excluded from the analysis if they had one of the following conditions:

- Inflammatory bowel disease or other chronic inflammatory or autoimmune diseases
- History of cancer within 5 years prior to admission
- Known liver disease of any other etiology
- Clinically significant cardio-vascular, pulmonary or renal co-morbidities
- Clinical and biochemical signs of active infection ($t^{\circ} > 37,5^{\circ}\text{C}$, viral syndrome, high CRP and high White Blood cell count)
- Administration of antibiotics, probiotics, glucocorticoids and nonsteroidal anti-inflammatory drugs during the two months preceding enrollment.

Abstinent AUD patient's characteristics (Supplementary table 1)

Abstinent AUD patients	
<i>Demographics</i>	
Gender (female/male)	8 (35%) / 15 (65%)
	Mean $\pm$ SEM
Age (years)	48.9 $\pm$ 11.4
Height (m)	1.75 $\pm$ 0.05
Weight (kg)	75.5 $\pm$ 11.3
BMI	24.4 $\pm$ 3.5
<i>Biological and imaging data</i>	
AST (IU/l)	45.2 $\pm$ 23.5
ALT (IU/l)	21.9 $\pm$ 8.2
γ -GT (IU/l)	136.4 $\pm$ 88.4
ALP (IU/l)	50.6 $\pm$ 16.9
Bilirubin (mg/dl)	0.4 $\pm$ 0.24

Albumin (g/l)	42.5 ± 2.1
CAP (dB/m)	227 ± 55
kPa	15.3 ± 10.3
CK18-M65 (U/l)	298.6 ± 96

Characteristics of the patient's subgroups based on clinical evaluation of ALD (Supplementary table 2)

	Non-progressive liver disease (n=50)		Progressive liver disease (n=50)	
Clinical parameters	No liver disease (n=20)	Simple steatosis (n=30)	Steato-hepatitis (n=25)	Steato-fibrosis (n=25)
	AST/ALT < 40 IU/L	AST/ALT < 40 IU/L	AST/ALT > 40 IU/L	AST/ALT > 40 IU/L
	CAP < 250 dB/m	CAP > 250 dB/m	CAP > 250 dB/m	CAP > 250 dB/m
	kPa < 7.6	kPa < 7.6	kPa < 7.6	kPa > 7.6
Biological and imaging data				
AST (IU/l)	21.95 ± 4.85	30.9 ± 9.5	98.56 ± 30.30	133.5 ± 79
ALT (IU/l)	19.15 ± 5.01	29.2 ± 11.8	97.64 ± 32.16	87.2 ± 51.44
γ-GT (IU/l)	40.26 ± 20.78	79.46 ± 46.10	187 ± 86.6	620 ± 467
ALP (IU/l)	69.53 ± 19.24	74.20 ± 15.45	80.72 ± 15.90	113.7 ± 36
Bilirubin(mg/dl)	0.5 ± 0.22	0.49 ± 0.20	0.59 ± 0.19	0.68 ± 0.28
Albumin (g/l)	47.7 ± 2.7	46.42 ± 2.63	47.27 ± 2.89	44.48 ± 3.42
CAP (dB/m)	206 ± 23	298 ± 34	315 ± 23	300 ± 49
kPa	4.53 ± 0.94	5.07 ± 1.09	5.67 ± 0.99	19.7 ± 10.8
CK18-M65 (U/l)	154.1 ± 53.3	189.8 ± 59.6	541.9 ± 171.9	713.7 ± 457.8

Examinations and sample collections in AUD patients (Supplementary Fig.1)

At admission, Fibroscan® (Echosense, Paris, France) combined with the controlled attenuation parameter (CAP) were obtained and a fasting blood sample (serum, plasma) was drawn. Esophagogastroduodenoscopy with distal duodenal biopsies was performed within 48 hours (details in supplementary material) and patients underwent an abdominal Doppler ultrasound on the third day as part of the routine work-up in the unit.

Fibroscan® measurements

Transient elastography measurements were performed using the Fibroscan® device (Echosens, Paris, France) by an experienced examiner blinded to the patient's data. Fibroscan® was considered as valid if at least 10 validated measurements were obtained with an interquartile range of less than 30%. The final result expressed in kPa was the median of all valid measurements obtained. We used the cut-offs for alcoholic patients proposed by Nguyen-Khac et al. (Aliment Pharmacol Ther. 2008; 28:1188-98), i.e. 7.6, 11, 19.5 kPa for F2, F3 and F4, respectively.

Controlled attenuation parameter (CAP) was obtained during the same procedure. We used a median cut-off of 250 dB/m to diagnose steatosis as suggested by Kalas T et al. (J Hepatol 2017; 66:1022–1030). An interquartile range of 50 dB/m was deemed acceptable to validate the result.

Upper gastro-intestinal endoscopy and biopsy specimens

The standardized protocol of the alcohol withdrawal unit foresees a routine upper gastro-intestinal endoscopy within 72 hours of admission by an experienced endoscopist. Patients were slightly sedated with midazolam and 20mg of butylhyosine was administered intravenously prior to the procedure in order to reduce intestinal peristaltic contractions. The endoscope was rapidly pushed under visual control into the distal duodenum. Care was taken to keep the transit time through the esophagus and the stomach as short as possible. Biopsies were taken from the distal duodenum with a single-use biopsy forceps (Boston Scientific, Galway, Ireland). During the first forceps passage, two duodenal biopsies were obtained for T lymphocyte isolation and placed in Eppendorf tubes containing medium RPMI 1640 FBS 10 % P/S 1 % on ice. Subsequent passages were performed to obtain additional biopsies which were preserved in RNA later (Invitrogen™, Thermo Fisher Scientific, Merelbeke, Belgium) or fixed in formalin. For obvious ethical reasons, we were able to collect only a limited amount of tissue per patient. To reach a sufficient number of lymphocytes (minimum 10⁴ T cells) per patient we were forced to use the whole biopsies specimens of a single patient. Therefore, the different analyses were performed in consecutively sampled duodenal biopsies of a representative cohorts of patients.

Histological classification of NAFLD patients

Liver biopsies from NAFLD patients were classified using the steatosis, activity and fibrosis (SAF) score (Bedossa P et al., Hepatology 2012).

Immunofluorescence

Intestinal samples were fixed in 10% formalin for 2 hours, washed and dehydrated in 30% sucrose for at least 4 hours and the included in OCT compound (Sakura, Kampenhout, Belgium). Five µm cryosections were rehydrated, blocked with phosphate buffer saline (PBS) 1x, 10% bovine serum albumin (BSA), and 3% milk. Sections were stained with the primary antibody anti-human CD8 (Mouse monoclonal anti-CD8, Clone C8/144B) 1/24 overnight at 4 °C followed by incubation with a secondary antibody conjugated with the fluorochrome Alexa Fluor 488 donkey anti-mouse at 1/1000. Before imaging, nuclei were counterstained with 4', 6-diamidin-2-phenylindole (DAPI). For CD8 Ki67 double-immunofluorescence, sections were first stained with the primary antibody anti-Ki67 (Mouse anti-CKi67, 556003, BD Biosciences) 1/250 overnight at 4 °C followed by incubation with a secondary antibody conjugated with the horseradish peroxidase (HRP) for one hour at room temperature. Next, sections were stained by using the tyramide system amplification (TSA) with the Alexa Fluor™ 488 tyramide reagent for 10 minutes. Thereafter, antibodies were stripped by using a Citrate buffer and microwave and sections were then blocked, stained with the primary antibody anti-CD8 1/24 overnight at 4 °C followed by incubation with a secondary antibody conjugated with the fluorochrome Alexa Fluor 594 donkey anti-mouse at 1/1000. Before imaging, nuclei were counterstained with 4', 6-diamidin-2-phenylindole (DAPI). Fluorescence multiplex immunostained slides were digitalized using a Panoramic 250 FlashIII scanner (3DHistech, Budapest, Hungary). Apoptosis of CD8+ T lymphocytes was further evaluated in cryosections. The terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed using an *in-situ* cell death detection kit (Roche, Vilvoorde, Belgium) following the manufacturer's instructions.

Immunohistochemistry

Since upper gastro-intestinal endoscopy is part of the standardized routine program of the alcohol withdrawal unit, biopsy specimens were routinely sent to the pathology department for examination by an experienced gastro-intestinal pathologist who generated a standard pathology report. After exclusion of any gastro-intestinal pathology susceptible to interfere with additional analyses of the specimens (e.g. celiac disease, Whipple's disease, collagenous sprue, inflammatory bowel disease, etc.), duodenal sections were stained overnight at 4°C with antibodies against CD4 (Mouse monoclonal anti-CD4, Clone 4B12) 1/50 and CD8 (Mouse monoclonal anti-CD8, Clone C8/144B) 1/24, and digitalized using a SCN400 slide scanner (Leica Biosystems, Wetzlar, Germany) at X20 magnification. Scanned slides were subjected to quantification analysis with the image analysis tool Author version 2017.2 (Visiopharm, Hørsholm, Denmark). On each slide, villi were first manually delineated and then automatically delineated along their perimeter. Stained pixels were detected at high resolution (x20) using a thresholding classification method. Thresholds were adjusted on representative stained vs unstained regions. The same parameters were kept constant for all slides. At least 3 villi were analysed per slide. Results were expressed as the mean percentage of the stained area normalized to the total surface area of the villi taken into consideration.

RNA Sequencing-Quality control

Quality control of raw reads was performed with FastQC v0.11.7, available online at: <https://eur03.safelinks.protection.outlook.com/?url=http%3A%2F%2Fwww.bioinformatics.babraham.ac.uk%2Fprojects%2Ffastqc&data=04%7C01%7Cluca.maccioni%40uclouvain.be%7C45b9082c0c1c4e0d869e08d88b322984%7C7ab090d4fa2e4ecfbc7c4127b4d582ec%7C0%7C0%7C637412395187814382%7CUnknown%7CTWFpGZsb3d8eyJWljojMC4wLjAwMDAiLCJQIjoiV2luMzliLCJBTiI6IklhaWwiLCJXVCi6Mn0%3D%7C3000&data=J%2Bwf%2FHmQ9rOuZChuv5i9RwuxJoL8ekhHaa%2FI9Ey39bo%3D&reserved=0>. Adapters were filtered with ea-utils fastq-mcf v1.05 (Erik Aronesty (2011), ea-utils: "Command-line tools for processing biological sequencing data": <https://eur03.safelinks.protection.outlook.com/?url=https%3A%2F%2Fgithub.com%2FExpressionAnalysis%2Ffeutils&data=04%7C01%7Cluca.maccioni%40uclouvain.be%7C45b9082c0c1c4e0d869e08d88b322984%7C7ab090d4fa2e4ecfbc7c4127b4d582ec%7C0%7C0%7C637412395187814382%7CUnknown%7CTWFpGZsb3d8eyJWljojMC4wLjAwMDAiLCJQIjoiV2luMzliLCJBTiI6IklhaWwiLCJXVCi6Mn0%3D%7C3000&data=ED4wkJzlibsHY35iE2%2FYN18tC32PjMeVAAxAlPT9bK8%3D&reserved=0>).

RNA sequencing and Gene set enrichment analysis (GSEA)

RNA from sorted duodenal T resident memory cells was isolated using QIAGEN RNeasy Plus Mini Kit and sequenced using the HiSeq4000 instrument (low output sequencing, paired and read, 50 bp). RNA quality was assessed on an Agilent 2100 Bioanalyzer and samples with an RNA Integrity Number (RIN) <7 were excluded from further analyses. Sequence libraries were prepared with the Lexogen QuantSeq 3' mRNA-Seq library prep kit according to the manufacturer protocol. Samples were indexed to allow for multiplexing. Library quality and size range was assessed using a Bioanalyzer (Agilent Technologies) with the DNA 1000 kit (Agilent Technologies, California, USA). Libraries were subsequently sequenced on an Illumina HiSeq4000 instrument. Single-end reads of 50 bp length were produced with a minimum of 1M reads per sample. Splice-aware alignment was performed with HiSAT2 against the human reference genome hg19. Reads mapping to multiple loci in the reference genome were discarded. Resulting BAM files were handled with Samtools v1.5 (Li H et al., Bioinformatics 2009). Quantification of reads per gene was performed with HT-seq Count v2.7.14. Count-based differential expression analysis was done with R-based Bioconductor package DESeq2

(Love MI et al., Genome Biology 2014). Reported p-values were adjusted for multiple testing with the Benjamini-Hochberg procedure, which controls false discovery rate (FDR). GSEA was performed with clusterProfiler (v.3.14.3) Bioconductor package and gene sets from Molecular Signature Database (msigdb v.7.1.1). p-values were adjusted for multiple testing.

Quantitative Real-Time Polymerase Chain Reaction

Primers were as follows:

ENPP7: forward (F)-5'- GTGAAGGCACGCTACATGAC-3' and reverse (R)-5'- CACCCCGTGGTTCTCGATA-3';

ASAH2: (F)-5'- GTGCCTTTAACCCCAGAGTCT-3' and (R)-5'- GTGCAGTCAGCTCGTCCAA-3';

CERS6: (F)-5'- ACATTCTTCAGCCTCCTGGAGTT-3' and (R)-5'- GCTCCCTGGTTCCAGGCCAC-3';

IL1RN: (F)-5'-CCCCACCACCAGTTTTGAGT-3'; (R)-5'-GCCTTCGTCAGGCATATTGG-3';

Rpl-19: (F)-5'-CAAGCGGATTCTCATGGAACA-3'; (R)-5'-TGGTCAGCCAGGAGCTTC T-3'.

Duodenal T cell isolation and immunophenotyping by flow cytometry (FACS Sorting)

CD8+ KLRG1(low) CD103+ T cells were isolated from distal duodenal biopsies using a modified version of the method previously described by Trapecar M. et al., AIDS Research and Human Retroviruses 2017 and FACS sorted using a BD FACSAria™ III sorter equipped with BD FACSDiva™ software. Briefly, duodenal biopsies were collected in 10% RPMI and the epithelial layer containing intra-epithelial lymphocytes was stripped by incubation with 1 mmol/L dithiothreitol and 5 mmol/L EDTA in calcium- and magnesium-free Hanks' balanced salt solution (HBSS) for 15 min at 37°C. Single-cell suspension from the epithelium was obtained by treatment with DNase I for 30 minutes at 37°C. The remaining lamina propria were washed with HBSS and digested with 1 mg/mL collagenase A (Roche Diagnostics Ltd., Indianapolis, IN) in HBSS supplemented with calcium, magnesium, and 1 mg/ml DNase I (Roche Diagnostics Ltd.) for 30 minutes at 37°C and mechanically disrupted by gentle pipetting until dissociation was complete. Mucosal cells released from the tissue samples were passed through a 70 µm cell strainer and washed with complete medium. Cell viability was assessed by trypan blue exclusion. Recovered mucosal cells were stained with the following antibodies:

Antibodies used for flow cytometry

Name	Company	Reference
CD8-PerCP-Vio700, human	Miltenyi Biotec	130-110-682
CD3-FITC, human, Clone REA613	Miltenyi Biotec	130-113-138
CD8 Antibody, anti-human, APC, REAfinity™	Miltenyi Biotec	130-110-817
CD197 (CCR7)-PE, human, Clone REA546	Miltenyi Biotec	130-119-583
CD45RA-APC-Vio770, human, Clone REA1047	Miltenyi Biotec	130-117-747
CD4-VioGreen, human, Clone REA623	Miltenyi Biotec	130-113-230
CD45-VioBlue, human	Miltenyi Biotec	130-110-637
CD103-APC, human, Clone REA803	Miltenyi Biotec	130-111-833
Anti-KLRG1-PE-Vio615, human	Miltenyi Biotec	130-120-427
CD57-PE-Vio770, human	Miltenyi Biotec	130-111-812
BV711 Mouse Anti-Human CD279 (PD-1)	BD Bioscience	564017
BV786 Mouse Anti-Human CD69	BD Bioscience	563834

Serum biomarkers of liver cell damage and microbial translocation

Serum cytokeratin 18 (CK18) was used to assess liver cell damage (CK18-M65 ELISA kit; TECOmedical AG, Sissach, Switzerland). Microbial translocation was determined using soluble CD14 (Human CD14 Quantikine ELISA kit sCD14, Bio-techne Ltd., Abingdon, United Kingdom) and Peptidoglycan Recognition Proteins (Human PGRPs ELISA kit, ThermoFisher, Merelbeke, Belgium), respectively. Serum levels of antimicrobial CHI3L1 were assessed by ELISA (ThermoFisher). All assays were performed in duplicate following the manufacturer's instructions.

Lipid extraction and targeted LC-MS analysis of sphingolipids

Lipids were obtained from plasma samples after liquid-liquid extraction adapted from the classical Folch method (Folch J et al., The Journal of Biological Chemistry 1957). Briefly, 500 μ L of a methanol solution and 1 mL of cold chloroform were mixed to 100 μ L of plasma and 275 μ L of milliQ water were added. The mixture was centrifuged at 16000 g for 15 min at 4°C. Deuterium labeled 18:1-d9 SM (Avanti Polar Lipid) at a concentration of 4 μ M was used as internal standard. The organic layer (lower phase, 200 μ L) was dried down under nitrogen at room temperature, resuspended in 100 μ L of methanol: isopropanol (1:1 v/v) and centrifuged for 10 min at 4°C and 16000 g. The supernatant was transferred into fresh glass vials. Liquid chromatography including the linear gradients was performed as described by Dewulf et al., The Journal of Biological Chemistry 2021, using an Agilent 1290 HPLC system, a C30 column (150 x 2 mm, particle size 2.6 μ m, pore diameter 150Å) (ThermoFisher) and two mobile phases: A [acetonitrile: water 60:40 v/v, 10 mM ammonium formate and 0.1 % formic acid] and B [isopropanol:acetonitrile:water 90:10:1 v/v/v, 10 mM ammonium formate and 0.1 % formic acid]. A high-resolution mass spectrometer system (Agilent 6550 ion funnel MS-qTOF) set in positive electrospray mode was used for detection. The area under the curve of each sphingolipid (m/z 677.5592 for SM d32:0 and m/z 733.6218 for SM d36:0) was normalized to 18:1-d9 SM standard, allowing a semi-quantitative analysis of the peaks. The indicated ions were fragmented using energy collisions of 10, 20 and 30 eV. The identification of the acyl composition is based on the occurrence of weak daughter ions that correspond in accurate mass to the sphinganine part of the moiety. MS2 generated spectra allowed the identification of the composition of sphingolipid isomers eluting at different retention times.

Cell culture

Sorted T resident memory cells (10^4 /well) were seeded on 96-well plates in RPMI-1640 (ThermoFisher) containing 10% fetal bovine serum (FBS) and antibiotics. They were stimulated for 24 hours with various concentrations of CA-074, Genipin (Santa Cruz) and C14 dihydroceramide (d18:0/14:0) (Sigma). Cell viability was determined using a colorimetric WST-1 assay (Roche). Caspases3/7 activity was assessed using the Caspase-Glo-3/7 assay (Promega, Leiden, Netherlands) according to the manufacturer's instructions.

LC-MS analysis of sphingolipids

Name	m/z	Retention time (min)
SM (d32:0) peak 1	677,5592	15
SM (d32:0) peak 2	677,5592	15,6
SM (d36:0) peak 1	733,6218	17,5
SM (d36:0) peak 2	733,6218	18

Mass to charge and retention time of the sphingomyelins (SM) identified by LC-MS analysis. ***Listing of genes in pathways up-regulated in TRM of AUD with progressive ALD***

Apoptosis

KEGG Apoptosis: CTSE, CTSH, LMNA, GZMB, TUBA1B, CTSL, ACTB, PIK3R1, CTSS, HTRA2, CTSD, BAX, NFKBIA, FOS, TRADD

GO Apoptotic signalling pathway: CYP1B1, TYROBP, ITGAM, CTSH, LGALS3, PLAUR, STYXL1, ANXA6, MMP9, PPP1R15A, GZMB, GSN, LMNA, CD74, CDKN1A

GO Positive regulation of cell death: CYP1B1, RGCC, GRN, TYROBP, GSN, CTSD, BAX, CDKN1A, HTRA2

Lysosomes

GO Lysosomal lumen: CTSE, GM2A, HSPA8, CD74, CTSL, CTSD, NAAA, NAGLU, GALNS, PPT1, CHD1, GAA, HSP90AA1, NPC2, HEXA, NSG1, PSAP, GLA, SCARB2, ASAH1, GLB1, GUSB

GO Secondary lysosomes: NCF2, FTH1, FTL, PIK3C3

Mitochondria and metabolism

GO Oxidative phosphorylation: NDUFA10, NDUFS2, NDUFS6, NDUFA9, NDUFB1, NDUFA2, ATP6AP1, NDUFB7, MT-ND2, NDUFB10, NDUFB5, NDUFB1, MT-ND6, GZMB, MT-ND4

GO ATP and metabolic process: HSPA8, PKM, ENO1, ABCD1, NDUFB7, ATP7A, ALDOA, COX5A, NDUFB1, UQCRL, NDUFS6, COX7A2, HSPA1B

Lipid metabolism

GO Blood microparticle: APOE, SERPING1, GSN, HSPA8, C1QB, HBA2, A2M, ACTB, HBB, HSPA1B, APOA4, ACTG1, CLIC1, CLU, HSPA1A, PFN1, MSN

GO Chylomicron: APOC1, APOE, LPL, APOA4

GO Low density lipoprotein particle: APOE, PLA2G7, MSR1

GO Protein lipid complex: APOC1, APOE, PLA2G7, LPL, MSR1, APOA4, CLU

GO Triglyceride rich plasma lipoprotein particle: APOC1, APOE, LPL, APOA4

GO Cellular response to lipoprotein particle stimulus: APOE, FCER1G, ITGB2, LPL

GO fatty acid biosynthetic process: APOC1, SCD, LPL

GO Fatty acid metabolic process: APOC1, CYP1B1, SCD, IRS2, LPL

GO Fatty acid transport: APOE, IRS2, ABCD1

GO Long chain fatty acid transport: APOE, IRS2, ABCD1

GO Monocarboxylic acid biosynthetic process: APOC1, CYP27A1, SCD, LPL

GO Protein containing complex remodeling: APOC1, APOE, PLA2G7, LPL, APOA4

GO Unsaturated fatty acid metabolic process: CYP1B1, SCD, TBXAS1, CD74, FABP5, ABCD1

Antimicrobial responses

GO Response to bacterium: LYZ, CHIT1, GRN, IL1RN, FCER1G

GO Defense response to bacterium: LYZ, CHIT1, FCER1G

GO Inflammatory response: CHI3L1, CYBB, LYZ, SPP1, APOE, GRN, PLA2G7, TYROBP, CCL22, IL1RN, ITGAM, FCER1G, ITGB2

GO Leukocyte mediated immunity: CHI3L1, CYBB, LYZ, CTSE, CHIT1, GRN, GM2A, TYROBP, SERPING1, PIGR, ITGAM, FCER1G, CTSH, ITGB2

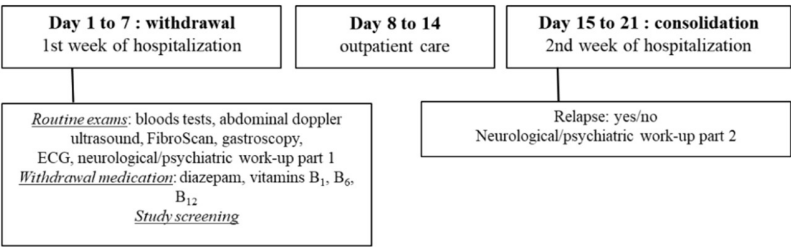
Correlations between plasma levels of lipids and liver parameters

Lipid	AST (IU/l)	ALT (IU/l)	CAP (dB/m)	CK18-M65 (U/l)
SM (d18:0/14:0)	r = 0.5192 p = 0.0273	r = 0.5160 p = 0.0284	r = 0.3329 p = 0.1770	r = 0.4527 p = 0.0202
SM (d20:0/16:0)	r = 0.4743 p = 0.0402	r = 0.5224 p = 0.0218	r = 0.3298 p = 0.1679	r = 0.5443 p = 0.0060

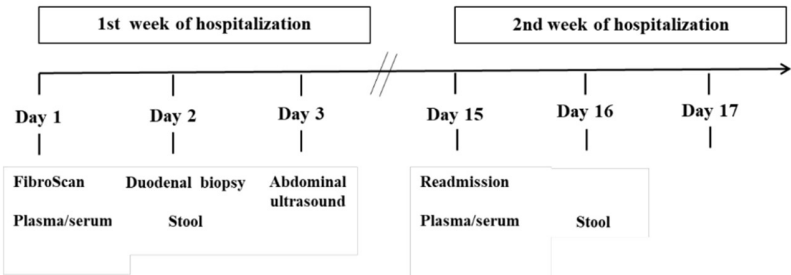
Correlations between plasma levels of lipids and top-ranked genes in lysosomes and lipid metabolism pathways in TRM

Gene	SM (d18:0/14:0)	SM (d20:0/16:0)
CTSB	r = 0.5261 p = 0.0083	r = 0.5078 p = 0.0113
GM2A	r = 0.3096 p = 0.1410	r = 0.32 p = 0.1274
CTSD	r = 0.1983 p = 0.3530	r = 0.4635 p = 0.0225
CTSL	r = 0.4553 p = 0.0254	r = 0.434 p = 0.0341
APOE	r = 0.4418 p = 0.0307	r = 0.3976 p = 0.0544
APOC1	r = 0.3879 p = 0.0611	r = 0.3519 p = 0.0917
LPL	r = 0.3156 p = 0.1130	r = 0.3469 p = 0.0968
CYP27A1	r = 0.3985 p = 0.0537	r = 0.4034 p = 0.0506
CYP1B1	r = 0.4651 p = 0.0220	r = 0.467 p = 0.0214
PLA2G7	r = 0.3079 p = 0.1432	r = 0.3139 p = 0.1352
CYBB	r = 0.4032 p = 0.0507	r = 0.3813 p = 0.0660
NCF2	r = 0.5019 p = 0.0125	r = 0.4539 p = 0.0259

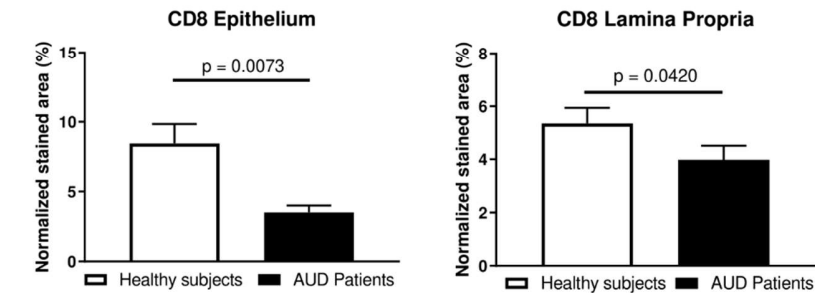
Supplementary figures



Schematic representation and analyses during the study

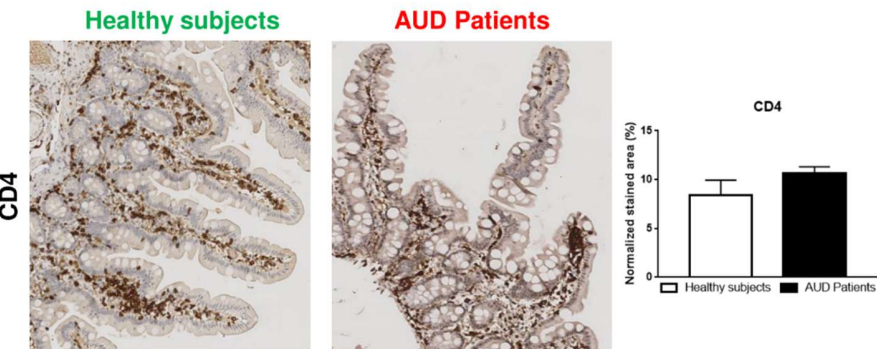


Supplementary Figure 1. Standardized working scheme of the alcohol withdrawal unit.



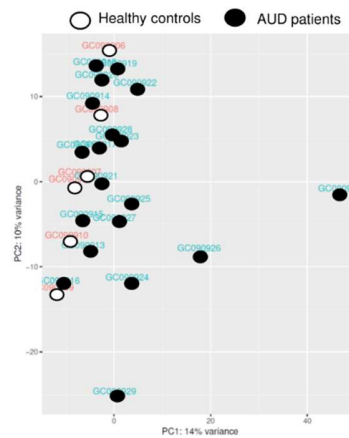
Supplementary Figure 2. Evaluation of CD8+ T cells in epithelium and lamina propria

Alcohol use disorder (AUD) patients (n=15) are characterized by a significative reduction of CD8+ T lymphocytes in both epithelium and lamina propria as compared to healthy controls (n=9).



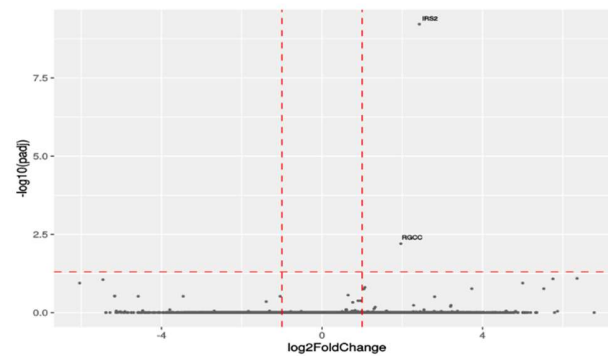
Supplementary Figure 3. Evaluation of CD8+ T cells in epithelium and lamina propria

Alcohol use disorder (AUD) patients (n=15) are characterized by a significative reduction of CD8+ T lymphocytes in both epithelium and lamina propria as compared to healthy controls (n=9).



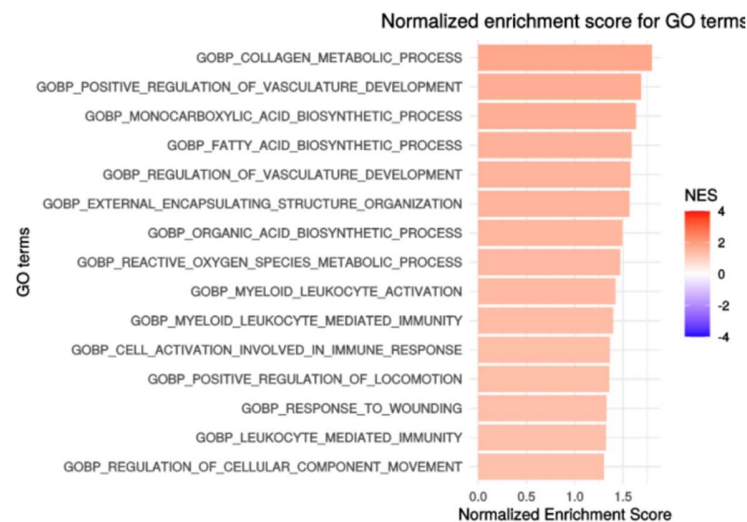
Supplementary Figure 4. Principal component analysis between healthy controls and alcohol use disorder (AUD) patients

Healthy controls vs Non-progressive ALD



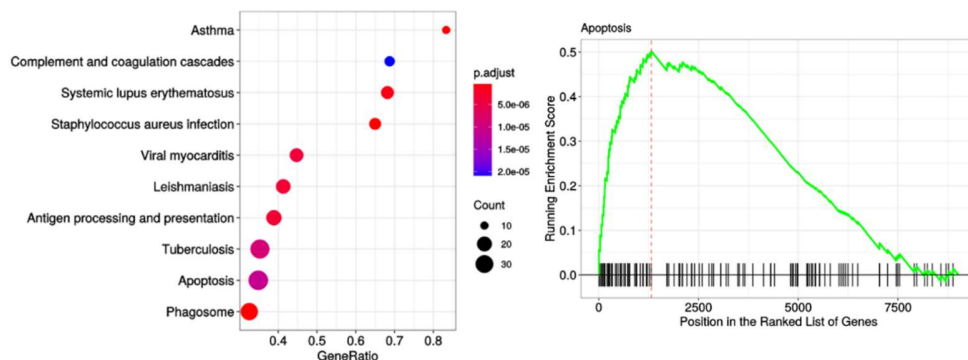
Supplementary Figure 5. Differential gene expression analysis between healthy controls and patients with non-progressive forms of alcohol-associated liver disease (ALD)

T resident memory cells from alcohol use disorder (AUD) patients with non-progressive ALD have a similar transcriptomic profile compared to healthy controls.



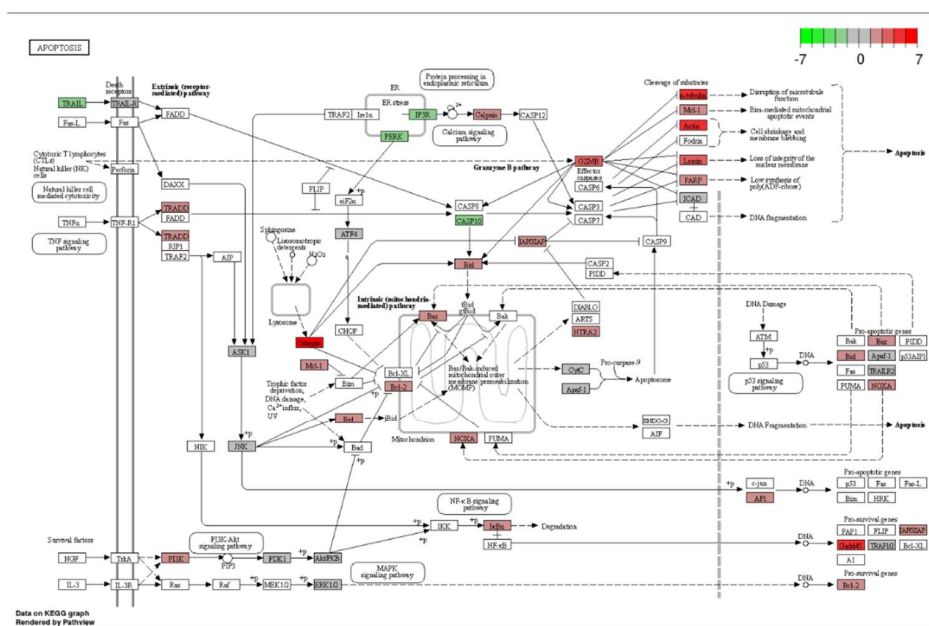
Supplementary Figure 6. Gene ontology (GO) gene sets significantly enriched in T resident memory lymphocytes (TRM) of patients with progressive alcohol-associated liver disease (ALD).

Fifteen top ranked GO biological processes terms in TRM from patients with an alcohol use disorder (AUD) with progressive ALD compared to controls.



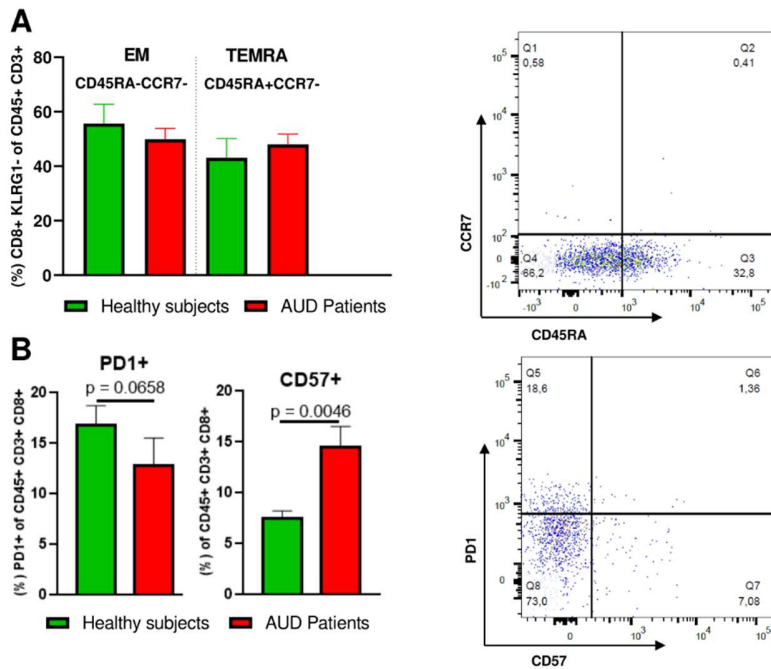
Supplementary Figure 7. KEGG analysis of T resident memory cells (TRM) in patients with progressive alcohol-associated liver disease (ALD)

Dot plot map (left) showing the ten top ranked KEGG pathways in TRM in patients with progressive ALD. Enrichment plot of Apoptosis KEGG pathway (right).



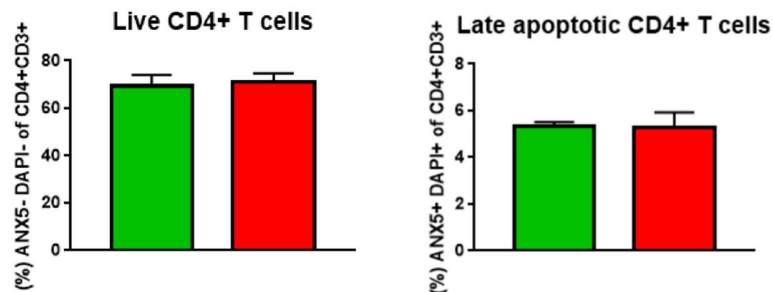
Supplementary Figure 8. KEGG_Apoptosis pathway visualization

T resident memory cells from alcohol use disorder (AUD) patients with progressive forms of alcohol-associated liver disease are characterized by up-regulation of genes (indicated in red) related to lysosomes and mitochondria and a down-regulation of genes (indicated in green) mainly related to the extracellular pathway of programmed cell death



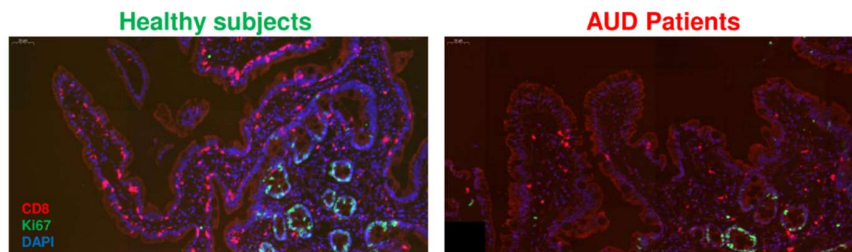
Supplementary Figure 9. Assessment of phenotypic markers in duodenal CD8+ T resident memory (TRM) cells

(A) Flow cytometry analysis showed similar proportions of effector memory (EM) and terminally-differentiated (TEMRA) CD8+ TRM in alcohol use disorder (AUD) patients (n=22) compared to controls (n=6). (B) Analysis showed reduced PD-1+ and increased CD57+ CD8+ TRM in AUD patients compared to controls.



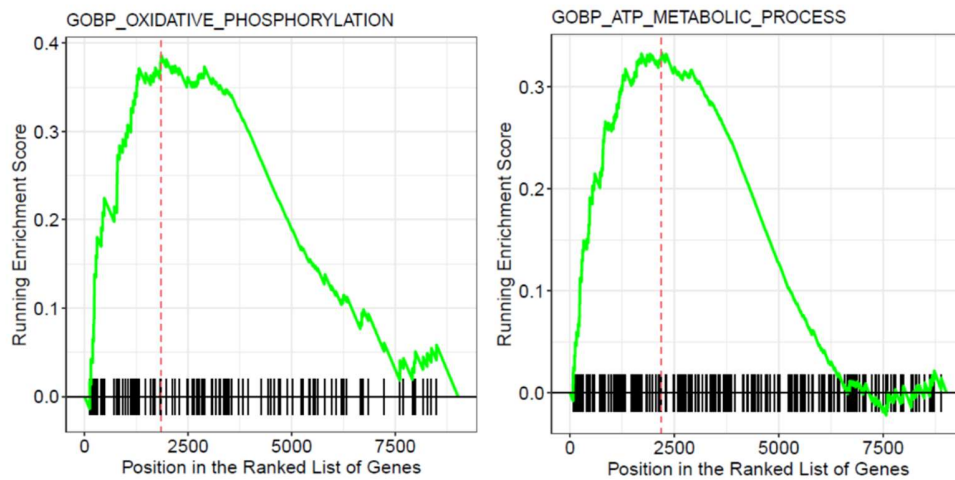
Supplementary Figure 10. Evaluation of apoptosis in duodenal CD4+ T cells

Alcohol use disorder (AUD) patients (n=16) are characterized by a similar proportion of live and late-apoptotic CD4+ T cells as compared to healthy controls (n=3).



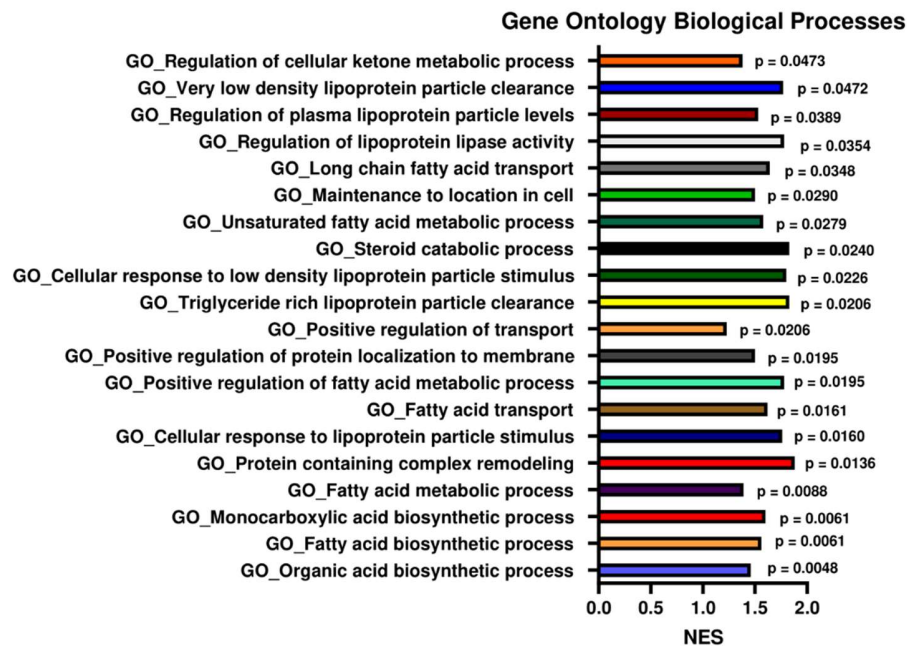
Supplementary Figure 11. Evaluation of proliferation in duodenal CD8+ T cells

Representative immunofluorescence showing no proliferation (Ki67+) of CD8+ T cells in AUD patients and healthy controls.



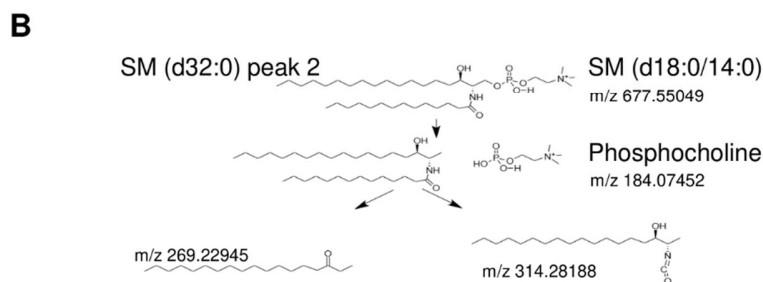
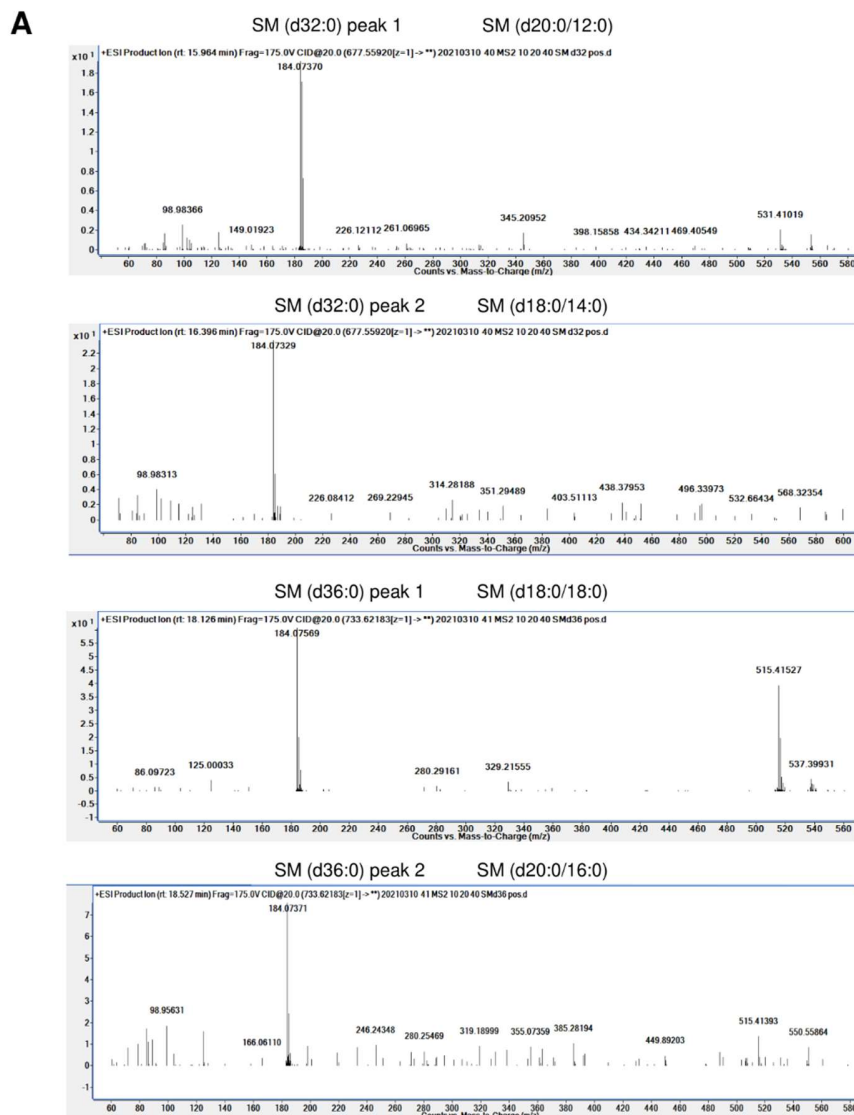
Supplementary Figure 12. Evaluation of mitochondrial pathways in CD8+ T resident memory (TRM) cells

Gene set enrichment analysis showed significant enrichment of gene sets linked to oxidative phosphorylation and ATP metabolic process in TRM cells from alcohol use disorder (AUD) patients with progressive alcohol-associated liver disease (ALD).



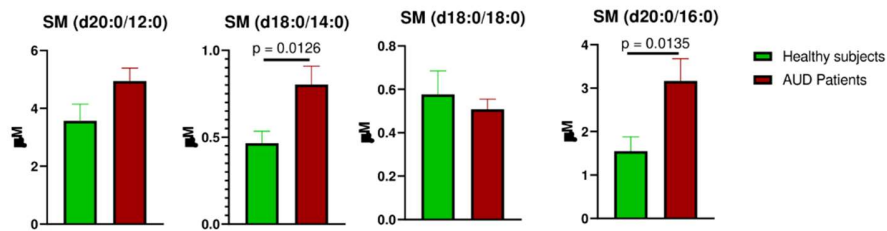
Supplementary Figure 13. Evaluation of lipid pathways related to biological processes in T resident memory (TRM) cells from patients with progressive liver disease

Gene set enrichment analysis showed significant enrichment of gene ontology biological processes gene sets linked to lipid metabolism in TRM cells from alcohol use disorder (AUD) patients with progressive alcohol-associated liver disease (ALD).



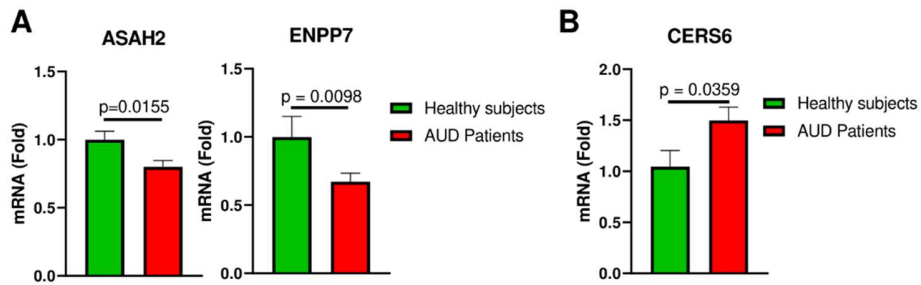
Supplementary Figure 14. Identification of the composition of sphingolipid isomers

(A) Representative collision induced dissociation (CID) spectra of sphingomyelin SM(d32:0) (top) and SM (d36:0) (bottom) isomers with CID of 20 eV. (B) Proposed general fragmentation pattern by LC-MS/MS technology of identified protonated [M+H]⁺ sphingomyelin SM (d18:0/14:0).



