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Habilitationsschrift

Prognose bestimmende inflammatorische Marker der Leberzirrhose

Zur Erlangung der Lehrbefähigung für das Fach
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von

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S. 93, Lit. 86, Abb. 4, Tab. 2

Referat (Zusammenfassung):

Die Leberzirrhose ist eine Erkrankung mit häufig progredientem Verlauf. Patienten entwickeln vermehrt Zirrhose-assoziierte Komplikationen (dekompensierte Leberzirrhose) und sterben letztendlich häufig am sogenannten akut-auf-chronischen Leberversagen mit Multiorganversagen. Bakterielle Infektionen und eine systemische Inflammationreaktion wind wesentliche Faktoren, welche den Verlauf der Erkrankung bestimmen. Allerdings sind die diagnostischen und therapeutischen Optionen bisher unzureichend, um die hohe Letalität der Zirrhose im Endstadium zu verbessern. Ziel der hier gezeigten Habilitationsarbeit war es daher, prognostische Parameter für Patienten mit fortgeschrittener Leberzirrhose zu evaluieren und damit eine Grundlage für die Anpassung von Management-Strategien für Patienten zu liefern. Über den Prognosescore CLIF-C ACLF Score war es möglich Patienten mit besonders hohem Sterberisiko und Dringlichkeit zur Anpassung der Behandlung zu identifizieren. Es zeigte sich, dass eine Dysfunktion der peritonealen neutrophilen Granulozyten für die hohe Rate an infektiösen Komplikation, insbesondere im Peritoneum, verantwortlich sein können und dass eine übermäßige Aktivierung von Neutrophilen und Monozyten mit Sequestration von Mikropartikeln im Aszites Ausdruck einer allgemeinen Immunaktivierung und schlechter Prognose sind. Zudem konnten neue PCR (polymerase chain reaction) basierte Methoden etabliert werden, über die eine rasche Identifikation und Charakterisierung von bakteriellen und fungalen Infektionen möglich ist.

Auf Grundlage dieser Ergebnisse sollte nun in Zukunft evaluiert werden, ob gezielte Anpassung der Diagnostik und Therapie die Prognose von Patienten mit fortgeschrittener Leberzirrhose verbessern kann.

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Abkürzungsverzeichnis

ACLF – akut-auf-chronisches Leberversagen (acute-on-chronic liver failure)

bactDNA – bakterielle DNA

CLIF-C OF score – CLIF-C organ failure score
CRP – C-reactives Protein

DAMP – Damage associated molecular patterns
DNA – Desoxyribonuklein Säure (Acid)

EASL – European Association for the Study of the Liver
EASL-CLIF – European Foundation for the Study of the Chronic Liver Failure
ESBL - Extended-Spectrum Beta-Lactamasen

FiO2 – Sauerstoffgehalt in der Einatemluft

HCC – hepatozelluläres Karzinom (hepatocellular carcinoma)
HVPG – hepatischer portosystemischer Druckgradient (hepatic venous portosystemic pressure gradient)

IL6 – Interleukin 6
IL1alpha – Interleukin 1alpha
IL10 – Interleukin 10
INR – internationalized normalized ratio

LPS – Lipopolysaccharid

MAP – mittlerer arterieller Druck im Blut
MELD – model of endstage liver disease
MRSA - Methicillin resistenter Staphylococcus aureus
MyD88 – Myeloid differentiation primary response protein MyD88

NOD – Nucleotide-binding oligomerization domain Rezeptoren

PAMP – Pathogen associated molecular patterns

PaO2 – Sauerstoffpartialdruck im Blut
PCR – Polymerase chain reaction
PCT – Procalcitonien
PMN – Polymorphnuclear neutrophils
PPR – pattern recognition receptor

RNA – Ribonukleinsäure (acid)
rRNA – ribsomale RNA

SBP – Spontan bakterielle Peritonitis
SIRS – Systemic inflammatory response syndrome
SOFA score – Sepsis related Organ Failure Assessment score
SpO2 – Sauerstoffsättigung im Blut

TIPS – transjugulärer intrahepatischer portosystemischer Stent
TLR4 – Toll-like receptor 4
TNFalpha – Tumor Necrosis Factor alpha
Traf6 – TNF receptor-associated factor 6
Trim – Tripartite motif (TRIM) family
T-RFLP – terminalen Restriktions Fragment Längen Polymorphismus

