Dissertation

Pathophysiological basis of neutrophil dysfunction in different chronic liver diseases

Subtitle: with special emphasis on bile acid interaction with neutrophil functions and gut microbiome composition

submitted by

Irina BALAZS

for the Academic Degree of

Doctor of Philosophy

(PhD)

at the

Medical University of Graz

Department of Gastroenterology and Hepatology

under the supervision of

Prof. Dr. Vanessa STADLBAUER-KÖLLNER

2021

DECLARATION

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Guidelines of the Medical University of Graz on Good Scientific Practice“.

Date

DISCLOSURES

Parts of this thesis were published in:

Leber B [§], Balázs I [§], Horvath A, Posch A, Streit A, Spindelböck W, Feldbacher N, Stiegler P, Stauber RE, Rechberger GN, Kollroser M, Sattler W, Nusshold C, Stadlbauer V. Direct acting antiviral therapy rescues neutrophil dysfunction and reduces hemolysis in hepatitis C infection. Translational Research. https://doi.org/10.1016/j.trsl.2020.12.005

[§] shared first authorship

Parts of this thesis were submitted for publication:

Balazs I, Horvath A, Leber B, Feldbacher N, Sattler W, Rainer F, Fauler G, Vermeren S, Stadlbauer V. Bile acids in liver cirrhosis promote neutrophil dysfunction.

Co-authors who contributed to these publications:

Angela Horvath [1, 2], Bettina Leber [3], Nicole Feldbacher [1], Andreas Posch [1], Andrea Streit [1], Walter Spindelböck [1], Philipp Stiegler [3], Rudolf E. Stauber [1], Gerald N. Rechberger [4, 5], Wolfgang Sattler [5, 6], Manfred Kollroser [7], Christoph Nusshold [8], Florian Rainer[1], Günter Fauler[9], Sonja Vermeren[10], Vanessa Stadlbauer[1]

[1] Department of Internal Medicine, Division of Gastroenterology and Hepatology, Medical University of Graz, Graz, Austria

[2] Center for Biomarker Research in Medicine (CBmed), Graz, Austria

[3] Department of Surgery, Division of Transplantation Surgery, Medical University of Graz, Graz, Austria

[4] Institute of Molecular Biosciences, University of Graz, Graz, Austria

[5] Center for Explorative Lipidomics, BioTechMed Graz, Graz, Austria

[6] Gottfried Schatz Research Center (for Cell Signaling, Metabolism and Aging), Division of Molecular Biology and Biochemistry, Medical University of Graz, Graz, Austria

[7] Diagnostic & Research Institute of Forensic Medicine, Medical University of Graz, Graz, Austria

[8] Division of Physiological Chemistry, Otto Loewi Research Center for Vascular Biology, Immunology and Inflammation, Medical University of Graz, Graz, Austria

[9] Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz, Graz, Austria

[10] Centre for Inflammation Research, University of Edinburgh, Edinburgh, UK

I confirm that all co-authors have agreed to include their published data in my dissertation. The manuscript “Direct acting antiviral therapy rescues neutrophil dysfunction and reduces hemolysis in hepatitis C infection” was published in peer-reviewed journal “Translational Research” by Elsevier Inc. under Creative Commons Attribution – NonCommercial – NoDerivs (CC BY-NC-ND 4.0) license, which allows to reuse portions or extracts from the article in other works for non-commercial purposes (https://creativecommons.org/licenses/by-nc-nd/4.0/). Furthermore, Elsevier allows including the manuscript in full or in part by the authors in their thesis/dissertation for non-commercial purposes without obtaining a written permission from Elsevier(https://www.elsevier.com/about/policies/copyright/permissions,https://www.elsevier.com/about/policies/copyright). The parts of published work are being reproduced identically in this dissertation.

Some of the experiments described in this dissertation were performed during my stay abroad at the lab of Dr. Sonja Vermeren (Centre for Inflammation Research, Queen’s Medical Research Institute, University of Edinburgh). This is indicated in the relevant parts of the dissertation.

Dr. Luke Tweedy (Beatson Institute, University of Glasgow) provided ImageJ plug-in “TrackMaxima” for the analysis of chemotaxis and Dr. Silvia Racedo (Medical University of Graz) measured total bile acids in bile and took representative immunofluorescence pictures of NETs with the microscope from the slides provided by me.

Other publications I co-authored during my PhD studies:

Horvath, A; Leber, B; Feldbacher, N; Steinwender, M; Komarova, I; Rainer, F; Blesl, A; Stadlbauer, V. The effects of a multispecies synbiotic on microbiome-related side effects of long-term proton pump inhibitor use: A pilot study. Sci Rep. 2020; 10(1): 2723-2723

Stadlbauer, V; Engertsberger, L; Komarova, I; Feldbacher, N; Leber, B; Pichler, G; Fink, N; Scarpatetti, M; Schippinger, W; Schmidt, R; Horvath, A. Dysbiosis, gut barrier dysfunction and inflammation in dementia: a pilot study. BMC Geriatr. 2020; 20(1):248-248

Stadlbauer, V; Komarova, I; Klymiuk, I; Durdevic, M; Reisinger, A; Blesl, A; Rainer, F; Horvath, A. Disease severity and proton pump inhibitor use impact strongest on faecal microbiome composition in liver cirrhosis. Liver Int. 2020

Stadlbauer, V; Horvath, A; Komarova, I; Schmerboeck, B; Feldbacher, N; Klymiuk, I; Durdevic, M; Rainer, F; Blesl, A; Stiegler, P; Leber, B. Dysbiosis in early sepsis can be modulated by a multispecies probiotic: a randomised controlled pilot trial. Benef Microbes. 2019; 10(3):265-278

Stadlbauer, V; Horvath, A; Komarova, I; Schmerboeck, B; Feldbacher, N; Wurm, S; Klymiuk, I; Durdevic, M; Rainer, F; Blesl, A; Stryeck, S; Madl, T; Stiegler, P; Leber, B. A single alcohol binge impacts on neutrophil function without changes in gut barrier function and gut microbiome composition in healthy volunteers. PLoS One. 2019; 14(2):e0211703-e0211703

“Esse quam videri”

Cicero

“To strive, to seek, to find, and not to yield”

Alfred Tennyson, 'Ulysses'

ACKNOWLEDGEMENTS

“If you want to go quickly, go alone. If you want to go far, go together.”

African proverb

I owe my deepest gratitude to my supervisor Prof. Dr. Vanessa Stadlbauer-Köllner. Without her trust, guidance, support and encouragement throughout my PhD way, this dissertation would not have materialized.

I would like to thank all my colleagues at the Medical University of Graz and the University Hospital of Graz, especially Dr. Angela Horvath, Dr. Bettina Leber, Nicole Feldbacher, Bianca Schmerböck, who were always by my side in going through any challenges PhD brings and in the moments of success.

I am thankful to Prof. Dr. Wolfgang Sattler for being in my thesis committee and guiding me through this project. I thank Dr. Christoph Nusshold for his help with proteome analysis, Dr. Silvia Racedo for the discussions and support and Dr. Günter Fauler for his help with serum bile acids measurements.

I am thankful to all DK MOLIN PhD students for friendship and scientific discussions, as well as to the DK MOLIN faculty members and the office for Doctoral Studies for their support.

My deepest appreciation goes to Dr. Sonja Vermeren and her lab team, especially Dr. Utsa Karmakar and Dr. Julia Chu, at the Centre for Inflammation Research (University of Edinburgh), where I was privileged to spend my scientific stay abroad. This was a life-changing experience for me, which helped me to grow professionally and personally and facilitated the development of my PhD project.

I am thankful to the Core Facilities of Center for Medical Research (ZMF), Medical University of Graz and Centre for Inflammation Research (CIR), University of Edinburgh for providing support with the technical part of the experiments and the analyses.

With my full heart I am grateful to my parents Prof. Dr. Irina Komarova and Dr. Andrei Komarov for their limitless support and encouragement, for love and trust in me, for their professional and personal advice. I am grateful to my granny, Valentina Novikova, who encouraged me and

supported with all her heart throughout my medical studies and who would have been proud of me now. I am incredibly thankful to my husband Alexander Balazs, who was motivating, supporting and taking care of me, while I was fully concentrated on science.

This PhD project was funded by the Austrian Science Fund FWF (W1241) and the Medical University of Graz through the PhD Program “Molecular Fundamentals of Inflammation (DK MOLIN)”.

TABLE OF CONTENTS

ABBREVIATIONS AND DEFINITIONS

AAT	Alpha-1-antitrypsin
ALT	Alanine aminotransferase
ANA	Anti-nuclear antibody
ANOSIM	Analysis of similarity
ANOVA	Analysis of variance
AP	Alkaline phosphatase
AST	Aspartate aminotransferase
BAs	Bile acids
BSA	Bovine serum albumin
cANCA	Antineutrophil cytoplasmic antibodies
CA	Cholic acid
CDCA	Chenodeoxycholic acid
CHRM3	Cholinergic Receptor Muscarinic 3
CI	Confidence interval
CsH	Cyclosporine H
DCA	Deoxycholic acid
EMR 2	Mucin-like hormone receptor 2
fMLF	N-formyl-met-leu-phe
FPR	Formyl peptide receptor
FXR	Farnesoid X receptor
GCA	Glycocholic acid
GCDCA	Glycochenodeoxycholic acid
GDCA	Glycodeoxycholic acid
GGT	Gamma-glutamyl transferase
GLCA	Glycolithocholic acid
GMFI	Geometric mean fluorescence intensity
GUDCA	Glycoursodeoxycholic acid
HBSS	Hanks' Balanced Salt Solution
HBV	Hepatitis B virus
HCC	Hepatocellular carcinome
HCV	Hepatitis C virus
HDL	High-density lipoprotein

HPLC-HRMS	High performance liquid chromatography-high resolution mass spectrometry
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
INR	International normalized ratio
IR	Interquartile range
LCA	Lithocholic acid
LPS	Lipopolysaccharide
MELD	Model for End-Stage Liver Disease
MPO	Myeloperoxidase
MW	Molecular weight
NASH	Non-alcoholic steatohepatitis
NE	Neutrophil elastase
NET	Neutrophil extracellular trap
NK-cells	Natural killer cells
NMMDS	Non-metric multidimentional scaling
OD	Optical density
OTU	Operatinal Taxonomic Unit
pANCA	Perinuclear anti-neutrophil cytoplasmic antibodies
PBC	Primary biliary cirrhosis
PBS	Phosphate-buffered saline
pI	Isoelectric point
PMA	Phorbol-12-myristat-13-acetat
PMN	Polymorphonuclear leukocytes
PNPP	Para-Nitrophenylphosphate
PSC	Primary sclerosis cholangitis
PPP	Platelet poor plasma
PXR	Pregnane X receptor
RA	Relative abundance
RDA	Redundancy analysis
RLU	Relative light units
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute

RT	Room temperature
SD	Standard deviation
SEM	Standard error of the mean
SIMPER	Similarity percentages breakdown
S1PR2	Sphingosine-1-phosphate receptor 2
SXR	Steroid and xenobiotic receptor
TCA	Taurocholic acid
TCDCA	Taurochenodeoxycholic acid
TDCA	Taurodeoxycholic acid
TGR5	Takeda-G-protein-receptor-5
TLCA	Taurolithocholic acid
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TUDCA	Tauroursodeoxycholic acid
UDCA	Ursodeoxycholic acid
VDR	Vitamin D receptor

LIST OF FIGURES

LIST OF TABLES

ZUSAMMENFASSUNG

Leberzirrhose ist das Endstadium aller chronischen Lebererkrankungen und wird häufig durch bakterielle Infektionen erschwert wird. Die angeborene Immunantwort, insbesondere die Funktion neutrophiler Granulozyten, ist bei Patient*innen mit Leberzirrhose beeinträchtigt. Dies führt zur Entwicklung infektiöser Komplikationen und zu einem höheren Mortalitätsrisiko. Es wird vermutet, dass bestimmte, noch nicht identifizierte, Serumbestandteile die Dysfunktion von neutrophilen Granulozyten bei Leberzirrhose fördern. Gallensäuren (BA) wurden als wichtige Moleküle zur Regulation der Funktion verschiedener Immunzellen identifiziert. Über den Einfluss von Gallensäuren auf neutrophile Granulozyten ist bisher noch wenig bekannt.
Ziel dieser Studie war es daher zu untersuchen, ob Änderungen der BA Zusammensetzung im Serum zur Zirrhose-assoziierten Neutrophilen-Dysfunktion beitragen können. Zusätzlich wurden mögliche Signalwege von BAs in neutrophilen Granulozyten, Wechselwirkungen zwischen BA und dem Darm-Mikrobiom und weitere, möglicherweise mit der Neutrophilen-Dysfunktion assoziierten Serumfaktoren untersucht.
Serum BAs wurden mit HPLC-HRMS gemessen. Die Neutrophilenfunktionen wurden mit Durchflusszytometrie, Chemilumineszenz, Immunfluoreszenzmikroskopie und mit ChemoTx®-Platten und ibidi µ-slides gemessen. Die Expression der BA Rezeptoren wurde mittels RT-qPCR und Durchflusszytometrie analysiert. Für die Darmmikrobiomanalyse wurde die Illumina MiSeq-Sequenzierungstechnik verwendet. Das Serumproteom wurde durch 2D-Gelelektrophorese analysiert.
Die Analyse der Serum BAs und der Funktion der im peripheren Blut zirkulierenden neutrophilen Granulozyten in den Kohorten von Patient*innen mit Leberzirrhose (n=109) und gesunden Kontrollpersonen (n=21) zeigte einen Zusammenhang einer höheren relativen Häufigkeit von Gesamt-Chenodesoxycholsäure (CDCA) mit einer beeinträchtigten Neutrophilen-Phagozytose. Eine niedrige relative Häufigkeit von nicht konjugierter Ursodesoxycholsäure (UDCA) war mit einer beeinträchtigten neutrophilen Phagozytosekapazität und ROS Produktion assoziiert. Neutrophile von gesunden Spender*innen zeigten eine erhöhte Produktion basaler ROS, wenn sie direkt mit Gesamt-Lithocholsäure (LCA) behandelt wurden, verringerten jedoch die ROS Produktion als Reaktion auf die Stimulation mit fMLF nach der Behandlung mit Gesamt-CDCA oder Gesamt-LCA und als Reaktion auf die Stimulation mit E. coli nach Behandlung mit Gesamt-CDCA oder Gesamt-Desoxycholsäure (DCA). Gesamt-CDCA und Gesamt-DCA verringerten reversibel die neutrophile Phagozytose von E. coli. Alle BAs mit Ausnahme von Cholsäure (CA), ihren Konjugaten und nicht konjugiertem UDCA hemmten die Chemotaxis von Neutrophilen

gegenüber fMLF. Nicht konjugierte LCA, CDCA und UDCA verzögerten die Apoptose von Neutrophilen. Gallenblasengalle von Patient*innen mit Leberzirrhose, aber nicht von Lebergesunden induzierte Bildung von NETs. Neutrophile exprimierten FPR1, VDR und TGR5 BA Rezeptoren. Die Serum-BA Zusammensetzung war bei Patient*innen mit Leberzirrhose signifikant mit der Zusammensetzung des Darm-Mikrobioms. Haptoglobin war das am stärksten herunterregulierte Protein im Serum von Patient*innen mit HCV-assoziierter Zirrhose. Eine Albuminintervention bei Patient*innen mit Leberzirrhose führte zu keiner stabilen Verbesserung der Neutrophilenfunktion. Serumferritin und Häm waren nicht mit der Neutrophilenfunktion assoziiert. pANCA, aber keine anderen Autoantikörper (cANCA, ANA), förderte die Bildung von NETs.

Diese Studie identifiziert Serum BAs, Haptoglobin und pANCA als potenzielle Faktoren für die Funktionsstörung von Neutrophilen bei Leberzirrhose. Daher können therapeutische Ansätze zur Modifizierung der BA Zusammensetzung im Serum potenzielle Wege zur Behandlung von Neutrophilenfunktionsstörungen und bakteriellen Infektionen bei Leberzirrhose sein.

ABSTRACT

Liver cirrhosis is the end stage of all chronic liver diseases, often complicated by bacterial infections. The innate immune response, in particular neutrophil response, is impaired in cirrhotic patients, leading to the development of infectious complications and higher risk of mortality. Yet unidentified serum components were discussed to promote neutrophil dysfunction in liver cirrhosis. Bile acids (BA) have been recently found to play role in function regulation of several types of immune cells. The impact of BAs on neutrophil function in patients with liver cirrhosis has not been studied in detail yet.

This study aimed to investigate, if changes in serum BA composition can contribute to the cirrhosis-associated neutrophil dysfunction. Additionally, possible signalling pathways of BAs in neutrophils, BA and gut microbiome interactions and other, potentially associated with neutrophil dysfunction serum factors, were studied.

Serum BAs were measured by HPLC-HRMS. Neutrophil functions were measured by flow cytometry, chemiluminescence, immunofluorescent microscopy and with ChemoTx plates and ibidi µ-slides. BA receptors expression was analyzed by RT-qPCR and flow cytometry. For gut microbiome analysis Illumina MiSeq sequencing technique was used. Serum proteome was analysed by 2D gel electrophoresis.

The analysis of serum BAs and circulating neutrophil function in the cohorts of cirrhotic patients (n=109) and healthy controls (n=21) showed the association of higher total chenodeoxycholic acid (CDCA) relative abundance (RA) with impaired neutrophil phagocytosis and the association of lower unconjugated ursodeoxycholic acid (UDCA) RA with impaired neutrophil phagocytosis and ROS production. Neutrophils from healthy donors exhibited increased basal ROS production, when directly treated with total lithocholic acid (LCA), but decreased ROS production in response to stimulation with fMLF after being treated with total CDCA or total LCA and in response to stimulation with E. coli after being treated with total CDCA or total deoxycholic acid (DCA). Total CDCA and total DCA reversibly decreased neutrophil phagocytosis of E. coli. All BAs, except for cholic acid (CA), its conjugates and unconjugated UDCA, inhibited chemotaxis of neutrophils towards fMLF. Unconjugated LCA, CDCA and UDCA delayed neutrophil apoptosis. Gallbladder bile of cirrhotic patients, but not healthy bile induced NETs formation. Neutrophils expressed FPR1, VDR and TGR5 BA receptors. Serum BA composition was significantly associated with gut microbiome composition in cirrhotic patients. Haptoglobin was the most downregulated protein in serum of patients with HCV-associated cirrhosis. Albumin intervention in cirrhotic patients did not cause a stable improvement in neutrophil function. Serum ferritin and heme were not associated with

neutrophil function. pANCA, but not other autoantibodies (cANCA, ANA), promoted NETs formation.

In summary, this study identifies serum BAs, as well as haptoglobin and pANCA as potential contributors to neutrophil dysfunction in liver cirrhosis. Therefore, therapeutic approaches aimed at modification of serum BA composition, may be potential ways to prevent and treat neutrophil dysfunction and bacterial infections in liver cirrhosis.

INTRODUCTION

Liver cirrhosis is a life-threatening chronic progressive liver disease, which develops prevalently as a consequence of chronic hepatitis B (HBV) or hepatitis C (HCV) virus infections, alcoholic and non-alcoholic steatohepatitis (NASH), as well as other reasons like alpha-1-antitrypsin deficiency, hemochromatosis, primary sclerosis cholangitis (PSC), Wilson's disease, etc. It is a late-stage fibrosis, characterized by scarring of the liver (1, 2). Being 11th leading cause of death in the world, it accounted for 2.2% of total deaths in 2016 (3) and 2.4 % of total deaths in 2017 (4), with annually increasing mortality rate (4, 5). Compensated patients with liver cirrhosis have an about 5-fold increased mortality risk and decompensated patients a 10-fold increased mortality risk compared to general population (6). Austria has the second highest age-standardized prevalence of liver cirrhosis and other chronic liver diseases in Europe with more than 1 100 cases per 100 000 people (7). All of the above underscores the importance to study the pathophysiology of liver cirrhosis and its complications in order to develop the efficient preventive and therapeutic strategies, which can improve the quality of life and save lives of many patients.

Liver cirrhosis and bacterial infections

Prognosis and survival rate in cirrhotic patients are greatly influenced by development of bacterial infectious complications, such as spontaneous bacterial peritonitis, urinary tract infections, pneumonia and others (8-10). Around one third of hospitalized cirrhotic patients are suffering from bacterial infections, which leads to the increased time of hospitalization (8), de-listing from liver transplantation (11), acute kidney injury (12), and finally 4-fold increased death rate compared to non-infected cirrhotic patients (9). 30% of patients, who develop bacterial infections, die within 1 month and more than 60% of cirrhotic patients die within 1 year after the infection was diagnosed (9).

Infections in cirrhotic patients are mostly caused by gram-negative bacteria (such as *Escherichia coli* and *Klebsiella Pneumoniae*) of intestinal origin, but the infections caused by gram-positive bacteria (such as *Staphylococcus aureus* and *Enterococci*) also can occur, particularly in hospitalized patients (13). Multidrug-resistant bacterial infections in liver cirrhosis are currently a growing problem worldwide, therefore, the development of alternative prevention and treatment methods for bacterial infections in liver cirrhosis is urgently needed (14, 15).

The important reason for the increased susceptibility to bacterial infections in cirrhotic patients is a progressive development of immune dysfunction (16). Innate immune dysfunction, including impaired neutrophil functionality, is a predominant part of cirrhosis-associated immune dysfunction and, therefore, is mainly responsible for the development of bacterial infectious complications in cirrhotic patients (17).

Neutrophil dysfunction in liver cirrhosis

Neutrophils are the largest population of leucocytes and are among the first cells of the innate immune system, which start fighting against the intruded pathogens. Neutrophils have a range of surface G protein coupled receptors, which enable them to sense the chemoattractants (inflammatory mediators or pathogen related molecules) and consequently migrate to the site of the intruded pathogen (mostly bacteria and fungi). This process of directed migration is called chemotaxis. As soon as neutrophils reach the site of infection, they use one of their defense mechanisms in order to protect host organism from the infectious agent. They can internalize the pathogen via the process called phagocytosis and kill it inside the phagolysosome with the microbicidal contents of their intracellular granules (e.g. various proteases) and reactive oxygen species (ROS). These substances can be also released into the extracellular space by neutrophils, which enable extracellular bacterial killing, but can also cause a tissue damage. Neutrophils produce different anti- and pro-inflammatory cytokines, which help to regulate the inflammatory and other physiological and pathophysiological processes. Furthermore, neutrophils were recently described to be able to extrude their DNA coated with histones and cytoplasmic and granular proteins (e.g. neutrophil elastase, myeloperoxidase, etc.), so called neutrophil extracellular traps (NETs), in order to catch, immobilize and kill infectious agents. Neutrophils are cells with a relatively short lifespan, which is extended in case of infection. Neutrophils undergo apoptosis and then are phagocytosed by macrophages and dendritic cells to promote resolution of inflammation (18-20).

All the above-mentioned functions allow neutrophils fighting bacterial infections. Cirrhotic patients' neutrophils have altered functionality, such as altered phagocytic ability, bacterial killing, chemotaxis, degranulation, ROS production and NETs formation (Figure 1). This results in their inability to mount an adequate antibacterial response and protect organism from infection.

Chemotaxis

Neutrophils from cirrhotic patients were shown to have a decreased chemotaxis ability, either, because of cellular defects or caused by impaired chemoattractant activity of cirrhotic serum (21-26). Neutrophils from patients with alcoholic liver cirrhosis showed reduced migration towards healthy serum as chemoattractant compared to healthy donor neutrophils. Serum from cirrhotic patients had less chemoattractant activity compared to healthy serum in the same study. No correlation with bacterial infections were found (21), however another study showed lower neutrophil migration ability in cirrhotic patients with previous bacterial infections compared to the ones without (25). Interestingly, another study showed that serum chemotactic inhibitory activity (which shows how effectively cirrhotic serum inhibits the chemoattractant activity of healthy serum mixed with zymosan A) was higher in alcoholic cirrhosis compared to non-alcoholic cirrhosis (23). Other studies also reported presence of serum chemotactic inhibitory activity in patients with cirrhosis (24, 26). Cirrhotic neutrophils had a decreased transendothelial migration in response to N-formyl-met-leu-phe (fMLF), increased adhesion to endothelial cells (HMEC-1) and an altered expression of adhesion receptors: higher CD11b and lower CD62L expression compared to healthy controls (22). Neutrophil chemotaxis towards casein in cirrhotic patients was comparable to healthy controls (27). Recent study revealed that swarming (coordinated neutrophil communication and recruitment) of cirrhotic patients' neutrophils in order to control *Candida albicans* hyphae growth was impaired compared to healthy controls (28). Decreased neutrophil migration towards IL-8 was shown in cirrhosis, probably due to the decreased expression of CXCR2 receptor (sense IL-8) (29).

Phagocytosis

Phagocytic function of neutrophils from cirrhotic patients was also shown to be impaired (25, 30-36). Defective phagocytosis of *S. aureus* was detected in isolated neutrophils in alcoholic liver cirrhosis (30, 36) and primary biliary cirrhosis (PBC) (36), as well as defective phagocytosis of *E. coli*, but to a less extent (30). Decrease in neutrophil phagocytosis of *E. coli* was more pronounced in cirrhotic patients, who had previously bacterial infections (25). Cirrhotic neutrophil phagocytic capacity (number of internalized bacteria per cell) of *E. coli* measured in whole blood or isolated neutrophils of patients with cirrhosis was also reported to be impaired (31, 32, 37). Percentage of phagocytic neutrophils was decreased in blood of cirrhotic patients (33, 35) and the degree of this dysfunction was increasing with increasing

severity of cirrhosis, but was not different between the etiologies of cirrhosis (alcoholic, HCV, autoimmune and other) (33). Phagocytic capacity of *E.coli* was reduced and a percentage of non-phagocytic neutrophils was increased in HCV-associated cirrhosis (34). No phagocytosis defect of *C. albicans* was shown in neutrophils of cirrhotic patients (27).

Killing capacity

Moreover, bacterial killing was also shown to be reduced in neutrophils from cirrhotic patients, which means that even those active neutrophils, which manage to engulf bacteria, cannot efficiently kill them (30, 38). In particular, intracellular killing of *S. aureus* and *E. coli*, which are a common cause of bacterial infections in liver cirrhosis, was shown to be impaired in alcoholic cirrhosis (30). In contrast, another study did not find any differences in neutrophil intracellular killing capacity of *S. aureus* in alcoholic cirrhosis and PBC, despite decreased total bacterial killing, which they explained by decreased percentage of phagocytic neutrophils (36). Neutrophils from cirrhotic patients showed impaired killing capacity not only of bacteria, but also of fungi, such as *Candida albicans* (28), however, another study found it unchanged (27).

ROS production and degranulation

Impaired neutrophil ROS production (31-35, 37, 39-42) and degranulation (30, 38) were described in liver cirrhosis and it might contribute to the impaired bacterial killing (30). Alteration of ROS production was characterized by elevated percentage of neutrophils with basal ROS production (so called resting burst) (31, 34, 35, 39) and elevated (37, 39) or reduced (41) intracellular basal ROS production (intracellular amount of ROS). Extracellular basal superoxide production was unchanged (30) or elevated in cirrhotic patients (27).

Increased percentage of neutrophils from cirrhotic patients respond to a low physiological stimulus like fMLF (31, 32, 35), which means that these neutrophils were previously primed by persistent low-grade stimulation with some priming agents, like lipopolysaccharide (LPS) or tumor necrosis factor alpha (TNF-alpha). The intracellular ROS pool of those neutrophils, which respond to fMLF stimulation, was shown to be slightly increased (39) or decreased (41) in patients with cirrhosis. Extracellular superoxide release in response to fMLF (40-42) and TNF-alpha (40) was decreased in patients with cirrhosis. Noticeably, priming of neutrophils from cirrhotic patients with TNF-alpha did not increase their response to fMLF, as it is usually the case with healthy donor neutrophils (40).

The number of neutrophils producing ROS in response to a potent stimulus *E. coli* was unchanged in cirrhotic patients (31, 33, 39), but their intracellular ROS amount was increased (39). Extracellular superoxide production in response to zymosan (structural component of yeast cell wall) was shown to be reduced in patients with cirrhosis (27, 30, 40, 43). Extracellular hydrogen peroxide levels produced by neutrophils in response to zymosan were not different or were higher in cirrhotic patients compared to healthy controls, which was reflected in the increased hydrogen peroxide/superoxide molar ratio (30). Interestingly, superoxide and hydrogen peroxide have different effects on cell apoptosis and necrosis: superoxide was described to inhibit apoptosis, while hydrogen peroxide can promote cell apoptosis via intracellular acidification, and even necrosis, when present in very high concentrations (44, 45). Nitric oxide production in response to opsonized zymosan was also increased in neutrophils from cirrhotic patients (43).

Changes in ROS production were different in cirrhotic patients with active infection: basal ROS production and ROS production in response to fMLF were shown to be unaltered, as well as the percentage of neutrophils, which responded with ROS production to *E. coli* stimulation; however, intracellular ROS pool generated in response to *E. coli* was decreased, which might support a concept that cirrhotic neutrophils are exhausted via prior low-grade stimulation and cannot mount an augmented ROS response, when they have to fight a real infection (39).

Above described changes were shown in liver cirrhosis of different etiologies, but most of the studies were performed with neutrophils from patients with alcoholic and HCV-associated cirrhosis. Several studies reported no dependence of ROS production on etiology of cirrhosis (33, 40). Contrasting findings were regarding the correlation of the changes in ROS production with cirrhosis severity: some studies reported no correlation (31, 33), while some studies found a correlation of these parameters (39, 40).

The enzyme intracellular contents (lysozyme, myeloperoxidase (MPO)) and their release from neutrophil granules upon stimulation with zymosan were reduced in neutrophils from cirrhotic patients; however, authors claimed that the release reduction was not dependent on the reduction of the enzymes level inside the granules (30). Another study showed that intracellular content of MPO was not altered in neutrophils from cirrhotic patients, but its extracellular release in response to fMLF was decreased (38). MPO activity was shown to be either decreased (40) or unchanged (38) in neutrophils from cirrhotic patients.

NETs formation

NETs formation is a recently discovered mechanism of neutrophil defense (46). To date only scarce data are available, which investigated the role of NETs formation in liver cirrhosis. One research group showed a significant decrease in NETs formation in response to phorbol-12-myristat-13-acetat (PMA) in patients with liver cirrhosis complicated with spontaneous bacterial peritonitis compared to healthy controls (47, 48).

Apoptosis

The current knowledge about neutrophil apoptosis in liver cirrhosis is also not very broad. Increased apoptosis rate and decreased viability of neutrophils isolated from neutropenic patients with viral liver cirrhosis 24 hours after isolation from whole blood were shown (49).

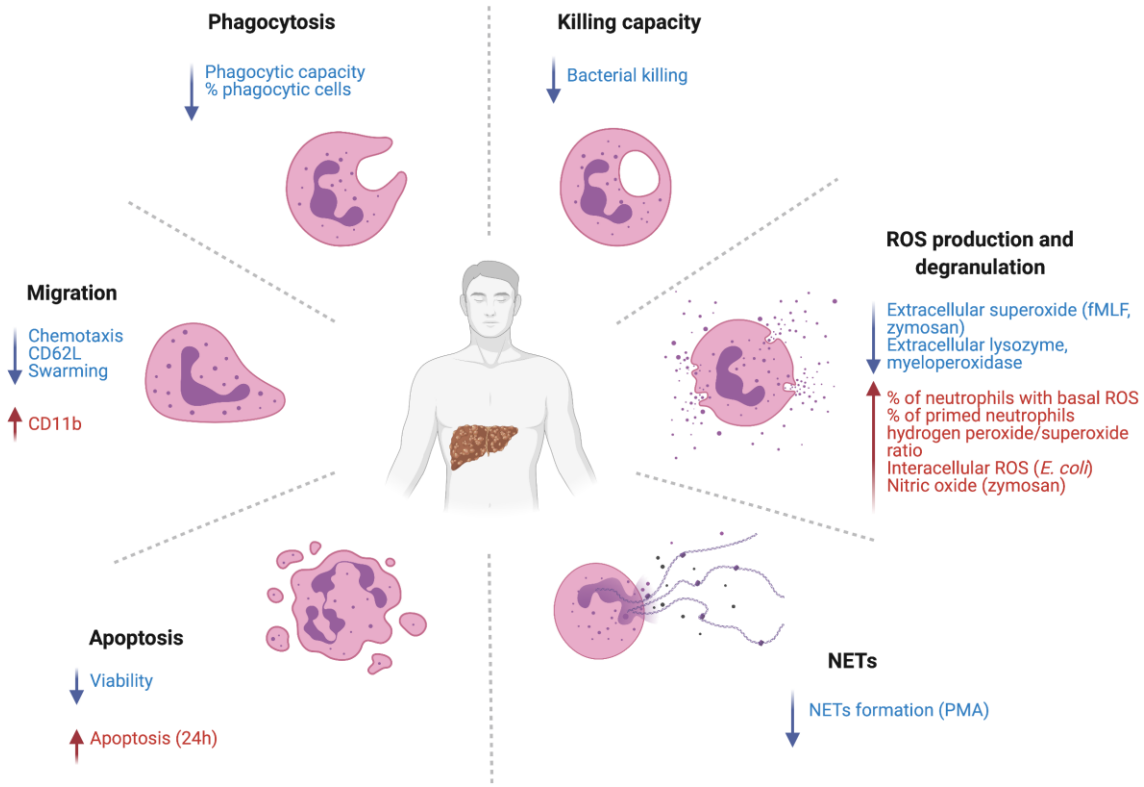


Figure 1. Summary of neutrophil dysfunction features in liver cirrhosis.
Only consistent throughout the literature findings are shown. Created with Biorender.com.

Thus, circulating neutrophils in liver cirrhosis exhibit multiple functional deficiencies, starting from the inability to migrate to the infection site and following inability to internalize and kill pathogens. The loss of neutrophil antimicrobial function, in particular changes in ROS production and phagocytosis, in cirrhosis contributes to the development of bacterial infections, organ failure and mortality (25, 31, 33).

This underscores the importance of identifying the mechanisms underlying neutrophil dysfunction in liver cirrhosis, which will allow developing target therapy for prevention and treatment of bacterial infections in liver cirrhosis and thereby decreasing mortality.

Cause of neutrophil dysfunction in liver cirrhosis

The reasons for the observed changes in neutrophil function in liver cirrhosis were questioned starting from the discovery of these functional defects. One of the concerns was either the neutrophil dysfunction can be explained by cellular or serum defects. There are multiple studies, which attempted to prove one or another of those concepts.

Proposed intrinsic defects

Some studies proposed that cellular defects of neutrophils are responsible for their dysfunction in liver cirrhosis. In one study, serum from patients with decreased neutrophil locomotion did not cause a similar chemotaxis defect in healthy control neutrophils (21). However, in the same study, serum from cirrhotic patients had a decreased chemoattractant activity for healthy donor neutrophils, compared to healthy donor serum, which can indicate that changes in serum content also potentially contribute to the decreased chemotaxis of patients' neutrophils (21). Reduced intracellular glutathione levels – detoxifier of hydrogen peroxide - were reported in neutrophils from patients with liver cirrhosis, which can explain the prevalence of hydrogen peroxide over superoxide. The lower levels of glutathione were associated with the higher levels of hydrogen peroxide production and lower hydrogen peroxide/superoxide ratio, degranulation and intracellular killing of *E. coli*. In this study, cirrhotic serum did not cause any changes in phagocytic function or intracellular bacterial killing of healthy donor neutrophils, suggesting underlying cellular defects, including intracellular glutathione deficiency (30).

Proposed serum defects

The majority of studies, which investigated neutrophil dysfunction, however, proposed that some changes in serum content of cirrhotic patients initiate neutrophil dysfunction development (Figure 2). Almost all of the above reviewed neutrophil function deficiencies were shown to be transferred with cirrhotic patients' serum to healthy donor neutrophils. Neutrophils from cirrhotic patients had a defect in migration towards zymosan only in presence of autologous plasma and not healthy control plasma and these defects varied between the etiologies of liver cirrhosis (defects were present in alcoholic and cryptogenic cirrhosis, but not PBC) (24). Healthy donor neutrophils were shown to have decreased killing capacity of *C. albicans* following incubation with cirrhotic serum (28). Decreased phagocytic capacity of *E. coli*, but unchanged (though there was tendency of increase) basal ROS production (percentage of neutrophils) were shown in healthy donor neutrophils after incubation with cirrhotic patients' plasma, which was dependent on cirrhosis severity, but was independent on cirrhosis etiology (50). In another study, incubation with cirrhotic plasma could promote the increase in number of healthy donor neutrophils with basal ROS production and the decrease in phagocytosis. Interestingly, neutrophil dysfunction of cirrhotic patients' neutrophils was reversible and neutrophils restored their functions following the incubation with healthy donor plasma (31). Neutrophil phagocytosis and intracellular killing of *S. aureus* were not affected in cirrhotic patients' neutrophils incubated with AB serum, despite being dysfunctional in presence of autologous serum (36). Patients' plasma also had an effect on healthy donor neutrophil degranulation, decreasing MPO release in response to fMLF (38). Our research group has recently showed that serum components more than 30kD in size are responsible for the changes in neutrophil phagocytic function (34).

The increased gut permeability in liver cirrhosis results in various bacteria and bacteria derived molecules, like endotoxins, getting from the gut lumen to systemic circulation (51). These substances in serum of cirrhotic patients were thought to have an influence on neutrophil function, via e.g. persistent neutrophil low-grade stimulation with following functional exhaustion.

Endotoxins

Endotoxin caused the increase in the percentage of neutrophils with basal ROS production in *in vitro* experiments with healthy donor neutrophils and decrease in phagocytic capacity in

experiments with cirrhotic patients' neutrophils. Plasma endotoxin removal strategies prevented deteriorating effects of patients' plasma on healthy donor neutrophils (31). However, despite *in vitro* shown effects of LPS on neutrophil function, the levels of bacterial endotoxin in plasma of cirrhotic patients did not correlate with the defects in phagocytic (35, 50) and basal ROS production (50) functions of neutrophils. Endotoxins receptors TLR2 and TLR4 were described to be upregulated in healthy donor neutrophils after incubation with cirrhotic plasma (50, 52) and in neutrophils from cirrhotic patients (32). TLR2 was upregulated only after incubation with plasma from patients with alcoholic cirrhosis, while TLR4 had higher expression in both alcoholic and viral cirrhosis plasma treated neutrophils (50). Inhibition of TLR2 and TLR4 decreased the number of neutrophils with basal ROS production caused by incubation with cirrhotic serum, but further impaired phagocytic capacity (52). Lipopolysaccharide-binding protein (LBP) levels were described to be elevated in patients with liver cirrhosis compared to healthy controls, being associated with lower neutrophil intracellular basal ROS production (39) and with the development of severe bacterial infections (53). LBP was shown to enhance LPS effects on immune cells (54).

Bacterial DNA

Bacterial DNA itself was also proposed to be responsible for neutrophil function changes in liver cirrhosis. Bacterial DNA was identified to be present in some patients with cirrhosis with no active infection (55-57), which was, however, not associated with Child-Pugh score and clinical characteristics of these patients (55). Presence of bacterial DNA in serum of cirrhotic patients was associated with higher levels of cytokines, such as TNF-alpha, IFN-gamma, IL-12 and nitric oxide (57). Interestingly, higher cytokine levels in serum of patients with bacterial DNA were independent on their LPS or LBP serum levels (58). In one study DNA sensing receptor in neutrophils TLR9 was shown to be higher expressed in patients' neutrophils compared to healthy control neutrophils (32). However, in other studies TLR9 was not shown to be upregulated in cirrhotic patients (50, 52) and circulating bacterial DNA measured in plasma of cirrhotic patients was associated neither with the changes in neutrophil phagocytic and basal ROS production functions, nor with mortality of patients (50).

Albumin

Albumin is synthesized in the liver and, therefore, its concentration is reduced in cirrhosis (59). Furthermore, the structure of albumin is also altered, mainly via oxidation (60). Albumin binding

capacity was shown to be decreased in patients with decompensated cirrhosis, which negatively correlated with MELD score (61) and increased mortality (62). Given the function of albumin to bind bacterial products, ROS, nitric oxide (63), the changes in its abundance and structure might contribute to the neutrophil dysfunction. Addition of albumin into the incubation media decreased the number of neutrophils with high basal ROS production and recovered phagocytic capacity defects caused by plasma from patients with liver cirrhosis (52). Albumin intervention in cirrhotic patients with spontaneous bacterial peritonitis reduced plasma levels of TNF-alpha, IL-6 and nitric oxide (64). Albumin administration in cirrhotic patients without spontaneous bacterial peritonitis decreased number of nosocomial infections (65). However, one study showed that cirrhotic patients with dysfunctional neutrophil phagocytosis and intracellular killing capacity had comparable to healthy controls serum albumin levels (30). Furthermore, *C. albicans* killing capacity in cirrhotic patients' neutrophils did not correlate with serum albumin levels (28).

Ammonia

Ammonia level in serum of cirrhotic patients predicts organ failure and mortality (66). Neutrophils from rats with ammonia supplementation and healthy donor neutrophils incubated with ammonia were shown to have impaired phagocytosis of *E. coli* and increased number of neutrophils with basal ROS production (67). Decreased neutrophil phagocytic activity correlated with increase in plasma ammonia level in cirrhotic patients (33).

Lipoproteins

Patients with cirrhosis were shown to have lower levels of high-density lipoprotein (HDL) cholesterol and apolipoprotein A1, which further decreased upon decompensation, correlating with increased levels of TNF-alpha, IL-8, IL-6, severe bacterial infection development and predicted patients' mortality (68). The important function of HDL similar to that of albumin is to neutralize LPS (69), therefore, its low abundance in cirrhosis can be a reason for higher LPS serum concentration and low-grade inflammation. Furthermore, HDL composition and function are altered in cirrhosis (70). Higher levels of IgG autoantibodies against oxidized low-density lipoproteins were correlated with higher integrated intracellular ROS production level in response to *E. coli* in neutrophils from patients with liver cirrhosis (39).

Cytokines

IL8 (28, 33, 35, 50) and IL10 (32, 35), but not TNF-alpha, IL-6 (32, 35) or IL-1-beta were increased in cirrhotic patients plasma (35). In other studies, also TNF-alpha (28), IL-6 (28, 33), IL-1-beta (33) were increased in cirrhotic patients and increasing numbers of neutrophils with basal ROS production were correlated with increasing TNF-alpha, IL-6, IL-8 and IL-10, whereas increase in ROS producing neutrophils in response to *E. coli* correlated with increased IL-1-beta, IL-8, IL-1 and IL-17 levels (33). IL-10 was shown to inhibit neutrophil phagocytosis and bactericidal activity (71). IL-33 intervention in cirrhosis improved neutrophil chemotaxis towards IL-8 (29).

Heme

Disturbances in iron metabolism are known in patients with liver cirrhosis (72). Iron metabolism parameters were shown to influence neutrophil function. Heme delayed apoptosis of neutrophils (73). Hemin was shown to activate neutrophil chemotaxis, ROS production and IL-8 expression (74). Another study showed in contrast impaired phagocytosis and migration of neutrophils treated with heme (75).

Serum opsonins

Opsonization of bacteria helps neutrophils to recognize the pathogen and promotes its phagocytosis and killing. The main opsonins are immunoglobulins and components of complement system (76). IgG, IgM, IgA levels, measured in serum from patients with and without neutrophil chemotaxis, phagocytosis or intracellular killing capacity defects, were not different (21, 30). In another study though, IgA was increased in patients with cirrhosis compared to healthy controls, but there was no correlation of IgA with the level of serum chemotactic inhibitory activity (23). No correlation of neutrophil locomotion defect with IgA and immune complexes, as well as serum levels of C3 and C5 was shown in one more study (24). However, one study showed the increased levels of IgA and IgG in cirrhotic patients with chemotactic inhibitory activity, where IgA removal restored normal chemotactic activity (26). Normal levels of C3 and C4 were shown in cirrhotic patients in another study (36), but decreased levels of them in another study (77). Defects in serum opsonization were found in patients with liver cirrhosis, due to deficiency of opsonization factors. It was not connected to the decreased levels of complement factors, IgA, IgM and IgG (78). Another study showed

increased levels of IgA, IgG and IgM in sera from cirrhotic patients. Opsonic effects of patients' sera for *E. coli* were shown decreased compared to controls in this study (77).

Other

EGF-like molecule containing mucin-like hormone receptor 2 (EMR 2) expression was shown to be increased in cirrhotic patients, to be dependent on liver cirrhosis severity and presence of bacterial infections, as well as to predict mortality. However, ligation of EMR 2 failed to improve phagocytic capacity of cirrhotic neutrophils, despite increasing the intracellular ROS production in response to *E. coli* (37). Dysfunctional neutrophils from patients with liver cirrhosis had decreased phospholipase C (which is involved in superoxide production) activity, however, the level of phospholipase C protein was increased in these neutrophils (40).

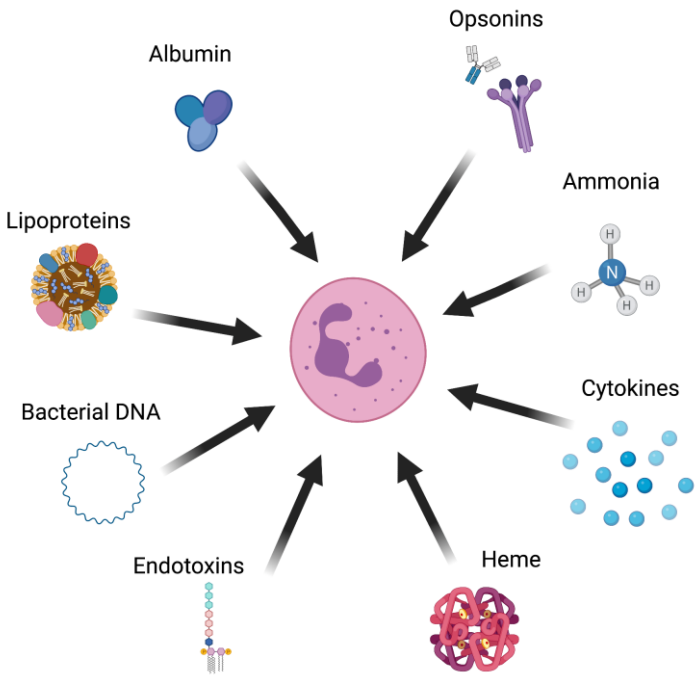


Figure 2. Proposed serum factors, which might cause neutrophil dysfunction in liver cirrhosis.
Created with Biorender.com.

The contribution of serum factors discussed above to the cirrhosis-associated neutrophil dysfunction is rather controversial, given contrasting findings in different studies regarding their

effects on neutrophils, as well as their association with bacterial infections and mortality in cirrhosis. Further investigations of causes for neutrophil deficiency in liver cirrhosis are needed. Particular attention should be at serum components larger than 30kDa in size (34). BAs are among these components, as in systemic circulation they are binding mainly to albumin and lipoproteins and, therefore, are found in the serum fraction larger than 30kD (79-81).

Bile acids in liver cirrhosis

Bile acids (BAs) are broadly known for their functions in gastrointestinal tract such as cholesterol elimination and lipid emulsification, but recently, since the discovery of first BA receptors, Farnesoid-X-Receptor (FXR) (82-84) in 1999 and Takeda-G-protein-receptor-5 (TGR5) in 2002 (85, 86), have been shown to play a role in many metabolic processes and immune response (87, 88).

Bile acid metabolism

Primary BAs chenodeoxycholic acid (CDCA) and cholic acid (CA) are synthesized in the liver from cholesterol. Before excretion into bile BAs are conjugated mostly with the amino acid glycine and less with taurine in humans, with the ratio about 3 to 1 (89). This alters the physicochemical properties of BAs, e.g. increase their solubility and ionization, make them resistant to Ca^{2+} precipitation (90).

There are two main BA synthetic pathways: classic (or neutral) and alternative (or acidic). Classic pathway is characterized by side-chain cleavage following steroid ring modification and in alternative pathway it is vice versa. The classic pathway: synthesis of CA (with microsomal sterol 12α-hydroxylase (CYP8B1)) and CDCA (without microsomal sterol 12α-hydroxylase (CYP8B1)), initiated by cholesterol 7α-hydroxylase (CYP7A1) (91). The alternative pathway: mainly responsible for CDCA synthesis, initiated by sterol 27-hydroxylase (CYP27A1). The alternative pathway does not contribute a lot to bile acid synthesis in healthy people (only about 9%), however, it has more importance in different pathological conditions (e.g. cirrhosis) and in neonates (91, 92).

After a meal, BAs are released into the duodenum, then in the distal intestine conjugated CA and CDCA are being deconjugated by intestinal bacterial bile salt hydrolase (93, 94). Various bacteria are known to have bile salt hydrolase activity, including *Bacteroides ovatus* (95),

Clostridium perfringens (96), *Enterococcus faecalis* (97), different strains of *Bifidobacterium* (98) and *Lactobacillus* (99, 100), *etc*. Further, deconjugated CA and CDCA are converted into secondary BAs deoxycholic acid (DCA) and lithocholic acid (LCA) respectively via 7α-hydroxylation by intestinal bacteria. LCA can be also formed by 7β-hydroxylation of ursodeoxycholic acid (UDCA) (101). Bacterial strains of *Clostridium* are mainly responsible for these processes (102). UDCA is formed through the epimerization of the 7α-hydroxyl groups in CDCA by intestinal bacteria (93). This involves such bacteria as *Clostridium absonum* (103), *Clostridium baratii* (104), strains from genera *Eubacterium* and *Ruminococcus* (105) and others. The overview of bile acid metabolism is shown in Figure 3.

Approximately 95 % of BAs (predominantly conjugated) are being reabsorbed in the intestine by the apical sodium-dependent BA transporter or through passive diffusion and around 0.5 mg/day of them enter systemic circulation (106).

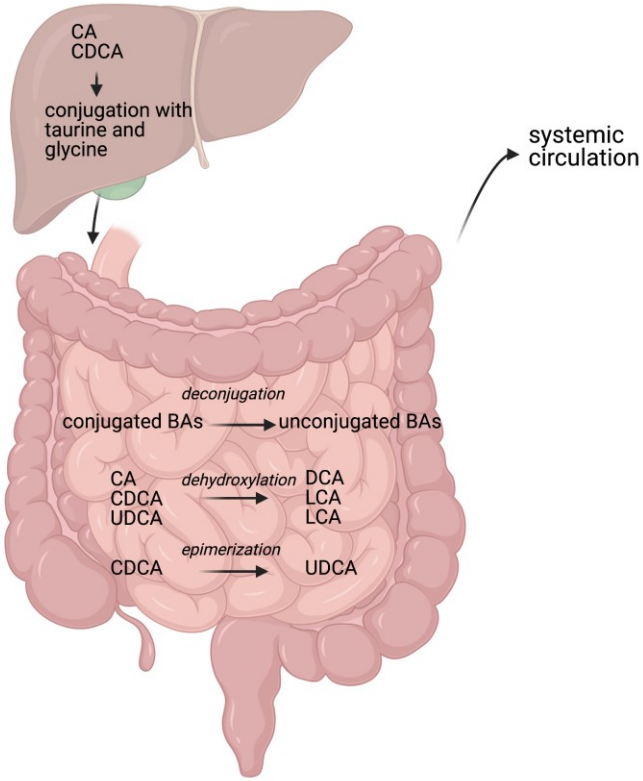


Figure 3. Overview of bile acid metabolism.
Created with Biorender.com.

Serum bile acids in liver diseases

Total serum BAs are elevated in liver diseases (107-113). This increase can be up to 100 times the normal concentration (107). Total BAs are represented in liver diseases mostly by conjugated BAs (107, 109), predominantly conjugates of CDCA (110). Glycochenodeoxycholic acid (GCDCA) is the most abundant BA in cirrhotic patients of different cirrhosis etiologies and is the most accurate diagnostic predictor of liver cirrhosis and predictor of mortality (113). Conjugates of CA and taurochenodeoxycholic acid (TCDCA) also predicted mortality of cirrhotic patients in this study. Levels of primary BAs and conjugated forms of UDCA, but not of secondary BAs were significantly increasing with increasing stage of cirrhosis (113). Another study showed that unconjugated DCA (secondary BA) was not elevated in patients with hepatic impairments (109). The ratio of glycine to taurine conjugated BAs was low in liver diseases (107, 114). The ratio of trihydroxy to dihydroxy BAs (107), as well as the ratio of 12α-hydroxylated to 12α-non-hydroxylated BAs (112) in serum of liver cirrhotic patients were decreased.

Different liver diseases are associated with different serum BA profiles. The highest levels of total BAs were found in cirrhosis, compared to hepatitis and liver cancer (108). When measured independent on presence or absence of UDCA treatment the levels of serum unconjugated UDCA and glycochenodeoxycholic acid (GUDCA) were higher in alcoholic liver disease and TCA level was higher in biliary tract diseases compared to viral hepatitis. Unconjugated DCA and UDCA were lower in biliary tract diseases than in viral hepatitis. In the absence of UDCA therapy, taurolithocholic acid (TLCA) was significantly lower in alcoholic liver disease, as well as unconjugated CDCA, unconjugated DCA and glycolithocholic acid (GLCA) levels were lower in patients with biliary tract diseases compared to viral hepatitis. Unconjugated UDCA level was higher in patients with alcoholic liver disease compared to viral hepatitis (115). In NASH the relative abundances of BAs were unchanged. Nevertheless, the absolute values of secondary BAs were higher than in healthy controls, taurine conjugated BAs increased 5.6-fold, glycine conjugated BAs increased 3.2-fold (111). There was no correlation between degree of steatosis and total BA levels in liver diseases (116).

The levels of serum BAs were stronger associated with mortality in cirrhosis than such parameters as presence of ascites, levels of albumin, pseudocholinesterase, bilirubin, as well as prothrombin time and nutritional state (117). Serum BAs alone could predict mortality comparable to Child-Pugh and Model for End-Stage Liver Disease (MELD) scores (113, 117).

Bile acid signalling

As mentioned above, BAs play a role not only in digestion. Circulating serum BAs were recently shown to participate in various processes including glucose metabolism and insulin signalling, as well as immune response and inflammation (118-120). BAs were shown to signal through a range of nuclear (FXR, pregnane X-receptor (PXR), vitamin D-receptor (VDR)) and G-protein-coupled receptors (TGR5, sphingosine-1-phosphate receptor 2 (S1PR2), formyl peptide receptors (FPRs), muscarinic receptors) broadly distributed in different tissues and immune cells (119). Immunosuppressive effects of BAs mediated through different signalling pathways were described in monocytes/macrophages (121, 122), lymphocytes (123, 124), dendritic cells (125).

Farnesoid X receptor (FXR) or NR1H4

FXR is a nuclear BA receptor which is expressed in different tissues including liver, intestine, kidney, adrenal gland (126), macrophages (127), CD4 and CD8 T-cells, B-cells, monocytes (128).

CDCA and its conjugated forms are most potent FXR agonists (82, 83). Unconjugated, glycine and taurine conjugated forms of LCA, DCA and conjugated forms of CA were also shown to activate FXR, whereas UDCA could not activate this receptor (82). However, in another study UDCA treatment was suggested to have FXR antagonistic properties (129). FXR antagonistic activity was described also for LCA (130).

FXR activation decreases proinflammatory cytokine expression (IL-1-beta, IL-6 (127, 131), TNF-alpha, INF-gamma, TGF-beta1 (127), Mcp-1 (131)) in mouse colonic mucosa, as well as in intestinal cells (IL-1-beta) and monocytes, dendritic cells (TNF-alpha) and macrophages (IL-1-beta, TNF-alpha, IL-6) (127), and increases expression of microbicidal genes in colon (NO synthase, cathelicidin) and ileum (Ang1) (131).

Pregnane X receptor (PXR)

Pregnane X receptor (PXR) or steroid and xenobiotic receptor (SXR) is a nuclear receptor, which is expressed in liver and intestine (132), CD4 and CD8 T-cells, B-cells, monocytes (128).

Unconjugated DCA, LCA and CDCA were described to activate this receptor (133). PXR/SXR signalling was shown to be protective against LCA toxicity (133, 134). PXR activation was also reported to have anti-inflammatory effects, suppressing TNF-alpha expression in monocytes (135). PXR signalling was also shown to supress T cell functions (136).

Vitamin D receptor (VDR)

VDR is also a nuclear receptor, which is expressed in such immune cells as monocytes, activated T-lymphocytes and B-lymphocytes (137), macrophages (138). Neutrophils express comparable to monocytes level of VDR mRNA (139).