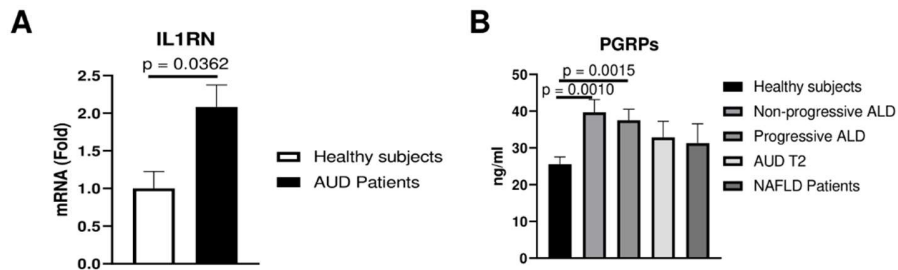
Supplementary Figure 15. Evaluation of plasma lipid levels by high-resolution LC-MS/MS

Targeted LC-MS analysis showed significant increased plasma levels of SM (d18:0/14:0) and SM (d20:0/16:0) in alcohol use disorder (AUD) patients (n=19) compared to healthy volunteers (n=8).



Supplementary Figure 16. Evaluation of key enzymes involved in sphingolipid metabolism in patients with an alcohol use disorder (AUD)

(A) Intestinal alkaline sphingomyelinase (ENPP7) and neutral ceramidase (ASAH2), enzymes involved in dietary sphingomyelin absorption, are down-regulated in distal duodenal biopsies from AUD patients (n=46) compared to controls (n=12). By contrast, Ceramide synthase 6 (CERS6) is up-regulated in the duodenal mucosa from AUD patients (n=46) compared to healthy volunteers (n=12) (B).



Supplementary Figure 17. Evaluation of duodenal inflammatory response and microbial translocation

(A) Up-regulation of IL1RN in the duodenum of alcohol use disorder patients (n=46) compared to controls (n=12). (B) Serum PGRPs levels significantly increased in actively drinking AUD patients with non-progressive (n=27) and progressive ALD (n=35) compared to controls (n=21).

Complementary discussion and conclusion

We have revealed that apoptosis of duodenal T resident memory lymphocytes is associated with lysosome dysfunction, altered lipid metabolism and diminution in mitochondria ROS production. We identified different intracellular molecules involved in this process, such as Cathepsin B and UCP2. We connected the phenomenon of cell death with increased serum levels of sphingomyelins. Stimulation with dihydroceramide (d18:0/14:0), cleavage product of the identified sphingomyelin SM (d18:0/14:0), dose-dependently reduced viability of intestinal TRM, supporting a potential interaction in the proximal small gut between circulating sphingomyelin and/or their cleavage products and gut T resident memory lymphocytes. Because of unavailability of several molecules, we could not test the potential effect of the identified sphingomyelins (d18:0/14:0) and (d20:0/16:0) as well as of dihydroceramide (d20:0/16:0).

Duodenal TRM up-regulated genes associated with lymphocyte activation and response to microbes. Interestingly, this response was not linked to pro-inflammatory mediators but to antimicrobial molecules as well as tolerogenic cytokine such as IL1 receptor antagonist (IL1RN).

In conclusion, we have revealed in chapter 3 a specific diminution of duodenal TRM in patients with progressive ALD due to cell death linked to lysosomal dysfunction, mitochondria, and lipid metabolism alterations (Fig.3.1). These changes were related to reduced immunosurveillance against microbes, especially in progressive ALD.

Elevated translocation of microbes and/or their products due to reduced TRM cells could lead to activation of inflammatory responses in the systemic circulation and/or in the liver. Different cell types in the blood and in the liver may be affected by different gut-derived products and could activate inflammatory pathways implicated in ALD progression. Systemic and hepatic immune pathways linked to microbial translocation will be investigated in the next two chapters.

The perspectives of chapter 3 are the following:

- Assessment of systemic and liver inflammatory responses caused by elevated translocation of gut-derived products

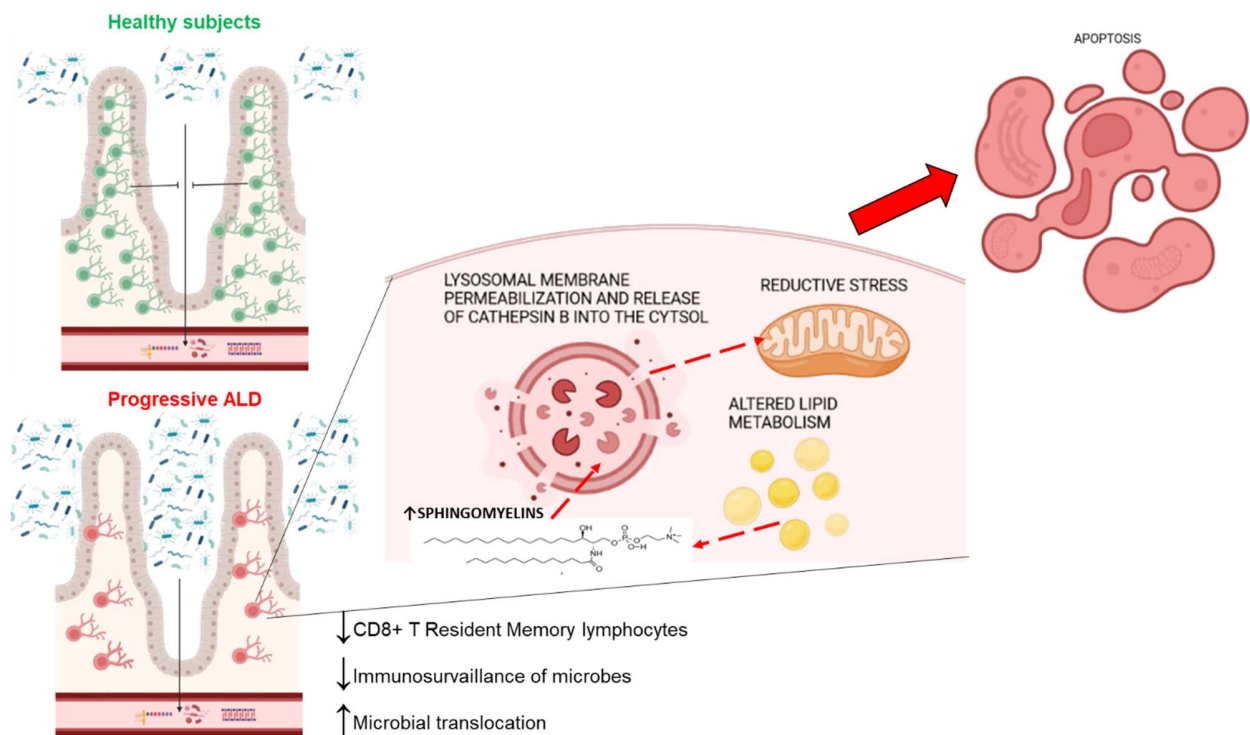


Fig.3.1 Schematic representation of the processes described in chapter 4. In patients with progressive alcohol-associated liver disease (ALD), apoptosis of duodenal CD8+ T resident memory (TRM) cells contribute to reduced immunosurveillance against microbes thus promoting microbial translocation. Cell death was related to dysfunctional lysosomes and mitochondria, changes in lipid metabolism and increased levels of sphingomyelins.

CHAPTER 4: TLR 2 activation in monocytes contributes to systemic inflammation and alcohol-associated liver disease in humans

Increased translocation of bacterial and fungal products into the blood circulation may trigger systemic inflammatory responses. Recent studies suggest that peripheral blood mononuclear cells (PBMCs) activation by microbial products promotes the release of inflammatory cytokines implicated in the development of systemic inflammation and potentially ALD progression. Among PBMCs, monocytes have a great potential to participate in this process. In this chapter we tested whether circulating inflammatory monocytes are recruited to the liver during inflammation and fibrosis, main mechanisms involved in alcohol-associated liver disease. disease progression in response to bacterial and fungal antigens in earlier stages of ALD.

The aim of this study was to evaluate the role of monocytes in the development of systemic inflammation in relation to elevated systemic translocation of microbes and/or their products and its involvement in ALD. We finally tested the relationship between monocytes recruitment into the liver, macrophages activation and liver cell damage.

We found that, in AUD patients, the number of blood monocytes increased. Monocytes from AUD patients **up-regulated IL1 β and IL8 together with TLR2 and down-stream AP-1 while fungal down-stream effector CARD9 was down-regulated**. According to TLR2 up-regulation, IL1 β and IL8 were actively secreted upon stimulation *in vitro* with the TLR2 ligand PGN. By contrast, *Candida albicans* stimulation leads to down-regulation of IL1 β and TNF- α , indicating a **reduced immune response against this fungus overrepresented in AUD patients**. In parallel, **IL1 β and IL8 were increased in ALD livers together with activation of intrahepatic macrophages and up-regulation of liver chemokines involved in monocytes attraction**, pointing to the contribution of circulating activated monocytes in ALD progression.

The results of this fourth chapter have been submitted

TLR 2 activation in monocytes contributes to systemic inflammation and alcohol-associated liver disease in humans

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Acknowledgements: This study was supported in part by NIH grants R01 AA24726, R37 AA020703, U01 AA026939, U01 AA026939-04S1, by Award Number BX004594 from the Biomedical Laboratory Research & Development Service of the VA Office of Research and Development, and a Biocodex Microbiota Foundation Grant (to B.S.) and services provided by NIH centers P30 DK120515 and P50 AA011999, by grants from Fond National de Recherche Scientifique Belgium (J.0146.17 and T.0217.18) and Action de Recherche Concertée (ARC), Université Catholique de Louvain, Belgium to P.S. We thank the IREC flow cytometry and imaging platforms for their support in analyzing the results.

Conflicts of interest: B.S. has been consulting for Ferring Research Institute, HOST Therapeutics, Intercept Pharmaceuticals, Mabwell Therapeutics, Patara Pharmaceuticals and Takeda. B.S.'s institution UC San Diego has received research support from Axial Biotherapeutics, BiomX, CymaBay Therapeutics, NGM Biopharmaceuticals, Prodigy Biotech and Synlogic Operating Company. B.S. is founder of Nterica Bio. UC San Diego has filed several patents with B.S. as inventor related to this work. P.S. received grant support from Gilead Sciences Belgium.

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ABSTRACT

Background and Aims: Monocytes activation by microbial products might contribute to systemic and liver inflammation in alcohol-associated liver disease (ALD). We explored the association between monocytes, microbial translocation, systemic inflammation and ALD.

Method: Alcohol use disorder (AUD) patients following a rehabilitation program were compared to healthy controls. We determined the circulating number and proportion of monocytes subsets by FACS. Activation of signaling pathways by gut-derived microbes was analyzed by qPCR in isolated monocytes. Cytokines secretion by monocytes and phagocytosis were assessed *in vitro*. Serum microbial translocation markers and cytokines were measured by ELISA and multiplex assay, respectively. ALD severity and liver inflammatory responses were analyzed in liver biopsies by various methods.

Results: In AUD patients, the number of blood monocytes increased compared to controls. Monocytes from AUD patients up-regulated IL1 β and IL8 together with TLR2 and down-stream AP-1 while fungal sensor CARD9 was down-regulated. IL1 β and IL8 were actively secreted upon stimulation *in vitro* with the TLR2 ligand Peptidoglycan. Exposure with *E. coli* confirmed preserved bacterial phagocytic activity. By contrast, *Candida albicans* stimulation leads to down-regulation of IL1 β and TNF- α compared to controls. Systemic cytokines and monocytes changes correlated with microbial translocation. Hepatic IL1 β and IL8 increased with ALD severity together with liver macrophages activation and up-regulation of chemokines involved in monocytes attraction.

Conclusion: Our results point to a contribution of activated monocytes to systemic inflammation and ALD. Monocytes likely infiltrate the liver, transform into monocyte-derived macrophages and release IL1 β and IL8 in response to Peptidoglycan and TLR2 activation.

INTRODUCTION

Alcohol abuse is a major public health problem and alcohol-associated liver disease (ALD) is the most prevalent type of chronic liver disease worldwide. Most individuals with an alcohol use disorder (AUD) develop simple steatosis which can progress to alcoholic steatohepatitis (ASH) characterized by hepatic inflammation and hepatocyte ballooning¹. Chronic ASH eventually leads to fibrosis and cirrhosis and in some cases hepatocellular carcinoma². The mechanisms leading to ALD progression remain incompletely understood.

Recent reports have highlighted the pivotal role of the gut-liver axis in the pathogenesis of ALD. In humans, alterations in the gut micro- and mycobiome together with elevated systemic translocation of either microbial products and/or microbes themselves are associated with ALD progression^{3,4}. Increased systemic translocation of bacterial products, such as Lipopolysaccharides (LPS) and Peptidoglycans (PGN) are associated with elevated plasma levels of inflammatory cytokines in AUD patients⁵.

Mechanistic analyses have shown that in AUD patients LPS and to a greater extent PGN can contribute to the activation of peripheral blood mononuclear cells (PBMCs). PBMCs are likely contributing to PGN-triggered release of IL1 β , IL8 and IL18 into the blood of AUD patients⁵. Intriguingly, a short period of abstinence was able to restore normal blood LPS levels, while the expression of PGN-associated receptors remained increased in the PBMCs, suggesting that PGN could contribute to the persistence of an elevated systemic inflammatory response observed in sober patients. By contrast, TNF- α and IL6 were not elevated in PBMCs indicating that their increased plasma levels might originate from other sources⁶. Among PBMCs, monocytes represent the cell type with the highest potential to secrete these cytokines⁷.

Circulating inflammatory monocytes are recruited to the liver during inflammation and fibrosis, principally involved in alcohol-associated liver disease progression⁸. Once in the liver, monocytes transform into monocyte-derived macrophages (MDM) which have been shown to play a key role in ALD development in animal models^{9,10}. However, animals only develop mild forms of ALD upon chronic alcohol feeding and do not resume the liver-damage pattern observed in humans¹¹. Therefore, investigations in human ALD are needed to evaluate the role of monocytes and MDMs in systemic inflammation and liver disease.

Human studies generally focused on the role of monocytes and monocyte-derived macrophages in late-stages of liver disease such as decompensated cirrhosis and severe alcoholic hepatitis^{12,13}. Little is known about the role of monocytes and monocyte-derived macrophages in the development of systemic inflammation and alcohol-associated liver disease progression in response to bacterial and fungal antigens in earlier stages of ALD.

The aim of our present study is to assess the potential role of monocytes in the development of systemic inflammation in relation to elevated systemic microbial translocation and its involvement in alcohol-associated liver disease progression in humans.

METHODS

Patients

Alcohol use disorder (AUD) patients (Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition criteria) admitted for elective alcohol withdrawal to a dedicated alcohol withdrawal unit followed a highly standardized and controlled 3-week detoxification and rehabilitation program (Supplementary Fig.1). They were compared with healthy volunteers (social drinkers consuming <20g of alcohol/day) matched for gender, age and BMI. All patients reported long-term (>1year) alcohol consumption (>60g/day) and were actively drinking until the day of admission. Clinical and baseline biochemical data were collected prospectively for all patients and they routinely underwent a large panel of investigations. Additional inclusion and exclusion criteria are available in Supplementary data. All. Due to methodological and logistical reasons, some analyses were only performed in representative cohorts of patients, as indicated in the figure legends.

Examinations and sample collections (Supplementary Fig.1)

On the day of admission, transient elastography (Fibroscan®, Echosense, Paris, France) was performed and a fasting blood sample (serum, plasma) was drawn. A transjugular liver biopsy was routinely proposed to all patients with suspicion of significant liver disease (FibroScan ≥ 7.6 kPa ($\geq F2$), or doppler ultrasound with dysmorphic liver or splenomegaly or alterations of portal vein flow).

Ethical aspects

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institution's human research and ethical committee (B403201422657). Written informed consent was obtained from all patients and healthy volunteers.

Isolation of human monocytes

The peripheral blood mononuclear cells (PBMCs) were isolated from blood by centrifugation on a Ficoll-Paque Plus gradient medium (GE Healthcare Biosciences AB, Uppsala, Sweden) (supplementary material). CD14⁺ monocytes were isolated by using anti-human CD14

MicroBeads (Miltenyi Biotec), according to manufacturer's protocol. Quality control by flow cytometry showed that at least 90% of the isolated cells were CD14+.

RNA extraction, reverse transcription and real-time quantitative polymerase chain reaction

RNA extraction from cells and tissue was performed by using TRIzol reagent (Invitrogen, Carlsbad, California). RNA was then purified by using columns of the Direct-zol™ RNA Microprep kit (Zymo Research, BaseClear Lab, The Netherlands). Messenger RNA (mRNA) was assessed by quantitative real-time polymerase chain reaction (PCR) and quantified as described. Primers are depicted in supplementary material.

Immunohistochemistry/ Immunofluorescence

Five µm formalin fixed liver sections were routinely stained with Hemalin-eosin and primary and secondary antibodies using standard methods followed by quantification (details in supplementary material).

Flow cytometry evaluation of monocyte subsets

Flow cytometric analysis was performed on isolated PBMCs stained with antibodies against CD14, CD16, CCR2, HLA-DR and CX3CR1 (listed in supplementary material) by using a BD FACSCanto™ II equipped with BD FACSDiva™ software.

Short-term cell culture and *in vitro* stimulation

To test *in vitro* Toll-like receptors activation upon LPS and PGN stimulation, monocytes from AUD patients and matched healthy controls were cultured in 96-well plates in RPMI 1640 culture medium (50,000 cells/well) containing L-glutamine, 10% FBS, penicillin (100 units/ml), and streptomycin (100 µg/ml) in a 5% CO₂-humidified incubator at 37 °C, and exposed to 1 ng/ml of either LPS (InvivoGen) or PGN (InvivoGen) or with increasing doses of *Candida albicans* extracts (InvivoGen) (10⁶, 500x10⁶ and 10⁷ cells/ml) for 24 h. Cells cultured in culture medium alone served as controls. Culture medium was recovered for measurements of IL1β, IL8 and TNF-α levels. RNA was extracted from stimulated monocytes as described above.

Phagocytosis assay

Phagocytosis was assessed by using dTomato-expressing *Escherichia coli* generated by transforming *E. coli* with the plasmid vector ptdTomato (details in supplementary material). Bacteria and monocytes isolated from AUD patients or healthy controls were co-cultured in a ratio 1:10 in RPMI-1640 medium supplemented with heat-inactivated fetal bovine serum, L-glutamine, and penicillin/streptomycin for 15 minutes to assess bacterial engulfment. Bacterial engulfment was evaluated by flow cytometry as previously described¹⁴ using a BD FACSCanto™ II equipped with BD FACSDiva™ software.

Determination of serum biomarkers of liver cell damage and microbial translocation

In addition to AST and ALT, serum cytokeratin 18 (CK18) was used to assess liver cell damage (CK18-M65 ELISA kit; TECOmedical AG, Sissach, Switzerland). Serum levels of CK18-M30 (CK18-M30 ELISA kit; TECOmedical AG, Sissach, Switzerland) were used to assess hepatocyte's apoptosis. Microbial translocation was determined using soluble CD14 (Human CD14 Quantikine ELISA kit sCD14, Bio-techne Ltd., Abingdon, United Kingdom) and Peptidoglycan Recognition Proteins (Human PGRPs ELISA kit, Thermofisher, Merelbeke, Belgium), respectively. All assays were performed in duplicate following the manufacturer's instructions.

Assessment of plasma levels of cytokines

Plasma concentrations of cytokines (IL8, IL18, GM-CSF, IL6 and TNF- α) were determined using the Meso Scale Discovery (MSD) U-Plex assay (Rockville, MD, USA), IL1 β levels which were assessed by using the IL-1 beta Human ELISA kit, high sensitivity (ThermoFisher, Merelbeke, Belgium), following the manufacturer's instructions.

Statistics

Data were analyzed using Graph Pad Prism 8 (GraphPad Software, San Diego, CA, USA) and presented as mean $\pm$ standard error of the mean (SEM) unless otherwise indicated. Normality was assessed by the Kolmogorov-Smirnov test followed by t-tests for normally distributed data, or the Wilcoxon test for nonnormally distributed data. Data were compared by one-way ANOVA for multiple groups, followed by Bonferroni's post hoc test for pairwise comparisons. Wilcoxon or paired t tests compared data of patients before and after abstinence. Pearson's or Spearman's correlation tests were used for correlations between data sets. A p value <0.05 was considered as statistically significant. Flow cytometry data were analyzed using FlowJo (TreeStar).

RESULTS

Study population

The study population consists of a cohort of 123, middle-aged, predominantly male AUD patients and 26 healthy volunteers. Demographic and biochemical data are provided in Table 1. All patients reported alcohol consumption until the evening prior to their admission. In sixty-two AUD patients with suspicion of significant liver disease (FibroScan ≥ 7.6 kPa ($\geq F2$), or doppler ultrasound with dysmorphic liver or splenomegaly or alterations of portal vein flow) a liver biopsy was performed. Forty eight percent ($n=30$) had early-stage ALD with a Metavir fibrosis score of F0/F1 and various degrees of steatosis on histology. Seven patients had a metavir score of F2 and 25 patients presented with advanced fibrosis ($\geq F3$) All patients had a preserved synthetic liver function and showed no clinical signs of liver decompensation.

Table 1. Baseline demographic and biochemical data of the study population

<i>Demographics</i>			
	Healthy volunteers (n = 26)	AUD patients (n = 123)	p value
Gender (female/male)	10 (38 %) / 16 (62 %)	39 (32 %) / 84 (68 %)	.5054
	Mean $\pm$ SEM		
Age (years)	46.8 $\pm$ 9.9	50.5 $\pm$ 8.2	.1713
BMI	25.6 $\pm$ 2.8	25.6 $\pm$ 3.8	.9671
<i>Biochemistry</i>			
	Mean $\pm$ SEM (normal range)		
AST (IU/l)	ND	90.5 $\pm$ 63 (<40)	-
ALT (IU/l)	ND	75.7 $\pm$ 49.7 (<40)	-
γ -GT (IU/l)	ND	350.3 $\pm$ 327.8 (<40)	-
ALP (IU/l)	ND	93.6 $\pm$ 34.7 (30-130)	-
Bilirubin (mg/dl)	ND	0.84 $\pm$ 0.39 (0.3-1.2)	-
Albumin (g/l)	ND	44.35 $\pm$ 4.18 (35-52)	-

Histology (n=62)

Fibrosis Metavir

No fibrosis F0/F1 n=30	Significant fibrosis F2 n=7	F3 n=10	F4 n=15
Presence of steatohepatitis n=18	n=7	n=6	n=11

Abbreviations: BMI, body mass index; AST, Aspartate transaminase; ALT, Alanine transaminase; γ -GT, gamma-glutamyltransferase; ALP, alkaline phosphatase

Circulating monocytes and systemic microbial translocation in AUD patients

Increased number of circulating monocytes together with increased proportions of intermediate and non-classical subsets characterized AUD patients

We first assessed whether there was a difference between number of circulating monocytes in AUD patients and healthy controls and found a significant ($p < 0.0001$) increase in the number of monocytes in AUD patients (Fig.1A). We then determined the three main subsets of monocytes by flow cytometry (Supplementary Fig.2). We observed a significant diminution of classical monocytes accompanied by a significant increase in both intermediate and non-classical monocytes (Fig.1B).

Expression of IL1 β and IL8 is up-regulated in monocytes of AUD patients

A previous report has shown that PBMCs from AUD patients were characterized by up-regulation of IL1 β , IL8 and IL18 while TNF- α and IL6 were not increased compared with PBMCs from healthy controls⁵. We isolated monocytes to verify whether these transcriptional changes also characterize monocytes in AUD patients. IL1 β and IL8 mRNA were significantly up-regulated (Fig.1C) while the expression of other pro-inflammatory cytokines remained unchanged in monocytes from AUD patients compared to healthy controls (supplementary fig.3A). In parallel, we found elevated blood levels of the inflammatory cytokines IL8 (Fig 1 C), IL6 and TNF- α (Supplementary Fig.3B) in our cohort of AUD patients compared with controls while IL1 β could not be detected in blood in both groups.

Since IL1 β and IL8 are transcriptionally activated by the transcription factor AP-1, we then assessed gene expression of c-Fos and c-Jun as components of the transcription factor AP-1¹⁵. We found a significantly increased gene expression of c-Jun, but not c-Fos, in monocytes from AUD patients compared to controls, pointing to a potential involvement of the transcription factor AP-1 (Fig.1D). By contrast, gene expression of NLRP3, a component of the inflammasome involved in cleavage and activation of pro-IL1 β ¹⁶, was similar between the two groups (Fig. 1D). Since NF- κ B pathway controls the expression of IL6 and TNF- α , which did not change in monocytes from AUD patients, we next assessed the mRNA levels of I- κ B, inhibitor of NF- κ B, and found up-regulation in AUD compared to controls (Supplementary Fig.3A), pointing to a possible restriction of the pathway.

These results indicate that an increased number of inflammatory-primed (i.e., the ability to produce IL1 β and IL8) monocytes accompanied by alterations in their subpopulations characterizes AUD patients as compared with healthy volunteers.

Figure 1

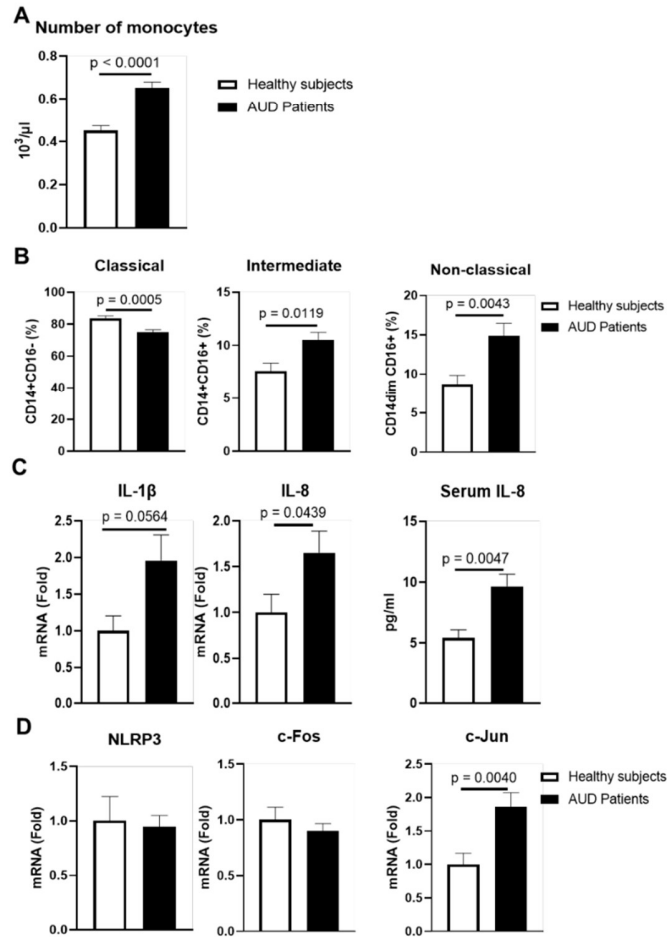


Fig.1 Evaluation of monocytes in alcohol use disorder (AUD) patients. Monocyte's count in the systemic circulation (A) showed a significant ($p < 0.0001$) increased number of monocytes in AUD patients ($n=62$) compared to matched healthy volunteers ($n=18$). (B) Flow cytometry analysis revealed diminution in the proportion of classical monocytes accompanied by increased proportion of both intermediate and non-classical monocytes in AUD patients ($n=24$) compared to healthy controls ($n=8$). Gene expression of inflammatory cytokines in monocytes showed up-regulation of IL1 β and IL8 in AUD patients ($n=24$) compared to controls ($n=8$) associated with increased serum levels of IL8. Serum IL1 β levels could not be detected with the ELISA kit used to assess IL1 β in the culture medium (C). Monocytes from AUD patients have similar gene expression of NLRP3 and analysis of the two component of the transcription factor AP-1, such as c-Fos and c-Jun, showed up-regulated of c-Jun in monocytes from AUD patients compared to controls while c-Fos did not change (D).

Elevated serum levels of Gram-positive translocation marker correlate with the proportion of intermediate monocytes

As anticipated from previous work, we also show a significant increase of soluble CD14 (sCD14) and Peptidoglycan-recognition Proteins (PGRPs), serum surrogate markers of microbial translocation (MT) for Gram- and Gram+ bacteria, respectively^{17,18} in this cohort of AUD patients (Fig. 2A). We next evaluated whether the alterations observed in the monocytes correlated with serum levels of microbial translocation markers. We found a positive correlation between intermediate monocytes and serum levels of PGRPs (Fig 2B), while no correlations between alterations in the monocytes and sCD14 levels were observed (supplementary table 1).

Since microbial translocation may drive systemic inflammation in mouse models of alcohol-associated liver disease and humans¹⁹, we also correlated plasma levels of cytokines with surrogate markers of microbial translocation. All three cytokines significantly correlated with sCD14 but not with PGRPs (supplementary table 2).

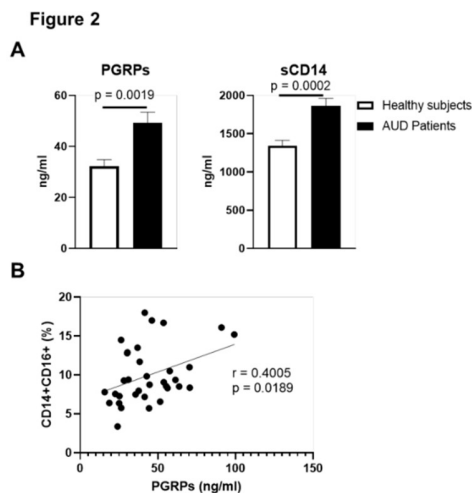


Fig.2 Evaluation of microbial translocation in relation to monocytes from alcohol use disorder (AUD) patients. Analysis of serum Peptidoglycan Recognition proteins (PGRPs) and soluble CD14 (sCD14), marker of Gram+ and Gram- translocation, respectively, showed significantly increased levels in AUD patients (n=24) compared to controls (n=8) (A). Analysis showed a significant positive correlation between proportion of C14+CD16+ intermediate monocytes and serum levels of PGRPs microbial translocation marker (B).

Peptidoglycan activation of TLR2 primes monocytes for increased IL1 β and IL8 production in AUD patients

We next evaluated gene expression of Toll-like receptor 2 (TLR2) and 4 (TLR4), which bind the Gram+ and Gram- products Peptidoglycan (PGN) and Lipopolysaccharide (LPS), respectively²⁰. TLR2 was up-regulated in monocytes from AUD patients (Fig.3A) while TLR4 gene expression did not change (Supplementary Fig.4A). In addition, TLR2 expression correlated with mRNA levels of IL1 β and IL8 ($r=0.3698$, $p=0.0406$; $r=0.6036$, $p=0.0003$, for IL1 β and IL8, respectively) indicating a potential link between TLR2 ligands and activation of monocytes.

To test the possible differential activation of monocytes from AUD patients by Gram+ and Gram- bacteria, we performed in vitro stimulation tests in monocytes isolated from AUD patients. We first evaluated the overall phagocytic capacity of monocytes to live *E. coli* and observed no differences between monocytes from AUD patients and healthy controls (Fig.3B) revealing a preserved bacterial phagocytic function.

The concept of trained immunity describes the long-term functional reprogramming of innate immune cells, which is evoked by exogenous or endogenous insults and which leads to an altered response towards a second challenge after the return to a non-activated state²¹. We first assessed whether monocytes from AUD patients were “trained” against bacterial antigens by stimulating them with bacterial products Peptidoglycan/PGN (Gram-positive bacteria) and LPS (Gram-negative bacteria). Monocytes from AUD patients secreted higher levels of IL1 β and IL8 in response to the TLR2 ligand Peptidoglycan (Fig.3C) compared with control monocytes. Intriguingly, peptidoglycan stimulation also increased gene expression of TLR2 and the inflammasome component NLRP3 in monocytes from AUD patients (Fig.3D). By contrast, stimulation with Lipopolysaccharide LPS lead to modest increase of TNF- α secretion (supplementary Fig.4C) without stimulating gene expression of its receptor TLR4 (supplementary Fig.4B).

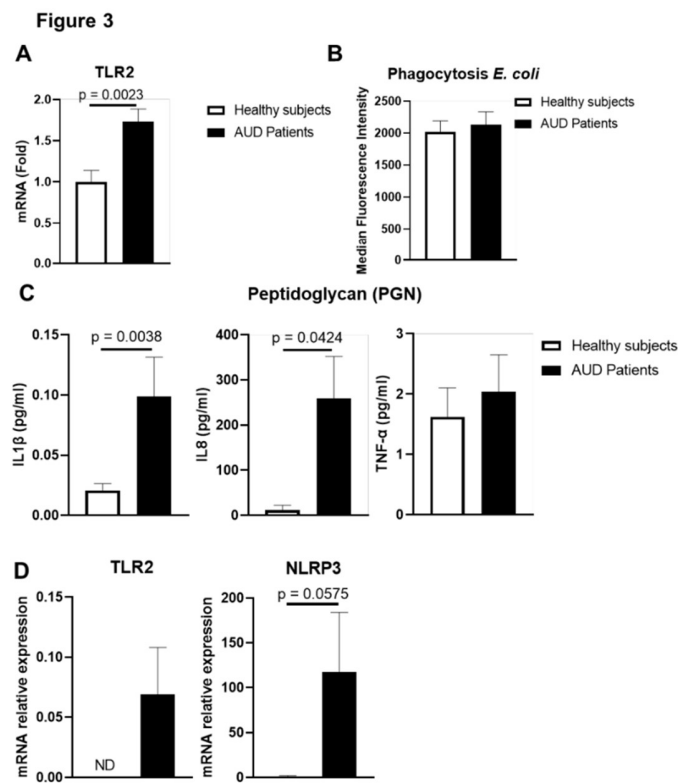


Fig.3 Assessment of monocytes activation by bacterial products in alcohol use disorder (AUD) patients. Toll-like receptor 2 (TLR2) was up-regulated in monocytes from AUD patients (n=24) compared to controls (n=8) (A). (B) Phagocytosis capacity was similar in monocytes from AUD patients (n=4) as compared to healthy controls (n=4). Peptidoglycan (PGN) stimulation (C) promoted increased secretion in the medium of IL1 β and IL8, but not TNF- α , in monocytes from AUD patients (n=8) compared to controls (n=8). (D) Peptidoglycan stimulation leads to up-regulation of TLR2 and NLRP3 in monocytes from AUD patients.

Reduced CARD9 is associated with a blunted response to fungi in monocytes of AUD patients

AUD patients are characterized by fungal overgrowth and a specific increased proportion of *Candida albicans*^{4,22}. C-type lectin receptors (CLRs) bind and respond to different fungal species. We therefore evaluated CLRs (e.g., Dectin1, Dectin2 and Mincle) mRNA levels and found no differences in monocytes of AUD compared to controls (Fig. 4A).

CARD9 is a signaling adaptor protein downstream of a variety of CLRs and plays a critical role in fungal immune surveillance. Following receptor engagement and recruitment of Syk kinase, CARD9 forms a complex with BCL10 and MALT1, which transduces non-Toll-like receptor signaling to the canonical NF- κ B pathway²³. We next assessed CARD9 gene expression and found down-regulation in monocytes from AUD patients compared with controls (Fig. 4A), pointing to a potential immune dysregulation against fungi.

To test this hypothesis, we stimulated monocytes with different doses of *Candida albicans* extracts. We found a down-regulation of IL1 β and TNF- α in monocytes from AUD patients compared to controls with the highest dose of the extracts, while IL8 mRNA did not change between the two groups (Fig. 4B), indicating a reduced immune response against this fungus.

Overall, these observations indicate that circulating monocytes are primarily activated by TLR2 ligands in AUD patients with subsequent priming for increased production of IL1 β and IL8.

Figure 4

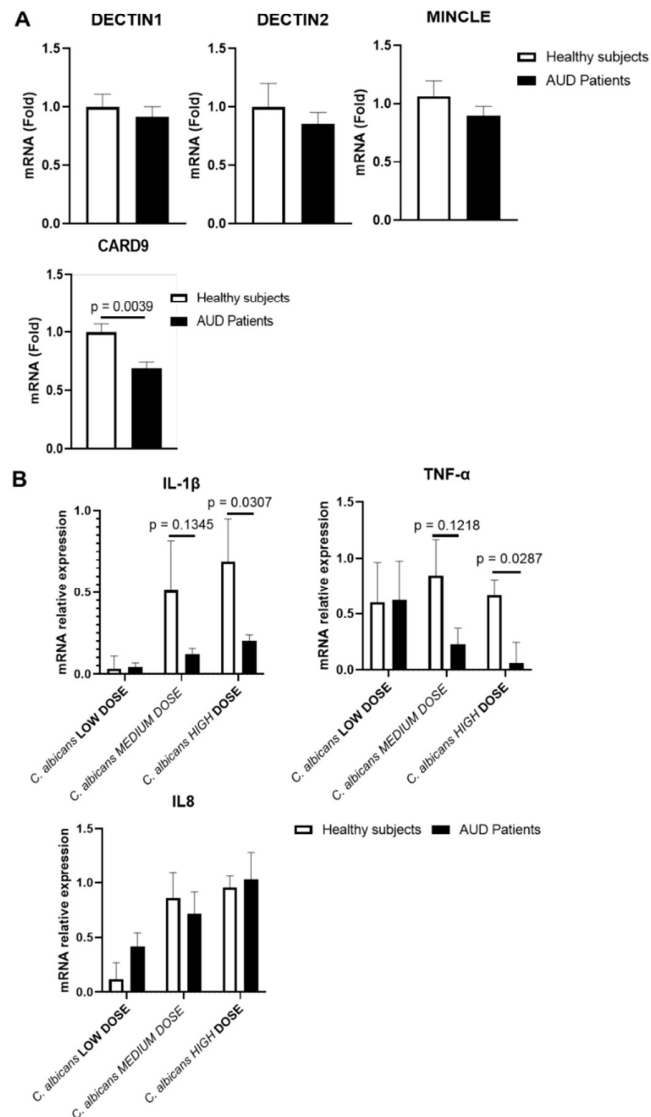


Fig.4 Assessment of monocytes activation by fungi in alcohol use disorder (AUD) patients. Monocytes from AUD patients (n=24) have similar gene expression of receptors Dectin1, Dectin2 and Mincle compared to healthy volunteers (n=8) while downstream CARD9 was down-regulated in AUD patients (A). Stimulation with increasing doses of *Candida albicans* showed reduced gene expression of IL1 β and TNF- α , but not IL8, in a dose-dependent manner (B) in monocytes from AUD patients compared to controls (n=7/group).

Monocyte-derived macrophages and alcoholic liver disease progression

Intrahepatic IL1 β and IL8 correlated with alcohol-associated liver disease severity

Chronic liver diseases including ALD, have been associated with activation of infiltrating macrophages with massive release of pro-inflammatory cytokines in the liver¹². We therefore assessed whether intrahepatic cytokines and markers of macrophages polarization correlated with markers related to liver disease severity. We subdivided AUD patient according to liver disease severity based on histology and we evaluated intrahepatic cytokines/chemokines and markers of macrophages activation in liver biopsies by qPCR and immunohistochemistry analysis. Based on histology, AUD patients were subdivided into three groups: steatosis (Metavir F0/F1, presence of steatosis, normal transaminases; n=12); steatohepatitis (Metavir F0/F1, steatosis, hepatocyte ballooning and elevated transaminases; n=18), significant fibrosis (Metavir $\geq$ F2; n=32) (supplementary Fig.5). To get a better appreciation of liver disease severity, we further measured serum levels of markers linked to liver cell damage and apoptosis, such as CK18-M65 and CK18-M30, respectively (Table 2).

Table 2. Serum levels of liver cell damage markers according to ALD stage.

	AST (IU/l)	ALT (IU/l)	CK18-M65 (U/l)	CK18-M30 (U/l)
	Mean $\pm$ SEM			
Steatosis	31.9 $\pm$ 5.9	30.6 $\pm$ 11.6	329.3 $\pm$ 96.3	193.9 $\pm$ 98
Steatohepatitis	133.4 $\pm$ 68.1	103.2 $\pm$ 48.2	718.8 $\pm$ 233	636.7 $\pm$ 408.5
Significant fibrosis	136.2 $\pm$ 89.6	89.8 $\pm$ 65.3	827.5 $\pm$ 575	813.4 $\pm$ 737.4

Liver gene expression of IL1 β increased significantly in patients with steatohepatitis and significant fibrosis compared to steatosis. IL8 raised progressively in the different stages of ALD (Fig.5A). Moreover, IL8 positively correlated with AST, CK18-M65 and CK18-M30 while IL1 β correlated with ALT levels and CK18-M30 (supplementary table 2), indicating a role of IL1 β and IL8 in ALD progression. No differences were found for TNF- α (Supplementary Fig.6). We next evaluated liver chemokines involved in recruitment of monocytes, such as monocyte chemoattractant protein 1 (MCP1, also known as CCL2) and CX3CL1. Both chemokines were up-regulated especially in AUD subjects with fibrosis (Fig.5B). We then assessed whether they correlated with hepatic IL1 β and IL8 and found a positive correlation between MCP1, but not CX3CL1, with both inflammatory mediators ($r=0.5253$ and $r=0.7156$, $p < 0.0001$, for IL1 β and IL8, respectively).

We finally assessed markers of macrophage activation such as iNOS and CD163. Liver gene expression of iNOS increased significantly in patients with steatohepatitis and especially in patients with significant fibrosis compared with steatosis (Fig.5C). Immunofluorescence showed that iNOS is principally found in CD68+ macrophages (Fig.5D). Similar results were observed for CD163+ macrophages (supplementary Fig.7A, B). We then evaluated the potential relationship between macrophage activation markers (e.g. iNOS and CD163) and chemoattraction. Both chemokines (MCP1 and CX3CL1) positively correlated with iNOS ($r=0.4460$, $p=0.0009$; $r=0.4895$, $p = 0.0002$, for MCP1 and CX3CL1, respectively) but not with CD163 (data not shown). Remarkably, staining in liver biopsies from AUD patients showed presence of two different subpopulations of CD68+ macrophages: one expressing the activation marker Triggering receptor expressed on myeloid cells 1 (TREM1) and one expressing CD9 (Fig.6), a marker of activated monocytes primed to infiltration into the

tissues²⁴. These results indicate that infiltration of different subpopulations of macrophages possibly coming from the circulation upon activation by microbial antigens.

Overall, these observations suggest that hepatic recruitment of monocytes and transformation into pro-inflammatory macrophages producing IL1 β and IL8 in response to TLR2 ligand Peptidoglycan could contribute to ALD progression.

Figure 5

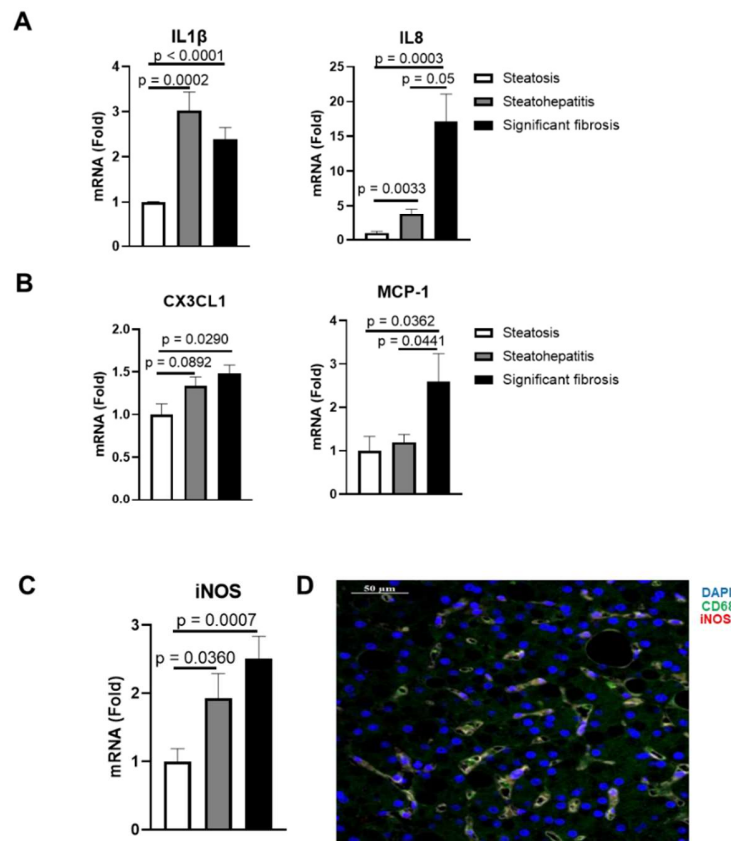


Fig.5 Evaluation of inflammatory markers related to monocyte-derived macrophages in livers of alcohol use disorder (AUD) subjects according to liver disease severity.

Hepatic gene expression of IL1 β increased significantly in AUD patients with steatohepatitis (n=18) and Fibrosis (n=29) compared of steatosis (n=3) while IL8 was progressively up-regulated according to liver disease severity (A). Hepatic expression of chemokines involved in monocyte's chemoattraction (CX3CL1 and MCP-1) increased significantly especially in AUD patients with fibrosis (B). Activation marker iNOS increased in AUD subjects with Steato-hepatitis (n=18) and Fibrosis(n=29) compared to steatosis (n=3) (C). Representative immunofluorescence staining in liver biopsies of CD68+ macrophages expressing iNOS and TREM1 activation markers (D).

Figure 6

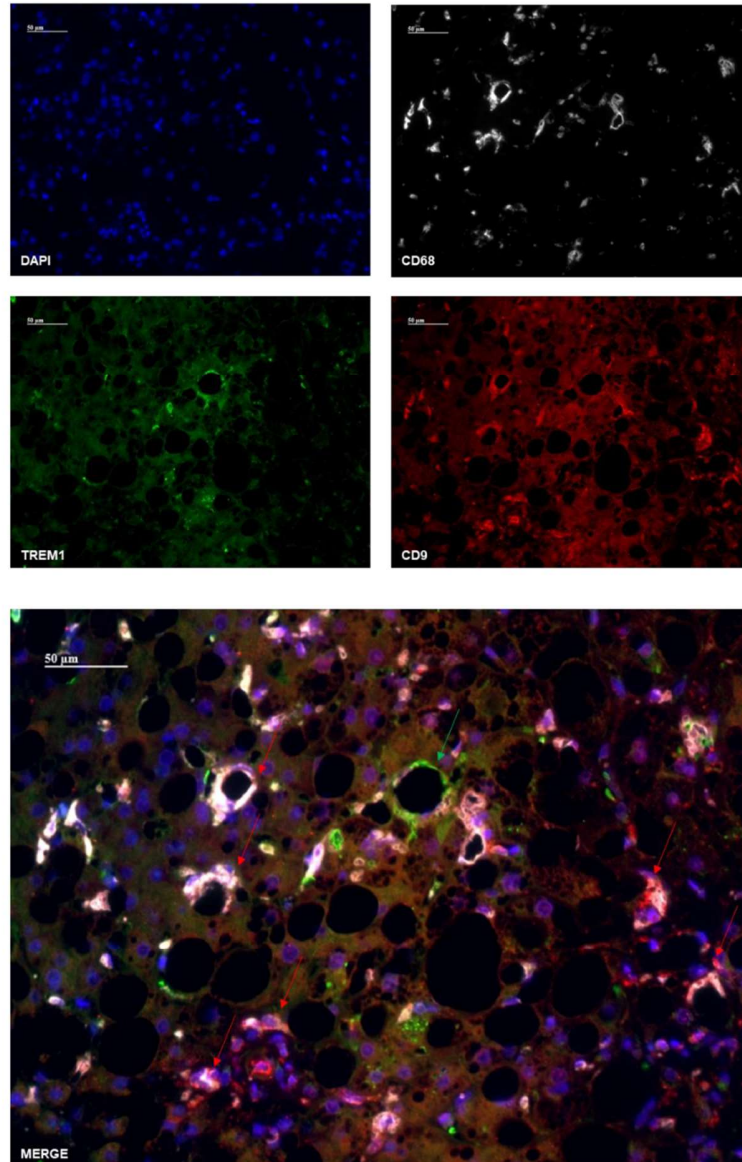


Fig.6 Representative immunofluorescence staining in liver biopsies of CD68+ macrophages expressing CD9 or TREM1 activation markers. Macrophages expressing CD9 are indicated with red arrows while macrophages expressing TREM1 are indicated with green arrows.

DISCUSSION

Our study demonstrates a higher activation of the TLR2/Inflammasome/AP-1 pathway in response to peptidoglycan in circulating monocytes that release key inflammatory mediators such as IL1 β and IL8 contributing to systemic inflammation. Furthermore, those activated monocytes retain the capacity to infiltrate the liver and transform into monocyte-derived macrophages that likely contribute to an inflammatory response already at early stages of ALD which might ultimately lead to progression of ALD.