LCA and GLCA were shown to be the most potent activators of VDR among BAs, however, relatively high concentrations are needed (140). VDR signalling was described to be important for T cell development and functions (141). Unconjugated LCA via VDR has an influence on T cells by decreasing production of IFN-gamma and TNF-alpha, decreasing expression of T-box protein expressed in T cells, Stat1 and Stat4 and decreasing STAT1 alpha/beta phosphorylation (123). The activation of VDR in neutrophils can suppress the expression of neutrophil genes like trappin-2/elafin/SKALP (inhibitor of elastase) and inhibit IL-1-beta expression (139).

TGR5 receptor (or GP-BAR1, or M-BAR)

The TGR5 receptor (or GP-BAR1, or M-BAR) is localized on the plasma membrane and is activated by unconjugated and taurine and glycine conjugated BAs (142). The tissues, which express this receptor, include placenta and spleen (both very high expression), muscles, lymph nodes, liver, monocytes (85), intestine (86).

Potency of BAs to activate TGR5 vary: LCA ≥ DCA > CDCA > CA; taurine-conjugated > unconjugated bile acids > glycine-conjugated BAs (142). EC 50 for LCA is 0.53 μM, TLCA – 0.33 μM, EC 50 for DCA – 1.01 μM, CDCA – 4.43μM, CA – 7.72 μM (85).

Activation of TGR5 reduced phagocytosis and increased IL-10/IL-12 ratio in macrophages (121). In sinusoidal endothelial cells activation of TGR5 signalling was shown to induce expression and activate endothelial NO synthase and induce NO production in liver (143). TGR5 signalling was also shown to be involved in monocytes differentiation into dendritic cells

with decreased IL-12 production (144). TGR5 signalling was linked to cholangiocyte proliferation and ROS production, as well as its antiapoptotic effects were described (145).

An association between TGR5 single-nucleotide polymorphism and PSC and ulcerative colitis was previously described (146). The anti-inflammatory effects of TGR5 activation in Kupffer cells can be protective in steatosis (147).

Sphingosine-1-phosphate receptor 2 (S1PR2 or S1P2)

Sphingosine-1-phosphate receptor 2 (S1PR2 or S1P2) is a G protein-coupled receptor, which is expressed e.g. in rodent hepatocytes (148), T and B lymphocytes, monocytes, macrophages, neutrophils (but depends on their activation status), eosinophils, dendritic cells (149), vascular smooth muscle cells, heart, liver, kidney, spleen, lung and brain (150).

Taurocholic acid (TCA), taurodeoxycholic acid (TDCA), tauroursodeoxycholic acid (TUDCA), glycocholic acid (GCA), glycodeoxycholic acid (GDCA) were shown to activate S1P2 receptor. TCA significantly activated S1P2, but not S1P1 and S1P3-5 receptors (148). S1PR2 plays role in regulation of *Cryptococcus neoformans* phagocytosis in alveolar macrophages (151). S1PR2 has been reported to be required for mast cell degranulation and chemotaxis (152).

G-protein coupled M3 muscarinic receptor (CHRM3)

G-protein coupled M3 muscarinic receptor (CHMR3) is expressed in e.g. gastrointestinal tissues (153), macrophages (154), neutrophils (155). TLCA can interact with muscarinic receptors in gastric chief cells (156) and TDCA and GDCA can interact with M3-muscarinic receptor in Chinese hamster ovary cells (157). Muscarinic receptors are involved in many physiological processes, as well as pathophysiology of various diseases (158).

Formyl peptide receptors (FPRs)

Formyl peptide receptors (FPR1, FPR2, FPR3) are membrane G-protein coupled receptors, which are expressed in e.g. monocytes, macrophages, dendritic cells (159), NK cells (160). Neutrophils were shown to express FPR1 and FPR2 (159). FPR1 plays a significant role in the regulation of neutrophil functions and it is the main receptor, which sense fMLF (159). CDCA (161) and DCA (162) can competitively inhibit binding of fMLF to FPR1 and as a consequence

inhibit monocyte and neutrophil chemotaxis and calcium flux in response to fMLF in a dose-dependent fashion (IC 50 for CDCA and DCA to inhibit monocyte chemotaxis was approximately 100 μM, minimum CDCA concentration to cause an inhibitory effect was 25 μM). The inhibitory effect of UDCA on monocyte chemotaxis was shown to be much weaker (UDCA at 100 μM inhibited less than 10% of fMLF induced chemotaxis). Conjugated forms of CDCA and UDCA had the same direction of effects, but were less efficient (TCDCA and GCDCA were inhibitors at 50 μM – 200 μM, TUDCA was an inhibitor only at 200 μM) (161). Unconjugated DCA inhibited neutrophil chemotaxis and calcium flux towards fMLF already at concentrations lower than 50 μM (162).

Membrane fluidity

Membrane fluidity or viscosity is defined by ability of lipid bilayer molecules to move within the membrane, which is important to maintain cell functionality (163). DCA was shown to cause a decrease in membrane fluidity in HCT116 cells after 1 hour treatment. In contrast, UDCA or CA did not exhibit this effect (164). However, another study showed that DCA, UDCA and TUDCA increased the plasma membrane fluidity of rat hepatocytes and HEK293T cells and this effect did not associate with cell apoptosis (165). TCA and TUDCA exhibited protective properties, preventing the decrease in membrane fluidity induced by LPS in rat erythrocytes, but did not change the level of hydroxyl radicals produced in response to LPS. TCDCA did not influence membrane fluidity changes caused by LPS, but decreased the hydroxyl radical production in LPS treated erythrocytes (166). BAs (CDCA, TCDCA, DCA, TDCA) were suggested to play a role in the development of cardiomyopathy in cholestatic liver disease. Their 1 mM concentration was associated with decrease in rat cardiac cell membrane fluidity (167). Interestingly, decrease in membrane fluidity was described in neutrophils from patients with liver diseases, e.g. viral hepatitis (168).

Thus, BAs were shown to affect functions of various immune cells via different pathways. However, the knowledge about BAs effects on neutrophil functions is scarce and contradictory.

Bile acids and neutrophils

Several studies described neutrophil function in rat or mouse cholestatic models, associating cholestasis with either increased (169) or decreased (170) ROS production, decreased bacterial killing (170), neutrophil adhesion (171) and increased migration (169), unchanged

(170) or increased (169) phagocytosis and unchanged degranulation (170), increased Mac-1 expression and L-selectin shedding (172). Bile, unconjugated LCA, CDCA, DCA and CA (at very high concentrations) potentiated ROS release in primed rat neutrophils. Only unconjugated LCA could cause superoxide production also in not pre-activated rat neutrophils (173). Priming effect of LCA on rat neutrophils activated with PMA, fMLF or calcium ionophore was shown, as well as LCA inhibitory activity against beta-glucuronidase release from fMLF activated neutrophils (174). However, the BA composition and functions in rodents were described to be significantly different compared to humans, therefore, it is difficult to make the conclusions about BA effects on human neutrophils based on the results from rat and mouse studies (175-177).

There are only a few studies describing BA effects on human neutrophils. Sera from patients with obstructive jaundice were shown to induce ROS production in healthy donor neutrophils (178). Individual BAs were studied only in regard to chemotaxis and intracellular calcium mobilization in human neutrophils. Unconjugated CDCA and UDCA were shown to reversibly inhibit chemotaxis of human neutrophils in response to fMLF, but these effects were not shown for LCA and CA (179). In other studies, unconjugated CDCA, UDCA (161) and DCA (162) inhibited chemotaxis towards fMLF in human neutrophils, but DCA did not inhibit chemotaxis towards C5a or IL-8 (162). Unconjugated and conjugated forms of CDCA, UDCA (at high concentrations) (161) and unconjugated DCA (reversibly) (162) were shown to inhibit calcium flux in healthy donor neutrophils in response to fMLF, but not in response to C5a or IL-8.

Hence, effects of BAs on human neutrophils are not yet well described. The results of a few existing studies are contradictory and describe only chemotaxis and calcium flux of neutrophils and no other neutrophil functions. This underscores the necessity to further investigate the effects of BAs on neutrophils as potential contributors to the cirrhosis-associated immune dysfunction.

HYPOTHESIS AND AIMS

Serum BAs, which have been recently shown to play role in immune response and which concentrations dramatically increase in cirrhotic patients, could be serum compounds responsible for human neutrophil dysfunction in liver cirrhosis.

The specific aims of this thesis were

1) To describe neutrophil phagocytosis and ROS production, as well as serum BA composition in cirrhotic patients and healthy controls and to study the relationship between neutrophil function changes and BA composition in liver cirrhosis.

2) To study the effects of pathophysiological concentrations of BAs on human neutrophil phagocytosis, ROS production, chemotaxis, NETs formation, apoptosis and viability in *in vitro* experiments.

3) To investigate possible pathways of BA effects on neutrophil function.

4) To study gut microbiome composition in cirrhotic patients depending on cirrhosis etiology and its relationship with serum BA composition.

5) To analyse the changes in serum protein content in cirrhotic patients compared to healthy controls in order to discover other possible serum factors, which contribute to neutrophil dysfunction in liver cirrhosis.

6) To study effects of albumin supplementation on neutrophil function in cirrhotic patients.

7) To analyse serum iron metabolism parameters in cirrhotic patients compared to healthy controls.

8) To study effects of various autoantibodies on NETs production.

MATERIAL AND METHODS

The description of some parts of Material and Methods section may be similar to those published in Leber B er al., 2020 (34) or submitted for publication (Balazs I et al., 2021).

Material

All reagents were from Sigma-Aldrich (St. Louis, Missouri, USA), if not stated otherwise. GLCA and Cyclosporine H (CsH) were from Santa Cruz Biotechnology (Dallas, Texas, USA). Phosphate-buffered saline (PBS), Hanks' Balanced Salt Solution (HBSS) and Roswell Park Memorial Institute (RPMI) 1640 supplemented with L-glutamine, Iscove's Modified Dulbecco's Medium (IMDM) were from Gibco™ Thermo Fisher Scientific (Waltham, Massachusetts, USA). TUDCA, TCA, GCA, TCDCA, GCDCA, DCA, TDCA, glycodeoxycholic acid (GDCA) were diluted in sterile distilled water; UDCA, GUDCA, CA, CDCA, LCA, TLCA, GLCA were diluted in DMSO, aliquoted and stored at -20°C (Table 1). Aliquots were used only once to avoid freeze-thaw cycles.

Table 1. Bile acids used in *in vitro* experiments with neutrophils.

Bile acid	**Company**	**Catalogue number**
Cholic acid	Sigma-Aldrich	C1129
Chenodeoxycholic acid	Sigma-Aldrich	C9377
Sodium deoxycholate	Sigma-Aldrich	D6750
Sodium glycocholate hydrate	Sigma-Aldrich	G7132
Sodium glycochenodeoxycholate	Sigma-Aldrich	G0759
Sodium glycodeoxycholate	Sigma-Aldrich	G9910
Glycolithocholic acid sodium salt	Santa Cruz Biotechnology	SC-396741
Glycoursodeoxycholic acid	Sigma-Aldrich	06863
Lithocholic acid	Sigma-Aldrich	L6250
Taurocholic acid sodium salt hydrate	Sigma-Aldrich	T4009

Sodium taurochenodeoxycholate	Sigma-Aldrich	T6260
Sodium taurodeoxycholate hydrate	Sigma-Aldrich	T0557
Sodium taurolithocholate	Sigma-Aldrich	T7515
Sodium tauroursodeoxycholate	Sigma-Aldrich	T0266
Ursodeoxyholic acid	Sigma-Aldrich	U5127

Human samples

Cirrhotic patients (n=109) with clinical/radiological/histological evidence of liver cirrhosis of any cause were recruited from the outpatient clinic at the Department of Gastroenterology and Hepatology or the Department of Transplantation Surgery at the University Hospital of Graz between 2012 and 2015. Along with healthy controls (n=21), patients were recruited within different clinical studies: NCT01607528, NCT02545309, NCT02545335 (ethic vote numbers: 23-096 ex 10/11, 25-006 ex 12/13, 26-569 ex 13/14). Baseline blood samples (before any study specific intervention) were taken from all the subjects for the analysis of neutrophil function (phagocytosis and ROS production), serum BA concentrations and serum protein composition. Baseline stool samples of 83 patients and 21 healthy controls from NCT01607528 study (ethic vote number: 23-096 ex 10/11) were used for gut microbiome analysis. Patients and healthy controls provided written informed consent and the studies were approved by the Medical University of Graz Institutional Review Board and carried out according to the Declaration of Helsinki. Presence of active infection, organ failure, malignancy, pregnancy, active alcohol intake, treatment with immunomodulating medications or antibiotics a month prior, treatment with UDCA and age under 18 were exclusion criteria.

The *in vitro* experiments with BAs, bile and autoantibodies were performed with neutrophils isolated from the blood of healthy volunteers, after they provided informed consent. This was approved by the Institutional Review Board of the Medical University of Graz (ethic vote number: 23-096 ex 10/11) and the local Lothian Research Ethics Committee (AMREC 15-HV-013). Blood from cirrhotic patients for the experiments, where BA receptor expression was analysed, was obtained from clinical study: NCT03080129 (ethic vote number: 29-280 ex 16/17). Baseline neutrophil function and serum BA composition from UDCA-treated cirrhotic patients were also analysed: 5 patients from NCT01607528 study (ethic vote number: 23-096

ex 10/11) and 1 patient from NCT02545309 study (ethic vote number: 25-006 ex 12/13). For the NETs formation experiments, bile from explanted livers from patients undergoing liver transplantation and healthy organ donors enrolled in the clinical study: NCT02545309 (ethic vote number: 26-569 ex 13/14) was used. Blood samples were also obtained from cirrhotic patients, who received albumin intervention (clinical study: NCT03214796, ethic vote number: 29-040 ex 16/1). None of healthy controls had active or chronic infectious or other diseases, current use of medications or pregnancy.

Neutrophil phagocytosis and ROS production (whole blood)

Peripheral blood was taken to the VACUETTE® tubes containing 3.8% sodium citrate (Greiner Bio-One, Kremsmünster, Austria).

The Phagotest® kit (Glycotope, Heidelberg, Germany) was used to determine the phagocytic capacity of neutrophils according to the manufacturer's instructions. In brief, 100µl of the whole blood was mixed with 20µl of stabilized and opsonized FITC-labelled *E. coli* suspension (2 x 10^9 bacteria/ml) in two tubes. One tube (control) stayed on ice; another one was incubated for 10 min at 37°C. Quenching solution was added to avoid counting of the cell membrane-attached not engulfed bacteria. After washing step cells were fixed and red blood cells were lysed. LSRII flow cytometer (BD Biosciences, San Jose, California, USA) with BD FACS Diva 6.2 software (BD Bioscience) were used to record 10,000 neutrophils. Further analysis was performed in FlowJo™ V10 software (BD Biosciences) and the the percentage of non-phagocytic neutrophils as well as phagocytic capacity of neutrophils were calculated as described in (35). In brief, phagocytic capacity was calculated as a weighted geometric mean fluorescence intensity (GMFI) of phagocytic neutrophil populations. FITC-negative neutrophils were defined as non-phagocytic neutrophils. The results were normalized to *E. coli* batch (average of 4-5 healthy controls per every *E. coli* batch used).

The Phagoburst® kit (Glycotope, Heidelberg, Germany) was used to determine the percentage of neutrophils that produce ROS in response to different stimuli and their intracellular ROS amount according to the manufacturer's instructions. In brief, 100µl of the whole blood was mixed with 20µl of either wash solution (no stimulus – shows neutrophil basal ROS production), fMLF (shows the ability of neutrophils to respond to the low physiological stimuli, reflecting their pre-activated status), PMA (ROS production in the presence of a very potent stimuli, served as a positive control) or stabilized and opsonized (non-labelled) *E. coli* suspension (1-

2 x 10^9 bacteria/ml) (ROS production in the presence of a potent physiological stimuli). Tubes were incubated for 10 min at 37°C. Fluorogenic substrate solution containing dihydrorhodamine 123 was added to all the tubes and incubated for 10 min at 37°C. Cells were fixed and red blood cells were lysed. LSRII flow cytometer (BD Bioscience) with BD FACS Diva 6.2 software (BD Bioscience) were used to record 10,000 neutrophils. Further analysis was performed in FlowJo™ V10 software (BD Biosciences).

HPLC-HRMS

Serum BA concentrations were determined with high performance liquid chromatography - high-resolution mass spectrometry (HPLC-HRMS), as described in (180). HPLC was performed with 10µl of deproteinized serum using a Nucleoshell C18 reversed phase column (2.7 µm, 50 * 2.0 mm) (Macherey-Nagel, Düren, Germany) mounted in an oven with a column switching unit (Mistraswitch, Maylab, Vienna, Austria, set to 25 °C). The autosampler was Accela Open AS (Thermo Fisher Scientific), the HPLC-pump was a 1250 Accela (Thermo Fisher Scientific). BA identification was performed with a Q Exactive hybrid quadrupole-orbitrap mass spectrometer (Thermo Fisher Scientific) with a heated electrospray ionization ion source with negative ionization. Linear calibration was performed and Xcalibur 2.3 software (Thermo Fisher Scientific) was used for BA concentrations quantification. The BA detection limit was 0.025 µmol/l.

Neutrophil isolation (Percoll method 1)

Neutrophils were isolated from human peripheral venous blood as described before (181). 36ml of whole blood was gently mixed with 4 ml warm sodium citrate 3.8% solution (Roth, Carlsruhe, Germany or Sigma-Aldrich). 40ml in total was centrifuged at 350xg for 20 min at room temperature (RT). Supernatant (platelet-rich plasma) was carefully collected without disturbing the cells and transferred to the glass tube. 220 µl of 1M calcium chloride were added per 10 ml of plasma and incubated at 37°C for 1 hour to prepare autologous recalcified plasma (serum). 6 ml of 6% dextran solution (Serva, Heidelberg, Germany or Sigma-Aldrich) were added to the tube with pelleted cells and 0.9% sodium chloride was added up to 50 ml and the tube was gently rolled to mix the content. Suspension was left standing upright for 30 min at RT until a clear upper interface was visible. Supernatant was collected without disturbing the pellet and centrifuged (350xg, 6 min, RT). Supernatant was discarded, cell pellet was resuspended in 3 ml 55% Percoll (Cytiva, Marlborough, Massachusetts, USA) and overlaid on

a 81%/70% Percoll gradient (3 ml each). After centrifugation (720xg, 20 min, RT) polymorphonuclear cell (PMN) layer was removed to another tube and washed twice with 50 ml of 1x Dulbecco's phosphate buffered saline (DPBS) without Ca^{2+} and Mg^{2+} (230xg, 6 min, room temperature) and resuspended in assay specific medium. Neutrophils were counted with either TC20™ Automated Cell Counter or Neubauer chamber and their viability was determined with trypan blue exclusion staining. All the reagents were sterile to keep cells intact. The method yields a neutrophil purity >95% according to Diff-Quik (Thermo Scientific) stained cytocentrifuge preparations.

Phagocytic function of isolated neutrophils

100 µl containing $5x10^5$ human neutrophils isolated with Percoll method 1 were resuspended in RPMI 1640 medium containing L-glutamine and 10% of autologous serum and treated with BAs or vehicle for 45 min at 37°C. The Phagotest™ kit (Celonic, Basel, Switzerland) was used to determine phagocytic capacity of isolated neutrophils according to the manufacturer's instructions and as described above. CytoFLEX LX flow cytometer (Beckman Coulter, Brea, California, USA) with CytExpert 2.3 software (Beckman Coulter) were used to record the data and further analysis was performed with FlowJo™ V10 software (BD Biosciences). The percentage of non-phagocytic neutrophils as well as phagocytic capacity of neutrophils were calculated as described in (35) and above.

ROS production of isolated neutrophils

ROS production was measured essentially as previously described (181). Human neutrophils right after isolation with Percoll method 1 were resuspended in PBS++ (Dulbecco's PBS supplemented with Ca^{2+} and Mg^{2+}, 1 g/l D-glucose and 4 mM sodium bicarbonate) to a concentration of $6.25x10^6$ cells/ml. Luminescence-grade 96 well plate (Nunc, Thermo Fisher Scientific or Greiner Bio-One) was pre-treated with 1% skimmed milk for 1 hour at RT and subsequently washed 2 times with PBS without Ca^{2+} and Mg^{2+} and 1 time with PBS++. 180 µl of cells were mixed with 225ul of horseradish peroxidase (18.75 U/ml) and luminol (150 µM) and added to the plate containing BA/vehicle and PBS++, fMLF (added after 45 min of luminescence reading; final concentration 1.45 µM) or heat-inactivated serum-opsonized *E. coli* (40 bacteria/cell). Total ROS production (sum of intracellular and extracellular ROS) was measured at 37ºC in real-time indirectly by chemiluminescence for 60 min in a plate reader (Cytation plate reader (BioTek, Swindon, UK) or a Lumistar Omega luminescence microplate

reader (BMG Labtech, Offenburg, Germany)). Relative light units per second (RLUs/s) or total RLUs integrated over the indicated periods of time were analyzed.

Chemotaxis (ChemoTx® disposable chemotaxis systems)

ChemoTx® disposable chemotaxis systems 300 µl 96-well microplates, 5.7 mm sites, pore size 3 µm (Neuro Probe, Gaithersburg, Maryland, USA) were used. Neutrophils were isolated with Percoll method 1 and resuspended in HBSS++ solution (HBSS with Ca^{2+} and Mg^{2+}, 0.05% bovine serum albumin (BSA) fatty acid and endotoxin free, 15mM Hepes (Roth)) to a concentration $1x10^7$ cells/ml. Cells were pre-treated with BAs/vehicle for 45 min at 37°C. In the lower wells 310 µl of 10 nM fMLF were added, filter was placed above and 50 µl of neutrophil suspension was added on the filter. After 1 hour at 37°C, not migrated cells were carefully wiped out from the top of the filter and the plate was centrifuged at 300xg for 10 min with the filter attached. Supernatant was carefully discarded with the pipette and cells were resuspended in 100 µl freshly prepared p-nitrophenyl phosphate (PNPP) solution (5mM PNPP (BioLabs, Ipswich, Massachusetts, USA), 0.1 M sodium acetate (Roth), 0.1% Triton X-100 (Roth)). Plates were incubated at 37°C for 2h. After that 5µl/well of 2 M sodium hydroxide was added to amplify the yellow color, which developed upon reaction of PNPP with alkaline phosphatase (allows to estimate the amount of migrated neutrophils). Absorbance at 450 nm was measured by Cytation plate reader (BioTek, Swindon, UK) within 24 hours. Negative control without chemoattractant in the lower chamber was performed to assess the level of random migration. Chemokinesis control with no chemoattractant gradient was also performed. The results were analysed as optical density (OD).

Chemotaxis (µ-slide chemotaxis)

To investigate the chemotaxis defect in more detail, µ-slide chemotaxis chambers (ibidi, Martinsried, Planegg, Germany) were used. Freshly isolated human neutrophils (Percoll method 1) were resuspended in HBSS++ buffer in a concentration of $5x10^6$ cells/ml. 35 µl of cells (with added BA/CsH/vehicle) were mixed with 75 µl collagen from rat tail (Roche, Basel, Switzerland), 5 µl of sodium bicarbonate buffer, 10 µl of 10x HBSS with Ca^{2+} and Mg^{2+} and phenol red. 6µl of this suspension was added to the capillary of µ-slide and removed with the pipette from another edge of capillary. Slides were incubated for 20 min at 37°C until collagen solidify. 65 µl of HBSS++ buffer with or without 50 nM fMLF were added to the slides to create a gradient and images of neutrophils were taken every 30 s for 30 min at 37°C with the inverted

RMDIB microscope (Leica, Newcastle, UK) with temperature-controlled chamber and an Orca camera (Hamamatsu, Welwyn Garden City, UK). Paths of individual neutrophils were tracked and analysis of the tracks was performed using “TrackMaxima” plug-in in ImageJ kindly provided by Luke Tweedy, Beatson Institute, University of Glasgow.

Neutrophil isolation (Polymorphprep method)

Blood was taken into 9 ml VACUETTE® tubes containing Lithium Heparin (Greiner Bio One) and then carefully placed on equal amount of Polymorphprep (Axis shield, Oslo, Norway) without mixing two phases. After centrifugation for 35 min at 500xg, upper layer of monocytes was carefully discarded with sterile Pasteur pipette and lower layer containing neutrophil granulocytes was washed in sterile HBSS without Ca^{2+} and Mg^{2+}. Red blood cell lysis buffer (Roche) was added for 10 min to lyse red blood cells. After that, cells were washed again, cell amount and viability were counted with trypan blue exclusion method by TC20™ Automated Cell Counter.

NETs formation

NETs formation assay was previously described (182). Neutrophils were isolated from healthy volunteers’ peripheral blood with Polymorphprep method. Isolated neutrophils were resuspended to the concentration $4x10^5$ cells/ml in RPMI 1640 media supplemented with 2% Human Serum Albumin (CSL Behring, Pennsylvania, USA) and L-Glutamine. 500 µl of cell suspension per well were added into 24 well plate with sterile glass round coverslips inside (13mm) (VWR, Darmstadt, Germany) and incubated for 1 hour at 37°C. To each well in duplicates either 100µl of 600 nM PMA (positive control), heat-inactivated *E. coli* $5x10^8$ cells/ml or RPMI 1640 (negative control) were added in presence or not of bile/vehicle/autoantibodies. Bile samples were added at 1:50 dilution, autoantibodies were added to the final concentration 12 µg/ml (Table 2). After incubation for 2 hours at 37°C 600µl of 8% paraformaldehyde were added to fix the cells for at least 15 min, coverslips were carefully washed with distilled water 1 time for 5 min. Coverslips were mounted with ProLong Gold antifade reagent with DAPI (Life Technologies, Carlsbad, USA) on the glass slides and kept at 4°C until the analysis by Olympus BX51 Fluorescence Microscope (Shinjuku City, Tokyo, Japan) at 600x total magnification. NETs-like structures percentage was quantified as NETs-like structures divided by total number of neutrophils. 10 fields of vision were analyzed per slide.

For representative pictures, main NET components like MPO and neutrophil elastase (NE) were stained with immunofluorescence. Staining was performed with rabbit anti-MPO (Cat.N. A039829, Dako, Jena, Germany) and mouse anti-NE (Cat.N. M0752, Dako) primary antibodies and anti-rabbit (Cat.N. ab96899, abcam, Cambridge, UK) and anti-mouse (Cat.N. 84540, Thermo Scientific) secondary antibodies. Coverslips were washed once with distilled water after fixation with paraformaldehyde and stained with 1:200 diluted primary antibodies for 1 hour at RT with subsequent washing with PBS (3 times for 5 min). After that coverslips were incubated with 1:300 diluted secondary antibodies cocktail for 30 min at RT in the dark with subsequent washing with PBS (3 times for 5 min). Coverslips were mounted on the glass slides with ProLong Gold antifade reagent with DAPI (Thermo Fisher Sicentific) and representative pictures were taken with Nikon A1R microscope (Minato City, Tokyo, Japan).

Table 2. List of antibodies used to test NETs formation.

Antigen	**Company**	**Catalogue number**
pANCA (anti-MPO antibody)	Dako	A039829
pANCA isotype control (anti-MPO isotype control)	Dako	X093602-2
cANCA (anti-PR3 antibody)	Immunotools	21336031
ANA (anti-citHistone3 antibody)	Abcam	ab5103

Apoptosis and viability

Human neutrophils isolated with Percoll method 1 were resuspended in IMDM media (supplemented with 10% autologous serum) to the concentration $1x10^7$ cells/ml. 75 µl of cells were mixed with 75 µl of BA/vehicle containing media and incubated for 3, 6, 9 or 12 hours at 37°C. 50 µl of cells were mixed with 230µl of annexin buffer (500 ml HBSS with Ca^{2+} and Mg^{2+}, 2.5 ml 1 M $CaCl_2$, 1:1000 FITC-annexin V (Roche)). Propidium iodide (PI) was added immediately prior to flow cytometry analysis. Analysis of necrotic and apoptotic cells was performed at the baseline and each time point by flow cytometer Attune NxT (Thermo Fisher Scientific) and FlowJo™ V10 software (BD Biosciences). Percentage of necrotic neutrophils (PI^+), early apoptotic neutrophils (PI^-Anx^+) and total apoptotic neutrophils (Anx^+) was analysed (Figure 4).

Neutrophil viability was also assessed separately after resuspending 5x10^5 cells in 100µl of PBS++ and incubating them with different concentrations of BA/vehicle for 1 hour at 37°C. PI was added to the final concentration 1 µg/ml right prior to flow cytometry analysis. Data were recorded using a CytoFLEX LX flow cytometer (Beckman Coulter) in combination with CytExpert 2.3 software (Beckman Coulter). FlowJo™ V10 software (BD Biosciences) was used to analyze the percentage of PI positive cells.

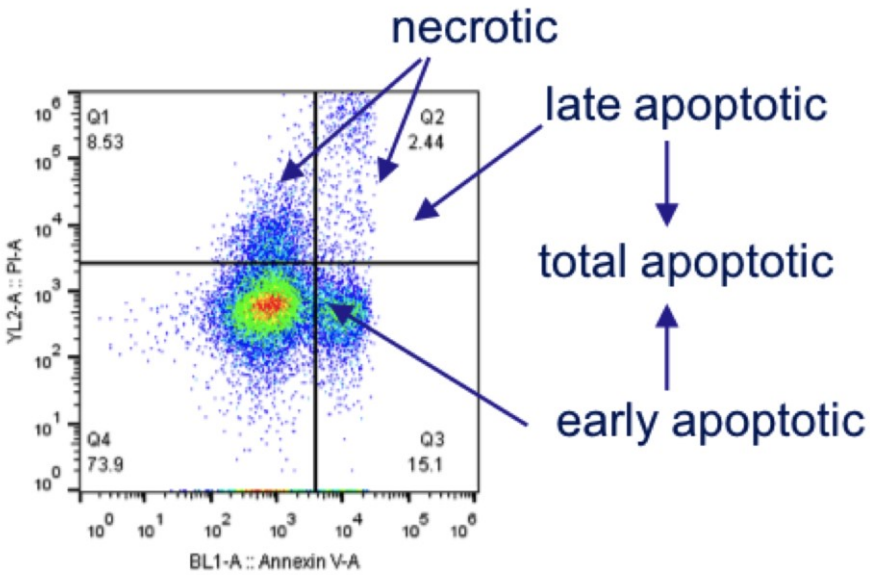


Figure 4. Gating strategy to identify neutrophil populations with necrosis, early apoptosis or late apoptosis.

Neutrophil isolation (Percoll method 2)

Method was described earlier (183). 36ml of whole blood was gently mixed with 4 ml warm sodium citrate 3.8% solution (Roth). 40ml in total was centrifuged at 270xg for 20 min at RT. Supernatant was collected and centrifuged (1000xg, 20 min, RT) to produce platelet poor plasma (PPP). To the red pellet 6 ml of 6% dextran solution (Serva) and 0.9% sodium chloride were added up to 50ml and gently rolled. Suspension was left standing upright for max. 30 min until a clear upper interface was visible. Supernatant was collected without disturbing the pellet and centrifuged (185xg, 6 min, RT). Supernatant was discarded; cell pellet was resuspended in 2 ml PPP and overlaid on a 51%/42% Percoll gradient. After centrifugation (225xg, 11 min, RT) PMN layer was transferred to another tube, resuspended in 2 ml of PPP and the tube was filled up to 40 ml with HBSS without Ca^{2+} and Mg^{2+}. Neutrophils were counted and their viability

was determined with TC20™ Automated Cell Counter. After centrifugation (420xg, 6 min, RT) cells were resuspended in assay specific medium.

Negative selection of neutrophils

Negative selection was used to improve neutrophil purity before RT-qPCR. Cells were pelleted by centrifugation (250xg, 5 min, RT) and resuspended in the column buffer (HBSS without Ca^{2+} and Mg^{2+}, 2% FCS Clone), then biotin antibodies cocktail (Table 3) was added. After 15 min of incubation at RT Anti-Biotin Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were added and incubated again for 15 min at RT. Cells were added to the magnetic column (Miltenyi Biotec), rinsed with column buffer prior. Cell count and viability were analysed by TC20™ Automated Cell Counter, purity and viability of neutrophils were assessed by LSRII flow cytometer (BD Biosciences) with BD FACS Diva 6.2 software (BD Bioscience). Neutrophil purity was >95%.

Table 3. List of antibodies used for negative selection of neutrophils.

Antigen	Company	Catalogue number
CD3	eBiosciences	13-0037-80
CD19	eBiosciences	13-0199-80
CD36	Thermo Fisher scientific	PA1-16815
CD56	eBiosciences	13-0567-80
CD235a	eBiosciences	13-9987-80

RT-qPCR

Neutrophils were isolated from peripheral blood of healthy volunteers with Percoll method 2. Neutrophils were negatively selected as described above to obtain a pure neutrophil fraction. Purity of neutrophil was assessed by flow cytometry after CD45 staining (BioLegend, San Diego, California, USA) and was >95%. Total RNA was isolated from neutrophils using TRIZOL Reagent (ambion, Austin, Texas, USA). DNAse treatment was performed with ambion DNA-free™ Kit (Thermo Fischer Scientific). RNA concentration and purity were assessed by NanoDrop 2000 (Wilmington, Delaware, USA). RNA was converted to cDNA using Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California, USA) simultaneously for all samples, including RT- controls (without reverse

transcriptase). RT PCR program was: 25°C – 10min, 37°C – 180 min, 85°C – 5 min. qPCR was performed using GoTaq® qPCR Master Mix (Promega, Madison, Wisconsin, USA) at 95°C for 2 min, then 45 cycles of 95°C for 15 sec and 60°C for 1 min and melting curve (15 sec 95°C, 5 sec 60°C, 0.5 sec 95°C) at BioRad 96 CFX (Hercules, California, USA). TGR5 (RefSeq ID: NM_170699), S1PR2 (RefSeq ID: NM_004230), VDR (RefSeq ID: NM_001017536), PXR (RefSeq ID: NM_022002), FXR (RefSeq ID: NM_001206978), FPR1 (RefSeq ID: NM_002029), FPR3 (RefSeq ID: NM_002030), CHRM3 (RefSeq ID: NM_000740) BA receptor primers and ACTB (RefSeq ID: NM 001101) and GAPDH (RefSeq ID: NM 002046) housekeeping gene primers were used to perform qPCR (Table 4). NTC and RT- controls were always run together with the samples, samples were run in triplicates. Standard curves with positive controls were performed for each gene to estimate primer efficiency. Ct values were normalized to the housekeeping genes and interplate calibrator and corrected for primer efficiency with LinRegPCR program (184).

Table 4. qPCR primers for RT-qPCR analysis of bile acid receptor expression in neutrophils.

Gene	Forward	Reverse
TGR5	GCTGCTTCTTCCTGAGCCTA	GTTGGGAGCCAAGTAGACGA
S1PR2	TCTCTACGCCAAGCATTATGTGC	TGGCCAACAGGATGATGGA
VDR	CTGACCCTGGAGACTTTGAC	TTCCTCTGCACTTCCTCATC
PXR	TTGCCCATCGAGGACCAGAT	GTCTCCGCGTTGAACACTGT
FXR	GACTTTGGACCATGAAGACCAG	GCCCAGACGGAAGTTTCTTATT
FPR1	TGGGAGGACATTGGCCTTTC	GGATGCAGGACGCAAACAC
FPR3	GCTAGTCCACGGAGTCACCT	GGTAGGATGGCACTGAAAGAGA
CHRM3	ATCGGTCTGGCTTGGGTCATCTC	AGCGGCCATACTTCCTCCTGTTG
ACTB	GTTGTCGACGACGAGCG	GCACAGAGCCTCGCCTT
GAPDH	AATGAAGGGGTCATTGATGG	AAGGTGAAGGTCGGAGTCAA

Flow cytometry

To determine BA receptors expression on protein level, extracellular (TGR5, FPR1, S1PR2) and intracellular (VDR) staining was performed. Neutrophil population was gated as CD16+CD14-CD45+ (Figure 5). When CD16 receptor shedding occurred, neutrophil population was gated based on SSC/CD45 properties. 100µl of whole venous blood from

healthy volunteers were placed into 5 ml FACs tube and washed once with 1 ml of staining buffer (PBS without Ca^{2+} and Mg^{2+}, 4% FBS). Then blood was lysed with red blood cell lysis buffer (Roche) for 20 min and washed with 2 ml staining buffer. Supernatant was carefully discarded with the pipette. Antibodies for extracellular staining were added (Table 5). After 20 min at RT in the dark, cells were washed with 1 ml of staining buffer and tubes with extracellular staining were fixed with 1x CellFIX (BD Bioscience). For further intracellular staining cells were then fixed with lysis/fixation solution (Thermo Fischer Scientific) for 20 min in the dark. Afterwards cells were twice washed with permeabilization buffer (Foxp3 staining buffer set, Thermo Fischer Scientific) and then incubated with antibodies in permeabilization buffer for 20 min in the dark (anti-VDR antibody (Table 5)). Cells were washed with 1 ml permeabilization buffer and resuspended in staining buffer. Unstained and fluorescence minus one (FMO) controls were always included. Compensation was performed with OneComp eBeads (eBioscience, San Diego, California, USA). Unstained cells were used for adjustment of FSC/SSC and PMT voltages. For the experiments to determine BA receptor expression in liver cirrhotic patients in whole blood the same protocol was used. LSRII flow cytometer (BD Bioscience) in combination with BD FACS Diva 6.2 software (BD Bioscience) was used to record 10,000 neutrophils. Analysis was performed with FlowJo™ V10 software (BD Bioscience).

To determine FPR1 expression in isolated neutrophils after BA treatment, cells were isolated with Percoll method 1. Cells were resuspended in PBS++ and treated with BAs for 45 min either at 37°C or on ice. fMLF was subsequently added (final concentration 1.45 µM) for 15 min. Cells were washed with ice-cold PBS++ (300xg, 5 min) and incubated with anti-hFPR1 antibody (1:50, R&D Systems, FAB3744A, APC) for 30 min on ice. Cells were washed with ice-cold PBS++, fixed with CellFIX and percentage of FPR1 expressing neutrophils was measured by Attune NxT (Thermo Fischer Scientific) and analysed with FlowJo™ V10 software (BD Bioscience).

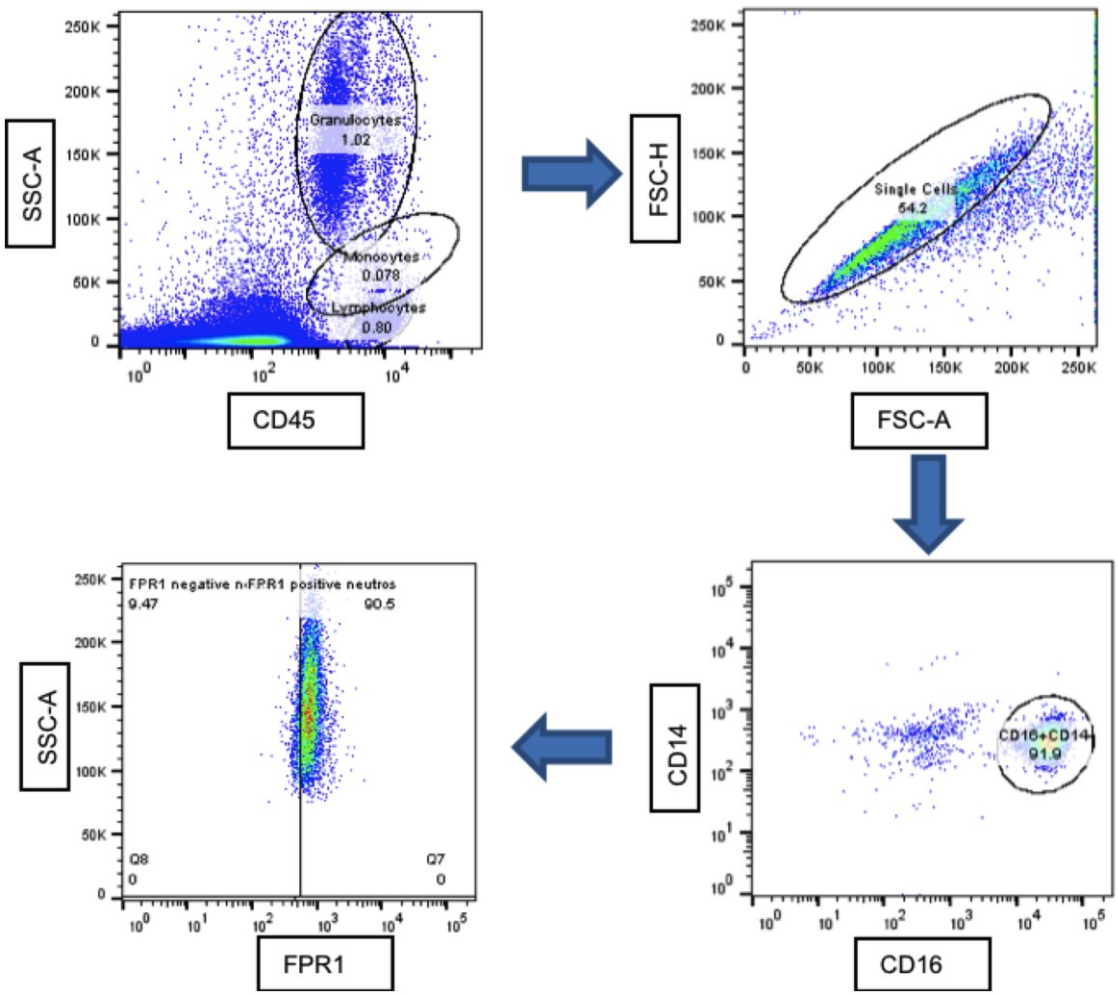


Figure 5. Flow cytometry gating strategy.

Neutrophil population was first gated based on SSC parameter and CD45 positivity. Then single cell population was determined and CD16+CD14- cells gated as neutrophils. Gate on negative population for BA receptor expression was set on FMO control and then copied to the stained sample (FPR1 receptor gating as an example on the picture).

Table 5. List of antibodies used for bile acid receptor staining in neutrophils.

Antigen	Company	Catalogue number	Dilution (fluorophore)
hFPR1	R&D Systems	FAB3744V	1:50 (Alexa Fluor® 405)
hGPBAR1	R&D Systems	FAB4286P	1:20 (PE)
hS1P2/EDG-5	R&D Systems	FAB3649R	1:50 (Alexa Fluor® 647)
CD14	Immunotools	21279143	1:20 (FITC)

CD14	BioLegend	301815	1:20 (Pacific Blue)
CD16	BioLegend	360725	1:20 (APC/Fire 750)
CD45	BioLegend	304026	1:50 (PerCP)
VDR	Santa Cruz Biotechnology	sc-13133	1:100 (Alexa Fluor® 488)

Microbiome analysis

Total DNA was isolated from frozen stool samples using MagnaPure LC DNA Isolation Kit III (Bacteria, Fungi) (Roche) according to manufacturer's instructions. Hypervariable regions V1-2 were amplified in a target specific PCR using the primers 27F and R357 (27F-AGAGTTTGATCCTGGCTCAG; R357-CTGCTGCCTYCCGTA) and sequenced with the Illumina MiSeq technique (Illumina, Eindhoven, The Netherlands) (185). This part was done in collaboration with the Core Facility for Molecular Biology at the Center for Medical Research (ZMF) in Graz.

For microbiome data analysis QIIME1 tools implemented in Galaxy (https://galaxy.medunigraz.at) were used for processing the generated FASTQ files. Pre-processing and Core Diversity Analysis was done using open reference operational taxonomic unit (OTUs) from SILVA database. PICRUSt (186) implemented in Galaxy was used to predict Kyoto Encyclopedia of Genes and Genomes (KEGG) functional pathway abundance. Rarefied unfiltered OTU table was used to calculate alpha (Chao1 index) and beta diversity. Rarefaction depth was 26688 reads (lowest sample read counts). In total 82 cirrhotic patients' samples (Child-Pugh A n=61, Child-Pugh B+C n=21) and 21 healthy controls were analysed for the differences in gut microbiome composition between the etiologies of cirrhosis (1 sample was lost during the pre-processing of the data). None of the patients received UDCA treatment. Patients' samples were grouped according to the etiology of cirrhosis (alcoholic n=47, hepatitis C n=16, other etiologies of cirrhosis n=19). Kruskal-Wallis test with FDR correction was used to obtain a group significance. Beta diversity was calculated by means of redundancy analysis (RDA) and ANOSIM in Calypso 7.14 (http://cgenome.net/calypso/). LDA-effect size (LEfSe) analysis was performed to determine differentially abundant taxa and microbiome functions.

Spearman correlation with Benjamini-Hochberg multiplicity correction was performed in R to study the linear relationship between 15 BA (absolute levels and relative abundancies) and 91 bacterial genera (genera with less than 1% as their maximum relative abundance were

excluded from the analysis) within the Child-Pugh A cirrhotic patients (n=61, no UDCA-treated patients). Spearman partial correlation was performed to account for confounding of etiology, age and sex. Mixed effect regression controlling for etiology influence, RDA at genus and OTU levels, Random Forest were performed in Calypso 7.14 (http://cgenome.net/calypso/) to further investigate the associations between BA and gut microbiome composition. Microbiome data was rarefied, normalized with total-sum normalization (TSS) and transformed with Square Root transformation for associations' analysis.

2D gel electrophoresis (2D-DIGE)

ProteoPrep Immunoaffinity Albumin and IgG depletion Kit was used to remove most of albumin and IgG from serum of healthy controls and cirrhotic patients according to the manufacturer's instructions. Further, protein content of these samples was determined by the bicinchoninic acid assay. 700 µg protein of these samples were precipitated via incubation with 10% ice-cold solution of TCA in acetone with 20 mM dithiothreitol for 60 minutes at −20°C. Samples were washed twice in ice-cold acetone and resuspended in labelling puffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, and 40 mM Tris, pH 8.5). Protein content was measured again with the same method and samples were stored at −80°C.

50 µg of protein from each serum sample were labelled with Cy dyes (Cy2 (green) or Cy3 (red)) as described previously (187). 350 µg of unlabelled proteins were mixed with the corresponding Cy labelled samples and isoelectric focusing on Immobiline IPG strips was performed. Further 2D-DIGE and image analysis were performed as described (187).

Analysis and quantification of protein spots were done using DeCyder-DIA software (Amersham Biosciences, Freiburg, Germany). Protein spots, which were differentially expressed, were tryptically digested and analyzed as described (187). Peptide extracts were dissolved in 0.1% formic acid and separated by nano-HPLC (Ultimate 3000, Dionex, Amsterdam, the Netherland). The sample, ionized in a Finnigan nano-ESI ion source (Finnigan MAT, San Jose, CA, USA) with NanoSpray tips (PicoTip Emitter, New Objective, Woburn, MA, USA), was analyzed in a LCQ Deca XPplus ion trap mass spectrometer (Thermo, San Jose, CA, USA). Further, the MS/MS data were analyzed with SpectrumMill version 2.7 (Agilent, Waldbronn, Germany).

Distance in mm to each protein spot from upper (molecular weight (MW)) and left (isoelectric point (pI)) border of each gel was measured. Experimental MW and pI of protein spots were interpolated from standard curve with sigmoidal curve fit. MW standards were log10 transformed. MW values were also linearly interpolated (with 250 kDa standard excluded from the analysis), which identified some additional proteins, which were further also included in the analysis. MW values were back transformed and the differences between experimental and theoretical MW and pI were calculated. Proteins were considered fitting (from those with ≥ 2-fold change identified in at least 2 gels out of 3 with the MS/MS score higher than 20), if their experimental MW was 20% higher or 10% lower than theoretical MW and their experimental pI was ± 2 of their theoretical pI. Proteins with MS/MS score ≥ 80, even if they did not fit with MW and pI, were still considered to account for excessive posttranslational modifications. Mean of fold regulation for all spots representing the same protein within each gel was calculated and the means from 2 or 3 gels were averaged for every protein. Proteins with highly variable fold regulation between the spots were excluded from the analysis. Significance was tested based on the 95% confidence interval (CI) – proteins were considered significantly regulated, when the 95% CI of fold change means across 3 gels did not include value of zero effect. Contribution of significantly differentially abundant proteins into different biological pathways was analyzed with the help of STRING V11 (188) and reactome pathway database (189).

Statistical analysis

Analysis was performed in IBM SPSS Statistics V25.0 (IBM, Armonk, New York, USA) and GraphPad Prism V9 (GraphPad Software, San Diego, CA, USA) using t test or Mann-Whitney U test for comparison of two groups of continuous variables depending on data distribution type. For analysis of data distribution type Shapiro-Wilk normality test was used. Pearson's chi-squared test was used to compare groups of categorical data. For a comparison of more than two groups, analysis of variance (ANOVA) with Tukey post hoc test or Kruskal-Wallis test with Bonferroni or Dunn's multiple comparisons test depending on data distribution type or Friedman test with Dunn's test for multiple comparisons (for paired data) were used. Outliers were detected with the ROUT method (Q=1%). Multivariate analysis was performed in R V4.0.3 software (190) in an integrated development environment RStudio V1.3.1093 (191) and Past V4.03 programs (192). Spearman's correlation coefficient with Benjamini-Hochberg adjustment for multiple tests ("psych" package (193)), partial Spearman's correlation coefficient ("ppcor" package (194)), simple and multiple linear regression were calculated to

assess the variables relationships. Missing values were omitted, log10 or cube root ("kader" package (195)) transformations were performed and outliers were removed, if the assumptions of linear regression were violated. An analysis of similarity (ANOSIM) based on Bray-Curtis distance was used to obtain the significance level of the groups differences, visualized by non-metric multidimensional scaling (NMMDS) based on Bray-Curtis distance ("vegan" (196), "ggplot2" (197) packages). The similarity percentages breakdown (SIMPER) procedure was used to evaluate each variable contribution to the groups' dissimilarities. The results are presented as mean ± standard deviation (SD), mean ± standard error of mean (SEM) or median ± interquartile range (IR), as indicated. $p < 0.05$ was considered statistically significant.

RESULTS

Some parts of the results are reported in (34) or Balazs et al, 2021 (submitted for publication).

Clinical data analysis

Patients' characteristics

Cirrhotic patients (n=109) and healthy controls (n=21) had comparable demographic characteristics, except for the sex distribution, with a bigger proportion of male patients in the cirrhotic group (Table 6).

Table 6. Demographic characteristics and liver function parameters of cirrhotic patients and healthy controls.
Reproduced from (Balazs et al, 2021, in revision).

Characteristic	Cirrhotic patients (n=109)	Healthy controls (n=21)	p-value
Age (years)	58±13	58±11	p=0.790
Sex (Male/Female, n)	77/32	9/12	p=0.014
Aetiology group (Alcoholic/HCV/Other, n)	54/32/23	-	-
Child-Pugh group (A/B+C, n)	79/30	-	-
Child-Pugh score	5±2	-	-
MELD score	10±6	-	-
AST (U/l)	49±43	23±9	p<0.001
ALT (U/l)	38±38	19±10	p<0.001
GGT (U/l)	112±160	19±15	p<0.001
AP (U/l)	105±71	59±23	p<0.001
Bilirubin (mg/dl)	1.3±1.3	0.5±0.2	p<0.001
INR (ratio)	1.2±0.3	1.0±0.1	p<0.001
Creatinine (mg/dl)	0.8±0.3	0.9±0.3	p=0.218

Albumin (g/dl)	4.1±0.9	4.3±0.2	p=0.071
Neutrophils (x10^9/l)	2.8±1.4	3.2±1.4	p=0.187

Median ± IR. ALT: Alanine transaminase; AST: Aspartate transaminase; AP: Alkaline phosphatase; GGT: Gamma-glutamyl transferase; INR: International Normalized Ratio; MELD: Model for End-Stage Liver Disease.

Cirrhotic patients were grouped according to the severity of cirrhosis (Child-Pugh A (n=79), Child-Pugh B+C (n=30)). Patients from Child-Pugh B+C group were characterized by significantly higher levels of aspartate transaminase (AST), bilirubin, International Normalized Ratio (INR) and significantly lower level of albumin (Table 7).

Table 7. Demographic characteristics and liver function parameters of cirrhotic patients depending on liver cirrhosis severity.
Reproduced from (Balazs et al, 2021, in revision).

Characteristic	Child-Pugh A (n=79)	Child-Pugh B+C (n=30)	p-value
Age (years)	58±14	55.5±8	p=0.134
Sex (Male/Female, n)	55/24	22/8	p=0.704
Aetiology (Alcoholic/HCV/Other, n)	37/25/17	17/7/6	p=0.619
Child-Pugh grade (n)	A –79	B – 26, C – 4	-
Child-Pugh score	5±1	8±1	p<0.001
MELD score	8±3	15±5	p<0.001
AST (U/l)	44.5±39	59±47	p=0.043
ALT (U/l)	39±44	35±22	p=0.235
GGT (U/l)	129±169	74.5±136	p=0.135
AP (U/l)	104±62	114.5±74	p=0.211
Bilirubin (mg/dl)	0.9±0.7	2.8±3.5	p<0.001
INR (ratio)	1.2±0.2	1.5±0.3	p<0.001
Creatinine (mg/dl)	0.8±0.3	0.8±0.2	p=0.111
Albumin (g/dl)	4.3±0.7	3.2±0.4	p<0.001
Neutrophils (x10^9/l)	2.8±1.4	2.7±1.5	p=0.820

Median ± IR. ALT: Alanine transaminase; AST: Aspartate transaminase; AP: Alkaline phosphatase; GGT: Gamma-glutamyl transferase; INR: International Normalized Ratio; MELD: Model for End-Stage Liver Disease.

Cirrhotic patients were grouped also according to the etiology of cirrhosis (alcoholic cirrhosis (n=54), HCV associated cirrhosis (n=32), other etiologies of cirrhosis (n=23), including alpha-1 antitrypsin deficiency (n=1), secondary sclerosing cholangitis in critically ill patients (n=2), HBV (n=4), hemochromatosis (n=2), non-alcoholic fatty liver disease (NAFLD) (n=5), medication associated (n=2), Wilson's disease (n=3) and cryptogenic (n= 4). Alcoholic cirrhotic patients were characterized with the highest MELD score, bilirubin and INR. HCV patients had the highest levels of AST, alanine transaminase (ALT) (Table 8).

Table 8. Demographic characteristics and liver function parameters of cirrhotic patients depending on liver cirrhosis etiology.
Reproduced from (Balazs et al, 2021, in revision).

Characteristic	**Alcoholic (n=54)**	**HCV (n=32)**	**Other (n=23)**	**p-value**
Age (years)	56±12	60±9	55±14	n.s.
Sex (Male/Female, n)	42/12	22/10	13/10	n.s.
Child-Pugh group (A/B+C, n)	37/17	25/7	17/6	n.s.
Child-Pugh score	6±2	5±1	5±2	n.s.
MELD score	12±6	8±4	10±7	$p=0.014^{\dagger}$
AST (U/l)	44±26	91±72	40±38	$p<0.001^{\dagger,\ddagger}$
ALT (U/l)	32.5±19	70±80	34±38	$p<0.001^{\dagger}$, $p=0.001^{\ddagger}$
GGT (U/l)	105.5±176	114.5±135	120.5±285	n.s.
AP (U/l)	109±74	94±45	105±100	n.s.
Bilirubin (mg/dl)	1.5±1.6	0.9±0.9	1.1±3.5	$p=0.030^{\dagger}$
INR (ratio)	1.3±0.3	1.1±0.3	1.2±0.3	$p=0.030^{\S}$
Creatinine (mg/dl)	0.8±0.3	0.8±0.2	0.8±0.3	n.s.
Albumin (g/dl)	4.0±1.1	4.0±0.8	4.5±1.0	n.s.
Neutrophils ($x10^9$/l)	2.9±1.4	2.6±1.0	3.1±2.8	n.s.

Median ± IR. MELD: Model for End-Stage Liver Disease; AST: Aspartate transaminase; ALT: Alanine transaminase; GGT: Gamma-glutamyl transferase; AP: Alkaline phosphatase; INR:

International Normalized Ratio. †Between HCV and alcoholic; ‡between HCV and other; §between other and alcoholic groups.

Neutrophil function in the whole blood of cirrhotic patients and healthy controls.

To assess the presence of neutrophil dysfunction, neutrophil phagocytosis and ROS production were analyzed in whole blood of cirrhotic patients and healthy controls. The cirrhotic cohort was characterized by a high proportion of non-phagocytic neutrophils compared to healthy controls, while cirrhotic neutrophil phagocytic capacity was not significantly different (Figure 6 A, B). ROS production by cirrhotic neutrophils was altered, which was reflected by a higher percentage of neutrophils with basal ROS production and ROS production in response to fMLF and lower percentage of neutrophils producing ROS in response to *E. coli* compared to healthy controls (Figure 6 C-E).

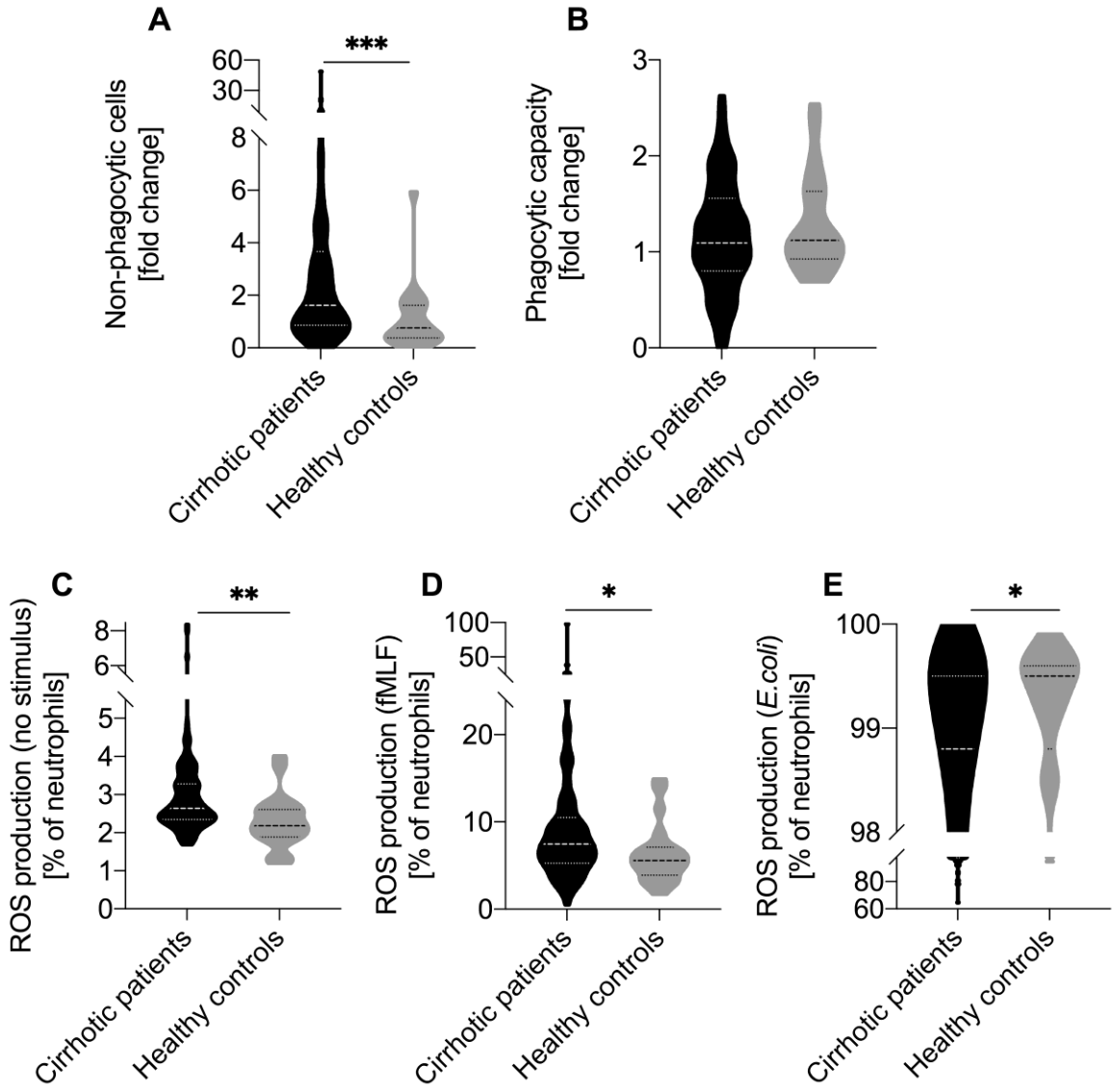


Figure 6. Defective neutrophil phagocytosis and ROS production in whole blood of cirrhotic patients.

(A, B) Phagocytosis of *E. coli* ($4x10^7$ bacteria/100µl blood). (A) Percentage of non-phagocytic neutrophils and (B) neutrophil phagocytic capacity normalized to *E. coli* batch; (C-E) ROS production. Percentage of neutrophils, which produced ROS (C) without any stimulus or after 10 min of (D) fMLF (0.8µM) or (E) *E. coli* (2-$4x10^7$ bacteria/100µl blood) stimulation. Truncated violin plots show the frequency distribution of measured parameters, the broken line indicates median, dotted lines indicate quartiles; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (Mann-Whitney U test). ROS: reactive oxygen species; fMLF: N-formyl-met-leu-phe. Reproduced from (Balazs et al, 2021, in revision).

However, the intracellular amount of ROS produced by these neutrophils was not significantly different between cirrhotic patients and healthy controls (Figure 7 A-C).

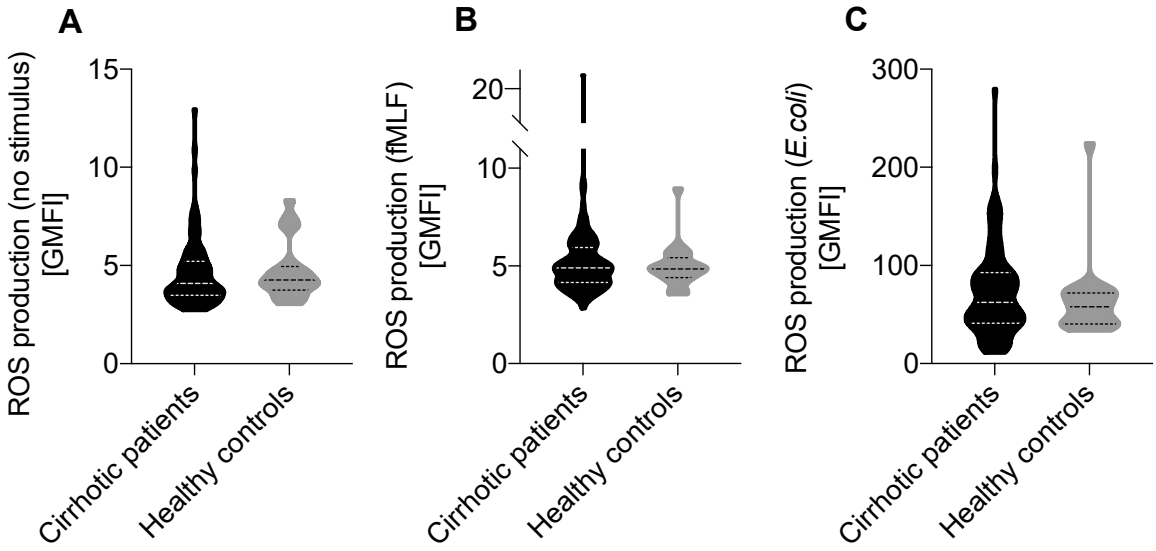


Figure 7. Intracellular ROS amount produced by neutrophils in whole blood is not different in cirrhotic patients compared to healthy controls.

(A-C) Intracellular ROS pool, defined as GMFI of neutrophils, which produced ROS (A) without a stimulus or after 10 min of (B) fMLF (0.8μM) or (C) *E.coli* (2-4x10^7 bacteria/100μl blood) stimulation. Truncated violin plots show the frequency distribution of measured parameters, the broken line indicates median, dotted lines indicate quartiles. Statistical analysis was by Mann-Whitney U test (A-C). ROS: reactive oxygen species; GMFI: geometric mean fluorescence intensity; fMLF: N-formyl-met-leu-phe. Reproduced from (Balazs et al, 2021, in revision).

Neutrophil function was also compared between severity and etiology groups of liver cirrhosis (Figures 8, 9). The percentage of non-phagocytic cells and basal ROS production were significantly higher already in Child-Pugh A patients compared to healthy controls (Figure 8 A, C). Neutrophil phagocytic capacity was significantly lower in Child-Pugh B+C cirrhotic patients compared to Child-Pugh A patients (Figure 8 B).

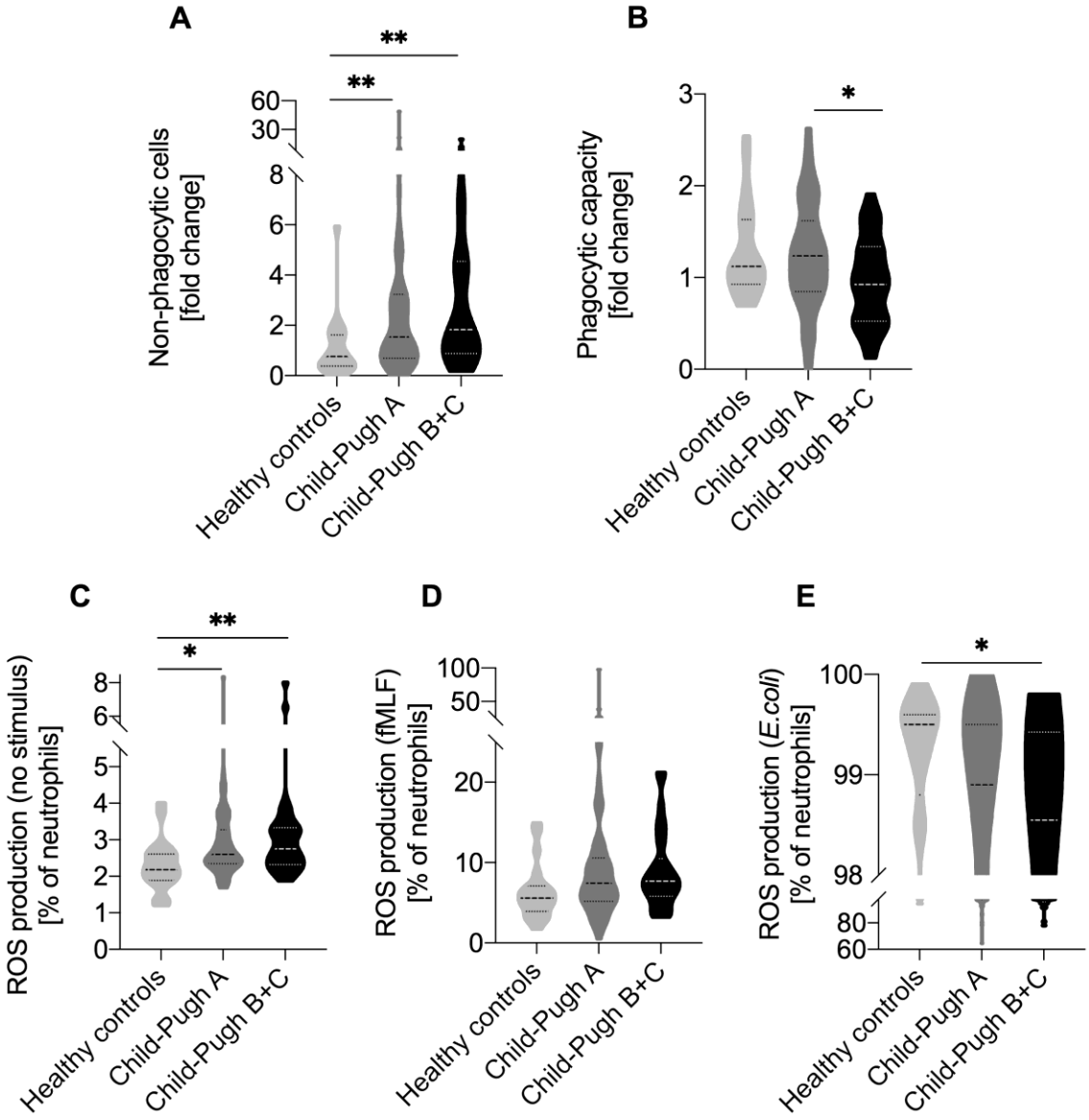


Figure 8. Neutrophil function in whole blood varies between the groups of liver cirrhosis severity.

(A-B) Phagocytosis of *E.coli* (4x10^7 bacteria/100µl blood). (A) Percentage of non-phagocytic neutrophils and (B) neutrophil phagocytic capacity normalised to *E.coli* batch; (C-E) ROS production. Percentage of neutrophils, which produced ROS (C) without a stimulus or after 10 min of (D) fMLF (0.8µM) or (E) *E.coli* (2-4x10^7 bacteria/100µl blood) stimulation. Truncated violin plots show the frequency distribution of measured parameters, the broken line indicates median, dotted lines indicate quartiles; *p < 0.05; **p < 0.01 (Kruskal-Wallis test with Dunn's multiple comparisons test). ROS: reactive oxygen species; fMLF: N-formyl-met-leu-phe. Reproduced from (Balazs et al, 2021, in revision).

Patients with HCV related liver cirrhosis had the worst phagocytic capacity compared to patients with alcoholic and other etiologies of cirrhosis groups (Figure 9 B).

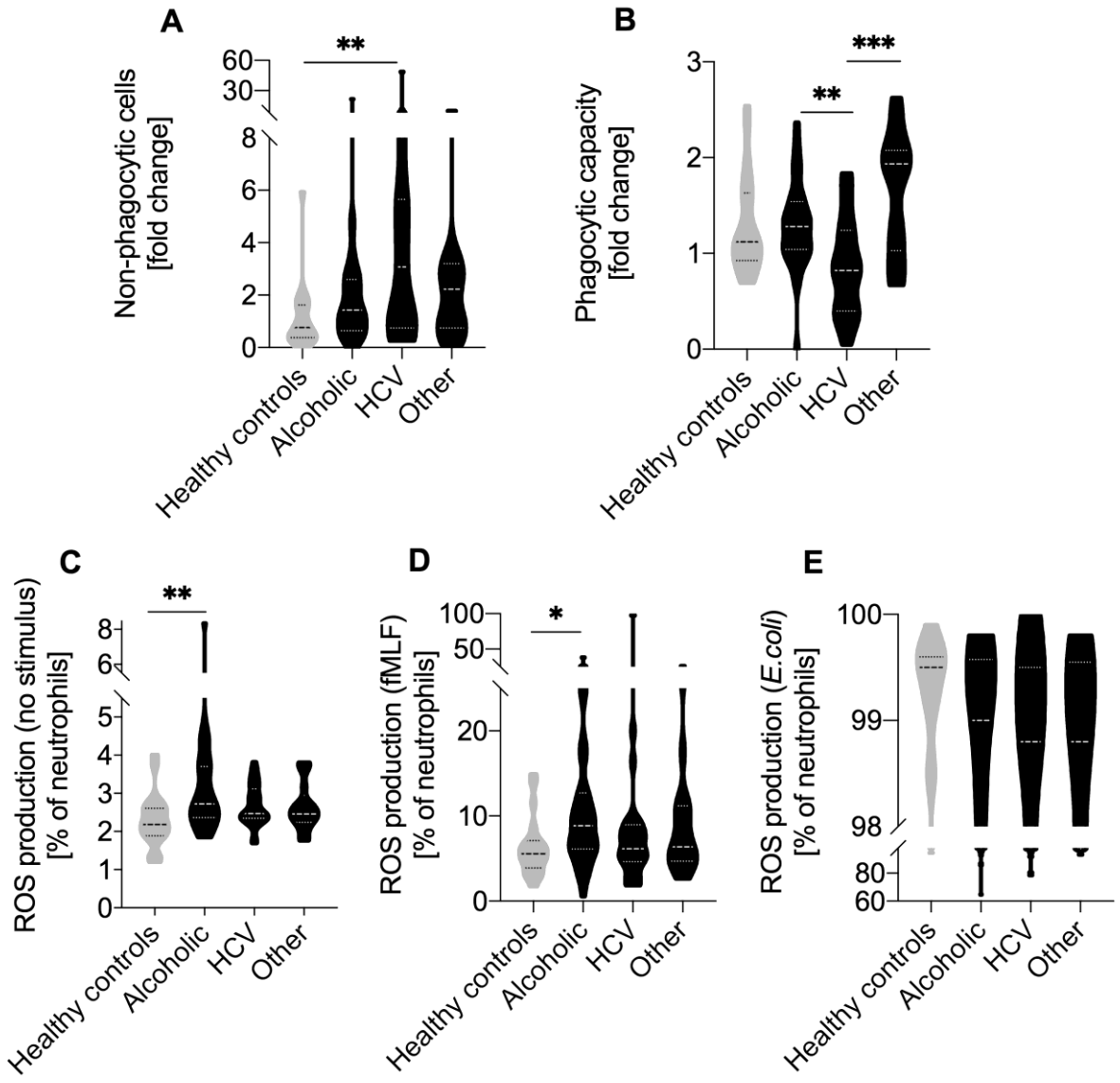


Figure 9. Neutrophil function in whole blood varies between the groups of liver cirrhosis etiology.

(A, B) Phagocytosis of *E.coli* ($4x10^7$ bacteria/100µl blood). (A) Percentage of non-phagocytic neutrophils and (B) neutrophil phagocytic capacity normalized to *E.coli* batch; (C-E) ROS production. Percentage of neutrophils, which produced ROS (C) without a stimulus or after 10 min of (D) fMLF (0.8µM) or (E) *E.coli* ($2\text{-}4x10^7$ bacteria/100µl blood) stimulation. Truncated violin plots show the frequency distribution of measured parameters, the broken line indicates

median, dotted lines indicate quartiles. Analysed within Child-Pugh A group of patients (n=79); *p < 0.05; **p < 0.01; ***p < 0.001 (Kruskal-Wallis test with Dunn's multiple comparisons test). ROS: reactive oxygen species; fMLF: N-formyl-met-leu-phe. Reproduced from (Balazs et al, 2021, in revision).

Bile acid composition in sera of cirrhotic patients and healthy controls.

To assess the differences in serum BA levels and composition between cirrhotic patients and healthy controls, the BA concentrations were determined by HPLC-HRMS (This was done in collaboration with Dr. Günter Fauler, Medical University of Graz). The explorative multivariate analysis was first performed with all fifteen BAs and their relative abundancies within the total BA pool. BA composition was visualized with NMMDS based on Bray-Curtis distance and the significance level of the observed differences was assessed by ANOSIM (Figure 10 A, B).

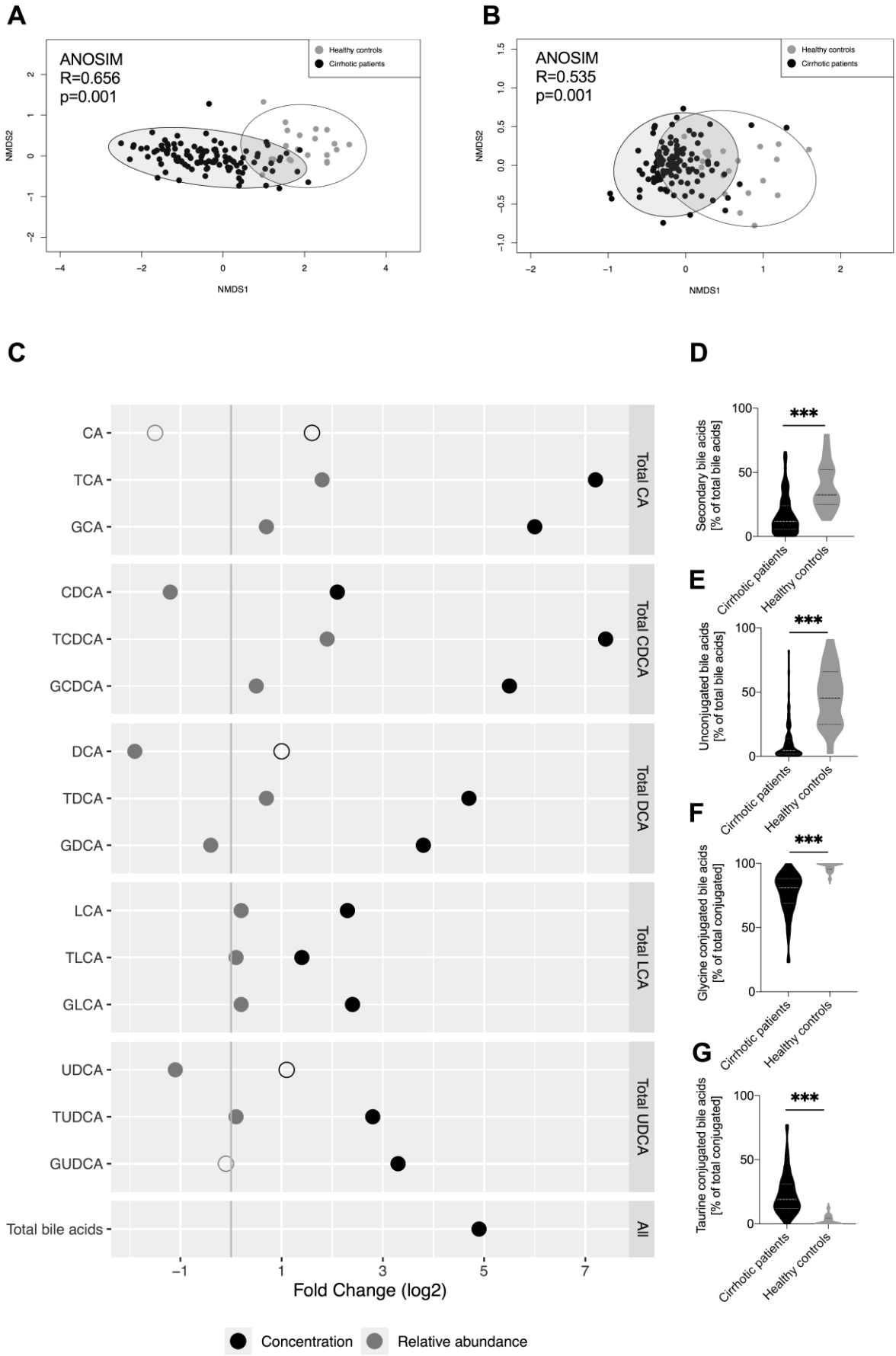

A
ANOSIM
R=0.656
p=0.001
Healthy controls
Cirrhotic patients
NMDS1
NMDS2
B
ANOSIM
R=0.535
p=0.001
Healthy controls
Cirrhotic patients
NMDS1
NMDS2
C
CA
TCA
GCA
CDCA
TCDCA
GCDCA
DCA
TDCA
GDCA
LCA
TLCA
GLCA
UDCA
TUDCA
GUDCA
Total bile acids
Total CA
Total CDCA
Total DCA
Total LCA
Total UDCA
All
Fold Change (log2)
Concentration
Relative abundance
D
Secondary bile acids [% of total bile acids]
***
Cirrhotic patients
Healthy controls
E
Unconjugated bile acids [% of total bile acids]
***
Cirrhotic patients
Healthy controls
F
Glycine conjugated bile acids [% of total conjugated]
***
Cirrhotic patients
Healthy controls
G
Taurine conjugated bile acids [% of total conjugated]
***
Cirrhotic patients
Healthy controls

Figure 10. Serum bile acid composition is altered in cirrhotic patients.

(A-G) Serum bile acids in 109 cirrhotic patients and 21 healthy controls. (A, B) Multivariate analysis of bile acid (A) concentrations and (B) relative abundances; (C) Fold change of the patients' samples compared to the healthy controls; (D-G) Relative abundance of different bile acid groups; (A, B) NMMDS and ANOSIM, ellipses: 95% CI; (C) vertical line is at zero (no change), circles: full – significant; empty – n.s. (Mann-Whitney U test/unpaired t-test); (D-G) truncated violin plots: frequency distribution, the broken line: median, dotted lines: quartiles, ***p < 0.001 (Mann-Whitney U test). ANOSIM: analysis of similarity; CA: cholic acid; TCA: taurocholic acid; GCA: glycocholic acid; CDCA: chenodeoxycholic acid; TCDCA: taurochenodeoxycholic acid; GCDCA: glycochenodeoxycholic acid; DCA: deoxycholic acid; TDCA: taurodeoxycholic acid; GDCA: glycodeoxycholic acid; LCA: lithocholic acid; TLCA: taurolithocholic acid; GLCA: glycolithocholic acid; UDCA: ursodeoxycholic acid; TUDCA: tauroursodeoxycholic acid; GUDCA: glycoursodeoxycholic acid. Reproduced from (Balazs et al, 2021, in revision).

According to SIMPER analysis GCDCA, TCDCA, DCA and GCA were most important variables for groups' dissimilarity (Figure 11 A, B).

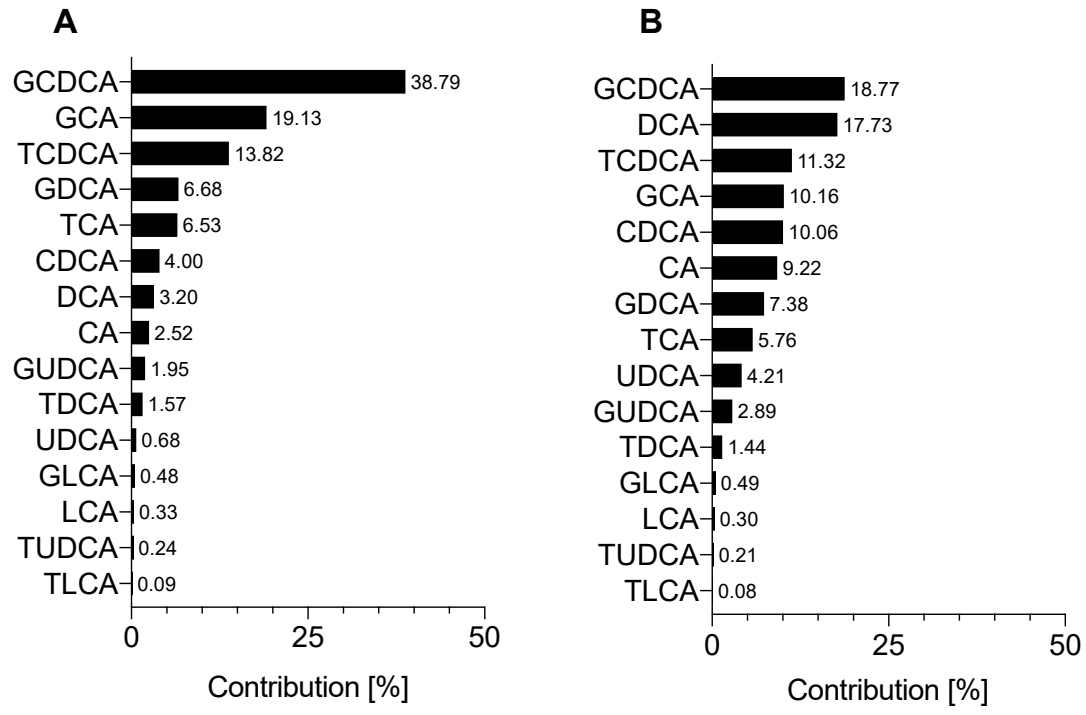


Figure 11. Contribution of different bile acids into cirrhotic patient and healthy control groups' dissimilarity.

(A, B) The similarity percentages breakdown (SIMPER) analysis was performed to analyse contribution of different bile acids into the dissimilarity in bile acid (A) concentrations and (B) relative abundances between the groups of cirrhotic patients and healthy controls. Bars indicate contribution of each bile acid (%). CA: cholic acid; TCA: taurocholic acid; GCA: glycocholic acid; CDCA: chenodeoxycholic acid; TCDCA: taurochenodeoxycholic acid; GCDCA: glycochenodeoxycholic acid; DCA: deoxycholic acid; TDCA: taurodeoxycholic acid; GDCA: glycodeoxycholic acid; ; LCA: lithocholic acid; TLCA: taurolithocholic acid; GLCA: glycolithocholic acid; UDCA: ursodeoxycholic acid; TUDCA: tauroursodeoxycholic acid; GUDCA: glycoursodeoxycholic acid. Reproduced from (Balazs et al, 2021, in revision).

Further, all fifteen BAs and their relative abundancies were analyzed separately. Univariate analysis showed higher concentrations of BAs in cirrhotic patients' sera, but lower relative abundance of total DCA (because of DCA and GDCA), CDCA and UDCA (Figure 10 C, Table 9). The relative abundances of secondary, unconjugated and glycine-conjugated BAs within the total BA pool were significantly lower in cirrhotic patients compared to healthy controls. The relative abundance of taurine-conjugated BAs was significantly higher in cirrhotic patients compared to healthy controls (Figure 10 D-G, Table 9).

Table 9. Serum bile acid concentrations, ratios and relative abundances in cirrhotic patients and healthy controls.
Reproduced from (Balazs et al, 2021, in revision).

Bile acid parameter	Cirrhotic patients (n=109)	Healthy controls (n=21)	p-value
Total bile acids (μmol/l)	53.340 (70.620)	1.748 (1.746)	p<0.001
CA (μmol/l)	0.742 (2.609)	0.232 (0.393)	p=0.232
TCA (μmol/l)	3.692 (5.505)	0.000 (0.000)	p<0.001
GCA (μmol/l)	10.460 (13.977)	0.139 (0.106)	p<0.001
CDCA (μmol/l)	1.518 (4.828)	0.341 (0.873)	p=0.001
TCDCA (μmol/l)	9.400 (17.932)	0.029 (0.051)	p<0.001
GCDCA (μmol/l)	22.861 (39.234)	0.484 (0.551)	p<0.001
DCA (μmol/l)	0.576 (0.729)	0.284 (0.316)	p=0.289
TDCA (μmol/l)	0.619 (1.053)	0.000 (0.000)	p<0.001

GDCA (µmol/l)	2.211 (3.145)	0.141 (0.133)	p<0.001
LCA (µmol/l)	0.094 (0.153)	0.000 (0.000)	p<0.001
TLCA (µmol/l)	0.040 (0.078)	0.000 (0.000)	p<0.001
GLCA (µmol/l)	0.130 (0.203)	0.002 (0.007)	p<0.001
UDCA (µmol/l)	0.122 (0.205)	0.042 (0.051)	p=0.150
TUDCA (µmol/l)	0.146 (0.269)	0.000 (0.000)	p<0.001
GUDCA (µmol/l)	0.732 (1.250)	0.054 (0.089)	p<0.001
CA RA (%)	1.78 (5.1)	9.61 (10.93)	p=0.076
TCA RA (%)	6.04 (5.54)	0.0 (0.0)	p<0.001
GCA RA (%)	18.62 (10.05)	10.73 (5.29)	p<0.001
CDCA RA (%)	3.52 (5.89)	11.54 (11.69)	p<0.001
TCDCA RA (%)	13.16 (10.11)	1.61 (2.56)	p<0.001
GCDCA RA (%)	40.49 (12.78)	27.87 (3.55)	p<0.001
DCA RA (%)	3.83 (6.47)	21.21 (13.65)	p<0.001
TDCA RA (%)	1.50 (1.73)	0.0 (0.0)	p<0.001
GDCA RA (%)	7.17 (7.11)	10.47 (5.57)	p=0.007
LCA RA (%)	0.32 (0.62)	0.0 (0.0)	p<0.001
TLCA RA (%)	0.08 (0.14)	0.0 (0.0)	p<0.001
GLCA RA (%)	0.48 (1.01)	0.07 (0.33)	p<0.001
UDCA RA (%)	0.65 (1.53)	4.42 (5.27)	p=0.023
TUDCA RA (%)	0.22 (0.28)	0.0 (0.0)	p<0.001
GUDCA RA (%)	2.14 (2.61)	2.47 (3.63)	p=0.181
Total CA (µmol/l)	14.893 (18.161)	0.371 (0.416)	p<0.001
Total CDCA (µmol/l)	33.778 (55.114)	0.854 (1.066)	p<0.001
Total DCA (µmol/l)	3.405 (4.394)	0.425 (0.335)	p<0.001
Total LCA (µmol/l)	0.264 (0.369)	0.002 (0.007)	p<0.001
Total UDCA (µmol/l)	1.000 (1.563)	0.097 (0.125)	p<0.001
Total CA RA (%)	26.44 (12.33)	20.34 (9.46)	p=0.033
Total CDCA RA (%)	57.17 (14.27)	41.02 (16.48)	p<0.001
Total DCA RA (%)	12.50 (12.77)	31.68 (16.35)	p<0.001
Total LCA RA (%)	0.88 (1.52)	0.07 (0.33)	p<0.001
Total UDCA RA (%)	3.01 (3.60)	6.89 (6.48)	p=0.059
Primary (µmol/l)	48.671 (68.068)	1.225 (1.420)	p<0.001
Secondary (µmol/l)	4.669 (5.191)	0.523 (0.378)	p<0.001

Secondary to primary (ratio)	0.250 (0.328)	0.851 (0.867)	p<0.001
Primary RA (%)	83.62 (14.63)	61.35 (18.03)	p<0.001
Secondary RA (%)	16.39 (14.63)	38.65 (18.03)	p<0.001
Unconjugated (µmol/l)	3.051 (7.240)	0.899 (1.527)	p=0.001
Conjugated (µmol/l)	50.289 (69.031)	0.849 (0.861)	p<0.001
Unconjugated to conjugated (ratio)	0.173 (0.488)	1.552 (2.106)	p<0.001
Unconjugated RA (%)	10.10 (13.48)	46.78 (22.95)	p<0.001
Conjugated RA (%)	89.90 (13.48)	53.22 (22.95)	p<0.001
Taurine conjugated (µmol/l)	13.896 (22.763)	0.029 (0.051)	p<0.001
Glycine conjugated (µmol/l)	36.393 (51.253)	0.820 (0.818)	p<0.001
Taurine to glycine conjugated (ratio)	0.368 (0.467)	0.026 (0.038)	p<0.001
Taurine conjugated RA (%)	22.47 (14.76)	2.38 (3.41)	p<0.001
Glycine conjugated RA (%)	77.53 (14.75)	97.62 (3.41)	p<0.001
Non-12-alpha hydroxylated (µmol/l)	35.042 (56.432)	0.953 (1.088)	p<0.001
12-alpha hydroxylated (µmol/l)	18.298 (20.309)	0.795 (0.711)	p<0.001
12-alpha hydroxylated to non-12-alpha hydroxylated (ratio)	0.748 (0.521)	1.475 (1.539)	p=0.001
Non-12-alpha hydroxylated RA (%)	61.06 (14.19)	47.98 (15.46)	p<0.001
12-alpha hydroxylated RA (%)	38.94 (14.19)	52.02 (15.46)	p<0.001
Hydrophilic (µmol/l)	15.893 (18.859)	0.468 (0.456)	p<0.001
Hydrophobic (µmol/l)	37.448 (56.243)	1.281 (1.368)	p<0.001
Hydrophilic to hydrophobic (ratio)	0.459 (0.287)	0.405 (0.228)	p=0.307
Hydrophilic RA (%)	29.45 (11.02)	27.23 (10.37)	p=0.307
Hydrophobic RA (%)	70.55 (11.02)	72.77 (10.37)	p=0.307

Mean (SD). RA: relative abundance (% of total bile acids*). CA: cholic acid; TCA: taurocholic acid; GCA: glycocholic acid; CDCA: chenodeoxycholic acid; TCDCA: taurochenodeoxycholic acid; GCDCA: glycochenodeoxycholic acid; DCA: deoxycholic acid; TDCA: taurodeoxycholic acid; GDCA: glycodeoxycholic acid; LCA: lithocholic acid; TLCA: taurolithocholic acid; GLCA: glycolithocholic acid; UDCA: ursodeoxycholic acid; TUDCA: tauroursodeoxycholic acid; GUDCA: glycoursodeoxycholic acid; total CA: CA + TCA + GCA; total CDCA: CDCA + TCDCA + GCDCA; total DCA: DCA + TDCA + GDCA; total LCA: LCA + TLCA + GLCA; total UDCA: UDCA + TUDCA + GUDCA; primary: total CA + total CDCA; secondary: total DCA + total LCA + total UDCA; unconjugated: CA + CDCA + DCA + LCA + UDCA; conjugated: taurine conjugated + glycine conjugated; taurine conjugated: TCA + TCDCA + TDCA + TLCA + TUDCA; glycine conjugated: GCA + GCDCA + GDCA + GLCA + GUDCA; *taurine conjugated

RA: % of conjugated; *glycine conjugated RA: % of conjugated; non-12-alpha hydroxylated: total CDCA + total LCA + total UDCA; 12-apha hydroxylated: total CA + total DCA; hydrophilic: total CA + total UDCA; hydrophobic: total CDCA + total DCA + total LCA

Differences in BA composition were assessed also between the groups of severity and etiology of cirrhosis. Multivariate analysis with NMMDS, ANOSIM and SIMPER and univariate analysis showed significant differences between all these groups (Figures 9-11, Tables 10-11).

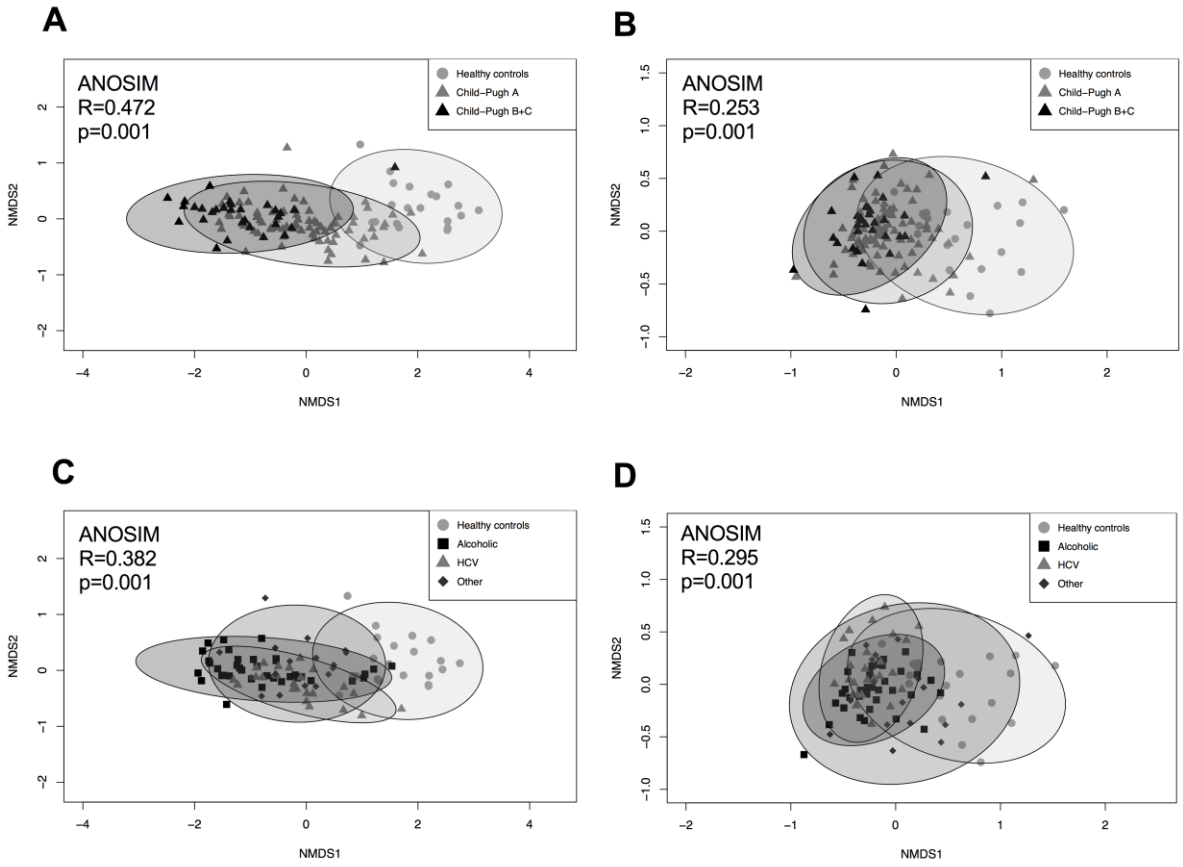


Figure 12. Serum bile acid composition is significantly variable depending on liver cirrhosis severity and etiology.

(A-D) Serum bile acids in 109 cirrhotic patients and 21 healthy controls. Multivariate analysis of fifteen bile acid (A, C) concentrations and (B, D) relative abundances (% of total bile acids). (C, D) For the analysis of etiology groups only Child-Pugh A patients (n=79) were included. (A-D) Ellipses indicate 95% CI. Statistical analysis was by NNMDS and ANOSIM based on Bray-Curtis distance. ANOSIM: analysis of similarity; NNMDS: non-metric multidimensional scaling. Reproduced from (Balazs et al, 2021, in revision).

GCDCA, TCDCA and GCA contributed most into the Child-Pugh groups' dissimilarity (Figure 13 A-F).

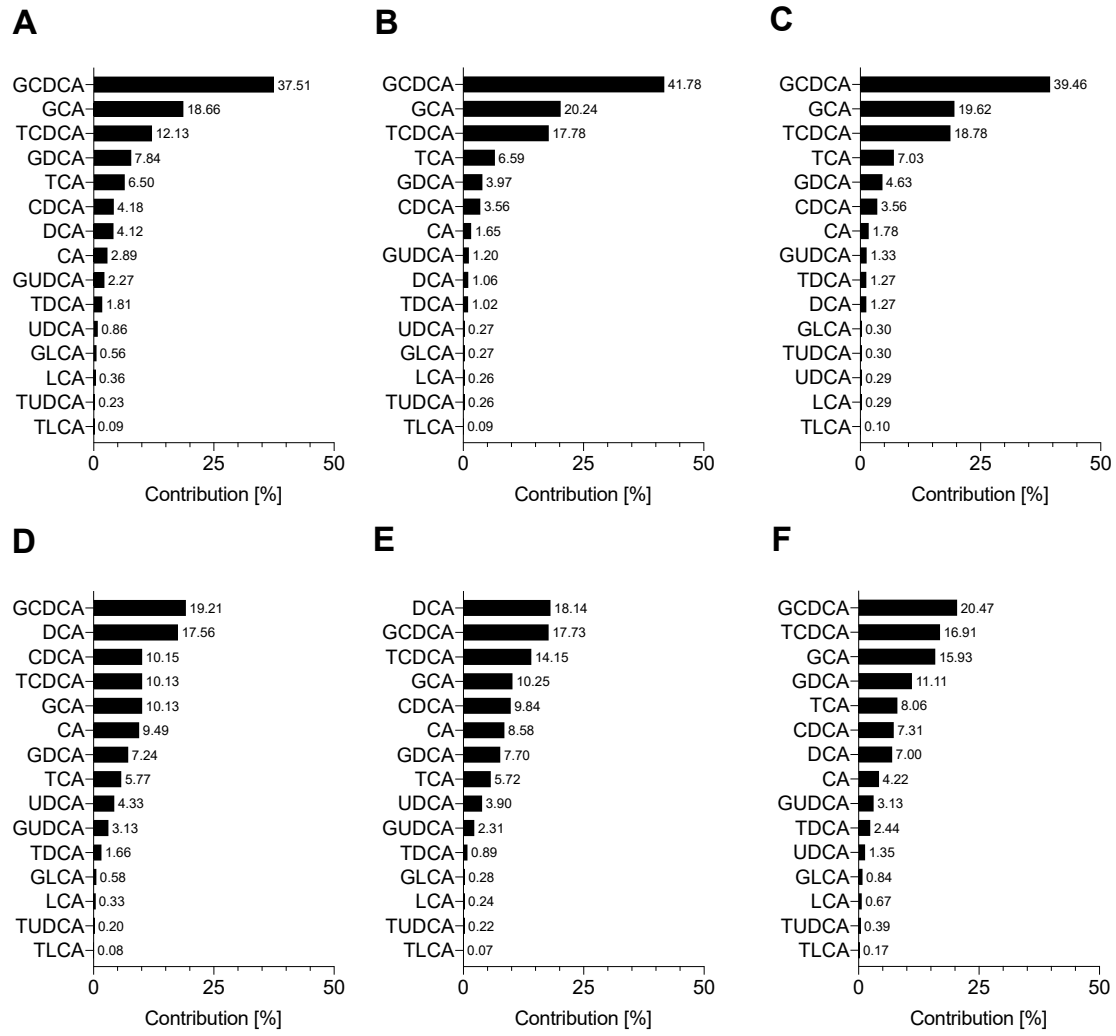


Figure 13. Contribution of different bile acids into liver cirrhosis severity groups' dissimilarity.

(A-F) The similarity percentages breakdown (SIMPER) analysis was performed to analyse contribution of different bile acids into the dissimilarity in bile acid (A-C) concentrations and (D-F) relative abundances between the groups of (A, D) Child-Pugh A and healthy controls, (B, E) Child-Pugh B+C and healthy controls, (C, F) Child-Pugh A and Child Pugh B+C. Bars indicate contribution of each bile acid (%). CA: cholic acid; TCA: taurocholic acid; GCA: glycocholic acid; CDCA: chenodeoxycholic acid; TCDCA: taurochenodeoxycholic acid; GCDCA: glycochenodeoxycholic acid; DCA: deoxycholic acid; TDCA: taurodeoxycholic acid;

GDCA: glycodeoxycholic acid; ; LCA: lithocholic acid; TLCA: taurolithocholic acid; GLCA: glycolithocholic acid; UDCA: ursodeoxycholic acid; TUDCA: tauroursodeoxycholic acid; GUDCA: glycoursodeoxycholic acid. Reproduced from (Balazs et al, 2021, in revision).

GCDCA, TCDCA and GCA contributed most into the multivariate differences between the groups of alcoholic and HCV cirrhosis, GCDCA, TCDCA, GCA and DCA contributed most into the multivariate differences between the groups of alcoholic and other etiologies of cirrhosis and between the groups of HCV-related cirrhosis and other etiologies of cirrhosis (Figure 14 A-F).

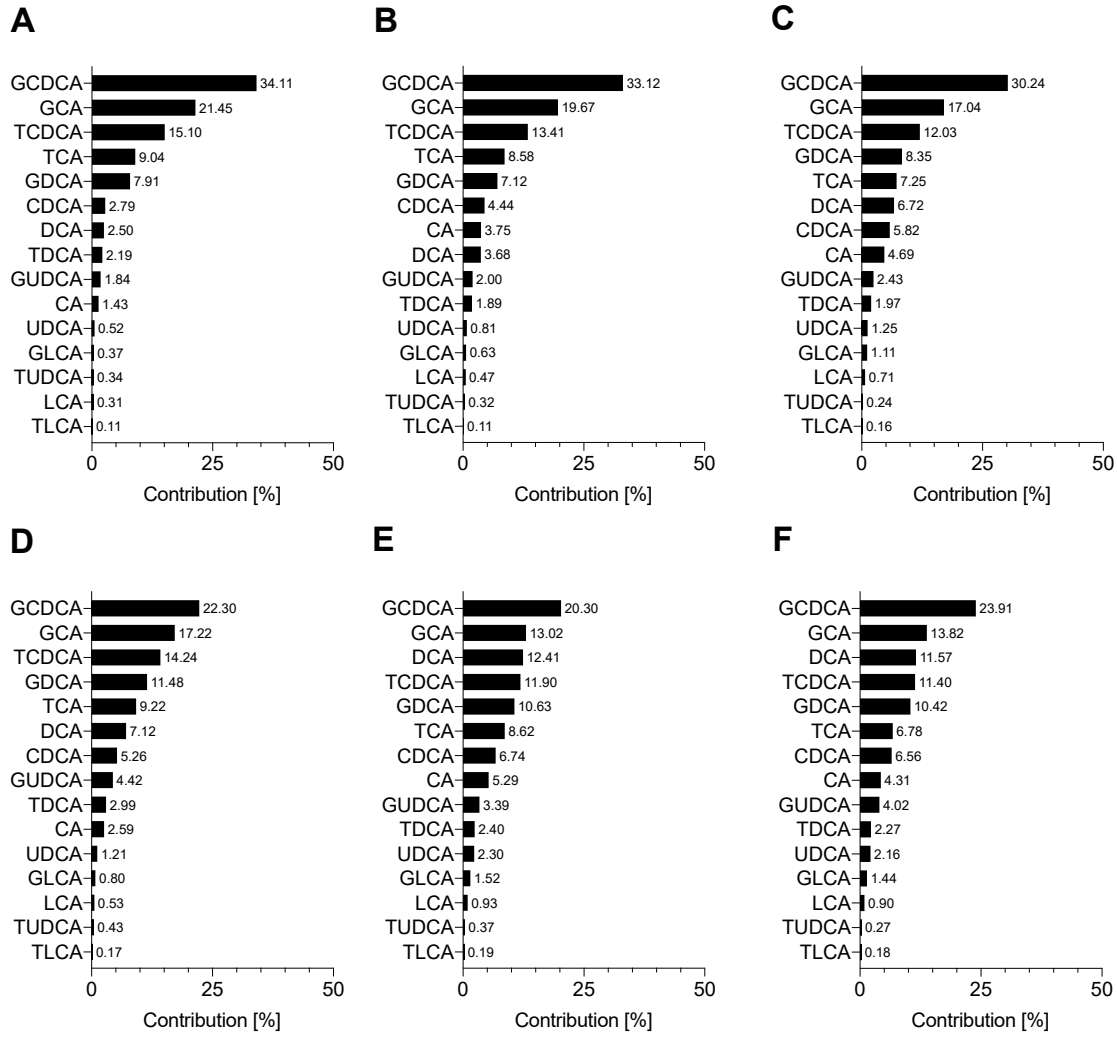


Figure 14. Contribution of different bile acids into liver cirrhosis aetiology groups' dissimilarity in bile acid composition between the groups of liver cirrhosis etiology.

(A-F) The similarity percentages breakdown (SIMPER) analysis was performed to analyse contribution of different bile acids into the dissimilarity in bile acid (A-C) concentrations and (D-F) relative abundances between the groups of (A, D) alcoholic and HCV, (B, E) alcoholic and other, (C, F) HCV and other aetiologies groups. Only Child-Pugh A patients (n=79) were analysed. Bars indicate contribution of each bile acid (%). CA: cholic acid; TCA: taurocholic acid; GCA: glycocholic acid; CDCA: chenodeoxycholic acid; TCDCA: taurochenodeoxycholic acid; GCDCA: glycochenodeoxycholic acid; DCA: deoxycholic acid; TDCA: taurodeoxycholic acid; GDCA: glycodeoxycholic acid; ; LCA: lithocholic acid; TLCA: taurolithocholic acid; GLCA: glycolithocholic acid; UDCA: ursodeoxycholic acid; TUDCA: tauroursodeoxycholic acid; GUDCA: glycoursodeoxycholic acid. Reproduced from (Balazs et al, 2021, in revision).

Patients with alcoholic cirrhosis had the highest level of total BAs with the prevalence, however, of hydrophilic BAs. HCV patients had the highest relative abundance of total CDCA, but the lowest level of unconjugated BAs (Table 11).

Table 10. Serum bile acid concentrations, ratios and relative abundances in patients with different severity of liver cirrhosis and healthy controls.
Reproduced from (Balazs et al, 2021, in revision).

Bile acid parameter	Child-Pugh A (n=79)	Child-Pugh B+C (n=30)	Healthy controls (n=21)	p-value
Total bile acids (µmol/l)	29.729 (31.170)	115.516 (102.186)	1.748 (1.746)	$p<0.001^{\Delta,\&,£}$
CA (µmol/l)	0.658 (2.858)	0.961 (1.819)	0.232 (0.393)	$p=0.017^{\&}$ $p=0.004^{£}$
TCA (µmol/l)	2.497 (4.307)	6.840 (6.986)	0.000 (0.000)	$p<0.001^{\Delta,\&,£}$
GCA (µmol/l)	6.391 (8.468)	21.172 (19.258)	0.139 (0.106)	$p<0.001^{\Delta,\&,£}$
CDCA (µmol/l)	0.940 (2.733)	3.038 (7.963)	0.341 (0.873)	$p<0.001^{\&,£}$
TCDCA (µmol/l)	4.159 (6.392)	23.200 (28.561)	0.029 (0.051)	$p<0.001^{\Delta,\&,£}$
GCDCA (µmol/l)	11.162 (12.550)	53.669 (62.884)	0.484 (0.551)	$p<0.001^{\Delta,\&,£}$
DCA (µmol/l)	0.625 (0.768)	0.446 (0.607)	0.284 (0.316)	n.s.
TDCA (µmol/l)	0.492 (0.714)	0.951 (1.613)	0.000 (0.000)	$p<0.001^{\Delta,\&}$
GDCA (µmol/l)	1.928 (2.509)	2.958 (4.372)	0.141 (0.133)	$p<0.001^{\Delta}$ $p=0.002^{\&}$
LCA (µmol/l)	0.077 (0.121)	0.137 (0.213)	0.000 (0.000)	$p<0.001^{\Delta}$ $p=0.001^{\&}$

TLCA (µmol/l)	0.024 (0.039)	0.083 (0.126)	0.000 (0.000)	$p<0.001^{\&}$ $p=0.014^{\Delta}$ $p=0.046^{£}$
GLCA (µmol/l)	0.102 (0.160)	0.206 (0.277)	0.002 (0.007)	$p<0.001^{\Delta,\&}$
UDCA (µmol/l)	0.109 (0.179)	0.158 (0.261)	0.042 (0.051)	n.s.
TUDCA (µmol/l)	0.083 (0.168)	0.311 (0.393)	0.000 (0.000)	$p<0.001^{\Delta,\&,£}$
GUDCA (µmol/l)	0.482 (0.661)	1.391 (2.007)	0.054 (0.089)	$p<0.001^{\Delta,\&}$ $p=0.022^{£}$
CA RA (%)	1.71 (5.19)	1.98 (4.93)	9.61 (10.93)	n.s.
TCA RA (%)	5.89 (5.84)	6.44 (4.71)	0.0 (0.0)	$p<0.001^{\Delta,\&}$
GCA RA (%)	18.01 (9.23)	20.24 (11.98)	10.73 (5.29)	$p=0.001^{\Delta,\&}$
CDCA RA (%)	3.30 (5.05)	4.10 (7.75)	11.54 (11.69)	$p=0.001^{\Delta}$ $p=0.002^{\&}$
TCDCA RA (%)	11.57 (8.81)	17.36 (12.13)	1.61 (2.56)	$p<0.001^{\Delta,\&}$
GCDCA RA (%)	40.02 (13.07)	41.75 (12.12)	27.87 (16.25)	$p=0.001^{\Delta,\&}$
DCA RA (%)	4.96 (7.22)	0.84 (1.63)	21.21 (13.65)	$p<0.001^{\Delta,\&}$ $p=0.001^{£}$
TDCA RA (%)	1.70 (1.83)	1.00 (1.30)	0.0 (0.0)	$p<0.001^{\Delta}$ $p=0.001^{\&}$
GDCA RA (%)	8.42 (7.22)	3.88 (5.71)	10.47 (5.57)	$p<0.001^{\&}$ $p=0.001^{£}$
LCA RA (%)	0.34 (0.67)	0.26 (0.44)	0.0 (0.0)	$p<0.001^{\Delta}$, $p=0.004^{\&}$
TLCA RA (%)	0.08 (0.15)	0.08 (0.13)	0.0 (0.0)	$p<0.001^{\&}$ $p=0.007^{\Delta}$
GLCA RA (%)	0.56 (1.16)	0.27 (0.37)	0.07 (0.33)	$p<0.001^{\Delta}$ $p=0.002^{\&}$
UDCA RA (%)	0.75 (1.68)	0.39 (1.02)	4.42 (5.27)	n.s.
TUDCA RA (%)	0.21 (0.28)	0.25 (0.29)	0.0 (0.0)	$p<0.001^{\Delta,\&}$
GUDCA RA (%)	2.50 (2.90)	1.19 (1.21)	2.47 (3.63)	n.s.
Total CA (µmol/l)	9.546 (12.286)	28.972 (23.223)	0.371 (0.416)	$p<0.001^{\Delta,\&,£}$
Total CDCA (µmol/l)	16.261 (17.634)	79.907 (86.247)	0.854 (1.066)	$p<0.001^{\Delta,\&,£}$
Total DCA (µmol/l)	3.045 (3.488)	4.355 (6.157)	0.425 (0.335)	$p<0.001^{\Delta}$ $p=0.009^{\&}$

Total LCA (µmol/l)	0.203 (0.273)	0.426 (0.519)	0.002 (0.007)	$p<0.001^{\Delta,\&}$
Total UDCA(µmol/l)	0.674 (0.886)	1.859 (2.436)	0.097 (0.125)	$p<0.001^{\Delta,\&}$ $p=0.008^{£}$
Total CA RA (%)	25.60 (12.46)	28.65 (11.88)	20.34 (9.46)	$p=0.027^{\&}$
Total CDCA RA (%)	54.88 (13.99)	63.20 (13.40)	41.02 (16.48)	$p<0.001^{\Delta,\&}$ $p=0.020^{£}$
Total DCA RA (%)	15.08 (13.36)	5.71 (7.87)	31.68 (16.35)	$p<0.001^{\Delta,\&,£}$
Total LCA RA (%)	0.98 (1.69)	0.61 (0.87)	0.07 (0.33)	$p<0.001^{\Delta}$ $p=0.001^{\&}$
Total UDCA RA (%)	3.46 (4.02)	1.83 (1.73)	6.89 (6.48)	n.s.
Primary (µmol/l)	25.807 (28.995)	108.879 (99.094)	1.225 (1.420)	$p<0.001^{\Delta,\&,£}$
Secondary (µmol/l)	3.921 (4.036)	6.639 (7.141)	0.523 (0.378)	$p<0.001^{\Delta,\&}$
Secondary to primary (ratio)	0.307 (0.362)	0.100 (0.124)	0.851 (0.867)	$p<0.001^{\Delta,\&,£}$
Primary RA (%)	80.49 (15.22)	91.85 (8.77)	61.35 (18.03)	$p<0.001^{\Delta,\&,£}$
Secondary RA (%)	19.51 (15.22)	8.15 (8.79)	38.65 (18.03)	$p<0.001^{\Delta,\&,£}$
Unconjugated (µmol/l)	2.410 (5.921)	4.739 (9.842)	0.899 (1.527)	$p<0.001^{\&}$ $p=0.029^{\Delta}$ $p=0.010^{£}$
Conjugated (µmol/l)	27.319 (29.778)	110.778 (100.693)	0.849 (0.861)	$p<0.001^{\Delta,\&,£}$
Unconjugated to conjugated (ratio)	0.190 (0.534)	0.128 (0.346)	1.552 (2.106)	$p<0.001^{\Delta,\&}$
Unconjugated RA (%)	11.06 (13.49)	7.57 (13.34)	46.78 (22.95)	$p<0.001^{\Delta,\&}$
Conjugated RA (%)	88.94 (13.49)	92.44 (13.34)	53.22 (22.95)	$p<0.001^{\Delta,\&}$
Taurine conjugated (µmol/l)	7.255 (10.962)	31.384 (34.205)	0.029 (0.051)	$p<0.001^{\Delta,\&,£}$
Glycine conjugated (µmol/l)	20.064 (22.032)	79.395 (76.405)	0.820 (0.818)	$p<0.001^{\Delta,\&,£}$
Taurine to glycine conjugated (ratio)	0.330 (0.428)	0.467 (0.553)	0.027 (0.038)	$p<0.001^{\Delta,\&}$
Taurine conjugated RA (%)	20.87 (14.18)	26.67 (15.64)	2.38 (3.41)	$p<0.001^{\Delta,\&}$
Glycine conjugated RA (%)	79.13 (14.18)	73.33 (15.64)	97.62 (3.41)	$p<0.001^{\Delta,\&}$
Non-12-alpha hydroxylated (µmol/l)	17.137 (18.250)	82.191 (88.266)	0.953 (1.088)	$p<0.001^{\Delta,\&,£}$

12-alpha hydroxylated (µmol/l)	12.591 (14.023)	33.327 (26.167)	0.795 (0.711)	$p<0.001^{\Delta,\&,£}$
12-alpha hydroxylated to non 12-alpha hydroxylated (ratio)	0.803 (0.540)	0.604 (0.445)	1.475 (1.539)	$p<0.001$ $^{\&}p=0.022^{\Delta}$
Non-12-alpha hydroxylated RA (%)	59.32 (14.20)	65.64 (13.32)	47.98 (15.46)	$p<0.001^{\&}$ $p=0.004^{\Delta}$
12-alpha hydroxylated RA (%)	40.68 (14.20)	34.36 (13.33)	52.02 (15.46)	$p<0.001^{\&}$ $p=0.004^{\Delta}$
Hydrophilic (µmol/l)	10.220 (12.680)	30.831 (23.949)	0.468 (0.456)	$p<0.001^{\Delta,\&,£}$
Hydrophobic (µmol/l)	19.509 (19.384)	84.687 (87.091)	1.281 (1.368)	$p<0.001^{\Delta,\&,£}$
Hydrophilic to hydrophobic (ratio)	0.444 (0.237)	0.497 (0.392)	0.405 (0.228)	n.s.
Hydrophilic RA (%)	29.07 (10.80)	30.48 (11.71)	27.23 (10.37)	n.s.
Hydrophobic RA (%)	70.93 (10.80)	69.53 (11.71)	72.77 (10.37)	n.s.