We show an increased number of circulating monocytes in AUD patients compared with healthy subjects. Under pathological conditions, it has been proposed that the bone marrow stem cells could produce myeloid cells to counteract the increased translocation of microbial products and other inflammatory stimuli²⁵. The increased production of monocytes may be caused by different cytokines and growth factors as well as by alcohol-associated endotoxemia. Activation of pattern-recognition receptors (PRRs), like Toll-like receptors, in myeloid progenitors by bacterial antigens could induce signaling pathways involved in cell proliferation, differentiation and migration to enhance the immune response against different insults²⁶. We found higher activation of TLR2 in response to peptidoglycan in monocytes and monocyte-derived macrophages linked to enhanced secretion of IL1 β and IL8. This cascade might also be activated in progenitor cells and then lead to enhanced production of monocytes with their subsequent release into the blood circulation. Classical monocytes are the first subset released into the blood circulation from the bone marrow and a small proportion of them further mature into intermediate monocytes, which finally convert to non-classical monocytes before homing into the tissues²⁷. We found a reduced proportion of classical monocytes accompanied by increased proportion of both intermediate and non-classical monocytes in actively drinking AUD patients. These results suggest an increased transition of classical to intermediate and non-classical monocytes. The expansion of intermediate and/or non-classical subsets has been well described in many different types of infectious and inflammatory diseases where they were associated with an increased release of pro-inflammatory cytokines²⁸. The mechanisms of this process are still not known but appear to be related to microbial stimuli and/or inflammatory conditions, which characterize also subjects with an alcohol use disorder.

Serum surrogate markers of microbial translocation such as PGRPs and sCD14 were elevated in AUD patients. Levels of Gram+ translocation marker PGRPs were correlated with proportion of intermediate monocytes. Those monocytes express high levels of MHC class II processing and presentation genes²⁹, this may reflect a link between translocation of bacterial products such as PGN, recognition and presentation of antigens to T cells with subsequent activation of adaptive immune responses. Phagocytosis represents a defense mechanism used by innate cells like monocytes to deal with bacterial translocation³⁰. Interestingly, bacterial phagocytic capacity was maintained in culture in monocytes from AUD patients compared to monocytes from healthy controls, indicating no defects in bacterial phagocytosis in monocytes at early ALD stages. Chronic alcohol consumption is also associated with gut fungal overgrowth with overrepresentation of *Candida albicans*⁴. The human intestine can become the source for systemic fungal products or fungal infection when the gut barrier is disrupted³¹. We here revealed an intriguing down-regulation of CARD9, an intracellular sensor playing a pivotal role in fungal immune surveillance. In accordance with this result, stimulation of monocytes with *Candida albicans* showed a reduced transcription of inflammatory mediators IL1 β and TNF- α in monocytes from AUD patients compared to controls.

During the different stages of alcoholic liver disease, monocytes influx the liver where they transform into monocyte-derived macrophages that are likely contributing to the progression of ALD, as shown in animal models of chronic alcohol exposure¹⁰. We found that expression

of hepatic inflammatory mediators, such as the pro-inflammatory cytokine IL1 β and especially the chemokine IL8, increased progressively in more severe forms of liver disease as compared to simple fatty liver. Moreover, both factors strongly correlated with markers of hepatocyte damage and apoptosis, pointing to their contribution in the early development of alcohol-associated liver disease in humans. Recruitment of monocytes appears to play an important role, since both chemokines MCP1 and CX3CL1 correlated with IL1 β and IL8 and associate with a more advanced disease state such as presence of significant fibrosis. Markers of macrophages activation (e.g., iNOS and CD163) increased in livers with steatohepatitis and fibrosis compared to simple hepatic steatosis, further supporting the concept of the pivotal role of macrophages activation in ALD. Increased release of IL1 β and IL8 possibly by monocyte-derived macrophages was triggered by TLR2 in response to PGN, similar to their circulating counterparts. This result points to the importance of TLR2 activation in macrophages in the early development of human alcoholic liver disease. It has been believed for many years that activation of TLR4 signaling is crucial in the pathogenesis of alcohol-induced liver injury³². Deficiency in TLR2 had no protective effect on alcohol-induced liver injury in a chronic ethanol-feeding mouse model³³. However, differences in the characteristics of murine and human circulating monocytes/tissue macrophages exist and this may influence the pathophysiological divergences of alcohol-associated liver disease present in mice and humans.

Here we investigated the potential contribution of monocyte-derived macrophages in ALD. However, liver macrophages are a highly heterogeneous population composed by monocyte-derived and resident macrophages (e.g. Kupffer cells)³⁴. Both cell types exhibit a tremendous plasticity, adapting their function in response to tissue-specific signals within their microenvironment³⁵. In disease states where resident macrophages are depleted or reduced by injury, infiltrating monocytes can transform into macrophages with similar characteristics of Kupffer cells³⁶. We identified two subpopulations of macrophages expressing either TREM1 or CD9. Especially CD9 is not expected to be expressed by liver resident macrophages³⁷. Therefore, it is conceivable that these CD9+ damage-associated macrophages are derived from circulating monocytes. Future studies combining flow cytometry analysis with single-cell approaches are needed to reveal specific inflammatory subsets contributing to ALD pathology.

A limitation of this study is that data are in part descriptive and/or based on correlations. Furthermore, since suspicion of significant liver disease was a prerequisite for liver biopsy, more severe stages might be overrepresented in the histology cohort. However, given the difficulties of accessing tissue from early-disease stage in humans, we believe our investigation contributes to a better comprehension of the role of circulating monocytes in the pathogenesis of ALD. The main strength of our study is the unique human cohort of a high number of heavily, actively drinking AUD subjects undergoing elective alcohol withdrawal in whom liver biopsies and blood collections have been performed in a strict, highly standardized clinical program.

In summary, the current study highlights the contribution of TLR2 activation in monocytes and monocyte-derived macrophages in the development of systemic inflammation and ALD progression. A deeper understanding of the connections between this pathway and other inflammatory mediators involved in human alcohol-associated liver disease progression would provide us with the identification of innovative therapeutic targets in early ALD stages.

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Supporting material/data

TLR 2 activation in monocytes contributes to systemic inflammation and alcohol-associated liver disease in humans

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Supplementary material

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- Methods
 - *Fibroscan® measurements*
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 - *Immunohistochemistry*
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Patients, methods

Inclusion-exclusion criteria

Additional inclusion criteria were first admission to the alcohol withdrawal unit and absence of previously diagnosed significant alcoholic liver disease.

Patients with diabetes were eligible for the study if they were on stable treatment (metformin, oral antidiabetics with or without insulin) and had a glycosylated hemoglobin level <7% at admission.

Patients were excluded from the analysis if they had one of the following conditions:

- Inflammatory bowel disease or other chronic inflammatory or autoimmune diseases
- History of cancer within 5 years prior to admission
- Known liver disease of any other etiology
- Clinically significant cardiovascular, pulmonary, or renal comorbidities
- Clinical and biochemical signs of active infection ($t > 37.5^{\circ}\text{C}$, viral syndrome, high C-reactive protein [CRP], and high white blood cell count)
- Administration of antibiotics, probiotics, glucocorticoids, and nonsteroidal anti-inflammatory drugs during the 2 months preceding enrollment

FibroScan measurements

Transient elastography measurements were performed using the FibroScan device (Echosens, Paris, France) by an experienced examiner blinded to the patient's data. FibroScan was considered as valid if at least 10 validated measurements were obtained with an interquartile range of <30%. The final result expressed in kPa was the median of all valid measurements obtained. We used the cutoffs for patients with alcoholism proposed by Nguyen-Khac et al. (Aliment Pharmacol Ther 2008; 28:1188-1198), i.e., 7.8, 11, and 19.5 kPa for F2, F3, and F4, respectively.

Transjugular liver biopsy and tissue sampling

Liver biopsy was performed using a transjugular liver access and biopsy needle (16G) set (Cook, Bjæverskov, Denmark). After administration of local anaesthesia to the neck, a catheter was introduced into the right jugular vein and guided under fluoroscopy into the right main hepatic vein. Separate tissue samples were taken and routinely fixed in formalin, snap-frozen in liquid nitrogen, and preserved in RNAlater solution (Invitrogen, Carlsbad, CA). After a complete routine histopathological examination of the biopsy samples by an experienced liver pathologist, surplus tissue was recovered for further research purposes.

Biopsy samples of ≥ 15 mm in length, including a minimum of 6 portal tracts, were considered suitable or when the pathologist could establish the degree of fibrosis with certainty on a smaller sample.

Immunofluorescence

Five-micrometer sections were prepared from formalin-fixes, paraffin-embedded livers rehydrated, blocked with phosphate buffer saline (PBS) 1x, 10% bovine serum albumin (BSA), and 3% milk. Sections were stained with the primary antibodies (Mouse monoclonal anti-CD68, Clone PG-M1) 1/100; Polyclonal antibody anti-iNOS (PA3-030A, Thermofisher, Merelbeke, Belgium) 1/200; Polyclonal antibody anti-TREM1 (MBS2016030, MyBioSource) 1/100; Monoclonal antibody anti-CD9 (ab236630, Abcam) 1/100; overnight at 4 °C followed by incubation with a secondary antibody conjugated with different fluorochromes at 1/1000. Before imaging, nuclei were counterstained with 4', 6-diamidin-2-phenylindole (DAPI). Fluorescence multiplex immunostained slides were digitalized using a Panoramic 250 FlashIII scanner (3DHitech, Budapest, Hungary).

Immunohistochemistry

Five-micrometer sections were prepared from formalin-fixed, paraffin-embedded livers and stained for conventional histology or stained overnight at 4°C with antibody against CD163 and digitalized using a SCN400 slide scanner (Leica Biosystems, Wetzlar, Germany) at X20 magnification. Scanned slides were subjected to quantification analysis with the image analysis tool Author version 2017.2 (Visiopharm, Hørsholm, Denmark). Stained pixels were detected at high resolution (x20) using a thresholding classification method. Thresholds were adjusted on representative stained vs not stained regions. The same parameters were kept constant for all slides. Results were expressed as the mean stained area in percentage normalized to the total surface area of the section.

Reverse-Transcription and quantitative Real-Time Polymerase Chain Reaction

Primers were as follows: IL1 β : forward (F)-5'- GCACGATGCACCTGTACGAT-3' and reverse (R)-5'- AGACATCACCAAGCTTTTTTGCT-3'; IL8: (F)-5'- CTAGGACAAGAGCCAGGAAGAAAC-3' and (R)-5'- CGGCCAGCTTGGAAGTCA-3'; IL6: (F)-5'- TGGCTGAAAAAGATGGATGC-3' and (R)-5'- TGCAGGAAGTGGATCAGGAC-3'; IL18: (F)-5'- CCAAGGAAATCGGCCTCTATT-3'; (R)-5'- CTTACACAGAGATAGTTACAGCCATACCT-3'; TNF- α : (F)-5'- ATCTTCTCGAACCCCGAGTGA-3'; (R)-5'- CGGTTTCAGCCACTGGAGCT-3'; TLR2: (F)-5'- TCCTGTGCCACC GTTTCC-3'; (R)-5'- GA GCTTTCCTGG GCTTCCTT-3'; TLR4: (F)-5'- GATTGCTCAGACCT GGCAGTT-3'; (R)-5'- TGTCTCCC ACTCCAGGTAAGT-3'; NLRP3: (F)-5'- TGCCCCGACCCAAACC-3'; (R)-5'- GAAGCCGTCATGAGGAAGA-3'; NOD2: (F)-5'- GCCACGGTGAAAGCGAAT-3'; (R)-5'- GGAAGCGAGACTGAGCAGACA-3'; MyD88: (F)-5'- CATATGCCTGAGCGTTTCGA-3'; (R)-5'- CATCGCGGTCAGACACACA-3'; MCP1: (F)-5'- GCTCAGCCAGATGCAATCAA-3'; (R)-5'- GCCTCTGCACTGAGATCTTCCT-3'; iNOS: (F)-5'- GGTGGAAGCGGTAAACAAAGG-3'; (R)-5'- TGCTTGGTGGCGAAGATGA-3'; IkB: (F)-5'- GCTGAAGAAGGAGCGGCTACT-3'; (R)-5'- TCGTACTCCTCGTCTTTCATGGA-3'; CX3CL1: (F)-5'- ACCACGGTGTGACGAAATG-3'; (R)-5'- TGTGATAGTGGATGAGCAAAGC-3'; Rpl-19: (F)-5'-CAAGCGGATTCTCATGGAACA-3'; (R)-5'-TGGTCAGCCAGGAGCTTC T-3'.

Antibodies used for flow cytometry

Name	Company	Reference
CD14-FITC, human	Miltenyi Biotec	130-110-518
CD16-APC, human	Miltenyi Biotec	130-113-389
CD192 (CCR2)-PE-Vio770, human	Miltenyi Biotec	130-109-597
Anti CX3CR1-PE, human	Miltenyi Biotec	130-096-432
HLA-DR-VioGreen(TM), human	Miltenyi Biotec	130-111-795

Phagocytosis assay

***E. coli* strain (GL1435):**

Genotype: TOP10/pSEVA251-pBioFab-dtomato

The red fluorescent protein dTomato is constitutively expressed from the synthetic pBioFab promoter on the pSEVA251 plasmid carrying a kanamycin resistance cassette.

Preparation of the bacterial suspensions:

A colony of GL1435 isolated on LB (Lysogeny Broth) agar supplemented with 50 µg/ml kanamycin was used to inoculate 5 ml of LB medium + 50 µg/ml kanamycin. This starter culture was grown overnight with shaking at 37°C before 1:200 dilution in fresh LB + 50 µg/ml kanamycin. Cells were grown at 37°C and harvested by centrifugation when OD600 reached ~ 0.4 (exponential phase). All subsequent steps were performed on ice. Cells were washed first with ice-cold RPMI and then with either RPMI or RPMI + 1% P/S, before final resuspension in RPMI or RPMI + 1% P/S. All centrifugation steps were performed at 3000 g for 8 min at 4°C. Serial dilutions of the RPMI and RPMI + 1% P/S bacterial suspensions were plated on LB + kanamycin plates for CFU (colony forming units) counting. For each condition, one aliquot was stored at 4°C and another aliquot was stored at -20°C. The next day, CFU were counted and red fluorescence from dTomato was verified by epifluorescence microscopy on a Nikon Ti2 equipped with a 100X phase contrast objective (Nikon Plan Apochromat Ph3 DM NA=1.45), an mCherry filter cube and a Prime 95b 25mm camera (Photometrics).

Supplementary table 1. Correlations between monocytes subsets and serum microbial translocation levels

	PGRPs		sCD14	
	r	p	r	p
Monocytes number	0.3107	0.1147	-0.0079	0.9687
Classical	-0.1979	0.2618	-0.0795	0.6550
Intermediate	0.4005	0.0189	0.0206	0.9078
Non-classical	0.0564	0.7515	0.0239	0.8929

Supplementary table 2. Correlations between plasma cytokines and serum microbial translocation marker sCD14

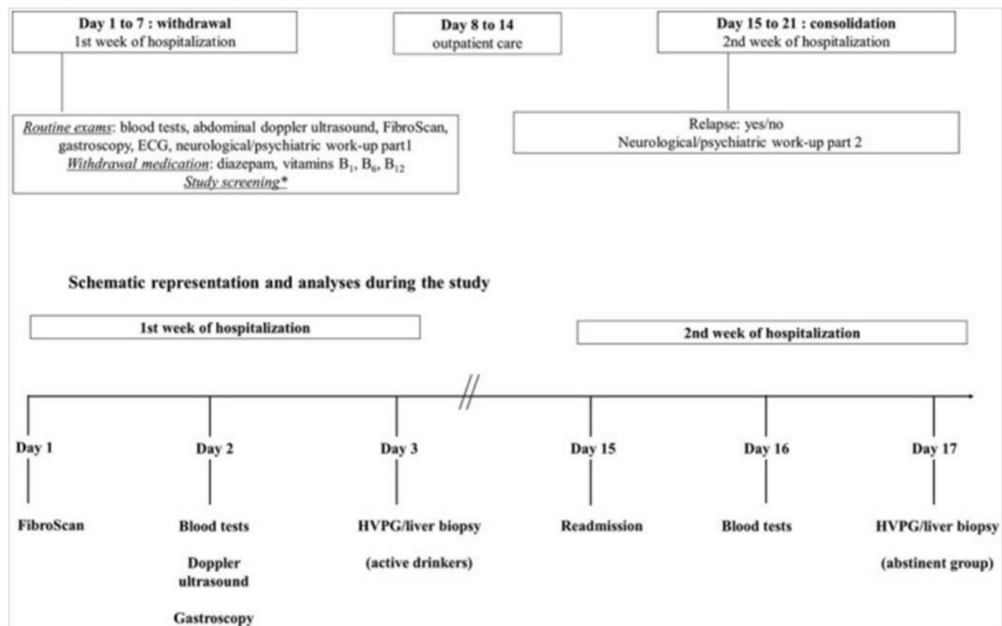
Cytokine	r	p
IL8	0.3605	0.0284
IL6	0.3521	0.0326
TNF-α	0.3530	0.0321

*Plasma levels of IL1β were not detectable with the ELISA kit used to measure IL1β in the medium

Supplementary table 3. Correlations between intrahepatic IL1β and IL8 and liver cell damage/apoptosis markers

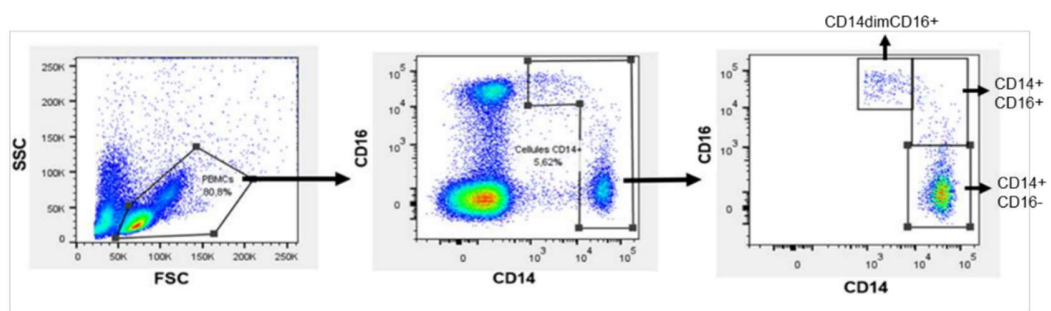
Liver cytokine	AST (IU/l)	ALT (IU/l)	CK18-M65 (U/l)	CK18-M30 (U/l)
IL1β	r=0.1619, p=0.4295	r=0.4096, p=0.0377	r=0.2570, p=0.1956	r=0.3978, p=0.0399
IL8	r=0.5205, p=0.0064	r=0.2794, p=0.1669	r=0.3896, p=0.0446	r=0.4430, p=0.0207

Supplementary figures



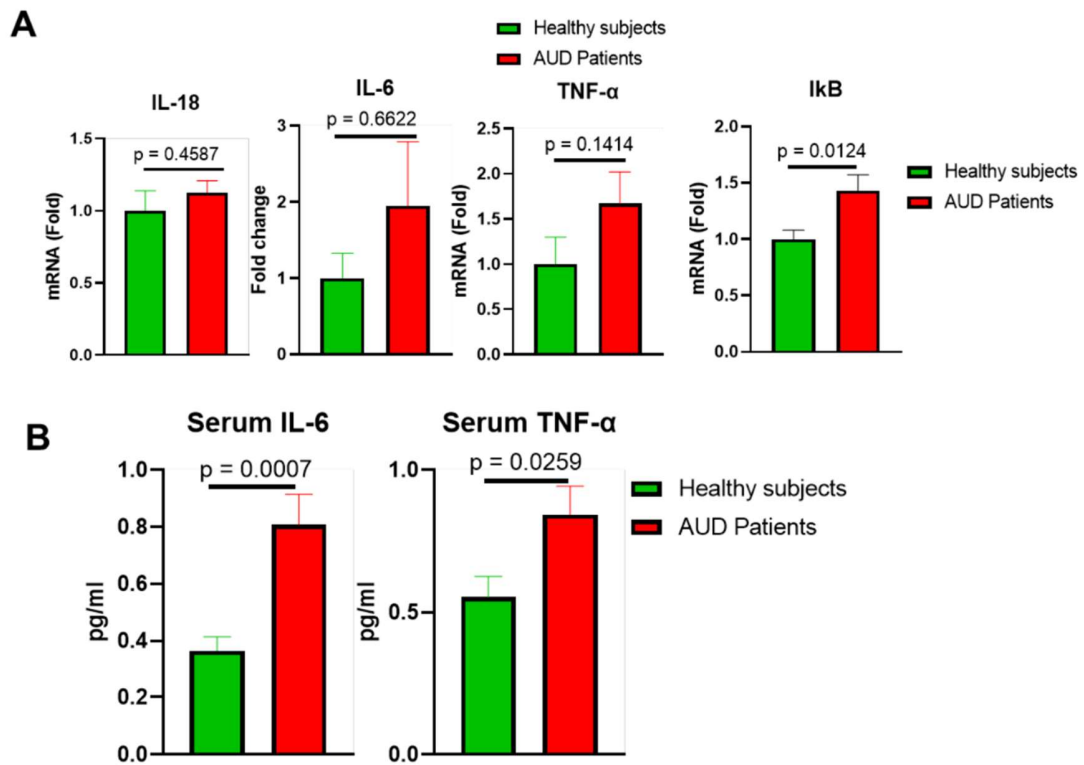
Supplementary Figure 1. Standardized working scheme of the alcohol withdrawal unit.

Alcohol use disorder (AUD) patients are undergoing a highly standardized clinical work-up with fixed sampling of biological specimens.



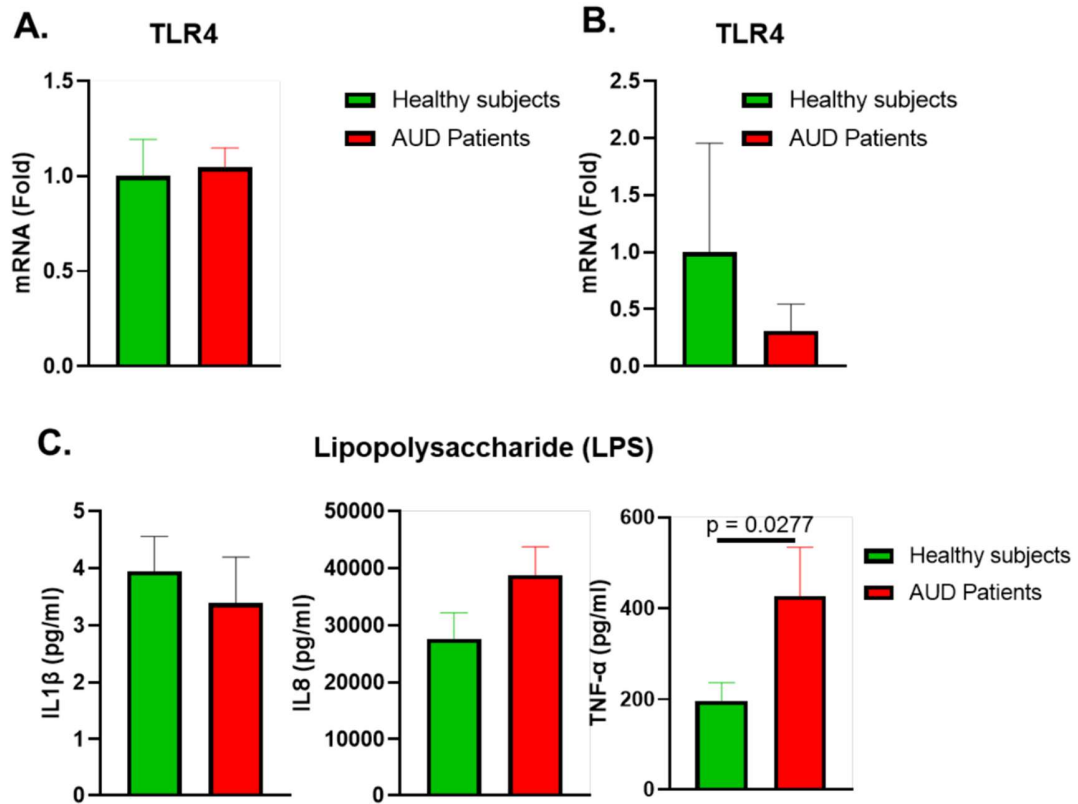
Supplementary Figure 2. Evaluation of monocytes proportion in alcohol use disorder (AUD) patients and healthy controls

Gating strategy used to assess the three subpopulations of monocytes: CD14+CD16- classical monocytes, CD14+CD16+ intermediate monocytes and CD14dimCD16+ non-classical monocytes



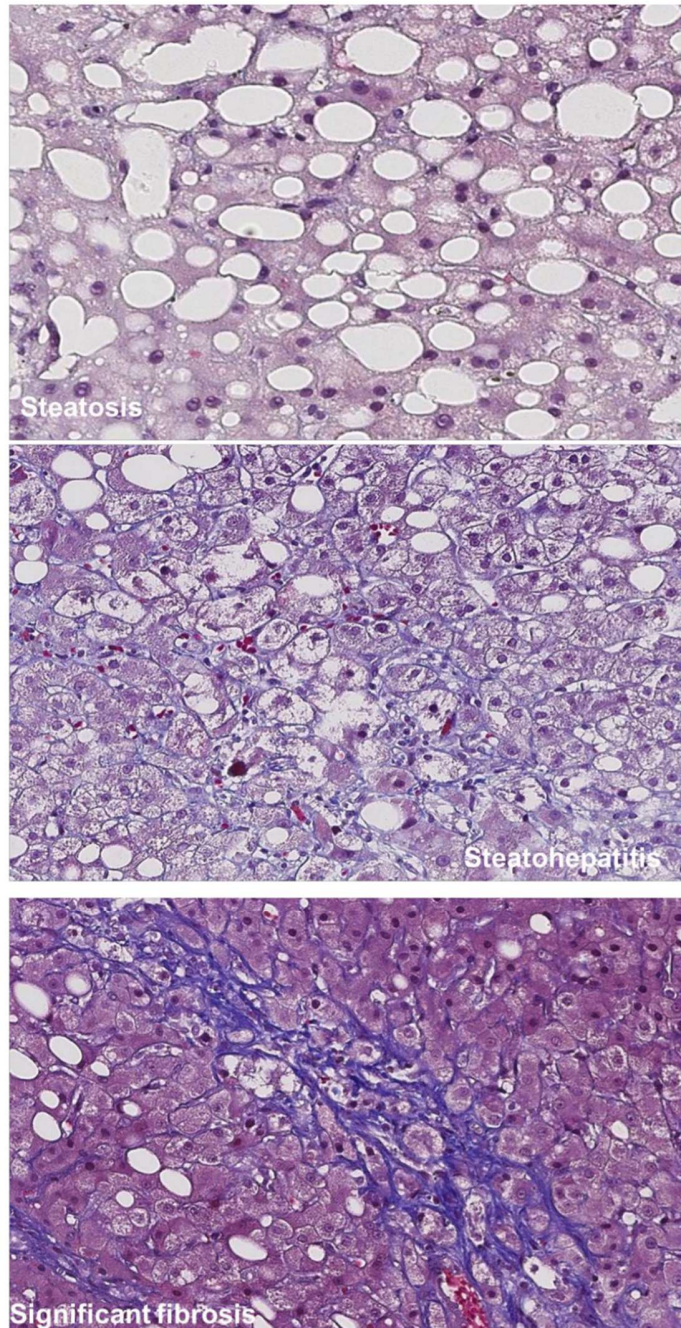
Supplementary Figure 3. Evaluation of inflammatory genes in alcohol use disorder (AUD) patients.

Monocytes from AUD patients have similar gene expression of IL18, IL6 and TNF- α (A) compared to healthy volunteers while I κ B, inhibitor of transcription factor NF κ B, was up-regulated in monocytes of AUD patients (n=24) compared to controls (n=8). (B) Serum levels of IL6 and TNF- α increased significantly ($p < 0.05$) in AUD patients (n=24) compared to controls (n=8).

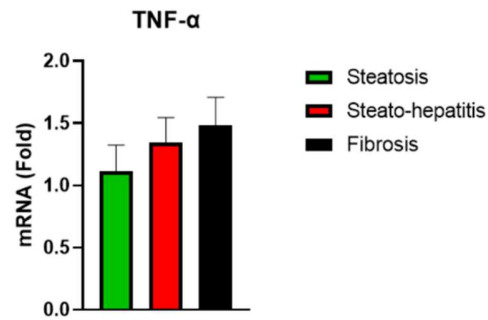


Supplementary Figure 4. Evaluation of Toll-like receptor activation in monocytes from patients with an alcohol use disorder (AUD).

(A.) Monocytes from AUD patients (n=24) have similar gene expression of TLR4 compared to healthy volunteers (n=8) at baseline which did not increase after stimulation in vitro with LPS (B). Lipopolysaccharide (LPS) stimulation lead to increased release of TNF-α in monocytes from AUD patients compared to healthy subjects while IL1β and IL8 did not change significantly (C).

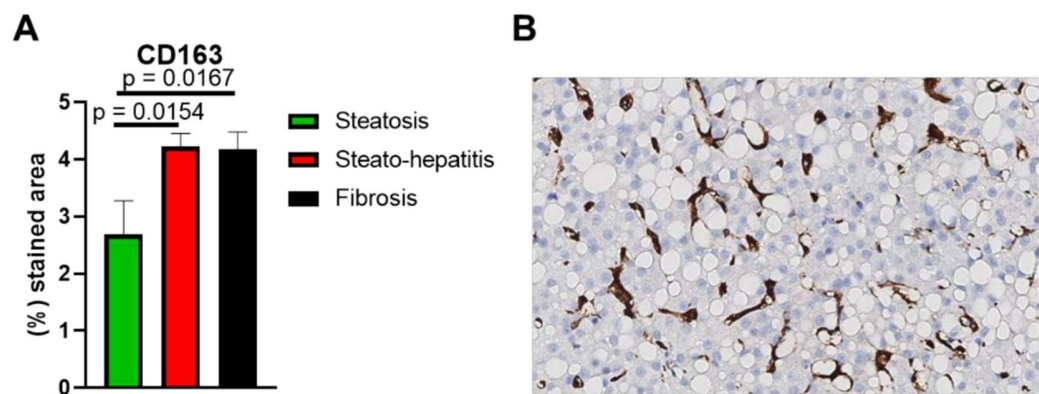


Supplementary Figure 5 Representative Masson's trichrome staining of AUD patients with steatosis (upper panel), steato-hepatitis (middle panel) and fibrosis (lower panel)



Supplementary Figure 6. Evaluation of inflammatory cytokines in livers of alcohol use disorder (AUD) at different disease stage.

Gene expression of TNF- α did not differ between the three groups.



Supplementary Figure 7. Evaluation of macrophages activation markers in livers of alcohol use disorder (AUD) patients at different disease stage.

CD163 (A) increased in AUD subjects with Steato-hepatitis (n=18) and Fibrosis(n=29) compared to steatosis (n=3). (B) Representative immunostaining of CD163 in liver biopsies from AUD patients.

Complementary discussion and conclusion

In this chapter we have demonstrated the role of TLR2 activation in circulating monocytes with subsequent release of IL1 β and IL8 in the development of systemic inflammation and ALD progression. Regarding liver disease, these two inflammatory mediators could be implicated in different mechanisms related to early ALD pathology. The inflammatory cytokine IL1 β is a well-known actor in liver cell damage, and it can be produced by resident macrophages (e.g., Kupffer cells) as well as by monocyte-derived macrophages. By contrast, increased liver gene expression of IL8 in relation to ALD severity could be explained by increased production of both macrophages and hepatocytes because hepatocytes are important producers of this important inflammatory mediator^{169,170}. We also showed in this study up-regulation of chemokines involved in monocytes recruitment as well as presence of macrophages expressing markers of monocyte-derived macrophages. However, due to the high plasticity that characterize both Kupffer cells and monocyte-derived macrophages, we cannot formally exclude that some of the macrophages we marked by immunofluorescence and immunohistochemistry in liver biopsies are resident populations.

In conclusion, we have revealed in chapter 4 a higher activation of the TLR2/Inflammasome/AP-1 pathway in response to peptidoglycan in circulating monocytes that release key inflammatory mediators such as IL1 β and IL8 contributing to systemic inflammation in AUD patients. Furthermore, those activated monocytes likely infiltrate the liver and transform into monocyte-derived macrophages activating an inflammatory response already at early stages of ALD which might ultimately lead to progression of ALD (Fig.4.1).

The perspective of chapter 4 is the following:

- Evaluation of other liver inflammatory responses in early ALD

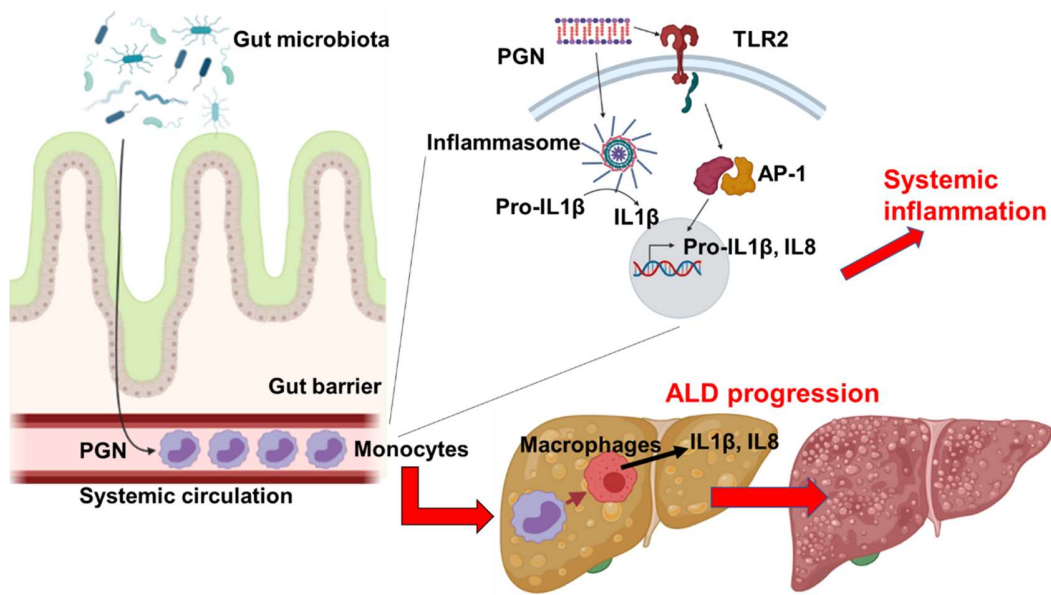


Fig.4.1 Schematic representation of the results obtained in the study described in chapter 4. We point to a contribution of activated monocytes to systemic and liver inflammation. Monocytes likely infiltrate the liver, transform into monocyte-derived macrophages and release IL1 β and IL8 in response to Peptidoglycan and TLR2 activation, ultimately leading to ALD progression.

CHAPTER 5: Deficient IL-6/Stat3 signaling, high TLR7, and Type I Interferons in early human alcoholic liver disease: a triad for liver damage and fibrosis

Alcohol-associated hepatic damage, impairment of liver regeneration and activation of liver immune response by gut-derived products have been suggested to play crucial roles in the development of progressive forms of ALD. However, to date, the relationship between these processes and the underlying mechanisms still remain incompletely understood. In the fifth chapter of this thesis, we evaluated the connection between progression to steatohepatitis and/or fibrosis and alcohol-related hepatic inflammatory responses. The principal aim of this study was to assess liver inflammatory and immune responses at early stages of ALD and to correlate them with liver damage and repair mechanisms and/or progression of fibrosis. We finally evaluated whether a short period of abstinence could reverse those early alcohol-associated changes in the liver.

We found that a proinflammatory response, including activation of TNF- α , IL-1 β , and NF- κ B, driven by liver resident macrophages, already occurred at very early stages of the disease. The early proinflammatory response was alleviated by short-term abstinence. By contrast, the **IL-6/Stat3 axis was blunted and Stat3 sequestered in the cytoplasm of hepatocytes in livers with increased apoptosis**. On the other hand, **livers that were able to overcome alcohol-associated Stat3 blockage, as indicated by preserved nuclear translocation of Stat3, retained some proliferative capacity**. TLR7 up-regulation occurred concomitantly with activation of interferon signaling predominantly in hepatocytes. **Activation of the TLR7–IFN axis strongly correlated with liver fibrosis markers and disease progression**.

The results of this sixth chapter have been published in *Hepatology communications* 2019, Volume 3, N. 7

Deficient IL-6/Stat3 Signaling, High TLR7, and Type I Interferons in Early Human Alcoholic Liver Disease: A Triad for Liver Damage and Fibrosis

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Mechanisms underlying alcohol-induced liver injury and its progression still remain incompletely understood. Animal models can only address some aspects of the pathophysiology that requires studies directly in humans, which are scarce. We assessed liver inflammatory and immune responses at early stages of alcoholic liver disease in a unique cohort of alcohol-dependent patients undergoing a highly standardized alcohol withdrawal program. In active drinkers, quantitative real-time polymerase chain reaction revealed alcohol-induced activation of tumor necrosis factor alpha, interleukin (IL)-1 β , and nuclear factor kappa B in liver tissue already at early disease stages. Double immunofluorescence staining indicated that this proinflammatory response was restricted to activated, CD68-positive macrophages. In parallel, down-regulation of IL-6, inhibition of the signal transducer and activator of transcription 3 (Stat3) pathway, as well as blunted cyclin D expression in hepatocytes, reduced proliferation and favored hepatocyte apoptosis. In addition, immunofluorescence and quantitative real-time polymerase chain reaction of liver tissue showed that alcohol also activated the toll-like receptor (TLR) 7–interferon (IFN) axis in hepatocytes, which was confirmed in alcohol-stimulated primary human hepatocytes and precision-cut liver slices *in vitro*. Activation of the TLR7–IFN axis strongly correlated with liver fibrosis markers and disease progression. Two weeks of abstinence attenuated the inflammatory response but did not allow recovery of the defective Stat3 pathway or effect on fibrosis-associated factors. **Conclusion:** In humans, inflammation, activation of the TLR7–IFN axis, and inhibition of Stat3-dependent repair mechanisms in early alcoholic liver disease pave the way for fibrosis development and ultimately disease progression. (*Hepatology Communications* 2019;3:867–882).

Alcohol is one of the leading causes of chronic liver disease and liver-related deaths worldwide.⁽¹⁾ The natural history of alcoholic liver disease (ALD) cannot be separated from the natural history of alcoholism and spans a wide spectrum of disease manifestations. Up to 90% of heavy drinkers develop steatosis, but only up to 40% of those progress to alcoholic steatohepatitis (ASH), and 10%–20%

Abbreviations: α -SMA, α -smooth muscle actin; ALD, alcoholic liver disease; ALT, alanine aminotransferase; ASH, alcoholic steatohepatitis; AST, aspartate aminotransferase; CK, cytokeratin; ELISA, enzyme-linked immunosorbent assay; HSC, hepatic stellate cell; IFN, interferon; IL, interleukin; IRF3, interferon regulatory factor 3; LBP, lipopolysaccharide-binding protein; LPS, lipopolysaccharide; MCP1, monocyte chemoattractant protein 1; mRNA, messenger RNA; NF- κ B, nuclear factor kappa B; PCLS, precision-cut liver slices; PCR, polymerase chain reaction; PHH, primary human hepatocyte; Stat3, signal transducer and activator of transcription 3; TLR, toll-like receptor; TNF, tumor necrosis factor.

Received November 12, 2018; accepted April 15, 2019.

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1364/supinfo.

Supported by Fond National de Recherche Scientifique Belgium (J.0146.17 and T.0217.18); and National Institute on Alcohol Abuse and Alcoholism (R01 AA024726-01).

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DOI 10.1002/hep4.1364

eventually develop cirrhosis.⁽²⁾ To date, the mechanisms that underlie alcohol-induced liver injury and progressive ALD still remain incompletely understood. Much of our current knowledge has been generated in animal models, which have intrinsic limitations that narrow extrapolation of data to humans. In contrast to humans, animals do not develop a true addiction,⁽³⁾ implying that alcohol administration to rodents requires artifices (e.g., specific diets, gavage, intragastric tubes) that do not mimic human drinking patterns. Ethanol metabolism and elimination is 5 times greater in mice than in humans, which precludes achieving high blood alcohol levels.⁽⁴⁾ Even more significantly, alcohol-induced organ damage in animals only partially reproduces the entire spectrum of the target organ damage observed in humans.^(5,6) Finally, differences exist between the murine and human immune systems as well as in the capacity of immune cells to respond to damage-associated molecular patterns, such as toll-like receptors (TLRs) that are expressed at the cell surface in macrophages (13 in rodents, 10 in humans).⁽⁷⁻⁹⁾ For these reasons and also because alcohol-induced inflammation, crucial in the onset and progression of ALD, implicates multiple hepatic cell types as well as innate and adaptive immune responses,^(10,11) studies directly conducted in humans are mandatory. However, human studies are scarce, and those available focus on severe alcoholic hepatitis, a condition for which liver biopsies and data are more readily available than for early ALD. Moreover, they require rigorously standardized conditions for patient selection and sampling to avoid as much bias as possible.

The aim of our study was to assess liver inflammatory and immune responses at early stages of ALD in humans and to correlate them with liver damage and repair mechanisms and/or progression of fibrosis. We also assessed whether a short period of abstinence could reverse those early alcohol-induced changes in the liver.

Materials and Methods

CLINICAL SETTING, PATIENTS, AND TISSUE AND BLOOD SAMPLING

Alcohol-dependent patients (Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition criteria) admitted to the alcohol withdrawal unit follow a highly standardized and controlled 3-week detoxification and rehabilitation program. They are taken care of by a multidisciplinary team consisting of a gastroenterologist, psychiatrist, psychologist, dietician, social assistant, and dedicated nursing team. At admission, a complete medication and medical history is taken, and a complete physical examination is performed, including collection of basic demographic data, such as age, gender, weight and height, and self-reported daily alcohol consumption. All patients reported long-term (>1 year) alcohol consumption (>60 g/day) and were actively drinking until the day of admission. They routinely and prospectively

Potential conflict of interest: Dr. Bataller is on the speakers' bureau for EchoSens. Dr. Lanthier advises Gilead and Promethera; he received grants from MSD and Abbvie and is on the speakers' bureau for Fresenius Kabi. Dr. Leciercq consults for Biocellvia and received grants from Genfit.

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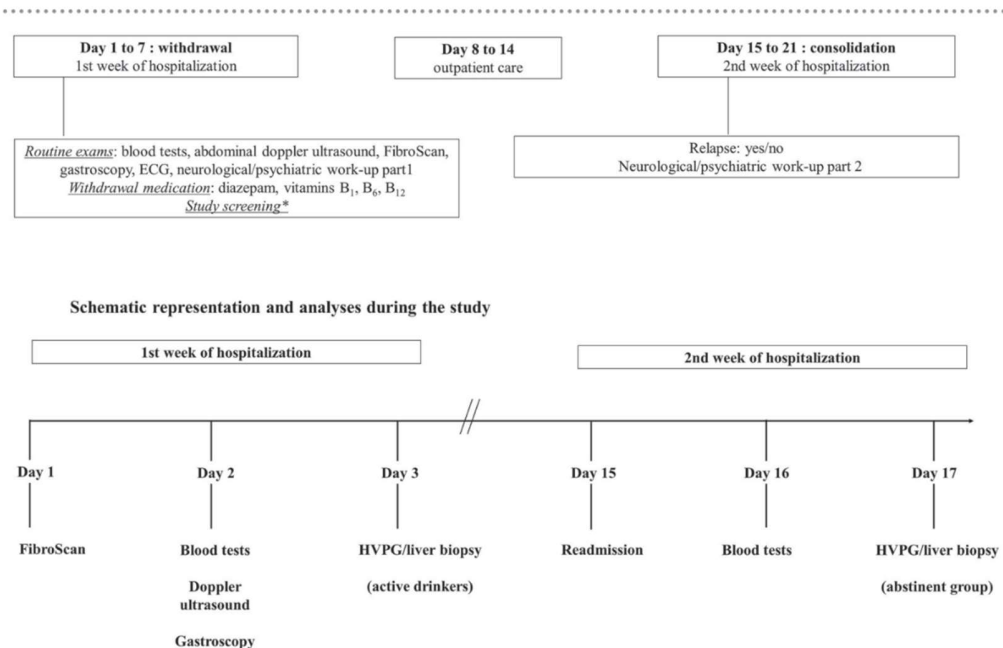


FIG. 1. Schematic representation of the standardized working scheme of patients admitted to the alcohol withdrawal unit for a 3-week detoxification program. Investigations and sampling are performed at predefined days as close as possible to the last drink (maximum 72-hour delay) in active drinkers and after at least 14 days of abstinence in the abstinence group.

underwent a large panel of investigations (Fig. 1). Transient elastography (FibroScan) was performed on the day of their admission. Fasting blood was drawn on the second day of admission during the first and second week of hospitalization and immediately centrifuged, and aliquots were stored at -80°C until use. A transjugular liver biopsy was routinely proposed to all patients with suspicion of significant liver disease (FibroScan $\geq$ F2 with increased aspartate aminotransferase [AST] and/or alanine aminotransferase [ALT]).

Patients who consented to a liver biopsy were eligible for the study and randomly assigned to undergo liver biopsy on the third day of admission (active drinking group) or during the second week of hospitalization after 14 days of abstinence (short-term abstinence group) (Fig. 1). Additional inclusion/exclusion criteria can be found in the Supporting Materials and Methods.

Histological normal liver samples and blood samples from healthy volunteers served as controls (details in the Supporting Materials and Methods).

ETHICAL ASPECTS

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institution's human research and ethical committee. Written informed consent was obtained from all patients.

REVERSE-TRANSCRIPTION AND QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

Tissue messenger RNA (mRNA) was assessed by quantitative real-time polymerase chain reaction (PCR) and quantified as described.⁽¹²⁾ Primers are depicted in Supporting Table S1.

RNA SEQUENCING

High-throughput transcriptome profiling by RNA sequencing was performed, followed by differential expression and functional enrichment analysis.

Transcriptomic footprint and canonical pathway analysis was applied to RNA sequencing data. Z score statistic was calculated (details in the Supporting Materials and Methods).

WESTERN BLOTTING

Western blot analysis was performed on whole cell extracts (Nuclear Extract Kit; Active Motif, La Hulpe, Belgium) according to standard electrophoresis, transfer, and detection techniques as described.⁽¹²⁾ Membranes were stripped (Fisher Scientific, Erembodegem, Belgium) and re-probed with several antibodies (Supporting Table S2).

QUANTIFICATION OF TRANSCRIPTION FACTOR ACTIVATION AND CASPASE 3 ACTIVITY

Signal transducer and activator of transcription 3 (Stat3) DNA-binding and caspase 3 activity were assessed in whole cell extracts using a TransAM detection kit (Active Motif) and Caspase-Glo-3/7 assay (Promega, Leiden, the Netherlands), respectively, according to the manufacturer's instructions.

HISTOLOGY, IMMUNOHISTOCHEMISTRY, AND IMMUNOFLUORESCENCE

Liver sections were stained with hematoxylin and eosin and Masson's trichrome blue (fibrosis) or incubated with primary and secondary antibodies and quantified by morphometric analysis (Supporting Table S2).

DETERMINATION OF BLOOD CYTOKINE LEVELS AND INFLAMMATORY MARKERS

Plasma cytokines, lipocalin 2, and serum amyloid A1 were assayed in duplicate with a multiplex immunoassay (Millipore, Molsheim, France) and Luminex xMap technology (Bio-Rad Laboratories, Hercules, CA) or enzyme-linked immunosorbent assay (ELISA) (Lipocalin-2/NGAL Human ELISA Kit EHLN2 and SAA Human ELISA Kit KHA0011; Invitrogen, Carlsbad, CA) following the manufacturer's instructions.

CELL CULTURE EXPERIMENTS

Human liver tissue for cell isolation was obtained from the charitable state-controlled foundation, Human Tissue and Cell Research, with informed patient consent and approved by the local ethics committee.

Isolation and culture of primary human hepatocytes (PHHs) and hepatic stellate cells (HSCs) were performed as described.⁽¹³⁾ In addition, we used the LX-2 human HSC cell line. Cells were incubated with serial alcohol concentrations for up to 24 hours.

PRECISION-CUT LIVER SLICES

Human liver tissue was obtained from patients who underwent partial hepatectomy for colorectal liver metastasis from the London Clinic (London, United Kingdom). The healthy portions of the liver specimen were harvested, and the preparation of precision-cut liver slices (PCLS) was performed as described.⁽¹⁴⁾ Each slice was maintained in culture for 24 hours or 72 hours with or without the addition of 100 mM or 250 mM ethanol (details in the Supporting Materials and Methods).

STATISTICS

Data are presented as mean $\pm$ standard error of the mean unless otherwise indicated. The Kolmogorov-Smirnov test was used to assess normal distribution of the data. Accordingly, the Student *t* test was performed for normally distributed data, and the Wilcoxon test for nonnormally distributed data. Pearson's or Spearman's correlation tests were used for correlations between data sets. A *P* value of less than 0.05 was considered as statistically significant.

Results

STUDY POPULATION

The study population consists of a typical cohort of 88 alcohol-dependent, middle-aged, predominantly male subjects. Demographic, biochemical, and histology data are depicted in Table 1. Most had high transaminases and gamma-glutamyltransferase levels. Two-thirds of the patients had early-stage ALD with a Metavir fibrosis score of $\leq$ F2 and various degrees of steatosis on

TABLE 1. BASELINE DEMOGRAPHIC AND BIOCHEMICAL DATA OF THE STUDY POPULATION

Demographics		Controls (n = 14)		Alcoholics (n = 88)	
Gender (female/male)		5 (35%)/9 (65%)		26 (29.5%)/62 (70.5%)	
		Mean ± Standard Deviation			
Age (years)		40 ± 11.7		49 ± 10.3	
Height (cm)		175 ± 8		171 ± 20	
Weight (kg)		70.3 ± 9.1		76.7 ± 16.4	
BMI		23 ± 2.9		26 ± 5.3	
Biochemistry		Mean ± Standard Deviation (normal range)			
AST (IU/L)		ND		116 ± 89 (<50)	
ALT (IU/L)		ND		80 ± 60 (<50)	
γ-GT (IU/L)		ND		476 ± 486 (<50)	
ALP (IU/L)		ND		108 ± 77 (30-120)	
Bilirubin (mg/dL)		ND		1.6 ± 2.5 (0.3-1.2)	
Albumin (g/dL)		ND		4.55 ± 3.55 (3.5-5.2)	
INR		ND		1 ± 0.2 (0.8-1.3)	
Alcoholics (n [%])					
Histology					
Fibrosis Metavir					
F0/F1		F2		F3	
42 (48%)		15 (17%)		16 (18%)	
F4				15 (17%)	
Sinusoidal Fibrosis					
None		Mild		Moderate	
30 (34%)		34 (39%)		17 (19%)	
Severe				7 (8%)	
Steatosis					
None		Mild (<33%)		Moderate (33%-66%)	
6 (7%)		24 (27%)		30 (34%)	
Severe (>66%)				28 (32%)	
Satellitosis					
Absent				Present	
71 (81%)				17 (19%)	
Diabetes (Type 2)					
Absent				Present	
77 (87.5%)				11 (12.5%)	

Abbreviations: γ-GT, gamma-glutamyltransferase; ALP, alkaline phosphatase; BMI, body mass index; INR, international normalized ratio; ND, not done.

histology. All patients with advanced fibrosis (≥F3) had a preserved synthetic liver function and showed no clinical signs of liver decompensation.

EARLY ACTIVATION OF PROINFLAMMATORY CYTOKINES AND NUCLEAR FACTOR KAPPA B IN MACROPHAGES/KUPFFER CELLS

Alcohol elicited a proinflammatory cytokine response in the liver of active drinkers, characterized

by increased tumor necrosis factor alpha (TNF-α) and interleukin (IL)-1β expression, down-regulation of IL-1 receptor antagonist expression, and activation of nuclear factor kappa B (NF-κB) (Fig. 2A-C). In addition, plasma levels of lipocalin-2 and serum amyloid A1, two inflammatory molecules associated with ALD,^(15,16) were elevated in active drinkers (Supporting Fig. S1). High plasma levels of macrophage inflammatory protein 1β, increased mRNA expression of the macrophage/Kupffer cell marker CD68 but not CD11b, and activated CD68-positive macrophages co-expressing NF-κB

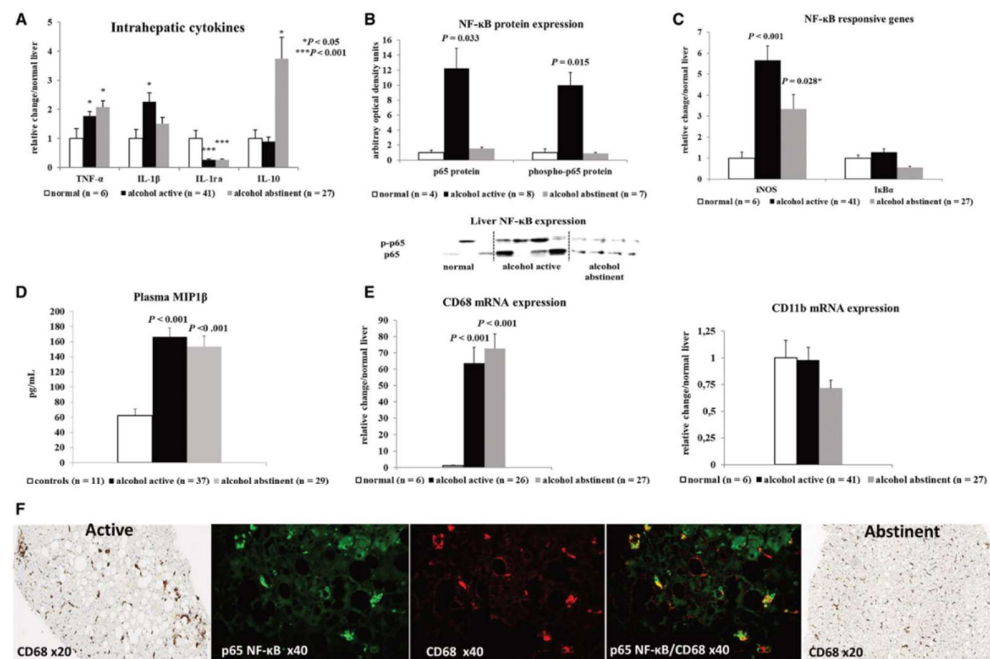


FIG. 2. Proinflammatory response in human liver tissue of actively drinking patients with alcoholism or after 14 days of abstinence. (A) Quantitative real-time PCR of principal pro-inflammatory and anti-inflammatory cytokines in liver biopsies. (B) Western blot of total and phosphorylated p65NF-κB expression in liver tissue. (C) Quantitative real-time PCR of NF-κB-regulated genes to confirm functional activation of NF-κB. (D) Plasma levels of macrophage inflammatory protein 1β. (E) Increased expression of CD68 but not of CD11b mRNA (representing infiltrating monocytes/macrophages) on quantitative real-time PCR. (F) Representative immunohistochemistry and double immunofluorescence staining of C68-positive macrophages and NF-κB. Activated macrophages in active drinkers form cell clusters around foci of hepatocyte steatonecrosis that co-localize with NF-κB. Normalization of the staining pattern of C68-positive macrophages after 14 days of abstinence. Indicated *P* values refer to normal liver or controls. Abbreviations: IkBα, nuclear factor kappa B inhibitor alpha; IL-1ra, interleukin-1 receptor antagonist; iNOS, inducible nitric oxide synthase; MIP1β, macrophage inflammatory protein 1β.

were found (Fig. 2D-F). These macrophages formed clusters around foci of steatonecrosis. Histological assessment and myeloperoxidase staining of liver sections showed only low numbers of neutrophils in early ASH (Supporting Fig. S1). These data suggest that macrophages are important for driving the proinflammatory response and likely correspond to Kupffer cells (i.e., the liver-resident macrophages).

Short-term abstinence attenuated the proinflammatory response together with up-regulation of the anti-inflammatory cytokine IL-10 (Fig. 2A-C). This response was accompanied by a normalization of the

CD68 staining pattern, suggesting reduced Kupffer cell activation (Fig. 2F).

INHIBITION OF STAT3 SIGNALING IN HEPATOCYTES IS ASSOCIATED WITH LOW PROLIFERATION AND HIGH APOPTOSIS AT EARLY STAGES OF ALD

Surprisingly, liver mRNA expression of the Stat3-induced proinflammatory IL-6 was suppressed by alcohol, as were Stat3, monocyte chemoattractant protein 1

(MCP1), and suppressor of cytokine signaling 3 mRNAs, the latter two being Stat3 response genes (Fig. 3A). Despite some persistent Stat 3 phosphorylation on western blotting, Stat3 DNA binding was decreased (Fig. 3B,C), which is in line with the predominant cytoplasmic localization of Stat3 in hepatocytes (Fig. 4A; Supporting Fig. S2). RNA sequencing and canonical pathway analysis confirmed down-regulation of Stat3 footprint and inhibition of the Stat3 pathway

in early but not severe ALD (Fig. 3D). Short-term abstinence did not allow for recovery of defective Stat3 signaling (Fig. 3A-C). Inhibition of Stat3 and repression of IL-6 and MCP1 mRNAs in the liver did not prevent plasma increase of IL-6 and MCP1, suggesting the contribution of cells outside the liver to production and blood release of these cytokines (Fig. 3E).

The proliferation markers Ki-67 and cyclin D identified two subgroups in active drinkers: one with

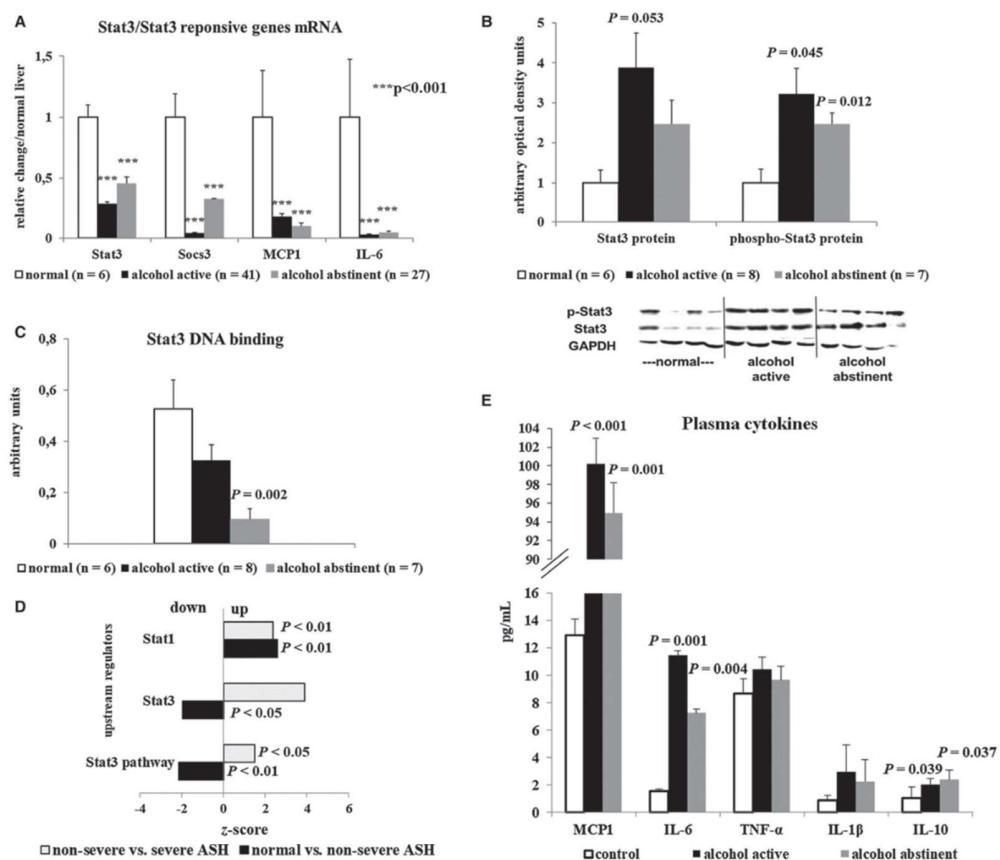


FIG. 3. Functional inhibition of the Stat3 pathway in liver tissue at early stages of ALD. (A) Down-regulation of Stat3 mRNA and of Stat3-regulated genes in the liver. (B,C) Western blot of total and phosphorylated Stat3 in liver tissue lysates showing increased expression of Stat3 (B) but decreased Stat3 DNA binding in patients with alcoholism (C). (D) RNA sequencing analysis confirming down-regulation of Stat3 footprint in early ALD in contrast to an increase with disease progression. (E) Increased blood levels of IL-6 and MCP1 in contrast to the blunted expression of both factors in the liver. Indicated *P* values refer to normal liver or controls. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Socs, suppressor of cytokine signaling.

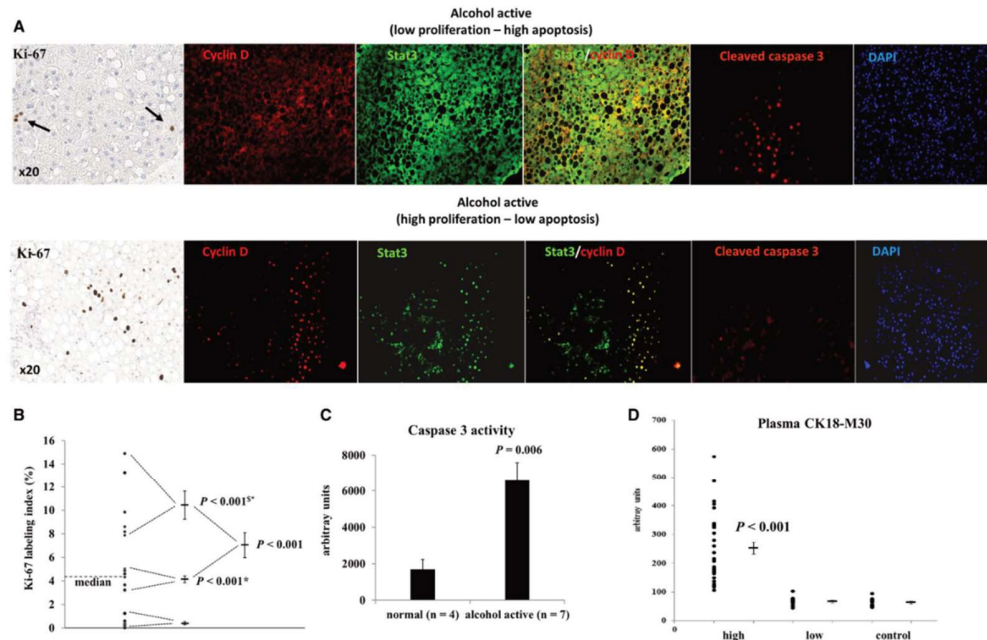


FIG. 4. Proliferation–apoptosis balance in early-stage ALD in actively drinking patients. (A) Representative immunohistochemistry of Ki-67, immunofluorescence of cyclin D, Stat3, and cleaved caspase 3 showing a low proliferation, high apoptosis (upper panel), and high proliferation, low apoptosis group (lower panel). Stat3 was sequestered in the cytosol of low proliferators, but co-localized with cyclin D in the nucleus of high proliferators. (B) Quantification of Ki-67 expression confirming the presence of a high, intermediate, and low proliferation group. *P* values refer to the low (*) and intermediate (S) proliferation group. (C) Caspase 3 activity in liver lysates of controls and patients with high cleaved caspase 3 nuclear expression confirming increased apoptosis. (D) Increased blood levels of the apoptotic cytokeratin 18 fragments M30 confirming the presence of a high and low apoptosis group. *P* value compares to the low apoptosis group and to controls. Abbreviation: DAPI, 4',6-diamidino-2-phenylindole.

low and one with high expression of both markers. More precise quantification of Ki-67 in hepatocytes distinguished three clusters of patients: (1) very low proliferation (Ki-67 $\leq 1\%$); (2) intermediate proliferation (Ki-67 = 4.3%; range 3.2%–5%); and (3) high and diffuse hepatocyte proliferation (Ki-67 $\geq 8\%$) (Fig. 4A,B; Supporting Fig. S3). No expression of Ki-67 and very low expression of cyclin D ($0.46\% \pm 0.19\%$) were found in normal livers. Strikingly, patients with foci of intense hepatocyte proliferation also showed nuclear staining of Stat3 co-localizing with nuclear cyclin D, a Stat3 responsive gene. When these livers with active proliferation were stained for cleaved caspase 3, no or minimal nuclear staining of caspase 3 was observed (Fig. 4A).

By contrast, Stat3 and cyclin D were sequestered in the cytoplasm in active drinkers with no or very low proliferation. In parallel, those livers exhibited intense clusters of cleaved caspase 3 nuclear staining together with increased liver caspase 3 activity, confirming activation of apoptosis (Fig. 4A,C). Nuclear caspase 3 co-localized with hepatocyte nuclear factor 4 on double immunofluorescence, suggesting that apoptosis principally occurred in hepatocytes (Supporting Fig. S3).

Cytokeratin (CK) 18 fragments are released following hepatocyte damage. Significantly increased levels of CK18-M65 and the caspase-cleaved fragment M30 were detected in the plasma of patients with alcoholism. Interestingly, one-third (32%) of patients had M30 levels close to control values, confirming,

as with cleaved caspase 3 expression, the existence of two subgroups of patients: one with increased hepatocyte apoptosis and one with minimal or no apoptosis (Fig. 4D; Supporting Fig. S4). Both markers correlated well with transaminases (Table 2), adding additional evidence for increased hepatocyte damage and apoptosis in actively drinking patients with alcoholism.

ALCOHOL DRIVES INDUCTION OF INTRACELLULAR TLR3 AND TLR7

Given the potential role of TLRs in driving “sterile” inflammation, we assessed the expression profile of various TLRs. Intriguingly, intracellular TLR3, TLR7, and myeloid differentiation 2 mRNA expression were strongly up-regulated in livers of active drinkers (Fig. 5A). RNA sequencing analysis showed that TLR7 footprint was already increased in early ASH and more so in severe ASH (Fig. 5B).

To determine whether alcohol directly induces TLR3 and TLR7 up-regulation, we cultured human PCLS with or without ethanol for up to 72 hours. The expression of TLR3 and TLR7 rose following alcohol treatment in a time- and dose-dependent manner (Fig. 5C). Similar results were obtained with alcohol-stimulated PHHs from two different donors (Fig. 5D), whereas TLR expression did not change in alcohol-stimulated cultured primary human HSCs or in the LX-2 human HSC cell line (Supporting Fig. S5).

Expression of membrane-bound TLRs, including TLR4, did not increase in livers of patients with alcoholism compared with control samples. In addition, CD14, a cofactor required for TLR4 activation, as well as lipopolysaccharide-binding protein (LBP) mRNAs, were down-regulated in patients with alcoholism (Fig. 5E). No increase in LBP plasma levels was found in patients with alcoholism compared with healthy controls (Fig. 5F). As a confirmation, alcohol stimulation of PHHs as well as human HSCs did not up-regulate TLR4 expression (Supporting Fig. S5).

ALCOHOL STRONGLY ACTIVATES INTERFERON SIGNALING IN HEPATOCYTES

Alcohol-induced interferon (INF)- β and INF- γ mRNA expression and RNA sequencing analysis indicated increased INF- γ footprint already in early ASH. Furthermore, activation of interferon signaling was suggested by canonical pathway analysis of RNA sequencing data and confirmed by quantitative real-time PCR of the IFN responsive genes 2'-5' oligoadenylate synthetase 1 and IFN-stimulated gene 6-16 (Fig. 4A,B). In cultured human PCLS, alcohol stimulated the expression of IFN- β and IFN- γ mRNAs (Fig. 4C).

Alcohol also induced phosphorylation of the interferon upstream transcription factor IRF3 (Fig. 4E). IRF3 nuclear staining was found principally in hepatocytes, but cytoplasmic expression was also seen

TABLE 2. CORRELATIONS BETWEEN INFLAMMATORY, FIBROSIS, AND LIVER DAMAGE MARKERS

Fibrosis Markers			
	IL-1 β	TLR7	IFN- β
Collagen 1	$r = 0.45$; $P = 0.0008$	$r = 0.605$; $P = 0.0002$	$r = 0.546$; $P < 0.0001$
α -SMA	$r = 0.423$; $P = 0.0017$	$r = 0.511$; $P = 0.002$	$r = 0.500$; $P = 0.0002$
Timp1	$r = 0.5756$; $P < 0.0001$	$r = 0.5673$; $P = 0.0011$	$r = 0.5979$; $P < 0.0001$
Serum Liver Damage Markers			
	IL-1 β	TLR7	IFN- β
AST	$r = 0.162$; NS	$r = 0.4917$; $P = 0.0017$	$r = 0.1839$; NS
ALT	$r = -0.0529$; NS	$r = 0.2747$; NS	$r = -0.0207$; NS
CK18-M30	$r = 0.2699$; NS	$r = 0.5783$; $P = 0.0012$	$r = 0.2008$; NS
CK18-M65	$r = 0.1299$; NS	$r = 0.4389$; $P = 0.01806$	$r = 0.0786$; NS
	CK18-M30	CK18-M65	
AST	$r = 0.476$; $P = 0.0013$	$r = 0.529$; $P = 0.0003$	
ALT	$r = 0.508$; $P = 0.0003$	$r = 0.555$; $P = 0.0001$	

Abbreviations: NS, not significant; Timp1, tissue inhibitor of metalloproteinase.

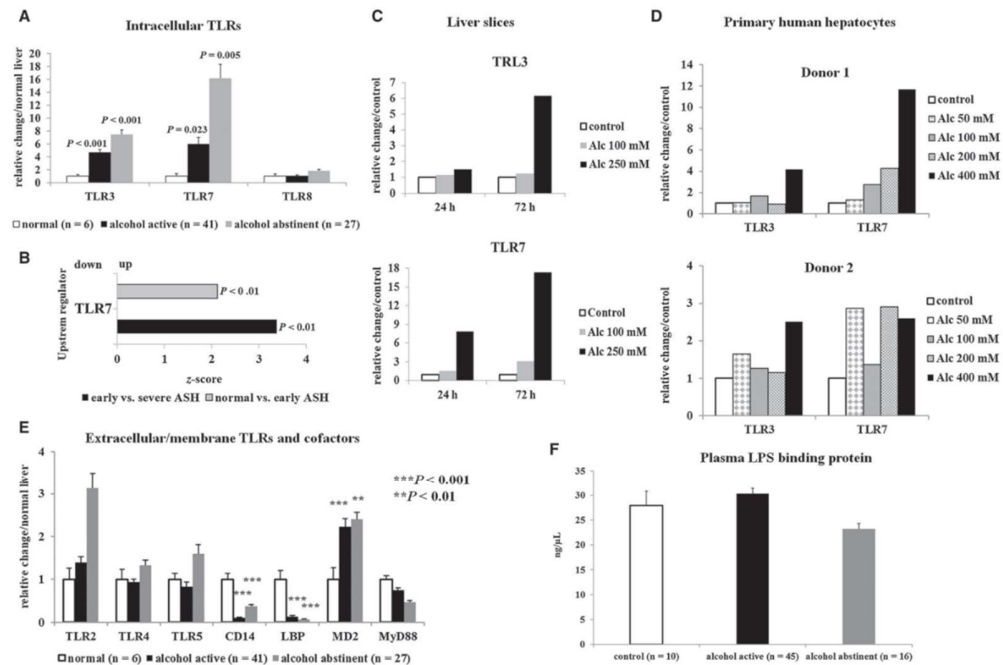


FIG. 5. Expression of intracellular and extracellular (membrane-bound) TLRs and their cofactors. (A) Quantitative real-time PCR showing up-regulation of intracellular TLR3 and TLR7 mRNA in liver tissue of patients with alcoholism who did not regress following abstinence. (B) RNA sequencing analysis indicating increased TLR7 footprint already in early AD. (C) Representative experiment showing induction of TLR3 and TLR7 mRNA in PCLS cultured for up to 72 hours with or without (control) addition of alcohol. (D) Stimulation of cultured PHHs with increasing doses of alcohol for 16 hours or 24 hours showing that already low doses of alcohol (50–100 mM) induce TLR7 mRNA. (E) Quantitative real-time PCR showing little variation of extracellular TLR mRNAs in liver tissue of patients with alcoholism, whereas TLR4-associated cofactors (CD14, LBP) were significantly down-regulated. (F) No increase of plasma LPS-binding protein was observed in actively drinking patients with alcoholism. Indicated *P* values refer to normal liver. Abbreviations: Alc, alcohol; MyD88, myeloid differentiation primary response 88.

in bile ducts, as confirmed by co-localization with CK7 (Fig. 4F). No co-localization between IRF3 and CD68 was observed, thus excluding activation of the IFN pathway in Kupffer cells (Fig. 4F). In addition, after alcohol stimulation, a dose-dependent increase of IFN- γ and IFN- β mRNA expression was only observed in PHHs (Fig. 4D), but not in primary human HSCs (Supporting Fig. S5). These observations confirm alcohol-induced IFN pathway activation primarily in hepatocytes.

Interestingly, IFN- β and IFN- γ significantly correlated with TLR7 expression in liver biopsies and in alcohol-stimulated PHHs, indicating a possible link between both pathways (Supporting Fig. S6).

IFN- γ was also detected in two-thirds of the plasma samples and tended to be higher in patients with alcoholism compared with controls (Supporting Fig. S6).

IL-1 β , IFN- β , AND TLR7 ARE ASSOCIATED WITH MORE SEVERE FIBROSIS AND DISEASE PROGRESSION

Patients with advanced fibrosis (F3/F4) expressed significantly higher levels of the fibrosis markers collagen 1, α -smooth muscle actin (α -SMA), and tissue inhibitor of metalloproteinase 1 compared with

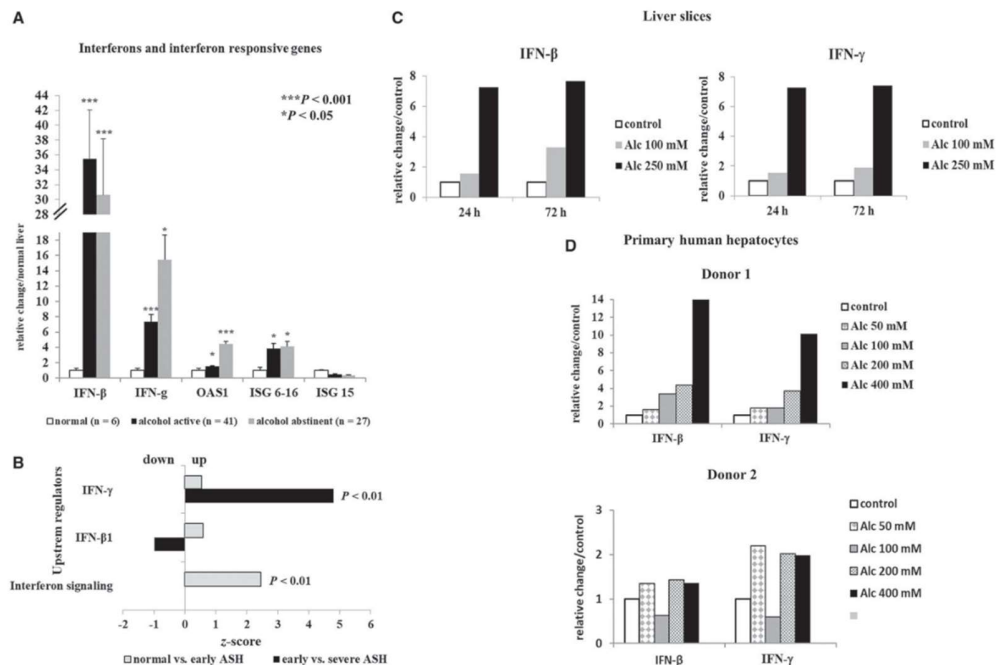


FIG. 6. Activation of the IFN pathway in hepatocytes. (A) Quantitative real-time PCR of IFN- β and IFN- γ and IFN-responsive gene mRNAs showing strong activation of IFN signaling in liver tissue of active drinkers that do not regress after 14 days of abstinence. (B) RNA sequencing analysis showing increased IFN- γ footprint and IFN signaling early in ALD. (C) Representative experiment showing induction of IFN- β and IFN- γ mRNA in PCLS cultured for up to 72 hours with or without (control) addition of alcohol. (D) Stimulation of cultured primary human hepatocytes with increasing doses of alcohol for 16 hours or 24 hours, showing that already low doses of alcohol (50–100 mM) induce IFN- β and IFN- γ mRNA expression. (E) Western blot of total and phosphorylated upstream IRF3, demonstrating increased levels in active drinkers. (F) Representative staining of IRF3 expression in livers of active drinkers. Immunofluorescence staining shows IRF3 nuclear staining in hepatocytes (HNF4+) as well as cytoplasmic staining in CK7-positive bile ducts, but no co-localization of IRF3 in CD68-positive Kupffer cells. Indicated P values refer to normal liver. Abbreviations: Alc, alcohol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HNF4, hepatocyte nuclear factor 4; ISG, IFN-stimulated gene; OAS1, 2'-5' oligoadenylate synthetase 1.

the minimal fibrosis group (F0-F2) (Fig. 7A). No significant difference was found between the minimal fibrosis group and control livers (not shown). Collagen 1 also correlated well with quantification of fibrosis on histology assessed by Masson's trichrome staining ($r = 0.5098$; $P = 0.0015$). We next addressed which of those investigated inflammatory factors with increased expression in actively drinking patients could contribute to fibrosis progression. Intriguingly, only IL-1 β , IFN- β , and TLR7 showed a significantly higher expression in livers with advanced fibrosis compared with the minimal

fibrosis group (Fig. 7B). All three factors strongly correlated with the liver tissue fibrosis markers (Table 2). RNA sequencing showed not only that TLR7 and IFN footprints further increased with disease severity (Figs. 5B and 6B), but also were significantly ($P < 0.01$) associated with more severe disease stages (Fig. 7C). Importantly, those factors did not regress following short-term abstinence (Figs. 5A and 6A). These observations suggest that they might be important players in promoting alcohol-induced disease progression. Furthermore, TLR7 correlated with AST and serum CK18-M30

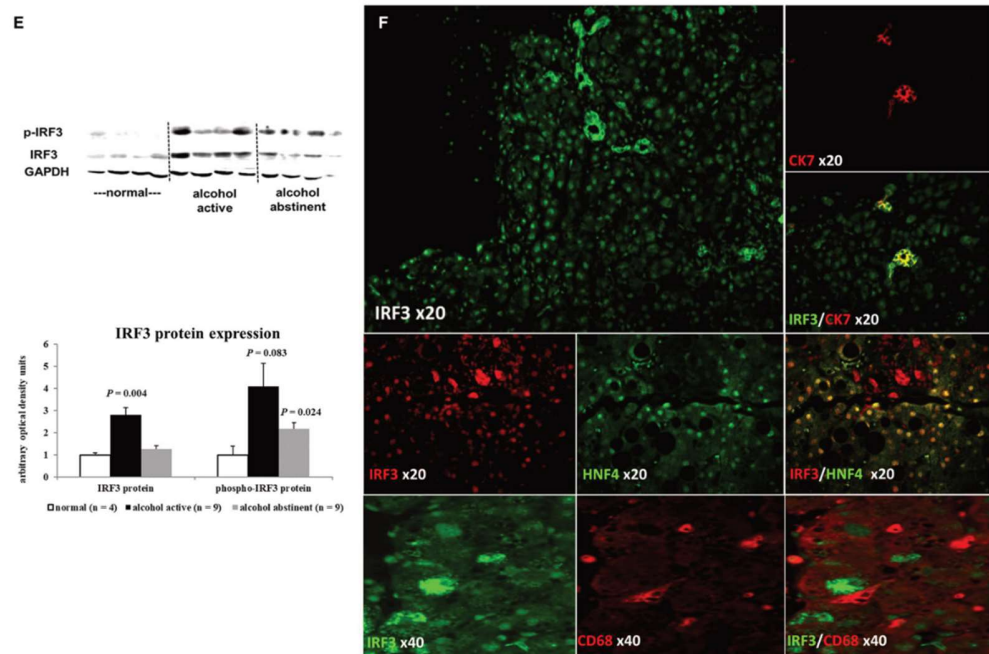


FIG. 6. Continued.

and M65 levels, markers of liver damage and/or apoptosis (Table 2), also linking it to liver damage. Surprisingly, RNA sequencing analysis showed that Stat3 footprints as well as the Stat3 signaling pathway were activated during progression of the disease, which is in sharp contrast to the inhibition of these factors in early ASH (Fig. 7C).

Discussion

Our work addresses alcohol-induced changes that occur at early stages of ALD in a unique human cohort. We show that a proinflammatory response, including activation of TNF- α , IL-1 β , and NF- κ B, driven by liver resident macrophages, already occurred at very early stages of the disease. The early proinflammatory response was alleviated by short-term abstinence. By contrast, the IL-6/Stat3 axis was blunted and Stat3 sequestered in the

cytoplasm of hepatocytes in livers with increased apoptosis. On the other hand, livers that were able to overcome alcohol-induced Stat3 blockage, as indicated by preserved nuclear translocation of Stat3, retained some proliferative capacity. In contrast to animal data, intracellular TLRs, in particular TLR7, increased at early stages in humans, whereas TLR4 did not. TLR7 up-regulation occurred concomitantly with activation of interferon signaling predominantly in hepatocytes. Recent evidence links TLR7 to alcohol-related neuroinflammation,^(17,18) whereas its role in alcohol-induced liver damage remains unclear. TLR7 recognizes synthetic imidazoquinoline-like molecules, guanosine analogs, and single-stranded RNA, leading to myeloid differentiation primary response 88/IRF-dependent induction of type I interferons.⁽¹⁹⁾

Two animal studies in cholestatic and nonalcoholic fatty liver disease seem in favor of a protective effect of TLR7 against disease progression and

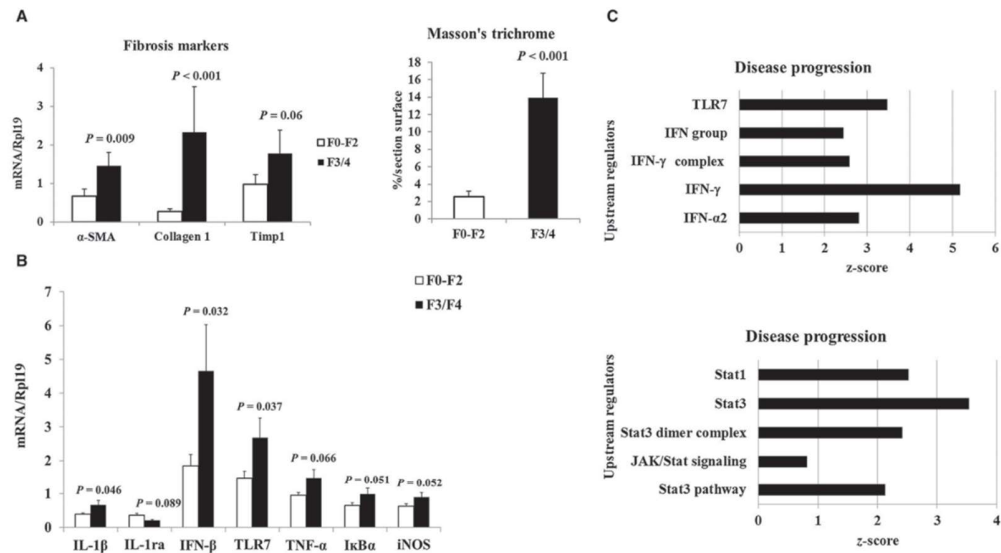


FIG. 7. Factors associated with liver fibrosis and disease progression. (A) Quantitative real-time PCR showing up-regulation of liver mRNA expression of markers α -SMA, collagen 1, and Timp1 and increased collagen deposition assessed by Masson's trichrome staining in the severe fibrosis group (F3/F4). (B) Liver mRNA expression of factors associated with more severe fibrosis. In particular, TLR7 and IFN- β strongly increased in the advanced fibrosis group. (C) RNA sequencing showing increased footprints of TLR7, IFNs, Stat1, and Stat3 as well as activation of the Stat3 signaling pathway with disease progression. *P* values compare the F3/F4 group to the F0-F2 group. Abbreviations: IkB α , nuclear factor kappa B inhibitor alpha; IL-1ra, interleukin-1 receptor antagonist; iNOS, inducible nitric oxide synthases factor; JAK, Janus kinase; Timp1, tissue inhibitor of metalloproteinase 1.

fibrosis.⁽²⁰⁻²²⁾ Expression of TLRs, including TLR7, was also elevated in a chronic ethanol-feeding model. However, specific TLR7 stimulation did not exacerbate liver inflammation and damage.⁽²³⁾ Animal studies concerning the IRF3 type I interferon axis yielded conflicting results. One study suggested a protective effect of IRF3 and type I interferon activation in parenchymal cells in ALD.⁽²⁴⁾ By contrast, two studies linked IRF3 activation with apoptotic signaling in hepatocytes and with transactivation of proinflammatory TNF- α in macrophages following chronic alcohol exposure.^(25,26)

Our results support that the TLR7 type I interferon axis has a deleterious effect in humans for several reasons. As an extension to our previous report of increased TLR7 levels in patients transplanted for end-stage ALD,⁽¹²⁾ the current data confirm that both factors are already up-regulated in early stages of the disease. TLR7 correlated well with AST and serum

CK18-M30 and M65 levels sustaining a link between TLR7 and liver damage. A good correlation between TLR7 and activation of the IFN cascade was found in hepatocytes *in vivo*. Alcohol stimulation of liver slices *ex vivo* as well as of cultured PHHs confirmed concomitant up-regulation of both TLR7 and IFNs, indicating a connection between the two pathways. In addition, TLR7 and type I IFNs were the only factors that correlated with liver fibrosis markers and more advanced stages of ALD, pointing to a role in disease progression. In contrast to animal data, we did not find a predominant implication of the TLR4 system in the pathological modifications of early ALD in humans.⁽²⁷⁻²⁹⁾ This finding further emphasizes the prominence of the pathophysiological role for TLR7 in human ALD. It is likely that sensitization to TLR ligands is regulated by multiple mechanisms, including activation by endogenous danger signals from damaged cells in ALD.⁽²⁹⁾ The latter may trigger immune

reactions in the liver that are different in humans and rodents in accordance with their different immune and TLR systems.^(9,10,30)

Endogenous danger signals released from damaged cells can also activate the inflammatory cascade. Although a proinflammatory response occurred in liver-resident macrophages, the IL-6/Stat3 pathway was surprisingly blunted in parenchymal cells. We and others have already reported decreased Stat3 activation in alcoholic cirrhosis and end-stage ALD.^(31,32) Here we show that this mechanism already operates at very early stages of ALD. Hepatocytes with deficient Stat3 activation were unable to mount a proliferative response and underwent apoptosis. On the contrary, those with preserved Stat3 nuclear translocation maintained their cell proliferation. Accumulating evidence suggests that the IL-6/Stat3 axis plays an important role in repairing ethanol-induced hepatocellular damage through induction of a variety of hepatoprotective genes in hepatocytes.⁽³³⁻³⁵⁾ Also, the absence of IL-6 in mice chronically exposed to alcohol promotes liver damage.⁽³⁶⁻³⁸⁾ Stat3 likely acts differently depending on the cell type and the environment. Its absence in nonparenchymal cells allows unrestricted inflammation to develop, whereas the suppression of Stat3 in hepatocytes favors liver damage.^(39,40) The absence of Stat3 in nonparenchymal cells and the deficient IL-6/Stat3 axis in hepatocytes in our study were associated with impaired liver repair and up-regulation of Kupffer cell-derived proinflammatory cytokines (TNF- α , IL-1 β). These findings are in line with the view that activation of the IL-6/Stat3 pathway exerts a protective effect at the early stage of ALD in humans. In more advanced fibrotic ALD, however, Stat3 is activated, which is supported by our data. *In vitro* evidence showed that Stat3 activation in HSCs could be deleterious and favor fibrosis progression.^(41,42) Because our study focused on early ALD, we did not investigate this possibility any further.

IL-10, an important anti-inflammatory cytokine, acts as a brake to control inflammation by specifically targeting activated macrophages or monocytes.^(34,35) Human monocytes activated by lipopolysaccharide (LPS) produce a high level of IL-10, which in turn tempers the production of proinflammatory cytokines, such as TNF- α and IL-1 β .⁽⁴³⁾ The absence of IL-10 in mice leads to more severe liver damage following LPS treatment or chronic ethanol feeding.^(44,45)

Up-regulation of IL-10 did not occur in human livers of active drinkers. As a consequence, the brake that is able to restrain production of proinflammatory TNF- α and IL-1 β is not fully activated. Thus, an inadequate IL-10 response combined with deficient liver repair mechanism driven by alcohol-induced Stat3 inhibition could pave the way for progression of liver damage.

In absence of any effective pharmacological therapy, long-term abstinence constitutes the cornerstone of ALD treatment. Unfortunately, frequent relapses after short periods of abstinence are common in patients with alcoholism. We therefore examined the effect of short-term abstinence on the evolution of the putative proinflammatory, profibrotic, and pro-liver damage factors. Importantly, the deficient IL-6/Stat3 axis in hepatocytes did not recover following short-term abstinence. Abstinence was also insufficient to significantly affect the TLR7/IFN axis associated with fibrosis progression.

An intrinsic limitation of this study is that data are in part descriptive and/or based on correlations, which do not formally prove a cause-and-effect relationship. However, given the difficulties of accessing tissue from early-stage disease, we believe this investigation adds significant insights into the early pathogenesis of ALD. Certainly, the main strength of the study is the unique patient cohort of a high number of heavily, actively drinking subjects undergoing elective alcohol withdrawal in whom liver biopsy had been performed in a strict, standardized clinical program.

Our observations affect clinical practice because they suggest that many unfavorable factors are already present at early stages in human ALD. Short-term abstinence does not lead to full recovery of impaired liver repair mechanisms and factors potentially associated with fibrosis progression. No biomarker allows distinguishing vulnerable patients from those with a more benign disease course; therefore, clinicians need to do their utmost to promote long-term abstinence in patients with alcohol abuse and signs of liver disease.

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Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1364/supinfo.

Deficient IL-6/Stat3 Signaling, High TLR7, and Type I Interferons in Early Human Alcoholic Liver Disease: A Triad for Liver Damage and Fibrosis

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Supporting material/data

Content:

Supporting methods

- Patients, methods, samples
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Supporting tables

- Table S1: qPCR primers
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Supporting Methods

Patients, methods, samples

Inclusion-exclusion criteria

Additional inclusion criteria were first admission to the alcohol withdrawal unit and absence of previously diagnosed significant alcoholic liver disease.

Patients with diabetes were eligible for the study if they were on stable treatment (metformin, oral antidiabetics with or without insulin) and had a glycosylated hemoglobin level <7% at admission.

Patients were excluded from the analysis if they had one of the following conditions:

- Inflammatory bowel disease or other chronic inflammatory or autoimmune diseases
- History of cancer within 5 years prior to admission
- Known liver disease of any other etiology
- Clinically significant cardiovascular, pulmonary, or renal comorbidities
- Clinical and biochemical signs of active infection (>37.5°C, viral syndrome, high C-reactive protein [CRP], and high white blood cell count)
- Administration of antibiotics, probiotics, glucocorticoids, and nonsteroidal anti-inflammatory drugs during the 2 months preceding enrollment

In addition, patients assigned to undergo liver biopsy during the second week of hospitalization and who relapsed during the week at home were excluded from the study.

FibroScan measurements

Transient elastography measurements were performed using the FibroScan device (Echosens, Paris, France) by an experienced examiner blinded to the patient's data. FibroScan was considered as valid if at least 10 validated measurements were obtained with an interquartile range of <30%. The final result expressed in kPa was the median of all valid measurements obtained. We used the cutoffs for patients with alcoholism proposed by Nguyen-Khac et al. (Aliment Pharmacol Ther 2008;28:1188-1198), i.e., 7.8, 11, and 19.5 kPa for F2, F3, and F4, respectively.

Transjugular liver biopsy and tissue sampling

Liver biopsy was performed using a transjugular liver access and biopsy needle (16G) set (Cook, Bjæverskov, Denmark). After administration of local anaesthesia to the neck, a catheter was introduced into the right jugular vein and guided under fluoroscopy into the right main hepatic vein. Separate tissue samples were taken and routinely fixed in formalin, snap-frozen in liquid nitrogen, and preserved in RNAlater solution (Invitrogen, Carlsbad, CA). After a complete routine histopathological examination of the biopsy samples by an experienced liver pathologist, surplus tissue was recovered for further research purposes.

Biopsy samples of ≥ 15 mm in length, including a minimum of 6 portal tracts, were considered suitable or when the pathologist could establish the degree of fibrosis with certainty on a smaller sample.

Normal liver tissue and control blood samples

Normal liver samples were derived from size-reduced liver grafts and/or hepatectomy specimens performed for resection of liver metastasis at distance of the tumor. A thorough

pathological analysis confirmed that all samples from the hepatectomy specimens were histologically normal and free of tumor.

Fasting control blood samples were obtained from healthy volunteers consuming <20 g of alcohol per day.

Precision-cut liver slices

Human liver tissue was obtained from patients who underwent partial hepatectomy for colorectal liver metastasis from The London Clinic, London. Immediately after surgical resection, the healthy portions of the liver specimen were harvested and kept on ice in sterile University of Wisconsin solution (ViaSpan; Bridge to Life, Ltd., London, UK) until slicing. Time from harvest to culture was kept to a minimum (3-10 hours), and the preparation of precision-cut liver slices (PCLS) was performed as described.⁽¹³⁾ Each slice was placed in a 12-well plate and cultured in Williams' medium E (WME) supplemented with glucose (Sigma, Gillingham, UK), penicillin, and streptomycin (Life technologies, Paisley, UK), with the addition of insulin-transferrin-selenium (Life Technologies, Paisley, UK), epidermal growth factor 1 nM (Life Technologies), corticosterone 1 μ M (Sigma), glucagon 100 nM (Sigma), and human AB serum 5% (Gemini Bio-Products, West Sacramento, CA). The medium was saturated with carbogen (95% O₂/5% CO₂) and the plates kept in sealed chambers at 37°C in a shaking incubator. After an initial preincubation of 2 hours to allow the recovery after the cut, each slice was maintained in culture for 24 or 72 hours with or without the addition of ethanol (100 or 250 mM), and the medium was replaced every day.

Immunohistochemistry and immunofluorescence

Five-micrometer sections were prepared from formalin-fixed, paraffin-embedded livers and stained for conventional histology or incubated with primary and secondary antibodies. Stained sections were scanned, and morphometrical quantification of fibrosis and labeled nuclei (Ki-67, cyclin D) were obtained from a digitalized image through a computer algorithm (Leica Microsystem Imaging Solution, Ltd., Cambridge, UK). The final result was adjusted to the surface of the section. Immunofluorescence (IF) pictures were taken with an Axiovert 200 fluorescence microscope (Carl Zeiss, Jena, Germany). Computer image analyses of immunostained sections were performed using Zeiss microscope coupled to an Axiocam camera (MR3, Carl Zeiss, Munich, Germany) and the AxioVision software (Zeiss). Nuclei were detected by incubation with 4',6-diamidino-2-phénylindole (DAPI) (cyan) (Vector Laboratories H-1200, Belgium).

RNA sequencing

High-throughput transcriptome profiling by RNA sequencing (RNA-seq) with liver samples provided by the InTeam Consortium Human Biorepository Core (University of Pittsburgh, Pittsburgh, PA) was performed. A total of 530 patients with different phenotypes were selected: 12 patients with early alcoholic steatohepatitis (ASH) and 18 patients with severe ASH (Maddrey's discriminant function [MDF] >32). Normal controls (n=10) were non-diseased livers obtained from biopsied single nodules, whose histology was without significant alterations. Differential expression analysis comparing normal versus early ASH and early ASH versus severe ASH livers was performed. Differential expressed genes with false discovery rate (FDR) <0.005 for normal versus early ASH and with FDR <0.0001 for early ASH versus severe ASH were selected for functional enrichment analysis. In order to detect toll-like receptor (TLR)-, interferon-, and signal transducer and activator of transcription (STAT)-related transcriptomic footprint, causal analytics algorithms were performed using Upstream Regulator prediction tool under the Ingenuity Pathway Analysis (IPA) software package (Qiagen, Inc.). In order to detect enrichment of interferon pathway,

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IPA-embedded Canonical Pathway prediction tool was used. Z score statistic was calculated as described elsewhere (PMID: 24336805).