Mean (SD). $^{\Delta}$Significant difference between Child-Pugh A and healthy group, $^{\&}$significant difference between Child-Pugh B+C and healthy controls; $^{£}$significant difference between Child-Pugh A and Child-Pugh B+C groups. RA: relative abundance (% of total bile acids*). CA: cholic acid; TCA: taurocholic acid; GCA: glycocholic acid; CDCA: chenodeoxycholic acid; TCDCA: taurochenodeoxycholic acid; GCDCA: glycochenodeoxycholic acid; DCA: deoxycholic acid; TDCA: taurodeoxycholic acid; GDCA: glycodeoxycholic acid; LCA: lithocholic acid; TLCA: taurolithocholic acid; GLCA: glycolithocholic acid; UDCA: ursodeoxycholic acid; TUDCA: tauroursodeoxycholic acid; GUDCA: glycoursodeoxycholic acid; total CA: CA + TCA + GCA; total CDCA: CDCA + TCDCA + GCDCA; total DCA: DCA + TDCA + GDCA; total LCA: LCA + TLCA + GLCA; total UDCA: UDCA + TUDCA + GUDCA; primary: total CA + total CDCA; secondary: total DCA + total LCA + total UDCA; unconjugated: CA + CDCA + DCA + LCA + UDCA; conjugated: taurine conjugated + glycine conjugated; taurine conjugated: TCA + TCDCA + TDCA + TLCA + TUDCA; glycine conjugated: GCA + GCDCA + GDCA + GLCA + GUDCA; *taurine conjugated RA: % of conjugated; *glycine conjugated RA: % of conjugated; non-12-alpha hydroxylated: total CDCA + total LCA + total UDCA; 12-apha hydroxylated: total CA + total DCA; hydrophilic: total CA + total UDCA; hydrophobic: total CDCA + total DCA + total LCA

Table 11. Serum bile acid concentrations, ratios and relative abundances in patients with different etiology of liver cirrhosis and healthy controls.

Reproduced from (Balazs et al, 2021, in revision).

Bile acid parameter	Alcoholic (n=37)	HCV (n=25)	Other (n=17)	Healthy controls (n=21)	p-value
Total bile acids (µmol/l)	44.721 (37.258)	14.760 (11.993)	19.111 (20.192)	1.748 (1.746)	$p<0.001^{\Delta,\&,£}$ $p=0.039^{®}$
CA (µmol/l)	0.647 (1.723)	0.087 (0.200)	1.524 (5.637)	0.232 (0.393)	$p<0.001^{®}$
TCA (µmol/l)	4.155 (5.648)	1.059 (1.462)	1.000 (1.869)	0.000 (0.000)	$p<0.001^{\Delta,\&,£}$
GCA (µmol/l)	10.448 (10.736)	2.609 (2.481)	3.124 (3.047)	0.139 (0.106)	$p<0.001^{\Delta}$ $p=0.006^{\&}$ $p=0.001^{£}$ $p=0.005^{®}$
CDCA (µmol/l)	1.143 (2.903)	0.322 (0.582)	1.408 (4.003)	0.341 (0.873)	$p=0.004^{\Delta}$ $p=0.034^{®}$
TCDCA (µmol/l)	6.592 (8.231)	2.281 (3.079)	1.625 (2.545)	0.029 (0.051)	$p<0.001^{\Delta,\&}$ $p=0.001^{£}$
GCDCA (µmol/l)	16.373 (14.389)	6.293 (4.946)	6.982 (12.119)	0.484 (0.551)	$p<0.001^{\Delta,\&}$ $p=0.001^{£}$ p=0.048°
DCA (µmol/l)	0.694 (0.787)	0.291 (0.359)	0.967 (0.992)	0.284 (0.316)	$p=0.026^{®}$ p=0.013^
TDCA (µmol/l)	0.751 (0.903)	0.266 (0.441)	0.261 (0.258)	0.000 (0.000)	$p<0.001^{\Delta,£}$ $p=0.003^{\&}$
GDCA (µmol/l)	2.751 (3.253)	1.138 (1.263)	1.296 (1.235)	0.141 (0.133)	$p<0.001^{\Delta}$ $p=0.001^{£}$ $p=0.007^{\&}$
LCA (µmol/l)	0.092 (0.114)	0.043 (0.057)	0.095 (0.184)	0.000 (0.000)	$p<0.001^{\Delta}$ $p=0.020^{\&}$ $p=0.013^{£}$
TLCA (µmol/l)	0.030 (0.041)	0.019 (0.038)	0.019 (0.036)	0.000 (0.000)	$p=0.003^{\Delta}$
GLCA (µmol/l)	0.096 (0.111)	0.071 (0.098)	0.157 (0.280)	0.002 (0.007)	$p<0.001^{\Delta,£}$ $p=0.002^{\&}$
UDCA (µmol/l)	0.140 (0.206)	0.033 (0.050)	0.153 (0.209)	0.042 (0.051)	n.s.

TUDCA (µmol/l)	0.142 (0.226)	0.026 (0.041)	0.039 (0.067)	0.000 (0.000)	$p<0.001^{\Delta}$ $p=0.037^{£}$
GUDCA (µmol/l)	0.667 (0.776)	0.222 (0.204)	0.459 (0.726)	0.054 (0.089)	$p<0.001^{\Delta}$ $p=0.009^{\&}$ $p=0.003^{£}$
CA RA (%)	1.64 (2.63)	0.54 (0.92)	3.56 (10.42)	9.61 (10.93)	$p=0.015^{\&}$
TCA RA (%)	7.09 (5.83)	4.90 (4.54)	4.73 (7.27)	0.0 (0.0)	$p<0.001^{\Delta,\&,£}$
GCA RA (%)	20.43 (7.24)	14.97 (10.37)	17.20 (10.36)	10.73 (5.29)	$p<0.001^{\Delta}$
CDCA RA (%)	2.89 (3.67)	2.67 (3.87)	5.10 (8.19)	11.54 (11.69)	$p=0.002^{\&}$ $p=0.006^{\Delta}$
TCDCA RA (%)	12.45 (9.77)	13.28 (7.73)	7.13 (6.82)	1.61 (2.56)	$p<0.001^{\Delta,\&}$ $p=0.025^{£}$
GCDCA RA (%)	38.24 (10.41)	47.26 (11.55)	33.22 (15.82)	27.87 (16.25)	$p<0.001^{\&}$ p=0.010^
DCA RA (%)	3.97 (5.74)	2.86 (3.95)	10.22 (10.87)	21.21 (13.65)	$p<0.001^{\Delta,\&}$
TDCA RA (%)	1.85 (1.90)	1.36 (1.79)	1.85 (1.81)	0.0 (0.0)	$p<0.001^{\Delta,£}$ $p=0.001^{\&}$
GDCA RA (%)	7.96 (6.84)	7.88 (6.86)	10.20 (8.58)	10.47 (5.57)	n.s.
LCA RA (%)	0.22 (0.37)	0.28 (0.37)	0.69 (1.24)	0.0 (0.0)	$p=0.002^{\Delta,\&,£}$
TLCA RA (%)	0.07 (0.10)	0.08 (0.15)	0.11 (0.21)	0.0 (0.0)	$p=0.007^{\Delta}$
GLCA RA (%)	0.32 (0.49)	0.44 (0.58)	1.23 (2.21)	0.07 (0.33)	$p<0.001^{£}$ $p=0.001^{\Delta}$ $p=0.002^{\&}$
UDCA RA (%)	0.57 (1.03)	0.38 (0.96)	1.70 (2.97)	4.42 (5.27)	$p=0.021^{\&}$
TUDCA RA (%)	0.27 (0.33)	0.14 (0.21)	0.17 (0.24)	0.0 (0.0)	$p<0.001^{\Delta}$ $p=0.022^{\&}$ $p=0.018^{£}$
GUDCA RA (%)	2.03 (1.96)	2.94 (3.84)	2.89 (3.04)	2.47 (3.63)	n.s.
Total CA (µmol/l)	15.250 (15.263)	3.755 (3.721)	5.649 (6.871)	0.371 (0.416)	$p<0.001^{\Delta}$ $p=0.002^{£}$ $p=0.019^{\&}$ $p=0.005^{®}$
Total CDCA (µmol/l)	24.108 (20.511)	8.895 (7.859)	10.015 (14.428)	0.854 (1.066)	$p<0.001^{\Delta,\&}$ $p=0.001^{£}$
Total DCA (µmol/l)	4.196 (4.457)	1.695 (1.867)	2.524 (1.781)	0.425 (0.335)	$p<0.001^{\Delta}$ $p=0.001^{£}$

Total LCA (µmol/l)	0.219 (0.243)	0.133 (0.164)	0.271 (0.422)	0.002 (0.007)	$p<0.001^{\Delta,£}$ $p=0.001^{\&}$
Total UDCA (µmol/l)	0.948 (1.057)	0.282 (0.259)	0.652 (0.892)	0.097 (0.125)	$p<0.001^{\Delta}$ $p=0.005^{£}$
Total CA RA (%)	29.16 (9.13)	20.42 (13.32)	25.49 (15.24)	20.34 (9.46)	$p=0.014^{\Delta}$
Total CDCA RA (%)	53.58 (9.77)	63.22 (13.20)	45.45 (16.46)	41.02 (16.48)	$p<0.001^{\&}$ $p=0.046^{®}$ p=0.001^
Total DCA RA (%)	13.77 (11.46)	12.11 (10.35)	22.28 (18.46)	31.68 (16.35)	$p<0.001^{\Delta,\&}$
Total LCA RA (%)	0.61 (0.74)	0.80 (0.86)	2.03 (3.18)	0.07 (0.33)	$p<0.001^{\Delta,\&,£}$
Total UDCA RA (%)	2.87 (2.85)	3.46 (4.19)	4.76 (5.61)	6.89 (6.48)	n.s.
Primary (µmol/l)	39.357 (34.990)	12.651 (11.101)	15.664 (18.832)	1.225 (1.420)	$p<0.001^{\Delta,\&,£}$
Secondary (µmol/l)	5.363 (5.078)	2.110 (2.097)	3.447 (2.153)	0.523 (0.378)	$p<0.001^{\Delta,£}$ $p=0.024^{\&}$
Secondary to primary (ratio)	0.246 (0.248)	0.222 (0.195)	0.565 (0.595)	0.851 (0.867)	$p<0.001^{\Delta,\&}$
Primary RA (%)	82.75 (13.05)	83.63 (11.88)	70.94 (20.27)	61.35 (18.03)	$p<0.001^{\Delta,\&}$
Secondary RA (%)	17.25 (13.05)	16.37 (11.88)	29.06 (20.27)	38.65 (18.03)	$p<0.001^{\Delta,\&}$
Unconjugated (µmol/l)	2.716 (4.960)	0.776 (0.945)	4.148 (10.325)	0.899 (1.527)	$p=0.004^{\Delta}$ $p=0.013^{£}$ $p=0.006^{®}$ p=0.020^
Conjugated (µmol/l)	42.005 (35.513)	13.984 (11.798)	14.963 (18.375)	0.849 (0.861)	$p<0.001^{\Delta,\&,£}$
Unconjugated to conjugated (ratio)	0.120 (0.160)	0.078 (0.081)	0.509 (1.087)	1.552 (2.106)	$p<0.001^{\Delta}$ $p=0.038^{£}$
Unconjugated RA (%)	9.30 (10.20)	6.73 (6.50)	21.27 (21.03)	46.78 (22.95)	$p<0.001^{\Delta,\&}$ $p=0.038^{£}$
Conjugated RA (%)	90.70 (10.20)	93.27 (6.50)	78.73 (21.03)	53.22 (22.95)	$p<0.001^{\Delta,\&}$ $p=0.038^{£}$
Taurine conjugated (µmol/l)	11.670 (14.119)	3.651 (4.675)	2.945 (4.409)	0.029 (0.051)	$p<0.001^{\Delta,\&}$ $p=0.001^{£}$

Glycine conjugated (µmol/l)	30.335 (26.488)	10.333 (7.683)	12.018 (15.443)	0.820 (0.818)	$p<0.001^{\Delta,\&,£}$
Taurine to glycine conjugated (ratio)	0.398 (0.557)	0.291 (0.205)	0.243 (0.336)	0.026 (0.038)	$p<0.001^{\Delta,\&}$ $p=0.001^{£}$
Taurine conjugated RA (%)	23.09 (15.79)	20.81 (11.25)	16.12 (13.93)	2.38 (3.41)	$p<0.001^{\Delta,\&}$ $p=0.001^{£}$
Glycine conjugated RA (%)	76.91 (15.79)	79.19 (11.25)	83.88 (13.93)	97.62 (3.41)	$p<0.001^{\Delta,\&}$ $p=0.001^{£}$
Non-12-alpha hydroxylated (µmol/l)	25.275 (21.202)	9.310 (7.971)	10.938 (15.077)	0.953 (1.088)	$p<0.001^{\Delta,\&}$ $p=0.001^{£}$
12-alpha hydroxylated (µmol/l)	19.446 (17.051)	5.451 (4.869)	8.173 (7.736)	0.795 (0.711)	$p<0.001^{\Delta,£}$ $p=0.001^{\&}$ $p=0.007^{®}$
12-alpha hydroxylated to non 12-alpha hydroxylated (ratio)	0.804 (0.339)	0.556 (0.383)	1.164 (0.842)	1.475 (1.539)	$p<0.001^{\&}$ $p=0.006^{£}$ $p=0.025^{®}$
Non-12-alpha hydroxylated RA (%)	57.06 (9.26)	67.47 (14.32)	52.24 (17.71)	47.98 (15.46)	$p<0.001^{\&}$ p=0.006^ $p=0.025^{®}$
12-alpha hydroxylated RA (%)	42.94 (9.26)	32.53 (14.32)	47.76 (17.71)	52.02 (15.46)	$p<0.001^{\&}$ p=0.006^ $p=0.025^{®}$
Hydrophilic (µmol/l)	16.198 (15.719)	4.037 (3.745)	6.300 (6.939)	0.468 (0.456)	$p<0.001^{\Delta}$ $p=0.001^{£}$ $p=0.005^{\&}$ $p=0.009^{®}$
Hydrophobic (µmol/l)	28.522 (22.430)	10.723 (8.610)	12.811 (15.248)	1.281 (1.368)	$p<0.001^{\Delta,\&,£}$
Hydrophilic to hydrophobic (ratio)	0.493 (0.194)	0.342 (0.199)	0.489 (0.324)	0.405 (0.228)	$p=0.043^{®}$
Hydrophilic RA (%)	32.03 (7.99)	23.88 (11.32)	30.24 (12.99)	27.23 (10.37)	$p=0.015^{®}$
Hydrophobic RA (%)	67.97 (7.99)	76.12 (11.32)	69.76 (12.99)	72.77 (10.37)	$p=0.015^{®}$

Mean (SD). Analyzed within Child-Pugh A group of patients. $^{\Delta}$Significant difference between alcoholic and healthy controls group, $^{\&}$significant difference between HCV and healthy controls; $^{£}$significant difference between other etiologies and healthy controls, $^{®}$significant difference between alcoholic and HCV group, °significant difference between alcoholic and other etiologies group, ^significant difference between HCV and other etiologies groups. RA: relative abundance (% of total bile acids*). CA: cholic acid; TCA: taurocholic acid; GCA:

glycocholic acid; CDCA: chenodeoxycholic acid; TCDCA: taurochenodeoxycholic acid; GCDCA: glycochenodeoxycholic acid; DCA: deoxycholic acid; TDCA: taurodeoxycholic acid; GDCA: glycodeoxycholic acid; LCA: lithocholic acid; TLCA: taurolithocholic acid; GLCA: glycolithocholic acid; UDCA: ursodeoxycholic acid; TUDCA: tauroursodeoxycholic acid; GUDCA: glycoursodeoxycholic acid; total CA: CA + TCA + GCA; total CDCA: CDCA + TCDCA + GCDCA; total DCA: DCA + TDCA + GDCA; total LCA: LCA + TLCA + GLCA; total UDCA: UDCA + TUDCA + GUDCA; primary: total CA + total CDCA; secondary: total DCA + total LCA + total UDCA; unconjugated: CA + CDCA + DCA + LCA + UDCA; conjugated: taurine conjugated + glycine conjugated; taurine conjugated: TCA + TCDCA + TDCA + TLCA + TUDCA; glycine conjugated: GCA + GCDCA + GDCA + GLCA + GUDCA; *taurine conjugated RA: % of conjugated; *glycine conjugated RA: % of conjugated; non-12-alpha hydroxylated: total CDCA + total LCA + total UDCA; 12-apha hydroxylated: total CA + total DCA; hydrophilic: total CA + total UDCA; hydrophobic: total CDCA + total DCA + total LCA

Relationship between whole blood neutrophil function and serum bile acid composition in cirrhotic patients.

To assess the relationship of the observed changes in BA composition and neutrophil function, the correlation analysis between those parameters within cirrhotic patients (n=109) with Benjamini-Hochberg multiplicity correction was performed. Further partial correlation analysis of significantly correlating variable pairs was performed to exclude the influence on correlation of confounding variables like severity and etiology of cirrhosis, age and sex of patients (Table 12).

Table 12. Significant correlations between bile acid concentrations and relative abundances and neutrophils phagocytosis and ROS production.

Parameters	**ρ**	**Corrected p-value**	**Partial ρ**	**p-value**
Total CDCA RA & phagocytic capacity	-0.335	p=0.010	-0.329	p=0.001
UDCA RA & non-phagocytic neutrophils	-0.335	p=0.022	-0.337	p=0.001

UDCA RA & ROS production (E. coli), %	0.326	p=0.022	0.343	p<0.001

Then multiple linear regression was carried out to study the nature of the relationship between significantly correlating variables. This analysis was also performed accounting for the above-mentioned confounders. In the models, where the BA and confounding variables were included together, BAs were still significant explanatory variables. When models containing all confounding explanatory variables with and without main explanatory variable (BA) were compared with likelihood ratio test, models were significantly different (Table 13).

Table 13. Multiple linear regression to identify, if bile acids are predictive for neutrophil function in cirrhotic patients.
Reproduced from (Balazs et al, 2021, in revision).

Phagocytic capacity ~ Total CDCA relative abundance + Etiology of cirrhosis + Child-Pugh group +Age + Sex

F (5,102) = 5.85, p <0.001, R^2 = 0.223

	Estimate	Std. Error	t-value	p-value
Total CDCA relative abundance	-126	37	-3.4	0.001
Etiology of cirrhosis	-0.61	6.47	-0.09	0.925
Child-Pugh group	-18.96	11.39	-1.66	0.100
Age	-0.52	0.55	-0.94	0.351
Sex	-34.75	10.95	-3.18	0.002

Non-phagocytic neutrophils ~ UDCA relative abundance + Etiology of cirrhosis + Child-Pugh group +Age + Sex

F (5,101) = 3.83, p = 0.003, R^2 = 0.159

	Estimate	Std. Error	t-value	p-value
UDCA relative abundance (cube root)	-1.68	0.43	-3.95	<0.001
Etiology of cirrhosis	0.06	0.06	1.10	0.273
Child-Pugh group	0.09	0.10	0.89	0.374
Age	-0.00	0.01	-0.02	0.981
Sex	0.11	0.10	1.11	0.268

ROS production (*E. coli*) ~ UDCA relative abundance+ Etiology of cirrhosis + Child-Pugh group +Age + Sex

F (5,88) = 2.85, p = 0.020, R^2 = 0.140

	Estimate	Std. Error	t-value	p-value
UDCA relative abundance (cube root)	3.48	1.27	2.75	0.007
Etiology of cirrhosis	-0.03	0.17	-0.19	0.853
Child-Pugh group	-0.40	0.30	-1.34	0.185
Age	-0.03	0.01	-2.15	0.034
Sex	0.04	0.30	0.15	0.882

Total CDCA RA and lower UDCA RA were significantly associated with decreased neutrophil phagocytosis. Lower UDCA RA was also predictive for the impairment of ROS production in response to *E. coli* (Figure 15).

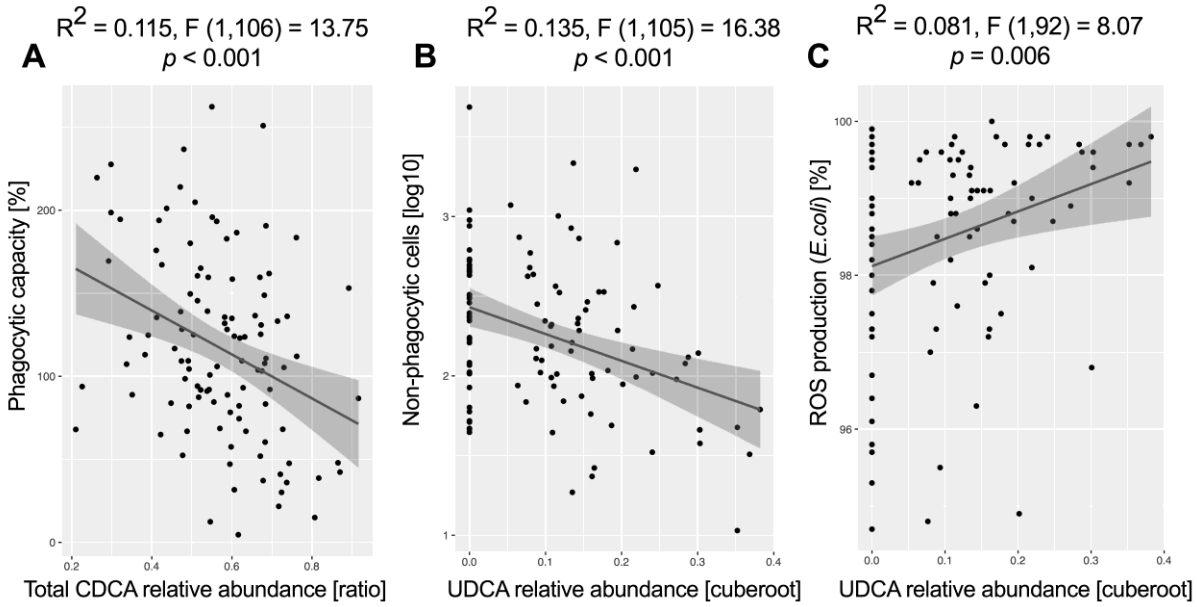


Figure 15. Changed BA composition in cirrhotic sera associates with dysregulated neutrophil functions.

(A-C) Linear regression analysis of associations between bile acid relative abundances and neutrophil functions in a cohort of cirrhotic patients (n=109). Only significantly correlated variables were analyzed with linear regression (partial correlation results: total CDCA relative abundance and phagocytic capacity (r=-0.329, p=0.001), UDCA relative abundance and non-phagocytic neutrophils (r=-0.337, p=0.001), UDCA relative abundance and ROS production (*E. coli*) (r=0.343, p<0.001)). Reproduced from (Balazs et al, 2021, in revision).

Albumin was shown to play a role in neutrophil dysfunction (52). Therefore, we decided to check, if there was an association between albumin and neutrophil phagocytosis and ROS production in this cirrhotic patients' cohort. Spearman partial correlation accounting for the influence of etiology and severity of liver cirrhosis, age and sex. Albumin did not significantly correlate with ROS production in response to *E. coli*, however, it correlated significantly with neutrophil phagocytic capacity (ρ = 0.213, p = 0.030) and non-phagocytic neutrophils amount (ρ = -0.309, p = 0.001). However, when albumin was included in the multiple linear regression model together with BAs and confounding variables, it was not significant explanatory variable for phagocytic capacity anymore, but was still a significant explanatory variable for the amount of non-phagocytic neutrophils. BAs remained significant explanatory variables even after addition of albumin as explanatory variable (Table 14).

Table 14. Multiple linear regression to identify, if albumin is predictive for neutrophil function in cirrhotic patients.

Phagocytic capacity ~ Total CDCA relative abundance + Albumin + Etiology of cirrhosis + Child-Pugh group +Age + Sex

F (6,101) = 5.38, $p < 0.001$, $R^2 = 0.242$

	Estimate	Std. Error	t-value	p-value
Total CDCA relative abundance	-114.04	37.47	-3.04	0.003
Albumin	18.55	11.57	1.60	0.112
Etiology of cirrhosis	-2.16	6.49	-0.33	0.740
Child-Pugh group	-0.50	16.14	-0.03	0.976
Age	-0.37	0.56	-0.66	0.513
Sex	-34.32	10.87	-3.16	0.002

Non-phagocytic neutrophils ~ UDCA relative abundance + Albumin + Etiology of cirrhosis + Child-Pugh group +Age + Sex

F (6,100) = 4.61, $p < 0.001$, $R^2 = 0.217$

	Estimate	Std. Error	t-value	p-value
UDCA relative abundance (cube root)	-1.38	0.43	-3.21	0.002
Albumin	-0.28	0.10	-2.71	0.008
Etiology of cirrhosis	0.09	0.06	1.58	0.117
Child-Pugh group	-0.20	0.14	-1.42	0.159
Age	-0.00	0.01	-0.53	0.600

Sex	0.12	0.10	1.26	0.212

Neutrophil function and BA composition in UDCA-treated and non-treated liver cirrhotic patients

To investigate effects of UDCA supplementation on neutrophil function in liver cirrhosis, a small cohort of patients receiving UDCA treatment (n = 6) was analyzed in comparison with not treated with UDCA patients (n = 79). UDCA-treated group included only Child-Pugh A patients, therefore only Child-Pugh A patients were analysed from UDCA non-treated group as well, that the comparison is not influenced by the severity of cirrhosis. Demographic characteristics and liver function parameters, including albumin and neutrophil count, were comparable between the groups. Only alkaline phosphatase was significantly higher and INR was significantly lower in UDCA-treated group (Table 15).

Table 15. Demographic characteristics and liver function parameters of cirrhotic patients with and without UDCA treatment.

Characteristic	UDCA non-treated (n=79)	UDCA-treated (n=6)	p-value
Age (years)	58±14	64±10	p=0.201
Sex (Male/Female, n)	55/24	2/4	p=0.068
Etiology (Alcoholic/HCV/Other, n)	37/25/17	0/0/6	p<0.001
Child-Pugh grade (n)	A –79	A - 6	-
Child-Pugh score	5±1	5±1	p=0.259
MELD score	8±3	8±5	p=0.274
AST (U/l)	44.5±39	41±24	p=0.424
ALT (U/l)	39±44	41±49	p=0.420
GGT (U/l)	129±169	134±440	p=0.613
AP (U/l)	104±62	166.5±144	p=0.031
Bilirubin (mg/dl)	0.9±0.7	1.3±1.4	p=0.625
INR (ratio)	1.2±0.2	1.1±0.1	p=0.024
Creatinine (mg/dl)	0.8±0.3	0.8±0.4	p=0.577
Albumin (g/dl)	4.3±0.7	4.3±0.5	p=0.409
Neutrophils ($x10^9$/l)	2.8±1.4	2.8±1.8	p=0.645

Median ± IR. ALT: Alanine transaminase; AST: Aspartate transaminase; AP: Alkaline phosphatase; GGT: Gamma-glutamyl transferase; INR: International Normalized Ratio; MELD: Model for End-Stage Liver Disease.

UDCA treatment significantly changed serum BA composition in cirrhotic patients. Absolute concentrations and relative abundances of total UDCA, as well as its individual forms increased in UDCA-treated patients. The largest increase was in GUDCA (Figure 16 A-H).

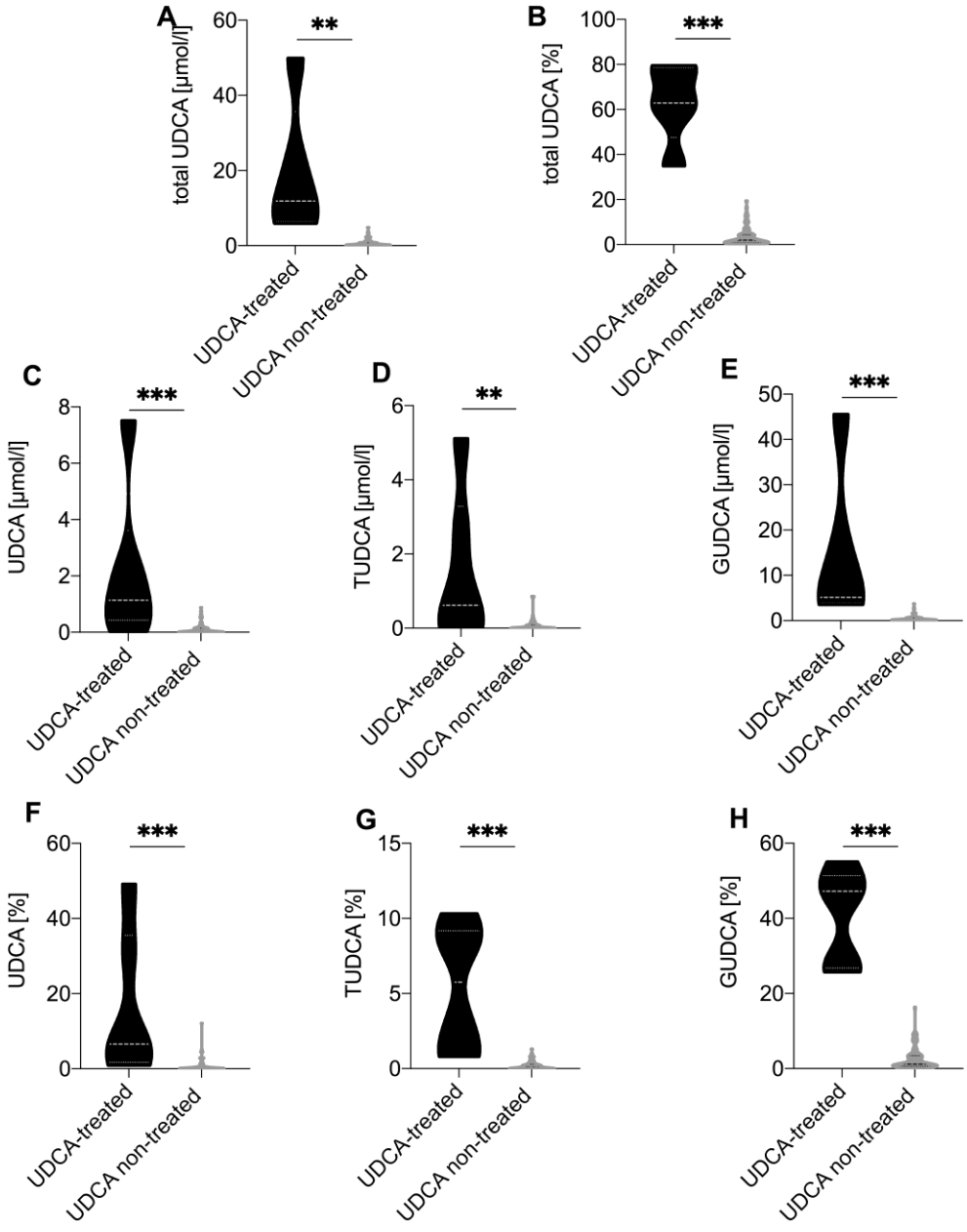


Figure 16. UDCA absolute concentrations and relative abundances were increased in UDCA-treated group.

(A-H) Serum bile acids in 79 UDCA non-treated cirrhotic patients and 6 UDCA-treated cirrhotic patients were measured with HPLC-HRMS. Total UDCA and its unconjugated and conjugated forms (A, C-E) absolute concentrations and (B, F-H) relative abundances; Truncated violin plots represent frequency distribution, the broken line indicates median, dotted lines indicate quartiles. **p < 0.01, ***p < 0.001 (Statistical analysis was by Mann-Whitney U test).

UDCA supplementation also changed relative abundances of total CA, total CDCA and total LCA and tended to decrease the absolute serum concentration of total CDCA (Figure 17). The most striking decrease in UDCA-treated patients was in GCDCA relative abundance (from 40% to 12%, p<0.001).

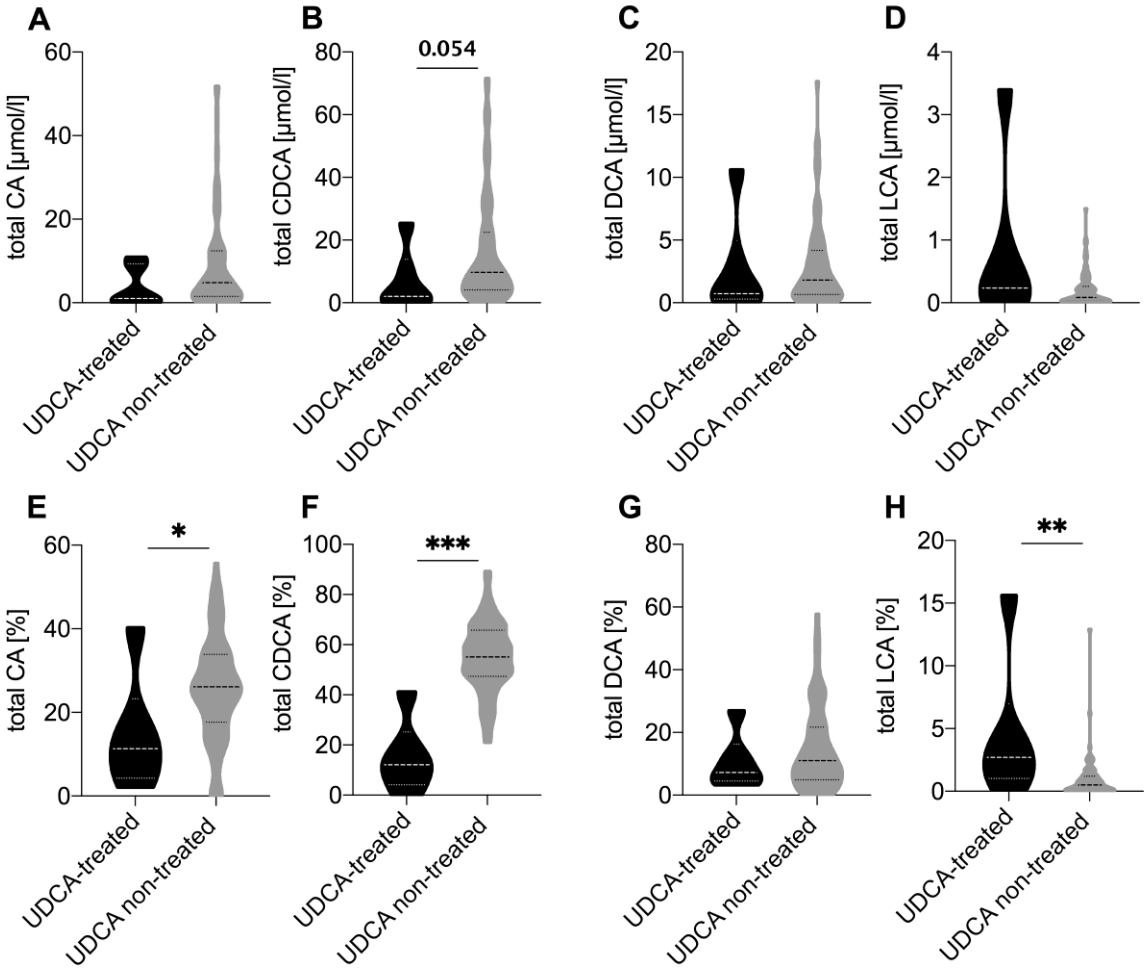


Figure 17. Total CA and total CDCA relative abundances were decreased and total LCA relative abundance was increased in UDCA-treated cirrhotic patients.

(A-H) Serum bile acids in 79 UDCA non-treated cirrhotic patients and 6 UDCA-treated cirrhotic patients were measured with HPLC-HRMS. Total CA, CDCA, DCA, LCA (A-D) absolute concentrations and (E-H) relative abundances; Truncated violin plots represent frequency distribution, the broken line indicates median, dotted lines indicate quartiles. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Statistical analysis was by Mann-Whitney U test).

Patients after UDCA treatment had a tendency for improvement of neutrophil phagocytosis (Figure 18 A, B) and a significantly lower intracellular basal ROS production and ROS production in response to fMLF (Figure 18 F, G). As UDCA-treated group included only patients with other etiologies of cirrhosis, the analysis of neutrophil function differences was performed also between UDCA-treated group and only patients with other etiologies of cirrhosis from UDCA non-treated group, however, the results were comparable to the ones described above.

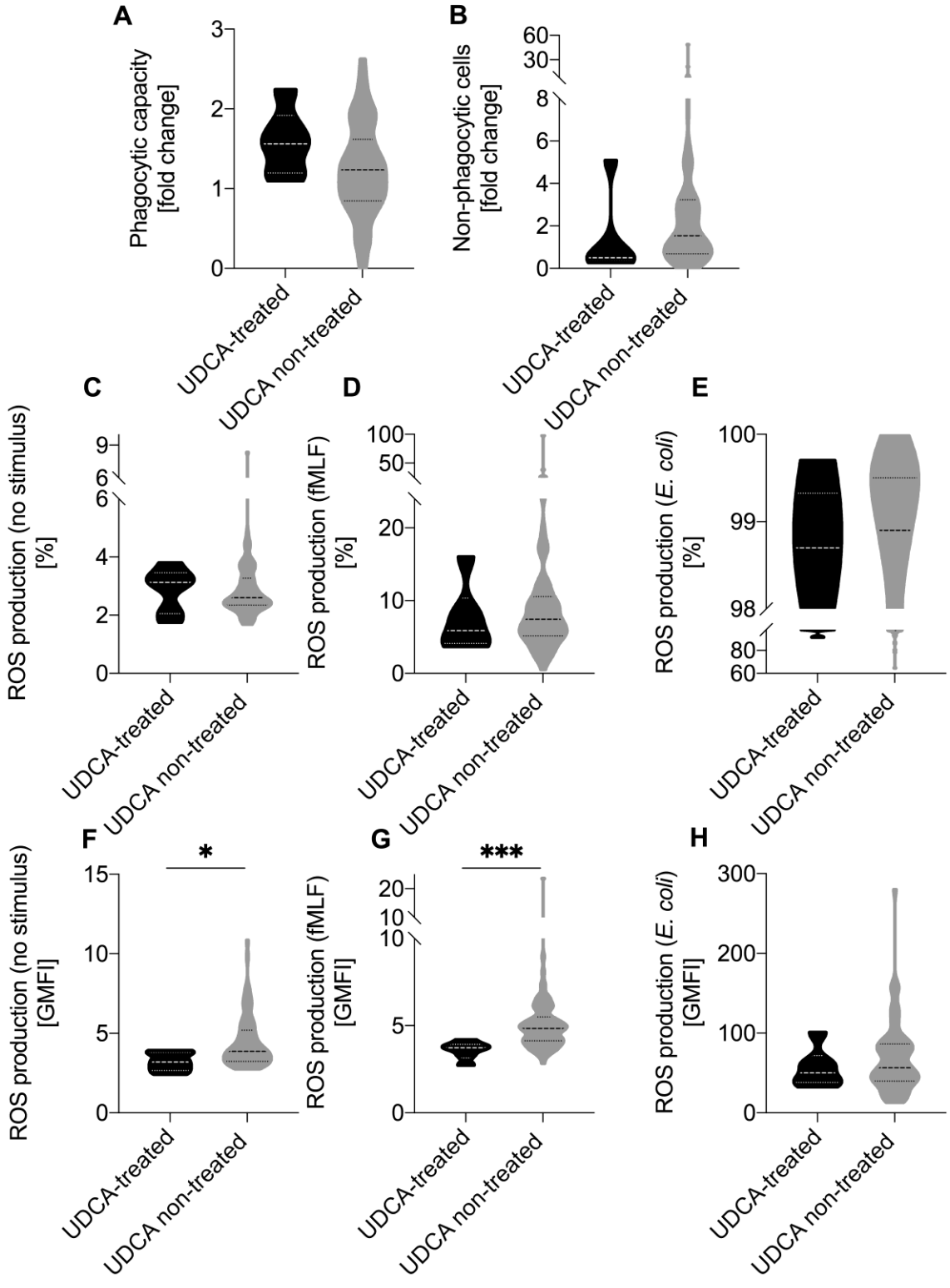


Figure 18. Neutrophil function of UDCA-treated patients compared to patients not treated with UDCA.

(A, B) Neutrophil phagocytosis of *E. coli* (4x10^7 bacteria/100µl blood). (A) Fold change of non-phagocytic neutrophils and (B) neutrophil phagocytic capacity related to *E. coli* batch controls;

(C-H) Neutrophils ROS production. (C-E) Percentage of neutrophils, which produced ROS (C) without any stimulus or in response to (D) fMLF (0.8 μM) or (E) *E. coli* (2-4x10^7 bacteria/100μl blood). (F-H) Intracellular ROS production (GMFI). Truncated violin plots represent the frequency distribution, the broken line represents median and dotted lines represent quartiles; *p < 0.05; ***p < 0.001 (Mann-Whitney U test).

Direct effects of bile acids on neutrophil functions

Further, direct effects of BAs on healthy human neutrophils were described. BAs were tested in a range of pathophysiological concentrations, found in cirrhotic patients (Table 16). Isolated human neutrophils were treated with either BA mix – the mixture of all 15 BAs in concentrations and relative abundances found in cirrhotic patients (Table 17), or with individual or total (mixture of unconjugated and conjugated BA forms) BAs.

Table 16. Pathophysiological bile acid concentrations used for the treatment of isolated neutrophils.
Reproduced from (Balazs et al, 2021, in revision).

Bile acid	Min/Max concentration in cirrhotic cohort (μM)	Concentration range used for neutrophil treatment (μM)
CA	0/23.4	5-100
TCA	0/27.9	
GCA	0/72.0	
CDCA	0/44.3	5-300
TCDCA	0/131.3	
GCDCA	0.3/287.1	
DCA	0/3.9	5-100
TDCA	0/7.1	
GDCA	0/16.4	
LCA	0/0.8	5-100
TLCA	0/0.4	
GLCA	0/1.4	
UDCA	0/1.4	5-100
TUDCA	0/1.4	

GUDCA	0/8.8	

CA: cholic acid; TCA: taurocholic acid; GCA: glycocholic acid; CDCA: chenodeoxycholic acid; TCDCA: taurochenodeoxycholic acid; GCDCA: glycochenodeoxycholic acid; DCA: deoxycholic acid; TDCA: taurodeoxycholic acid; GDCA: glycodeoxycholic acid; LCA: lithocholic acid; TLCA: taurolithocholic acid; GLCA: glycolithocholic acid; UDCA: ursodeoxycholic acid; TUDCA: tauroursodeoxycholic acid; GUDCA: glycoursodeoxycholic acid.

Table 17. Bile acid concentrations and relative abundances in "bile acid mix". Reproduced from (Balazs et al, 2021, in revision).

Bile acid	Mean + 2SD concentration in cirrhosis (μM)*	RA in liver cirrhosis (%)*
TUDCA	0.684	0.2
GUDCA	3.232	2.1
UDCA	0.532	0.7
TCA	14.702	6.0
GCA	38.413	18.6
CA	5.960	1.8
TCDCA	45.264	13.2
GCDCA	101.329	40.5
CDCA	11.174	3.5
TDCA	2.725	1.5
GDCA	8.501	7.2
DCA	2.034	3.8
TLCA	0.196	0.1
GLCA	0.536	0.5
LCA	0.400	0.3

RA: relative abundance; CA: cholic acid; TCA: taurocholic acid; GCA: glycocholic acid; CDCA: chenodeoxycholic acid; TCDCA: taurochenodeoxycholic acid; GCDCA: glycochenodeoxycholic acid; DCA: deoxycholic acid; TDCA: taurodeoxycholic acid; GDCA: glycodeoxycholic acid; LCA: lithocholic acid; TLCA: taurolithocholic acid; GLCA: glycolithocholic acid; UDCA: ursodeoxycholic acid; TUDCA: tauroursodeoxycholic acid;

GUDCA: glycoursodeoxycholic acid.*Final concentrations and relative abundances, which influenced neutrophils in in vitro experiments.

Phagocytosis

Neutrophils were pre-treated with BAs/vehicle for 45 min at 37°C and after that incubated with *E. coli* for 10 min (Figure 19 A). BA mix did not significantly affect neutrophil phagocytosis (Figure 19 B). Total CDCA and total DCA reduced phagocytosis of *E. coli* (Figure 19 C, D). However, neutrophils restored their phagocytic capacity immediately after total CDCA or total DCA were washed out (Figure 19 E-G).

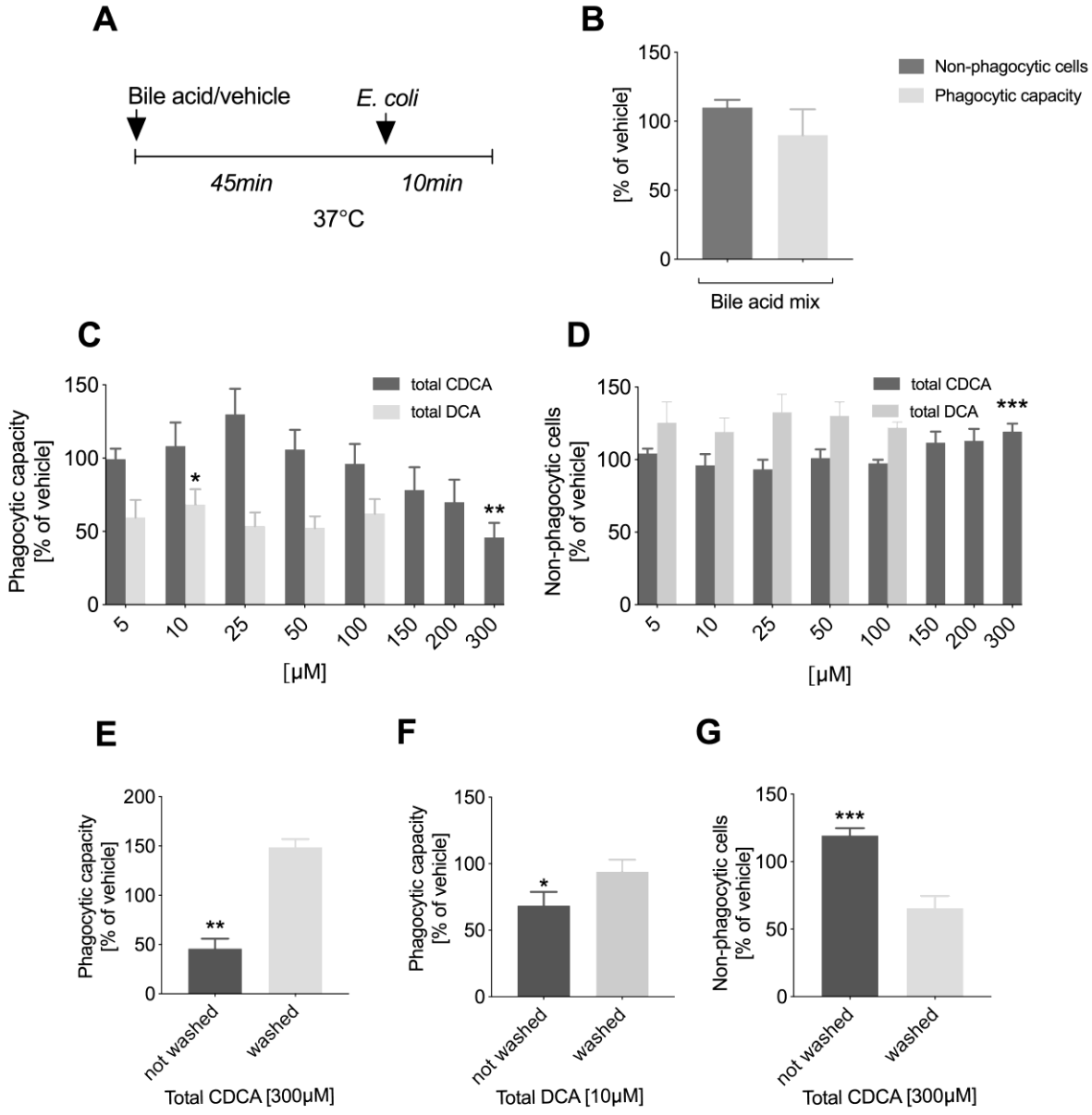


Figure 19. Total CDCA and total DCA inhibit neutrophil phagocytosis.

(A) Experimental setup. (B-G) $5x10^5$ isolated healthy donor neutrophils were pre-incubated with bile acids and then allowed to phagocytose $4x10^7$ *E. coli*. (E-G) Bile acids were or were not washed out following pre-incubation with the cells for 45 minutes. Phagocytic capacity (B, C, E, F) and percentage of non-phagocytic neutrophils (B, D, G) were measured by flow cytometry. The responses were normalized to the response of vehicle-treated (PBS or different concentrations of DMSO) neutrophils. A minimum of four separate experiments are combined in these graphs; error bars, SEM; *p<0.05,**p<0.01,***p<0.001 (unpaired t-test). CDCA: chenodeoxycholic acid; DCA: deoxycholic acid. Reproduced from (Balazs et al, 2021, in revision).

If tested separately, unconjugated and conjugated forms of CDCA and DCA did not significantly impact on phagocytic function of neutrophils (Figure 20 A-C).

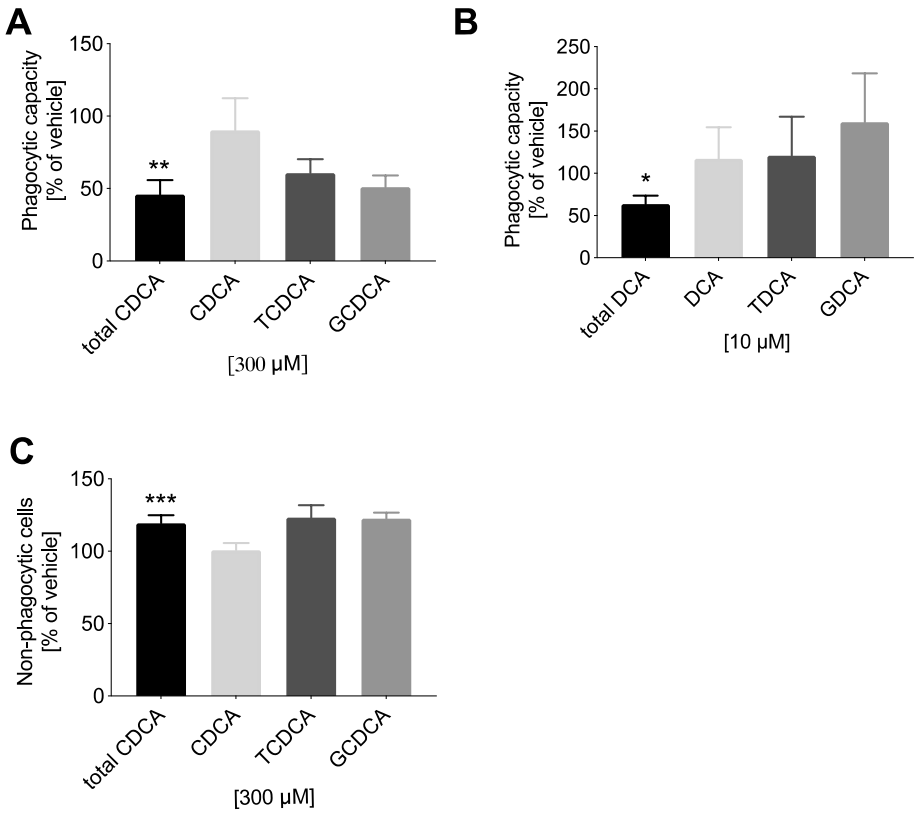


Figure 20. Phagocytosis in neutrophils treated with individual unconjugated and conjugated forms of bile acids.

(A-C) 5x10^5 neutrophils were pre-incubated with bile acids of different concentrations and then allowed to phagocytose 4x10^7 *E.coli*. (A, B) Phagocytic capacity and (C) percentage of neutrophils that had not internalised any bacteria were measured by flow cytometry. The responses were normalised to the response of vehicle-treated (PBS or DMSO of different concentrations) neutrophils. A minimum of 5 separate experiments are combined in these graphs; error bars, SEM; *p < 0.05; **p < 0.01, ***p < 0.001 (unpaired t-test). CDCA: chenodeoxycholic acid; TCDCA: taurochenodeoxycholic acid; GCDCA: glycochenodeoxycholic acid; DCA: dexycholic acid; TDCA: taurodeoxycholic acid; GDCA: glycodeoxycholic acid. Reproduced from (Balazs et al, 2021, in revision).