RNA sequencing followed by transcriptomic footprint analysis of target genes (Ingenuity Pathway and Ingenuity Canonical Pathways analysis)

According to disease severity

Stat1:

Normal versus early ASH

ACOD1, ALDH1A3, ANGPT2, ARG1, BAX, CCL20, CCL5, CCND1, CDKN1A, CEACAM1, CEBPD, CX3CL1, CXCL10, CXCL8, CXCL9, CYP3A5, DCSTAMP, EGR1, FOS, FOXO1, IFIT2, IFIT3, IL12B, IRF8, ISG15, ISL1, MAFA, MDM2, OAS1, PDX1, PRKCE, PTN, SHH, SREBF1, TRAF2, TYMP, UBD

Early versus severe ASH

ALDH1A3, BAD, BCL2L1, CCL20, CCND3, CDKN1A, CXCL8, CYP1A2, CYP2C8, CYP2E1, EGR1, FCGR1A, HIF1A, ICAM1, IFI16, IL15RA, INS, KCTD12, MDM2, MUC1, MYC, NEURL3, PCDH17, PDGFA, PDGFRB, PECAM1, RNF213, RORC, RUNX1, S100A10, SHH, SLC2A2, SMAD2, SOCS1, SOCS3, STAT3, TNFRSF10B, TNFSF10, TP53BP2, UBD

Stat3:

Normal versus early ASH

ACTA2, ADM, ALDH1A3, ANGPT2, ARG1, BEX1, BOC, C5AR1, CCL20, CCL5, CCND1, CD226, CDKN1A, CEACAM1, CEBPA, CEBPD, CHI3L1, CX3CL1, CXCL10, CXCL8, CXCL9, CXCR4, DLL1, EGR1, F3, FERMT2, FFAR2, FLRT3, FLT1, FOS, FOXA3, GADD45G, HERC5, HES5, ID2, IFIT2, IFIT3, IGFBP1, IHH, IKZF3, IL12B, IL1RN, IL6R, IL7, ISG15, LBP, MAF, MAP2, MX2, NAMPT, NRP2, OAS1, PCSK9, PGR, PHLDA1, PRDM1, PTN, PTPN13, REG1A, RET, RORA, S1PR1, SGK1, SHH, SLC1A3, SNAI2, SOD2, SREBF1, SYNPO, TAGLN, TFF3, TNFSF11, TRIM14, TRPM3, WNT5A, ZFP36

Early versus severe ASH

ACTA2, AHSG, ALDH1A3, AMER1, BATF, BCL2, BCL2L1, BOC, CAT, CCL20, CDKN1A, CDKN2A, CDKN2B, CEBPA, COL1A1, COL1A2, COL3A1, COL5A1, CTGF, CXCL6, CXCL8, CXCR4, CYP26A1, EGR1, ESR1, F3, FCGR1A, FERMT2, FUT4, GADD45A, GAP43, HAVCR1, HIF1A, ICAM1, IFI16, IHH, IKBKE, IL13RA2, ITGAV, ITGB1, ITGB6, JAG1, JUNB, LCAT, LIF, MAP2, MCL1, mir21, MMP2, MMP7, MUC1, MYC, NAMPT, NEURL3, NOTCH3, PCSK1, PECAM1, PHLDA1, PLSCR1, PROM1, RALGDS, REN, RORC, RUNX1, SERPINB1, SERPINE1, SERPINE2, SHH, SLC1A3, SLC28A2, SLC2A1, SOCS1, SOCS3, SOD2, STAT3, STC2, TAGLN, THBS1, TIMP1, TNFRSF10B, TNFSF10, VCAN, VIM, WNT5A, ZFP36

TLR7:

Normal versus early ASH

CCL20, CCL5, CDKN1A, CHST9, CPEB2, CXCL10, CXCL8, CXCL9, IFIT3, IL12B, ISG15, MX2, NFKBIA, PLAT, SELE, SOD2

Early versus severe ASH

BCL2, CCL20, CD44, CDKN1A, CREB5, CXCL1, CXCL8, HIVEP2, ICAM1, IER3, JUNB, KCNMA1, NR1H4, SOCS1, SOD2, TNFAIP3, TRPM2, ZC3H12A

Interferon- γ (IFN- γ):

Normal versus early ASH

ABCB1, ACOD1, ACTA2, ADM, AGRN, ALDH1A3, AMER2, ANKS1A, ARG1, ASS1, ATP2A2, BAX, BBC3, BCAN, BMP6, C5AR1, CAPN3, CAV1, CCL20, CCL5, CCND1, CD4, CDH22, CDKN1A, CEACAM1, CEBPA, CEBPD, CHST3, CLEC4E, CX3CL1, CXCL10, CXCL8, CXCL9, CXCR4, CYB561, CYP3A4, DCT, DDB2, DDR2, DLL1, DUSP1, E2F1, EGR1, EHF, ELN, F3, FAM107A, FBLN1, FKBP5, FLT1, FOS, FOXO1, GAS6, GPR83, IFIT2, IFIT3, IL12B, IL18RAP, IL1RN, IL32, IL36G, IL6R, IL7, IL7R, IRF8, ISG15, ISL1, ITGA6, ITGB3, ITPR1, KLF6, MAFA, MDM2, MERTK, mir103, mir23, MRC1, MUC2, MX2, NAMPT, NEDD9, NFKBIA, NGF, NOD2, NTRK2, OAS1, OCLN, P2RY1, P2RY14, PAM, PCSK2, PDX1, PHLDA1, PIGR, PLA2G5, PPARD, PRDM1, PRKCQ, PSEN2, PSME3, PTN, PTPRB, RHOB, RUNX2, SCG5, SCUBE1, SELE, SEPT4, SHH, SIX1, SLC16A9, SLC1A3, SLC7A5, SMC1B, SMURF1, SNAI2, SNAP25, SOCS2, SOD2, SREBF1, TAP2, TGFB3, TICAM1, TIMP3, TIMP4, TNFSF11, TP73, TRAF2, TREM2, TSC22D3, TXNIP, TYMP, UBD, VCAM1, VIPR1, WNT5A, ZBTB16, ZFP36, ZPR1

Early versus severe ASH

ABLM3, ACSS1, ACTA2, ACTB, ADGRG1, AGRN, AHCY, ALDH1A3, ALDH1L1, ALDH7A1, AQP1, AQP11, AQP9, ARNT2, ATP1A1, AZGP1, BCL2, BCL2L1, CARD6, CAT, CCL20, CCL28, CD200, CD44, CD5L, CD63, CDKN1A, CDKN2A, CDKN2B, CEBPA, CELSR1, CFTR, CHST11, CHST3, CLIP2, COL1A1, COL1A2, COL5A2, CRLF1, CTGF, CTSK, CXCL1, CXCL6, CXCL8, CXCR4, CYB5A, CYP1A2, CYP27A1, CYP2C9, CYP2E1, DBP, DDX5, DIO1, EGR1, EHF, ELN, F2R, F3, FAAH, FAH, FCGRI1A, FLRT2, GBP7, GCHFR, GLIPR2, GLS, GNG7, GPER1, GPR146, GPRC5B, HAAO, HIF1A, HSP90AA1, HSP90AB1, ICAM1, IER3, IFI16, IFNGR1, IFNGR2, IKBKE, IL15RA, IL18, IL1RL1, IL27, IL32, INS, ITGA6, ITGAV, ITGB1, JAG1, JAK3, JUNB, KCNMA1, KCTD12, KLF6, KRT19, LAMC1, LAMC2, LGALS3, LIF, LIMK1, LOX, MAFF, MAP3K8, MDM2, MMP2, MST1R, MUC1, MYC, MYH9, NAMPT, NCR3LG1, NEDD9, NEURL3, NFKB2, NOTCH3, NQO1, NUPR1, PAPP, PCDH17, PCSK1, PCTP, PDGFA, PEA15, PECAM1, PHGDH, PHLDA1, PMEL, POLR2F, PPARD, PRDX2, PSMA2, PSME3, PTHLH, RAP2B, RGN, RORC, RUNX2, S100A10, SCG5, SELENOP, SEMA7A, SEPT4, SERPINA5, SERPINE1, SERPINH1, SHH, SLC12A2, SLC1A3, SLC28A2, SLC29A1, SLC2A1, SLC2A2, SLC46A1, SLC6A1, SLC6A6, SOCS1, SOCS3, SOD2, SOD3, SPP1, SQSTM1, ST13, STAT3, TGFB2, TGFB3, THBS1, THY1, TIMP1, TIMP3, TJP1, TLR5, TNFRSF10A, TNFRSF10B, TNFRSF12A, TNFSF10, TRIB2, TXNIP, UBD, VDR, VIPR1, VSNL1, WNT5A, ZFP36

IFN- β 1:

Normal versus early ASH

ACOD1, CCL5, CDKL2, CDKN1A, CLEC4E, COL12A1, CRYAB, CX3CL1, CXCL10, CXCL8, CXCL9, DLL1, E2F1, FOS, GLP2R, HERC5, IFIT2, IFIT3, IL12B, ISG15, ITGB3, LAMB1, NFKBIE, NID2, NOD2, OAS1, OGN, PDK4, SHH

Early versus severe ASH

AGL, ANXA5, BCL2, BCL2L1, CAPN2, CARD6, CDKN1A, CLSTN1, CRYAB, CTSK, CXCL8, CYP1A1, CYP1A2, CYP2E1, F2R, FBLN2, FBN1, FKBP9, GAS1, GBP7, GPC4, IFI16, IFNGR1, IL27, ITGAV, LAMB1, mir21, MMP2, MYC, NOD1, NPTX1, PMEL, PTCH1, SHH, SOCS1, SPRY1, THBS1, THBS2, TIMP1, TIMP2, TNFRSF10A, TNFSF10, UCHL1, VCL, VDR

Stat3 pathway:

Normal versus early ASH

FGFR1, MAPK10, TGFB3, CDKN1A, FLT1, NTRK3, NTRK2, SOCS2

Early versus severe ASH

NDUFA13, STAT3, PDGFRB, TGFB2, MYC, PDGFRA, SOCS3, BCL2, CDKN1A, SOCS1, RAC1, IGF2R

IFN signaling:

OAS1, IFNAR2, IFIT3, ISG15, BAX, PIAS1

According to disease progression

TLR7:

ATF3, BCL2, CCL20, CD44, CDKN1A, CREB5, CXCL1, CXCL8, ETS2, HIVEP2, ICAM1, IER3, IFIT1, IL1R1, IL27, JUNB, NR1H4, PTX3, SOCS1, SOD2, STAT1, TNFAIP3, TP53, TRIM38

IFN group:

ADAMTS1, B2M, CD44, CD58, ICAM1, IFI16, IFIT1, IL1R1, IL27, LCN2, PECAM1, PML, SERPINE1, SOCS1, SPP1, STAT1, TLR5, TP53, XDH

IFN- γ complex:

BCL2L1, CDKN1A, CDKN2A, CHI3L1, CXCL8, ESR1, ICAM1, LGALS9, LIF, NR4A3, PML, SOCS1, SOCS3, STAT1, TGFB2, TNFSF10

IFN- γ :

ABCA6, ABI1, ACLY, ACO1, ACSS1, ACTB, ADA2, ADGRG1, ADRA2A, AGRN, AHCY, ALDH1L1, ALDH7A1, ALKAL2, APP, AQP1, AQP11, AQP9, ARNT2, ARPP19, ATF3, ATP1A1, AZGP1, B2M, BCL2, BCL2L1, BIRC3, BRIP1, C5AR1, CALB1, CAPN3, CARD6, CAT, CCL20, CCL23, CCL28, CCND1, CCND3, CCNO, CCR6, CD44, CD5L, CDKN1A, CDKN2A, CDKN2B, CFTR, CHST11, CLEC5A, CLIP2, CRLF1, CSF1, CTGF, CTSC, CTSD, CTSK, CXCL1, CXCL5, CXCL6, CXCL8, CYB5A, CYP27A1, CYP2E1, CYP3A4, DCT, DDX5, DEFB1, DPP4, DUSP5, EEF1A1, EGLN3, EGR3, ETS2, F2R, F2RL1, FAAH, FAH, FCGR1A, FCGR2A, FCGR2B, FHL2, FOXP3, FTH1, FXN, FZD1, GAS6, GBP7, GCHFR, GDF15, GGT5, GJA1, GLIPR2, GLS, GNA13, GNA14, GNB4, GPER1, GPRC5B, GPT, HAAO, HLAB, HMGA1, HSPG2, ICAM1, IDH3B, IDO2, IER3, IFI16, IFIT1, IFNGR1, IFNGR2, IL12RB2, IL13RA2, IL17RB, IL1R1, IL27, IL36G, IL6R, IRF2, ITGA6, ITGAM, ITGAV, ITGB1, ITGB2, JAK3, JUN, JUNB, JUND, KCTD12, KLF2, KLF6, KMO, KRAS, KRT19, LAMC2, LARGE1, LCN2, LGALS3, LGALS9, LIF, LIMK1, LOX, LYN, LZTS1, MAFF, MAP3K8, MDM2, MITF, MLANA, MMP11, MMP2, MRAS, MST1R, MYH9, NCAM1, NOTCH1, NOTCH3, NR5A2, PAPP, PCDH17, PCSK1, PCTP, PDGFA, PEA15, PECAM1, PIM3, PKM, PLAUR, PMEL, PML, POLR2F, PPARG, PRDX2, PRKCD, PSME3, PTX3, RAB17, RAB36, RAP2B, RARRES1, RB1, RGN, RIPK2, RNF114, RORC, RUNX3, SBNO2, SCG5, SDC4, SEMA7A, SERPINB9, SERPINE1, SLC11A1, SLC12A2, SLC16A3, SLC1A2, SLC29A1, SLC37A4, SLC46A1, SLC6A1, SLC6A6, SLC7A11, SLFN5, SNAP25, SOAT1, SOCS1, SOCS3, SOD2, SOD3, SPP1, STAT1, STAT3, TAPBP, TGFB1, TGFB2, TGFB1, TGFB2, THOP1, TJP1, TLR5, TNFAIP2, TNFRSF12A, TNFSF10, TP53, TRPC4, TSHR, TSPAN33, TYRP1, UBD, UBE2D1, UNC5B, VDR, VIPR1, VSNL1, ZPR1, ZYX

IFN- α 2:

B2M, BAG3, BCL2, CBFB, CDKN1A, CDKN2A, CDKN2B, CLRN3, CYB5A, DPP4, EGLN3, ENPP1, GEM, HLAB, IFI16, IFIT1, IL12RB2, LYN, MDM2, MET, MYRF,

PLA2G1B, PLSCR1, PML, RASSF1, RB1, SERPINB9, SOCS1, SOCS3, STAT1, TGM2, TNFSF10, TP53, UBD

Stat1:

B2M, BCL2L1, CCL20, CCND1, CCND3, CCR6, CDKN1A, CXCL8, CYP2E1, DPP4, EGLN3, FCGR1A, FCGR2B, FGF7, FOXP3, HAPLN3, HES1, ICAM1, IFI16, IFIT1, IL12RB2, IL1R1, IRF2, JUN, KCTD12, LCN2, MDM2, PCDH17, PDGFA, PDGFRB, PECAM1, RORC, RUNX1, SLAMF8, SLFN13, SLFN5, SMAD2, SOAT1, SOCS1, SOCS3, STAT1, STAT3, TIGD6, TNFSF10, TP53, UBD, USP30-AS1

Stat3:

AMER1, BATF, BCL2, BCL2L1, C5AR1, CAT, CCDC88A, CCL20, CCND1, CD46, CDKN1A, CDKN2A, CDKN2B, CHI3L1, CTGF, CXCL6, CXCL8, DPP4, EGR3, EPAS1, ESR1, FCGR1A, FERMT2, FOXP3, FUT4, GJA1, HTR2A, ICAM1, IFI16, IFIT1, IL13RA2, IL1R1, IL6R, IL6ST, ITGAM, ITGAV, ITGB1, ITGB2, JUNB, LCAT, LIF, LYZ, MCL1, MITF, MMP2, MMP7, MRAS, NES, NOTCH1, NOTCH3, NR4A2, NRP1, PCSK1, PECAM1, PEX1, PLAUR, PLSCR1, PML, PROM1, RBPJ, RORC, RUNX1, SERPINB1, SERPINB9, SERPINE1, SLFN13, SLFN5, SNAI1, SOCS1, SOCS3, SOD2, STAT1, STAT3, STC2, TGFB1, THBD, TNFSF10, TP53, VCAN, VIM

Stat3 dimer:

BCL2L1, CDKN1A, ICAM1, JUN, SOCS3, SOD2

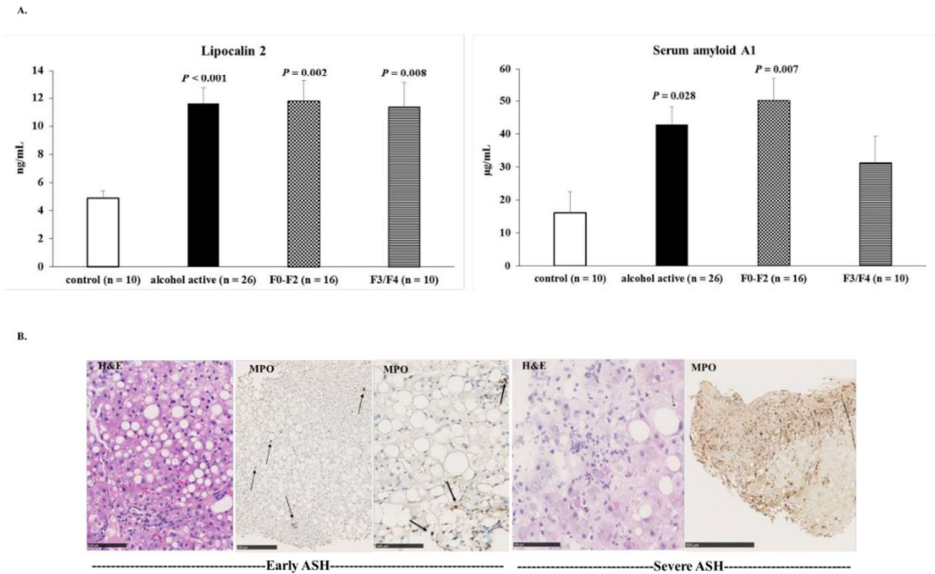
Jak/Stat signaling:

JUN, FGFR2, PIAS3, KRAS, PIK3C2A, PIAS2, SOCS4, SOCS3, MRAS, PIK3R4, RAF1, JAK3, PIK3R5, PIK3CB, AKT2, STAT3, GNAQ, PIK3CD, STAT1, KLB, CDKN1A, SOCS1, BCL2L1, RRAS

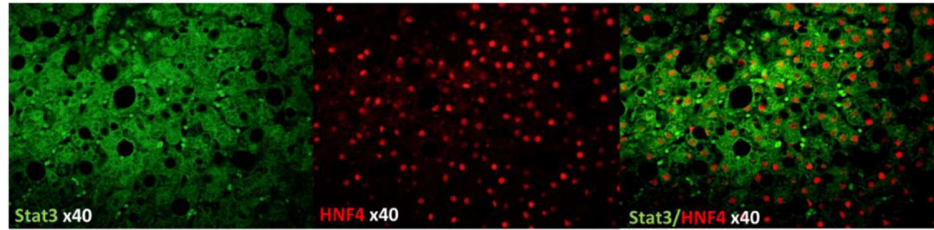
Stat3 pathway:

FGFR2, PIAS3, KRAS, PDGFRB, SRC, SOCS4, PDGFRA, SOCS3, MRAS, RAF1, DDR1, BMPR2, NDUFA13, STAT3, TGFB2, BCL2, CDKN1A, MAPK13, SOCS1, TGFB1, RRAS, RAC1

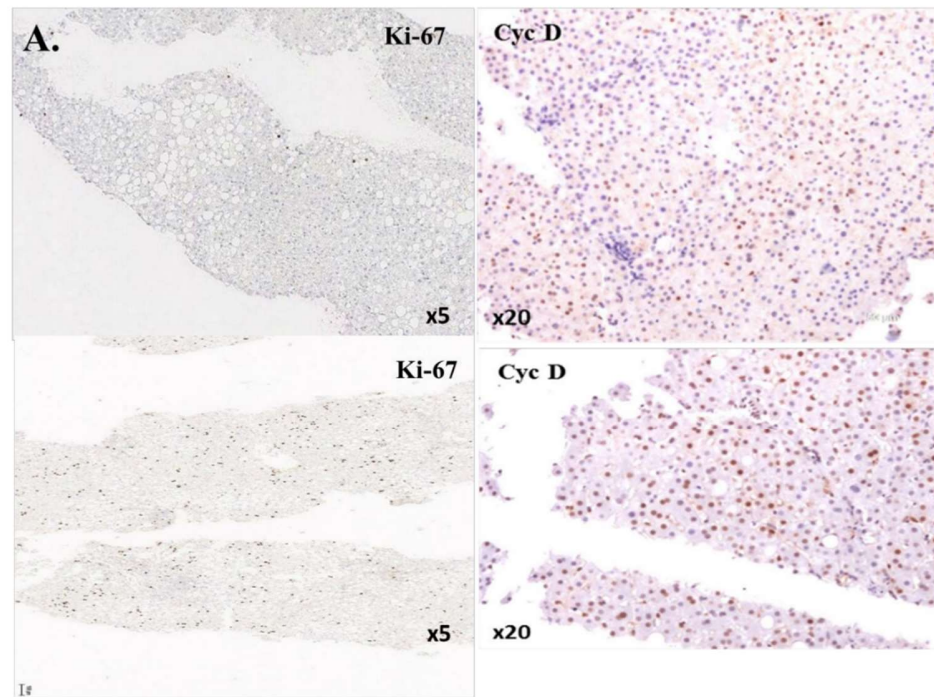
Supporting figures



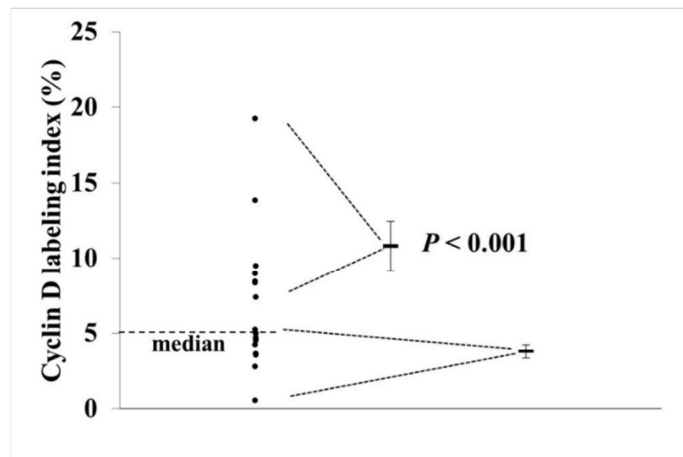
Supporting FIG. S1. (A) Blood levels of the inflammatory markers lipocalin 2 and serum amyloid A1 in active drinkers (black columns). The active drinking group was then analyzed according to the absence (F0-F2) or presence of advanced fibrosis (F3/F4). P values compare to healthy controls (white columns). (B) Representative hematoxylin and eosin (H&E) and myeloperoxidase (MPO) staining in patients with early alcoholic steatohepatitis (ASH) showing low numbers of MPO-positive neutrophils (arrows). Patients with the clinical syndrome of severe ASH (Maddreys's discriminant function >32) and massive neutrophil infiltration were used as a positive control.



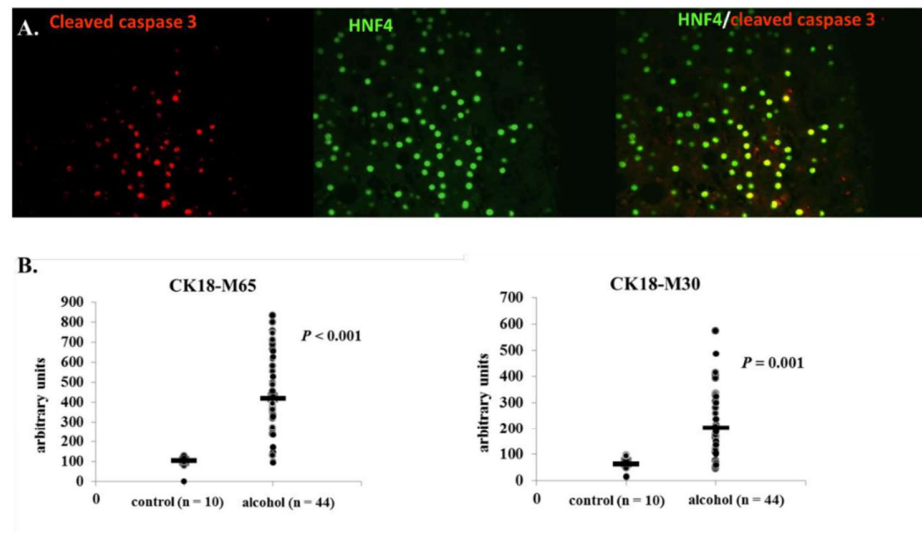
Supporting FIG. S2. Immunofluorescence of signal transducer and activator of transcription 3 (Stat3) in active drinkers with a low proliferation index showing sequestration of Stat3 in the cytoplasm of hepatocytes (hepatocyte nuclear factor [HNF] 4+).



B.

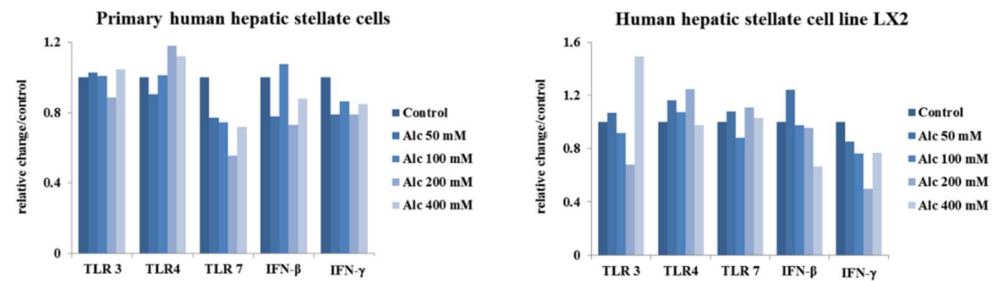


Supporting FIG. S3. (A) Immunohistochemistry of the proliferation markers Ki-67 and cyclin D (Cyc D) in active drinkers with low and high proliferation. (B) Quantification of nuclear Cyc D expression confirming the existence of a low (below median value) and a high (above median value) proliferation group.

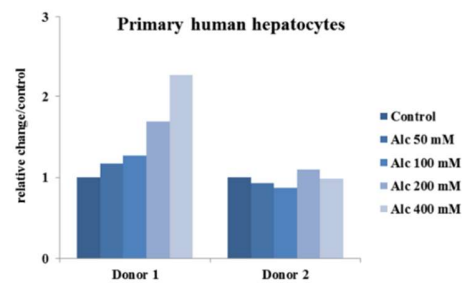


Supporting FIG. S4. (A) Immunofluorescence of cleaved caspase 3 in active drinkers with low proliferation and high apoptosis showing nuclear co-localization of caspase 3 with the hepatocyte marker hepatocyte nuclear factor 4 (HNF4). (B) Elevated plasma levels of the cytokeratin (CK) 18 fragments M65 and M30 indicating hepatocyte damage and apoptosis, respectively, in active drinkers. *P* values compare to healthy controls.

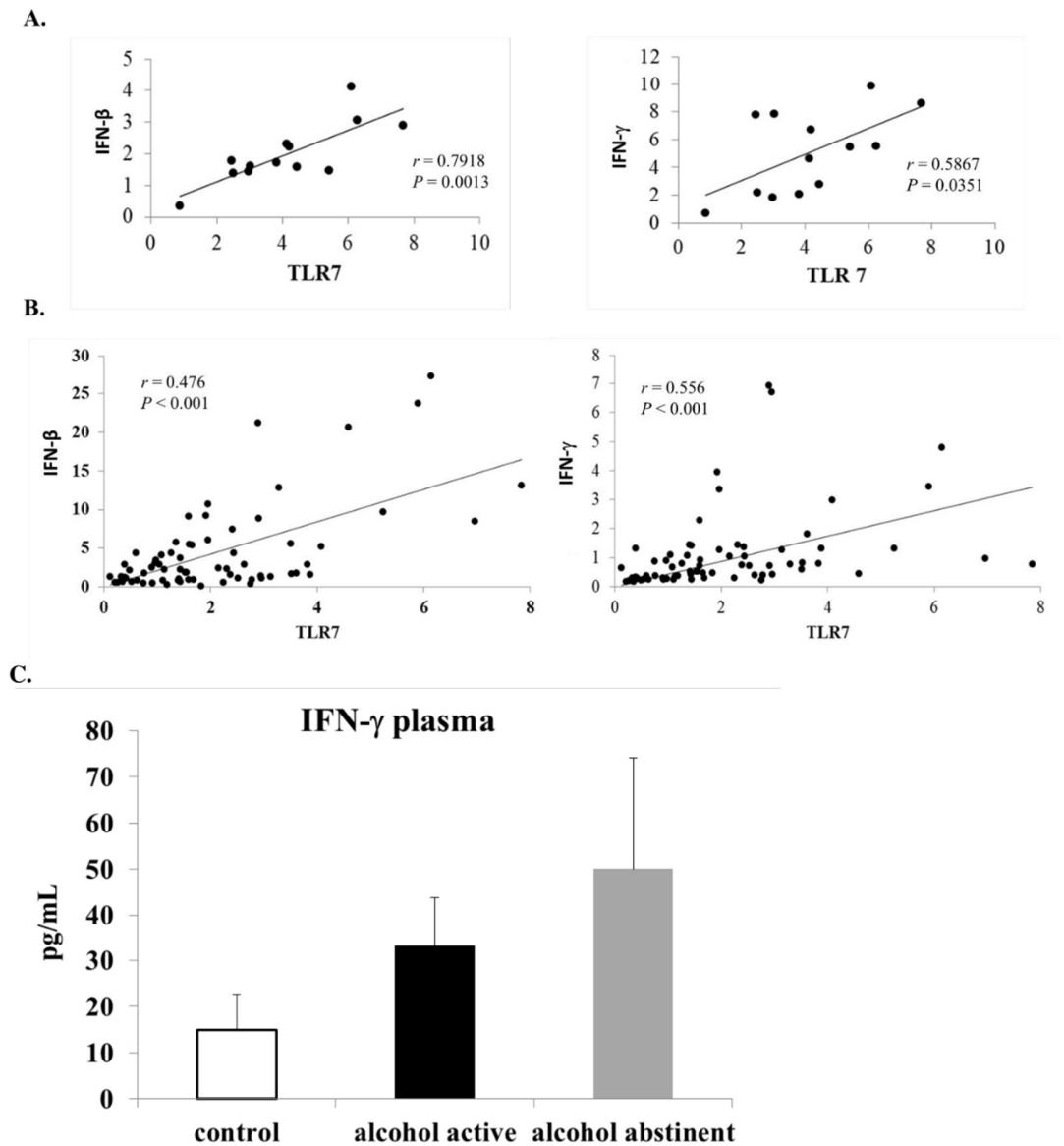
A.



B.



Supporting FIG. S5. Representative experiments of (A) toll-like receptor (TLR) and interferon (IFN) mRNA expression in human hepatic stellate cells and (B) TLR4 mRNA expression in human primary hepatocytes stimulated *in vitro* with various concentrations of alcohol (Alc).



Supporting FIG. S6. (A) Correlation between toll-like receptor 7 (TLR7) and interferon (IFN)- β or - γ mRNA expression in cultured human primary hepatocytes stimulated with increasing doses of ethanol. (B) Correlation between TLR7 and IFN- β or - γ mRNA expression in liver biopsies of alcohol-dependent patients. (C) Plasma levels of IFN- γ in active drinkers and after short-term abstinence (14 days).

Supporting tables

Supporting TABLE S1. Primer sequences used for quantitative PCR

Gene	Access Number	Sense	Antisense
TNF- α	NM_000594	ATCTTCTCGAACCCCGAGTGA	CGGTTTCAGCCACTGGAGCT
IL-1 β	NM_000576	GCACGATGCACCTGTACGAT	AGACATCACCAAGCTTTTGTGCT
IL-1ra		CCCCACCACCAGTTTTGAGT	GCCTTCGTGAGGCATATTGG
IL-6	NM_000600	TGGCTGAAAAAGATGGATGC	TGCAGGAAGTGGATCAGGAC
IL-10	NM_000572	CCAGGGCACCCAGTCTGA	TCGGAGATCTCGAAGCATGTT
iNos		GGTGGAAGCGGTAACAAAGG	TGCTTGGTGGCGAAGATGA
I κ Ba	NM_020529	GCTGAAGAAGGAGCGGCTACT	TCGTACTCCTCGTCTTTCATGGA
CD68		CTCATCATCGGCCTGATCCT	CGGATGATGCAGAAAGCAATAA
CD11b		GGGTCATTTCGTACGTAATTGG	TGTTCCACCAGCTGGCTTAGATG
TLR2	NM_003264	TCCTGTGCCACC GTTTCC	GA GCTTTCCTGG GCTTCCTT
TLR3	NM_003265	CCGAAGGGT GGCCCTTA	TTGCT TAGATCCAGA ATGGTCAAG
TLR4	NM_138554	GATTGCTCAGACCT GGCAGTT	TGTCCTCCC ACTCCAGGTAAGT
TLR5	NM_003268.5	CTGAGCTTCAACTATATCAG	TTGCTGATGGCATTGCTAAAG
TLR7	NM_016562	AGCCCTTTCAGAAG TCCAAGTTC	GGTGAG CTTGCGGGTT TGT
TLR8	NM_016610	TCCCAAACCTT TCTATGATGC TTACAT	GGTGGTAG CGCAGCTCAT TT
CD14	NM_000591	CGCTCCGAGATGCATGTG	AGCCCAGCGAACGACAGA
LBP		GGCTCAGTGTGGCCACTAATG	CACTTTTACCTTTCTGGCTTCA
MD2		ATGTTACCATTCTCTGTTT	CTAATTTGAATTAGGTTG
MyD88	NM_002468	CATATGCCTGAGCGTTTCGA	CATCGCGGTCAGACACACA
IFN- β	NM_002176	CCAACAAGTGTCTCCTCCAAATT	GTAGGAATCCAAGCAAGTTGTAGCT
IFN- γ		CCAACGCAAAGCAATACATGA	CCTTTTTCGCTTCCCTGTTTAA
OAS1		CCACCTGCTTCACAGAACTACAGA	AGGCGGATGAGGCTCTTGA
ISG 6-16		CAGCAGCGTCGTCATAGGTAAT	CCTCATCCTCCTCACTATCGAGAT
ISG 15		TGGCGGGCAACGAATT	GGGTGATCTGCGCCTTCA
Stat3	NM_139276	GG AGGAGGCATT CGGAAAG	TCGTTGGTGTC ACACAGAT
Socs3	NM_003955	TTCAGCATCTCTGTGCGAAGAC	CGGCAGCTGGGTGACTTT
MCP1	NM_002982	GCTCAGCCAGATGCAATCAA	GCCTCTGCACTGAGATCTTCCT
α -SMA	NM_001613	CAAGTGATCACCATCGGAAATG	GACTCCATCCCGATGAAGGA
Collagen 1	NM_000088	GCAAGACAGTGATTGAATACAAAACC	ACGTCGAAGCCGAATTCCT
Timp 1	NM_003254	GCTGACATCCGGTTCGTCTAC	GTTGTGGGACCTGTGGAAGTATC
Rpl19	NM_000981	CAAGCGGATTCTCATGGAACA	TGGTCAGCCAGGAGCTTCTT
GAPDH	NM_001256799	GGGTGTGAACCATGAGAAGTATGA	GGTGCAGGAGGCATTGCT

Supporting TABLE S2. Primary and secondary antibodies and their working conditions

Primary Antibody	Type	Conditions	Source (Cat. No.)
NF-κB p65	IF/WB: rabbit polyclonal	WB: 1/1000; ON at 4°C IF: 1/200; 1 hour at 37°C	Santa-Cruz (SC-372), Heidelberg, Germany
NF-κB p-p65	WB: rabbit polyclonal	1/2000; ON at 4°C	Santa-Cruz (SC-33020)
CD68	IHC: mouse monoclonal IF: goat polyclonal	1/30; 1 hour at RT 1/50; 1 hour at 37°C	Dako (PGM1), Glostrup, Denmark Santa-Cruz (SC-7082)
IRF3	WB: rabbit monoclonal IF: rabbit polyclonal	WB: 1/1000; ON at 4°C IF: 1/50; 2 hours at 37°C	Cell Signaling (D83B9), Danvers, MA, USA Abcam (ab25950), Cambridge, UK
p-IRF3	WB: rabbit monoclonal	WB: 1/1000; ON at 4°C	Cell Signaling (4D4G)
CK7	IF: mouse monoclonal	IF: 1/100; 30 minutes at RT	Abcam (ab9020)
Stat3	WB/IF: rabbit polyclonal	WB: 1/1000; 2 hours at RT IF: 1/25; 45 minutes at 37°C	Santa Cruz (sc-482)
p-Stat3	WB: mouse monoclonal	1/1000; ON at 4°C	Cell Signaling
HNF4	IF: mouse monoclonal	1/200; 2 hours at 37°C	Perseus Proteomics (PPH1415), Tokyo, Japan
Cyclin D	IF/IHC: rabbit monoclonal	IHC: ready to use; 2 hours at 37°C IF: ready to use; 1 hour at 37°C	Ventana (790-4508) Tucson, AZ, USA
Ki-67	IHC: mouse monoclonal	1/200; 1 hour at RT	Dako (Mib-1)
Cleaved caspase 3	IF: rabbit polyclonal	1/100; 2 hours at 37°C	Cell Signaling (9661)
<u>Myeloperoxidase (MPO)</u>	IHC: rabbit polyclonal	1/1800; 1 hour at RT	Dako (Agilent A0398)
Secondary Antibody			
Anti-goat IgG/HRP	Rabbit polyclonal	1/200; 1 hour at RT	Dako (P0449)
Dako REAL EnVision-HRP	Goat anti-rabbit	Ready to use; 1 hour at RT	Dako (K4002)
Anti-rabbit IgG (Alexa Fluor 488)	Goat polyclonal	1/200; 30 minutes at RT	Invitrogen (R37116), Merelbeke, Belgium
Anti-rabbit/mouse IgG (Alexa Fluor 594)	Goat polyclonal	1/200; 30 minutes at RT	Invitrogen (R37117/R37121)
Anti-goat IgG (Alexa Fluor 594)	Donkey polyclonal	1/200; 30 minutes at RT	Invitrogen (A-11058)

Abbreviations: IF, immunofluorescence; IgG, immunoglobulin; IHC, immunohistochemistry; ON, overnight; RT, room temperature; WB, western blot.

Complementary discussion and conclusion

The compensatory proliferative capacity of the liver has been extensively studied in animals undergoing partial hepatectomy but data about liver regeneration in disease conditions are particularly scarce. We here demonstrated that a blunted IL-6/Stat3 axis related to increased hepatocytes apoptosis. On the other hand, livers that were able to overcome alcohol-associated Stat3 blockage, as indicated by preserved nuclear translocation of Stat3, retained some proliferative capacity. In parallel, activation of pro-inflammatory macrophages leads to release of IL1 β in the liver thus contributing to chronic inflammation. TLR7 up-regulation occurred concomitantly with activation of interferon signaling predominantly in hepatocytes. Activation of the TLR7–IFN axis strongly correlated with liver fibrosis markers and disease progression (Fig.5.1).

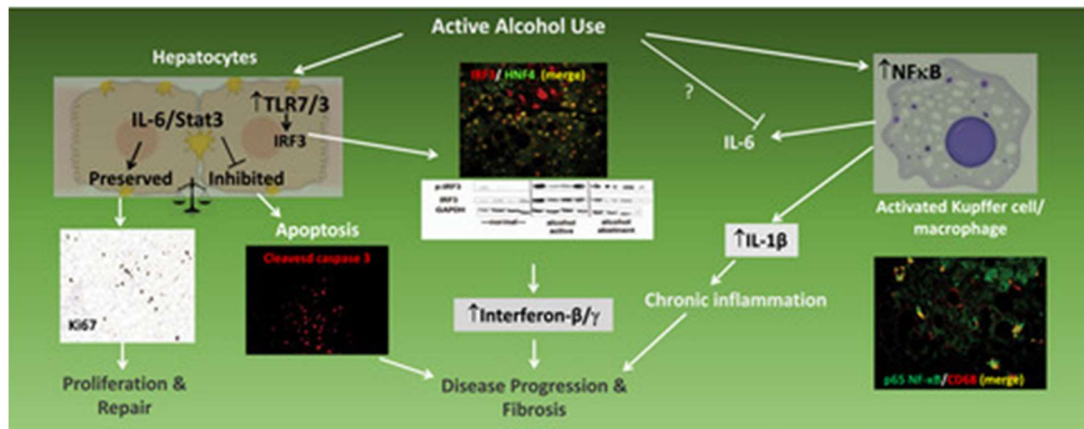


Fig.5.1 Schematic representation of the findings obtained in the study described in chapter 6. Activation of liver macrophages with release of IL1 β , blunted IL6/Stat3 linked to impaired liver regeneration and increased apoptosis together with activation of TLR7/Interferon I axis in hepatocytes all contribute to alcohol-associated liver disease (ALD) progression and fibrosis development.

GENERAL DISCUSSION AND PERSPECTIVES

GENERAL DISCUSSION

The results of this PhD thesis strongly support the key role of gut barrier dysfunctions in the progression of ALD at early pre-cirrhotic stages. We comprehensively uncover the complex relationship existing between intestinal barrier alterations and activation of systemic and liver inflammatory responses implicated in disease progression in humans. As ALD is a silent disease at early disease stage and since identification of more progressive forms is not only crucial for the management of AUD patients but also for the correct control of disease progression, we used a combination of non-invasive techniques for the assessment of liver fibrosis and cell damage. In this regard, we found in the first chapter that the circulating levels of the biomarker K18-M65 can be used to distinguish with a very high-performance patients with progressive ALD (steatohepatitis and fibrosis) from the benign forms of the disease. High K18-M65 levels are primarily markers of hepatocytes damage and could be used to detect “silent” severe forms of liver disease. Other studies have reported the diagnostic value of K18-M65 in discriminating progressive forms of fatty liver disease with different etiology, namely patients with benign non-alcoholic fatty liver disease (NAFLD) from non-alcoholic steatohepatitis (NASH)^{171,172,173}. Our study extends current data on the use of circulating cytokeratin 18 levels also to early stages of ALD. We then used the high diagnostic power of our integrated approach in the next chapters for monitoring liver disease progression and/or regression in AUD patients as well as to identify the relationships between intestinal changes and ALD.

The principal goal of the second study was to evaluate changes in the gut barrier in AUD patients and secondly, how they relate to liver disease. Increased intestinal paracellular and vascular permeability, as measured by the ⁵¹Cr-EDTA and fecal albumin, respectively, were found only in a minority of AUD patients. These two measurements did not correlate each other, supporting data obtained in animal models that indicate independent regulation of epithelial and vascular barriers in the gut¹¹³. Leaky gut did not associate with elevated systemic translocation of microbes and/or their products, result that leads us to ask what in the intestinal barrier can drive the real “leakiness” to translocation of microbes and/or their products in the blood circulation. Since the gut immune system plays an important role in immunosurveillance against microbes, we therefore explored the duodenal immune

system and found profound changes in the number and functions of gut immune effector cells, such as macrophages and T lymphocytes. A diminution of these immune cells together with the potential presence of a “tolerant-immunosuppressive status” linked to increased expression of regulatory and anti-inflammatory cytokines was strikingly related to elevated translocation of bacterial and fungal products. In addition, these phenomena (e.g., reduction of duodenal immune cells and elevated microbial translocation) were associated with increased attachment of microbes to the duodenal mucosa with predominant shift towards certain specific bacteria coming from the oral cavity. Changes in the proximal small intestine (gut immune system, duodenal microbiota, and elevated translocation of microbes and/or their products) strongly correlated with liver cell damage and progressive ALD, supporting that defective gut immunosurveillance against microbes could be a key event in the pathogenesis of alcohol-associated liver disease in humans.

The reduction of duodenal T cells could be explained by different mechanisms, which were therefore explored in the third chapter. We here revealed that a specific reduction together with functional changes of duodenal CD8⁺ T resident memory lymphocytes (TRM) in progressive ALD correlated with elevated microbial translocation and liver disease progression. TRM cells undergo apoptosis at least partially related to lysosome dysfunction and altered lipid metabolism. Regarding the lysosomes, we found that lysosomal membrane permeabilization with subsequent release of Cathepsin B was a driver of apoptosis. Our result open the intriguing possibility that in AUD patients lysosome stability is an important regulator of the maintenance of memory CD8⁺ T cells¹⁷⁴ in the duodenal mucosa. On top of lysosomal alterations, we found reduced mitochondrial ROS production in TRM of patients with progressive liver disease linked to uncoupling by the uncoupling protein 2 (UCP2). Lower levels of mitochondrial ROS in intestinal CD8⁺ T lymphocytes, a condition known as a “reductive stress”¹⁷⁵, might impact their survival. Under normal conditions, the maintenance of correct ROS levels in mitochondria is important to initiate protective signaling cascades related to cell proliferation, differentiation, immune responses and survival^{175,176}. Further studies should allow to gain more insight into the complex interaction between redox homeostasis, metabolic status, cell death and immune responses in human intestinal CD8⁺ TRM.

Apoptosis of duodenal TRM strongly contributed to elevated systemic microbial translocation in patients with progressive ALD. Consequent activation of systemic and liver immune responses by translocated gut-derived products were evaluated in the last two chapters of this PhD thesis. Our study in the fourth chapter demonstrated a higher activation of the TLR2/Inflammasome/AP-1 pathway in response to peptidoglycan in increased numbers of circulating monocytes. Monocytes from AUD patients differentially release key inflammatory mediators such as IL1 β and IL8 contributing to systemic inflammation in AUD patients. In addition, levels of Gram+ translocation marker PGRPs were correlated with proportion of intermediate monocytes. Those monocytes express high levels of MHC class II processing and presentation genes¹⁷⁷, and this result may reflect a link between translocation of bacterial products such as peptidoglycan, recognition and presentation of antigens to T cells with subsequent activation of adaptive immune responses in the circulation. By contrast of peptidoglycan-related immune response, we revealed an intriguing down-regulation of CARD9, an intracellular sensor playing a pivotal role in fungal immune surveillance¹⁷⁸. In accordance with this result, stimulation of monocytes with *Candida albicans* showed a reduced transcription of inflammatory mediators IL1 β and TNF- α in monocytes from AUD patients compared to controls. Future analyses could help us to elucidate mechanisms implicated in immunosurveillance against fungi and relative mechanism of translocation of fungi and/or their products in ALD. Circulating inflammatory monocytes are recruited to the liver during inflammation and fibrosis, principally involved in alcohol-associated liver disease progression⁵⁰. Once in the liver, monocytes transform into monocyte-derived macrophages (MDM) which have been shown to play a key role in ALD development in animal models^{63, 156}. Remarkably, expression of hepatic inflammatory mediators, such as the pro-inflammatory cytokine IL1 β and especially the chemokine IL8, increased progressively in more severe forms of liver disease as compared to simple fatty liver. Moreover, both factors strongly correlated with markers of hepatocyte damage and apoptosis, pointing to their contribution in the early development of alcohol-associated liver disease in humans. Recruitment of monocytes appears to play an important role, since liver chemokines MCP1 and CX3CL1 correlated with IL1 β and IL8 and associate with a more advanced disease state such as presence of significant fibrosis. Targeting IL1 β may represent a future therapeutic target to ameliorate alcohol-associated liver disease. Interventions aiming at blocking IL1 β signaling in rodents

chronically fed with ethanol markedly attenuated alcohol-induced liver inflammation, steatosis, and damage¹⁷⁹. Canakinumab, an anti-IL1 β antibody, is now in phase II of a clinical trial for the treatment of AUD patients with severe AH¹⁸⁰. However, it is important to remember that a similar treatment with antibodies anti-TNF- α was proved inefficient and dangerous in AH patients¹⁵⁷. Targeting IL1 β may encounter the same failure, since this cytokine also play a key role in controlling and coordinating intestinal and liver immune responses against microbes. Identifying and targeting multiple signaling pathways in well-characterized patients according to ALD stage and degrees of gut barrier dysfunction may represent the future of the interventions for ALD.

We finally assessed liver inflammatory and immune responses at early stages of ALD and to correlate them with liver damage and repair mechanisms and/or progression of fibrosis. We showed that a proinflammatory response, including production of TNF- α and IL-1 β by liver macrophages, already occurred at very early stages of the disease. The early proinflammatory response was alleviated by short-term abstinence. By contrast, the IL-6/ Stat3 axis was blunted and Stat3 sequestered in the cytoplasm of hepatocytes in livers with increased apoptosis. On the other hand, livers that were able to overcome alcohol-associated Stat3 blockage, as indicated by preserved nuclear translocation of Stat3, retained some proliferative capacity. In contrast to animal data, intracellular TLRs, in particular TLR7, increased at early stages in humans, whereas TLR4 did not⁴⁸. The fact that these inflammatory signaling pathways are differentially activated in hepatocytes point to the important role of parenchymal liver cells in ALD. TLR7 is an intracellular receptor able to recognize single-stranded ribonucleic acids. Induction of TLR7-Interferon I axis is particularly important in antiviral immune responses as well as in sensing both single-stranded ribonucleic acids from dying cells and certain miRNAs¹⁸¹. We here investigated only the possibility that increased expression of TLR7 could be linked to danger-associated molecular patterns (DAMPs) released by dying hepatocytes, because of “sterile” inflammation. Regarding the possible implication of gut-derived viruses, a recent investigation has looked at the fecal virome in AUD patients and in patients with severe AH. In this study, main divergences were found predominantly in patients with severe AH¹³⁰. However, as shown for the gut bacteria in chapter 2, changes in intestinal viruses related to the proximal small gut may be different and potentially linked to ALD progression in early pre-cirrhotic stages. The third possibility is that

miRNAs could be delivered to hepatocytes with the consequent activation of TLR7/Interferon I cascade. These possible interactions clearly deserve future investigations.

GENERAL CONCLUSION AND ADVANCES IN THE FIELD

Overall, the data obtained in this PhD work are in favor of the key role of gut barrier dysfunctions in the pathogenesis of early human ALD progression. We here revealed for the first time that reduced immunosurveillance against microbes was driven by the diminution of duodenal CD8⁺ T resident memory lymphocytes as well as macrophages and possibly by the presence of a pro-tolerogenic status in the mucosa of patients with progressive ALD. Interestingly, duodenal immune alterations and elevated translocation of microbes and/or their products occurred independently on the presence of increased intestinal paracellular and vascular permeability. These results are pointing the specific contribution of impaired gut immunity in elevated microbial translocation in early stages of ALD.

Previous data in the field of ALD already described the contribution of increased intestinal permeability and changes in the gut microbiota in elevated translocation of microbes and/or their products, a process implicated in the activation of systemic and liver immune responses. However, these studies principally used animal models of ALD, which present intrinsic limitations (e.g., differences in intestinal immune system and microbiota between animals and humans) for the evaluation of the gut-liver axis. Moreover, studies in humans generally consider advanced stages of ALD. Last, but not least, previous studies in humans lacked a standardization of the clinical settings for the evaluation of alcohol-associated intestinal alterations in actively drinking and abstinent subjects. By contrast to animal models, we showed that increased intestinal permeability is not absolutely required for translocation of microbes and/or their products to occur in early ALD. However, microbial translocation associated with duodenal immune and microbiota changes suggesting that additional elements of the gut barrier need to be impaired to finally make this process possible. In addition, thanks to the high standardization of the alcohol withdrawal program, we were able to investigate gut barrier dysfunctions in actively drinking and abstinent patients. Strikingly, we found that increased intestinal permeability was reversed to the level of the healthy controls upon short-term abstinence, which was not the case for duodenal

immune changes, microbial translocation and liver cell damage. These results further support the relationship between duodenal reduced immunosurveillance, translocation of microbes and/or their products and ALD progression in early disease stages.

As a consequence of elevated translocation of microbes and/or their products, we revealed differential activation of circulating monocytes and liver inflammatory responses in AUD patients. Increased number of circulating monocytes activated through TLR2 by translocated PGN contributed to systemic and liver inflammatory responses via IL1 β and IL8 secretion. Furthermore, monocytes infiltrate the liver already in early ALD stages and they transform into pro-inflammatory macrophages, a process strongly associated with liver fibrosis and disease progression (Figure 1).

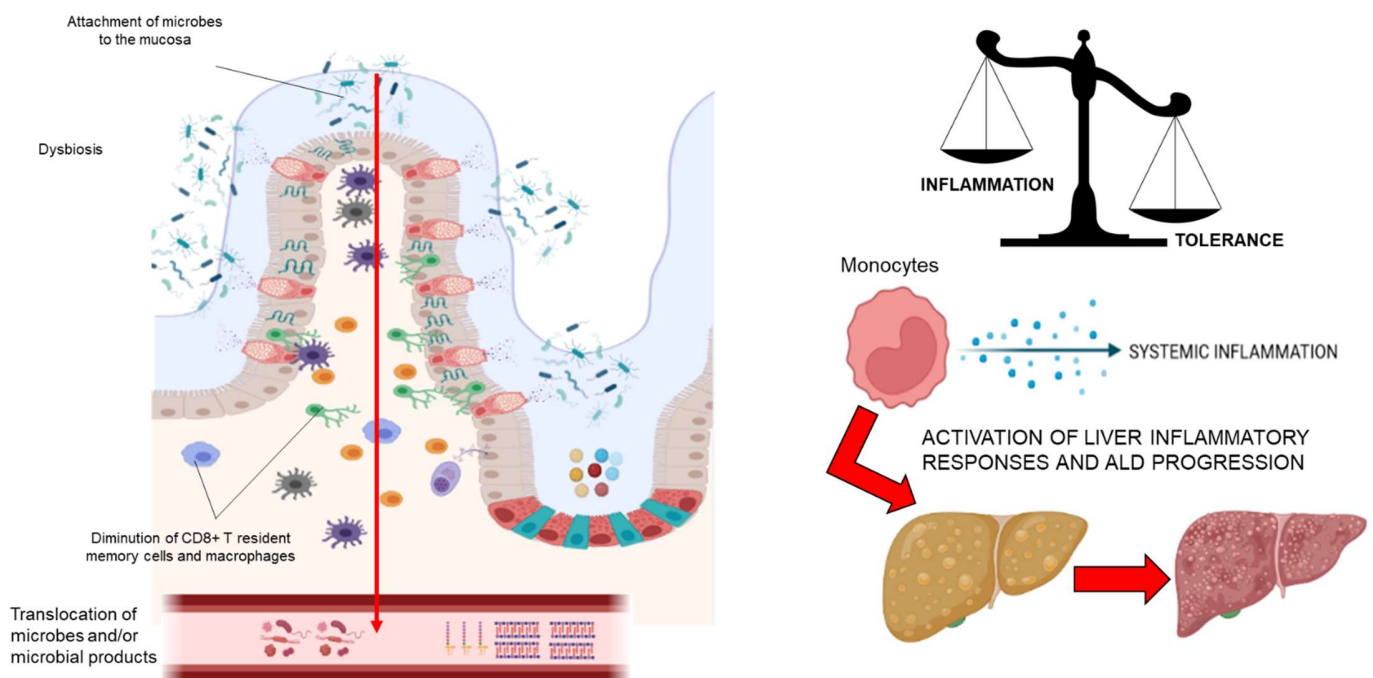


Figure 1. Gut barrier dysfunctions in early ALD. Reduced immunosurveillance against microbes due to a diminution of CD8+ T resident memory lymphocytes as well as macrophages and the presence of an immune tolerant status are associated with dysbiosis and elevated translocation of microbes and/or their products in the portal and systemic circulation. Increased translocation of microbes and/or their products contribute to activation of monocytes and liver inflammatory responses, processes implicated in ALD.

A limitation of these studies is that data are in part descriptive and/or based on correlations. Furthermore, since suspicion of liver disease was a prerequisite for liver biopsy, more severe stages might be overrepresented in cohorts where liver biopsies were analyzed. Ex vivo and in vitro approaches have been taken to explore mechanisms related to the observations made in humans. However, all these approaches do not allow to formally establish a cause-effect relationship.

PERSPECTIVES

To try to overcome the intrinsic limitations we faced during this PhD work while studying human alcohol-associated gut barrier dysfunctions and ALD pathogenesis, other mechanisms and cell types implicated in the gut-liver axis remain to be investigated implying the use of novel technologies and systems. Four major topics should be investigated.

1. Evaluation of metabolites possibly linked to gut barrier dysfunctions and ALD using a Multi-Omics approach

Although leaky gut was not related to microbial translocation and liver cell damage, we showed in study 2 that increased intestinal paracellular permeability, mainly observed in the proximal small gut, associated with fecal microbiota changes and with ALD progression (in particular liver fibrosis, figure 1.1). These observations raise the question on how the fecal microbiota could promote leaky gut at distant site not directly in contact with these microbes. The microbiota could produce different metabolites that, once in the circulation, could reach the proximal small intestine thus contributing to leaky gut. In accordance to this hypothesis, metabolomics analysis of volatile compounds in the stools of AUD patients has shown in a previous study the presence of different phenolic metabolites as well indoles associated with increased intestinal permeability¹²⁷. This observation supports the potential role of gut-derived metabolites in the development of leaky gut in AUD patients. A future analysis of metabolites by untargeted and targeted metabolomics approaches in the stools and in the systemic circulation is needed to test our hypothesis. Metabolites identified could be tested in vitro systems (see below) to show a potential cause-effect-relationship.

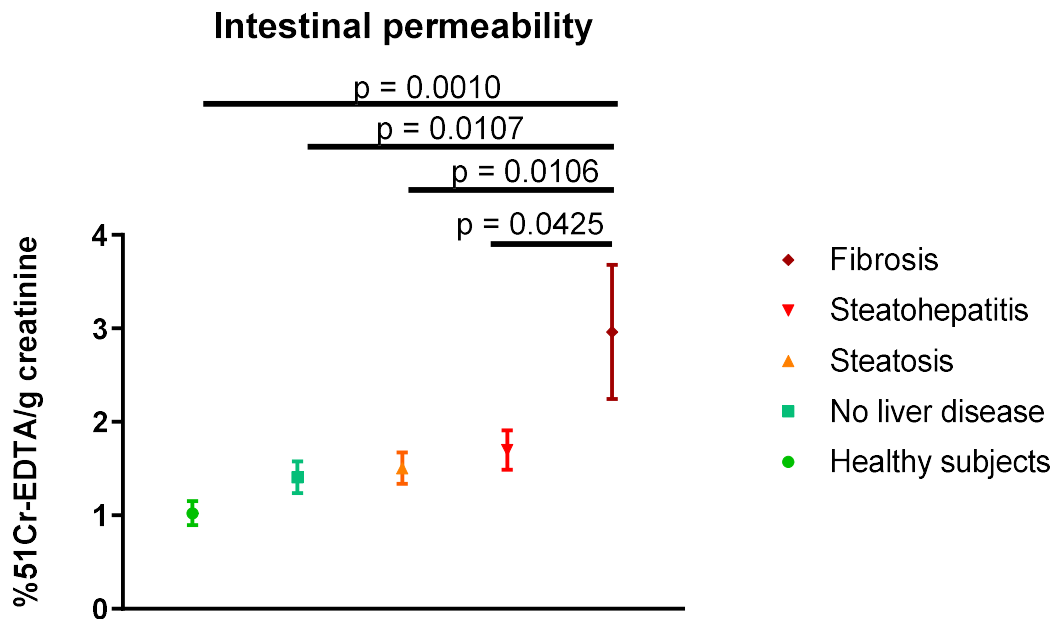


Fig.1.1 Intestinal paracellular permeability during the different stages of alcohol-associated liver disease.

In this PhD work, we evaluated the lipids in the circulation and in the stools which differentiate progressive ALD by using an untargeted lipidomics approach. Increased levels of certain saturated sphingomyelins correlated with changes in intestinal TRM, this suggests that these lipids may influence TRM function. Additional evidence suggest that these sphingolipids are likely not derived from increased absorption, raising the possibility that they are locally produced. Analysis of the duodenal sphingolipid composition by a targeted lipidomics approach should help us to decipher local changes in sphingolipids. Sphingolipid levels should then be analyzed together with transcriptomic and proteomic data obtained from biopsies of the same patients, to decipher molecular pathways alterations that may promote this potential accumulation. Bioinformatics integration of these big data is mandatory to identify the connections between the various omics information and their relationship with ALD.

2. Evaluation of the duodenal tolerogenic status and TCR repertoire in TRM

Since duodenal TRM had increased expression of the anti-inflammatory cytokine IL1RN, we have hypothesized that a tolerogenic environment could be present in the proximal small intestine of AUD patients with progressive ALD. To test this hypothesis, gene and protein expression of regulatory duodenal cytokines, such as

IL10 and TGF- β 1, on duodenal biopsies together with single-cell RNA sequencing on isolated duodenal cell suspensions could be performed to identify which cell types differentially produce these cytokines.

Additional mechanisms might be required on top of the presence of an immune tolerogenic status in the mucosa to facilitate translocation of microbes and/or their products in the circulation. A diverse TCR repertoire is required for proper response against invading pathogens. We have hypothesized that reduced number of duodenal TRM results in decreased diversity and recognition of specific pathogens in the intestine which might contribute to liver disease progression. To address this hypothesis, the next steps should consist in:

- Analysis of TCR repertoire together with single-cell transcriptomic analysis to characterize T resident memory cells heterogeneity
- Evaluation by high-throughput proteomics of proteins from duodenal microbes potentially recognized by TRM
- Evaluation of TRM responsiveness to the identified microbial proteins

3. Evaluation of the changes in the intestinal epithelium in AUD patients

Assessment of gut barrier dysfunction has been performed by measuring markers of intestinal permeability, microbial translocation and especially looking at immune responses in the proximal small intestine. However, as introduced in the section 1.5.2, beside the intestinal immune system, the gut epithelium represents a first layer of defense against potential invading pathogens⁸⁶. Duodenal biopsies in actively drinking patients revealed reduced villi length compared to controls. This alteration was found to be independent from intestinal permeability changes. We further found increased crypt length, also known as crypt hyperplasia, in the duodenum of AUD patients compared to controls (Fig.1.2).

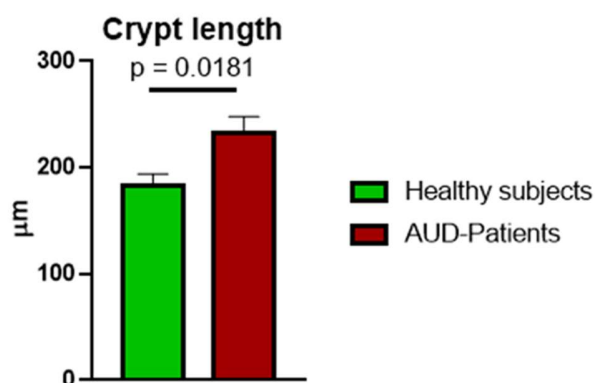


Fig.1.2 Evaluation of crypt length in the duodenum of alcohol use disorder (AUD) patients showed increased crypt length in the duodenum of AUD patients compared to controls.

AUD patients had a significantly higher ratio of Ki67 (a marker of proliferation) positive cells per crypt in the duodenal epithelium indicating an increase in proliferation in the duodenal epithelium of AUD patients (Fig.1.3). Intriguingly, AUD patients have a significantly lower ratio of stem cell niches with a Ki67+ nuclei per crypt compared to the control group (Fig.1.3) and the proliferation was mainly confined to the transit amplifying zone. In parallel, we found a significantly higher number of goblet cells per crypt and villi (Fig.1.4). All these changes suggest a profound alteration of the proliferation/differentiation program in the duodenal epithelium of AUD patients.

This preliminary investigation did not allow us to investigate the presence and the real proliferative capacity of the adult intestinal stem cells in AUD patients. Therefore, using LGR5 as adult stem cell marker combined with Ki67 could allow us to verify if

the adult stem cells are still present in AUD patients and if they maintain or not their normal proliferative activity. In addition, since correct renewal is fundamental for gut epithelial functions, it will be important to investigate which pathways (for example EGF, Wnt/ β -Catenin etc.) drive increased proliferation in the duodenal epithelium of active drinkers.

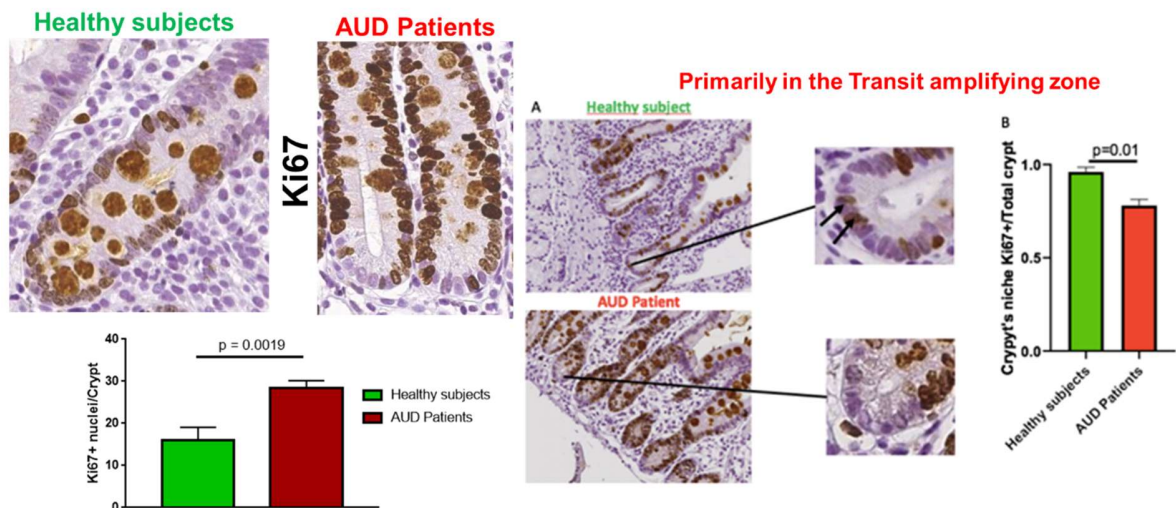


Fig.1.3 Increased proliferation in the crypt of alcohol use disorder (AUD) patients compared to controls (left) was primarily linked to proliferation in the transit-amplifying zone (right).

Expression of duodenal transcription factors implicated in goblet cell differentiation⁸⁸, such as SPDEF (SAM Pointed Domain Containing ETS Transcription Factor) and KLF4 (Krüppel-like factor 4), could be evaluated to identify differentiation programs altered in the duodenal epithelium of AUD patients.

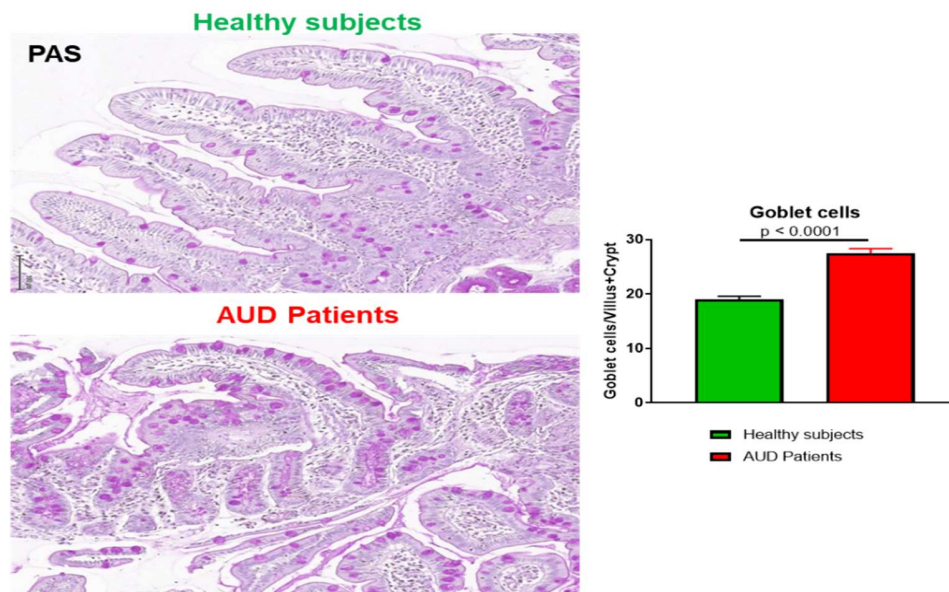


Fig.1.4 Periodic Acid Schiff (PAS) staining marking mucus-producing goblet cells showed increased number of goblet cells in the duodenum of alcohol use disorder (AUD) patients compared to controls.

Based on these results, we propose the hypothesis that changes in the proliferation/differentiation programs are a “first hit” in the physiopathology of gut barrier dysfunction in AUD patients. Modifications in the intestinal metabolic microenvironment and subsequent promotion of a tolerant immune status could be seen as a “second hit” in the cascade of events implicated in the dysfunctions of host protective mechanisms of the intestinal barrier. These phenomena would allow microbial invasion and translocation into the blood circulation (Fig.1.5).

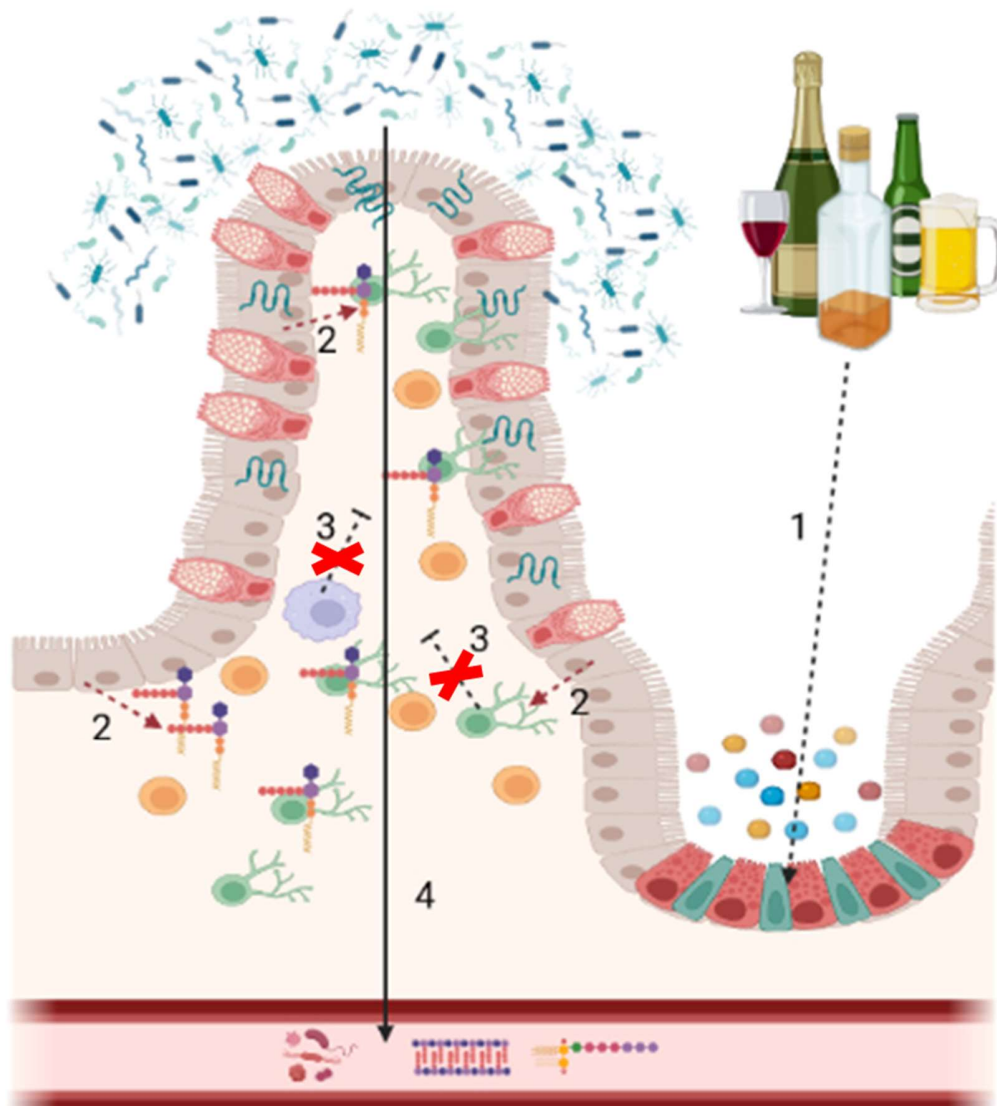


Fig.1.5 New hypothesis of alcohol-associated gut barrier dysfunction. Ethanol and/or ethanol metabolites may provoke alterations in the intestinal epithelium (1) such as changes in the proliferation/differentiation program. In certain AUD patients with progressive ALD, epithelial dysfunction may lead to synthesis and accumulation of metabolites in the duodenum (2). These metabolites could interfere with immune cells functions in the small gut (3), ultimately allowing microbes to proliferate, attach to the mucosa and translocate into the circulation (4).

4. Development of a new system allowing to test a potential cause-effect relationship between the observed gut changes in AUD patients

At this stage, we have no formal proof of a cause-effect relationship between all the observed changes in the intestinal barrier in relation to ALD progression. Studies in humans have the intrinsic limitations that a direct modulation of the different mechanisms and factors potentially implicated in the physiopathology is not possible for obvious ethical reasons. To continue to study more in depth the mechanisms and cause-effect relationships existing in humans between the observed changes, we have therefore started to develop promising models reproducing important features characterizing in vivo the intestinal epithelium. These in vitro models are called enteroids and can be derived from primary stem cells obtained from distal duodenal biopsies. Enteroids will enable us to perform targeted interventions and modulations of the environment (inhibitors, agonists and antagonists, RNA interference, use of lipids or aqueous metabolites identified by lipidomics or metabolomics etc.) to investigate more thoroughly the signaling pathways implicated not only in the differentiation and proliferation programs, but also to characterize the functionality of differentiated cells from AUD patients. Intestinal enteroids are limited by the fact that only epithelial cells are present. Therefore, co-culture of organoids with isolated immune cells from the same patients could help to identify mechanisms underlying these potential interactions. In addition, pre-conditioning of epithelial or immune cells with ethanol or other factors and subsequent use of the pre-conditioned medium could establish a cause-effect relationship between these changes.

Thanks to this innovative tool, we will be able to address the following questions:

- 1.) Is ethanol and/or its metabolites contributing to the changes in proliferation and/or differentiation in the duodenal epithelium of AUD patients?
- 2.) Which are the changes in epithelial cells functions contributing to the overall impairment of the intestinal barrier in AUD patients?
- 3.) Are there cause-effect relationships between alterations in the intestinal epithelium and gut immune system?

These studies will help us decode the key molecular mechanisms involved in alcohol-associated gut barrier dysfunction and liver disease progression during early human ALD. Understanding the cause-and-effect relationships between host and microbial

changes would provide us with the identification of molecular culprits which could be targeted to prevent and/or ameliorate ALD.

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ANNEXES

ANNEX 1 Host factors in dysregulation of the gut barrier function during alcohol-associated liver disease



Review

Host Factors in Dysregulation of the Gut Barrier Function during Alcohol-Associated Liver Disease

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Citation: Maccioni, L.; Leclercq, I.A.; Schnabl, B.; Stärkel, P. Host Factors in Dysregulation of the Gut Barrier Function during Alcohol-Associated Liver Disease. *Int. J. Mol. Sci.* **2021**, *22*, 12687. <https://doi.org/10.3390/ijms222312687>

Academic Editor: Takemi Akahane

Received: 25 October 2021

Accepted: 23 November 2021

Published: 24 November 2021

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Abstract: Chronic alcohol consumption and alcohol-associated liver disease (ALD) represent a major public health problem worldwide. Only a minority of patients with an alcohol-use disorder (AUD) develop severe forms of liver disease (e.g., steatohepatitis and fibrosis) and finally progress to the more advanced stages of ALD, such as severe alcohol-associated hepatitis and decompensated cirrhosis. Emerging evidence suggests that gut barrier dysfunction is multifactorial, implicating microbiota changes, alterations in the intestinal epithelium, and immune dysfunction. This failing gut barrier ultimately allows microbial antigens, microbes, and metabolites to translocate to the liver and into systemic circulation. Subsequent activation of immune and inflammatory responses contributes to liver disease progression. Here we review the literature about the disturbance of the different host defense mechanisms linked to gut barrier dysfunction, increased microbial translocation, and impairment of liver and systemic inflammatory responses in the different stages of ALD.

Keywords: intestinal barrier; chronic alcohol consumption; gut-liver axis; intestinal immunity; inflammation

1. Introduction

Chronic alcohol consumption is one of the leading causes of chronic liver disease and liver-related deaths worldwide. Although more than 90–95% of people with excessive alcohol drinking develop fatty liver, only a minority (10–20%) of subjects with an alcohol-use disorder (AUD) ultimately progress to more advanced stages of alcohol-associated liver disease (ALD) (e.g., severe alcohol-associated hepatitis and decompensated cirrhosis) and its related complications [1]. Some factors that are correlated with disease progression have been identified, including sex, drinking patterns, genetics, obesity, and dietary factors. Those factors either increase susceptibility to liver damage or aggravate disease by acting synergistically with alcohol [2,3]. The interaction between obesity and alcohol, in particular for mild or moderate consumption, is controversial in humans [4]. The incidence of obesity and the associated metabolic syndrome is increasing worldwide. A recent cohort study showed that patients with excessive alcohol consumption have increased prevalence of obesity and metabolic syndrome [5], which might increase the risk for liver disease progression. It has been reported that moderate alcohol consumption increases the risk of development of hepatic steatosis in association with obesity. In contrast, moderate alcohol consumption reduces mortality in normal body mass index (BMI) and overweight individuals. This beneficial effect was, however, lost in obese subjects [6]. In rodents, the synergism between alcohol and obesity might be linked to alterations in immune responses, metabolism, and gut microbiota composition [3]. Recent animal data where alcohol binges have been added on top of a non-alcoholic steatohepatitis model support a synergistic effect

for liver damage (for a detailed review see [7]). Even if some advances have been made in our understanding of the factors involved in the development of ALD, there are still no FDA-approved therapies for treating patients with ALD and abstinence is considered the most effective treatment option [2]. Drugs aiming at reducing the immune responses have been studied for patients with severe forms of liver disease, especially in severe alcohol-associated hepatitis [2]. However, none of these approaches have proven to be safe and efficient [2].

The pathophysiology of gut barrier dysfunction associated with alcohol abuse is likely multifactorial. Changes in the intestinal microbiome, including bacteria, fungi, and viruses, have been associated with ALD [8–11]. This so-called intestinal dysbiosis together with increased intestinal permeability is thought to lead to microbial translocation. Microbes and/or pathogen-associated molecular patterns (PAMPs) reach the liver and serve as a trigger for the initiation of an inflammatory response. Consequently, studies targeted the gut microbes as part of the intestinal barrier for the potential treatment of ALD with some success (for a more detailed review see [12]). Other reports have described how targeting intestinal permeability might prove beneficial also as a potential treatment for liver diseases [13].

This general concept primarily derived from animal studies is, however, challenged by observations made in humans indicating that only approximately 40% of subjects with alcohol abuse have measurable intestinal dysbiosis [14]. Moreover, increased intestinal permeability in humans does not seem to be a prerequisite for microbial translocation to occur [15]. These observations imply that additional factors beyond microbiota changes and intestinal permeability are likely implicated in the pathophysiology of ALD. They also highlight the difficulties with extrapolating animal data to human pathology for several reasons: (1) animals have a natural aversion to alcohol, requiring ways of administration that do not mimic human alcohol-seeking behavior [16,17]; (2) rodents have a 5-times faster ethanol metabolism, making it difficult to reach stable high alcohol levels [18]; (3) compared to humans, rodents are characterized by profound differences in their immune system [19] and their microbiota [20]; and (4) animals only develop mild forms of ALD upon chronic alcohol feeding and even the most “sophisticated models” do not resume the liver-damage pattern observed in humans [21].

Currently, detailed mechanisms of gut barrier dysfunction, especially in humans, are not available, and we generally lack a cause–effect relationship between changes in the intestine and ALD progression. Most of the available data is based on a series that have included only small numbers of patients which do not allow to consider heterogeneity and potential high variability in humans. The functions of the gut barrier are also ensured by factors that are specific to the host, such as the mucus, antimicrobial molecules, and immune cells. Emerging evidence suggests that dynamic modifications in the intestinal epithelium and immune responses as well as reduced immunosurveillance of microbes occur in parallel with microbial translocation and are associated with liver disease severity [22]. Because of the interaction between the microbiota and the host it is not entirely possible to dichotomize ALD pathophysiology into microbial changes and alterations in the host. However, a comprehensive overview of the host-related intestinal alterations linked to the different stages of ALD is lacking. Therefore, this review aims to highlight the advances made in the comprehension of the host factors involved in the maintenance of the intestinal barrier in healthy conditions and the changes linked to alcohol abuse and ALD progression, with specific attention paid to the data described from humans.

2. The Gut Barrier

2.1. Gut Microbiome

The gut is the natural habitat for 100 trillion microorganisms (bacteria, archaea, fungi, and viruses) [23]. This community of microbes’ genome—the microbiome—encodes 100 times more genes than the human genome [24]. These microbes exert a remarkable influence on the host during homeostasis and disease. Indeed, the gut microbiota can mod-

ulate multiple physiological functions of the host, such as strengthening gut integrity and shaping the intestinal epithelium, harvesting energy [25], protecting against pathogens [26], and regulating immune response [27].

The gut microbiome represents our first line of defense against potential pathogens [28]. Intestinal microbes compete with pathogens for nutrients and produce antimicrobial molecules called bacteriocins [29], which shape the composition of the gut microbiota. Commensal microbes further contribute to the development of the mucosal immune system via direct interactions with host epithelial and immune cells [30]. In addition, the gut microbiome is responsible for a number of vital metabolic and signaling functions for the host, such as biotransformation of primary bile acids [31], production of short-chain fatty acids [25], synthesis of all B vitamins and vitamin K [32], as well as synthesis of essential amino acids [33].

2.2. Intestinal Epithelium

The principal functions of the intestinal barrier are nutrient absorption and defense against invading pathogens and potentially harmful substances. Mucosal cell types dynamically contribute to a balance between protective immunity and prevention of excessive immune responses against nutrients and commensal microbes. A first single-layer of cells, called the intestinal epithelium, facilitate absorption of nutrients while preventing microbial attachment and their translocation into the blood circulation [34].

The small intestinal epithelium is organized in villi and crypts of Lieberkühn. Adult stem cells reside at the base of the crypts and give rise to all absorptive and secretory cells making up the epithelial layer [35]. Gut barrier functions are insured by different epithelial cell types. Secretory goblet cells produce mucus—a viscous fluid enriched in mucin glycoproteins that form a first physical barrier against luminal microbes. Commensal bacteria also degrade glycans to extract the energy content, which then they share with the host in a mutualistic relationship [36]. Moreover, goblet cells produce antimicrobial peptides, such as trefoil factor [37] and resistin-like molecule beta [38], to dampen the mucosal attachment of microbes. In addition, they are also able to present bacterial antigens to dendritic cells via the so-called “goblet cells antigen-associated passages” (GAPs), thus educating the intestinal immune system [39]. Paneth cells, which reside at the base of the crypts, contribute to gut barrier function by producing and releasing into the crypt lumen antimicrobial molecules such as antimicrobial peptides, the regenerating islet-derived 3 (Reg3) family of proteins, lysozyme, and secretory phospholipase A2 [40]. Microfold cells, also known as M cells, are located in the follicle-associated epithelium. They uptake antigens and microorganisms from the lumen and deliver to organized lymphoid tissues within the mucosa [41]. Absorptive enterocytes also contribute to defense mechanisms by regulating paracellular permeability with tight junction proteins and directly recognizing a variety of PAMPs with different pattern recognition receptors, including Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-containing proteins (NODs) [42]. Furthermore, a dense glycocalyx consisting of extended and heavily glycosylated membrane mucins covers the surface of enterocytes, reinforcing the protective properties of the intestinal mucus [43].

2.3. Intestinal Immune System

The mucosal immune system plays an important role in orchestrating intestinal defense mechanisms against potential pathogenic microorganisms by coordinating innate and adaptive responses. Immune cells strategically localize in different compartments of the gut mucosa according to the expression of different receptors and the capacity to sense directly or indirectly microbes or their products.

Macrophages are in part found under the epithelium in the lamina propria where they engulf and kill invading microorganisms and subsequently expose their antigens to adaptive immune cells. Unlike phagocytes in other parts of the body, gut macrophages produce large amounts of regulatory cytokines, such as IL-10, and contribute to maintain

a tolerant status in the intestine while avoiding excessive inflammatory reactions [44]. The intestinal macrophages are continuously replaced by circulating monocytes. However, a recent report sheds light on the existence of long-lived tissue-resident gut macrophages that are not replaced by infiltrating monocytes [45].

Dendritic cells (DCs) orchestrate mucosal immunity by acquiring antigens in the epithelium through goblet or microfold epithelial cells or by directly capturing and sampling luminal antigens. They then processed antigens to T cells and B cells in the mucosa or in organized secondary lymphoid structures known as Peyer's Patches, as well as in mesenteric lymph nodes [46]. Tolerogenic dendritic cells promote differentiation of regulatory T cells (Treg) [47], which in turn are able to induce tolerogenic DCs via IL-10, TGF- β , and other surface molecules [48]. DCs in gut-associated lymphoid tissue (GALT) and mesenteric lymph nodes further promote differentiation of B cells into immunoglobulin A (IgA)-producing plasma cells and memory B cells. Dimers of immunoglobulin A are transported through intestinal epithelial cells and secreted into the lumen where it binds commensal and pathogenic microbes, without initiating a pro-inflammatory response [49], and therefore contributes to mucosal defense.

Recent investigations have revealed the existence of CD8+ and CD4+ T resident memory cells (TRM) and their importance in the maintenance of gut microbial immune surveillance. These lymphocytes are localized in the intestinal epithelium and lamina propria and allow the fast initiation of targeted immune responses against potential invaders [50] (Figure 1A).

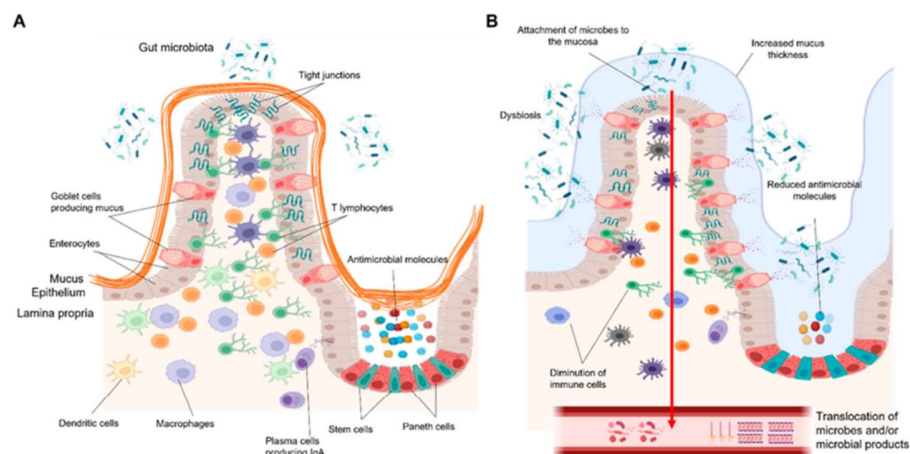


Figure 1. (A) The small intestinal barrier in healthy conditions. The intestinal barrier is composed of three main layers: the mucus, the epithelium, and the lamina propria. The epithelium, which is overlaid by a thin and discontinuous mucus layer, together with enterocytes and specialized cells constitute a first line of defense. Immune cells in the mucosa further fine-tune the defense mechanisms against pathogens. Macrophages underlying the epithelium regulate immune response by phagocytosis of microbes and production of large amounts of anti-inflammatory cytokines, thus preventing excessive immune responses. Dendritic cells capture, process, and present microbial antigens to different adaptive immune cells. T lymphocytes rapidly act against pathogens by killing infected cells, producing cytokines, and coordinating immune responses. (B) The small intestinal barrier in patients with an alcohol-use disorder. Chronic alcohol consumption is associated with alterations in the composition of the gut microbiota, increased attachment of microbes to the intestinal mucosa, and their translocation into the portal and systemic circulation. This process is enhanced by impairment of various epithelial and immune defense mechanisms. A loose, thickened mucus layer as well as reduced production of antimicrobial molecules and a diminution of macrophages and T lymphocytes all contribute to the failing gut barrier in AUD patients. Figures were created with [Biorender.com](https://www.biorender.com) (14 November 2021).

3. Gut Barrier Dysfunction in the Context of Chronic Alcohol Consumption

Alcohol abuse is associated with small intestinal bacterial overgrowth [51], alterations in the composition of the intestinal micro- and mycobiota, also called as gut dysbiosis [8,52], and increased microbial translocation [53]. In addition, both experimental and clinical evidence show modifications at multiple levels of gut barrier function, including the epithelium and immune system, which might shape the microbial changes, allow microbes to translocate to the portal circulation, and eventually participate in alcohol-associated liver disease progression. We here focus on the dysfunctional defense mechanisms in the different compartments and cell types that finally contribute to the overall failure of the gut barrier.

3.1. Alcohol-Associated Changes in the Gut Microbiome

Most of the studies have investigated the changes in the microbiota composition looking specifically at bacteria. Chronic alcohol consumption can lead to intestinal bacterial overgrowth [51], compositional changes in the bacterial microbiota [54], and elevated systemic levels of bacterial-derived products [53]. Alterations in the bacterial microbiota and their associations with chronic alcohol consumption in humans have been reviewed in more detail in [55].

Recent reports indicate alcohol-associated overgrowth of gut fungal populations [56], with reduced fungal diversity and a proportional increase in *Candida albicans* (*C. albicans*) and *Malassezia restricta* (*M. restricta*) in patients with alcohol-use disorder [57]. Regarding the enteric viruses, only one study by Jiang et al. has evaluated the fecal virome of patients with ALD at different disease stage (e.g., severe alcohol-associated hepatitis, alcohol-use disorder) in comparison to healthy controls [11]. Patients with severe alcohol-associated hepatitis were characterized by a marked increase in mammalian viruses, such as Parvoviridae and Herpesviridae [11]. In addition, many bacteriophages were more abundant in patients with severe alcohol-associated hepatitis, including *Staphylococcus*, *Escherichia*, *Enterobacteria*, and *Enterococcus* phages [11].

Gut dysbiosis is likely one factor that contributes to increased microbial translocation, either directly or in an indirect manner. Overrepresented pathogenic microbes can attach and invade the host by yet not completely elucidated mechanisms. Moreover, loss of commensal microbes involved in different metabolic functions (e.g., transformation of bile acids, synthesis of vitamins, short-chain fatty acids and amino acids) can negatively affect the intestinal epithelium and immune system [30].

3.2. Alcohol-Associated Changes in the Intestinal Epithelium

Several independent studies have shown alcohol-induced disruption of tight junction proteins (e.g., zonula occludens 1 (ZO-1), occludin, and claudins) and subsequent increased paracellular permeability, also called leaky gut, primarily confined to the proximal small intestine (for a more detailed review, see [58]). However, a leaky gut was observed in only a fraction of AUD patients at early ALD stages (steatohepatitis, steato-fibrosis) [14,15]. In contrast to animal models and in presence of more advanced stages of liver disease, such as decompensated cirrhosis, where additional factors (e.g., Portal hypertension) lead to increased paracellular permeability [55], we found that leaky gut alone could not explain increased systemic microbial translocation at these early disease stages in AUD patients [15]. These observations indicate that other defense mechanisms need to fail in order to allow microbes and/or their products to cross the intestinal barrier.

Both animal models and subjects with an alcohol-use disorder are characterized by increased production of mucus in the proximal small intestine [59]. Mucin-2 deficiency in mice leads to amelioration of alcohol-induced liver disease. The protective effect was demonstrated to be dependent on changes in the composition of the microbiota and increased expression of Reg3 antimicrobial molecules, resulting in reduced bacterial overload and translocation in mice [59]. Moreover, glycosylation of the mucins in the intestine is impaired since Fut2, an enzyme involved in α 1-2-fucosylation at the cell surface of ente-

rocytes and mucus [60], is downregulated in AUD patients [61]. Membrane and secreted α 1–2-linked fucose can be cleaved by bacterial fucosidase, and the liberated L-fucose is utilized by certain bacteria [62]. Fut2 deficiency could therefore induce overgrowth of potential pathogens in the mucosa. Deficiency of Fut2 exacerbates chronic ethanol-induced liver injury, steatosis, and inflammation in mice [61], supporting its protective role against alcohol-induced liver disease.

Chronic alcohol consumption reduces gene and protein expression of Reg3 γ and Reg3 β in murine small intestine and duodenal Reg3 γ in AUD patients, indicating impaired function of specialized Paneth cells [54]. Deficiency in Reg3 lectins in mice enhances bacterial translocation into the liver with subsequent activation of the immune responses [54]. Furthermore, Reg3 knockout mice chronically exposed to ethanol had worsened liver injury linked to increased bacterial translocation [51]. Interestingly, diminution of the antimicrobial molecules Reg3 and Human defensin 5 (own unpublished data) are associated with increased number of mucosa-associated bacteria [51] and increased systemic microbial translocation in actively drinking subjects compared to matched healthy subjects [15].

The maintenance of a correct balance between proliferation and differentiation programs is crucial for functions of the gut barrier, such as correct absorption of nutrients and coordination of defense mechanisms against invading microbes. Mice chronically fed ethanol are characterized by damage of adult stem cells via dysregulation of Wnt/ β -Catenin signaling and reduced expression of stem cell markers such as Lgr5 and Bmi1 [63]. In contrast, animal models of chronic and binge alcohol consumption show crypt hyperplasia (increased crypt length) with enhanced proliferation through the Wnt/ β -Catenin and Erk1/2 signaling pathways [64]. Duodenal biopsies in actively drinking subjects also revealed reduced villi length [15] and increased crypt hyperplasia (own unpublished data). The causes of the alterations in the small intestinal epithelium in humans are still incompletely understood. They might be related to modifications in the proliferation/differentiation program, which remain to be elucidated. Epithelial alterations could influence both defective defense mechanisms as well as malabsorption of nutrients, a well-known characteristic of patients with an alcohol-use disorder [65].

3.3. Alcohol-Induced Alterations in the Intestinal Immune System

Chronic alcohol consumption is associated with impairment of both innate and adaptive immune responses. How these alterations affect attachment and invasion of microbes in the gut mucosa in the different stages of ALD is still incompletely understood.

3.3.1. Macrophages

Recent finding from our laboratory showed a reduced number of macrophages in the duodenum of AUD patients associated with elevated microbial translocation [15]. Whether this reduced number of intestinal macrophages is due to a decrease in resident macrophages or of infiltrating monocytes is still not known. Macrophages in the lamina propria that produce TNF- α are increased in the proximal small intestine of mice chronically fed ethanol as well as in AUD patients [66]. The pro- (IL1 β) and anti-inflammatory cytokines related to macrophages (IL-10 and TGF β) are upregulated in distal duodenal biopsies from actively drinking subjects compared to the controls [15]. Which subsets of macrophages produce these inflammatory mediators with opposite roles (inflammatory vs. tolerogenic) remain unknown as well as its significance for defense mechanisms. Whether and how these changes relate to ALD also requires further investigation.

3.3.2. Dendritic Cells

Dendritic cells, together with macrophages, orchestrate immune responses in the gut barrier through presentation of the processed antigens to adaptive immune cells. Few studies have investigated the impact of alcohol abuse on dendritic cells. Acute alcohol intake alters the function and cytokine production of human monocyte-derived DCs in the blood circulation [67]. Moreover, chronic alcohol consumption is associated with changes

in the distribution, immunophenotype, and secretion of inflammatory mediators [68]. Rhesus macaques chronically exposed to ethanol show a reduced number of bone marrow and circulating pools of myeloid dendritic cells, with suppression of costimulatory molecule such as CD83, which may attenuate the ability of DCs to promote T cell expansion [69]. These data support the detrimental effect of alcohol abuse on the function of dendritic cells. However, no study has characterized the effect of chronic alcohol consumption on intestinal DCs in mice and humans related to or suffering from ALD.

3.3.3. Plasma Cells

The number of intestinal IgA-secreting plasma cells is reduced in experimental models of ethanol-induced liver disease in mice [70]. In humans, studies aimed at investigating IgA during ALD are scarce and contradictory. In one study, patients with decompensated cirrhosis had reduced intestinal secretion of IgA compared to compensated alcohol-associated cirrhosis [71]. By contrast, two other studies reported similar levels of IgA secretion [72] and IgA-secreting plasma cells [73]. Characterization of IgA levels with different methods as well as characterization of IgA-plasma cells at different stages of ALD and possible impact of a period of abstinence before admission may partially explain these conflicting observations. Future studies are needed to elucidate the role of IgA in the development of early and late stages of alcohol-associated liver disease.

3.3.4. T Cells

Intestinal T cells may play an important role in gut barrier dysfunction during ALD. Recent findings from our laboratory show a reduced number of T lymphocytes in the duodenum of actively drinking subjects associated with elevated microbial translocation [15]. Gene expression of Th1- and Th17-related cytokines remained unchanged in the mucosa of AUD patients compared to the controls [15]. These data rule out mucosal T cell pro-inflammatory responses as a significant driver of microbial translocation and point to a potential dysfunction in adaptive immunity. Pathophysiological mechanisms underlying the loss of intestinal T cells with identification of subsets that are specifically reduced could potentially pave the way for novel therapeutic targets directed towards improving gut barrier function. Impaired adaptive immunity linked to defective microbial immunosurveillance is a characteristic not only confined to early stages of ALD but is also common to more advanced liver disease, such as alcoholic hepatitis and cirrhosis. Reduced mucosa-associated invariant T (MAIT) cells, which play an important role against bacterial infections, have been observed in advanced ALD along with compromised antibacterial and cytotoxic responses [74]. Taken together, these data point to the contribution of different cell types to the overall dysfunctional defense mechanisms in the gut barrier during different stages of alcohol-associated liver disease (Figure 1B).

4. How Can Gut Barrier Dysfunction Contribute to ALD Progression?

The intestine and liver communicate via bidirectional links through the biliary tract, portal vein, and systemic circulation. The liver synthesizes and transports primary bile acids and antimicrobial molecules to the intestinal lumen through the bile duct. In the gut, the host and microbes metabolize endogenous (bile acids and amino acids) and exogenous substrates (dietary and environmental factors) into products that are then transported to the liver through the portal vein. Furthermore, the systemic circulation connects the gut-liver axis by transporting liver metabolites into the intestine [58]. Many of those products of metabolites (e.g., bile acids, fatty acids, ethanol metabolites) might act as signaling molecules at the local and distant sites where they activate various signaling processes (e.g., cell growth and differentiation, host metabolism, and immune responses) involved in regulation of the intestinal barrier [75] (Figure 2). Changes in the levels and in the structure of these signaling molecules, for example, a different proportion of secondary and primary bile acids, can negatively affect the processes (e.g., intestinal permeability, epithelial differentiation, absorption of nutrients and immunity) essential

for defense mechanisms in the gut. As a result, breakdown of gut barrier function allows microbes and/or their products to get into the liver and systemic circulation, where they are recognized by different pattern recognition receptors in various cell types with subsequent activation of immune responses. According to the stage of ALD, the cell types, and signaling pathways involved, activation of a pro-inflammatory response against microbial antigens might be beneficial or detrimental for liver disease progression [76]. We here discuss some recent findings that support the link between gut barrier dysfunction, systemic inflammation, and impaired hepatic immunity in the pathogenesis of early and advanced stages of ALD.