Total CA, total UDCA and total LCA did not directly affect the healthy neutrophil phagocytic function in these experiments (Figure 21 A, B).

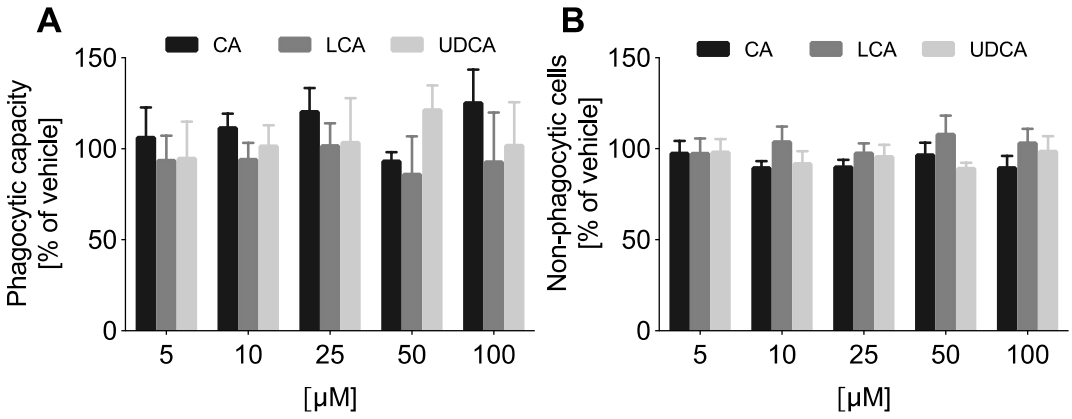


Figure 21. Total CA, total LCA and total UDCA did not significantly influence phagocytosis of neutrophils.

(A, B) 5x10^5 neutrophils were pre-incubated with bile acids of different concentrations and then allowed to phagocytose 4x10^7 *E.coli*. (A) Phagocytic capacity and (B) percentage of neutrophils that had not internalized any bacteria were measured by flow cytometry. The responses were normalised to the response of vehicle-treated (different concentrations of DMSO) neutrophils. A minimum of 4 separate experiments are combined in these graphs; error bars, SEM. Statistical analysis was by unpaired t-test. CA: cholic acid; LCA: lithocholic acid; UDCA: ursodeoxycholic acid. Reproduced from (Balazs et al, 2021, in revision).

ROS production

Total ROS production (sum of intracellular and extracellular ROS) was measured in isolated human neutrophils treated with BAs/vehicle and stimulated with PBS++, fMLF or *E. coli* (Figures 22 A, 26 A). BA mix and total LCA induced ROS production by unstimulated neutrophils (Figure 22 B-E).

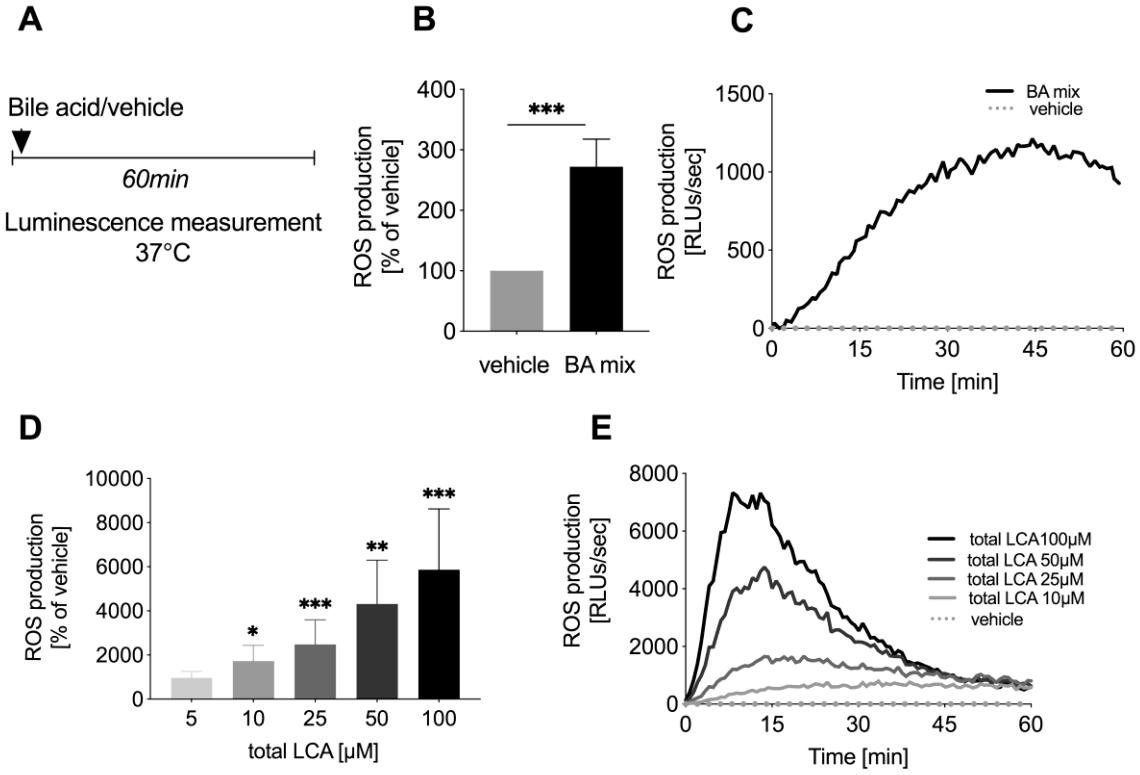


Figure 22. Total LCA induce ROS production by unstimulated neutrophils.

(A) Experimental setup. (B-E) ROS production of isolated healthy donor neutrophils after treatment with bile acid (BA)/vehicle. (C, E) Representative examples and (B, D) total ROS production over time. ROS data were normalised and compared to vehicle-treated control (PBS) and represent a minimum of four separate experiments. Data are presented for bile acids, which significantly influenced ROS production; error bars, SEM; *p < 0.05, **p < 0.01, ***p < 0.001 (unpaired t-test). ROS: reactive oxygen species; BAs: bile acids; RLUs: relative light units; LCA: lithocholic acid. Part of these experiments (panels B, C) was performed during my stay abroad at the lab of Dr. Sonja Vermeren (Centre for Inflammation Research, Queen's Medical Research Institute, University of Edinburgh). Reproduced from (Balazs et al, 2021, in revision).

When unconjugated and conjugated forms of LCA were tested separately, GLCA induced the strongest ROS production response (Figure 23 A).

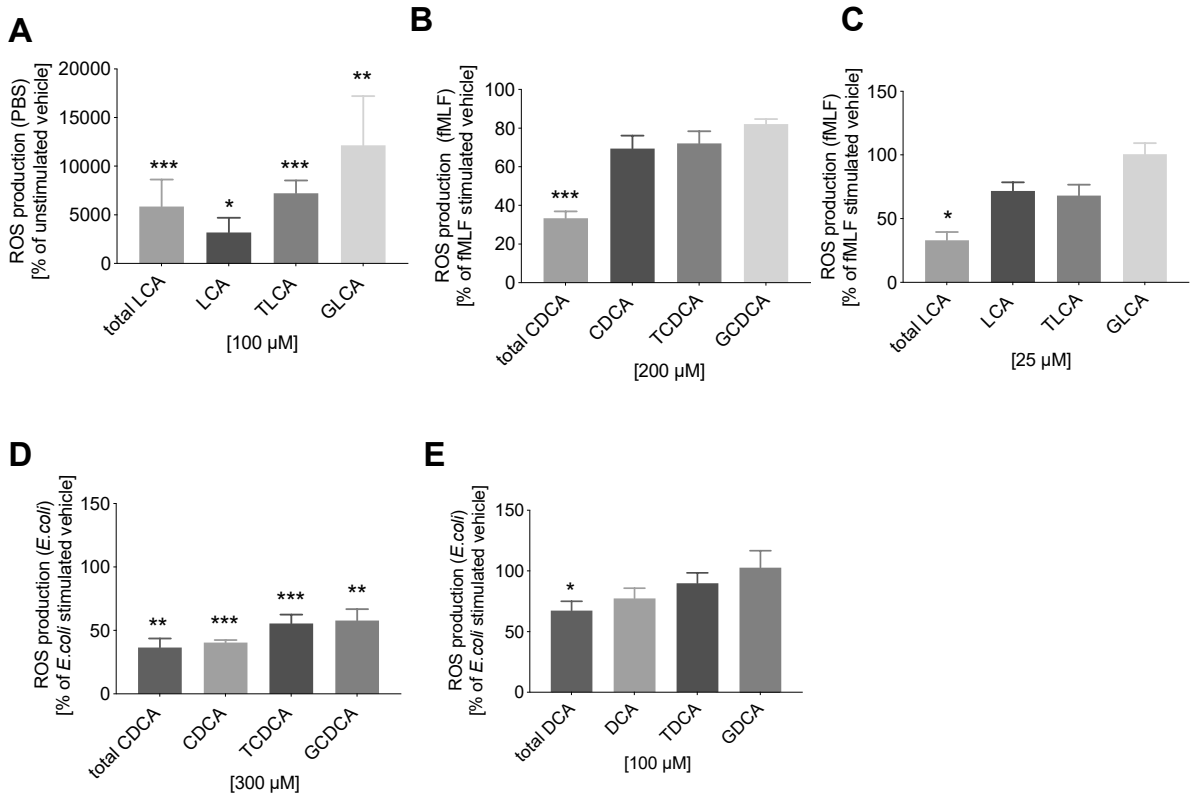


Figure 23. ROS production in neutrophils treated with individual unconjugated and conjugated forms of bile acids.

(A-E) ROS production after treatment with bile acid/vehicle and stimulation with (A) PBS, (B, C) fMLF [1.45µM] or (D, E) *E.coli* [40 bacteria/cell] was measured by chemiluminescence. Total ROS production over time is presented. For ease of viewing, total ROS data were normalized to vehicle-treated (PBS or different concentrations of DMSO) (A) unstimulated or (B-E) stimulated control and represent a minimum of 4 separate experiments; error bars, SEM; *p < 0.05, **p < 0.01, ***p < 0.001 (unpaired t-test). ROS: reactive oxygen species; fMLF: N-formyl-met-leu-phe, LCA: lithocholic acid; TLCA: taurolithocholic acid; GLC: glycolithocholic acid; CDCA: chenodeoxycholic acid; TCDCA: taurochenodeoxycholic acid; GCDCA: glycochenodeoxycholic acid; DCA: dexycholic acid; TDCA: taurodeoxycholic acid; GDCA: glycodeoxycholic acid. Reproduced from (Balazs et al, 2021, in revision).

No other BAs induced ROS production by unstimulated neutrophils (Figure 24 A).

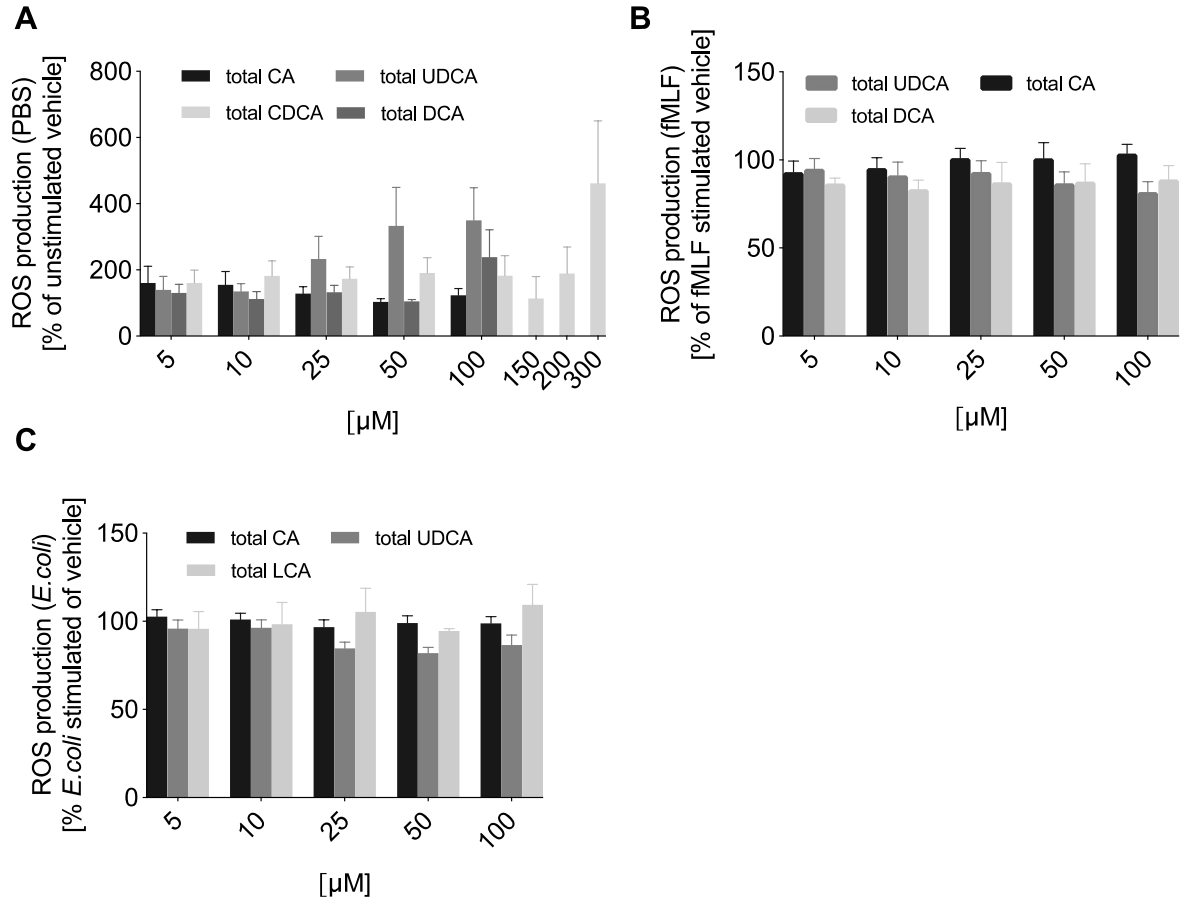


Figure 24. Bile acids and neutrophil ROS production.

(A-C) ROS production after treatment with bile acid/vehicle and stimulation with (A) PBS, (B) fMLF [1.45µM] or (C) *E.coli* [40 bacteria/cell] was measured by chemiluminescence. Total ROS production over time is presented. For ease of viewing, total ROS data were normalised to vehicle-treated (PBS or different concentrations of DMSO) (A) unstimulated or (B, C) stimulated control and represent a mean of a minimum of 4 separate experiments; error bars, SEM. Statistical analysis was by unpaired t-test. ROS: reactive oxygen species; fMLF: N-formyl-met-leu-phe; CA: cholic acid; CDCA: chenodeoxycholic acid; UDCA: ursodeoxycholic acid; DCA: dexycholic acid; LCA: lithocholic acid. Reproduced from (Balazs et al, 2021, in revision).

BA mix did not influence ROS production by fMLF or *E. coli* stimulated neutrophils (Figure 25 A, B).

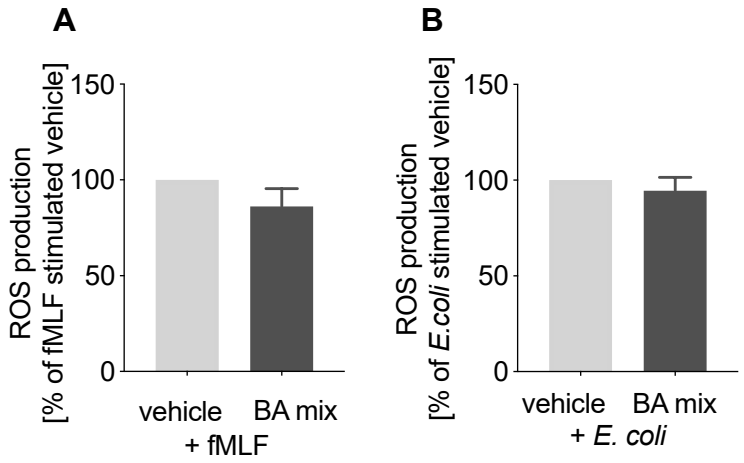


Figure 25. ROS production in neutrophils treated with bile acid mix.

(A, B) ROS production after treatment with bile acid (BA) mix/vehicle and stimulation with (A) fMLF [1.45μM] or (B) *E.coli* [40 bacteria/cell] was measured by chemiluminescence. Total ROS production over time is presented. For ease of viewing, total ROS data were normalised to vehicle-treated (PBS) stimulated control and represent a minimum of 4 separate experiments; error bars, SEM. Statistical analysis was by unpaired t-test. ROS: reactive oxygen species; fMLF: N-formyl-met-leu-phe; BAs: bile acids. Reproduced from (Balazs et al, 2021, in revision).

Total CDCA and total LCA reduced ROS production of neutrophils in response to fMLF in a dose-dependent manner (Figure 26 B, D, F). Total CDCA (also its unconjugated and conjugated forms separately) and total DCA reduced ROS production of neutrophils in response to *E. coli* (Figure 26 C, E, G, Figure 23 D, E). Total CA and total UDCA did not significantly influence ROS production by fMLF or *E. coli* stimulated neutrophils (Figure 24 B, C).

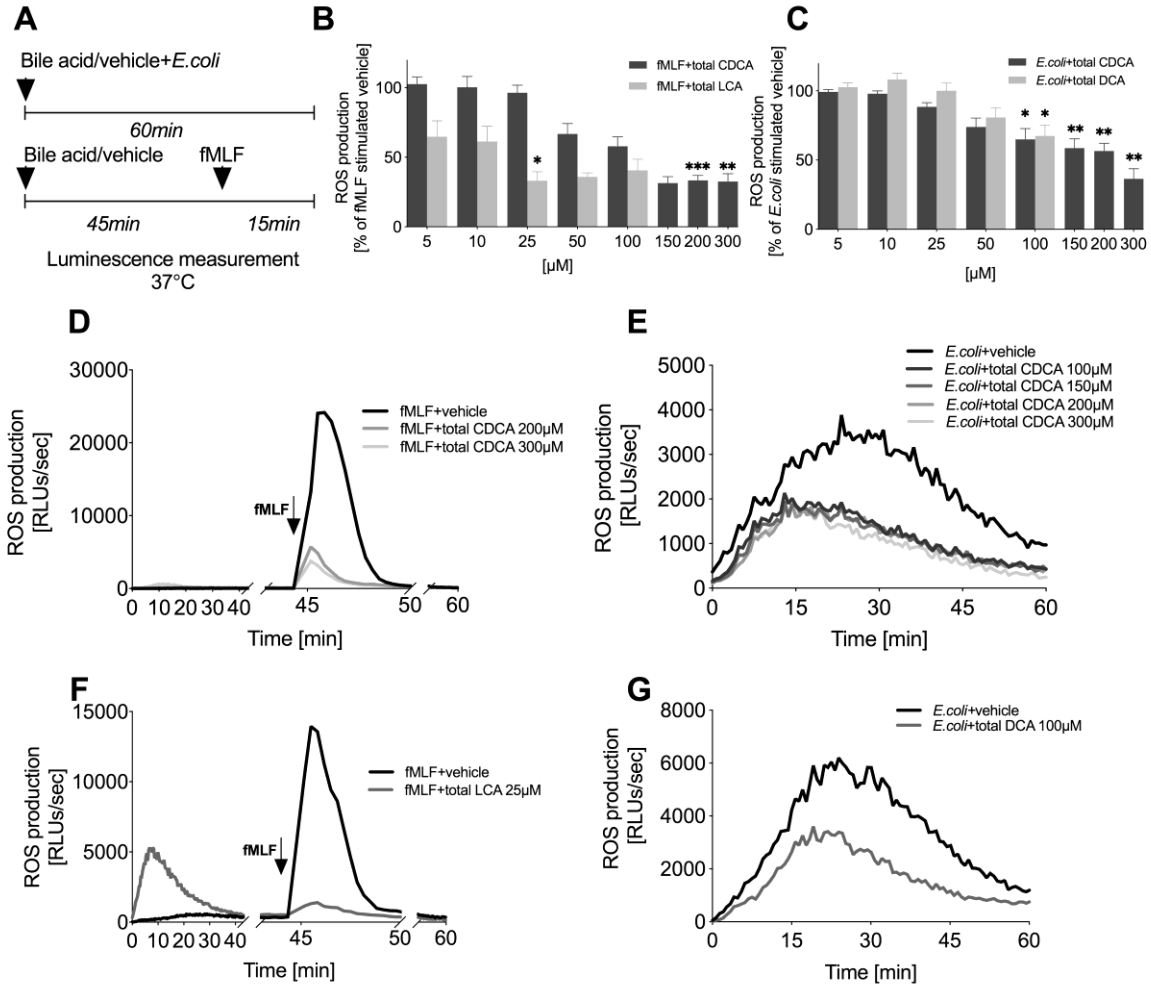


Figure 26. Bile acids inhibit neutrophil ROS production in response to fMLF and *E. coli*. (A) Experimental setup. (B-G) ROS production of isolated healthy donor neutrophils after treatment with bile acid/vehicle and stimulation with (B, D, F) fMLF [1.45µM] or (C, E, G) *E. coli* [40 bacteria/cell]. (D-G) Representative examples and (B, C) total ROS production over time. Total ROS data were normalized and compared to vehicle-treated (PBS or different concentrations of DMSO) stimulated control and represent a minimum of four separate experiments. Data are presented for bile acids, which significantly influenced ROS production; error bars, SEM; *p < 0.05,**p < 0.01,***p < 0.001 (unpaired t-test). fMLF: N-formyl-met-leu-phe; ROS: reactive oxygen species; CDCA: chenodeoxycholic acid; LCA: lithocholic acid; DCA: deoxycholic acid; RLUs: relative light units.Reproduced from (Balazs et al, 2021, in revision).

Chemotaxis

To further investigate the effects of BAs on neutrophil function, chemotaxis of neutrophils pre-treated with BAs/vehicles towards fMLF was measured with ChemoTx® plates. BA mix significantly inhibited chemotaxis of healthy donor neutrophils compared to vehicle control (Figure 27 A). Further, all 15 individual BAs were screened for their ability to influence neutrophil chemotaxis at the concentration 50 µM or their average serum concentrations in cirrhotic cohort, analyzed in this study (Table 17). All BAs, except for all forms of CA and unconjugated UDCA, inhibited chemotaxis of neutrophils at 50 µM concentration. LCA and its conjugates were the most potent inhibitors, supressing up to 95% of neutrophil chemotactic activity in response to fMLF (Figure 27 B). However, when healthy donor neutrophils were incubated with cirrhotic concentrations (Table 17) of these BAs, only treatment with GCDCA resulted in a significant inhibition of chemotaxis (Figure 27 C).

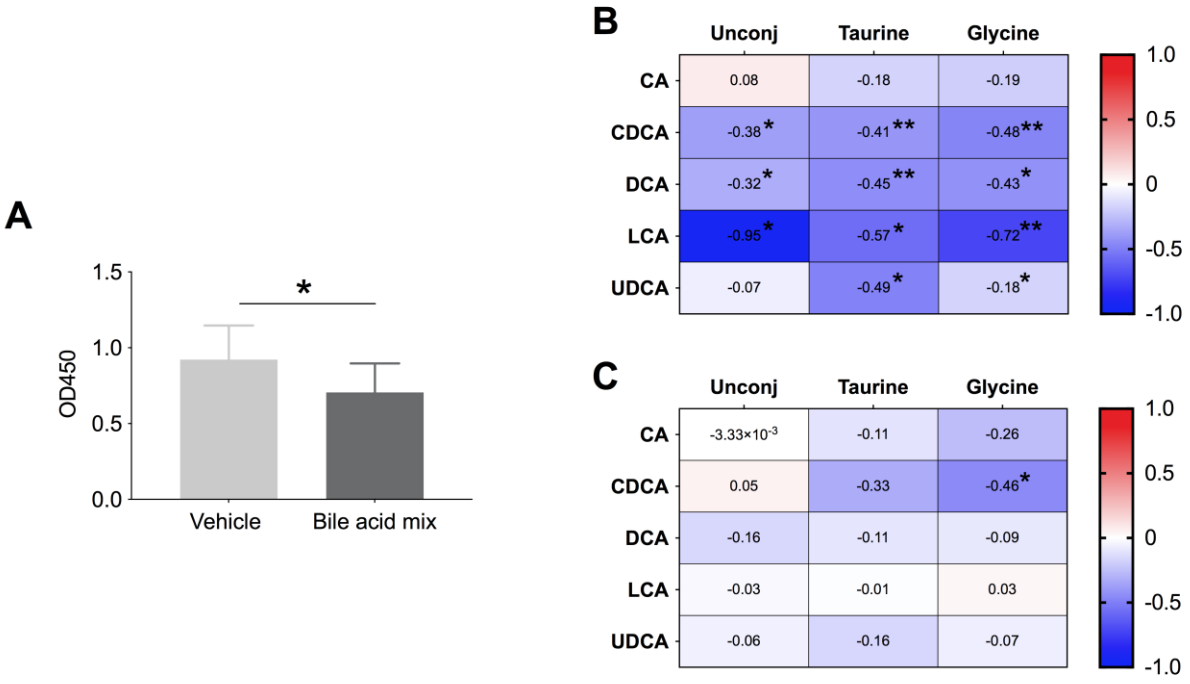


Figure 27. Bile acids inhibit chemotaxis towards fMLF.

(A-C) Neutrophils were pre-incubated with different bile acids/vehicles at 50 µM or at cirrhotic concentrations (Table 17) and allowed to migrate towards fMLF [10nM] for 1 hours at 37°C. The number of migrated neutrophils was estimated based on OD450 (color change developed upon reaction of PNPP with alkaline phosphatase released by migrated neutrophils). Results are shown either as OD450 (A) or as a fold change compared to vehicle control (B, C). Data represent a mean of a minimum 3 separate experiments ((C) GUDCA fold change is based only on 2 observations, therefore, no statistical test could be performed). error bars, SEM (A);

*p < 0.05,**p < 0.01 (paired t-test). These experiments were performed during my stay abroad at the lab of Dr. Sonja Vermeren (Centre for Inflammation Research, Queen's Medical Research Institute, University of Edinburgh).

To investigate the neutrophil chemotaxis defect after BAs treatment in more detail a live-imaging of neutrophils in µ-slide chemotaxis chambers was performed. GCDCA at 100 µM (cirrhotic concentration (Table 17)) was chosen for these experiments, as it was the only BA, which showed a significant effect on neutrophil chemotaxis, when tested in cirrhosis relevant concentrations (Figure 27 C). Furthermore, GCDCA was the most abundant BA in our cirrhotic cohort and contributed most in serum BA composition difference between cirrhotic patients and healthy controls.

GCDCA impaired neutrophil chemotaxis via interfering with directedness of chemotaxis (Figure 28 A, B) and neutrophils treated with GCDCA had decreased migration speed and, therefore, distance travelled (Figure 28 A-C).

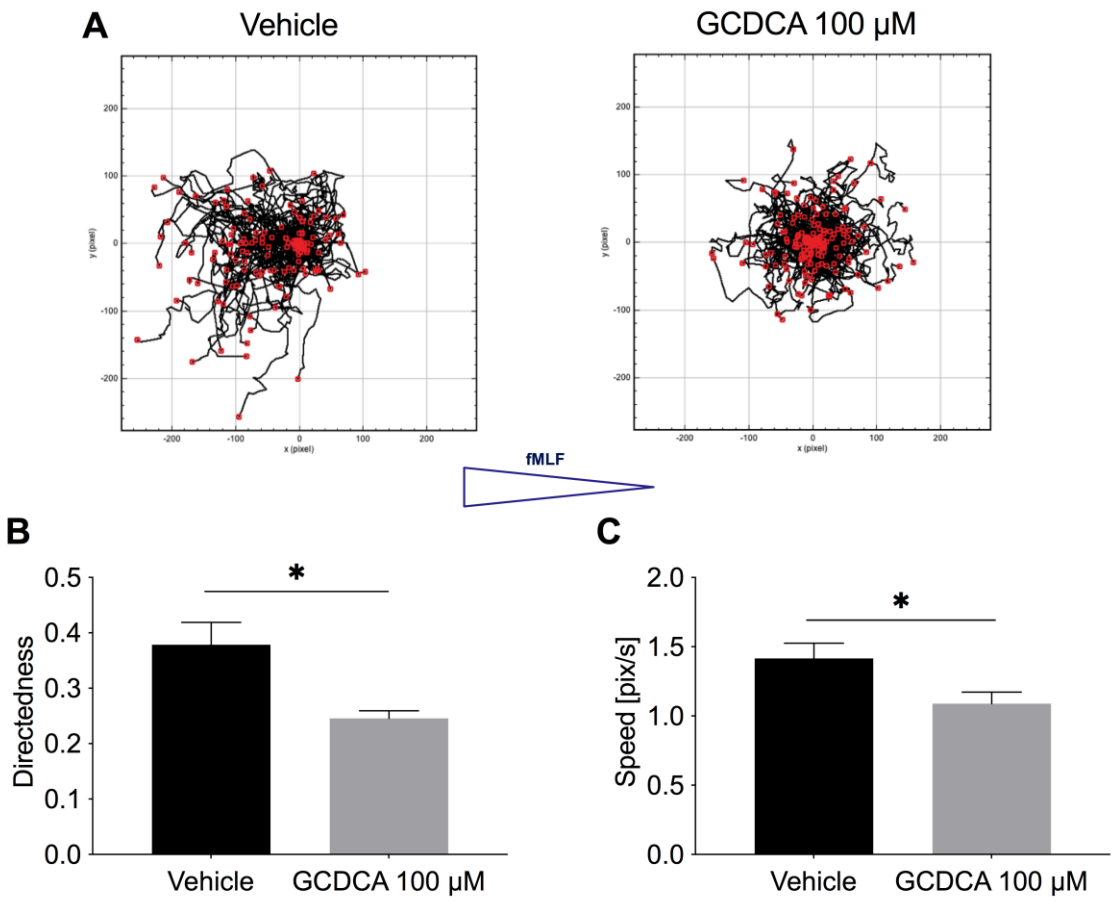
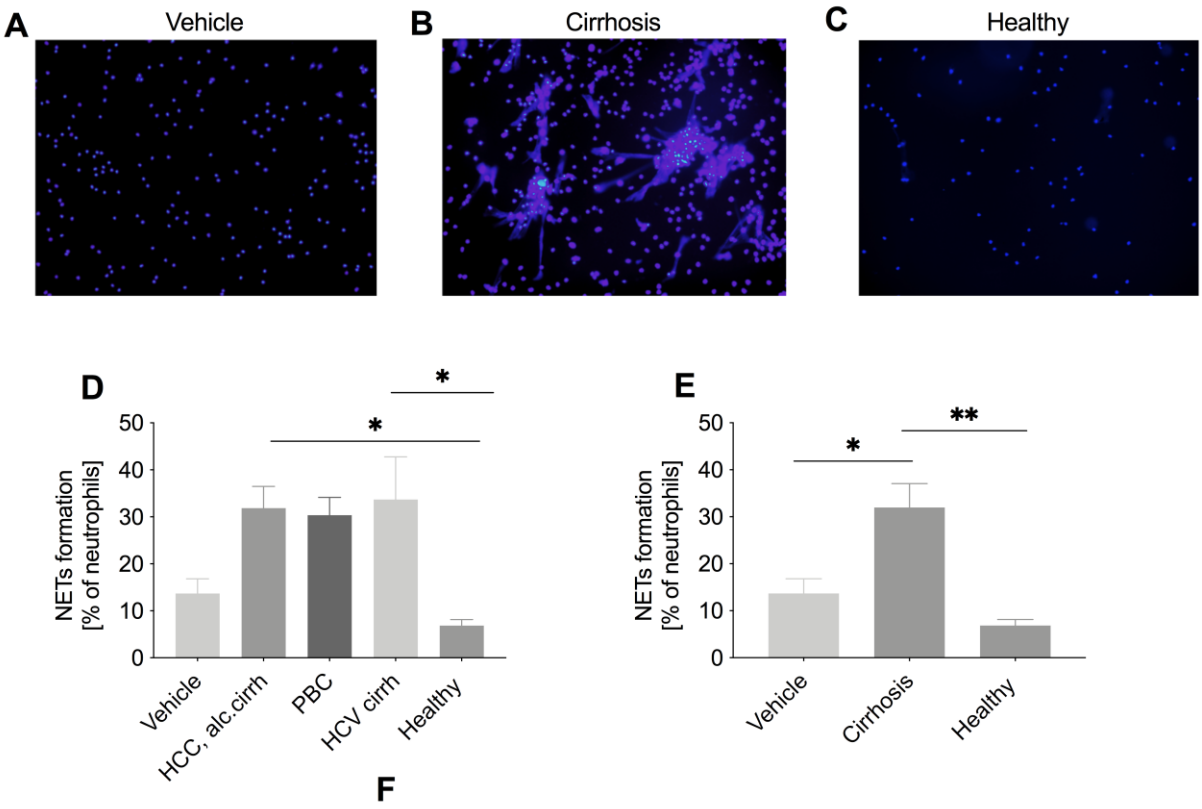


Figure 28. GCDCA impairs neutrophil chemotaxis via interfering with migration directedness and speed.

(A-C) Neutrophils were pre-treated with GCDCA/vehicle for 20 min and their migration towards fMLF [50 nM] was observed in real time for 30 min at 37°C with the help of inverted microscope. Analysis of migration tracks of each neutrophil and summary statistics were performed with ImageJ plugin "Track Maxima" (provided by Luke Tweedy, Beatson Institute, University of Glasgow). (A) Representative spider plots are shown to visualize neutrophil migration types. (B, C) Mean directedness and speed were calculated for all neutrophils from each of 5 separate experiments (per condition). error bars, SEM (B, C). *$p < 0.05$,**$p < 0.01$ (unpaired t-test). These experiments were performed during my stay abroad at the lab of Dr. Sonja Vermeren (Centre for Inflammation Research, Queen's Medical Research Institute, University of Edinburgh).

NETs formation

To investigate the effects of BAs on NETs formation, gallbladder bile (1:50 diluted not to affect the viability of neutrophils) from 3 patients with liver diseases (PBC, HCV associated cirrhosis, HCC + alcoholic cirrhosis) and gallbladder bile from 2 healthy donors was incubated with healthy donor neutrophils and NETs formation was assessed microscopically. Bile from cirrhotic patients induced significantly higher NETs formation compared to healthy bile (Figure 29 A-E). Bile total BA concentrations differed largely between patients, however, bile from patient with HCV associated cirrhosis had a total BA concentration comparable to healthy bile (Figure 29 F). Therefore, it seems that not the absolute BA concentration in bile, but rather BA composition or composition of other components of bile, are responsible for induction of NETs formation by patients' bile.



F

	Total BA, mM
HCC, alc.cirrh	11.6
PBC	0.75
HCV cirrh	51.85
Healthy 1	56.05
Healthy 2	58.95

Figure 29. Bile of cirrhotic patients induce NETs formation compared to healthy bile.
(A-E) Healthy donor neutrophils (n=4) were incubated with 1:50 diluted bile of 3 cirrhotic patients (PBC, HCV associated cirrhosis, HCC + alcoholic cirrhosis) and bile of 2 healthy donors (mean NETs formation per neutrophil donor is presented for 2 healthy bile donors for convenience). (A-C) Representative pictures of DAPI stained neutrophils (20x magnification) after incubation with vehicle/bile from cirrhotic patient/bile from healthy donor. (E) NETs formation results from 3 cirrhotic patients presented in panel D are pooled together for better visualization. (F) Total BA concentration in bile of cirrhotic patients and healthy controls (measured by Silvia Racedo, Medical University of Graz). (D, E) Data are shown as mean with SEM and represent minimum of 4 independent experiments. *p<0.05, **p<0.01. Statistical analysis was by ordinary one-way ANOVA with Tukey's multiple comparisons test.

Apoptosis and viability

BA influence on healthy donor neutrophil apoptosis and viability was studied with flow cytometry (Figures 30-33).

BA mix (Table 17) tended to delay neutrophil apoptosis at 9-hour time point and significantly delayed apoptosis at 12-hour time point. LCA (50 μM) tended to delay neutrophil apoptosis already after 6 hours of incubation and significantly delayed apoptosis after 9 and 12 hours (Figure 30 C-E). CDCA and UDCA (both 50 μM) also delayed apoptosis after 12 hours of incubation with healthy donor neutrophils (Figure 27 E). CA and DCA did not influence neutrophil apoptosis at 50 μM. Significant changes of apoptosis at baseline and 3-hour time point caused by CA, UDCA, CDCA and LCA were within 1-2% of cells, which is not physiologically relevant (Figure 30 A, B).

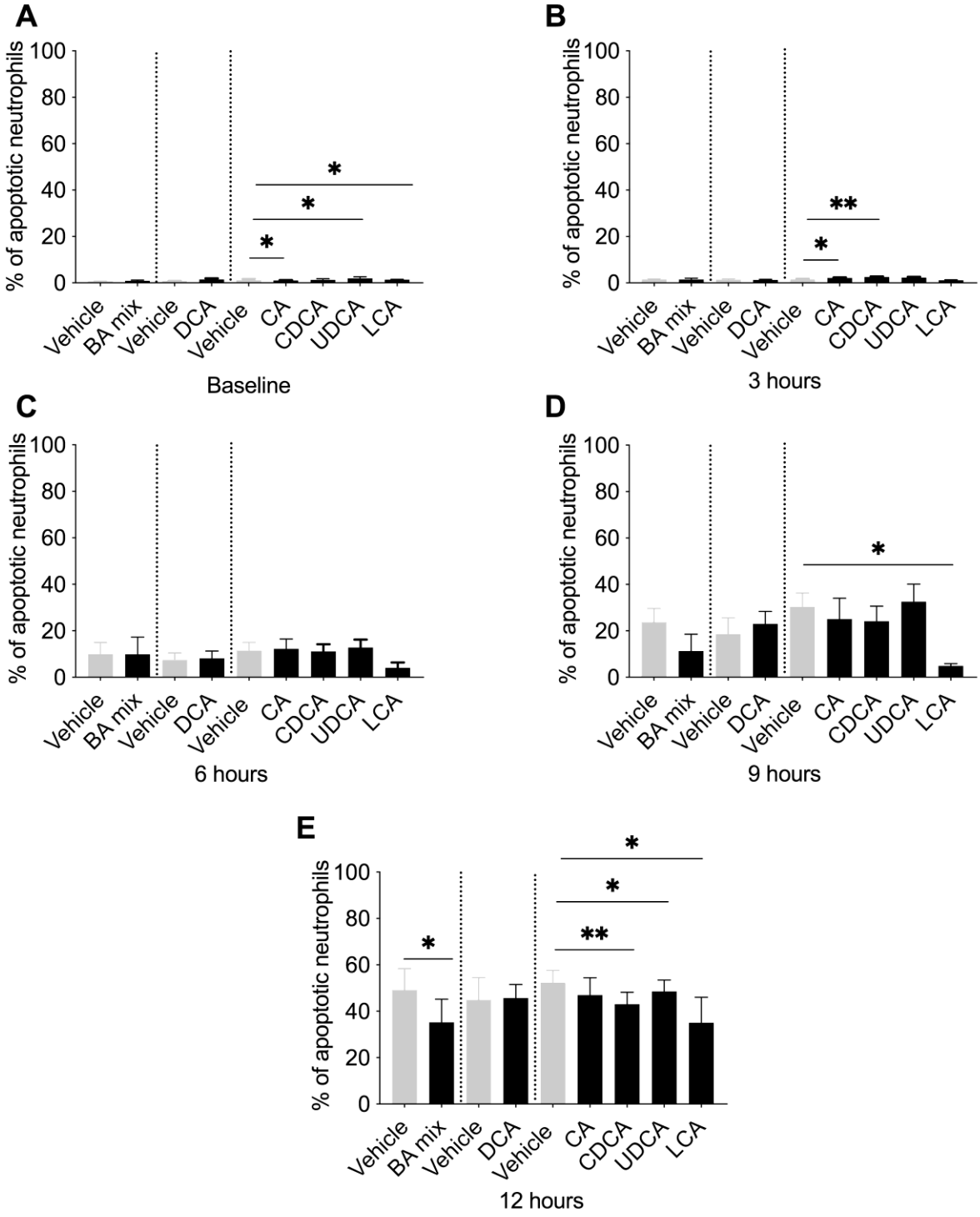


Figure 30. Bile acids delay early apoptosis.

(A-E) Neutrophils were incubated with bile acid mix or unconjugated bile acids at 50 μM/ vehicles (separate vehicles for BA mix (DMSO), DCA (distilled water) and other bile acids (DMSO)) up to 12 hours at 37°C. At baseline and further every 3 hours neutrophils were

stained with FITC-Annexin V and propidium iodide and percentage of Anx+PI- cells was recorded with flow cytometry. Data are represented as mean with SEM of at least 3 separate experiments. *$p < 0.05$,**$p < 0.01$ (paired t-test). These experiments were performed during my stay abroad at the lab of Dr. Sonja Vermeren (Centre for Inflammation Research, Queen's Medical Research Institute, University of Edinburgh).

When total apoptosis was analysed (early + late apoptosis + secondary necrosis), the changes were comparable with the early apoptosis alone – BA mix delayed neutrophil apoptosis at 12-hour time point and LCA delayed apoptosis at 9 and 12-hour time points (Figure 31 D, E).

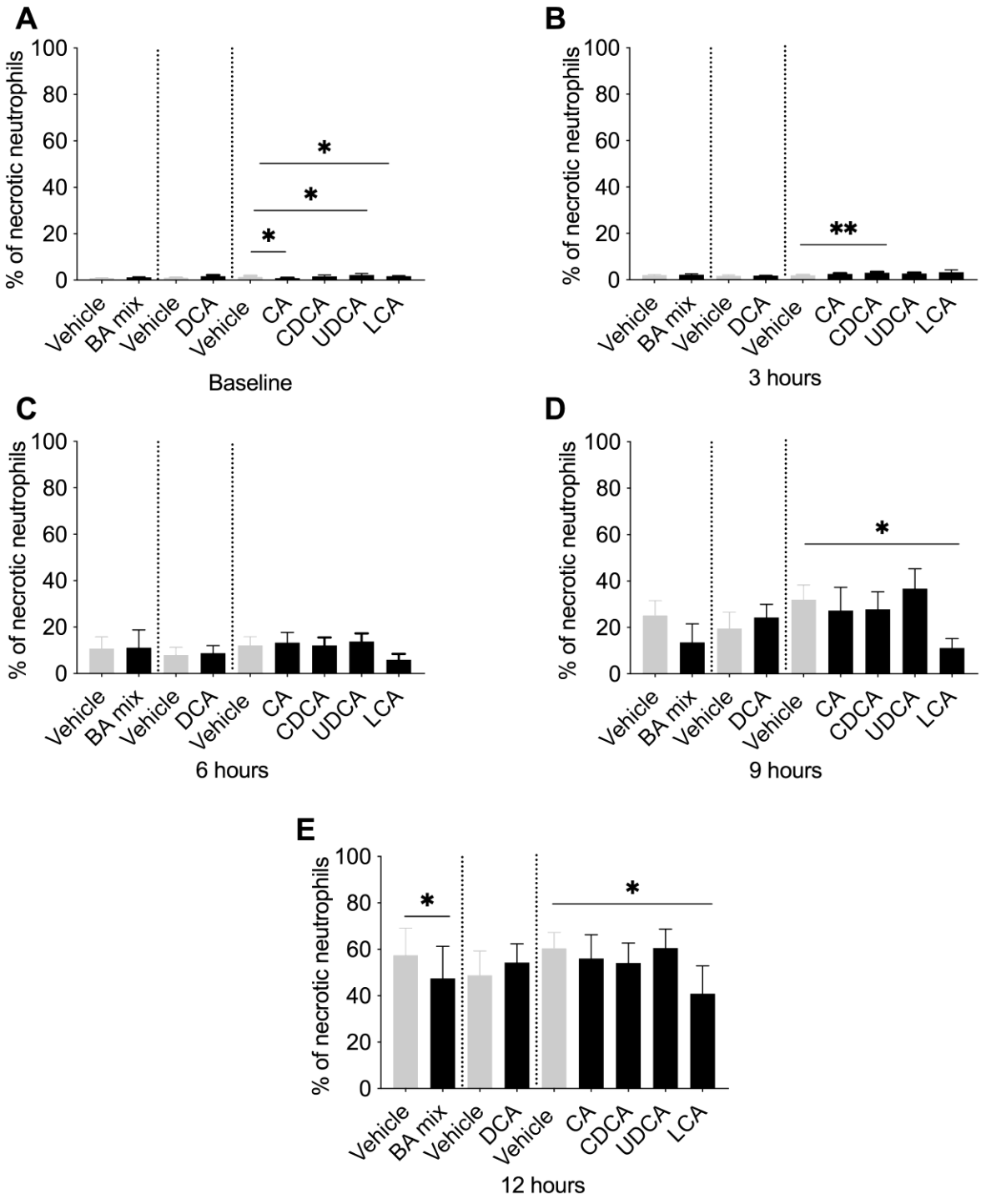


Figure 31. Bile acids delay total apoptosis.

(A-E) Neutrophils were incubated with bile acid mix or unconjugated bile acids at 50 μM/ vehicles (separate vehicles for BA mix (DMSO), DCA (distilled water) and other bile acids (DMSO)) up to 12 hours at 37°C. At baseline and further every 3 hours neutrophils were stained with FITC-Annexin V and propidium iodide and percentage of Anx+PI- & Anx+PI+ cells

was recorded with flow cytometry. Data are represented as mean with SEM of at least 3 separate experiments. *p < 0.05,**p < 0.01 (paired t-test). These experiments were performed during my stay abroad at the lab of Dr. Sonja Vermeren (Centre for Inflammation Research, Queen's Medical Research Institute, University of Edinburgh).

BA mix and unconjugated BAs at 50 µM were not cytotoxic for neutrophils throughout 12 hours of treatment, except for DCA, which showed 1% increase in the number of dead neutrophils at the baseline, which is not physiologically relevant and which was not proven at later time points (Figure 32 A).

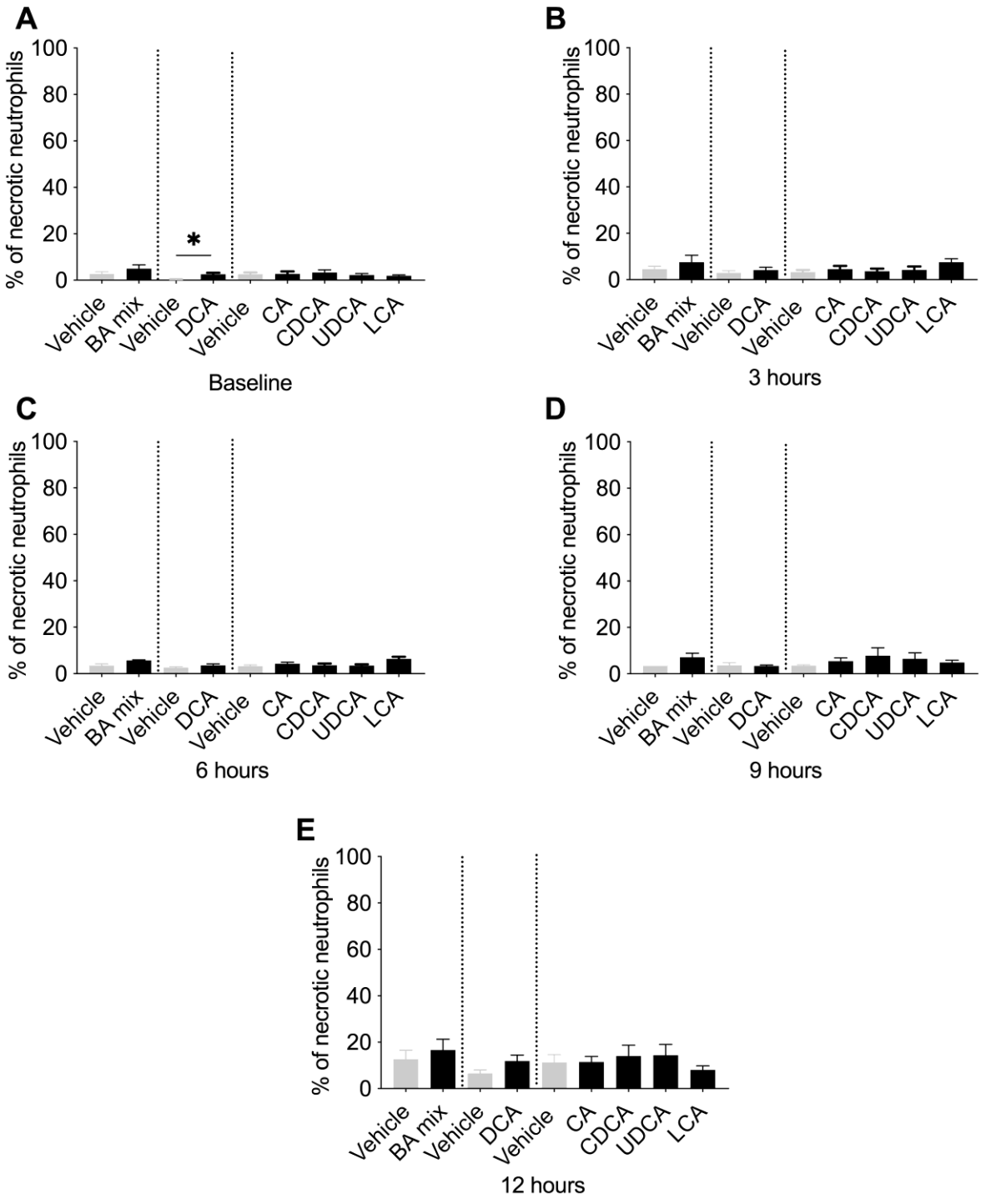


Figure 32. Bile acids do not influence viability of neutrophils.

(A-E) Neutrophils were incubated with bile acid mix or unconjugated bile acids at 50 μM/ vehicles (separate vehicles for BA mix (DMSO), DCA (distilled water) and other bile acids (DMSO)) up to 12 hours at 37°C. At baseline and further every 3 hours neutrophils were stained with FITC-Annexin V and propidium iodide and percentage of Anx-PI+ & Anx+PI+ cells

was recorded with flow cytometry. Data are represented as mean with SEM of at least 3 separate experiments. *p < 0.05,**p < 0.01 (paired t-test). These experiments were performed during my stay abroad at the lab of Dr. Sonja Vermeren (Centre for Inflammation Research, Queen's Medical Research Institute, University of Edinburgh).

To account for cytotoxicity of total BAs used in phagocytosis and ROS production experiments with healthy donor neutrophils, neutrophil viability was determined after 1-hour incubation with different concentrations of total BAs (Figure 33). No significant effect of total BAs on neutrophil viability was found, except for slightly increased number of dead neutrophils after incubation with total CDCA at 200 µM (Figure 33).

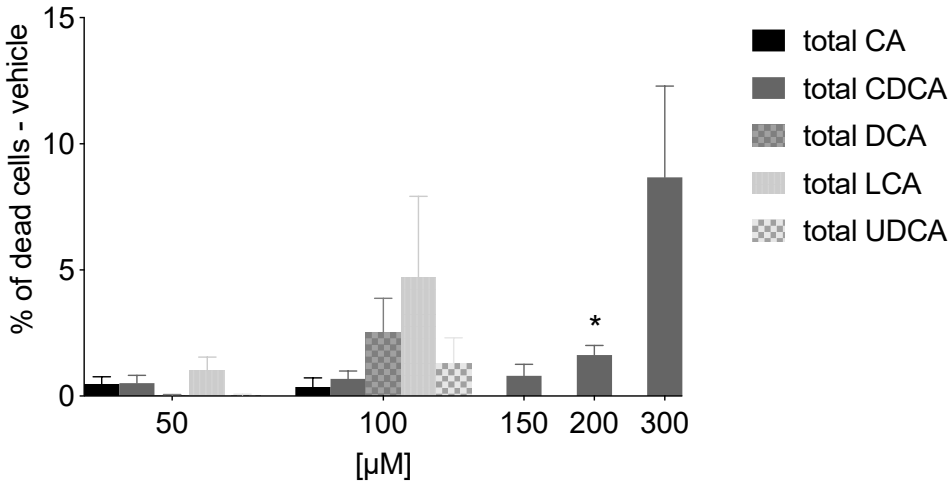


Figure 33. Viability of neutrophils after bile acid treatment.
Isolated neutrophils were stained with propidium iodide after 1 hour treatment with bile acids/vehicle (PBS or different concentrations of DMSO) and analysed by flow cytometry. Bars show mean percentage of dead neutrophils (propidium iodide positive) normalised to vehicle and represent a minimum of 4 separate experiments; error bars, SEM; *p<0.05 (unpaired t-test). Reproduced from (Balazs et al, 2021, in revision).

Mechanism of bile acid effects

BAs were shown to regulate immune cell function via range of surface and nuclear receptors (119). To test whether neutrophil function is in similar manner regulated by BAs via BA receptors, the expression of 8 BA receptors (surface receptors: TGR5, S1PR2, FPR1, FPR3, CHRM3; nuclear receptors: PXR, FXR, VDR) were tested in purified healthy donor neutrophils

with qPCR and in whole blood of healthy donors by flow cytometry. TGR5, S1PR2, VDR and FPR1 receptors were expressed by neutrophils on gene level and TGR5, VDR and FPR1 receptors were also detectable on protein level (Figure 34 A, B).

To study if the expression of BA receptors in neutrophils is different in cirrhosis, the neutrophil expression of TGR5, VDR and FPR1 receptors was measured in whole blood of cirrhotic patients. No significant differences were detected, however, FPR1 expression tended to decrease in cirrhotic patients compared to healthy controls (Figure 34 C). Furthermore, treatment with GCDCA tended to decrease FPR1 expression in isolated healthy donor neutrophils (Figure 34 D).

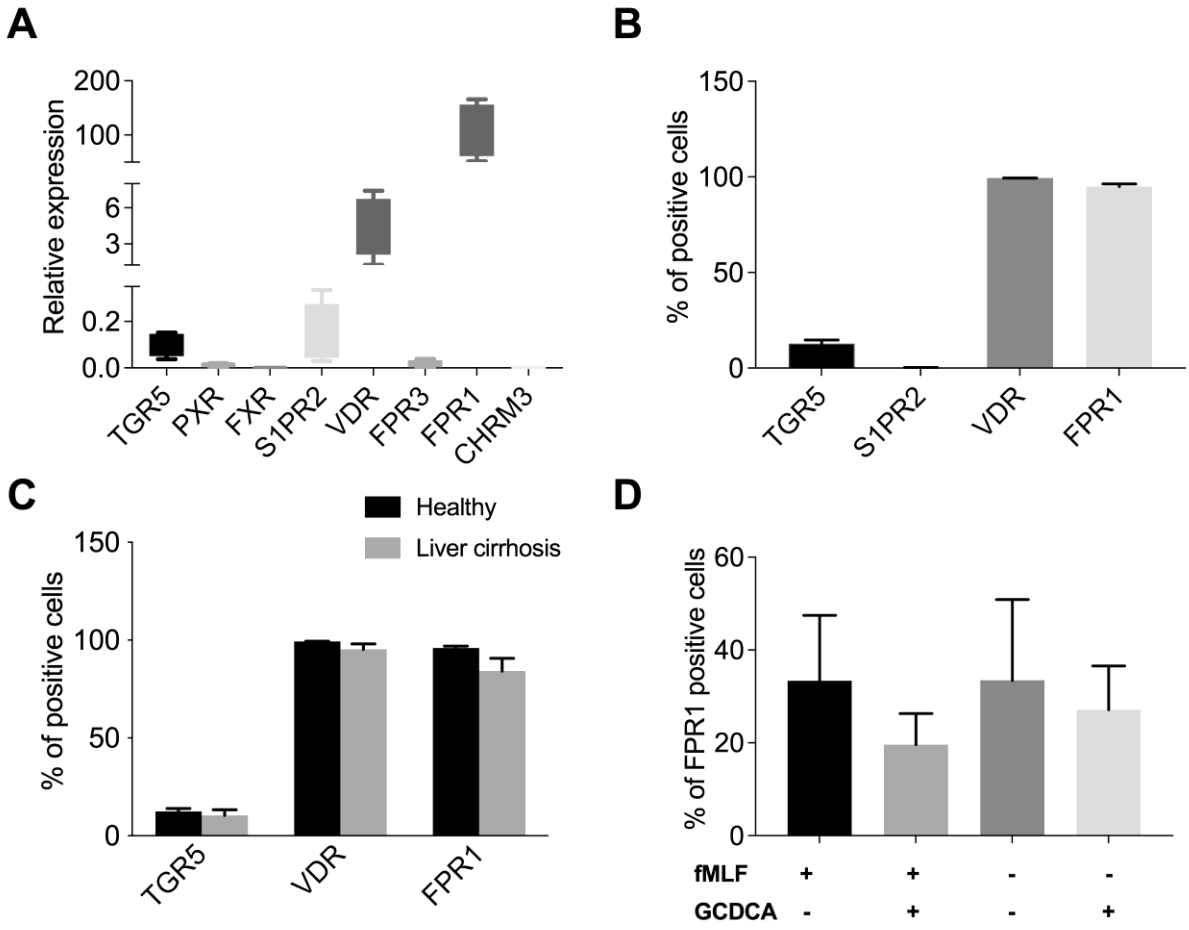


Figure 34. Bile acid receptors expression in neutrophils.