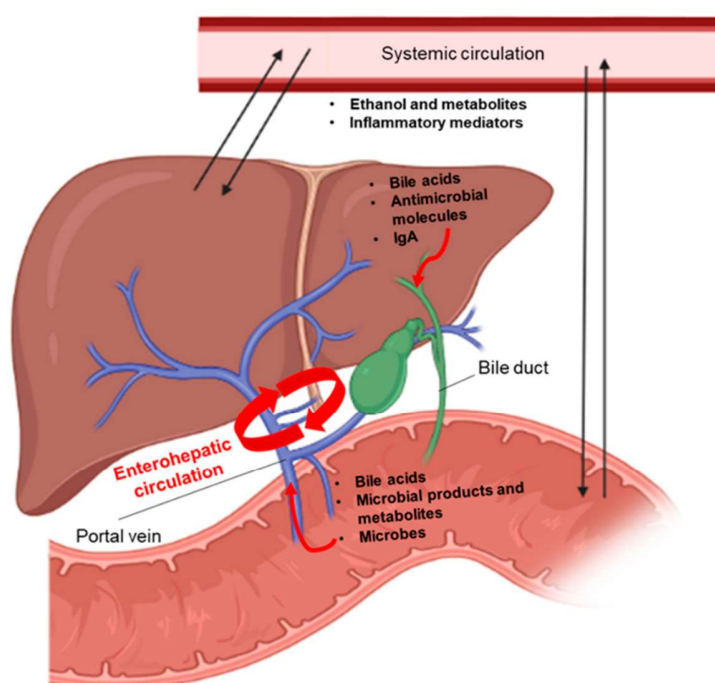


Figure 2. Bidirectional communication between the intestine and the liver. The liver produces primary bile acids, antimicrobial molecules, and IgA that are released in the intestine through the bile duct. In the intestine, these molecules contribute to shape the microbiota. In addition, primary bile acids are converted into secondary bile acids by the gut microbiota. Bile acids, which are reabsorbed in the terminal ileum, microbial products and metabolites, as well as viable microbes are transported to the liver through the portal vein. Once in the liver, they are implicated in triggering immune and inflammatory responses that might lead to liver disease. Moreover, the gut–liver axis is connected via the systemic circulation where ethanol, ethanol-derived metabolites, as well as other inflammatory mediators (cytokines, metabolites, etc.) can reach the two organs, thus influencing their functions. Figures were created with [Biorender.com](https://biorender.com) (14 November 2021).

4.1. Systemic Inflammatory Responses

Increased systemic translocation of bacterial products, such as lipopolysaccharides (LPS) and peptidoglycans (PGN), have been shown to be associated with elevated plasma levels of inflammatory cytokines (IL1 β , IL8, IL18, TNF- α , and IL6) in AUD patients [77]. Mechanistic analyses have shown that in AUD patients, LPS and to a greater extent PGN can contribute to the activation of peripheral blood mononuclear cells (PBMCs).

PBMCs are likely contributing to PGN-triggered release of IL1 β , IL8, and IL18 into the blood [77]. In contrast, TNF- α and IL6 were not elevated in PBMCs, suggesting that their increased plasma levels might originate from other sources [78]. Inflammatory chemokine monocyte chemoattractant protein 1 (MCP-1) levels in the plasma are increased in actively drinking subjects compared to the controls [79]. This may promote monocyte recruitment and infiltration into the liver where they transform into inflammatory monocyte-derived macrophages. This process has been observed in mice chronically fed ethanol [80] and it is believed to play an important role in the early pathogenesis of ALD [81]. Future studies are needed to clarify the pathways linked to this process. In contrast to the early stages of alcohol-associated liver disease, severe alcohol-associated hepatitis is characterized by massive hepatic infiltration of neutrophils coming from the circulation [81]. However, a recent investigation found that alcohol-associated hepatitis patients with more neutrophils had a better outcome than those with less neutrophil infiltration [82], probably due to defective bacterial clearance and impaired liver regeneration.

4.2. Impairment of Immune Responses Related to Microbes in the Liver

Increasing evidence supports the role of unbalanced immunity in the different stages of ALD. When the gut barrier is disrupted, higher amounts of microbes and/or their products translocate to the liver [55], leading to the activation of the hepatic immune system through pattern recognition receptors like Toll-like receptors (TLRs) [83]. TLRs are expressed widely in immune and non-immune cells [84] in the liver and their activation by different PAMPs coming from the intestine contributes to ALD progression [85].

It has been proposed that activation of the TLR4 signaling pathway by LPS plays a pivotal role in disease progression. Mice deficient in TLR4, CD14, and LPS-binding protein (LBP) show resistance to alcohol-induced liver injury [86]. Other studies in mice show the pathophysiological importance of TLR4 in resident macrophages (e.g., Kupffer cells), monocyte-derived macrophages, and hepatic stellate cells [87]. The concept of TLR4 activation with subsequent TNF- α release has conducted to translational approaches such as the anti-TNF- α therapies for patients with severe alcoholic hepatitis. The disappointing outcome of these clinical trials [88] highlight two main facts in the field of alcohol-associated liver disease: firstly, data obtained in animal models of ALD cannot be necessarily extrapolated to humans [16]; secondly, but not in order of importance, inflammatory responses can be beneficial or detrimental depending on the cell type involved and stage of liver disease. In this context, we have recently revealed that intracellular TLRs, in particular TLR7, increased at early stages of ALD in humans, whereas TLR4 did not, and was correlated with liver disease severity [79]. TLR7 upregulation occurred concomitantly with activation of interferon signaling predominantly in hepatocytes [79]. In contrast, specific TLR7 activation did not exacerbate liver disease in a chronic ethanol-feeding model [89].

Kupffer cells and monocyte-derived macrophages also exhibit different functions in different stages of ALD. Animal models show an important role of TLR4 activation with release of pro-inflammatory cytokines in alcohol-induced liver injury [90]. Pro-inflammatory cytokines produced by hepatic macrophages also seem to be important in the early pathogenesis of ALD [91]. However, defective macrophages in more advanced stages of liver disease may not allow optimal clearance of microbes by phagocytosis, leading to severe infections, a feature often encountered in patients with severe alcohol-associated hepatitis [92] and decompensated cirrhosis [93].

5. Conclusions and Future Directions

Gut barrier dysfunction is increasingly recognized as a main driver of alcohol-associated liver disease progression. A complex dynamic disturbance of defense mechanisms allows microbes and/or microbial antigens to translocate into the liver and systemic circulation, thus provoking immune responses contributing to liver damage.

Several studies have revealed the dual roles of inflammatory and tolerogenic immune responses in microbial immunosurveillance in the intestine and liver according to the stage

of ALD. Many pathophysiological aspects of the complex host–microbe interactions need to be dissected and future translational investigations should focus on getting a better insight into the dynamics between microbiota, intestinal, and hepatic cell types. Functional impairment of different specialized cell types and pathways contribute to the overall failure of the gut barrier and ALD progression. In the absence of effective pharmacological therapy, abstinence represents the only option for ALD treatment. We found that a short period of abstinence normalized increased intestinal permeability. However, serum levels of microbial translocation and liver cell damage markers remained significantly higher than control levels [15]. These results indicate that abstinence alone is not sufficient to improve all the modifications in the intestinal barrier linked to ALD. In this review, we have highlighted the fact that multiple cell types of the gut barrier are involved in mucosal dysfunction in a manner dependent on liver disease severity. Targeting only one aspect of alcohol-associated intestinal barrier dysfunction could not be sufficient to improve and/or reverse ALD. Therefore, multi-targeted and personalized therapy appears to be the only solution to finally treat these patients. For this purpose, standardized clinical studies are needed to stratify AUD patients according to their degree of gut barrier dysfunction, liver disease severity, and systemic inflammatory response. Identification of novel biomarkers related to these pathophysiological conditions will help clinicians to subdivide patients into subgroups linked to disease state and potentially treat them in a targeted fashion.

Innovative technological developments, such as metagenomic and metatranscriptomics, single-cell analysis, spatial transcriptomics, and mass spectrometry-based technologies, will help us to identify promising biomarkers and finally understand the complexity of the different factors and pathways involved in gut barrier dysfunction during ALD. Generation of small intestinal enteroids from primary adult stem cells and co-culture with different subsets of gut immunocytes from the same patients will help us to study the dynamics of the immune–epithelium crosstalk involved in defense mechanisms against microbes. A deep understanding of the causal links between changes in the gut microbiome and intestinal barrier dysfunction at different stages of alcohol-associated liver disease will provide us with the development of new diagnostic and prognostic tools and with the identification of new therapeutic targets.

Funding: This study was supported in part by NIH grants R01 AA24726, (to B.S. and P.S.) and services provided by NIH centers P30 DK120515 and P50 AA011999. PS also received grants from Fond National de Recherche Scientifique Belgium (J.0146.17 and T.0217.18) and Action de Recherche Concertée (ARC), Université Catholique de Louvain, Belgium.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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ANNEX 2 Lipidomics for prediction of progressive alcohol-associated liver disease

Different factors could promote alcohol-related gut barrier dysfunction and alcohol-associated liver disease. Recent studies suggest that host and microbial metabolites could play a role in the impairment of intestinal barrier during ALD described in chapter 2. Lipids control several processes implicated in ALD pathogenesis (e.g., steatosis, inflammation, and fibrosis) and are therefore potential mediators of the gut-liver axis. The principal aim of this ancillary study was to evaluate lipid profiles in the serum and in the stools of AUD patients subdivided according to liver disease stage compared to matched controls. The second aim of this study was to identify lipid species that could differentiate and predict progressive ALD. We finally test whether alterations in lipid profiles was associated with changes in microbial pathways related to lipid metabolism.

We found that **changes in lipidomic profiles in both serum and stools strongly associated with progressive ALD**. When looking into the different species able to differentiate progressive ALD, we revealed only **9 species differentially present in both serum and stools in AUD patients with progressive ALD** compared to the benign forms of liver disease. Among these lipids, certain **fatty acids and especially saturated sphingomyelins** predict **severity of ALD**.

The results of this study have been published in *Metabolites*, May 2022, Vol.12, Issue 5.

Article

Lipidomics for the Prediction of Progressive Liver Disease in Patients with Alcohol Use Disorder

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Citation: Gao, B.; Zeng, S.; Maccioni, L.; Shi, X.; Armando, A.; Quehenberger, O.; Zhang, X.; Stärkel, P.; Schnabl, B. Lipidomics for the Prediction of Progressive Liver Disease in Patients with Alcohol Use Disorder. *Metabolites* **2022**, *12*, 433. <https://doi.org/10.3390/metabo12050433>

Academic Editor: Nimer Assy

Received: 20 April 2022

Accepted: 4 May 2022

Published: 11 May 2022

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Abstract: Alcohol-related liver disease is a public health care burden globally. Only 10–20% of patients with alcohol use disorder have progressive liver disease. This study aimed to identify lipid biomarkers for the early identification of progressive alcohol-related liver disease, which is a key step for early intervention. We performed untargeted lipidomics analysis in serum and fecal samples for a cohort of 49 subjects, including 17 non-alcoholic controls, 16 patients with non-progressive alcohol-related liver disease, and 16 patients with progressive alcohol-related liver disease. The serum and fecal lipidome profiles in the two patient groups were different from that in the controls. Nine lipid biomarkers were identified that were significantly different between patients with progressive liver disease and patients with non-progressive liver disease in both serum and fecal samples. We further built a random forest model to predict progressive alcohol-related liver disease using nine lipid biomarkers. Fecal lipids performed better (Area Under the Curve, AUC = 0.90) than serum lipids (AUC = 0.79). The lipid biomarkers identified are promising candidates for the early identification of progressive alcohol-related liver disease.

Keywords: random forest; EPA; sphingomyelin; steatosis; alcohol-associated liver disease

1. Introduction

Chronic alcohol consumption induces steatosis through the acceleration of hepatic lipogenesis, deceleration of lipid breakdown, and defective export of hepatic lipids [1]. The majority of patients with alcohol use disorder develops hepatic steatosis. Alcohol-related steatosis is reversible upon cessation of alcohol consumption. However, only 10–20% of patients with heavy and daily drinking will develop progressive liver disease and cirrhosis. Various modifiers affect the progression of alcohol-related liver disease, such as the pattern of alcohol consumption, gender, age, race, genetics, nutritional factors, drugs, obesity, smoking, and viral infections [1]. Although the progression of alcohol-related liver disease is generally characterized from the histological point of view, the mechanisms that underlie the histological progression are still not well understood. In addition, there is currently no early prediction marker available to identify those who will develop liver fibrosis or cirrhosis. The ability to distinguish progressive liver disease from non-progressive liver

disease would allow for the early and aggressive treatment of the underlying alcohol use disorder, which is an integral part for therapy of alcohol-related liver disease.

As a common hepatic change due to alcohol abuse, steatosis can invoke metabolic changes. The liver plays a key role in lipid metabolism, such as taking up fatty acids from dietary intake or the synthesis of fatty acids, which can be used as energy sources through β -oxidation or substrates for triglyceride biosynthesis in hepatocytes [2]. Fatty acid β -oxidation is inhibited by ethanol, increasing the availability of long-chain fatty acids and enhancing their esterification [3]. Dysregulation of lipid influx and efflux induced by ethanol further leads to the accumulation of lipids in hepatocytes.

Tracer studies demonstrated that alcohol consumption induces alterations in *de novo* lipogenesis [4,5]. Chronic alcohol consumption increased hepatic free fatty acids, ceramide metabolites, and decreased the level of acyl-CoA in a mouse model [6]. In addition, tissue-specific changes in fatty acids induced by ethanol were also reported in a mouse model [7]. Dysregulation of the serum lipid profile was found in a patient cohort of 59 excessive alcohol drinkers [8]. Although the serum lipidome has been studied to some extent, the fecal lipidome is not well characterized in patients with alcohol use disorder. It is not clear whether fecal lipids can be used to predict progressive alcohol-related liver disease. In addition, fecal samples present an advantage over serum samples, as they can be collected in a non-invasive way.

In the present study, we performed a serum and fecal lipidomics analysis in a cohort of 49 subjects, including non-alcoholic controls and patients with non-progressive and progressive alcohol-related liver disease. We aimed to: (1) reveal the alteration of serum and fecal lipids in two patient groups compared with control subjects; (2) identify serum and fecal lipid biomarkers that distinguish between progressive and non-progressive alcohol use disorder; and (3) build a random forest model to predict progressive alcohol-related liver disease.

2. Results

2.1. Patient Cohort

A total of 17 non-alcoholic controls, 16 patients with non-progressive alcohol-related liver disease, and 16 patients with progressive alcohol-related liver disease, based on our clinical classification, were included in this study. CK-18-M65 supported this classification, with 33% of patients in the non-progressive, and 93% in the progressive, alcohol-related liver disease group showing levels below or above 400 U/L, respectively. Patient characteristics are summarized in Table 1. Patients with steato-hepatitis or steato-fibrosis had a preserved synthetic liver function and showed no clinical signs of liver decompensation. There was no significant difference in age, body mass index, and gender between control subjects and the two patient groups. Compared with patients with non-progressive liver disease, patients with progressive liver disease showed significantly higher levels of ALT, AST, GGT, total bilirubin, CAP, and CK18-M65 (Table 1).

2.2. Serum Lipidome

Hierarchical clustering of the serum lipidome showed the profile of serum lipids from control subjects was different from patients with non-progressive and progressive liver disease (Figure 1A, Supplementary File). A partial least square discriminant analysis (PLSDA) showed that control groups were separated from the two patient groups (Figure 1B). A Kruskal–Wallis rank sum test for component 1 showed a p -value of 1.2×10^{-6} between three groups (Nemenyi-tests for pairwise comparison between non-progressive liver disease vs. controls p -value = 0.0006; progressive liver disease vs. controls p -value = 1.6×10^{-6} ; progressive liver disease vs. non-progressive liver disease p -value = 0.41). For component 2, the comparison between the three groups showed a p -value of 0.002 (Nemenyi-tests for pairwise comparison between non-progressive liver disease vs. controls p -value = 0.038; progressive liver disease vs. controls p -value = 0.0019; progressive liver disease vs. non-progressive liver disease p -value = 0.61).

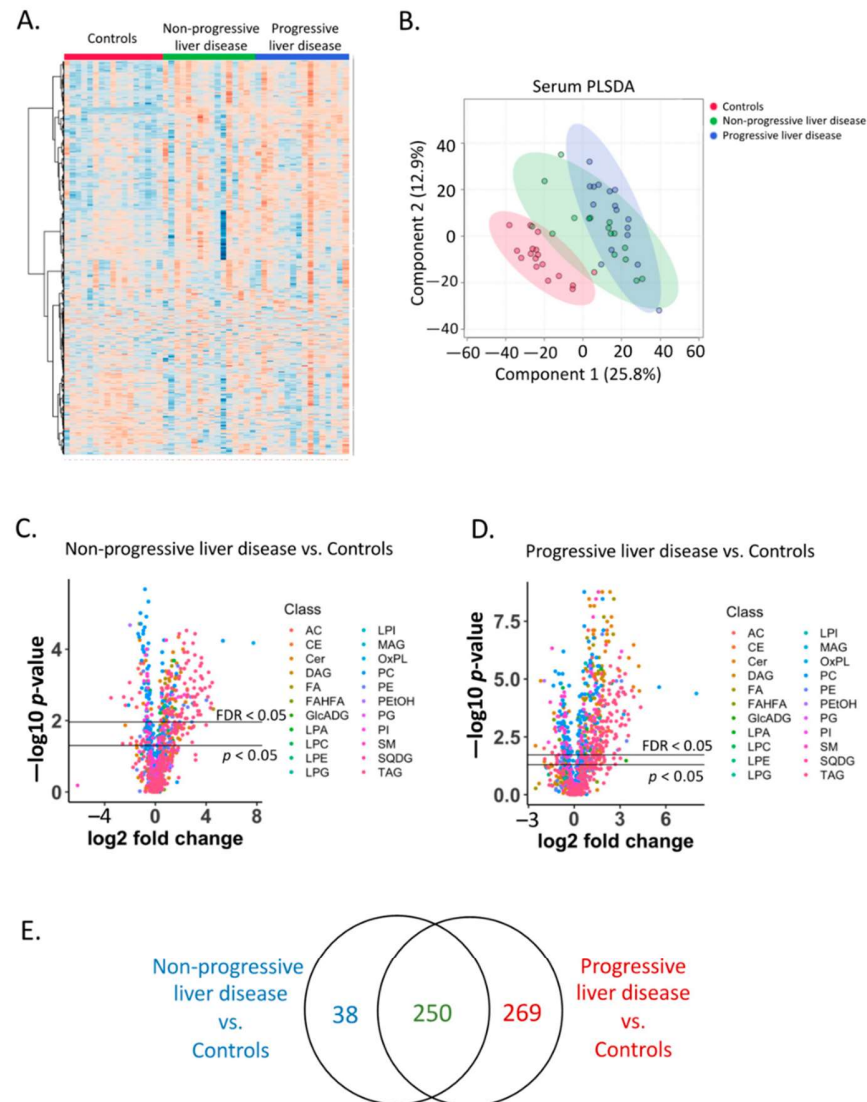


Figure 1. Serum lipidome. (A) Heatmap of the serum lipidome in the control and two patient groups. (B) Partial least squares-discriminant analysis of the serum lipidome. (C) Volcano plot of the serum lipidome in control subjects and patients with non-progressive liver disease. Different colors represent different lipid classes. Fold change: non-progressive liver disease ($n = 16$)/controls ($n = 17$). (D) Volcano plot of the serum lipidome in control subjects and patients with progressive liver disease. Different colors represent different lipid classes. Fold change: progressive liver disease ($n = 16$)/controls ($n = 17$). (E) Venn diagram of significant serum lipids (adjusted $p\text{-value} < 0.05$). Dark blue: number of unique significant lipids between non-progressive liver disease and controls; Red: number of unique significant lipids between progressive liver disease and controls; Dark green: shared significant lipids.

Table 1. Subject characteristics.

	Non-Alcoholic Controls	Non-Progressive Liver Disease	Progressive Liver Disease	p-Value
Clinical parameter				
Total <i>n</i>	17	16	16	
Age, years, <i>n</i> = 48	38 (27–71)	37 (27–58)	41 (28–59)	0.470
Body Mass Index (BMI), kg/m ² , <i>n</i> = 48	22 (19–29)	22 (19–31)	24 (18–31)	0.381
Gender (male), <i>n</i> (%), <i>n</i> = 48	14 (88)	11 (69)	14 (88)	0.292
Laboratory parameter				
Albumin (g/dL), <i>n</i> = 27		4.7 (4.2–5.2)	4.7 (3.9–5.2)	0.558
Alkaline phosphatase (U/L), <i>n</i> = 28		65 (38–101)	81 (47–113)	0.072
ALT (U/L), <i>n</i> = 32		19 (11–37)	78 (37–184)	<0.001
AST (U/L), <i>n</i> = 32		25 (15–36)	81 (46–283)	<0.001
Total bilirubin (mg/dL), <i>n</i> = 29		0.3 (0.2–1.1)	0.5 (0.3–0.9)	0.031
GGT (U/L), <i>n</i> = 29		31 (4–213)	139 (11–952)	0.012
Platelet counts ($\times 10^9$ /L), <i>n</i> = 28		268 (165–339)	220 (21–434)	0.270
Creatinine (mg/dL), <i>n</i> = 29		0.8 (0.5–1.0)	0.8 (0.6–1.2)	0.406
International normalized ratio, <i>n</i> = 29		1.0 (0.9–1.2)	0.9 (0.8–1.0)	0.115
Fibroscan (kpa), <i>n</i> = 32		4.8 (3.1–6.6)	6.0 (3.2–7.0)	0.122
CAP, (dB/m), <i>n</i> = 32		254 (148–325)	314 (222–381)	
CAP > 250 dB/m, <i>n</i> (%)		9 (56)	15 (94)	<0.001
Fecal albumin (μ g/L), <i>n</i> = 43	16.9 (4.7–66.6)	56.8 (10.5–504.4)	31.2 (2.2–98.1)	0.002
CK18-M65 (U/L), <i>n</i> = 38	166 (104–282)	332 (158–616)	592 (316–1576)	<0.001
sCD14 (ng/mL), <i>n</i> = 38	1376 (1074–1810)	1710 (1046–2570)	1745 (1191–2266)	0.033

Values are presented as medians with ranges in parentheses (·). ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl-transferase; CAP: controlled attenuation parameter. Fecal albumin: Non-progressive liver disease vs. controls: *p*-value < 0.001; Progressive liver disease vs. controls: *p*-value = 0.209; Progressive liver disease vs. Non-progressive liver disease: *p*-value = 0.118. CK18-M65: Non-progressive liver disease vs. controls: *p*-value = 0.084; Progressive liver disease vs. controls: *p*-value < 0.001; Progressive liver disease vs. Non-progressive liver disease: *p*-value = 0.006. sCD14: Non-progressive liver disease vs. controls: *p*-value = 0.053; Progressive liver disease vs. controls: *p*-value = 0.040; Progressive liver disease vs. Non-progressive liver disease: *p*-value = 0.991.

Out of 1266 annotated serum lipids, 481 showed a *p*-value < 0.05 and 288 showed an adjusted *p*-value < 0.05 when comparing patients with non-progressive liver disease with controls (Figure 1C). When comparing patients with progressive liver disease with controls, 640 serum lipids showed a *p*-value < 0.05 and 519 showed an adjusted *p*-value < 0.05 (Figure 1D). A total of 250 serum lipids were found to be significantly different (adjusted *p*-value < 0.05) in both patients with non-progressive liver disease and in patients with progressive liver disease compared with controls (Figure 1E). Compared with control subjects, 38 serum lipids were significantly different (adjusted *p*-value < 0.05) only in patients with non-progressive liver disease; meanwhile, 269 serum lipids were significantly different (adjusted *p*-value < 0.05) only in patients with progressive liver disease (Figure 1E).

2.3. Fecal Lipidome

Hierarchical clustering of the fecal lipidome showed that the profile of fecal lipids from control subjects was different from the two patient groups (Figure 2A, Supplementary File). A PLSDA plot showed control groups were generally separated from the two patient groups (Figure 2B). A Kruskal–Wallis rank sum test for component 1 showed a *p*-value of 5.7×10^{-7} between the three groups (Nemenyi-tests for pairwise comparison between non-progressive liver disease vs. controls *p*-value = 0.0081; progressive liver disease vs. controls *p*-value = 2.7×10^{-7} ; progressive liver disease vs. non-progressive liver disease *p*-value = 0.052). For component 2, a comparison between the three groups showed a *p*-value of 0.0008 (Nemenyi-tests for pairwise comparison between non-progressive liver disease vs. controls *p*-value = 0.24; progressive liver disease vs. controls *p*-value = 0.00048; progressive liver disease vs. non-progressive liver disease *p*-value = 0.087).

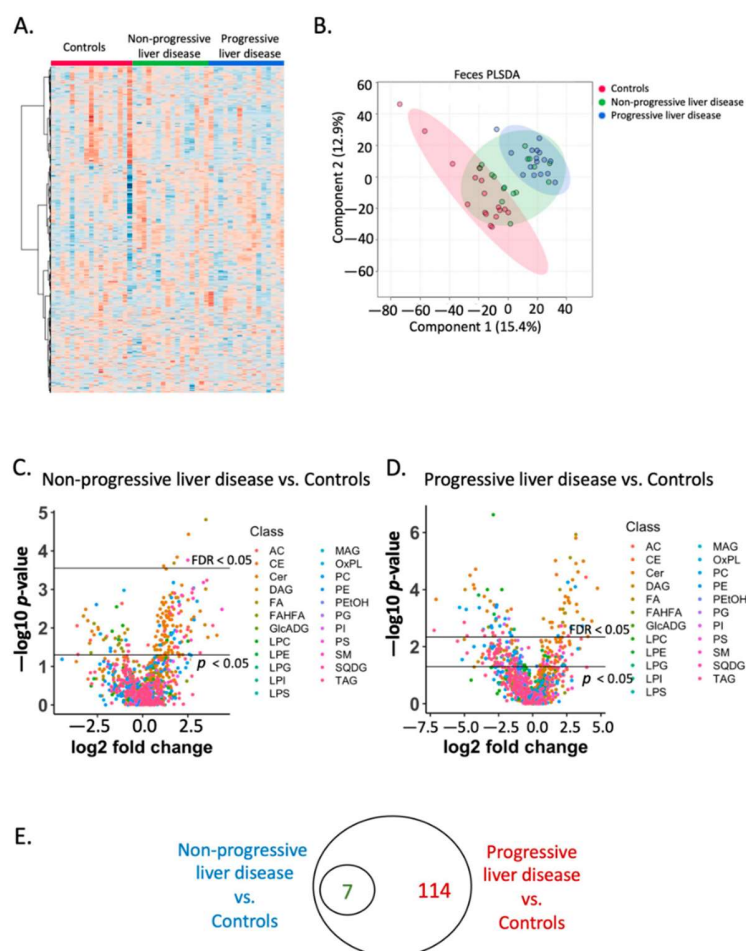


Figure 2. Fecal lipidome. (A) Heatmap of the fecal lipidome in the control and two patient groups. (B) Partial least squares-discriminant analysis of the fecal lipidome. (C) Volcano plot of the fecal lipidome in control subjects and patients with non-progressive liver disease. Different colors represent different lipid classes. Fold change: non-progressive liver disease ($n = 16$)/controls ($n = 17$). (D) Volcano plot of the fecal lipidome in control subjects and patients with progressive liver disease. Different colors represent different lipid class. Fold change: progressive liver disease ($n = 16$)/controls ($n = 17$). (E) Venn diagram of significant fecal lipids (adjusted $p\text{-value} < 0.05$). Red: number of unique, significant lipids between progressive liver disease and controls; Dark green: shared significant lipids.

Out of 1219 fecal lipids, 218 showed a $p\text{-value}$ less than 0.05 and seven showed an adjusted $p\text{-value}$ less than 0.05 when comparing patients with non-progressive liver disease with controls (Figure 2C). When comparing patients with progressive liver disease with controls, 357 fecal lipids showed a $p\text{-value} < 0.05$ and 121 showed an adjusted $p\text{-value}$ less than 0.05 (Figure 1D). Compared with controls, seven fecal lipids were found to be significantly different (adjusted $p\text{-value} < 0.05$) in both patients with non-progressive liver disease and in patients with progressive liver disease; meanwhile, 114 fecal lipids were

found to be significantly different (adjusted p -value < 0.05) only in patients with progressive liver disease (Figure 2E).

2.4. Lipid Biomarkers for Progressive Liver Disease

In order to find the biomarkers that can distinguish progressive liver disease from non-progressive liver disease, we compared serum and fecal lipids in the two patient groups. A total of 93 serum lipids showed p -values < 0.05 , with 10 decreased in progressive liver disease and 83 increased in progressive liver disease compared with non-progressive liver disease (Figure 3A). One serum lipid, SM (d36:0), was significantly increased in progressive liver disease, with an adjusted p -value < 0.05 (Figure 3A). A total of 106 fecal lipids showed p -values < 0.05 , with 96 decreased and 10 increased in progressive liver disease compared with non-progressive liver disease (Figure 3B). One fecal lipid, PC 35:1; PC17:0-18:1, was significantly increased in progressive liver disease with an adjusted p -value < 0.05 (Figure 3B). Notably, nine lipids were found to be significantly different in both the serum and feces (p -value < 0.05) (Figure 3C). These lipids were increased in the serum of patients with progressive liver disease compared with non-progressive liver disease (Figure 3D). In the fecal samples, five out of nine lipids were increased; meanwhile, the remaining four lipids were decreased in patients with progressive liver disease compared with non-progressive liver disease (Figure 3E).

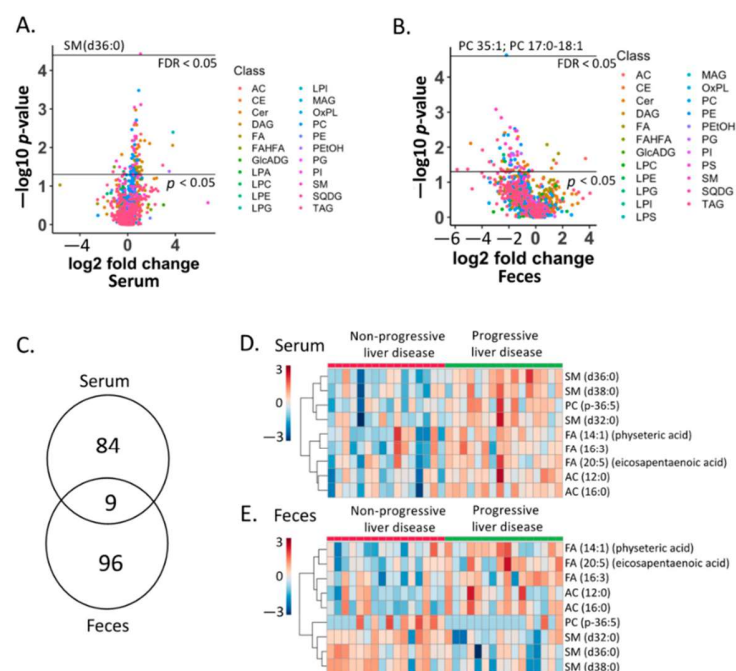


Figure 3. Comparison between progressive liver disease and non-progressive liver disease. (A) Volcano plot of the serum lipidome for the two patient groups. Different colors represent different lipid class. Fold change: progressive liver disease ($n = 16$)/non-progressive liver disease ($n = 16$). (B) Volcano plot of the fecal lipidome for the two patient groups. Different colors represent different lipid class. Fold change: progressive liver disease ($n = 16$)/non-progressive liver disease ($n = 16$). (C) Venn diagram of significant fecal lipids (p -value < 0.05). (D) Heatmap of nine serum lipid biomarkers. (E) Heatmap of nine fecal lipid biomarkers. SM: sphingomyelin; PC: phosphatidylcholine; FA: fatty acid; AC: acylcarnitine.

2.5. Association of Lipid Biomarkers with Clinical Parameters

We further examined the association of the nine lipid biomarkers with 14 clinical parameters. A Spearman correlation analysis showed that acylcarnitines, phosphatidylcholine, and sphingomyelins in the serum samples correlated with multiple clinical parameters, including BMI, AST, ALT, GGT, alkaline phosphatase, total bilirubin, international normalized ratio, platelet counts, fecal albumin, CAP, sCD14, and CK18-M65 (Figure 4A). However, fatty acids in the serum samples did not correlate with any clinical parameters (Figure 4A). In contrast, all of the nine lipid biomarkers in the fecal samples, including fatty acids, were significantly correlated with multiple clinical parameters (Figure 4B).

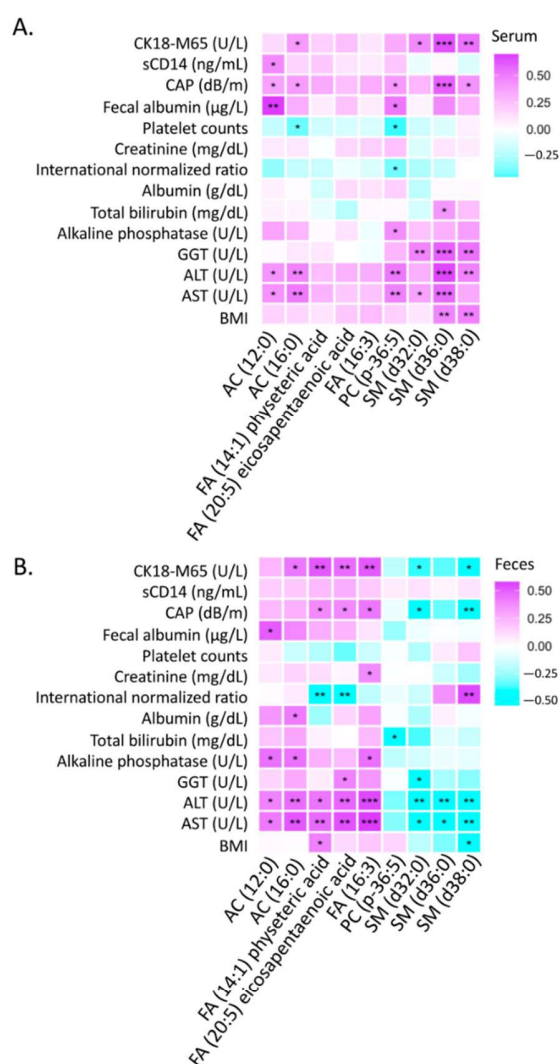


Figure 4. Spearman correlation between nine lipid biomarkers and clinical parameters. (A) Serum lipids. (B) Fecal lipids. *: p -value < 0.05; **: p -value < 0.01; ***: p -value < 0.001.

2.6. Prediction of Progressive Liver Disease

To distinguish progressive liver disease from non-progressive liver disease, we built a random forest model using the identified nine lipid biomarkers (Figure S1). The area under the curve achieved 0.79 when using nine serum lipids as variables (Figure 5A). Interestingly, AUC achieved 0.90 when using nine fecal lipids as variables (Figure 5A). Among the nine fecal lipids, the most important variable was eicosapentaenoic acid (EPA) (Figure 5B), which was significantly increased (p -value < 0.05) in serum and feces from patients with progressive liver disease compared with non-progressive liver disease (Figure 3D,E). The fecal level of EPA was positively correlated with AST, ALT, GGT, CAP, and CK18-M65, and negatively correlated with the international normalized ratio (Figure 4B).

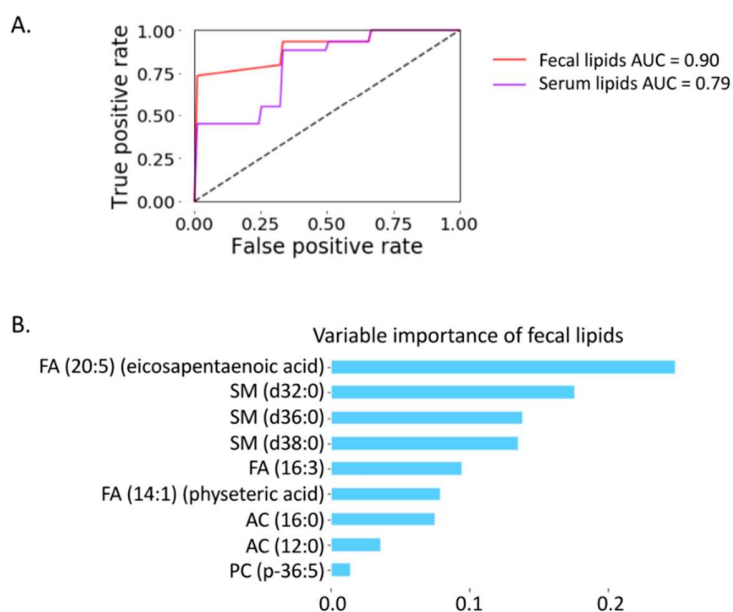


Figure 5. Prediction of progressive liver disease using nine lipid biomarkers. (A) Random forest model. Red: nine fecal lipids AUC = 0.90; Purple: nine serum lipids AUC = 0.79. (B) Variable importance.

2.7. Microbial Lipid Pathways

Given that nine fecal lipids showed better predictive power than serum lipids, we further investigated the potential contribution of gut microbiota to the host fecal lipidome. A total of 26 microbial lipid pathways were detected by shotgun metagenomic analysis (Figure 6A). A Lefse analysis showed that two microbial pathways were enriched in patients with progressive liver disease compared with non-progressive liver disease, including the LPSSYN-PWY superpathway of lipopolysaccharide biosynthesis and TEICHOICACID-PWY teichoic acid (poly-glycerol) biosynthesis (Figure 6B).

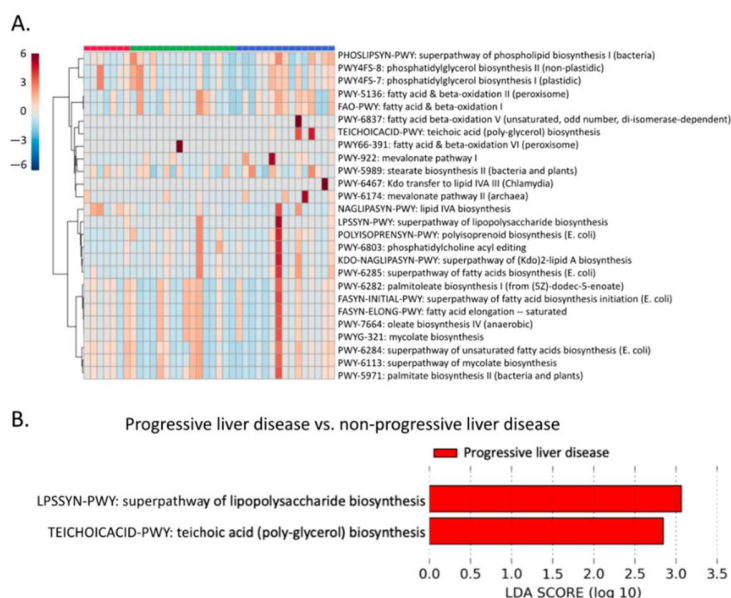


Figure 6. Microbial lipid metabolism. (A) Fecal microbial lipid pathways detected using metagenomic sequencing of fecal DNA. (B) Enriched fecal microbial lipid pathways in patients with progressive liver disease compared with non-progressive liver disease. LDA: Linear Discriminant Analysis.

3. Discussion

Although alcohol-related steatosis is generally considered as rather benign and reversible, it can further develop into steatohepatitis with fibrosis and progression to cirrhosis. The identification of effective and non-invasive biomarkers for progressive liver disease as early as possible during the disease course is important as it likely impacts patient management. In our present study, we identified lipid biomarkers in both fecal and serum samples. Interestingly, fecal lipids performed better than serum lipids to predict progressive liver disease (Figure 5A). Since fecal samples can be collected in a non-invasive way, they can serve as an alternative for the currently widely used serum biomarkers. It is noteworthy that the changing trajectory of fecal lipids could not fully represent the changing trajectory of circulating lipids. As shown in Figure 3D,E, although the changing direction for five lipids was consistent in both serum and fecal samples, including FA (14:1), FA (20:5), FA (16:3), AC (12:0), and AC (16:0), the other four lipids showed an opposite changing direction in serum and fecal samples.

As an omega-3 polyunsaturated fatty acid, EPA was the most important variable in our random forest model to predict progressive liver disease (Figure 5B). An increased level of EPA was found in both serum and fecal samples of patients with progressive liver disease compared with non-progressive liver disease (Figure 3D,E). The impact of EPA on alcohol-related liver disease is not well-understood. However, in non-alcoholic fatty liver disease, supplementation of EPA at 1.8 or 2.8 g/L showed no benefit in blood or hepatic markers of non-alcoholic steatohepatitis in a phase II clinical trial with 243 subjects enrolled at 37 sites in North America [9].

Three sphingomyelin species were increased in serum samples, while they were decreased in the fecal samples of patients with progressive liver disease compared with non-progressive liver disease (Figure 3D,E). Sphingolipids are ubiquitous constituents of the cell membrane. Hydrolysis of membrane sphingomyelins by acid sphingomyelinases produces ceramides, which serve as a second messenger in the sphingomyelin signaling

pathway and regulate a wide range of cellular responses [10]. Acid sphingomyelinases could be activated by alcohol, which has been proven to be a regulator of steatosis, fibrosis, lipotoxicity, and endoplasmic reticulum stress [11].

Although lipid metabolism is mainly controlled by the host, intestinal microbes also affect host lipid composition, including the production of sphingolipids, polyunsaturated fatty acid-derived metabolites, and hydroxy fatty acids [12–15]. Bacterial biosynthesis of saturated long chain fatty acids was reduced by chronic ethanol feeding in a mouse model [16]. In the present study, we found that two microbial lipid pathways were enriched in progressive liver disease compared with non-progressive liver disease, including lipopolysaccharide biosynthesis and teichoic acid (poly-glycerol) biosynthesis (Figure 6B). An *in vitro* study in primary hepatocytes showed that lipopolysaccharide potentiated the effects of the ethanol on the sphingomyelin cycle [17]. In addition, lipopolysaccharide is an important mediator of alcohol-related liver disease. Bacterial lipopolysaccharide translocates to the liver, which is facilitated by a disrupted gut barrier. Lipopolysaccharide binds to its receptor Toll-like receptor 4 in the liver, which contributes to the progression of alcohol-related disease [18]. Thus, increased microbial lipopolysaccharide biosynthesis could contribute to disease progression. In addition to lipopolysaccharide, which is found in the outer membrane of Gram-negative bacteria, the biosynthesis of teichoic acids was also increased, which is mostly found within the cell wall of Gram-positive bacteria (Figure 6B). Teichoic acids include both lipoteichoic acids and wall teichoic acids. Lipoteichoic acids are the agonist of Toll-like receptor 2 [19]. The activation of Toll-like receptor 2 also contributes to alcohol-related liver disease [20].

The limitations of this study include that the sample size is relatively small, the information on polymorphism, alcohol dehydrogenase (ADH), or Cytochrome P450 2E1 enzymes in patients is not available; liver tissue in this patient cohort is not available either. Although we used a cross-validation approach in the random forest model, our findings need to be confirmed in an independent, larger patient cohort using targeted lipid analysis. Despite this, we identified that lipid biomarkers, especially fecal lipids, showed great power to predict progressive liver disease at an early stage. This will allow physicians to initiate aggressive treatment of the underlying alcohol use disorder, which is currently the most important intervention for alcohol-related liver disease.

4. Materials and Methods

4.1. Patients

A total of 32 alcohol use disorder (AUD) patients (Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition criteria) admitted for elective alcohol withdrawal to a dedicated alcohol withdrawal unit were recruited for the study. Inclusion and exclusion criteria has been described in our previous study [21]. Stool samples were collected from the first bowel movement after admission. They were compared to a total of 17 nonalcoholic controls, who were social drinkers and consumed less than 20 g of alcohol per day, matched for gender, age, and BMI. During the two months preceding enrollment, nonalcoholic controls or patients with alcohol use disorder did not take immunosuppressive medication or antibiotics.

AUD patients were split into non-progressive liver disease and progressive liver disease based on clinical parameters as described in our previous publication [21]. The determination of blood cytokeratin 18, soluble CD14, and fecal albumin levels has been described in our previous study [21].

4.2. Lipidomics Analysis

Serum and fecal sample extraction was performed as described in our previous publication [22]. Fecal samples (10 mg) were homogenized at 1500 rpm for 30 s using a GenoGrinder 2010 (SPEX SamplePrep, Metuchen, NJ, USA). Serum samples (20 µL) were extracted without homogenization. Lipidomics data acquisition was performed as described in our previous publication [22]. LC-MS raw data files were converted to ABF

files using ABF converter (<https://www.reifycs.com/AbfConverter/>, accessed on 4 March 2021) and then processed by MS-DIAL version 2.94 [23] and MS-FLO [24] as described in our previous publication [22]. For compound identification, retention time-*m/z* libraries and MS/MS spectra databases were used as uploaded to MassBank of North America. Features present in at least 50% of samples in each group were reported.

4.3. Shotgun Metagenomics Analysis

Due to the sample availability, fecal DNA was extracted from stool samples in seven control subjects, 16 patients with non-progressive liver disease, and 15 patients with progressive liver disease using a FastDNA Spin Kit for Soil (MP-Biomedicals, Irvine, CA, USA) as described in our previous publication [25]. Illumina HiSeq 4000 generating 150 bp paired-end reads was used for shotgun metagenomics sequencing. Quality control of reads was performed using KneadData version 0.7.2 (<https://huttenhower.sph.harvard.edu/kneaddata/>, accessed on 4 March 2021), followed by The HMP Unified Metabolic Analysis Network 2 (HUMAN2) version 0.11.1 (<https://huttenhower.sph.harvard.edu/humann2/>, accessed on 4 March 2021) for the profiling of microbial pathways [26]. MetaCyc database was used for the fecal microbial pathway analysis [27]. Each of the HUMAN2 abundance output was normalized into relative abundance. A linear discriminant analysis (LDA) effect size (LEfSe) was used in this study for the biomarker discovery [28].

4.4. Statistical Analysis

R (version 3.6.2) (<https://cran.r-project.org/bin/windows/base/old/3.6.2/>, accessed on 4 March 2021) was used for statistical analysis. A Kruskal–Wallis and Mann–Whitney–Wilcoxon test was used for the comparison of three groups and two groups, respectively. The Benjamini–Hochberg procedure was used to control the false discovery rate. Heatmap and PLS-DA plots were generated using MetaboAnalyst 4.0 [29]. The Spearman correlation was conducted to correlate clinical parameters with serum or fecal lipids. A random forest model was built on the H₂O platform (<https://www.h2o.ai>, accessed on 6 April 2021) to predict the progressive liver disease using serum and fecal lipids. The lipidomics dataset was split into training and test datasets (80:20 stratified splits). We performed a stratified 5-fold cross-validation on the training set to choose the tuning parameters for the model.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/metabo12050433/s1>, Figure S1: Boxplot of nine fecal lipids used for the prediction of progressive liver disease. Supplementary File: Serum and fecal lipidome.

Author Contributions: Conceptualization, B.G. and B.S.; methodology, B.G.; software, B.G.; validation, X.Z.; formal analysis, B.G., S.Z., A.A. and O.Q.; investigation, B.G. and L.M.; resources, B.S.; data curation, B.G. and X.S.; writing—original draft preparation, B.G.; writing—review and editing, B.S. and P.S.; visualization, B.G.; supervision, B.S.; project administration, B.S. and P.S.; funding acquisition, B.S. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported in part by NIH grants R01 AA24726, R01AA020703, U01 AA026939, by Award Number BX004594 from the Biomedical Laboratory Research & Development Service of the VA Office of Research and Development (to B.S.) and services provided by NIH centers P30 DK120515 and P50 AA011999. P.S. received grant support from Fond National de Recherche Scientifique Belgium (J.0146.17 and T.0217.18) and Action de Recherche Concertée (ARC), Université Catholique de Louvain, Belgium.

Institutional Review Board Statement: The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institution's human research and ethical committee (Commission d'Ethique Biomedicale Hospitalo-Facultaire, Université Catholique de Louvain, Brussels, Belgium; B403201422657).

Informed Consent Statement: Written informed consent was obtained from all patients and controls.

Data Availability Statement: Data is available upon request. Contact information: Bernd Schnabl, (M.D., Department of Medicine, University of California San Diego, Email beschnabl@health.ucsd.edu). The data are not publicly available due to privacy restrictions.

Acknowledgments: We acknowledge the High Performance Computing Center of Nanjing University of Information Science and Technology for their support of this work.

Conflicts of Interest: B.S. has been consulting for Ferring Research Institute, HOST Therapeutics, Intercept Pharmaceuticals, Mabwell Therapeutics, Patara Pharmaceuticals, and Takeda. B.S.'s institution, UC San Diego, has received grant support from Axial Biotherapeutics, BiomX, CymaBay Therapeutics, NGM Biopharmaceuticals, and Synlogic Operating Company. B.S. is the founder of Nterica Bio. UC San Diego has filed several patents with B.S. as the inventor related to this work.

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Complementary discussion and conclusion

We here identified lipid differentially present in both serum and stools in AUD patients with progressive liver disease. However, it is important to notice that we used a completely untargeted approach to identify these lipid species. There are some limitations in the untargeted lipidomics approach we used: first, we do not really measure the levels of the lipids but we estimate that these lipids could be differentially present compared to another group; second, the identification of the lipid species is based on a software that matches the mass to charge and retention time in the liquid chromatography to libraries which cannot differentiate between possible isomers present in the sample¹⁸². Targeted approach with the use of standards in a specific concentration is needed to confirm the increased levels of these lipids.