(A) Relative expression of main BA receptors in purified healthy donor neutrophils was determined with qPCR. (B-D) Percentage of (B-D) healthy donor neutrophils or (C) cirrhotic patient neutrophils, which express BA receptors, was quantified with flow cytometry in (B, C)

whole blood or (D) in isolated neutrophils. (D) Neutrophils were additionally stimulated with fMLF or/and GCDCA/vehicle on ice. Data represent a minimum of 4 separate experiments. (A) Whiskers show minimum and maximum values. (B-D) Mean with SEM. Statistical analysis was by unpaired t-test. Part of these experiments (panel D) was performed during my stay abroad at the lab of Dr. Sonja Vermeren (Centre for Inflammation Research, Queen's Medical Research Institute, University of Edinburgh).

The inhibition of neutrophil chemotaxis towards fMLF by BAs, especially via decrease in chemotaxis directedness, could be a result of FPR1 (fMLF receptor) blockage by BAs, which would prevent it from sensing chemoattractant (fMLF). Preliminary experiments were performed to investigate whether BAs (GCDCA) exhibit effects on chemotaxis similar to inhibitory effects of a potent FPR1 inhibitor CsH. Treatment of neutrophils with both GCDCA and CsH resulted in decreased chemotaxis directedness – neutrophils were moving in random directions, rather than towards fMLF. However, the effects of GCDCA and CsH differed. While neutrophils treated with GCDCA failed to migrate towards fMLF and at the same were characterized by decreased distance travelled, CsH interfered prevalently with directedness of chemotaxis, with the moving ability being visually intact (Figure 35 A-D).

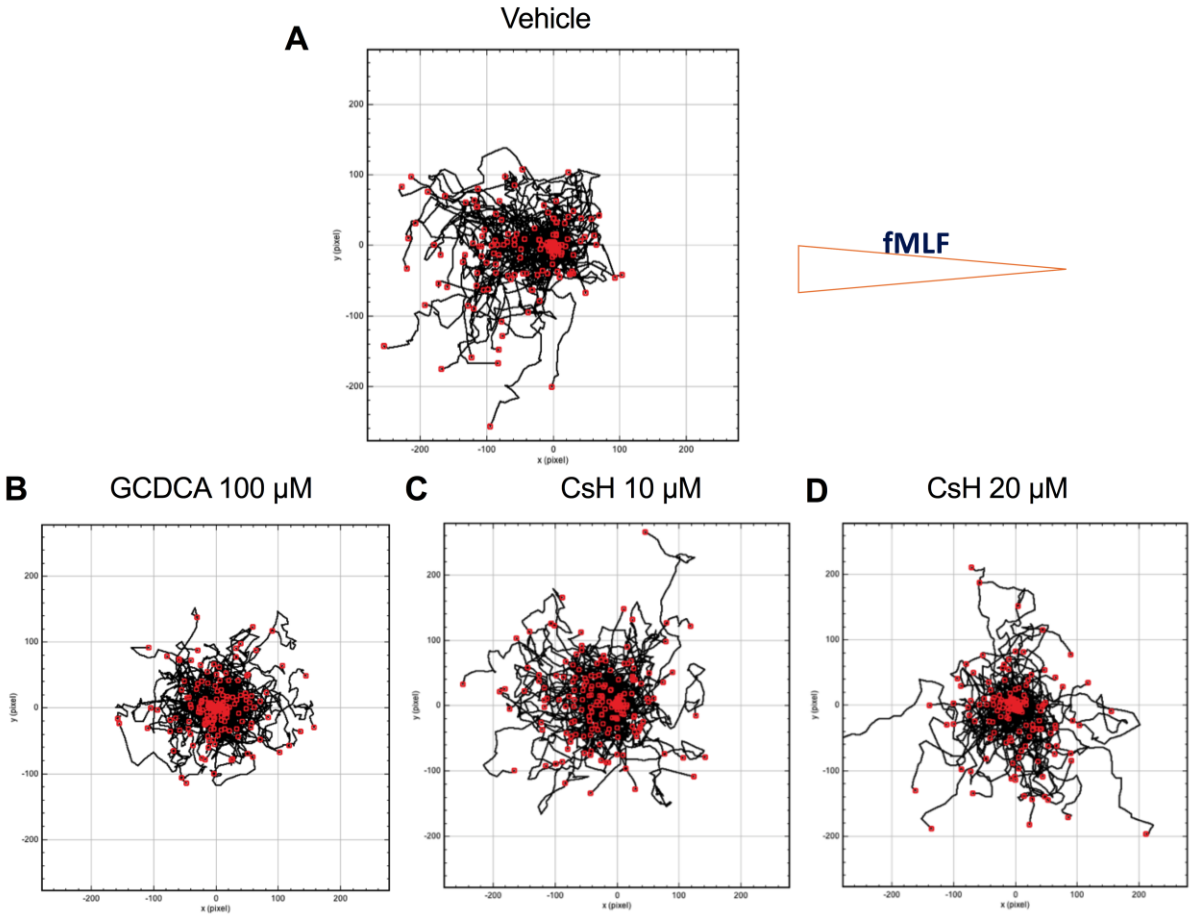


Figure 35. Bile acids effect on neutrophil chemotaxis differs from the effect of Cyclosporine H.

(A-D) Neutrophils were pre-treated with GCDCA/vehicle/CsH for 20 min and their migration towards fMLF [50 nM] was observed in real time for 30 min at 37°C with the help of inverted microscope. Analysis of migration tracks of each neutrophil and summary statistics were performed with ImageJ plugin "Track Maxima" (provided by Luke Tweedy, Beatson Institute, University of Glasgow). Representative spider plots are shown to visualize neutrophil migration types (only one experiment was performed for CsH 10 μM and for CsH 20 μM, therefore, no statistical analysis was possible). These experiments were performed during my stay abroad at the lab of Dr. Sonja Vermeren (Centre for Inflammation Research, Queen's Medical Research Institute, University of Edinburgh).

Bile acids and gut microbiome interactions in liver cirrhosis

BA metabolism is known to have close interactions with gut microbiome (102). The gut microbiome mediates secondary BA production via deconjugation of glycine and taurine conjugated BAs, as well as dihydroxylation of primary BAs (93). Therefore, the gut microbiome regulates BA composition in systemic circulation. BA in turn can regulate microbial community diversity due to their direct bactericidal effects and via FXR signalling, which induce transcription of antimicrobial agents (93). Therefore, it was of interest to study, whether there are associations between microbiome changes and BA composition changes in liver cirrhosis, as well as to study the gut microbiome composition changes between the groups of liver cirrhosis etiology.

Gut microbiome composition in liver cirrhosis depends on etiology

Cirrhotic patients were grouped according to the etiology of cirrhosis (alcoholic n=47, HCV n=16, other etiologies of cirrhosis n=19). Patients with cirrhosis had significantly decreased alpha diversity in comparison with healthy controls according to Chao1 index. No differences in alpha diversity between cirrhosis etiologies groups were found (Figure 36).

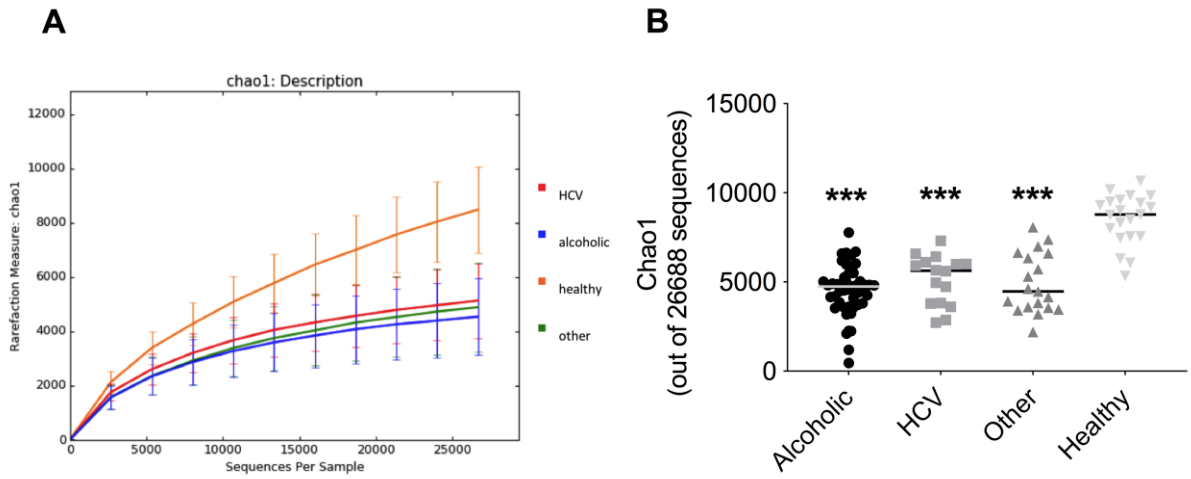


Figure 36. Rarefaction curve and alpha diversity changes between liver cirrhosis etiology groups and healthy controls.

(A) Rarefaction curve. (B) Chao1 index. Horizontal line indicates median. ***p<0.001 compared to healthy; statistical analysis was by Kruskall-Wallis test with Dunn's multiple comparisons test.

Redundancy analysis showed significant clustering of etiology groups (p=0.001). ANOSIM did not reveal significant differences between the groups. Analysis was performed at OTU level (Figure 37).

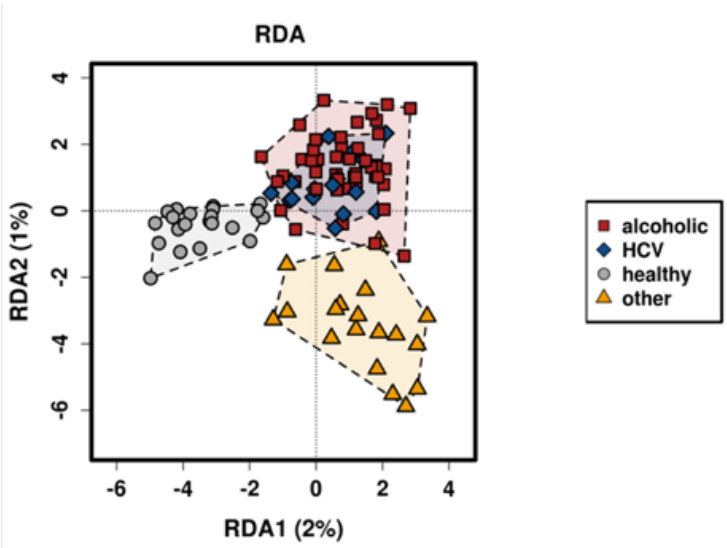


Figure 37. Redundancy analysis showed significant clustering of cirrhosis etiology groups and healthy controls at OTU level.

Redundancy analysis was performed at OTU level to analyse beta diversity of gut microbiome in different etiologies of cirrhosis and healthy controls.

Further group significance analysis showed 3 significantly different phyla, 6 classes, 9 orders, 20 families, 42 genera and 23 species (OTUs) (Tables 18-23). Most of the significant differences were compared to healthy controls. However, there were several differences at different levels also between the etiologies of cirrhosis.

The phylum Verrucomicrobia relative abundance was significantly lower in alcoholic cirrhosis group compared to other etiologies of cirrhosis (p=0.004) (Table 18).

Table 18. Taxonomic differences between different etiologies of liver cirrhosis and healthy controls at Phylum level.

Phylum	FDR corrected p value	Healthy (mean)	Other (mean)	HCV (mean)	Alcoholic (mean)
Tenericutes	0.002	0.001	0.0009	3.51E-05	5.82E-05
Other	0.018	0.015	0.010	0.0105	0.0102
Verrucomicrobia	0.018	0.0004	0.0005	0.0002	8.29E-05

The class Verrucomicrobiae was significantly lower abundant in alcoholic group in comparison with other etiologies of cirrhosis group (p=0.004) (Table 19).

Table 19. Taxonomic differences between the etiologies of liver cirrhosis and healthy controls at Class level.

Class	FDR corrected p value	Healthy (mean)	Other (mean)	HCV (mean)	Alcoholic (mean)
Bacilli	0.002	0.006	0.041	0.037	0.044
Mollicutes	0.002	0.001	0.0009	3.51E-05	5.82E-05
Firmicutes;Other	0.003	0.005	0.002	0.002	0.003
Other	0.016	0.015	0.010	0.011	0.010
Verrucomicrobiae	0.016	0.0004	0.0005	0.0002	8.21E-05

Clostridia	0.023	0.512	0.425	0.408	0.417

Order Verrucomicrobiales was significantly lower in alcoholic group compared to other etiology group (p=0.004) (Table 20).

Table 20. Taxonomic differences between the etiologies of liver cirrhosis and healthy controls at Order level.

Order	FDR corrected p value	Healthy (mean)	Other (mean)	HCV (mean)	Alcoholic (mean)
Lactobacillales	0.004	0.006	0.041	0.037	0.044
NB1-n	0.004	0.001	0.0009	9.37E-06	4.07E-05
Firmicutes;Other;Other	0.005	0.005	0.002	0.002	0.003
Thermoanaerobacterales	0.014	4.10E-05	2.08E-06	0	9.57E-06
Mollicutes RF9	0.017	0.0002	3.33E-05	2.58E-05	1.75E-05
Unassigned;Other;Other;Other	0.019	0.015	0.010	0.011	0.010
Verrucomicrobiales	0.019	0.0004	0.0005	0.0002	8.21E-05
Clostridiales	0.029	0.512	0.425	0.408	0.417
Rhodobacterales	0.032	1.61E-05	6.25E-06	2.34E-06	1.59E-06

Family Peptostreptococcaceae was significantly higher in alcoholic group compared to other etiology group (p=0.014) (Table 21).

Table 21. Taxonomic differences between the etiologies of liver cirrhosis and healthy controls at Family level.

Family	FDR corrected p value	Healthy (mean)	Other (mean)	HCV (mean)	Alcoholic (mean)
Christensenellaceae	0.005	0.030	0.004	0.004	0.004
NB1-n;Other	0.005	0.001	0.0009	9.37E-06	4.07E-05

Clostridiales vadinBB60 group	0.005	0.002	0.0007	7.73E-05	0.0003
Firmicutes;Other;Other;Other	0.005	0.005	0.002	0.002	0.003
Peptostreptococcaceae	0.005	0.023	0.004	0.008	0.009
Lactobacillaceae	0.005	0.003	0.024	0.012	0.009
Ruminococcaceae	0.005	0.233	0.128	0.153	0.146
Streptococcaceae	0.005	0.003	0.011	0.025	0.016
Thermoanaerobacteraceae	0.011	4.10E-05	1.97E-06	0	9.57E-06
Peptococcaceae	0.018	0.0004	2.96E-05	0.0002	0.0001
Unassigned;Other;Other;Other;Other	0.019	0.015	0.010	0.012	0.010
Micrococcaceae	0.028	8.21E-05	0.0002	0.0004	0.001
Verrucomicrobiaceae	0.031	0.0004	0.0004	0.0002	8.21E-05
Clostridiales;Family XIII	0.031	0.001	0.0004	0.001	0.0008
Mollicutes RF9;uncultured bacterium	0.033	0.0002	3.16E-05	2.11E-05	1.75E-05
Rhodobacteraceae	0.033	1.61E-05	5.92E-06	2.34E-06	1.59E-06
Clostridiaceae 1	0.046	0.005	0.005	0.003	0.002
Enterococcaceae	0.047	0.0001	0.004	9.84E-05	0.019
Comamonadaceae	0.047	5.17E-05	3.94E-06	0	1.04E-05
Defluviitaleaceae	0.047	0.0002	4.73E-05	4.22E-05	8.53E-05

Genus Prevotella from Prevatellaceae family was significantly higher in other etiology group compared to alcoholic (p=0.012) and HCV (p=0.014) groups. Genus Akkermansia from Verrucomicrobiaceae family was significantly lower in alcoholic cirrhosis compared to other etiologies of cirrhosis (p=0.022) (Table 22).

Table 22. Taxonomic differences between the etiologies of liver cirrhosis and healthy controls at Genus level. *Genera which were also shown to be significantly discriminative in LefSE analysis are marked in bold.

Genus	FDR corrected p value	Healthy (mean)	Other (mean)	HCV (mean)	Alcoholic (mean)
***Coprococcus 2**	0.010	0.004	0.0007	0.0001	0.0005
***Christensenellaceae R-7 group**	0.010	0.030	0.004	0.004	0.004
Ruminococcaceae UCG-014	0.010	0.028	0.010	0.003	0.005
NB1-n;Other;Other	0.010	0.001	0.0009	9.37E-06	4.07E-05
***[Eubacterium] coprostanoligenes group**	0.010	0.007	0.004	0.002	0.003
Christensenellaceae;uncultured	0.010	0.0002	1.18E-05	6.79E-05	7.41E-05
***Peptoclostridium**	0.010	0.020	0.003	0.006	0.008
Firmicutes;Other;Other;Other;Other	0.010	0.005	0.002	0.002	0.003
***Streptococcus**	0.010	0.003	0.011	0.025	0.016
***Lactobacillus**	0.010	0.003	0.024	0.012	0.009
***Anaerotruncus**	0.010	0.002	0.0006	0.0004	0.0008
uncultured	0.010	0.005	0.003	0.005	0.0003
Clostridiales vadinBB60 group;Other	0.011	0.0008	0.0005	1.41E-05	0.0001
***Blautia**	0.011	0.014	0.036	0.029	0.029
Intestinibacter	0.011	0.0001	2.37E-05	2.58E-05	3.19E-05
Terrisporobacter	0.012	0.0002	2.76E-05	8.20E-05	8.37E-05
Peptostreptococcaceae;Other	0.012	0.003	0.0004	0.002	0.001
[Eubacterium] oxidoreducens group	0.012	0.0001	5.52E-05	4.45E-05	5.26E-05
Lachnospiraceae UCG-006	0.012	1.43E-05	3.35E-05	0	1.59E-06
***Ruminococcaceae UCG-010**	0.012	0.001	0.0003	0.0001	0.0003
Marvinbryantia	0.012	0.0004	0.0001	0.0002	9.25E-05
***Ruminococcaceae NK4A214 group**	0.012	0.007	0.003	0.003	0.002
Ruminococcaceae UCG-005	0.013	0.005	0.002	0.005	0.002

Gelria	0.014	4.10E-05	1.97E-06	0	9.57E-06
Peptococcaceae;uncultured	0.018	0.0002	9.86E-06	4.68E-06	6.38E-05
uncultured bacterium	0.021	0.0008	0.0001	6.32E-05	0.0002
Unassigned;Other;Other;Other; Other;Other	0.026	0.015	0.010	0.012	0.010
***Ruminiclostridium 6**	0.026	0.008	0.0005	0.004	0.004
***Ruminococcaceae UCG-011**	0.028	0.0004	1.97E-05	3.51E-05	0.0004
Prevotella 7	0.033	0.009	0.019	1.87E-05	0.0005
***[Eubacterium] xylanophilum group**	0.035	0.003	0.0006	0.001	0.0006
***Rothia**	0.037	8.21E-05	0.0002	0.0004	0.001
Clostridiaceae 1;Other	0.037	0.000196 272	0.000396 393	0.000100 701	0.000147 488
Ruminococcaceae;Other	0.041	0.025	0.012	0.016	0.015
Akkermansia	0.041	0.0004	0.0004	0.0002	8.21E-05
***Clostridium sensu stricto 1**	0.042	0.005	0.002	0.003	0.002
***Lachnospiraceae NC2004 group**	0.044	0.002	0.0004	0.0004	0.0008
Veillonella	0.046	0.0003	0.003	0.007	0.011
Ruminococcaceae UCG-009	0.046	0.0001	1.18E-05	3.75E-05	4.62E-05
Mollicutes RF9;uncultured bacterium	0.046	0.0002	3.16E-05	2.11E-05	1.75E-05
Rhodobacteraceae;uncultured	0.046	1.61E-05	5.92E-06	2.34E-06	1.59E-06
Catenisphaera	0.049	0.001	0	1.41E-05	0

LefSe analysis revealed 23 discriminative features, which reached absolute LDA score>2 (Figure 38).

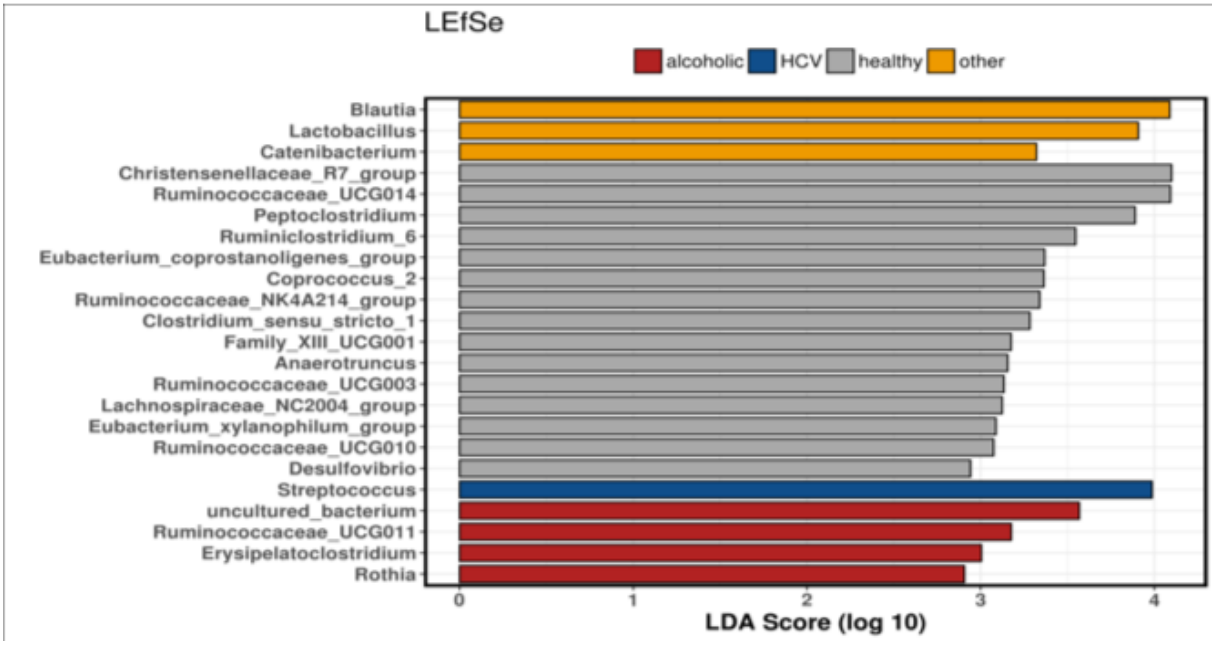


Figure 38. LefSe analysis to find discriminative features between the etiologies of liver cirrhosis and healthy controls at Genus level.

LefSE analysis was performed in Calypso at genus level. Features with LDA score>2 are shown.

23 OTUs were significantly differently abundant between the groups, but all showed the difference only between healthy group and cirrhosis groups and not between the etiologies of cirrhosis. All of these OTUs were higher abundant in healthy controls compared to cirrhotic patients (Table 23).

Table 23. Taxonomic differences between the etiologies of liver cirrhosis and healthy controls at OTU level.

OTU	FDR corrected p value	Healthy (mean)	Other (mean)	HCV (mean)	Alcoholic (mean)
Faecalibacterium prausnitzii	0.013	0.810	0	0	0
Massilioclostridium coli	0.013	8.905	1.053	0	0
Campylobacter hominis	0.013	1	0.526	0	0
Oscillibacter valericigenes	0.013	15.048	2.053	0	0.106

Intestinimonas butyriciproducens	0.014	3.381	1	3.188	0.298
Romboutsia timonensis	0.016	25.524	3.474	7.188	10.766
Paraburkholderia sacchari	0.018	20.714	14.211	0	0
Oscillibacter ruminantium GH1	0.018	2.905	0.158	0	0.596
Romboutsia timonensis	0.018	57.1904762	9.26315789	14.0625	13.8510638
Oscillibacter valericigenes	0.018	6.381	0	0	0
Ruminococcus faecis	0.018	0.762	0	0.125	0
Faecalibacterium prausnitzii	0.018	1.143	0.053	0	0.064
Ruminococcus bromii	0.018	3.381	0	0	0.043
Geosporobacter ferrireducens	0.018	0.905	0	0	0.064
Ruminococcaceae bacterium	0.023	2.191	0.316	0.125	0.149
Clostridium disporicum	0.023	0.667	0	0	0.064
Romboutsia timonensis	0.029	22.238	1.947	8.375	7.128
Intestinibacter bartlettii	0.031	0.714	0	0.063	0.170
Romboutsia timonensis	0.031	1.095	0	0	0.234
Geosporobacter ferrireducens	0.043	3.238	0.158	0.125	0.404
Geosporobacter ferrireducens	0.043	2.095	0.105	0.063	0.043
Faecalibacterium prausnitzii	0.043	1.191	0.105	0	0.170
Thermoanaerobacterium thermosaccharolyticum	0.045	17.524	0.737	0.063	5.085

Microbiome functions were predicted with PICRUSt. RDA analysis of PICRUSt data showed that functional characteristics of gut microbiome significantly varied between the groups of cirrhosis etiology and healthy controls (p=0.023) (Figure 39). ANOSIM did not reveal any significant differences.

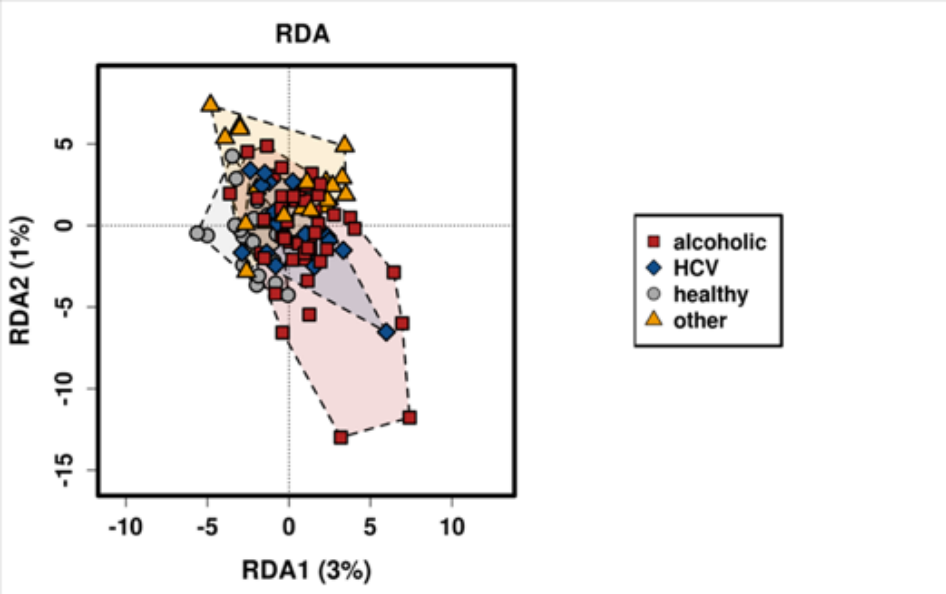


Figure 39. RDA revealed significant clustering based on microbiome functional content differences between the cirrhosis etiology groups.

RDA analysis was performed to study the differences in microbiome functional content between the etiologies of liver cirrhosis and healthy controls (with normalized PICRUSt data).

LefSe analysis of PICRUSt data revealed 12 differentially abundant pathways between etiologies of cirrhosis and healthy controls. Alcoholic etiology of cirrhosis was associated with galactose metabolism, glycan degradation and protein kinases, HCV was associated with polyketide sugar synthesis and propanoate metabolism, other etiologies of cirrhosis were associated with glycosyltransferases (Figure 40).

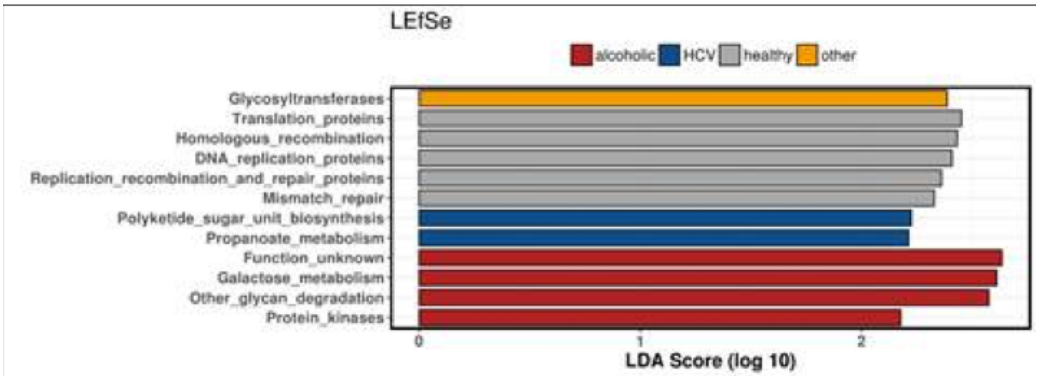
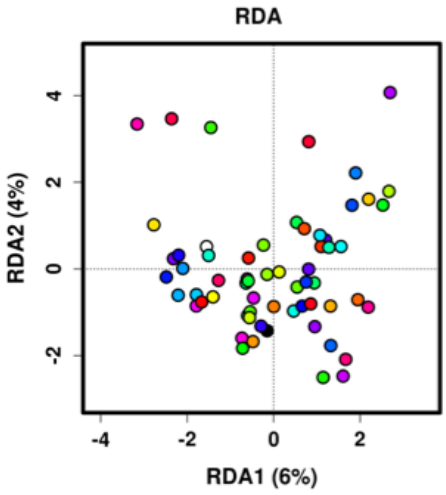


Figure 40. LefSe analysis revealed 12 differentially abundant pathways.

LefSe analysis was performed to study the differences in microbiome functional content between the etiologies of liver cirrhosis and healthy controls (with normalized PICRUSt data). LefSe analysis revealed 12 differentially abundant pathways (LDA score>2).

Association of BA composition and gut microbiome composition in liver cirrhosis

RDA at genus level identified GUDCA and DCA as the most important explanatory variables for interindividual gut microbiome composition variance in Child-Pugh A cirrhotic patients (n=61) (Figure 41).



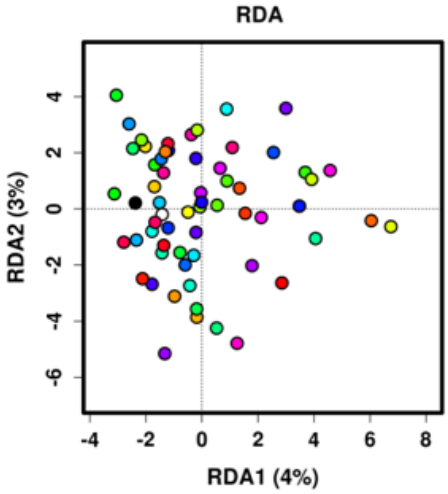
RDA Significance

	Variance	F	P	Signif
Healthy_cirrhosis	0.00			
Child_Pugh	0.00			
Etiology	3.94	1.13	0.001	***
Sex	1.93	1.11	0.262	
Age	1.82	1.05	0.354	
TUDCA	2.05	1.18	0.233	
GUDCA	2.92	1.68	0.012	*
TCA	1.51	0.87	0.649	
GCA	2.19	1.26	0.130	
TCDCA	2.04	1.17	0.243	
UDCA	1.62	0.93	0.577	
GCDCA	2.00	1.15	0.240	
TDCA	2.13	1.22	0.154	
GDCA	1.37	0.79	0.706	
CA	2.85	1.64	0.098	
TLCA	1.89	1.09	0.315	
GLCA	1.38	0.79	0.666	
CDCA	2.09	1.20	0.210	
DCA	2.57	1.48	0.036	*
LCA	1.68	0.97	0.452	

Figure 41. RDA at genus level identified GUDCA and DCA to be the most important explanatory variables for interindividual gut microbiome composition differences in liver cirrhosis.

RDA was performed in Calypso. All 15 BA (absolute concentrations) were included in the model as explanatory variables. Etiology, sex and age were also included in the model to account for their contribution to the analysis results.

RDA at OTU level showed GCDCA, DCA and CA as the most important explanatory variables (Figure 42).



RDA Significance

	Variance	F	P	Signif
Healthy_cirrhosis	0.00			
Child_Pugh	0.00			
Etiology	26.93	1.08	0.001	***
Sex	13.89	1.11	0.170	
Age	14.30	1.15	0.103	
TUDCA	12.57	1.01	0.437	
GUDCA	14.81	1.19	0.058	
TCA	11.84	0.95	0.617	
GCA	13.39	1.07	0.216	
TCDCA	12.24	0.98	0.531	
UDCA	11.07	0.89	0.840	
GCDCA	16.03	1.28	0.023	*
TDCA	12.74	1.02	0.401	
GDCA	11.80	0.94	0.620	
CA	19.96	1.60	0.004	**
TLCA	12.91	1.03	0.340	
GLCA	10.53	0.84	0.839	
CDCA	13.14	1.05	0.308	
DCA	16.38	1.31	0.017	*
LCA	13.89	1.11	0.166	

Figure 42. RDA at OTU level identified GCDCA, DCA and CA to be the most important explanatory variables for gut microbiome composition differences in liver cirrhosis. RDA was performed in Calypso. All 15 BA (absolute concentrations) were included in the model as explanatory variables. Etiology, sex and age were also included in the model to account for their contribution to the analysis results.

Spearman correlation analysis with Benjamini-Hochberg multiplicity correction was performed to investigate the relationships between 15 BA absolute concentrations and relative abundances with 91 bacterial genera. Further Spearman partial correlation was applied to significantly correlated variable pairs to account for the possible confounding of cirrhosis etiology, age and sex.

Genus Butyricimonas abundance significantly increased with TDCA and GDCA concentrations increase. Genera Ruminococcaceae NK4A214 group and Ruminococcaceae UCG-005 abundance significantly decreased when UDCA concentration was elevated (Table 24).

Table 24. Spearman partial correlation of bacterial genera and bile acids (absolute concentrations) within Child-Pugh A cirrhotic patients (n=61) controlling for confounding of liver cirrhosis etiology, age and sex.

Genus	UDCA	TDCA	GDCA	TLCA

Butyricimonas		R=0.508 P=<0.001	R=0.458 P=<0.001	
D_3__Gastranaerophilales;Other;Other				R= 0.464 P=<0.001
Ruminococcaceae NK4A214 group	R= -0.626 P<0.001			
Ruminococcaceae UCG-005	R= -0.473 P<0.001			

Genus Blautia abundance significantly increased with increasing GUDCA relative abundance. Genus Ruminococcaceae NK4A214 group abundance significantly decreased with the increase of UDCA relative abundance. Genus Ruminococcaceae UCG-014 increased with increasing TDCA relative abundance (Table 25). No significant correlations at OTU level were revealed.

Table 25. Spearman partial correlation of bacterial genera and bile acids (relative abundance) within Child-Pugh A cirrhotic patients (n=61) controlling for confounding of liver cirrhosis etiology, age and sex.

Genus	**UDCA**	**TDCA**	**GUDCA**
Blautia			R=0.467 P<0.001
Ruminococcaceae NK4A214 group	R= -0.579 P<0.001		
Ruminococcaceae UCG-014		R= 0.494 P<0.001	

Further mixed effect regression model was carried out in Calypso with significantly correlated variable pairs controlling for etiology influence (with absolute BA concentrations). It showed that UDCA can predict the Ruminococcaceae_UCG005 (p=0.012) and Ruminococcaceae NK4A214 group (p=0.008) genera levels and TDCA (p<0.001) and GDCA (p=0.004) can predict Butyricimonas genus levels. Random Forest identified these bacterial genera in the top 5 associated features with mentioned above BAs. Association of TDCA with Butyricimonas (importance 6.36) and Prevotella_9 (importance 5.04) genera abundance was shown by LASSO regularized regression.

Serum protein composition analysis

In order to screen for serum proteins, which can potentially modify neutrophil function in liver cirrhosis, serum protein composition in albumin and IgG depleted serum samples from HCV cirrhotic patients with poor neutrophil phagocytic capacity (sera from 3 different patients were pooled together for the analysis) and healthy controls (sera from 3 different healthy controls were pooled together for the analysis) were analyzed with 2D gel electrophoresis and gas chromatography–mass spectrometry (2D gel electrophoresis and mass spectrometry were performed by Angela Horvath, Christoph Nusshold and Gerald Rechberger, all from Medical University of Graz). Table 26 summarizes all differentially abundant proteins between HCV cirrhotic and healthy control serum samples.

Table 26. Differences in protein expression in serum of CHC patients compared to healthy controls.

Names and UniProt database accession numbers of the identified proteins, along with their spot number in gels, number of acquired spectra, amount of distinct peptides, distinct summed MS/MS score, percent of amino acid coverage, theoretical and experimental molecular weight (MW) and isoelectric point (pI), as well as their fold regulation in CHC patients compared to healthy controls are given. Differentially-expressed (≥ 2-fold) proteins with fitting MW and pI (max. deviation of “MW exper.” = 20% higher or 10% lower of “MW theor.”; max. deviation of “pI exper.” = ± 2 of “pI theor.”) that were identified in at least two out of three gels with a minimal MS/MS search score of 20 were selected. In addition all other differentially expressed proteins with an MS/MS search score of ≥ 80 were included in the table to account for proteins that could represent potential splice variants or harbour excessive posttranslational modifications. These protein spots (marked with “*”), however, were not included in further analyses. Reproduced from (34) with permission of Elsevier Inc.

Protein Name	UniProt accession number	Spot	Spectra	Distinct peptides	Distinct summed MS/MS	% AA coverage	MW theor. (Da)	MW exper. (Da)	pI theor.	pI exper.	Regulation control/HCV (Cy2/Cy3)

					score						
Alpha-2-macroglobulin	**P01023.3**	125*	162	30	**492.08**	21.50	164714.3	184450.0	6.03	9.55	2.53
		130	9	7	**92.65**	4.60	164714.3	184450.0	6.03	5.55	-2.33
		133*	124	28	**464.75**	19.20	164714.3	184450.0	6.03	9.40	2.23
		137*	41	18	**275.07**	12.80	164714.3	177228.1	6.03	3.39	5.04
		141*	26	13	**202.47**	8.80	164714.3	177228.1	6.03	3.49	2.28
		258*	103	18	**297.72**	12.70	164714.3	133967.9	6.03	9.67	2.53
		267*	68	16	**265.18**	10.70	164714.3	133967.9	6.03	9.37	2.31
		92*	55	18	**282.31**	13.90	164714.3	117228.1	6.03	3.15	5.25
		108*	79	22	**371.35**	18.00	164714.3	192230.8	6.03	9.36	2.14
		110*	115	32	**541.93**	26.20	164714.3	192230.8	6.03	9.51	2.60
		124	6	3	**42.28**	1.70	164714.3	184450.0	6.03	5.59	-2.01
		126	19	10	**147.19**	7.10	164714.3	177228.1	6.03	5.47	-2.08
		144	3	1	**20.33**	0.80	164714.3	177228.1	6.03	5.68	-2.53
		281*	60	15	**235.66**	10.90	164714.3	142863.4	6.03	9.39	2.81
		113*	90	25	**402.66**	18.50	164714.3	170507.0	6.03	3.31	5.28
Ceruloplasmin	**P00450.1**	258*	10	7	**100.10**	6.50	123058.7	133967.9	5.44	9.67	2.53
		126*	3	2	**26.74**	2.50	123058.7	177228.1	5.44	5.47	-2.08
		281	76	15	**253.94**	17.30	123058.7	142863.4	5.44	9.39	2.81
		295*	34	9	**144.54**	9.50	123058.7	122345.0	5.44	4.65	-2.73
Ig mu chain C region	**P01871.3**	423*	14	8	**114.73**	19.00	49990.3	98643.6	6.35	5.03	-2.10
		464*	55	14	**232.49**	34.90	49990.3	93934.5	6.35	5.55	2.11
		484*	68	15	**247.03**	38.20	49990.3	93934.5	6.35	5.76	2.23
		513*	65	14	**242.73**	35.30	49990.3	83697.2	6.35	6.44	-2.04
		543*	30	11	**177.54**	26.70	49990.3	83697.2	6.35	6.93	-2.27
		552*	50	13	**216.75**	32.00	49990.3	83697.2	6.35	6.68	-2.28
		561*	31	13	**206.75**	32.00	49990.3	83697.2	6.35	6.78	-3.04
		577*	27	12	**185.37**	28.70	49990.3	80117.3	6.35	3.08	2.41
		857	4	3	**42.69**	5.90	49990.3	53817.7	6.35	6.13	-2.67
		868	22	5	**72.45**	11.20	49990.3	54738.4	6.35	6.38	-2.74
		996	48	9	**161.59**	24.50	49990.3	45782.7	6.35	6.16	2.81
		370*	72	10	**153.56**	25.40	49990.3	115546.9	6.35	6.25	-2.92
		438*	41	8	**122.77**	20.10	49990.3	93934.5	6.35	4.89	-2.20
		455*	179	15	**257.96**	38.20	49990.3	93934.5	6.35	6.04	2.19
		505*	143	14	**243.91**	36.50	49990.3	87554.3	6.35	5.98	2.85
		567*	53	13	**181.23**	31.40	49990.3	85589.1	6.35	6.58	-2.08
		649*	23	7	**105.97**	16.80	49990.3	76786.0	6.35	5.98	-3.97

		770_2*	14	6	**81.02**	14.30	49990.3	60819.2	6.35	6.25	5.53
		778	12	6	**84.93**	14.60	49990.3	59733.8	6.35	6.37	2.18
		853	4	1	**20.46**	2.40	49990.3	54738.4	6.35	6.40	-3.15
		951	87	10	**169.09**	26.30	49990.3	52046.7	6.35	5.89	7.10
		318*	19	8	**127.88**	20.10	49990.3	112392.1	6.35	6.28	-2.42
		411*	108	13	**224.90**	31.80	49990.3	87554.3	6.35	6.49	2.39
		444*	37	8	**134.66**	20.10	49990.3	87554.3	6.35	6.71	-2.37
		447*	132	15	**264.15**	38.20	49990.3	89597.1	6.35	5.91	3.46
		451*	127	14	**231.81**	35.30	49990.3	87554.3	6.35	6.09	2.56
		458*	141	15	**267.25**	38.20	49990.3	85589.1	6.35	6.31	2.04
		462*	36	9	**145.59**	23.40	49990.3	85589.1	6.35	6.86	-2.47
Ig alpha-2 chain C region	**P01877.3**	707*	94	8	**148.98**	25.20	37324.4	64271.1	5.72	5.40	2.37
		714*	67	6	**104.13**	19.10	37324.4	63086.3	5.72	5.80	2.53
		724*	46	8	**138.32**	25.20	37324.4	61936.2	5.72	5.98	2.72
		743*	35	6	**102.15**	19.10	37324.4	61936.2	5.72	6.13	3.96
		769*	35	6	**102.80**	19.10	37324.4	58678.8	5.72	6.22	7.32
		780*	31	6	**103.92**	19.10	37324.4	61936.2	5.72	3.05	4.73
		649*	55	7	**110.97**	20.50	37324.4	76786.0	5.72	5.98	-3.97
		749*	61	7	**103.09**	20.50	37324.4	61936.2	5.72	5.86	2.77
		775_2*	98	7	**109.69**	21.10	37324.4	60819.2	5.72	6.16	3.79
Ig gamma-1 chain C region	**P01857.1**	927*	45	8	**127.19**	33.00	36618.7	52920.7	8.92	9.15	5.30
		830*	22	6	**96.07**	22.40	36618.7	55863.8	8.92	9.28	5.17
Immunoglobulin lambda-like polypeptide 5	**B9A064.2**	1320	24	5	**77.98**	30.30	23405.2	26369.9	9.65	9.76	3.29
		1479*	39	6	**96.38**	39.70	23405.2	24776.6	9.65	5.86	5.54
		1513*	38	6	**86.81**	35.00	23405.2	25085.4	9.65	5.91	4.22
		1576*	6	4	**43.59**	18.60	23405.2	22740.2	9.65	5.32	3.17
Ig lambda-2 chain C regions	**P0CG05.1**	1344*	48	6	**101.58**	82.00	11464.5	25085.4	6.94	5.76	3.86
		1353*	30	5	**81.06**	63.20	11464.5	24776.6	6.94	6.22	4.84
		1479*	57	6	**102.17**	82.00	11464.5	24776.6	6.94	5.86	5.54
		1500*	43	6	**96.20**	82.00	11464.5	23587.0	6.94	6.07	4.66
		1511*	30	5	**82.80**	67.90	11464.5	22740.2	6.94	6.43	4.57
		1521*	26	6	**85.37**	82.00	11464.5	22740.2	6.94	6.88	3.73

		1510*	39	5	**80.22**	63.20	11464.5	24776.6	6.94	5.32	2.87
		1544*	43	5	**81.52**	63.20	11464.5	23300.6	6.94	6.25	4.85
		1565*	51	5	**80.73**	63.20	11464.5	23877.7	6.94	6.80	3.64
		1572*	35	5	**80.08**	63.20	11464.5	22740.2	6.94	6.55	4.26
		1580*	49	5	**82.58**	63.20	11464.5	25085.4	6.94	5.69	3.20
Ig kappa chain C region	**P01834.1**	1381*	31	5	**93.57**	79.20	11779.8	23587.0	5.58	5.92	4.57
		1382*	27	5	**97.55**	79.20	11779.8	23877.7	5.58	5.76	3.74
		1389*	36	6	**103.95**	85.80	11779.8	23018.3	5.58	8.94	4.71
		1393*	34	6	**106.67**	85.80	11779.8	23300.6	5.58	9.30	4.38
		1395*	24	5	**93.56**	79.20	11779.8	23587.0	5.58	5.40	3.52
		1410*	71	5	**98.89**	79.20	11779.8	22466.0	5.58	7.72	3.81
		1412*	28	5	**98.47**	79.20	11779.8	22740.2	5.58	6.22	4.04
		1414*	42	5	**95.84**	79.20	11779.8	22466.0	5.58	5.92	4.75
		1415*	39	5	**97.00**	79.20	11779.8	22466.0	5.58	7.51	5.46
		1424*	30	5	**95.19**	79.20	11779.8	22195.8	5.58	6.99	3.54
		1429*	47	5	**97.28**	79.20	11779.8	22740.2	5.58	6.87	5.12
		1502*	83	6	**108.06**	85.80	11779.8	23587.0	5.58	7.61	5.39
		1508*	80	5	**97.30**	79.20	11779.8	23018.3	5.58	7.46	4.97
		1511*	110	6	**108.68**	85.80	11779.8	22740.2	5.58	6.43	4.57
		1520*	48	5	**96.78**	79.20	11779.8	22740.2	5.58	5.89	5.92
		1521*	79	5	**96.38**	79.20	11779.8	22740.2	5.58	6.88	3.73
		1530*	47	5	**95.36**	79.20	11779.8	24472.4	5.58	8.85	4.40
		1544*	40	5	**95.31**	79.20	11779.8	23300.6	5.58	6.25	4.85
		1549*	84	6	**109.71**	85.80	11779.8	23587.0	5.58	7.61	3.94
		1557*	92	6	**114.45**	85.80	11779.8	23300.6	5.58	7.42	4.92
		1565*	46	6	**108.15**	85.80	11779.8	23877.7	5.58	6.80	3.64
		1572*	72	6	**107.82**	85.80	11779.8	22740.2	5.58	6.55	4.26
		1576*	30	5	**90.34**	70.70	11779.8	22740.2	5.58	5.32	3.17
		1594*	54	6	**106.52**	85.80	11779.8	22466.0	5.58	5.85	5.69
Apolipoprotein A-I	**P02647.1**	1450*	71	14	**249.93**	50.90	30777.3	21152.6	5.56	5.03	2.74
		1453*	104	19	**332.61**	64.40	30777.3	20900.8	5.56	5.15	2.93
		1576*	19	8	**130.27**	31.40	30777.3	22740.2	5.56	5.32	3.17
		1636*	137	15	**252.44**	53.10	30777.3	21929.5	5.56	5.07	-3.39
Complement factor H	**P08603.4**	125*	12	9	**131.82**	6.60	143772.3	184450.0	6.22	9.55	2.53
		130	8	4	**47.64**	2.70	143772.3	184450.0	6.22	5.55	-2.23
		124	37	16	**257.80**	14.40	143772.3	184450.0	6.22	5.59	-2.01
		126	87	27	**453.26**	26.60	143772.3	177228.1	6.22	5.47	-2.08

		83	19	8	**119.65**	6.90	143772.3	184450.0	6.22	5.57	-2.12
Serotransferrin	**P02787.3**	137*	6	6	**96.05**	9.40	79344.7	177228.1	6.86	3.39	5.04
		370*	15	8	**137.04**	13.00	79344.7	115546.9	6.86	6.25	-2.92
		400*	16	7	**101.22**	11.70	79344.7	103774.0	6.86	3.64	-2.82
		464	8	5	**75.12**	8.00	79344.7	93934.5	6.86	5.55	2.11
		484	18	8	**119.71**	13.00	79344.7	93934.5	6.86	5.76	2.23
		513	74	20	**340.62**	29.30	79344.7	83697.2	6.86	6.44	-2.04
		543	244	41	**721.91**	54.10	79344.7	83697.2	6.86	6.93	-2.27
		552	200	36	**649.28**	53.10	79344.7	83697.2	6.86	6.68	-2.28
		561	222	43	**744.20**	60.30	79344.7	83697.2	6.86	6.78	-3.04
		566	3	2	**39.46**	4.10	79344.7	80117.3	6.86	5.40	-3.58
		577*	62	18	**306.76**	25.50	79344.7	80117.3	6.86	3.08	2.41
		868*	18	6	**88.53**	9.00	79344.7	54738.4	6.86	6.38	-2.74
		876*	27	7	**128.20**	11.80	79344.7	52046.7	6.86	6.68	-2.59
		136*	30	11	**173.73**	18.60	79344.7	158373.1	6.86	6.76	-2.15
		455	159	23	**403.33**	33.50	79344.7	93934.5	6.86	6.04	2.19
		505	106	23	**391.52**	36.90	79344.7	87554.3	6.86	5.98	2.85
		537*	472	38	**697.16**	58.80	79344.7	85589.1	6.86	9.09	-2.50
		567	328	37	**667.91**	53.70	79344.7	85589.1	6.86	6.58	-2.08
		113*	8	6	**85.61**	9.50	79344.7	170507.0	6.86	3.31	5.28
		411	268	36	**663.53**	51.80	79344.7	87554.3	6.86	6.49	2.39
		444	325	39	**707.79**	54.10	79344.7	87554.3	6.86	6.71	-2.37
		447	78	19	**336.74**	29.50	79344.7	89597.1	6.86	5.91	3.46
		451	69	21	**357.06**	31.90	79344.7	87554.3	6.86	6.09	2.56
		458	182	31	**567.13**	43.50	79344.7	85589.1	6.86	6.31	2.04
		462	228	33	**595.34**	46.50	79344.7	85589.1	6.86	6.86	-2.47
Plasminogen	**P00747.2**	344	25	7	**110.11**	11.10	93306	109384.9	7.14	7.12	-2.10
		243	69	14	**196.72**	15.30	93306	122345.0	7.14	7.08	-2.39
		268	68	15	**237.40**	17.50	93306	91722.2	7.14	7.17	-2.70
		274	49	9	**139.67**	8.60	93306	118860.4	7.14	7.30	-2.61
Complement factor B	**P00751.2**	370	211	23	**384.77**	28.70	86900.6	115546.9	6.68	6.25	-2.92
		318	182	24	**416.07**	33.20	86900.6	112392.1	6.68	6.28	-2.42
Ig alpha-1 chain C region	**P01876.2**	374*	40	8	**102.91**	21.50	38509.8	106515.3	6.08	5.18	-2.32
		464*	11	6	**86.08**	17.50	38509.8	93934.5	6.08	5.55	2.11

636*	22	8	**122.09**	23.70	38509.8	72201.1	6.08	5.98	-2.81
682*	18	8	**116.67**	22.30	38509.8	66750.6	6.08	5.18	2.26
707*	186	11	**201.27**	36.80	38509.8	64271.1	6.08	5.40	2.37
714*	155	8	**137.08**	24.30	38509.8	63086.3	6.08	5.80	2.53
724*	125	11	**188.86**	38.80	38509.8	61936.2	6.08	5.98	2.72
743*	81	9	**148.86**	32.80	38509.8	61936.2	6.08	6.13	3.96
769*	84	9	**154.31**	30.80	38509.8	58678.8	6.08	6.22	7.32
780*	67	8	**135.89**	24.30	38509.8	61936.2	6.08	3.05	4.73
809*	16	7	**103.46**	21.80	38509.8	57652.9	6.08	5.15	-2.20
832*	15	6	**86.81**	16.90	38509.8	57652.9	6.08	5.09	-3.04
857*	54	8	**131.24**	24.30	38509.8	53817.7	6.08	6.13	-2.67
868*	175	8	**130.87**	24.30	38509.8	54738.4	6.08	6.38	-2.74
874*	52	5	**85.25**	20.90	36618.7	54738.4	8.92	7.75	3.93
880*	47	5	**82.67**	17.20	36618.7	55683.8	8.92	7.54	3.31
898*	40	7	**99.13**	27.80	36618.7	52920.7	8.92	9.43	6.72
996	6	3	**47.47**	7.90	38509.8	45782.7	6.08	6.16	2.81
1049	5	3	**46.33**	7.90	38509.8	43722.9	6.08	4.50	-2.84
1050	7	3	**43.50**	9.30	38509.8	43066.7	6.08	5.15	-11.91
1057	12	5	**75.80**	14.10	38509.8	43066.7	6.08	4.72	-4.52
1083	4	2	**27.08**	5.00	38509.8	43066.7	6.08	5.52	-25.30
1091	3	2	**29.43**	5.00	38509.8	41796.9	6.08	5.73	-9.40
295*	38	6	**92.56**	16.90	38509.8	122345.0	6.08	4.65	-2.73
370*	38	7	**102.39**	19.50	38509.8	115546.9	6.08	6.25	-2.92
438*	20	6	**85.57**	16.10	38509.8	93934.5	6.08	4.89	-2.20
455*	49	7	**111.97**	22.30	38509.8	93934.5	6.08	6.04	2.19
505*	42	8	**121.57**	24.30	38509.8	87554.3	6.08	5.98	2.85
616_2*	83	6	**97.36**	19.50	38509.8	75205.5	6.08	5.59	-3.56
625_2*	114	8	**135.79**	24.30	38509.8	75205.5	6.08	5.71	-3.7
639*	32	7	**105.38**	22.30	38509.8	75205.5	6.08	5.83	-9.98
641_2*	88	7	**109.13**	20.90	38509.8	76786.0	6.08	5.89	-9.71
643*	63	8	**127.52**	24.30	38509.8	66750.6	6.08	4.28	-2.60
649*	112	9	**145.84**	25.70	38509.8	76786.0	6.08	5.98	-3.97
740*	353	12	**196.71**	42.70	38509.8	61936.2	6.08	5.25	2.21
749*	171	9	**139.06**	25.70	38509.8	61936.2	6.08	5.86	2.77
762*	316	12	**196.25**	42.70	38509.8	61936.2	6.08	5.59	2.48
765*	306	9	**147.72**	29.40	38509.8	61936.2	6.08	6.07	2.50
767*	248	12	**198.33**	42.70	38509.8	60819.2	6.08	5.77	3.08
770_1*	345	11	**169.43**	36.20	38509.8	60819.2	6.08	6.25	5.53
775_1*	208	11	**171.18**	41.30	38509.8	60819.2	6.08	6.16	3.79
778*	155	10	**155.62**	27.70	38509.8	59733.8	6.08	6.37	2.18
782*	41	5	**83.07**	15.50	38509.8	61936.2	6.08	5.98	3.72
92*	20	7	**99.62**	19.50	38509.8	117228.1	6.08	3.15	5.25
983	14	3	**31.52**	7.00	38509.8	45782.7	6.08	4.56	-7.17
1153	19	3	**40.26**	7.00	38509.8	43066.7	6.08	5.25	-5.24
447*	25	8	**115.30**	24.30	38509.8	89597.1	6.08	5.91	3.46

		590*	29	5	**82.67**	15.50	38509.8	73678.1	6.08	5.60	-11.54
		706*	219	12	**198.37**	42.70	38509.8	61936.2	6.08	5.60	2.87
		710*	242	11	**190.99**	41.30	38509.8	61936.2	6.08	5.26	3.17
		719*	238	12	**211.86**	47.30	38509.8	60819.2	6.08	5.44	3.51
		815*	24	6	**96.22**	17.50	38509.8	60819.2	6.08	6.49	-7.24
		998	5	2	**26.02**	5.00	38509.8	44394.0	6.08	4.49	-7.64
		1121	5	2	**28.49**	5.00	38509.8	45080.4	6.08	5.38	-27.72
Alpha-1-acid glycoprotein 1	**P02763.1**	400*	43	5	**81.32**	24.30	23739.3	103774.0	4.93	3.64	-2.82
		1013*	26	7	**130.44**	40.70	23739.3	44394.0	4.93	3.30	-3.89
		333*	14	5	**82.71**	27.30	23739.3	105515.3	4.93	3.81	-2.82
		1003*	37	7	**125.39**	30.80	23739.3	46501.5	4.93	3.22	-4.88
Complement component C7	**P10643.2**	420	4	2	**35.46**	3.90	96711.6	93934.5	6.10	4.68	-2.81
		318	32	14	**212.47**	19.80	96711.6	112392.1	6.10	6.28	-2.42
Afamin	**P43652.1**	423*	101	19	**282.86**	25.50	71007.7	98643.6	5.64	5.03	-2.10
		438*	116	13	**208.29**	17.60	71007.7	93934.5	5.64	4.89	-2.20
		743*	30	7	**107.56**	10.00	71007.7	61936.2	5.64	4.16	-2.98
Inter-alpha-trypsin inhibitor heavy chain H4	**Q14624.4**	423	9	5	**73.64**	5.20	103583.8	98643.6	6.52	5.03	-2.10
		295	54	13	**231.01**	16.30	103583.8	122345.0	6.52	4.65	-2.73
		438	47	11	**186.19**	12.20	103583.8	93934.5	6.52	4.89	-2.20
Serum albumin	**P02768.2**	636	108	22	**377.22**	32.00	71362.3	72201.1	5.92	5.98	-2.81
		566	3	2	**23.69**	2.60	71362.3	80117.3	5.92	5.40	-3.58
		616_3	11	4	**59.23**	6.40	71362.3	75205.5	5.92	5.59	-3.56
		641_3	19	7	**105.56**	12.60	71362.3	76786.0	5.92	5.89	-9.71
		649	9	4	**50.79**	5.20	71362.3	76786.0	5.92	5.98	-3.97
		762	95	18	**317.24**	28.00	71362.3	61936.2	5.92	5.59	2.48
		872*	68	21	**343.44**	32.50	71362.3	54738.4	5.92	7.36	2.15
Hemopexin	**P02790.2**	714	7	5	**80.89**	13.40	52417.1	63086.3	6.55	5.80	2.53
		724	6	3	**48.87**	8.20	52417.1	61936.2	6.55	5.98	2.72
		743	2	2	**30.14**	6.90	52417.1	61936.2	6.55	6.13	3.96

		522*	30	9	**158.15**	25.90	52417.1	85589.1	6.55	5.21	-2.07
		566	55	12	**187.39**	22.00	52417.1	80117.3	6.55	5.40	-3.58
		616_1*	213	16	**266.19**	33.30	52417.1	75205.5	6.55	5.59	-3.56
		625_1*	143	16	**267.54**	35.70	52417.1	75205.5	6.55	5.71	-3.71
		639*	144	18	**299.81**	45.40	52417.1	75205.5	6.55	5.83	-9.98
		641_1*	98	17	**258.30**	37.80	52417.1	76786.0	6.55	5.89	-9.71
		649*	46	10	**172.17**	26.40	52417.1	76786.0	6.55	5.98	-3.97
		562*	97	15	**255.96**	37.00	52417.1	75205.5	6.55	5.38	-3.90
		575*	90	17	**271.18**	35.20	52417.1	73678.1	6.55	5.47	-6.99
		590*	162	19	**320.84**	41.70	52417.1	73678.1	6.55	5.60	-11.54
Alpha-1-antitrypsin	**P01009.3**	780*	78	16	**275.19**	40.90	46906.8	61936.2	5.37	3.05	4.73
		809*	20	10	**174.29**	29.60	46906.8	57652.9	5.37	5.15	-2.20
		820*	23	8	**141.15**	26.50	46906.8	58678.0	5.37	4.78	-3.75
		821	108	19	**343.71**	41.60	46906.8	56654.9	5.37	4.97	-2.63
		832*	45	11	**196.65**	26.30	46906.8	57652.9	5.37	5.09	-3.04
		848	261	22	**418.32**	55.00	46906.8	56654.9	5.37	4.75	-2.56
		857	67	15	**259.20**	45.20	46906.8	53817.7	5.37	6.13	-2.67
		868	19	6	**112.31**	19.80	46906.8	54738.4	5.37	6.38	-2.74
		899	1	1	**20.39**	4.50	46906.8	52920.7	5.37	7.17	2.46
		938	2	2	**25.56**	6.20	46906.8	50363.8	5.37	4.29	-3.26
		983	2	2	**32.66**	8.10	46906.8	51194.7	5.37	4.69	-11.44
		1010	2	2	**38.42**	6.20	46906.8	45080.4	5.37	4.78	-10.74
		1027	3	2	**34.18**	6.20	46906.8	45080.4	5.37	4.94	-5.61
		295*	11	5	**84.92**	14.30	46906.8	122345.0	5.37	4.65	-2.73
		782*	56	10	**172.43**	24.80	46906.8	61936.2	5.37	5.98	3.72
		835	7	3	**46.76**	6.60	46906.8	56654.9	5.37	5.13	-2.01
		873	8	3	**51.81**	6.90	46906.8	53817.7	5.37	6.67	-4.07
		893	105	12	**204.77**	25.50	46906.8	53817.7	5.37	5.04	-3.96
		908	241	24	**435.28**	59.00	46906.8	52920.7	5.37	4.92	-2.04
		927*	46	10	**175.64**	30.80	46906.8	52920.7	5.37	9.15	5.30
		992	55	8	**141.29**	21.70	46906.8	55683.8	5.37	3.80	-2.21
		700*	57	10	**178.21**	25.80	46906.8	58678.8	5.37	4.12	-5.18
		732*	82	13	**235.06**	32.20	46906.8	57652.9	5.37	3.06	4.14
		781	62	10	**190.13**	29.60	46906.8	52920.7	5.37	4.80	-2.66
		815*	10	5	**83.14**	18.40	46906.8	60819.2	5.37	6.49	-7.24
		848	107	14	**233.60**	34.40	46906.8	54738.4	5.37	5.04	-2.78
		1113	16	6	**85.13**	14.50	46906.8	42424.9	5.37	4.61	-4.33
		1134	15	6	**88.41**	16.00	46906.8	41182.5	5.37	4.76	-3.26
		1745*	109	14	**247.06**	33.90	46906.8	16508.2	5.37	5.66	-30.21

Vitamin D-binding protein	**P02774.1**	809	125	23	**396.35**	59.20	54560.2	57652.9	5.40	5.15	-2.20
		820	2	1	**22.00**	3.50	54560.2	58678.0	5.40	4.78	-3.75
		832	106	18	**322.72**	38.30	54560.2	57652.9	5.40	5.09	-3.04
		740	23	6	**89.19**	11.30	54560.2	61936.2	5.40	5.25	2.21
		835	132	19	**328.03**	42.60	54560.2	56654.9	5.40	5.13	-2.01
		700	29	3	**41.81**	5.20	54560.2	58678.8	5.40	4.12	-5.18
		781	148	21	**360.07**	50.20	54560.2	52920.7	5.40	4.80	-2.66
		848	5	2	**33.30**	4.00	54560.2	54738.4	5.40	5.04	-2.78
Angiotensinogen	**P01019.1**	809	14	5	**94.64**	13.10	53438.5	57652.9	5.87	5.15	-2.20
		835	15	4	**72.35**	9.60	53438.5	56654.9	5.87	5.13	-2.01
		781	10	5	**98.03**	13.10	53438.5	52920.7	5.87	4.80	-2.66
Alpha-2-HS-glycoprotein	**P02765.1**	820*	51	7	**108.02**	20.90	40122.7	58678.0	5.43	4.78	-3.75
		368*	121	7	114.45	18.5	40122.7	112392.1	5.43	6.793	-2.35
		800*	90	6	94.07	13	40122.7	58678.8	5.43	4.254	-2.74
Beta-2-glycoprotein 1	**P02749.3**	857*	15	5	**97.12**	19.70	39609.7	53817.7	9.11	6.13	-2.67
		868*	40	9	**157.88**	32.70	39609.7	54738.4	9.11	6.38	-2.74
		876*	24	5	**93.53**	20.00	39609.7	52046.7	9.11	6.68	-2.59
		853*	30	5	**92.21**	16.80	39609.7	54738.4	9.11	6.40	-3.15
		778*	47	9	**155.61**	32.40	39609.7	59733.8	9.11	6.37	2.18
		706*	12	5	**90.18**	20.20	39609.7	61936.2	9.11	5.60	2.87
		815*	38	7	**127.59**	22.30	39609.7	60819.2	9.11	6.49	-7.24
Haptoglobin	**P00738.1**	938	8	4	**63.95**	10.00	45889.1	50363.8	6.13	4.29	-3.26
		970	5	4	**63.06**	10.50	45889.1	47990.9	6.13	5.27	2.35
		983	61	13	**228.12**	27.50	45889.1	51194.7	6.13	4.69	-11.44
		1010	80	14	**248.41**	27.50	45889.1	45080.4	6.13	4.78	-10.74
		1013*	16	6	**100.88**	13.30	45889.1	44394.0	6.13	3.30	-3.89
		1027	56	10	**173.41**	22.40	45889.1	45080.4	6.13	4.94	-5.61
		1049	30	6	**102.36**	13.30	45889.1	43722.9	6.13	4.50	-2.84
		1050	131	12	**213.51**	22.60	45889.1	43066.7	6.13	5.15	-11.91
		1053	4	2	**29.63**	5.10	45889.1	43066.7	6.13	4.60	-3.61
		1057	26	8	**137.71**	15.20	45889.1	43066.7	6.13	4.72	-4.52
		1066	51	10	**175.46**	22.40	45889.1	43066.7	6.13	5.27	-14.30

		1083	73	14	**242.84**	24.60	45889.1	43066.7	6.13	5.52	-25.30
		1091	44	10	**168.00**	19.20	45889.1	41796.9	6.13	5.73	-9.40
		1138	51	12	**217.36**	26.60	45889.1	38297.8	6.13	5.52	-12.27
		1160*	30	10	**161.68**	22.90	45889.1	33328.3	6.13	5.76	-8.80
		1577*	97	8	**147.17**	12.30	45889.1	15572.2	6.13	5.70	-35.82
		1584*	58	8	**137.25**	12.30	45889.1	15572.2	6.13	5.33	-29-99
		1590*	90	9	**158.52**	13.50	45889.1	15036.7	6.13	6.16	-17.98
		1618*	28	6	**100.34**	9.30	45889.1	14351.2	6.13	5.70	-16.57
		1626*	50	8	**138.99**	12.30	45889.1	14184.6	6.13	6.19	-8.37
		983	138	13	**227.88**	27.50	45889.1	45782.7	6.13	4.56	-7.17
		1014	178	14	**247.19**	24.60	45889.1	44394.0	6.13	4.89	-22.36
		1031	9	4	**63.26**	8.10	45889.1	44394.0	6.13	4.53	-3.29
		1043	131	13	**235.97**	27.50	45889.1	45080.4	6.13	5.10	-20.75
		1076	154	14	**258.69**	24.60	45889.1	47990.9	6.13	5.25	-11.00
		1112	91	13	**228.91**	24.60	45889.1	47990.9	6.13	5.38	-26.26
		1121	41	8	**132.15**	15.20	45889.1	45080.4	6.13	5.65	-21.82
		1153	95	13	**228.08**	26.60	45889.1	43066.7	6.13	5.25	-5.24
		1202	22	6	**101.27**	13.50	45889.1	38297.8	6.13	5.65	-7.31
		1673*	86	12	**214.39**	25.30	45889.1	17503.0	6.13	6.16	-13.36
		1692*	178	10	**185.10**	14.50	45889.1	16508.2	6.13	5.71	-33.45
		1693*	73	5	**91.55**	7.30	45889.1	17097.7	6.13	5.35	-31.30
		1702*	75	7	**123.74**	9.30	45889.1	16702.3	6.13	6.13	-19.91
		1724*	49	6	**110.35**	9.30	45889.1	15391.6	6.13	5.68	-14.00
		1735*	136	8	**138.08**	12.30	45889.1	15755.0	6.13	6.13	-7.40
		948*	13	6	**90.57**	12.00	45889.1	49553.4	6.13	3.87	-2.49
		998	88	12	**195.56**	21.10	45889.1	44394.0	6.13	4.49	-7.64
		1005	127	14	**244.65**	24.60	45889.1	44394.0	6.13	4.61	-29.39
		1018	152	12	**218.72**	22.60	45889.1	43722.9	6.13	4.76	-35.94
		1045	117	13	**230.36**	24.60	45889.1	43066.7	6.13	4.98	-17.45
		1097	138	14	**247.71**	24.60	45889.1	46501.5	6.13	5.23	-17.17
		1113	79	12	**214.72**	22.60	45889.1	42424.9	6.13	4.61	-4.33
		1120	17	6	**93.10**	13.50	45889.1	39992.3	6.13	4.36	-3.50
		1121	96	15	**264.24**	29.50	45889.1	45080.4	6.13	5.38	-27.72
		1132	22	7	**112.54**	13.00	45889.1	45080.4	6.13	5.57	-14.46
		1134	104	14	**246.20**	24.60	45889.1	41182.5	6.13	4.76	-3.26
		1166	22	6	**88.92**	11.30	45889.1	42424.9	6.13	5.63	-7.56
		1745*	186	11	**204.18**	16.00	45889.1	16508.2	6.13	5.66	-30.21
		1748*	77	9	**160.29**	13.50	45889.1	16127.1	6.13	5.26	-26.36
		1749*	137	11	**189.43**	14.70	45889.1	16316.5	6.13	6.09	-16.80
Alpha-1-antichymotrypsin	**P01011.2**	938	5	2	**31.83**	4.40	47821.2	50363.8	5.33	4.29	-3.26
		1049	2	2	**26.87**	4.40	47821.2	43722.9	5.33	4.50	-2.84

		643*	114	11	**185.94**	26.00	47821.2	66750.6	5.33	4.28	-2.60
Haptoglobin-related protein	**P00739.2**	983*	35	7	**125.63**	17.50	39542.3	51194.7	6.64	4.69	-11.44
		1010	52	7	**129.57**	17.50	39542.3	45080.4	6.64	4.78	-10.74
		1050	80	7	**122.81**	17.50	39542.3	43066.7	6.64	5.15	-11.91
		983*	51	7	**121.36**	17.50	39542.3	45782.7	6.64	4.56	-7.17
		1014	95	7	**130.55**	17.50	39542.3	44394.0	6.64	4.89	-22.36
		1043	68	7	**128.76**	17.50	39542.3	45080.4	6.64	5.10	-20.75
		1076*	87	7	**132.51**	17.50	39542.3	47990.9	6.64	5.25	-11.00
		1112*	52	7	**125.69**	17.50	39542.3	47990.9	6.64	5.38	-26.26
		1153	52	7	**121.43**	17.50	39542.3	43066.7	6.64	5.25	-5.24
		1673*	48	6	**108.81**	14.90	39542.3	17503.0	6.64	6.16	-13.36
Serum paraoxonase/ arylesterase 1	**P27169.3**	983*	22	6	**103.45**	19.40	39901.7	51194.7	5.08	4.69	-11.44
		1010	13	5	**93.74**	16.90	39901.7	45080.4	5.08	4.78	-10.74
		998	3	3	**44.00**	7.80	39901.7	44394.0	5.08	4.49	-7.64
		1113	18	4	**70.55**	11.80	39901.7	42424.9	5.08	4.61	-4.33
		1134	9	3	**51.88**	9.20	39901.7	41182.5	5.08	4.76	-3.26
Zinc-alpha-2-glycoprotein	**P25311.2**	983*	12	6	**100.69**	25.80	34486.3	51194.7	5.71	4.69	-11.44
		1049*	39	9	**145.35**	28.80	34486.3	43722.9	5.71	4.50	-2.84
		1053*	31	10	**165.98**	39.20	34486.3	43066.7	5.71	4.60	-3.61
		1057*	60	11	**182.44**	37.20	34486.3	43066.7	5.71	4.72	-4.52
		1031*	34	6	**104.51**	25.80	34486.3	44394.0	5.71	4.53	-3.29
		1120	30	8	**132.18**	30.50	34486.3	39992.3	5.71	4.36	-3.50
		1134	7	2	**30.76**	6.70	34486.3	41182.5	5.71	4.76	-3.26
		1673*	24	6	**103.06**	25.80	34486.3	17503.0	5.71	6.16	-13.36
Apolipoprotein A-IV	**P06727.3**	1010	11	4	**52.52**	9.00	45398.2	45080.4	5.28	4.78	-10.74
		1027	61	17	**292.72**	40.40	45398.2	45080.4	5.28	4.94	-5.61
		1050	24	8	**124.54**	20.40	45398.2	43066.7	5.28	5.15	-11.91
		1014	4	4	**51.08**	7.00	45398.2	44394.0	5.28	4.89	-22.36
		1134	53	12	**200.38**	30.00	45398.2	41182.5	5.28	4.76	-3.26
Complement C3	**P01024.2**	1049*	36	11	**187.01**	7.10	188685.1	43722.9	6.02	4.50	-2.84
		1053*	19	7	**120.33**	4.70	188685.1	43066.7	6.02	4.60	-3.61

		1144*	33	12	**193.31**	7.50	188685.1	38297.8	6.02	4.57	-3.49
		1363*	34	6	**100.75**	3.90	188685.1	24776.6	6.02	6.96	3.44
		649*	50	12	**204.95**	8.30	188685.1	76786.0	6.02	5.98	-3.97
		1031*	48	9	**151.65**	5.40	188685.1	44394.0	6.02	4.53	-3.29
		1455*	251	23	**403.92**	14.40	188685.1	25399.0	6.02	6.94	3.96
		579*	135	25	**444.90**	15.40	188685.1	72201.1	6.02	7.39	-5.39
		1120*	237	20	**364.30**	12.50	188685.1	39992.3	6.02	4.36	-3.50
		1482*	107	7	**108.00**	4.10	188685.1	26369.9	6.02	6.86	3.97
Clusterin	**P10909.1**	1144*	75	10	**179.05**	16.70	53064.2	38297.8	5.89	4.57	-3.49
		1172*	66	11	**199.68**	21.80	53064.2	37224.5	5.89	4.69	-3.11
		1198*	63	9	**147.65**	20.00	53064.2	35692.6	5.89	4.81	-4.93
		1226*	51	8	**140.17**	16.40	53064.2	36703.8	5.89	4.62	-3.47
		1284*	35	10	**159.16**	22.00	53064.2	35692.6	5.89	4.77	-6.12
		1272*	57	9	**152.60**	21.60	53064.2	32011.9	5.89	4.30	-3.56
		1303*	41	8	**139.62**	16.40	53064.2	31173.1	5.89	4.61	-5.08
Apolipoprotein E	**P02649.1**	1198	10	4	**60.36**	12.90	36267.6	34247.3	5.65	4.94	-4.55
		1301	23	6	**81.79**	19.50	36267.6	32881.5	5.65	4.98	-2.34
Apolipoprotein M	**O95445.2**	1450	3	2	**24.09**	7.90	21595.2	21152.6	5.66	5.03	2.74
		1453	3	2	**32.86**	12.20	21595.2	20900.8	5.66	5.15	2.93
		1563	5	2	**28.31**	8.50	21595.2	21152.6	5.66	5.13	-3.34
		1576	2	2	**22.28**	8.50	21595.2	22740.2	5.66	5.32	3.17
		1636	24	2	**32.12**	8.50	21595.2	21929.5	5.66	5.07	-3.39
Transthyretin	**P02766.1**	1718*	14	6	**118.65**	63.90	16000.8	11894.8	5.52	5.15	-3.74
		1720*	36	8	**148.73**	68.70	16000.8	11616.3	5.52	5.46	-5.93
		1826*	90	9	**173.00**	68.70	16000.8	13069.5	5.52	5.44	-4.32

Haptoglobin was the most significantly downregulated protein in serum of HCV associated cirrhotic patients compared to healthy controls (Figure 43).

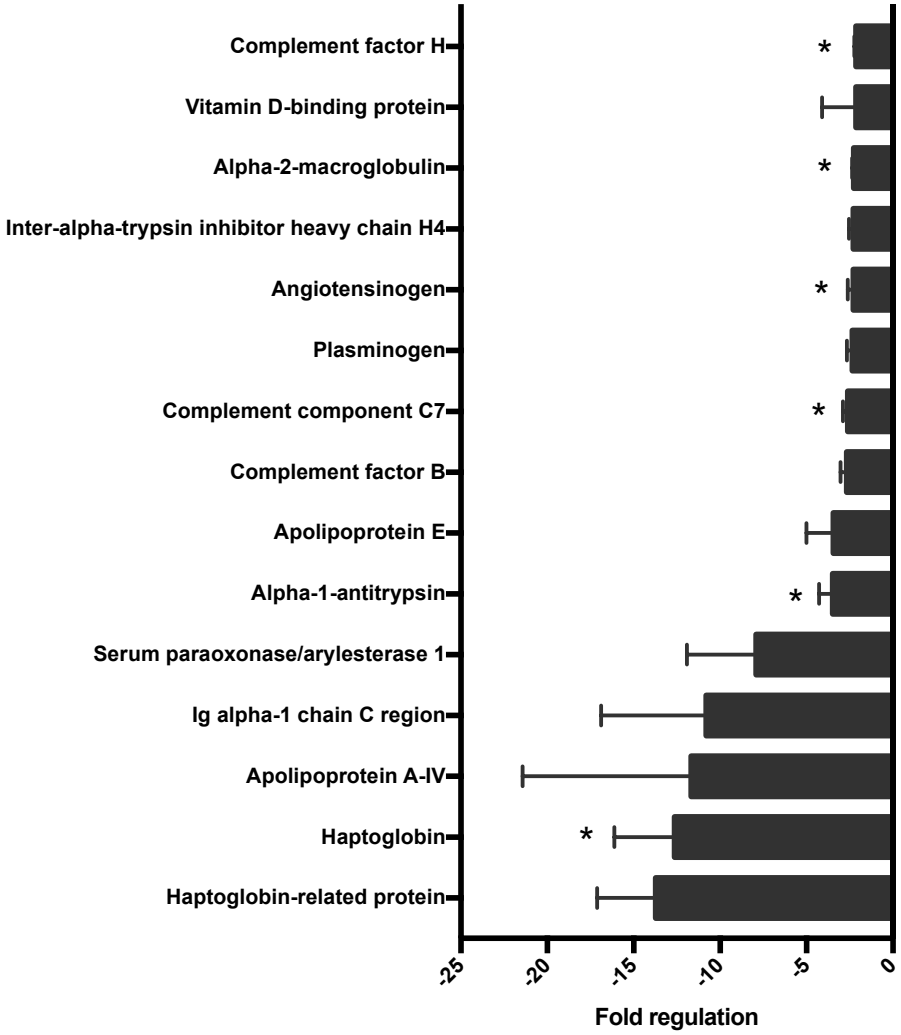


Figure 43. Differences in protein expression in serum of CHC patients compared to healthy controls.

Only proteins identified in at least 2 out of three gels with fitting MW and pI (see Table S2) were used for analysis. Mean of fold regulation for all spots representing the same protein within each gel was calculated and the means from 2 or 3 gels were averaged for every protein. Fold regulation is presented as mean + SD. Proteins with highly variable fold regulation between the spots were excluded from the analysis. Significantly downregulated proteins are marked with "*" (95% CI does not include the value of zero effect). Reproduced from (34) with permission of Elsevier Inc.

Analysis of biological pathways with the help of STRING database and reactome pathway database revealed that 4 of identified significantly differentially abundant proteins are involved

in innate immune system functioning (Complement component C7, Haptoglobin, Complement factor H, Alpha-1-antitrypsin (AAT)), including neutrophil degranulation (Haptoglobin, AAT).

Effects of albumin intervention on neutrophil function in liver cirrhosis

Albumin was shown to improve neutrophil function *in vitro* (52). In order to investigate, if albumin supplementation in cirrhotic patients may improve their neutrophil function, neutrophil phagocytosis and ROS production were measured in whole blood of 7 patients (baseline n=7, 24 hours n=6, 48 hours n=4) with decompensated liver cirrhosis treated with albumin intravenously. Neutrophil function was measured at the baseline, 24 and 48 hours after albumin infusion.

Significant differences were detected only in the number of neutrophils, which produced ROS in response to *E. coli*. This parameter was increased 24 hours after albumin intervention (p=0.024), but came back to the baseline level 48 hours after albumin intervention (Figure 44 E).

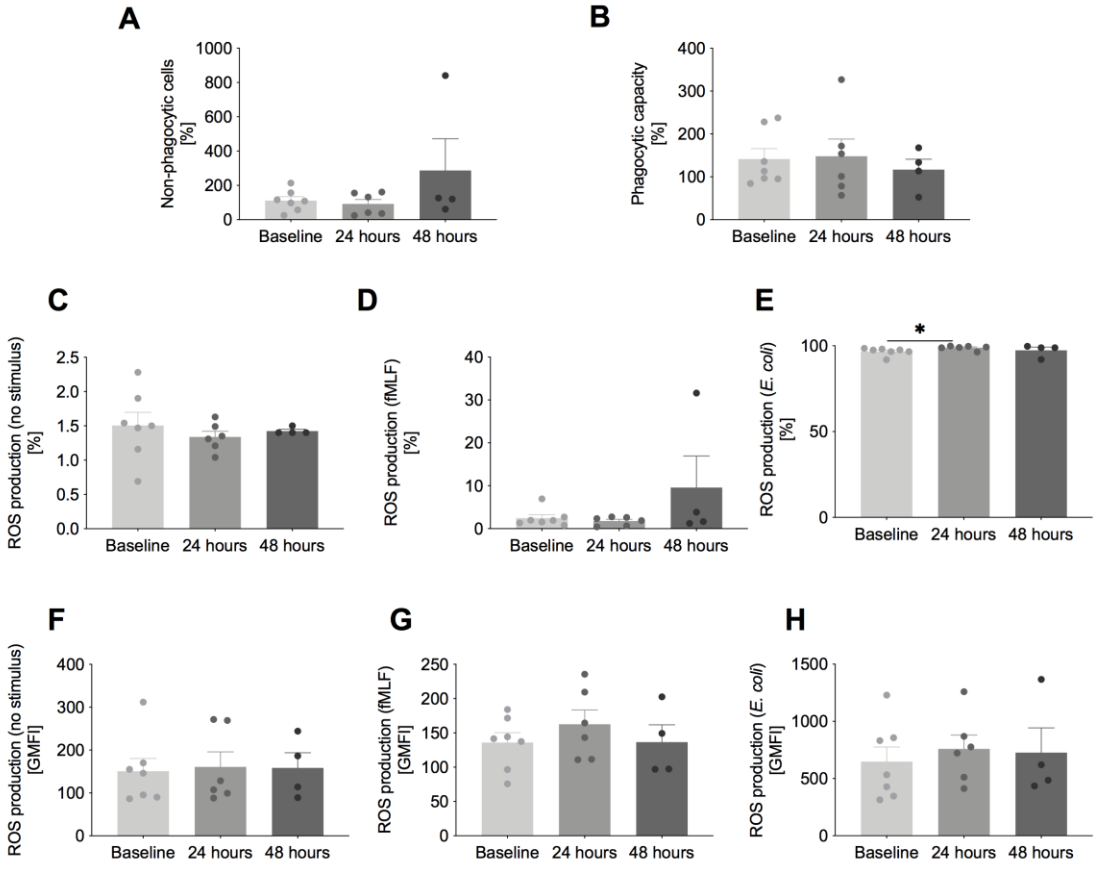


Figure 44. Neutrophil function in cirrhotic patients at the baseline, 24 hours and 48 hours after albumin intervention.

(A, B) Neutrophil phagocytosis of *E. coli* ($4x10^7$ bacteria/100µl blood). (A) Fold change of non-phagocytic neutrophils and (B) neutrophil phagocytic capacity (% of *E. coli* batch controls); (C-H) Neutrophils ROS production. (C-E) Percentage of neutrophils, which produced ROS (C) without any stimulus or in response to (D) fMLF (0.8 µM) or (E) *E. coli* ($2\text{-}4x10^7$ bacteria/100µl blood). (F-H) Intracellular ROS production (GMFI). Bars, mean; error bars, SEM; *$p < 0.05$ (Friedman test with Dunn's test for multiple comparisons (n=4 analyzed)).

Iron metabolism parameters and neutrophil function

As iron metabolism parameters, such as heme, were shown to be associated with the neutrophil function changes (75), the analysis of ferritin and heme levels was performed in serum of cirrhotic patients and healthy controls.

Ferritin and heme levels were increasing with increasing stage of cirrhosis (Figure 45 A, B). No associations between ferritin and heme levels and neutrophil phagocytosis and ROS production were found in cirrhotic patients with correlation analysis (performed within Child-Pugh A cirrhotic patients).

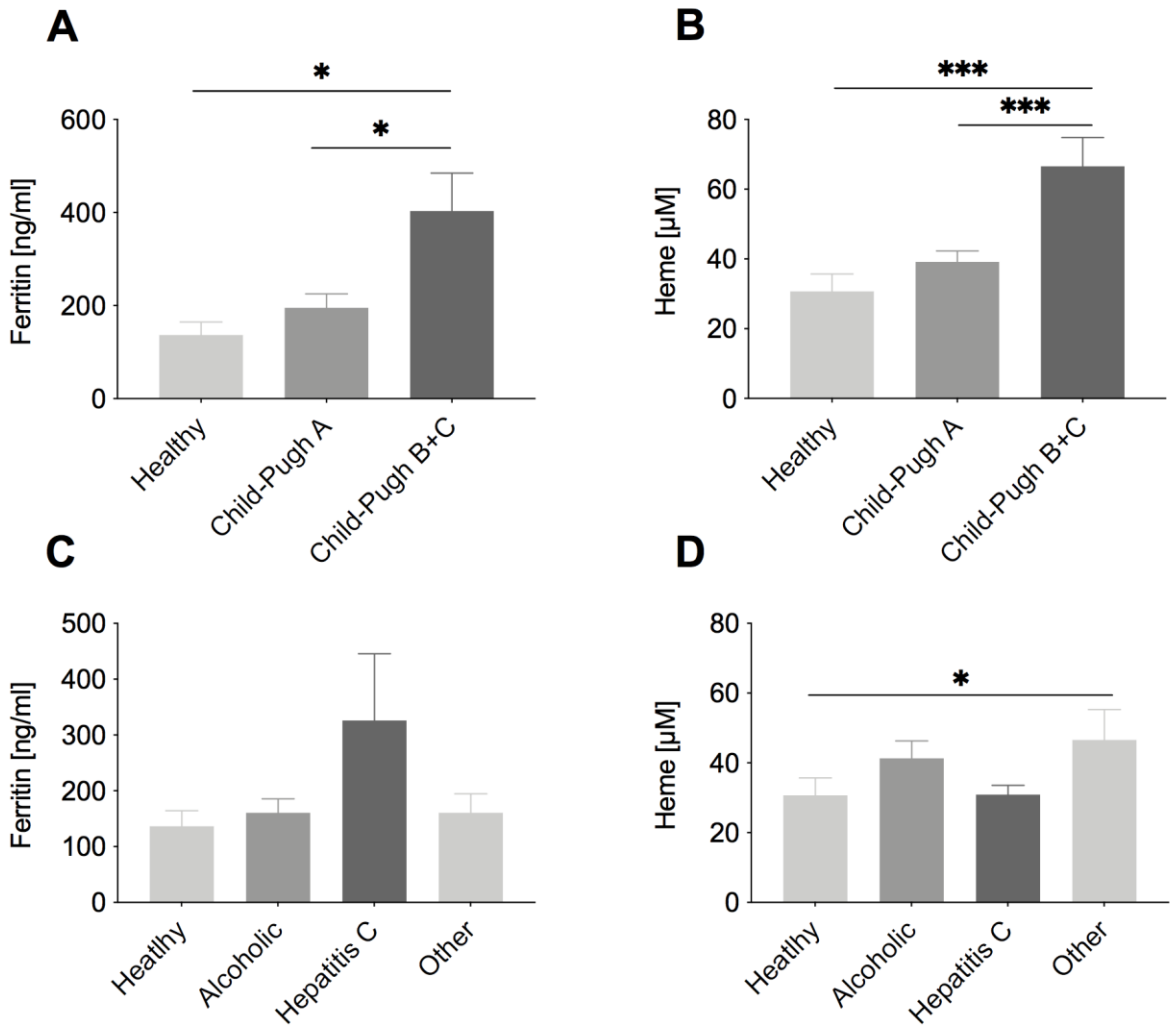


Figure 45. Iron metabolism parameters in different severity and etiology groups of liver cirrhosis.

(A, C) Ferritin and (B, D) heme levels were measured in serum of 106 (heme) or 95 (ferritin) cirrhotic patients and 21 healthy controls. (C, D) Analysis of differences between these parameters in different etiologies of cirrhosis groups was performed within Child-Pugh A patients (n=67 for ferritin, n=77 for heme). Bars, mean; error bars, SEM; *p < 0.05, **p < 0.01, ***p < 0.001. Statistical analysis was by Kruskal-Wallis test with Dunn's multiple comparisons test.

Autoantibodies and NETs formation

ANCA are present in serum of patients with autoimmune hepatitis, PSC and PBC (198) and were shown to induce NETs formation (199). Therefore, the influence of different types of anti-neutrophil autoantibodies, such as pANCA, cANCA and ANA on NETs formation was studied.

pANCA, but not cANCA or ANA, induced NETs formation in healthy donor derived unstimulated neutrophils (Figure 46 A-C). No significant influence of pANCA on NETs formation in response to *E. coli* or PMA was found (Figure 46 B).

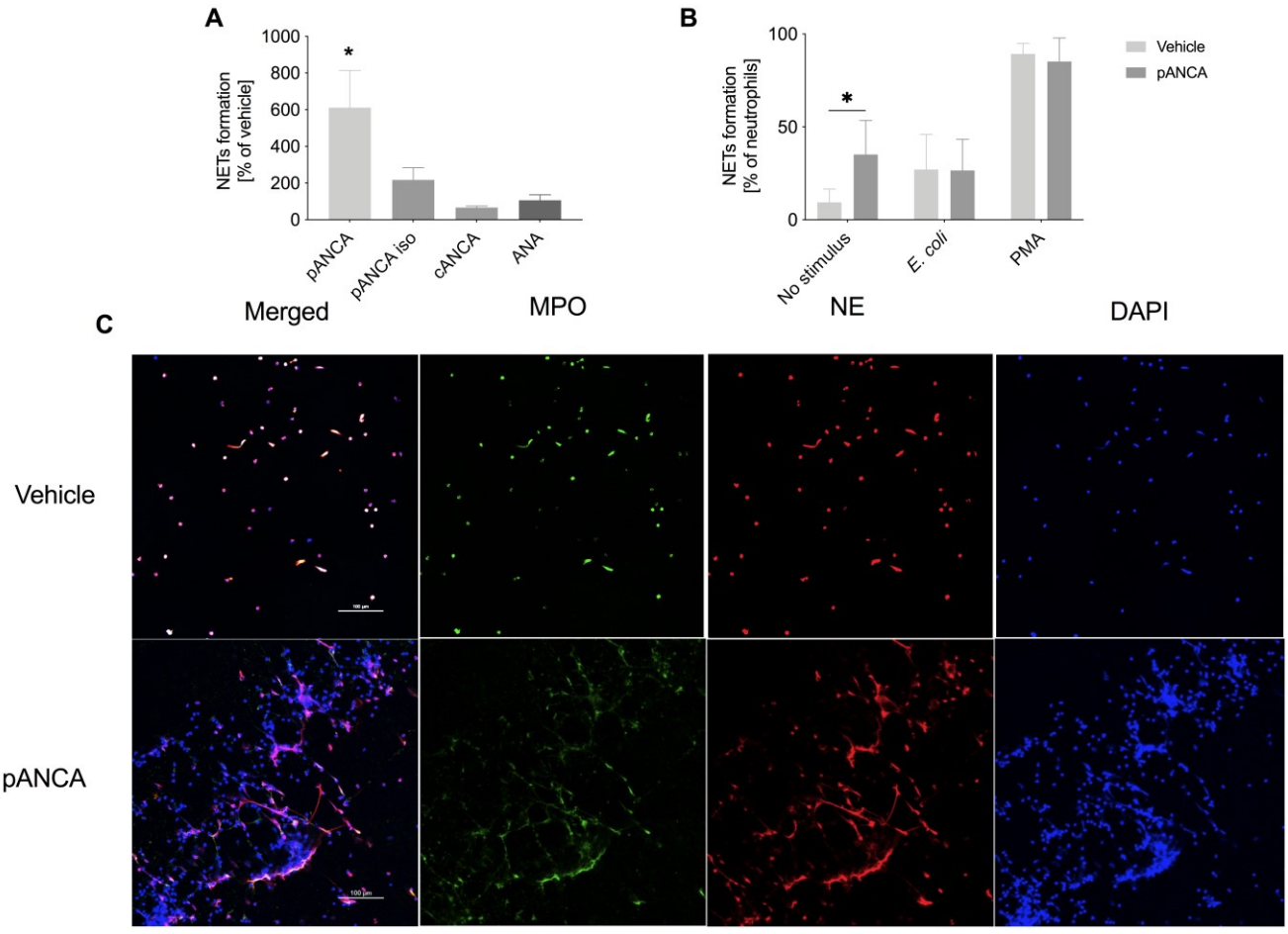


Figure 46. pANCA induce NETs formation.

(A-C) Healthy donor neutrophils were incubated with pANCA, pANCA isotype control, cANCA or ANA in presence (B) or absence (A, C) of *E. coli* or PMA and a percentage of NETs formation was quantified. (C) Representative immunofluorescence pictures of neutrophils incubated with pANCA/vehicle and stained with DAPI, anti-MPO and anti-NE antibodies (Scale bars: 100µm) (pictures were taken by Silvia Racedo, Medical University of Graz). (A) Data are represented as percentage of vehicle and compared to vehicle. (A, B) Data are shown as mean with SEM

and represent minimum of 4 independent experiments. *$p<0.05$. Statistical analysis was by unpaired t-test.

DISCUSSION

Some results were discussed in a similar manner to my first author publications (Balazs et al 2021, in revision) or (34).

Within my thesis I aimed to identify serum components, which contribute to neutrophil dysfunction in liver cirrhosis. Serum BA composition was found to be associated with neutrophil function deficiency in cirrhotic patients and BAs modulated healthy donor neutrophil functions upon direct treatment. Moreover, serum proteome and iron metabolism parameters, as well as the effects of albumin supplementation in cirrhotic patients were described. This study suggests that BA may be serum components, which contribute to the development of neutrophil function deficiency in cirrhotic patients and, therefore, can be a potential target for therapeutic intervention in cirrhosis to improve patients' life quality and survival.

Changes in neutrophil function are known during the course of liver cirrhosis (17). Findings about phagocytic function of neutrophils are rather consistent, showing the decrease in neutrophil phagocytosis in cirrhotic patients (25, 30-36), similarly to our results.

In contrast, the nature of alterations of neutrophil ROS production in liver cirrhosis is controversial. Although most of the studies describe the elevated number of neutrophils with basal ROS production (31, 34, 35, 39) and ROS production in response to physiological stimulus with low potency like fMLF (31, 32, 35), as was also shown in cirrhotic cohort analyzed within this study, the results on ROS production activity are highly vary. The reason for that is most probably the variety of methods and experimental conditions used to study ROS production in cirrhotic patients, which often measure different types of ROS.

The majority of studies measured neutrophil ROS production in liver cirrhosis with either flow cytometry (e.g. based on conversion of DHR 123 to R 123, or similar) (37, 39), cytochrome c reduction (40-42) or luminol-based (41) assays. Flow cytometry-based assays measure only intracellular ROS production, detecting mixture of different ROS, however, with almost no sensitivity towards superoxide (e.g. in case of DHR) (200). Cytochrome c reduction assay is able mainly to detect extracellular ROS and, in contrast, detects only superoxide (201). Luminol-based assays can be used to measure both intracellular or total ROS production (mixture of intracellular and extracellular ROS) depending on presence or absence of horseradish peroxidase and detect the mixture of different ROS (201, 202). Therefore, when

the literature is separated based on methodology used for ROS production assessment, the results become much more consistent. In our cirrhotic cohort, we studied intracellular ROS pool of neutrophils by flow cytometry, which was unchanged in response to fMLF or *E. coli* or without stimulus.

Consistent with previous studies (107-110, 112, 113), our cirrhotic patients‘ cohort had highly increased serum concentrations of BAs, with GCDCA being the most elevated and the most abundant in cirrhosis and DCA, which concentration was unaltered in cirrhosis. Interestingly, BA composition was cirrhosis etiology dependent with the most striking increase in total CDCA RA in HCV-associated cirrhosis, which was also characterized by the worst phagocytic function of neutrophils.

The associations between total CDCA and UDCA RA and phagocytic function of neutrophils in cirrhotic patients were present even after correcting for confounding of cirrhosis severity and etiology and including serum albumin levels as an explanatory variable into the multiple regression model. Albumin was previously shown to play a role in neutrophil dysfunction, including phagocytosis and basal ROS production (52) and to be associated with bacterial infections in cirrhosis (65). Indeed, lower albumin concentration in serum was associated with impaired neutrophil phagocytosis in our cirrhotic cohort.

However, our findings show that BAs are stronger predictors of neutrophil phagocytosis impairment than albumin. When total CDCA RA was included into the model together with albumin, albumin was no more a significant predictor of neutrophil phagocytic capacity. However, it was still a significant predictor of non-phagocytic neutrophil amount along with UDCA RA. This crosstalk between BAs and albumin might be explained by the fact that BAs are being transported in systemic circulation via binding to albumin (79, 80). Albumin has several identified BA specific binding sites (203, 204). Previously described albumin dysfunction in cirrhosis (205) might change its affinity to BAs. Furthermore, in conditions of albumin deficiency, BAs change their transporter preferences to lipoproteins, which in turn might facilitate their interaction with cells and tissues, including neutrophils (81) and potentially cause their higher intracellular accumulation. Therefore, serum concentrations of BAs we measure in cirrhosis might underestimate the concentrations neutrophils are really exposed to, making such low concentrated BAs as LCA and UDCA more pathophysiologically relevant. As some of the lipoproteins have been also shown to be deficient in serum of cirrhotic patients

(70), the pathophysiological importance of other potential BA protein transporters should be taken into consideration in future studies.

In support of the results of UDCA being associated with phagocytic function and ROS production, neutrophils of cirrhotic patients, who were receiving UDCA supplementation, tended to improve their phagocytic function and their basal intracellular ROS production and their ROS production in response to fMLF significantly decreased. The low number of patients with UDCA treatment was, however, a limitation for this analysis.

We could prove associations of CDCA with phagocytic capacity in the experiments, when healthy donor neutrophils were directly treated with pathophysiologically relevant serum concentrations of total CDCA, as well as different forms of CDCA, in the absence of other confounding factors. This suggests the causal relationship between CDCA and neutrophil function. Furthermore, CDCA inhibited response of neutrophils to fMLF (ROS production and chemotaxis). This has been already shown that different forms of CDCA inhibit chemotaxis and calcium flux of neutrophils (161, 179) and antagonism of FPR1 receptor (fMLF receptor) was suggested as a mechanism of these changes (161), which is supported by the findings of inhibited FPR1 signaling in neutrophils from cirrhotic patients (38). A model for BA recognition by FPR1 receptor was also previously proposed (206).

However, neutrophil expression of FPR1 receptor has been shown to be unchanged in cirrhotic patients (38), which is similar to our findings. Furthermore, according to our experiments, nature of GCDCA effects on neutrophil chemotaxis are different from the effects of most potent and selective (207, 208) FPR1 inhibitor Cyclosporine H. Moreover, in higher concentrations CDCA was previously shown also to activate calcium flux of neutrophils (209). The mechanism behind it needs further investigations.

Interestingly, along with anti-inflammatory CDCA effects discussed above, we observed its pro-inflammatory properties, represented by the ability to delay neutrophil apoptosis, which keeps neutrophils in the circulation longer and prevents resolution of inflammation. Therefore, the mechanism of CDCA effects on neutrophil function is not straightforward and might involve several pathways, which should be investigated in further studies.

Overall, CDCA, especially its glycine conjugated form, seems to be the most clinically relevant BA in serum of cirrhotic patients, as it is highly abundant and contributes most to the BA

composition dissimilarity between cirrhotic patients and healthy controls and we could show its association with impaired neutrophil response analyzing data from cirrhotic patients, as well as after direct neutrophil treatment with this BA. This makes it the most promising target for therapeutic intervention in cirrhosis.

Despite being associated with better neutrophil phagocytosis and ROS production in clinical dataset analysis, UDCA and its conjugates did not show direct effects on these neutrophil functions in the experiments with healthy donor neutrophils. UDCA might improve phagocytic and ROS production neutrophil functions in the experiments with neutrophils from cirrhotic patients with already present dysfunction or with neutrophils treated prior with toxic BAs like CDCA, which could be of interest for future studies. Furthermore, as shown in the results of the comparison of BA composition between UDCA-treated and not treated patients in this study, UDCA might indirectly exhibit beneficial effects on neutrophil function via reducing the GCDCA abundance.

Another BA type, CA and its conjugates, although being significantly higher concentrated and abundant in cirrhotic patients compared to healthy controls, was not associated with neutrophil function neither in clinical setup, nor in the experiments with healthy donor neutrophils. This might allow speculating that this BA either does not play a role in cirrhosis-associated neutrophil dysfunction or plays role indirectly via, for example, regulation of overall BA composition and other BA relative abundances. UDCA and CA are the most hydrophilic BAs (210), characterized by low toxicity (94, 211). Furthermore, UDCA and CA were associated with the restoration of membrane fluidity changes caused by LPS (166).

Notably, high concentration of TUDCA (50 µM) and to a less extent GUDCA inhibited neutrophil chemotaxis towards fMLF, similar results were previously shown, although with higher concentrations of UDCA and its conjugates (161). The same concentration of unconjugated UDCA slightly delayed apoptosis of neutrophils in our experiments. However, the effects of such high concentrations of UDCA and its conjugates are most likely not relevant for circulating neutrophils, given that the sum of maximum concentrations of these BAs in cirrhotic cohort analysed in this study was only 11.6 µM.

Secondary hydrophobic (210, 212) BAs, DCA and LCA and their conjugates, were not associated with neutrophil function in clinical data, however, influenced neutrophil function in *in vitro* experiments. There DCA suppressed phagocytic capacity of neutrophils and ROS

production in response to *E. coli*. DCA was previously described to inhibit neutrophil chemotaxis and calcium mobilization (162). Similar to CDCA, antagonism of FPR1 on neutrophils was proposed as a potential mechanism of these effects (162). As in case of CDCA, DCA was also shown to activate calcium mobilization at higher concentrations (209). DCA *in vitro* effects on neutrophils may be also explained via decrease of membrane fluidity, as was shown in HCT116 cells treated with DCA (164). However, it should be further investigated, if these immunosuppressive effects can play a role in cirrhosis-associated neutrophil dysfunction, as total DCA was significantly lower abundant in liver cirrhotic patients compared to healthy controls, although the absolute concentrations of TDCA and GDCA were significantly increased.

Our results demonstrated the ability of LCA and its conjugates to trigger ROS production in neutrophils, which may explain the elevated level of basal ROS production in neutrophils from cirrhotic patients. LCA and its conjugates are known to be the strongest ligands for TGR5 receptor and TGR5 activation is known to trigger ROS production (85, 145). We could show that neutrophils have a low expression of TGR5, which suggests a possible mechanism of ROS production in response to LCA. Furthermore, LCA inhibited neutrophil ROS production in response to fMLF, when fMLF was added after 45 min of LCA treatment. This could be a consequence of neutrophil exhaustion, because of active ROS production caused by LCA prior to addition of fMLF. All forms of LCA also inhibited neutrophil chemotaxis towards fMLF, although at concentration higher (50 µM), than serum concentrations of these BA in cirrhotic patients. The mechanism behind this should be approached in future studies.

The explanation of LCA ability to delay neutrophil apoptosis might be connected to the ability of LCA to cause ROS production in neutrophils. Superoxide was shown to inhibit apoptosis (44, 45), therefore, if ROS produced in response to LCA are represented mainly by superoxide, this could be the reason for delayed neutrophil apoptosis in presence of LCA. This would be of interest for further studies, which ROS species neutrophils mainly produce in response to LCA, as well, as if LCA increase intracellular or extracellular ROS production.

However, as LCA and its conjugates' concentrations in serum even in cirrhotic patients are very low (sum of maximum concentrations in cirrhotic cohort analyzed in this study was 2.6 µM), the contribution of LCA effects on ROS production, chemotaxis and apoptosis of neutrophils caused at its much higher concentrations is not yet clear. Though all LCA forms are highly hydrophobic BAs and their neutrophil intracellular concentration might depend on

serum protein and lipoprotein composition and might be underestimated, when measured in serum, as discussed above. In this case much higher concentrations of LCA might reach neutrophils and cause the effects we observed in *in vitro* experiments. Furthermore, the pathophysiological meaning of these effects might be more relevant for the gut local immunity, where the concentrations of BA are much higher than in serum (213).

Although BA composition in bile is most likely different from BA composition in serum, our finding of increased NETs production by healthy donor neutrophils after treatment with bile from cirrhotic patients indicates that either BAs or other bile components, which are present in cirrhotic patients' bile, are capable of inducing NETs production. Another possibility would be that the "healthy" bile has some components, which inhibit NETs formation. To date only one study investigated NETs formation in patients with cirrhosis and they found that these patients have a decreased ROS production in response to PMA, which is, however, is not reflected in the representative pictures, which are published in their manuscript (47) and is not directly comparable with our experimental setup. Another study showed increased levels of neutrophil elastase in serum of cirrhotic patients (214), which could be a consequence of increased NETs formation and, therefore, indirectly goes in line with our findings.

Besides, above discussed G protein-coupled receptors, FPR1 and TGR5, high neutrophil expression of nuclear VDR receptor was found in this study. However, here an immediate response of neutrophils to BA stimulation was observed, which occurred within first 15 min of treatment. Signaling through nuclear receptors usually takes longer, for example, functional response via VDR receptor was reached only after 3 hours of cell stimulation (123), on the other hand signaling via surface receptors, like FPR1 (215), or unspecific mechanisms, like changes in membrane fluidity (165), require only minutes. Therefore, in context of the experiments performed within this study, VDR signaling is most likely not involved in the BA effects on neutrophils, which were observed. However, further studies with different experimental setup, e.g. longer BA treatment, might reveal new aspects of VDR signaling of BA in neutrophils.

This study identified that BA composition changes might affect neutrophil function in liver cirrhosis. As BA are metabolites of gut microbiota and gut microbiome shapes serum BA composition (93), this was of interest to study the gut microbiome composition in cirrhotic patients' cohort, in which serum BA levels were also analyzed. It was found that genus Akkermansia was significantly lower abundant in alcoholic liver cirrhosis compared to other

etiologies of liver cirrhosis. Akkermansia was shown to be a “good” bacterium, which promotes intestinal barrier integrity (216). Akkermansia abundance was shown to be affected by BA (217), therefore, peculiarities of BA composition in alcoholic cirrhosis might potentially cause the decrease of its abundance. Interestingly, genus Prevotella, which was significantly higher in other etiologies of cirrhosis group compared to alcoholic and HCV groups, was previously shown to be associated with plasma BA composition (218). We found high abundance of genus Streptococcus as mainly attributed to HCV-associated cirrhosis. It has been previously shown to be involved in primary BAs metabolism (219, 220) and, therefore, might explain the highest serum CDCA level of these patients.

Interestingly, GCDCA RA and GUDCA (absolute concentrations) were the most important BAs, which were associated with interindividual microbiome composition differences in cirrhotic patients, after controlling for confounding of severity and etiology of liver disease. As discussed above we found total CDCA and UDCA being associated with neutrophil function alterations in both cirrhotic patients’ cohort analysis and after direct treatment of healthy donor neutrophils, which suggests that microbiome alterations in liver cirrhosis lead to altered serum CDCA/UDCA ratio and as a consequence neutrophil dysfunction, which can be also facilitated by altered CDCA transport in systemic circulation due to altered BA transporters’ properties and abundance.

UDCA is a result of 7α/β-isomerization of CDCA by gut microbiota (93), e.g. by *Clostridium absonum* (103), *Clostridium baratii* (104), strains from genera Eubacterium and Ruminococcus (105). Based on our findings it seems like this metabolic process is disturbed in liver cirrhosis, resulting in decreased UDCA production and accumulation of not transformed CDCA. Therefore, the modulation of microbiome composition in liver cirrhosis with e.g. probiotic supplements, which aim at restoration of the balance in abundance of bacteria with 7α - and 7β -hydroxysteroid dehydrogenase activity, which are responsible for epimerization of CDCA to UDCA (102), might represent a promising therapeutic strategy in cirrhosis.

In this study we found genus Ruminococcaceae to be negatively correlated with UDCA absolute concentration and relative abundance and genus Blautia to be positively correlated with GUDCA relative abundance in cirrhotic patients. Both involved in BA transformation, mainly conversion from primary to secondary BA, and were shown to be decreased in cirrhosis (221). Investigating the role of these bacterial species in UDCA metabolism might be also considered in future studies. It has been previously suggested that preceding deconjugation

might play role in epimerization process (104), therefore, further studies might more closely analyze, whether the modulation of abundance of bacteria with bile salt hydrolase activity, which are responsible for BA deconjugation, might be taken into consideration when developing therapeutic strategies for cirrhotic patients. The approach to administer a combination of bacterial strains with bile salt hydrolase activity and with 7α - and a 7β - hydroxysteroid dehydrogenase activity in order to increase UDCA production was already proposed (222).

Notably, the analysis of serum BA composition changes in UDCA-treated cirrhotic patients revealed much higher concentrations and RA of all UDCA forms, with the biggest increase of GUDCA and much lower concentration and relative abundance of total CDCA with the largest decrease in GCDCA relative abundance compared to cirrhotic patients, which were not treated with UDCA. Therefore, oral BA supplementation represents another therapeutic approach to restore serum CDCA/UDCA ratio in cirrhotic patients.

As serum protein composition seems to affect neutrophil function and play a role in serum BA transport, we did an exploratory analysis of the changes in all proteins in albumin & IgG depleted serum of cirrhotic patients compared to healthy controls. Indeed, protein composition analyses identified several proteins, which were significantly downregulated in serum of cirrhotic patients and notably most of them were identified to be involved in innate immune response.