We found increased levels of saturated sphingomyelins only in the blood circulation while they decreased in fecal samples of patients with progressive ALD compared with non-progressive liver disease. These lipids can be produced and/or transported in different organs where they are transformed into dihydroceramides, which serve as a second messenger in the sphingomyelin signaling pathway and regulate a wide range of cellular responses (e.g., apoptosis, proliferation, fatty acid metabolism, lysosome and mitochondrial functions)¹⁸³. We here did not investigate the possible interaction of these lipids with cells implicated in intestinal barrier dysfunction. Since different cell types in the intestine (e.g., T cells) use lipids for their functions¹⁸⁴, it is possible that sphingomyelins and/or their cleavage products could interfere with T cells in the gut thus contributing to intestinal barrier dysfunction. The hypothesis of potential interaction between sphingomyelin and intestinal T lymphocytes will be tested in part in the chapter 3 of this thesis.

In conclusion, we have identified in this study the lipids specific of patients with progressive ALD compared to the benign forms of liver disease.

The perspectives of this study are the following:

- Evaluation of circulating lipid levels by targeted lipidomics
- Assessment of potential interaction between sphingolipid and intestinal T cells

ANNEX 3 Liver alterations are not improved by inulin supplementation in alcohol use disorder patients during alcohol withdrawal: A pilot randomized, double-blind, placebo-controlled study

Liver alterations are not improved by inulin supplementation in alcohol use disorder patients during alcohol withdrawal: A pilot randomized, double-blind, placebo-controlled study



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Summary

Background Emerging evidence highlights that targeting the gut microbiota could be an interesting approach to improve alcohol liver disease due to its important plasticity. This study aimed to evaluate the effects of inulin supplementation on liver parameters in alcohol use disorder (AUD) patients (whole sample) and in a subpopulation with early alcohol-associated liver disease (eALD).

Methods Fifty AUD patients, hospitalized for a 3-week detoxification program, were enrolled in a randomized, double-blind, placebo-controlled study and assigned to prebiotic (inulin) versus placebo for 17 days. Liver damage, microbial translocation, inflammatory markers and 16S rDNA sequencing were measured at the beginning (T1) and at the end of the study (T2).

Findings Compared to placebo, AST ($\beta = 8.55$, 95% CI [2.33;14.77]), ALT ($\beta = 6.01$, 95% CI [2.02;10.00]) and IL-18 ($\beta = 113.86$, 95% CI [23.02;204.71]) were statistically significantly higher in the inulin group in the whole sample at T2. In the eALD subgroup, inulin supplementation leads to specific changes in the gut microbiota, including an increase in *Bifidobacterium* and a decrease of *Bacteroides*. Despite those changes, AST ($\beta = 14.63$, 95% CI [0.91;28.35]) and ALT ($\beta = 10.40$, 95% CI [1.93;18.88]) at T2 were higher in the inulin group compared to placebo. Treatment was well tolerated without important adverse events or side effects.

Interpretation This pilot study shows that 17 days of inulin supplementation versus placebo, even though it induces specific changes in the gut microbiota, did not alleviate liver damage in AUD patients. Further studies with a larger sample size and duration of supplementation with adequate monitoring of liver parameters are needed to confirm these results. Gut2Brain study: <https://clinicaltrials.gov/ct2/show/NCT03803709>

Funding Fédération Wallonie-Bruxelles, FRS-FNRS, Fondation Saint-Luc.

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Keywords: Alcohol use disorder; Prebiotics; Inulin; Alcohol-associated liver disease; Gut microbiota; Inflammation

eBioMedicine 2022;80:104033

Published online xxx
<https://doi.org/10.1016/j.ebiom.2022.104033>

Introduction

Alcohol-associated liver disease (ALD) is one of the leading causes of chronic liver disease worldwide. Although most patients with an alcohol use disorder (AUD) present with steatosis, only 10–20% develop progressive forms of liver disease and its related complications.

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Research in context

Evidence before the study

Alcohol use disorder (AUD) patients displayed concomitant liver and gut microbiota alterations. Recent studies have demonstrated the potential role of the gut-liver axis in alcohol liver disease progression. Inulin is considered a prebiotic and promotes the growth of beneficial bacteria especially *Bifidobacteria*. Modulating the gut microbiota could be an interesting approach to improve inflammatory status and liver damage in AUD.

Added value of the study

In this pilot interventional randomized placebo-controlled study we demonstrate that 17 days of inulin supplementation in AUD patients has no beneficial effect on liver, microbial translocation or inflammatory markers over alcohol abstinence alone even in patients with early alcohol-associated liver disease (eALD). Although inulin administration does modulate the microbiota inducing specific changes in its composition, AUD patients supplemented with inulin had higher level of AST, ALT and IL-18 after 17 days of supplementation compared to placebo. In particular, in AUD patients presenting an eALD, inulin further decreased microbial diversity of an already disturbed microbiota at baseline.

Implications of all the available evidence

Inulin does modulate the gut microbiota in a similar way independently of the severity of liver disease. However, this prebiotic intervention did not add any additional benefit of alcohol abstinence alone regarding improvement of liver function or inflammatory markers. Further studies with a larger sample size and duration of supplementation with accurate monitoring of liver parameters are needed to confirm these results.

Currently, the pathophysiological mechanisms implicated in liver disease progression are not completely understood and there is no drug approved for treatment of ALD.¹

Recent reports have highlighted the potential role of the gut-liver axis in ALD progression. Gut barrier dysfunction together with alterations in the composition of the intestinal microbiota as well as elevated systemic microbial translocation have been associated with ALD progression.²

Murine models of chronic ethanol exposure have been used to discover new potential therapeutic targets at the frontier of the gut-liver axis in ALD.³ Manipulations designed to restore gut barrier function, thus preventing microbial translocation, or alleviating dysbiosis all improved liver disease in animals.^{4–8} However, these data cannot necessarily be extrapolated to human pathology for several reasons. Animals have a natural aversion to alcohol,^{9,10} a 5 times faster ethanol

metabolism,¹¹ and profound differences in their immune system¹² and their microbiota¹³ compared to humans. Animals do only develop mild forms of ALD upon chronic alcohol feeding and do not resume the liver-damage pattern observed in humans.¹⁴ Clinical studies targeting the gut microbiota in AUD patients are scarce and generally focused on patients with severe alcoholic hepatitis and decompensated cirrhosis.¹⁵ Little is known about the impact of a gut microbiota modifying strategy in AUD patients on earlier non-cirrhotic disease stages of ALD. One potential way of modulating the gut microbiota in those patients might be the use of prebiotics. Prebiotics are defined as 'substrates that are selectively used by host microorganisms conferring a health benefit'¹⁶ meaning that they promote the growth of some specific bacteria. Of particular interest, inulin-type fructan (ITF) is known to favour *Bifidobacterium* and *F. prausnitzii* two well-recognized beneficial bacteria which are decreased in AUD patients.^{17–19} It has been shown that *Bifidobacteria* negatively correlate with pro-inflammatory cytokines and improve intestinal health in humans.^{20,21} *F. prausnitzii*, a butyrate producer, exhibits anti-inflammatory properties both *in vitro* and *in vivo* studies.^{22–23} ITF supplementation and subsequent microbial modulation, have been shown to exert beneficial effects on gut barrier function, reduce microbial translocation and thus attenuate systemic inflammation.^{15,18,24,25} We therefore designed a randomized, double-blind, placebo-controlled study to modulate the gut microbiota of AUD patients using a 3-week inulin supplementation. Since the liver is closely connected to the gut via the portal vein, we hypothesized that restoring the microbial balance with subsequent improvement of the gut barrier function could exert a beneficial effect on ALD development.

The principal aim of this sub-study was to investigate the effect of inulin supplementation on liver parameters and systemic inflammation in AUD subjects. The secondary objective was to study the effect of supplementation in a subgroup of patients with early alcohol-associated liver disease (eALD).

Methods

Study design and ethics

The Gut2Brain study was a randomized, double blind, placebo-controlled trial. Each subject was randomly assigned to daily intake of prebiotic inulin (Fibruline®; Inulin group) or maltodextrin (Placebo group). To reduce potential gastrointestinal side effects, the dose of inulin or maltodextrin increased gradually from 4 to 16 g per day during the 17 days of treatment (4 g from day 3 to day 4; 8 g from day 5 to day 14 and 16 g from day 15 to day 19 of the detoxification program; Supplementary Figure 1). We have previously shown that 16 g

of inulin per day was well tolerated and had a bifidogenic effect in obese patients.^{18,26,27}

The trial protocol was published on protocols.io (dx.doi.org/10.17504/protocols.io.bvs2n6ge).

This study was approved by the ethics committee of the hospital (Nb: 190616V1). All participants signed informed consent prior to inclusion and the trial was registered in the clinicaltrials.gov registry (ClinicalTrials.gov identifier: NCT03803709). Due to an administrative problem beyond our control, a slight delay between enrolment of study participants and registration on the website had occurred. During this period, the trial was conducted as if the trial had been registered, and only six patients were enrolled in that first phase (October–December 2018). In this study, we focused on the outcomes of the Gut2Brain study related to liver and inflammation (primary outcome see²⁸).

Patients

Fifty AUD patients undergoing elective alcohol withdrawal were recruited between October 2018 and December 2019. They followed a 3-week highly standardized alcohol-detoxification and rehabilitation program (Supplementary Figure 1) in an academic hospital (Brussels, Belgium). This program includes 1 week in the hospital, followed by 1 week at home and another week in the hospital (Supplementary Figure 1).

AUD patients were diagnosed by a psychiatrist according to the *Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition*.

Eighteen to 65 years old male or female subjects who were actively drinking until at least 48 h prior to admission were included in the study. The following exclusion criteria applied: presence of another addiction (except tobacco), inflammatory bowel disease, chronic inflammatory diseases (such as rheumatoid arthritis), cancer, obesity (BMI ≥ 30 kg/m²), diabetes, bariatric surgery, or severe cognitive impairment (Mini Mental State Examination (MMSE) < 24). Patients with known cirrhosis or significant hepatic fibrosis ($\geq F2$) detected by Fibroscan (>7.6 kPa) immediately after admission were also excluded from the study. Patients who have been taking antibiotics, probiotics or prebiotics during the 3 months prior to enrolment or who had regularly used non-steroidal anti-inflammatory drugs or glucocorticoids during the month prior to enrolment were excluded.

Randomization and masking

The randomization was performed using the method of randomly permuted blocks via the website www.randomization.com, by a researcher not clinically involved to ensure the double blind. The treatment allocation sequence was conserved in a sealed envelope kept in a separate location, inaccessible to the investigators, and was only made available at the time of database lock.

Neither the patient nor the investigators knew which boxes contained inulin or placebo until unblinding at the end of the study.

Examinations and sample collections

A Fibroscan[®] (Echosense, Paris, France) combined with the controlled attenuation parameter (CAP) was performed at admission and repeated after 19 days. Fasting blood samples were drawn and stool samples collected twice on day 2 (T1) and 19 (T2) after the admission. Blood samples were centrifuged at 1000g for 15 min at 4°C. Plasma, serum and stool samples were stored at -80 °C until use.

Inflammatory markers

Plasma concentrations of inflammatory markers (IL-18, MCP-1, IFN- γ , IL-8, IL-10, TNF- α , IL-6) and Fibroblast growth factor 21 (FGF-21) were determined using the Meso Scale Discovery (MSD) U-PLEX assay (Rockville, MD, USA) following the manufacturer's instructions.

Microbial translocation and biomarkers of liver cell damage

Microbial translocation was determined in plasma using Lipopolysaccharide Binding Protein (LBP), soluble CD14 (sCD14) (Human LBP duoset ELISA and Human CD14 Quantikine ELISA kit sCD14, Biotechne Ltd, Abingdon, United Kingdom) and Peptidoglycan Recognition Proteins (Human PGRPs ELISA kit, Thermofisher, Merelbeke, Belgium). Liver damage was assessed by measuring serum Keratin 18 (K18) (K18-M65 ELISA kit; TECOMedical AG, Sissach, Switzerland). All assays were performed in duplicate following the manufacturer's instructions. Routine biochemical analyses were performed by the clinical biochemistry laboratory of the hospital.

Classification of patients according to severity of liver disease

Currently, non-invasive testing allows for a good estimation of steatosis and fibrosis. Controlled attenuation parameter measurements > 250 dB/m is a generally accepted cut-off for steatosis^{29,30} and liver elasticity measurements by Fibroscan reliably predict fibrosis stage.³¹ In addition, a novel biomarker, keratin 18 (K18), correlates with histological changes³² and has the potential to distinguish severe forms of ALD from less severe ones.^{33,34} Our own studies also showed that K18-M65 can be used to discriminate patients with simple steatosis from those that have progressed to early stages of alcohol-associated liver disease or steato-fibrosis.² Validation of those findings in a cohort of 200 patients identified a level of K18-M65 > 270 U/L as a cut-off with excellent sensitivity and specificity (own unpublished data). Therefore, AUD patients were classified according to the severity of liver disease using

these clinical biomarkers. Briefly, patients with a normal or increased CAP values (> 250 dB/m)^{39,40} but normal transaminases, normal bilirubin and K18-M65 < 270 U/L were considered as having minimal liver disease (minimal liver involvement or simple steatosis). Patients with CAP values > 250 dB/m AND K18-M65 > 270 U/L and/or AST/ALT > 40 U/L were considered as having progressed to early alcohol-associated liver disease (eALD). In addition to CAP values, the presence of steatosis was further confirmed by Doppler ultrasound examination performed on the second day after admission. According to the predefined inclusion/exclusion criteria, patients with liver stiffness values > 7.6 kPa on Fibroscan at admission (significant fibrosis) were excluded from the study.

Patients' care and monitoring

Patients are taken care of by a multidisciplinary team consisting of a gastroenterologist, a psychiatrist, a psychologist, a dietician, a social assistant, and a dedicated nursing team. At admission, a complete medication and past medical history was taken, and a complete physical examination was performed including collection of basic demographic data such as age, gender, weight, and height. The amount of alcohol consumed the week before hospitalization was evaluated with the time line follow-back approach as previously described.^{35,36} During hospitalization, patients underwent daily clinical monitoring by the nursing and medical team following the standardized scheme of the unit. All adverse events considered as not related to alcohol withdrawal were recorded. Psychological support was provided throughout the study.

Potential gastro-intestinal side effects of inulin were assessed by a questionnaire commonly used to evaluate the symptoms of irritable bowel syndrome.³⁷ This questionnaire assessed the presence of abdominal pain and bloating, satisfaction about intestinal transit and whether gastrointestinal symptoms affected daily life by using visual analogic scale from 0 to 100. Patients were also asked about the frequency of stools and completed the Bristol Stool Form Scale (BSFS)³⁸ allowing to identify stool types ranging from the hardest (type 1) to the softest (type 7). Evaluation was performed at admission, during the second week of treatment and at the end of the study.

Dietary intake was evaluated by a trained dietician at admission of week 1 and week 3. During the week at home, dietary changes were monitored using a food diary. Detailed results of the dietary aspects are published elsewhere.^{28,39} Dietary advice was provided "on demand" at the end of study. All patients received standard hospital diet during hospitalization.

16S rRNA sequencing and data analysis

Stool samples were collected at Day 2 (T1) and at the end of the intervention (Day 19 – T2). They were collected

and stored immediately at -20°C and then transferred to -80°C within 5 to 10 h. Genomic DNA was extracted from the faeces using a QIAamp DNA Stool Mini Kit (Qiagen, Germany), including a bead-beating step and following the protocol Q.⁴⁰ The composition of the gut microbiota was analysed by Illumina sequencing of the 16S rRNA gene and qPCR of 16S rDNA was used to quantify the abundance of total bacteria and *Bifidobacterium* spp. (see supplementary Material-Microbiota analysis).

Statistical analysis

Data were presented as mean $\pm$ standard error of the mean (SEM) or mean $\pm$ standard deviation (SD). The efficacy analysis has been conducted in the modified intention to treat (mITT) population including all the randomized patients that completed the study. According to data distribution, baseline parameters were compared using T-test or Mann–Whitney U test. Linear regression models adjusted for gender, the quantity of ethanol consumed during the second week of the program and the baseline measurement of the outcome were performed to analyse the difference between placebo and inulin groups at T2.

For the gut microbiota analysis only the phyla, families, and genera with an average relative abundance superior to 0.1% were analysed. We used a Mann–Whitney U test in R to compare the relative abundance between groups and the within group analyses were evaluated using a Wilcoxon paired test.

Spearman partial correlations (adjusted for gender and the quantity of ethanol consumed during the second week of the program) were performed to assess the relationships between biological outcomes and microbial data. A *p*value $< .05$ was considered as statistically significant. Statistical analyses were performed using SAS version 9.4 and Graphpad Prism 8.0. As this was a pilot study with a small sample size, the multiplicity of outcomes was not taken into account.

Sample size

Sample size was estimated using G*Power based on the bifidogenic effect of inulin.⁴⁸ Therefore, we estimated that a total sample size of 50 participants, with a 20% drop out during the study and 20 patients in each group completing the study provides 80% power to observe an effect size of 0.34 (target difference, SD: 4.25) for the relative abundance of *Bifidobacterium* genus using a power calculation test with a 0.05 two-sided significance level.

Role of funding source

The funders had no role in collection, analysis, and interpretation of the data or in the writing of this publication.

Results

Study population

To reach the final sample, 150 patients were screened, 77 did not meet the inclusion criteria and 23 refused to participate. Finally, 50 AUD patients were randomized. Among those, 44 patients had complete biological data and composed the modified intention to treat (ITT) population (Figure 1). Compliance with the nutritional supplementation was 96% in the placebo group and 98% in the inulin group. The sociodemographic characteristics and biological parameters at baseline are presented in Tables 1 and 2. There was a women predominance in the inulin group (20% in placebo vs 48% in the inulin group, Chi-Square test $p = 0.11$) and they had one additional criterion in the DSM-5 classification compared to the placebo group (7.9 ± 1.9 in placebo vs 9.3 ± 1.4 in inulin group, Mann Whitney U test $p = 0.02$). During the second week of the detoxification program, among the patients who completed the study, 7 patients relapsed in the placebo group vs 10 in the inulin group (32% vs 45% respectively, Fisher's test $p = 0.53$). Patients who relapsed in placebo group consumed 53 g/d alcohol on average vs 74 g/d in inulin group (Mann Whitney U test, $p = 0.47$).

We first studied the effect of inulin supplementation compared to the placebo group in the whole sample ($n = 50$).

Then, we stratified the population according to the severity of the liver disease (minimal liver disease (LD) vs early ALD) to study the effect of inulin supplementation in patients with more severe liver disease.

Inulin supplementation did not improve liver damage, microbial translocation, and inflammatory markers over abstinence alone

The univariate analysis highlighted that inulin supplementation did not have a beneficial effect on liver enzymes, microbial translocation, and inflammatory markers (Table 3). Since an imbalance between the groups was initially observed between gender (50% of woman in inulin group vs 23% in placebo group), we adjusted the analysis for this parameter as well as for the amount of ethanol consumed during the intermediate week and the baseline parameter. Indeed, these parameters are known to influence the recovery of liver and inflammatory parameters.^{41,42} Linear regression analysis revealed that the levels of AST and ALT at T2 were higher in the inulin group than in the placebo group (Linear regression models $\beta = 8.55$, 95% CI [2.33; 14.77], $p = 0.008$ and $\beta = 6.01$, 95% CI [2.02; 10.00] $p = 0.004$ respectively; Table 3). Five patients (22.7%) in the inulin group had an AST value above the upper limit of normal, while all patients in the placebo group returned to a normal value at T2 (Fisher test, $p = 0.047$). CAP values and FGF-21 were not

significantly different between the placebo group and the inulin group (Table 3).

No difference was observed neither for microbial translocation nor for inflammatory markers between groups except for IL-18. The adjusted linear regression analysis showed a statistically significant higher level of IL-18 in inulin group compared to placebo group at T2 (Linear regression model $\beta = 102.85$, 95% CI [1.46; 190.25], $p = 0.02$; Table 3).

Taken together, our results suggest that 17 days of inulin supplementation has no beneficial effect on liver, bacterial translocation, and inflammatory markers in our population of AUD patients during a period of 3 weeks of abstinence.

The impact of inulin supplementation in AUD patients with early alcohol-associated liver disease (eALD)

We have previously shown that the gut microbiota of patients with more severe liver disease was particularly impaired.² Therefore, we wanted to study the effect of inulin supplementation in a subgroup of patients with eALD. For this exploratory analysis, we stratified the study population into patients with minimal LD and eALD according to clinical parameters. As by definition, patients with eALD were characterized by elevated AST, ALT, CAP, and serum K18-M65 compared to minimal LD patients. In addition, we found that among the upregulated markers, two of them, FGF-21, and IL-8, also distinguished minimal LD from eALD with statistically significantly higher levels in the latter one (Supplementary Figure 2).

Inulin supplementation induced different changes in gut microbiota composition at the phylum, family and genus level in minimal LD and eALD groups

Microbiota analysis at baseline revealed that the eALD group displayed a decreased richness since the Chao-1 index was statistically significantly decreased compared to minimal LD patients (Supplementary Figure 3a). A trend was also observed towards a lower number of species observed and a lower Shannon index ($p = 0.09$ and $p = 0.10$ respectively; Supplementary Figure 3a). Total bacteria measured by qPCR, was not different between the groups (Supplementary Figure 3b). The LEfSE analysis showed that Firmicutes and especially Clostridia were higher in minimal LD group while Proteobacteria were statistically significantly higher in eALD patients (Supplementary Figure 3c and d). At the genus level, the relative abundance of *NK4A214* group and *UCG-005* from *Oscillospira* family, *Ruminococcus* and *Fusicatibacter* were statistically significantly higher in minimal LD group while *Flavonifractor* and *Veillonella* were statistically significantly increased in eALD (Supplementary Figure 3d). The effects of inulin supplementation on the gut microbiota composition have been

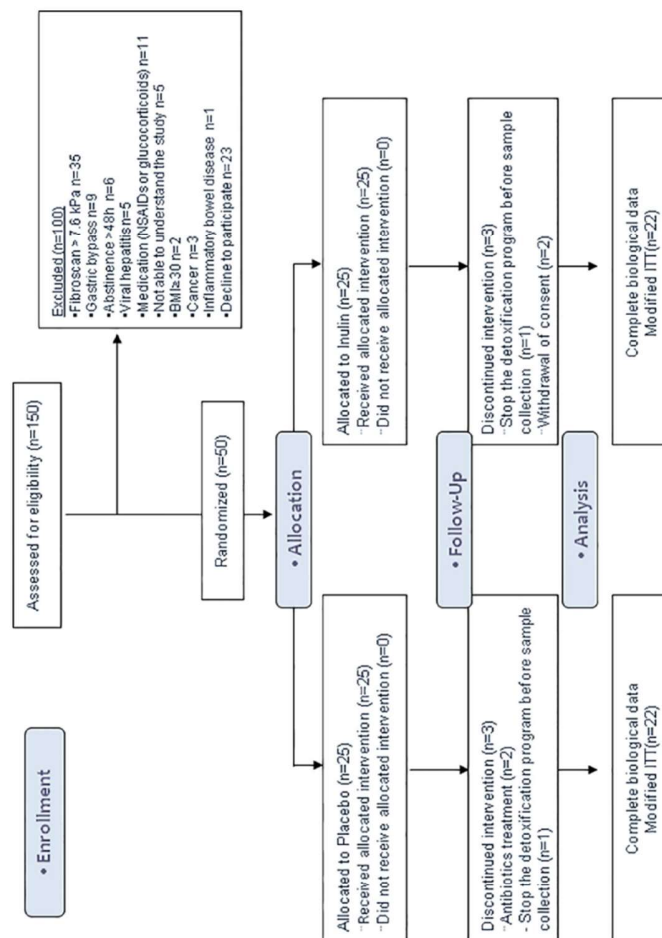


Figure 1. CONSORT flow diagram of the gut2Brain study.

	Placebo n = 22	Inulin n = 22
Sociodemographic characteristics		
Age (y)	45.8 [41.0; 56.3]	47.8 [38.9; 55.9]
Gender, n (%)		
Male	17 (77.3)	11 (50.0)
Female	5 (22.7)	11 (50.0)
Marital status, n (%)		
Couple/ married	10 (45.4)	7 (32.0)
Single	8 (36.4)	12 (52.0)
Separated/divorced	4 (18.2)	3 (16.0)
Educational level, n (%)		
Primary	2 (9.1)	2 (9.1)
Secondary	9 (40.9)	6 (27.3)
University	11 (50.0)	14 (63.6)
Clinical examination		
Weight (kg)	71.2 ± 10.2	73.4 ± 14.7
BMI (kg/m ²)	23.3 ± 3.5	24.4 ± 3.1
MMSE score	28.7 ± 1.2	27.7 ± 2.9
Smoking, n (%)	18 (81.8)	16 (72.7)
Alcohol history		
DSM-5 AUD score	7.9 ± 1.9	9.3 ± 1.4
Age of loss of control (years)	29.5 [25.0; 36.0]	32.0 [22.0; 40.0]
Number of alcohol withdrawal cures	2.5 ± 2.4	2.0 ± 2.1
Duration of drinking habit (years)	16.4 [5.7; 21.2]	14.9 [5.3; 22.4]
Alcohol consumption (g/day)	130.0 [85.6; 178.5]	143.3 [96.0; 180.0]

Table 1: Baseline characteristics of participants in the modified ITT population.
 Data are described as Mean ± standard deviation or numbers (n) with (%). Age, age of loss of control, duration of drinking habits and alcohol consumption are described as median [Q1; Q3].
 AUD, Alcohol use disorders; Alcohol Use Disorders Test; BMI, Body mass index; DSM-5, Diagnostic and Statistical Manual of Mental Disorders fifth edition; MMSE, Mini Mental State Examination

already published elsewhere, showing namely an increase in *Bifidobacterium* as a “signature” of inulin intake.²⁸ We compared the change from baseline of *Bifidobacterium* between placebo and the inulin group. *Bifidobacterium* increased statistically significantly in the inulin group compared to placebo in eALD as well as minimal LD group (Figure 2a and b). The bifidogenic effect of inulin was therefore confirmed in both groups. Inulin supplementation induced a statistically significant decrease of all α diversity indexes compared to placebo in the eALD group (Figure 2c) while only the number of observed species was decreased in the minimal LD group (Figure 2d). In the eALD group, analysis of phylum and family levels of bacteria revealed minor changes in those who received placebo while an increase of *Bifidobacteriaceae* (Actinobacteriota) and a decrease of *Bacteroidaceae* (Bacteroidota) was found in patients supplemented with inulin (Supplementary Figure 4a). In the minimal LD group, inulin supplementation has no effect at phylum level, but similar changes were observed at family level with an additional increase of *Veillonellaceae* family (Supplementary Figure 4b). At the genus level,

prebiotic treatment also increased *Dialister* whereas *Bacteroides*, *Ruminococcus torques* and *Dorea* decreased in the eALD group (Supplementary Table 1). In the placebo group, *Fusicatenibacter*, *Oscillospiraceae* UCG-002, *Monoglobus*, *Alistipes* and *Lachnospiraceae* ND3007 increased statistically significantly between T1 and T2 while *Oscillibacter*, *Flavonifractor*, *Colidextribacter* and *Sutterella* decreased (Supplementary Table 1).

The genera modified between T1 and T2 in minimal LD group are presented in Supplementary Table 2. *Bifidobacterium* and *Veillonella* increased statistically significantly after 17 days of inulin supplementation. In contrast, *Lachnospiraceae* UCG-003 and *Bacteroides* decreased statistically significantly (Supplementary Table 2). In the placebo group, *Lachnospiraceae* NK4A136 group increased while *Blautia*, *Eubacterium eligens* group and *Acidaminococcus* decreased after 17 days (Supplementary Table 2).

Globally, inulin supplementation exacerbated the decrease in α -diversity but did not impact the bacteria shown to be already altered at baseline in eALD patients.

	Placebo <i>n</i> = 22	Inulin <i>n</i> = 22
Liver related markers		
AST, IU/L	32.0 [25.0; 76.0]	36.0 [22.0; 70.0]
ALT, IU/L	32.5 [22.0; 62.0]	32.5 [19.0; 78.0]
γGT, IU/L	71.5 [52.0; 185.0]	63.0 [47.0; 131.0]
Liver elasticity, kPa	5.5 [4.5; 6.0]	5.7 [4.6; 6.3]
CAP, dB/m	267.0 [200.0; 312.0]	307.0 [234.0; 333.0]
K18-M65, pg/ml	215.0 [164.6; 322.9]	278.7 [162.3; 417.5]
FGF-21, pg/ml	2073.0 [1633.0; 3915.0]	2632.4 [2048.0; 4769.1]
Biochemical markers		
Bilirubin, mg/dL	0.40 [0.30; 0.60]	0.40 [0.30; 0.50]
Albumin, g/L	46.0 [44.0; 48.0]	47.0 [46.0; 48.0]
Creatinine mg/dl	0.78 [0.67; 0.84]	0.76 [0.66; 0.86]
Bacterial translocation		
LBP, ng/ml	18.6 [18.0; 20.1]	19.1 [18.1; 19.7]
sCD14, ng/ml	1601.4 [1470.0; 1845.6]	1739.2 [1516.0; 2081.3]
PGRP, ng/ml	44.9 [35.7; 57.1]	49.5 [37.2; 74.9]
Inflammation		
IL-18, pg/ml	462.8 [368.4; 656.6]	496.3 [370.6; 679.1]
IL-8, pg/ml	5.3 [4.0; 7.5]	5.4 [4.9; 8.5]
MCP-1, pg/ml	249.2 [215.5; 335.5]	240.4 [221.0; 285.6]
TNFα, pg/ml	2.1 [1.9; 3.1]	3.0 [2.0; 4.2]
IL-6, pg/ml	4.2 [2.3; 5.8]	4.3 [3.0; 6.9]
IL-10, pg/ml	1.5 [0.6; 2.1]	1.7 [1.2; 2.6]

Table 2: Liver related and biochemical markers at baseline.
 Data are described as median [Q1; Q3].
 AST, Aspartate transaminase; ALT, Alanine transaminase; CAP, controlled attenuation parameter; K18-M65, Serum Keratin 18; sCD14, soluble CD14.

	Placebo <i>n</i> = 22 T2	Inulin <i>n</i> = 22 T2	Adjusted difference at T2 (inulin vs placebo) β [95% CI]	P ¹
Liver related markers (<i>normal range</i>)				
AST (<40 IU/L)	24.9 ± 7.4	31.6 ± 17.7	8.55 [2.33; 14.77]	0.008
ALT (<40 IU/L)	12.2 ± 6.8	16.6 ± 12.8	6.01 [2.02; 10.00]	0.004
CAP (< 250 dB/m)	226.4 ± 39.5	243.9 ± 57.3	4.76 [-23.98; 33.51]	0.73
K18-M65 (<270 pg/ml)	221.1 ± 109.7	306.9 ± 204.9	74.48 [-1.89; 150.84]	0.06
FGF-21, pg/ml	2240.2 ± 1409.9	2590.3 ± 1515.7	-164.70 [-867.38; 537.97]	0.64
Bacterial translocation				
LBP, ng/ml	18.7 ± 1.6	19.1 ± 1.7	0.46 [-0.30; 1.22]	0.23
sCD14, ng/ml	1406.8 ± 282.2	1647.8 ± 448.7	94.71 [-92.61; 282.04]	0.31
PGRP, ng/ml	50.2 ± 18.5	58.9 ± 24.7	2.39 [-7.61; 12.40]	0.63
Inflammation				
IL-18, pg/ml	474.4 ± 208.4	545.3 ± 239.5	102.85 [15.46; 190.25]	0.02
IL-8, pg/ml	5.3 ± 2.2	5.5 ± 2.2	0.22 [-1.09; 1.53]	0.73
MCP-1, pg/ml	249.1 ± 81.8	240.0 ± 87.5	-2.71 [-47.15; 41.7]	0.90
TNFα, pg/ml	2.5 ± 1.2	3.1 ± 1.5	0.20 [-0.41; 0.80]	0.51
IL-6, pg/ml	4.7 ± 2.7	4.7 ± 2.9	-0.31 [-1.90; 1.27]	0.69
IL-10, pg/ml	1.8 ± 1.1	1.9 ± 1.0	-0.03 [-0.59; 0.53]	0.92

Table 3: Effect of inulin supplementation on liver function, bacterial translocation, and inflammatory markers in the global sample.
¹ Linear regression model adjusted for gender, the baseline measurement of the outcome and the quantity of ethanol consumed during the second week of the program. AST, Aspartate transaminase; ALT, Alanine transaminase; CAP, controlled attenuation parameter; FGF-21, Fibroblast growth factor 21; K18-M65, Serum keratin 18; sCD14, soluble CD14.

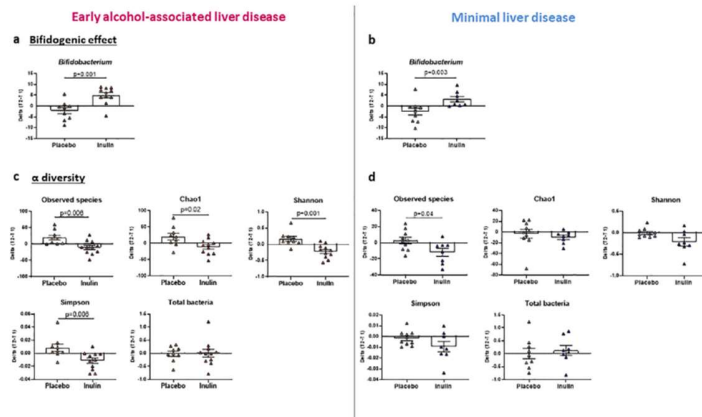


Figure 2. Effect of inulin supplementation on gut microbiota composition in minimal liver disease and early ALD patients. Early alcohol-associated liver disease (eALD): $n = 9$ and $n = 11$ in placebo and inulin groups respectively. Minimal liver disease: $n = 10$ and $n = 8$ in placebo and inulin groups respectively (a, b) Change in relative abundance of *Bifidobacterium* between T1 and T2. (c, d) Changes in alpha-diversity indexes: Number of observed species, Chao-1, Shannon, Simpson and total bacteria measured by qPCR. Mann-Whitney U tests were performed to compare placebo and inulin groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are expressed as mean $\pm$ SEM.

Inulin supplementation did not improve liver damage, microbial translocation, and inflammatory markers in the subgroup of patient suffering from early alcohol-associated liver disease

We compared liver enzymes, microbial translocation and inflammatory markers between inulin and placebo at T2 and found no difference (Table 4). However, the adjusted linear regression models revealed that AST and ALT at T2 remained higher in the inulin group compared to placebo (Linear regression models $\beta = 14.63$, 95% CI [0.91; 28.35], $p = 0.038$ and $\beta = 10.40$, 95% CI [1.93; 18.88] $p = 0.02$ respectively; Table 4). No statistically significant differences were observed between placebo and inulin for the other markers after adjustment.

Overall, our data do not support a beneficial effect of inulin supplementation compared with placebo, even in the sub-group of patients with more severe liver disease at baseline.

Link between liver alterations, inflammatory markers, and microbiota in the subgroup of patient with eALD

To investigate whether changes in liver or inflammatory markers after treatment could be related to certain bacterial genera, we performed partial correlations adjusted for gender and the amount of alcohol consumed during the second week in the eALD group. We found moderate negative correlations between the variations of Shannon and Simpson indexes, the variation of

Fusicatenibacter and the variation of AST level ($r = -0.49$, $p = 0.03$; $r = -0.47$, $p = 0.04$ and $r = -0.46$, $p = 0.048$ respectively; Supplementary Figure 4). The variation of Shannon and Simpson indexes, *Oscillospira* *UCG-002*, and *Alistipes* were negatively associated with the variation of the translocation marker sCD14 level ($r = -0.49$, $p = 0.03$; $r = -0.50$, $p = 0.027$; $r = -0.55$, $p = 0.01$ and $r = -0.59$, $p = 0.007$ respectively) while *Sutterella* was positively associated ($r = 0.46$, $p = 0.048$). A strong positive correlation was observed between *Oscillibacter* and IL-10 while *Colidextribacter* were moderately correlated with IL-10 ($r = 0.60$, $p = 0.007$; $r = 0.48$, $p = 0.04$ respectively). No correlation between alpha diversity indexes or bacterial genera and IL-18 levels was observed (supplementary Figure 4).

Tolerability and safety

Tolerability and safety endpoints are described in Table 5. No difference was observed for abdominal pain, bloating, stool frequency, the total score of gastrointestinal tolerance or the creatinine level between placebo and inulin group at the end of the study. Only the BSFS score was statistically significantly higher in inulin group compared to placebo (3.2 ± 1.5 vs 4.5 ± 3.3 in placebo and inulin group, respectively). However, a BSFS score of 4.5 is considered as a normal score.³⁸ One patient complained of diarrhoea in the inulin group which has been considered as possibly related to the compound. Three patients reported being constipated

	Placebo n = 9 T2	Inulin n = 13 T2	Adjusted difference at T2 (inulin vs placebo) β [95% CI]	p [†]
Liver related markers (normal range)				
AST (<40 IU/L)	27.7 $\pm$ 7.5	37.4 $\pm$ 20.6	14.63 [0.91; 28.35]	0.04
ALT (<40 IU/L)	16.3 $\pm$ 9.3	21.8 $\pm$ 13.7	10.40 [1.93; 18.88]	0.02
CAP (< 250 dB/m)	240.6 $\pm$ 37.4	256.1 $\pm$ 58.1	0.20 [-43.54; 43.93]	0.99
K18-M65	265.0 $\pm$ 140.2	390.5 $\pm$ 217.8	123.41 [-30.93; 277.76]	0.11
FGF-21, pg/ml	2589.6 $\pm$ 1903.5	2457.2 $\pm$ 1218.2	-258.20 [-1428.38; 911.98]	0.65
Bacterial translocation				
LBP, ng/ml	19.1 $\pm$ 1.4	18.7 $\pm$ 1.2	-0.25 [-1.31; 0.82]	0.63
sCD14, ng/ml	1293.1 $\pm$ 271.6	1639.8 $\pm$ 423.2	210.04 [-88.54; 508.61]	0.16
PGRP, ng/ml	43.0 $\pm$ 7.7	60.8 $\pm$ 23.2	3.01 [-10.85; 16.86]	0.65
Inflammation				
IL-18, pg/ml	457.9 $\pm$ 179.5	503.9 $\pm$ 222.8	99.97 [-15.84; 215.78]	0.09
IL-8, pg/ml	6.2 $\pm$ 2.6	5.2 $\pm$ 2.0	-0.39 [-2.24; 1.47]	0.66
MCP-1, pg/ml	282.5 $\pm$ 95.0	210.3 $\pm$ 49.3	-45.67 [-102.64; 11.29]	0.11
TNF α , pg/ml	2.4 $\pm$ 1.3	2.6 $\pm$ 0.8	0.06 [-0.60; 0.72]	0.85
IL-6, pg/ml	5.3 $\pm$ 2.9	4.3 $\pm$ 3.1	-1.12 [-3.19; 0.94]	0.27
IL-10, pg/ml	2.0 $\pm$ 1.3	1.6 $\pm$ 0.8	0.23 [-0.43; 0.88]	0.47

Table 4: Effect of inulin supplementation on liver function, bacterial translocation, and inflammatory markers in patients with early alcohol-associated liver disease.

[†] Linear regression model adjusted for gender, the baseline measurement of the outcome and the quantity of ethanol consumed during the second week of the program. AST, Aspartate transaminase; ALT, Alanine transaminase; CAP, controlled attenuation parameter; K18-M65, Serum cytokeratin 18; sCD14, soluble CD14.

	Placebo n=22				Inulin n=22			
	Study entry		End of the study		Study entry		End of the study	
Abdominal pain, n (%)	8	(34.8)	2	(11.1)	13	(59.1)	7	(31.8)
Bloating, n (%)	8	(34.8)	6	(33.3)	14	(63.6)	10	(45.5)
Stool frequency, mean $\pm$ SD	1.7 $\pm$ 1.2		1.5 $\pm$ 0.9		1.5 $\pm$ 0.9		1.9 $\pm$ 1.4	
Stool consistency, mean $\pm$ SD	4.7 $\pm$ 1.6		3.2 $\pm$ 1.5		5.2 $\pm$ 1.9		4.5 $\pm$ 3.3	
Global score of GI symptoms, mean $\pm$ SD	106.6 $\pm$ 80.5		53.2 $\pm$ 62.1		152.5 $\pm$ 78.7		70.7 $\pm$ 74.4	
Diarrhoea, n (%)	0	(0.00)	0	(0.00)	0	(0.00)	1	(4.5)
Constipation, n (%)	0	(0.00)	3	(13.6)	0	(0.00)	0	(0.00)
Creatinine (mg/dl)	0.77 $\pm$ 0.15		0.89 $\pm$ 0.16		0.75 $\pm$ 0.12		0.92 $\pm$ 0.16	
Allergic contact dermatitis, n (%)	0	(0.00)	0	(0.00)	0	(0.00)	1	(4.5)
Serious adverse events, n (%)	0	(0.00)	0	(0.00)	0	(0.00)	0	(0.00)

Table 5: Tolerability and safety assessments

in the placebo group. One patient developed an allergic contact dermatitis that was not related to the intervention. No severe adverse events have been reported during the study.

Discussion

This work aimed at studying the effect of inulin supplementation on the gut-liver axis in AUD patients during alcohol withdrawal. We hypothesized that inulin, through restoration of gut dysbiosis, could improve liver damage in AUD patients especially in a subgroup of patients with eALD defined by clinical parameters. We demonstrated that 17 days of inulin supplementation did not elicit additional benefit over abstinence alone on liver disease and inflammatory parameters in AUD patients. Surprisingly, we observed even a less pronounced reduction of AST, ALT and IL-18 in patients supplemented with inulin compared to placebo. Even after stratifying patients according to the severity of liver disease, we did not find a benefit of inulin in the subgroup of patient suffering from more severe ALD at baseline. AST and ALT remained statistically significantly higher in inulin group than in placebo group at the end of the intervention after adjustment for gender and the quantity of ethanol consumed during the second week of the detoxification program.

Our findings that inulin does not improve liver function or inflammatory markers in the specific context of AUD is in opposite to animal studies where some benefits have been observed^{43–46} after alcohol feeding. In obese or diabetic patients inulin supplementation also decreased circulating cytokines and ameliorated liver function tests as did oligofructose supplementation, a short chain ITF, in patients with non-alcoholic steatohepatitis.^{27,47–49}

The less pronounced decrease of ALT and AST in our patients occurred in parallel to higher IL-18 levels in inulin supplemented subjects than in placebo subjects at T2. This is in line with the proposal that IL-18 could

play a specific role in liver injury.^{50,51} One might postulate that some specific inulin-related changes in the gut microbiota composition could be responsible for this intriguing result. However, we did not find any correlation between the changes in IL-18 levels and changes in genera abundance. Alternatively, we did not assess changes in metabolites from the gut microbiota or bile acids that potentially could explain the changes observed in IL-18. Some studies have revealed that *Bifidobacterium* can produce ethanol through the fermentation of fibres, which is susceptible to partially counteract the beneficial effect of inulin.^{52,53} It would therefore be of interest to ensure that inulin does not promote the growth of ethanol-producing bacteria, which could explain the smaller decrease not only in liver enzymes but also in IL-18. This effect might be especially relevant in an AUD population.

Our previous study showed that patients with eALD had a particularly altered microbiota.² We here observed that inulin supplementation reduced microbial richness compared to placebo in these patients in line with other studies showing a decrease in microbial diversity with inulin supplementation.^{54,55} Decreased microbial diversity has often been associated with a pathological context in the literature^{56,57} including ALD.^{2,58} In our study, this decrease in diversity was negatively correlated with the decrease AST and sCD14 levels in eALD patients and as such might rather be an unfavourable outcome. Early ALD patients are characterized by an overrepresentation of the phylum Proteobacteria compared to minimal LD patient. Proteobacteria has been shown to be increased in patients with liver cirrhosis^{59,60} and in non-alcoholic steatohepatitis patients with advanced fibrosis and cirrhosis.⁶¹ Alcohol use has also been associated with an increase in Proteobacteria considered to be responsible for endotoxemia and hepatic inflammation.^{62–64} At least in our context, inulin was not able to counteract this potentially negative impact.

At the genus level, inulin increased the abundance of *Bifidobacterium* and *Dialister* while it decreased *Bacteroides*, *Ruminococcus torques* group, *Dorea* and *Eubacterium ruminantium* group. Most of these changes have already been observed in a different context^{19,65} but their significance in AUD and ALD is not clear. Addolorato *et al* showed that *Bacteroides* was enriched in AUD patients and that this is associated with circulating LPS.⁵⁸ By contrast, a preclinical study in mice fed a Lieber De Carli diet found that liver alterations due to alcohol are associated with a low level of *Bacteroides*.⁶⁶ *Bifidobacteria*, a core genus in human gut microbiota, are generally associated with good health outcome including improvement of gut barrier function and reduced liver alterations.^{20,21,67} Our group previously reported that AUD patients had a decreased abundance of *Bifidobacteria* compared to healthy individuals¹⁷ but another study showed an increased faecal abundance of *Bifidobacterium* in AUD patients with cirrhosis.⁶⁸ Inulin supplementation in dysbiotic TLR5-KO mice induced hepatic alterations via the increase of butyrate⁶⁹ indicating a possible negative impact that results from butyrate production via inulin fermentation. It is conceivable that potential beneficial effects of inulin only apply to particular pathological contexts^{70,71} and that promoting *Bifidobacteria*, in the context of AUD patients might not be beneficial.

Multiple alterations of the intestinal barrier have been associated with chronic alcohol consumption.^{27,8} It is possible that inulin is not the right compound to restore dysbiosis or that a prebiotic approach alone is not sufficiently powerful to achieve this objective. One small randomized, placebo-controlled study⁷² and one small open-label study⁷³ used FMT to modulate the microbiota in patients with advanced forms of ALD (cirrhosis and severe alcoholic hepatitis, respectively). They showed some beneficial biological and behavioural effects in the FMT groups indicating that a more "radical" change of the microbiota in patients with advanced ALD might be needed to observe improvements of biological and behavioural parameters. Our study took a different approach looking for benefits of microbiota modulation using a prebiotic in patients with less severe forms of liver disease who followed an in-hospital detoxification program. Given these profound differences in study design, a direct comparison of the results of the studies is not possible. It is also possible that the duration of the supplementation and the dose of inulin were not sufficient to induce significant changes in biological parameters. Indeed, to avoid side effects, the dose of inulin was progressively increased from 4 to 16g/d during the study, thus patients were supplemented with 16g only for 5 days. In addition, we recruited a highly selected population already on abstinence therapy. As abstinence alone already has a strong impact on liver and inflammatory parameters,^{2,74} this could mask an additional positive effect of inulin.

Finally, we have chosen maltodextrin as a placebo. Although a study in mice showed that maltodextrin could lead to a decrease in the gut barrier function,⁷⁵ no significant changes were observed in microbial translocation markers neither in the microbial composition.

The principal strength of our pilot study is the blinded-randomized-placebo controlled design. However, some limitations do apply. Since gender is known to influence the biological parameters including liver damage,^{41,42} a higher proportion of females in the inulin group could have impacted the results. Although relapse rates were similar between both groups of treatment, we cannot exclude that this parameter could have influenced liver recovery. To limit the consequences of these potential biases, we adjusted the linear regression models for both variables. The sample size for the subgroup analyses was small which could have impacted the results.

Overall, our pilot study shows that modulation of the microbiota can be obtained via inulin in AUD patients. However, 17 days of inulin supplementation versus placebo did not have a beneficial effect on liver recovery after short-term alcohol withdrawal. Further studies with a larger sample size and longer duration of supplementation are needed to confirm the results. Future trials targeting the gut microbiota in AUD subjects, especially those where a decrease in bacterial diversity could be anticipated, should be planned and performed carefully with a particular attention given to liver alterations.

Contributors

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Data sharing statement

The study protocol is available on protocol.io (dx.doi.org/10.17504/protocols.io.bvs2n6ge). The statistical analysis plan is provided in supplementary data files. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. The accession number

for the raw data generated with the 16S rRNA gene sequencing reported in this paper is BioProject PRJNA745947 (SRA) and data are available through the following link <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA745947/>.

Declaration of interests

The authors declare no competing interests.

Acknowledgements

We are grateful to the study participants for their participation in the study. We thank Alejandra Ruiz Moreno, as well as the nurses of the Unité Intégrée d'Hépatologie of Saint-Luc Hospital for their technical help. We also thank Isabelle Blave and Bouazza Es Saadi for their excellent technical and experimental assistance in this study. We thank Cosucra for kindly providing the inulin. We thank Ana Beloqui for her help with the MSD cytokines assays. Finally, we are grateful to the INRAE MIGALE bioinformatics facility (MIGALE, INRAE, 2020, Migale bioinformatics Facility, doi: 10.15454/1.5572390655343293E12) for providing help and computing and storage resources.

This study was supported by the Fédération Wallonie-Bruxelles (Action de Recherche Concertée ARC18-23/092). SL is supported by ARC. PS is supported by grants from Fond National de Recherche Scientifique Belgium (FRS-FNRS J.0146.17 and T.0217.18). NMD is a recipient of grants from the Fonds de la Recherche Scientifique (FRS-FNRS, convention PDR T.0068.19 and convention PINTMULTI R.8013.19 (NEURON, call 2019)). PdT is a recipient of other grant support from the Fondation Saint-Luc.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2022.104033.

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