We found haptoglobin to be the most downregulated protein in serum of cirrhotic patients compared to healthy controls. Our group has previously found associations of haptoglobin with neutrophil function (34). Angiotensinogen decrease was shown in liver cirrhosis already (223) and might mean that angiotensinogen is used up for the angiotensin II production, which can contribute to the increased vasoconstriction and fibrosis development (224). AAT deficiency can be a genetic disorder, however, changes in serum AAT were also reported in patients with liver disease without presence of AAT genetic disorder, with AAT being normal or increased (225). This protein protects tissues from enzymes of inflammatory cells, especially neutrophil elastase and proteinase-3 (226, 227). The interpretation of decreased AAT in serum of cirrhotic patients in this study is limited, as their AAT genotypes were not determined. Alpha-2-macroglobulin was already measured in cirrhotic patients and its concentration varies depending on the stage of fibrosis. The higher stage of liver fibrosis was associated with the higher level of alpha-2-macroglobulin and it was shown to be upregulated in patients with HCV

associated liver cirrhosis (228). This is in contrast to our finding, as we found it being downregulated in cirrhotic patients' serum, maybe the overall decrease in synthetic liver function in cirrhotic patients we included to the analysis could be the reason for this discrepancy. We found also two complement system proteins to be downregulated, however, according to previous studies, it rather does not play a role in cirrhosis-associated neutrophil dysfunction (24). Thus, our finding of dysregulated serum composition of proteins involved in innate immune response in cirrhosis might be another explanation of the neutrophil function deficiency and should be further investigated for its crosstalk with serum BA composition and as a potential therapeutic target.

Interestingly, albumin intervention in cirrhotic patients in our hands showed only a transient small increase in the number of neutrophils producing ROS in response to *E. coli* 24 hours after albumin intervention, which was not stable and was already not detectable after 48 hours. This may be attributed to the limited sample size. The clinical benefits of such transient changes in neutrophil function need to be studied in future larger studies. Previous studies showed the beneficial effects of albumin administration for overall cirrhotic patients' survival (229) and lower bacterial infections' rate (65), however, effects on neutrophil function have not been shown yet.

Iron metabolism parameters, such as ferritin and heme, might be also possible factors that regulate neutrophil function in liver cirrhosis. It has been previously shown that ferritin-containing fraction of serum significantly decreased neutrophil phagocytosis (230). Another study also showed that high serum ferritin levels can be related to PMN phagocytosis and chemotaxis impairment (231). Some authors link ferritin to the reduction of ROS formation (232). Heme was shown to have pro-inflammatory properties, inducing human neutrophil chemotaxis and cytoskeleton reorganization and triggering the oxidative burst (74, 233), however, inhibiting phagocytosis (75). In this clinical data analysis, we did not find any associations between neutrophil function and heme and ferritin levels in cirrhotic patients.

Increased ANCA levels were shown in liver cirrhosis and were associated with disease severity and risk of infections (234). Moreover, ANCAs were described to induce NETs release (199, 235) and NETs can induce autoantibodies formation (236). This study performed a comparative analysis between different types of autoantibodies and their effects on NETs production of healthy donor neutrophils. Our finding that pANCA, but not cANCA or ANA

autoantibodies cause NETs formation might be a basis for future targeted treatment of dysregulated NETs formation in cirrhosis.

Additionally, it has been shown in this study that the effects of BAs on neutrophil phagocytosis were reversible and could be restored when BAs were washed out. This goes in line with the previous studies, which showed both reversibility of BA effects (161, 179) and reversibility of neutrophil dysfunction in general (31). This makes it feasible to develop further strategies aimed at restoration of neutrophil function via therapeutic modulation of serum BA and protein compositions, which on the long run will allow to improve prognosis and survival of cirrhotic patients.

In conclusion, neutrophil dysfunction in patients with liver cirrhosis is a multifactorial problem. The results of my thesis suggest that altered serum BA and protein compositions might significantly contribute to the observed changes in cirrhotic patients' neutrophil response and, therefore, represent potential targets for combined immunomodulatory therapeutic strategies in liver cirrhosis, aiming at prevention and treatment of bacterial infections (Figure 47).

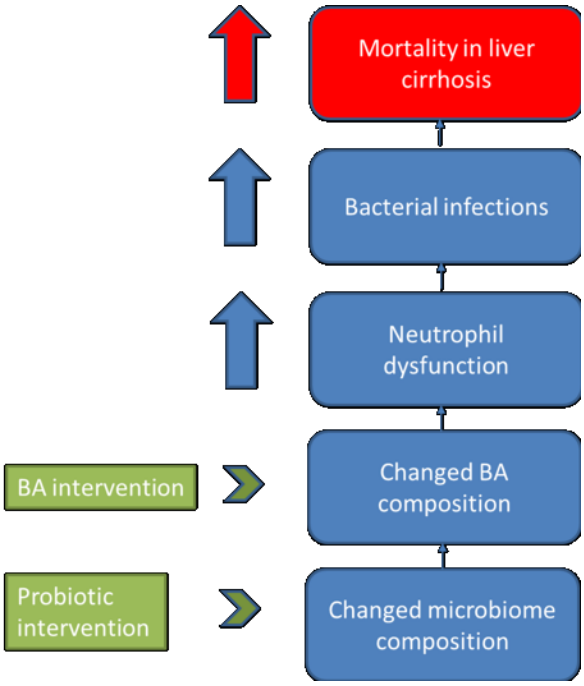


Figure 47. Summary on serum bile acid role in liver cirrhosis.

BIBLIOGRAPHY

1. Schuppan D, Afdhal NH. Liver cirrhosis. Lancet. 2008;371(9615):838-51.
2. Wiegand J, Berg T. The etiology, diagnosis and prevention of liver cirrhosis: part 1 of a series on liver cirrhosis. Dtsch Arztebl Int. 2013;110(6):85-91.
3. Global Health Estimates 2016: Deaths by Cause, Age, Sex, by Country and by Region, 2000-2016. Geneva: World Health Organisation; 2018.
4. Collaborators GBDC. The global, regional, and national burden of cirrhosis by cause in 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet Gastroenterol Hepatol. 2020;5(3):245-66.
5. Mokdad AA, Lopez AD, Shahraz S, Lozano R, Mokdad AH, Stanaway J, et al. Liver cirrhosis mortality in 187 countries between 1980 and 2010: a systematic analysis. BMC Med. 2014;12:145.
6. Fleming KM, Aithal GP, Card TR, West J. All-cause mortality in people with cirrhosis compared with the general population: a population-based cohort study. Liver Int. 2012;32(1):79-84.
7. Pimpin L, Cortez-Pinto H, Negro F, Corbould E, Lazarus JV, Webber L, et al. Burden of liver disease in Europe: Epidemiology and analysis of risk factors to identify prevention policies. J Hepatol. 2018;69(3):718-35.
8. Borzio M, Salerno F, Piantoni L, Cazzaniga M, Angeli P, Bissoli F, et al. Bacterial infection in patients with advanced cirrhosis: a multicentre prospective study. Dig Liver Dis. 2001;33(1):41-8.
9. Arvaniti V, D'Amico G, Fede G, Manousou P, Tsochatzis E, Pleguezuelo M, et al. Infections in patients with cirrhosis increase mortality four-fold and should be used in determining prognosis. Gastroenterology. 2010;139(4):1246-56, 56 e1-5.
10. Bajaj JS, O'Leary JG, Reddy KR, Wong F, Olson JC, Subramanian RM, et al. Second infections independently increase mortality in hospitalized patients with cirrhosis: the North American consortium for the study of end-stage liver disease (NACSELD) experience. Hepatology. 2012;56(6):2328-35.
11. Reddy KR, O'Leary JG, Kamath PS, Fallon MB, Biggins SW, Wong F, et al. High risk of delisting or death in liver transplant candidates following infections: Results from the North American Consortium for the Study of End-Stage Liver Disease. Liver Transpl. 2015;21(7):881-8.

12. Fasolato S, Angeli P, Dallagnese L, Maresio G, Zola E, Mazza E, et al. Renal failure and bacterial infections in patients with cirrhosis: epidemiology and clinical features. Hepatology. 2007;45(1):223-9.
13. Irvine KM, Ratnasekera I, Powell EE, Hume DA. Causes and Consequences of Innate Immune Dysfunction in Cirrhosis. Front Immunol. 2019;10:293.
14. Piano S, Singh V, Caraceni P, Maiwall R, Alessandria C, Fernandez J, et al. Epidemiology and Effects of Bacterial Infections in Patients With Cirrhosis Worldwide. Gastroenterology. 2019;156(5):1368-80 e10.
15. Fernandez J, Prado V, Trebicka J, Amoros A, Gustot T, Wiest R, et al. Multidrug-resistant bacterial infections in patients with decompensated cirrhosis and with acute-on-chronic liver failure in Europe. J Hepatol. 2019;70(3):398-411.
16. Albillos A, Lario M, Alvarez-Mon M. Cirrhosis-associated immune dysfunction: distinctive features and clinical relevance. J Hepatol. 2014;61(6):1385-96.
17. Bernsmeier C, van der Merwe S, Perianin A. Innate immune cells in cirrhosis. J Hepatol. 2020;73(1):186-201.
18. Ley K, Hoffman HM, Kubes P, Cassatella MA, Zychlinsky A, Hedrick CC, et al. Neutrophils: New insights and open questions. Sci Immunol. 2018;3(30).
19. Nauseef WM, Borregaard N. Neutrophils at work. Nat Immunol. 2014;15(7):602-11.
20. Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P, Halbwachs-Mecarelli L. Neutrophils: molecules, functions and pathophysiological aspects. Lab Invest. 2000;80(5):617-53.
21. Rajkovic IA, Yousif-Kadaru AG, Wyke RJ, Williams R. Polymorphonuclear leucocyte locomotion and aggregation in patients with alcoholic liver disease. Clin Exp Immunol. 1984;58(3):654-62.
22. Fiuza C, Salcedo M, Clemente G, Tellado JM. Granulocyte colony-stimulating factor improves deficient in vitro neutrophil transendothelial migration in patients with advanced liver disease. Clin Diagn Lab Immun. 2002;9(2):433-9.
23. Onishi S, Saibara T, Maeda T, Yamamoto Y, Ito K. Serum inhibition of complement derived leukocyte chemotaxis and levels of immunoglobulin A subclass in alcoholic liver disease. Gastroenterol Jpn. 1989;24(3):284-9.
24. Campbell AC, Dronfield MW, Toghill PJ, Reeves WG. Neutrophil function in chronic liver disease. Clin Exp Immunol. 1981;45(1):81-9.
25. Fiuza C, Salcedo M, Clemente G, Tellado JM. In vivo neutrophil dysfunction in cirrhotic patients with advanced liver disease. J Infect Dis. 2000;182(2):526-33.

26. Vanepps DE, Strickland RG, Williams RC. Inhibitors of Leukocyte Chemotaxis in Alcoholic Liver-Disease. Am J Med. 1975;59(2):200-7.
27. Laffi G, Carloni V, Baldi E, Rossi ME, Azzari C, Gresele P, et al. Impaired superoxide anion, platelet-activating factor, and leukotriene B4 synthesis by neutrophils in cirrhosis. Gastroenterology. 1993;105(1):170-7.
28. Knooihuizen SAI, Alexander NJ, Hopke A, Barros N, Viens A, Scherer A, et al. Loss of Coordinated Neutrophil Responses to the Human Fungal Pathogen, Candida albicans, in Patients With Cirrhosis. Hepatol Commun. 2021;5(3):502-15.
29. Artru F, Bou Saleh M, Maggiotto F, Lassailly G, Ningarhari M, Demaret J, et al. IL-33/ST2 pathway regulates neutrophil migration and predicts outcome in patients with severe alcoholic hepatitis. J Hepatol. 2020;72(6):1052-61.
30. Rajkovic IA, Williams R. Abnormalities of neutrophil phagocytosis, intracellular killing and metabolic activity in alcoholic cirrhosis and hepatitis. Hepatology. 1986;6(2):252-62.
31. Mookerjee RP, Stadlbauer V, Lidder S, Wright GA, Hodges SJ, Davies NA, et al. Neutrophil dysfunction in alcoholic hepatitis superimposed on cirrhosis is reversible and predicts the outcome. Hepatology. 2007;46(3):831-40.
32. Stadlbauer V, Mookerjee RP, Hodges S, Wright GA, Davies NA, Jalan R. Effect of probiotic treatment on deranged neutrophil function and cytokine responses in patients with compensated alcoholic cirrhosis. J Hepatol. 2008;48(6):945-51.
33. Taylor NJ, Vijay GKM, Abeles RD, Auzinger G, Bernal W, Ma Y, et al. The severity of circulating neutrophil dysfunction in patients with cirrhosis is associated with 90-day and 1-year mortality. Aliment Pharm Ther. 2014;40(6):705-15.
34. Leber B, Balázs I, Horvath A, Posch A, Streit A, Spindelböck W, et al. Direct acting antiviral therapy rescues neutrophil dysfunction and reduces hemolysis in hepatitis C infection. Translational Research.
35. Horvath A, Leber B, Schmerboeck B, Tawdrous M, Zettel G, Hartl A, et al. Randomised clinical trial: the effects of a multispecies probiotic vs. placebo on innate immune function, bacterial translocation and gut permeability in patients with cirrhosis. Aliment Pharmacol Ther. 2016;44(9):926-35.
36. De Fernandez MA, Clark A, Triger DR. Neutrophil phagocytic and bactericidal function in primary biliary cirrhosis and other chronic liver diseases. Clin Exp Immunol. 1987;67(3):655-61.
37. Huang CH, Jeng WJ, Ho YP, Teng W, Hsieh YC, Chen WT, et al. Increased EMR2 expression on neutrophils correlates with disease severity and predicts overall mortality in cirrhotic patients. Sci Rep. 2016;6:38250.

38. Boussif A, Rolas L, Weiss E, Bouriche H, Moreau R, Perianin A. Impaired intracellular signaling, myeloperoxidase release and bactericidal activity of neutrophils from patients with alcoholic cirrhosis. Journal of Hepatology. 2016;64(5):1041-8.
39. Bruns T, Peter J, Hagel S, Herrmann A, Stallmach A. The augmented neutrophil respiratory burst in response to Escherichia coli is reduced in liver cirrhosis during infection. Clin Exp Immunol. 2011;164(3):346-56.
40. Garfia C, Garcia-Ruiz I, Solis-Herruzo JA. Deficient phospholipase C activity in blood polimorphonuclear neutrophils from patients with liver cirrhosis. Journal of Hepatology. 2004;40(5):749-56.
41. Rolas L, Makhezer N, Hadjoudj S, El-Benna J, Djerdjouri B, Elkrief L, et al. Inhibition of mammalian target of rapamycin aggravates the respiratory burst defect of neutrophils from decompensated patients with cirrhosis. Hepatology. 2013;57(3):1163-71.
42. Rolas L, Boussif A, Weiss E, Letteron P, Haddad O, El-Benna J, et al. NADPH oxidase depletion in neutrophils from patients with cirrhosis and restoration via toll-like receptor 7/8 activation. Gut. 2018;67(8):1505-16.
43. Masini E, Mugnai L, Foschi M, Laffi G, Gentilini P, Mannaioni PF. Changes in the production of nitric oxide and superoxide by inflammatory cells in liver cirrhosis. Int Arch Allergy Immunol. 1995;107(1-3):197-8.
44. Clement MV, Pervaiz S. Intracellular superoxide and hydrogen peroxide concentrations: a critical balance that determines survival or death. Redox Rep. 2001;6(4):211-4.
45. Li PF, Dietz R, von Harsdorf R. Differential effect of hydrogen peroxide and superoxide anion on apoptosis and proliferation of vascular smooth muscle cells. Circulation. 1997;96(10):3602-9.
46. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. Science. 2004;303(5663):1532-5.
47. Agraz-Cibrian JM, Segura-Ortega JE, Delgado-Rizo V, Fafutis-Morris M. Alterations in neutrophil extracellular traps is associated with the degree of decompensation of liver cirrhosis. J Infect Dev Ctries. 2016;10(5):512-7.
48. Agraz-Cibrian JM, Delgado-Rizo V, Segura-Ortega JE, Maldonado-Gomez HA, Zambrano-Zaragoza JF, Duran-Avelar MJ, et al. Impaired neutrophil extracellular traps and inflammatory responses in the peritoneal fluid of patients with liver cirrhosis. Scand J Immunol. 2018;88(5):e12714.
49. Kusaba N, Kumashiro R, Ogata H, Sata M, Tanikawa K. In vitro study of neutrophil apoptosis in liver cirrhosis. Intern Med. 1998;37(1):11-7.

50. Tritto G, Bechlis Z, Stadlbauer V, Davies N, Frances R, Shah N, et al. Evidence of neutrophil functional defect despite inflammation in stable cirrhosis. J Hepatol. 2011;55(3):574-81.
51. Tsiaoussis GI, Assimakopoulos SF, Tsamandas AC, Triantos CK, Thomopoulos KC. Intestinal barrier dysfunction in cirrhosis: Current concepts in pathophysiology and clinical implications. World J Hepatol. 2015;7(17):2058-68.
52. Stadlbauer V, Mookerjee RP, Wright GA, Davies NA, Jurgens G, Hallstrom S, et al. Role of Toll-like receptors 2, 4, and 9 in mediating neutrophil dysfunction in alcoholic hepatitis. Am J Physiol Gastrointest Liver Physiol. 2009;296(1):G15-22.
53. Albillos A, de-la-Hera A, Alvarez-Mon M. Serum lipopolysaccharide-binding protein prediction of severe bacterial infection in cirrhotic patients with ascites. Lancet. 2004;363(9421):1608-10.
54. Dentener MA, Von Asmuth EJ, Francot GJ, Marra MN, Buurman WA. Antagonistic effects of lipopolysaccharide binding protein and bactericidal/permeability-increasing protein on lipopolysaccharide-induced cytokine release by mononuclear phagocytes. Competition for binding to lipopolysaccharide. J Immunol. 1993;151(8):4258-65.
55. Such J, Frances R, Munoz C, Zapater P, Casellas JA, Cifuentes A, et al. Detection and identification of bacterial DNA in patients with cirrhosis and culture-negative, nonneutrocytic ascites. Hepatology. 2002;36(1):135-41.
56. Frances R, Benlloch S, Zapater P, Gonzalez JM, Lozano B, Munoz C, et al. A sequential study of serum bacterial DNA in patients with advanced cirrhosis and ascites. Hepatology. 2004;39(2):484-91.
57. Frances R, Zapater P, Gonzalez-Navajas JM, Munoz C, Cano R, Moreu R, et al. Bacterial DNA in patients with cirrhosis and noninfected ascites mimics the soluble immune response established in patients with spontaneous bacterial peritonitis. Hepatology. 2008;47(3):978-85.
58. Gonzalez-Navajas JM, Bellot P, Frances R, Zapater P, Munoz C, Garcia-Pagan JC, et al. Presence of bacterial-DNA in cirrhosis identifies a subgroup of patients with marked inflammatory response not related to endotoxin. J Hepatol. 2008;48(1):61-7.
59. Spinella R, Sawhney R, Jalan R. Albumin in chronic liver disease: structure, functions and therapeutic implications. Hepatol Int. 2016;10(1):124-32.
60. Oettl K, Stadlbauer V, Petter F, Greilberger J, Putz-Bankuti C, Hallstrom S, et al. Oxidative damage of albumin in advanced liver disease. Bba-Mol Basis Dis. 2008;1782(7-8):469-73.

61. Klammt S, Mitzner S, Stange J, Brinkmann B, Drewelow B, Emmrich J, et al. Albumin-binding function is reduced in patients with decompensated cirrhosis and correlates inversely with severity of liver disease assessed by model for end-stage liver disease. Eur J Gastroen Hepat. 2007;19(3):257-63.
62. Jalan R, Schnurr K, Mookerjee RP, Sen S, Cheshire L, Hodges S, et al. Alterations in the Functional Capacity of Albumin in Patients with Decompensated Cirrhosis Is Associated with Increased Mortality. Hepatology. 2009;50(2):555-64.
63. Arroyo V, Garcia-Martinez R, Salvatella X. Human serum albumin, systemic inflammation, and cirrhosis. J Hepatol. 2014;61(2):396-407.
64. Chen TA, Tsao YC, Chen A, Lo GH, Lin CK, Yu HC, et al. Effect of intravenous albumin on endotoxin removal, cytokines, and nitric oxide production in patients with cirrhosis and spontaneous bacterial peritonitis. Scand J Gastroenterol. 2009;44(5):619-25.
65. Fernandez J, Angeli P, Trebicka J, Merli M, Gustot T, Alessandria C, et al. Efficacy of Albumin Treatment for Patients with Cirrhosis and Infections Unrelated to Spontaneous Bacterial Peritonitis. Clin Gastroenterol Hepatol. 2019.
66. Shalimar, Sheikh MF, Mookerjee RP, Agarwal B, Acharya SK, Jalan R. Prognostic Role of Ammonia in Patients With Cirrhosis. Hepatology. 2019;70(3):982-94.
67. Shawcross DL, Wright GA, Stadlbauer V, Hodges SJ, Davies NA, Wheeler-Jones C, et al. Ammonia impairs neutrophil phagocytic function in liver disease. Hepatology. 2008;48(4):1202-12.
68. Trieb M, Rainer F, Stadlbauer V, Douschan P, Horvath A, Binder L, et al. HDL-related biomarkers are robust predictors of survival in patients with chronic liver failure. J Hepatol. 2020;73(1):113-20.
69. Levine DM, Parker TS, Donnelly TM, Walsh A, Rubin AL. In vivo protection against endotoxin by plasma high density lipoprotein. Proc Natl Acad Sci U S A. 1993;90(24):12040-4.
70. Trieb M, Horvath A, Birner-Gruenberger R, Spindelboeck W, Stadlbauer V, Taschler U, et al. Liver disease alters high-density lipoprotein composition, metabolism and function. Biochim Biophys Acta. 2016;1861(7):630-8.
71. Laichalk LL, Danforth JM, Standiford TJ. Interleukin-10 inhibits neutrophil phagocytic and bactericidal activity. FEMS Immunol Med Microbiol. 1996;15(4):181-7.
72. Milic S, Mikolasevic I, Orlic L, Devcic E, Starcevic-Cizmarevic N, Stimac D, et al. The Role of Iron and Iron Overload in Chronic Liver Disease. Med Sci Monit. 2016;22:2144-51.

73. Arruda MA, Rossi AG, de Freitas MS, Barja-Fidalgo C, Graca-Souza AV. Heme inhibits human neutrophil apoptosis: involvement of phosphoinositide 3-kinase, MAPK, and NF-kappaB. J Immunol. 2004;173(3):2023-30.
74. Graca-Souza AV, Arruda MA, de Freitas MS, Barja-Fidalgo C, Oliveira PL. Neutrophil activation by heme: implications for inflammatory processes. Blood. 2002;99(11):4160-5.
75. Martins R, Maier J, Gorki AD, Huber KV, Sharif O, Starkl P, et al. Heme drives hemolysis-induced susceptibility to infection via disruption of phagocyte functions. Nat Immunol. 2016;17(12):1361-72.
76. van Kessel KP, Bestebroer J, van Strijp JA. Neutrophil-Mediated Phagocytosis of Staphylococcus aureus. Front Immunol. 2014;5:467.
77. Ono Y, Watanabe T, Matsumoto K, Ito T, Kunii O, Goldstein E. Opsonophagocytic dysfunction in patients with liver cirrhosis and low responses to tumor necrosis factor-alpha and lipopolysaccharide in patients' blood. J Infect Chemother. 2004;10(4):200-7.
78. Wyke RJ, Rajkovic IA, Williams R. Impaired opsonization by serum from patients with chronic liver disease. Clin Exp Immunol. 1983;51(1):91-8.
79. Rudman D, Kendall FE. Bile acid content of human serum. II. The binding of cholanic acids by human plasma proteins. J Clin Invest. 1957;36(4):538-42.
80. Kramer W, Buscher HP, Gerok W, Kurz G. Bile salt binding to serum components. Taurocholate incorporation into high-density lipoprotein revealed by photoaffinity labelling. Eur J Biochem. 1979;102(1):1-9.
81. Ceryak S, Bouscarel B, Fromm H. Comparative binding of bile acids to serum lipoproteins and albumin. J Lipid Res. 1993;34(10):1661-74.
82. Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kliewer SA, et al. Bile acids: natural ligands for an orphan nuclear receptor. Science. 1999;284(5418):1365-8.
83. Makishima M, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, et al. Identification of a nuclear receptor for bile acids. Science. 1999;284(5418):1362-5.
84. Wang H, Chen J, Hollister K, Sowers LC, Forman BM. Endogenous bile acids are ligands for the nuclear receptor FXR/BAR. Mol Cell. 1999;3(5):543-53.
85. Kawamata Y, Fujii R, Hosoya M, Harada M, Yoshida H, Miwa M, et al. A G protein-coupled receptor responsive to bile acids. J Biol Chem. 2003;278(11):9435-40.
86. Maruyama T, Miyamoto Y, Nakamura T, Tamai Y, Okada H, Sugiyama E, et al. Identification of membrane-type receptor for bile acids (M-BAR). Biochem Biophys Res Commun. 2002;298(5):714-9.

87. Chavez-Talavera O, Tailleux A, Lefebvre P, Staels B. Bile Acid Control of Metabolism and Inflammation in Obesity, Type 2 Diabetes, Dyslipidemia, and Nonalcoholic Fatty Liver Disease. Gastroenterology. 2017;152(7):1679-94 e3.
88. Gruner N, Mattner J. Bile Acids and Microbiota: Multifaceted and Versatile Regulators of the Liver-Gut Axis. Int J Mol Sci. 2021;22(3).
89. Chiang JYL. Bile acid metabolism and signaling in liver disease and therapy. Liver Res. 2017;1(1):3-9.
90. Hofmann AF, Hagey LR. Bile acids: chemistry, pathochemistry, biology, pathobiology, and therapeutics. Cell Mol Life Sci. 2008;65(16):2461-83.
91. Chiang JY. Bile acid metabolism and signaling. Compr Physiol. 2013;3(3):1191-212.
92. Pandak WM, Kakiyama G. The acidic pathway of bile acid synthesis: Not just an alternative pathway(). Liver Res. 2019;3(2):88-98.
93. Wahlstrom A, Sayin SI, Marschall HU, Backhed F. Intestinal Crosstalk between Bile Acids and Microbiota and Its Impact on Host Metabolism. Cell metabolism. 2016;24(1):41-50.
94. Hofmann AF. The continuing importance of bile acids in liver and intestinal disease. Arch Intern Med. 1999;159(22):2647-58.
95. Yoon S, Yu J, McDowell A, Kim SH, You HJ, Ko G. Bile salt hydrolase-mediated inhibitory effect of Bacteroides ovatus on growth of Clostridium difficile. J Microbiol. 2017;55(11):892-9.
96. Gopal-Srivastava R, Hylemon PB. Purification and characterization of bile salt hydrolase from Clostridium perfringens. J Lipid Res. 1988;29(8):1079-85.
97. Chand D, Panigrahi P, Varshney N, Ramasamy S, Suresh CG. Structure and function of a highly active Bile Salt Hydrolase (BSH) from Enterococcus faecalis and post-translational processing of BSH enzymes. Biochim Biophys Acta Proteins Proteom. 2018;1866(4):507-18.
98. Kim GB, Yi SH, Lee BH. Purification and characterization of three different types of bile salt hydrolases from Bifidobacterium strains. J Dairy Sci. 2004;87(2):258-66.
99. Allain T, Chaouch S, Thomas M, Vallee I, Buret AG, Langella P, et al. Bile-Salt-Hydrolases from the Probiotic Strain Lactobacillus johnsonii La1 Mediate Anti-giardial Activity in Vitro and in Vivo. Front Microbiol. 2017;8:2707.
100. Prete R, Long SL, Gallardo AL, Gahan CG, Corsetti A, Joyce SA. Beneficial bile acid metabolism from Lactobacillus plantarum of food origin. Sci Rep. 2020;10(1):1165.
101. Ridlon JM, Harris SC, Bhowmik S, Kang DJ, Hylemon PB. Consequences of bile salt biotransformations by intestinal bacteria. Gut Microbes. 2016;7(1):22-39.
102. Ridlon JM, Kang DJ, Hylemon PB. Bile salt biotransformations by human intestinal bacteria. J Lipid Res. 2006;47(2):241-59.

103. Macdonald IA, White BA, Hylemon PB. Separation of 7 alpha- and 7 beta-hydroxysteroid dehydrogenase activities from clostridium absonum ATCC# 27555 and cellular response of this organism to bile acid inducers. J Lipid Res. 1983;24(9):1119-26.
104. Lepercq P, Gerard P, Beguet F, Raibaud P, Grill JP, Relano P, et al. Epimerization of chenodeoxycholic acid to ursodeoxycholic acid by Clostridium baratii isolated from human feces. FEMS Microbiol Lett. 2004;235(1):65-72.
105. Lepercq P GP, Béguet F, Grill J-P, Relano P, Cayuela C, Juste C. Isolates from normal human intestinal flora but not lactic acid bacteria exhibit 7a- and 7b-hydroxysteroid dehydrogenase activities. Microb Ecol Health Dis. 2004;16(4):195–201.
106. Chiang JYL, Ferrell JM. Bile Acid Metabolism in Liver Pathobiology. Gene Expr. 2018;18(2):71-87.
107. Neale G, Lewis B, Weaver V, Panveliwalla D. Serum bile acids in liver disease. Gut. 1971;12(2):145-52.
108. Sadaruddin A, Zuberi SJ. Total serum bile acids in liver disease. JPMA The Journal of the Pakistan Medical Association. 1981;31(4):82-4.
109. Luo L, Aubrecht J, Li D, Warner RL, Johnson KJ, Kenny J, et al. Assessment of serum bile acid profiles as biomarkers of liver injury and liver disease in humans. PloS one. 2018;13(3):e0193824.
110. Greco AV, Mingrone G. Serum bile acid concentrations in mild liver cirrhosis. Clin Chim Acta. 1993;221(1-2):183-9.
111. Ferslew BC, Xie G, Johnston CK, Su M, Stewart PW, Jia W, et al. Altered Bile Acid Metabolome in Patients with Nonalcoholic Steatohepatitis. Dig Dis Sci. 2015;60(11):3318-28.
112. Amuro Y, Endo T, Higashino K, Uchida K, Yamamura Y. Serum, fecal and urinary bile acids in patients with mild and advanced liver cirrhosis. Gastroenterol Jpn. 1981;16(5):506-13.
113. Liu N, Feng J, Lv Y, Liu Q, Deng J, Xia Y, et al. Role of bile acids in the diagnosis and progression of liver cirrhosis: A prospective observational study. Exp Ther Med. 2019;18(5):4058-66.
114. Murphy GM, Ross A, Billing BH. Serum bile acids in primary biliary cirrhosis. Gut. 1972;13(3):201-6.
115. Sugita T, Amano K, Nakano M, Masubuchi N, Sugihara M, Matsuura T. Analysis of the serum bile Acid composition for differential diagnosis in patients with liver disease. Gastroenterol Res Pract. 2015;2015:717431.
116. Alm R, Carlson J, Eriksson S. Fasting serum bile acids in liver disease. A comparison with histological features. Scandinavian journal of gastroenterology. 1982;17(2):213-8.

117. Mannes GA, Thieme C, Stellaard F, Wang T, Sauerbruch T, Paumgartner G. Prognostic significance of serum bile acids in cirrhosis. Hepatology. 1986;6(1):50-3.
118. Ahmad TR, Haeusler RA. Bile acids in glucose metabolism and insulin signalling - mechanisms and research needs. Nat Rev Endocrinol. 2019;15(12):701-12.
119. Fiorucci S, Biagioli M, Zampella A, Distrutti E. Bile Acids Activated Receptors Regulate innate immunity. Frontiers in Immunology. 2018;9.
120. Zhu C, Fuchs CD, Halilbasic E, Trauner M. Bile acids in regulation of inflammation and immunity: friend or foe? Clin Exp Rheumatol. 2016;34(4):S25-S31.
121. Haselow K, Bode JG, Wammers M, Ehlting C, Keitel V, Kleinebrecht L, et al. Bile acids PKA-dependently induce a switch of the IL-10/IL-12 ratio and reduce proinflammatory capability of human macrophages. J Leukoc Biol. 2013;94(6):1253-64.
122. Wammers M, Schupp AK, Bode JG, Ehlting C, Wolf S, Deenen R, et al. Reprogramming of pro-inflammatory human macrophages to an anti-inflammatory phenotype by bile acids. Sci Rep. 2018;8(1):255.
123. Pols TWH, Puchner T, Korkmaz HI, Vos M, Soeters MR, de Vries CJM. Lithocholic acid controls adaptive immune responses by inhibition of Th1 activation through the Vitamin D receptor. PLoS One. 2017;12(5):e0176715.
124. Keane RM, Gadacz TR, Munster AM, Birmingham W, Winchurch RA. Impairment of human lymphocyte function by bile salts. Surgery. 1984;95(4):439-43.
125. Ichikawa R, Takayama T, Yoneno K, Kamada N, Kitazume MT, Higuchi H, et al. Bile acids induce monocyte differentiation toward interleukin-12 hypo-producing dendritic cells via a TGR5-dependent pathway. Immunology. 2012;136(2):153-62.
126. Huber RM, Murphy K, Miao B, Link JR, Cunningham MR, Rupar MJ, et al. Generation of multiple farnesoid-X-receptor isoforms through the use of alternative promoters. Gene. 2002;290(1-2):35-43.
127. Vavassori P, Mencarelli A, Renga B, Distrutti E, Fiorucci S. The bile acid receptor FXR is a modulator of intestinal innate immunity. J Immunol. 2009;183(10):6251-61.
128. Schote AB, Turner JD, Schiltz J, Muller CP. Nuclear receptors in human immune cells: expression and correlations. Mol Immunol. 2007;44(6):1436-45.
129. Mueller M, Thorell A, Claudel T, Jha P, Koefeler H, Lackner C, et al. Ursodeoxycholic acid exerts farnesoid X receptor-antagonistic effects on bile acid and lipid metabolism in morbid obesity. J Hepatol. 2015;62(6):1398-404.
130. Yu J, Lo JL, Huang L, Zhao A, Metzger E, Adams A, et al. Lithocholic acid decreases expression of bile salt export pump through farnesoid X receptor antagonist activity. J Biol Chem. 2002;277(35):31441-7.

131. Gadaleta RM, van Erpecum KJ, Oldenburg B, Willemsen EC, Renooij W, Murzilli S, et al. Farnesoid X receptor activation inhibits inflammation and preserves the intestinal barrier in inflammatory bowel disease. Gut. 2011;60(4):463-72.
132. Qiao E, Ji M, Wu J, Ma R, Zhang X, He Y, et al. Expression of the PXR gene in various types of cancer and drug resistance. Oncol Lett. 2013;5(4):1093-100.
133. Xie W, Radominska-Pandya A, Shi Y, Simon CM, Nelson MC, Ong ES, et al. An essential role for nuclear receptors SXR/PXR in detoxification of cholestatic bile acids. Proc Natl Acad Sci U S A. 2001;98(6):3375-80.
134. Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, et al. The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. Proc Natl Acad Sci U S A. 2001;98(6):3369-74.
135. Wallace K, Cowie DE, Konstantinou DK, Hill SJ, Tjelle TE, Axon A, et al. The PXR is a drug target for chronic inflammatory liver disease. J Steroid Biochem Mol Biol. 2010;120(2-3):137-48.
136. Dubrac S, Elentner A, Ebner S, Horejs-Hoeck J, Schmuth M. Modulation of T lymphocyte function by the pregnane X receptor. J Immunol. 2010;184(6):2949-57.
137. Provvedini DM, Tsoukas CD, Deftos LJ, Manolagas SC. 1,25-dihydroxyvitamin D3 receptors in human leukocytes. Science. 1983;221(4616):1181-3.
138. Kizaki M, Norman AW, Bishop JE, Lin CW, Karmakar A, Koeffler HP. 1,25-Dihydroxyvitamin D3 receptor RNA: expression in hematopoietic cells. Blood. 1991;77(6):1238-47.
139. Takahashi K, Nakayama Y, Horiuchi H, Ohta T, Komoriya K, Ohmori H, et al. Human neutrophils express messenger RNA of vitamin D receptor and respond to 1alpha,25-dihydroxyvitamin D3. Immunopharmacol Immunotoxicol. 2002;24(3):335-47.
140. Makishima M, Lu TT, Xie W, Whitfield GK, Domoto H, Evans RM, et al. Vitamin D receptor as an intestinal bile acid sensor. Science. 2002;296(5571):1313-6.
141. Kongsbak M, Levring TB, Geisler C, von Essen MR. The vitamin d receptor and T cell function. Front Immunol. 2013;4:148.
142. Duboc H, Tache Y, Hofmann AF. The bile acid TGR5 membrane receptor: from basic research to clinical application. Dig Liver Dis. 2014;46(4):302-12.
143. Keitel V, Reinehr R, Gatsios P, Rupprecht C, Gorg B, Selbach O, et al. The G-protein coupled bile salt receptor TGR5 is expressed in liver sinusoidal endothelial cells. Hepatology. 2007;45(3):695-704.

144. Deutschmann K, Reich M, Klindt C, Droge C, Spomer L, Haussinger D, et al. Bile acid receptors in the biliary tree: TGR5 in physiology and disease. Biochim Biophys Acta Mol Basis Dis. 2018;1864(4 Pt B):1319-25.
145. Reich M, Deutschmann K, Sommerfeld A, Klindt C, Kluge S, Kubitz R, et al. TGR5 is essential for bile acid-dependent cholangiocyte proliferation in vivo and in vitro. Gut. 2016;65(3):487-501.
146. Hov JR, Keitel V, Schrumpf E, Haussinger D, Karlsen TH. TGR5 sequence variation in primary sclerosing cholangitis. Dig Dis. 2011;29(1):78-84.
147. Pols TW, Noriega LG, Nomura M, Auwerx J, Schoonjans K. The bile acid membrane receptor TGR5 as an emerging target in metabolism and inflammation. J Hepatol. 2011;54(6):1263-72.
148. Studer E, Zhou X, Zhao R, Wang Y, Takabe K, Nagahashi M, et al. Conjugated bile acids activate the sphingosine-1-phosphate receptor 2 in primary rodent hepatocytes. Hepatology. 2012;55(1):267-76.
149. Blaho VA, Hla T. An update on the biology of sphingosine 1-phosphate receptors. J Lipid Res. 2014;55(8):1596-608.
150. Kwong E, Li Y, Hylemon PB, Zhou H. Bile acids and sphingosine-1-phosphate receptor 2 in hepatic lipid metabolism. Acta Pharm Sin B. 2015;5(2):151-7.
151. McQuiston T, Luberto C, Del Poeta M. Role of sphingosine-1-phosphate (S1P) and S1P receptor 2 in the phagocytosis of Cryptococcus neoformans by alveolar macrophages. Microbiology (Reading). 2011;157(Pt 5):1416-27.
152. Jolly PS, Bektas M, Olivera A, Gonzalez-Espinosa C, Proia RL, Rivera J, et al. Transactivation of sphingosine-1-phosphate receptors by FcepsilonRI triggering is required for normal mast cell degranulation and chemotaxis. J Exp Med. 2004;199(7):959-70.
153. Saternos HC, Almarghalani DA, Gibson HM, Meqdad MA, Antypas RB, Lingireddy A, et al. Distribution and function of the muscarinic receptor subtypes in the cardiovascular system. Physiol Genomics. 2018;50(1):1-9.
154. Koarai A, Traves SL, Fenwick PS, Brown SM, Chana KK, Russell RE, et al. Expression of muscarinic receptors by human macrophages. Eur Respir J. 2012;39(3):698-704.
155. Carmona-Rivera C, Purmalek MM, Moore E, Waldman M, Walter PJ, Garraffo HM, et al. A role for muscarinic receptors in neutrophil extracellular trap formation and levamisole-induced autoimmunity. JCI Insight. 2017;2(3):e89780.
156. Raufman JP, Zimniak P, Bartoszko-Malik A. Lithocholyltaurine interacts with cholinergic receptors on dispersed chief cells from guinea pig stomach. Am J Physiol. 1998;274(6):G997-1004.

157. Raufman JP, Chen Y, Zimniak P, Cheng K. Deoxycholic acid conjugates are muscarinic cholinergic receptor antagonists. Pharmacology. 2002;65(4):215-21.
158. Wess J, Eglen RM, Gautam D. Muscarinic acetylcholine receptors: mutant mice provide new insights for drug development. Nat Rev Drug Discov. 2007;6(9):721-33.
159. Dorward DA, Lucas CD, Chapman GB, Haslett C, Dhaliwal K, Rossi AG. The role of formylated peptides and formyl peptide receptor 1 in governing neutrophil function during acute inflammation. Am J Pathol. 2015;185(5):1172-84.
160. Kim SD, Kim JM, Jo SH, Lee HY, Lee SY, Shim JW, et al. Functional expression of formyl peptide receptor family in human NK cells. J Immunol. 2009;183(9):5511-7.
161. Chen X, Yang D, Shen W, Dong HF, Wang JM, Oppenheim JJ, et al. Characterization of chenodeoxycholic acid as an endogenous antagonist of the G-coupled formyl peptide receptors. Inflamm Res. 2000;49(12):744-55.
162. Chen X, Mellon RD, Yang L, Dong H, Oppenheim JJ, Howard OM. Regulatory effects of deoxycholic acid, a component of the anti-inflammatory traditional Chinese medicine Niuhuang, on human leukocyte response to chemoattractants. Biochem Pharmacol. 2002;63(3):533-41.
163. Los DA, Murata N. Membrane fluidity and its roles in the perception of environmental signals. Biochim Biophys Acta. 2004;1666(1-2):142-57.
164. Jean-Louis S, Akare S, Ali MA, Mash EA, Jr., Meuillet E, Martinez JD. Deoxycholic acid induces intracellular signaling through membrane perturbations. J Biol Chem. 2006;281(21):14948-60.
165. Sousa T, Castro RE, Pinto SN, Coutinho A, Lucas SD, Moreira R, et al. Deoxycholic acid modulates cell death signaling through changes in mitochondrial membrane properties. J Lipid Res. 2015;56(11):2158-71.
166. Hino Y, Kumashiro R, Hamada T, Tanaka S, Abe H, Sata M, et al. Protective Effects of Bile-Acids against Lipopolysaccharide-Induced Membrane Fragility in Rat Erythrocytes. J Clin Biochem Nutr. 1991;11(2):111-22.
167. Gazawi H, Ljubuncic P, Cogan U, Hochgraff E, Ben-Shachar D, Bomzon A. The effects of bile acids on beta-adrenoceptors, fluidity, and the extent of lipid peroxidation in rat cardiac membranes. Biochem Pharmacol. 2000;59(12):1623-8.
168. Fan XG, Zhang Z. The determination of neutrophil membrane fluidity in patients with hepatitis B: a fluorescence polarization study. APMIS. 1997;105(4):309-12.
169. Levy R, Schlaeffer F, Keynan A, Nagauker O, Yaari A, Sikuler E. Increased neutrophil function induced by bile duct ligation in a rat model. Hepatology. 1993;17(5):908-14.

170. Tjandra K, Woodman RC, Swain MG. Impaired neutrophil microbicidal activity in rat cholestasis. Gastroenterology. 1997;112(5):1692-8.
171. Swain MG, Tjandra K, Kanwar S, Kubes P. Neutrophil Adhesion Is Impaired in a Rat Model of Cholestasis. Gastroenterology. 1995;109(3):923-32.
172. Gujral JS, Farhood A, Bajt ML, Jaeschke H. Neutrophils aggravate acute liver injury during obstructive cholestasis in bile duct-ligated mice. Hepatology. 2003;38(2):355-63.
173. Dahm LJ, Hewett JA, Roth RA. Bile and Bile-Salts Potentiate Superoxide Anion Release from Activated, Rat Peritoneal Neutrophils. Toxicol Appl Pharm. 1988;95(1):82-92.
174. Dahm LJ, Roth RA. Differential-Effects of Lithocholate on Rat Neutrophil Activation. J Leukocyte Biol. 1990;47(6):551-60.
175. Li J, Dawson PA. Animal models to study bile acid metabolism. Biochim Biophys Acta Mol Basis Dis. 2019;1865(5):895-911.
176. Thakare R, Alamoudi JA, Gautam N, Rodrigues AD, Alnouti Y. Species differences in bile acids I. Plasma and urine bile acid composition. J Appl Toxicol. 2018;38(10):1323-35.
177. Thakare R, Alamoudi JA, Gautam N, Rodrigues AD, Alnouti Y. Species differences in bile acids II. Bile acid metabolism. J Appl Toxicol. 2018;38(10):1336-52.
178. Ohshio G, Miyachi Y, Kudo H, Niwa Y, Manabe T, Tobe T. Effects of sera from patients with obstructive jaundice on the generation of oxygen intermediates by normal polymorphonuclear leukocytes. Liver. 1988;8(6):366-71.
179. Santoro P, Raimondi F, Annunziata S, Paludetto R, Annella T, Ciccimarra F. Unconjugated bile acids modulate adult and neonatal neutrophil chemotaxis induced in vitro by N-formyl-met-leu-phe-peptide. Pediatr Res. 2002;51(3):392-6.
180. Amplatz B, Zohrer E, Haas C, Schaffer M, Stojakovic T, Jahnel J, et al. Bile acid preparation and comprehensive analysis by high performance liquid chromatography-high-resolution mass spectrometry. Clin Chim Acta. 2017;464:85-92.
181. Karmakar U, Chu JY, Sundaram K, Astier AL, Garside H, Hansen CG, et al. Immune complex-induced apoptosis and concurrent immune complex clearance are anti-inflammatory neutrophil functions. Cell Death Dis. 2021;12(4):296.
182. Brinkmann V, Laube B, Abu Abed U, Goosmann C, Zychlinsky A. Neutrophil extracellular traps: how to generate and visualize them. J Vis Exp. 2010(36).
183. Haslett C, Guthrie LA, Kopaniak MM, Johnston RB, Jr., Henson PM. Modulation of multiple neutrophil functions by preparative methods or trace concentrations of bacterial lipopolysaccharide. Am J Pathol. 1985;119(1):101-10.
184. Ruijter J. M. IA, Gunst Q.D. LinRegPCR. Nucleic Acids Research 37: e45; 2009.

185. Klymiuk I, Bilgilier C, Stadlmann A, Thannesberger J, Kastner MT, Hogenauer C, et al. The Human Gastric Microbiome Is Predicated upon Infection with Helicobacter pylori. Front Microbiol. 2017;8:2508.
186. Langille MG, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat Biotechnol. 2013;31(9):814-21.
187. Nusshold C, Kollroser M, Kofeler H, Rechberger G, Reicher H, Ullen A, et al. Hypochlorite modification of sphingomyelin generates chlorinated lipid species that induce apoptosis and proteome alterations in dopaminergic PC12 neurons in vitro. Free Radic Biol Med. 2010;48(12):1588-600.
188. Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, et al. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res. 2019;47(D1):D607-D13.
189. Jassal B, Matthews L, Viteri G, Gong C, Lorente P, Fabregat A, et al. The reactome pathway knowledgebase. Nucleic Acids Res. 2020;48(D1):D498-D503.
190. R Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing; 2020.
191. RStudio Team. RStudio: Integrated Development Environment for R. Boston, MA: RStudio, PBC; 2020.
192. Hammer Ø, Harper, D.A.T., Ryan, P.D. PAST: Paleontological statistics software package for education and data analysis. Palaeontologia Electronica. 2001;4(1):9.
193. Revelle W. psych: Procedures for Psychological, Psychometric, and Personality Research. R package version 1.8.10 ed. Evanston, Illinois: Northwestern University; 2018.
194. Kim S. ppcor: Partial and Semi-Partial (Part) Correlation. R package version 1.1. ed2015.
195. Eichner G. kader: Kernel Adaptive Density Estimation and Regression. 2017.
196. Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, et al. vegan: Community Ecology Package. R package version 2.5-5 ed2019.
197. Wickham H. ggplot2: Elegant Graphics for Data Analysis: Springer-Verlag New York; 2016.
198. Roozendaal C, de Jong MA, van den Berg AP, van Wijk RT, Limburg PC, Kallenberg CG. Clinical significance of anti-neutrophil cytoplasmic antibodies (ANCA) in autoimmune liver diseases. J Hepatol. 2000;32(5):734-41.
199. Kessenbrock K, Krumbholz M, Schonermarck U, Back W, Gross WL, Werb Z, et al. Netting neutrophils in autoimmune small-vessel vasculitis. Nat Med. 2009;15(6):623-5.

200. Hempel SL, Buettner GR, O'Malley YQ, Wessels DA, Flaherty DM. Dihydrofluorescein diacetate is superior for detecting intracellular oxidants: comparison with 2',7'-dichlorodihydrofluorescein diacetate, 5(and 6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate, and dihydrorhodamine 123. Free Radic Biol Med. 1999;27(1-2):146-59.
201. Dikalov S, Griendling KK, Harrison DG. Measurement of reactive oxygen species in cardiovascular studies. Hypertension. 2007;49(4):717-27.
202. Rajecky M, Lojek A, Ciz M. Differentiating between intra- and extracellular chemiluminescence in diluted whole-blood samples. Int J Lab Hematol. 2012;34(2):136-42.
203. Roda A, Cappelleri G, Aldini R, Roda E, Barbara L. Quantitative aspects of the interaction of bile acids with human serum albumin. J Lipid Res. 1982;23(3):490-5.
204. Takikawa H, Sugiyama Y, Hanano M, Kurita M, Yoshida H, Sugimoto T. A novel binding site for bile acids on human serum albumin. Biochim Biophys Acta. 1987;926(2):145-53.
205. Bernardi M, Angeli P, Claria J, Moreau R, Gines P, Jalan R, et al. Albumin in decompensated cirrhosis: new concepts and perspectives. Gut. 2020;69(6):1127-38.
206. Ferrari C, Macchiarulo A, Costantino G, Pellicciari R. Pharmacophore model for bile acids recognition by the FPR receptor. J Comput Aided Mol Des. 2006;20(5):295-303.
207. Wenzel-Seifert K, Seifert R. Cyclosporin H is a potent and selective formyl peptide receptor antagonist. Comparison with N-t-butoxycarbonyl-L-phenylalanyl-L-leucyl-L-phenylalanyl-L- leucyl-L-phenylalanine and cyclosporins A, B, C, D, and E. J Immunol. 1993;150(10):4591-9.
208. de Paulis A, Ciccarelli A, de Crescenzo G, Cirillo R, Patella V, Marone G. Cyclosporin H is a potent and selective competitive antagonist of human basophil activation by N-formyl-methionyl-leucyl-phenylalanine. J Allergy Clin Immunol. 1996;98(1):152-64.
209. Schepetkin IA, Khlebnikov AI, Kirpotina LN, Quinn MT. Antagonism of human formyl peptide receptor 1 with natural compounds and their synthetic derivatives. Int Immunopharmacol. 2016;37:43-58.
210. Heuman DM. Quantitative estimation of the hydrophilic-hydrophobic balance of mixed bile salt solutions. J Lipid Res. 1989;30(5):719-30.
211. Perez MJ, Briz O. Bile-acid-induced cell injury and protection. World J Gastroenterol. 2009;15(14):1677-89.
212. Thomas C, Pellicciari R, Pruzanski M, Auwerx J, Schoonjans K. Targeting bile-acid signalling for metabolic diseases. Nat Rev Drug Discov. 2008;7(8):678-93.
213. Chen ML, Takeda K, Sundrud MS. Emerging roles of bile acids in mucosal immunity and inflammation. Mucosal Immunol. 2019;12(4):851-61.

214. Stanley AJ, McGregor IR, Dillon JF, Bouchier IAD, Hayes PC. Neutrophil activation in chronic liver disease. Eur J Gastroen Hepat. 1996;8(2):135-8.
215. Castaldo M, Zollo C, Esposito G, Ammendola R, Cattaneo F. NOX2-Dependent Reactive Oxygen Species Regulate Formyl-Peptide Receptor 1-Mediated TrkA Transactivation in SH-SY5Y Cells. Oxid Med Cell Longev. 2019;2019:2051235.
216. Grander C, Adolph TE, Wieser V, Lowe P, Wrzosek L, Gyongyosi B, et al. Recovery of ethanol-induced Akkermansia muciniphila depletion ameliorates alcoholic liver disease. Gut. 2018;67(5):891-901.
217. Hagi T, Geerlings SY, Nijsse B, Belzer C. The effect of bile acids on the growth and global gene expression profiles in Akkermansia muciniphila. Appl Microbiol Biotechnol. 2020;104(24):10641-53.
218. Gu Y, Wang X, Li J, Zhang Y, Zhong H, Liu R, et al. Analyses of gut microbiota and plasma bile acids enable stratification of patients for antidiabetic treatment. Nat Commun. 2017;8(1):1785.
219. Li M, Liu S, Wang M, Hu H, Yin J, Liu C, et al. Gut Microbiota Dysbiosis Associated with Bile Acid Metabolism in Neonatal Cholestasis Disease. Sci Rep. 2020;10(1):7686.
220. Salvioli G, Salati R, Bondi M, Fratalocchi A, Sala BM, Gibertini A. Bile acid transformation by the intestinal flora and cholesterol saturation in bile. Effects of Streptococcus faecium administration. Digestion. 1982;23(2):80-8.
221. Kakiyama G, Pandak WM, Gillevet PM, Hylemon PB, Heuman DM, Daita K, et al. Modulation of the fecal bile acid profile by gut microbiota in cirrhosis. J Hepatol. 2013;58(5):949-55.
222. Lepercq P, Hermier D, David O, Michelin R, Gibard C, Beguet F, et al. Increasing ursodeoxycholic acid in the enterohepatic circulation of pigs through the administration of living bacteria. Br J Nutr. 2005;93(4):457-69.
223. Wernze H, Spech HJ, Muller G. Studies on the activity of the renin-angiotensin-aldosterone system (RAAS) in patients with cirrhosis of the liver. Klin Wochenschr. 1978;56(8):389-97.
224. Simoes ESAC, Miranda AS, Rocha NP, Teixeira AL. Renin angiotensin system in liver diseases: Friend or foe? World J Gastroenterol. 2017;23(19):3396-406.
225. Deam D, Byron K, Ratnaike S. Alpha-1-antitrypsin in liver disease. Ann Clin Biochem. 1989;26 (Pt 2):122-4.
226. Travis J, Salvesen GS. Human plasma proteinase inhibitors. Annu Rev Biochem. 1983;52:655-709.

227. Mason DY, Cramer EM, Masse JM, Crystal R, Bassot JM, Breton-Gorius J. Alpha 1-antitrypsin is present within the primary granules of human polymorphonuclear leukocytes. Am J Pathol. 1991;139(3):623-8.
228. Ho AS, Cheng CC, Lee SC, Liu ML, Lee JY, Wang WM, et al. Novel biomarkers predict liver fibrosis in hepatitis C patients: alpha 2 macroglobulin, vitamin D binding protein and apolipoprotein AI. J Biomed Sci. 2010;17:58.
229. Caraceni P, Riggio O, Angeli P, Alessandria C, Neri S, Foschi FG, et al. Long-term albumin administration in decompensated cirrhosis (ANSWER): an open-label randomised trial. Lancet. 2018;391(10138):2417-29.
230. Cantinieaux B, Janssens A, Boelaert JR, Lejeune M, Vermylen C, Kerrels V, et al. Ferritin-associated iron induces neutrophil dysfunction in hemosiderosis. J Lab Clin Med. 1999;133(4):353-61.
231. Park W, Jang DH, Kim SH, Han CS, Shin WS, Kim HY, et al. Impaired polymorphonuclear leukocyte function in chronically hemodialyzed patients with iron overload. Korean J Intern Med. 1988;3(1):64-71.
232. Orino K, Lehman L, Tsuji Y, Ayaki H, Torti SV, Torti FM. Ferritin and the response to oxidative stress. Biochem J. 2001;357(Pt 1):241-7.
233. Porto BN, Alves LS, Fernandez PL, Dutra TP, Figueiredo RT, Graca-Souza AV, et al. Heme induces neutrophil migration and reactive oxygen species generation through signaling pathways characteristic of chemotactic receptors. J Biol Chem. 2007;282(33):24430-6.
234. Papp M, Sipeki N, Vitalis Z, Tornai T, Altorjay I, Tornai I, et al. High prevalence of IgA class anti-neutrophil cytoplasmic antibodies (ANCA) is associated with increased risk of bacterial infection in patients with cirrhosis. J Hepatol. 2013;59(3):457-66.
235. Nakazawa D, Shida H, Tomaru U, Yoshida M, Nishio S, Atsumi T, et al. Enhanced formation and disordered regulation of NETs in myeloperoxidase-ANCA-associated microscopic polyangiitis. J Am Soc Nephrol. 2014;25(5):990-7.
236. Shida H, Hashimoto N, Kusunoki Y, Hattanda F, Ogawa Y, Hayashi T, et al. Anti-neutrophil extracellular trap antibody in a patient with relapse of anti-neutrophil cytoplasmic antibody-associated vasculitis: a case report. BMC Nephrol. 2018;19(1):145.