VRE - *Vancomycin-resistenten Enterokokken*

1 Einführung in die Thematik

1.1 Definition der Leberzirrhose

Die Leberzirrhose beschreibt das Endstadium chronischer Lebererkrankungen, welche durch einen anhaltenden Schädigungsprozess zur fibrinösen Regeneration mit Septenbildung und Regeneratknoten führen (1). Die histologischen Kriterien einer Zirrhose bestehen aus konfluierenden Septen, Gallengangsproliferaten und Gefäßektasien. Das Laennec Systems graduiert das Ausmaß der Fibrosierung und erlaubt die Einteilung in weitere histologische Substadien. Das Vorliegen dünner Septen definiert ein histologisch frühes Stadium, mindestens zwei breite Septen das fortgeschrittene Stadium und mindestens ein sehr breites Septum bzw. Knotenbildung das Endstadium der Zirrhose (2). Diese histologische Einteilung korreliert mit den klinischen Schweregraden und dem Ausmaß der portalen Hypertension (2).

1.2 Epidemiologie und Ätiologie der Leberzirrhose

In Europa leiden 29 Mio. Menschen und in Deutschland ca. fünf Millionen Menschen an einer chronischen Lebererkrankung und Leberzirrhose. Damit gehören fortgeschrittene Lebererkrankungen zu den häufigsten nicht-übertragbaren chronischen Erkrankungen. Mit fast 20.000 Todesfällen pro Jahr (2% aller Todesfälle) haben Lebererkrankungen in der Letalitätsstatistik einen ähnlichen Stellenwert wie der Schlaganfall und zählen zu den 10 häufigsten Todesursachen in Deutschland (Statistisches Bundesamt, Fachserie 12, Reihe 4, 2015). Die Leberzirrhose macht mehr als 2/3 dieser Leber-assoziierten Todesfälle aus (3). Trotz signifikanter Verbesserungen in der Diagnostik und Behandlung von chronischen Lebererkrankungen sind in den letzten Jahren nicht nur die Inzidenz, sondern auch die Zahl der Todesfälle in einigen Regionen von Europa, insbesondere Osteuropa, Finnland und Großbritannien deutlich angestiegen (3, 4). Der zunehmende Alkoholismus spielt dabei eine entscheidende Rolle (4). Zusammen mit den chronisch viralen Hepatitiden B und C gehören diese mit 60-85% zu den häufigsten Auslösern der Zirrhose (European Liver Transplant Registry 2012). In Zukunft wird sich das Verhältnis deutlich zugunsten der alkoholischen und nicht-alkoholischen Fettlebererkrankungen verschieben. Schuld daran sind zum einen die neuen und sehr effektiven antiviralen Therapien der Hepatitis C und zum anderen die hauptsächlich in West Europa sowie in Nord-Amerika zunehmenden metabolischen Erkrankungen und Adipositas. Die nicht-alkoholische Steatohepatitis als Ursache eines hepatozelluläres Karzinoms (HCC) stieg in Nordamerika von 8.3% im Jahr 2002 auf 13.5% im Jahr 2012 an und verzeichnete damit den deutlichsten Anstieg aller Ätiologien (5).

1.3 Natürlicher Verlauf der Leberzirrhose

1.3.1 Chronisch progredienter Verlauf

Die Leberzirrhose zeigt in vielen Fällen einen progredienten Spontanverlauf, der in der Regel in einer Dekompensation der Leberfunktion mit anschließendem akut-auf-chronischem Leberversagen (ACLF) und schlussendlich Tod mündet. Bei aktiver Grunderkrankung, beispielsweise im Rahmen einer chronischen Virushepatitis C oder B, liegt die jährliche Dekompensationsrate der Leberzirrhose bei 4-10% (6). Entscheidend für die Reduktion des Progressions-Risikos ist die adäquate Behandlung der zugrundeliegenden Lebererkrankung. Sowohl Patienten(innen) mit chronischer Hepatitis C Virusinfektion als auch mit alkoholischer Leberkrankung zeigen nach vollständiger Viruselemination bzw. Abstinenz eine Verbesserung der Leberfunktion und Steigerung des Langezeitüberlebens (7-9).

In den frühen Stadien verläuft die Erkrankung in der Regel klinisch inapparent. Bei weiterer Progression treten Symptome auf, die Ausdruck typischer Zirrhose-assoziierter Komplikationen sind. Spätestens ab diesem Stadium muss die Lebererkrankung als eine Systemerkrankung betrachtet werden, da prinzipiell alle Organe von Komplikationen betroffen sein können (Abb. 1) (10).

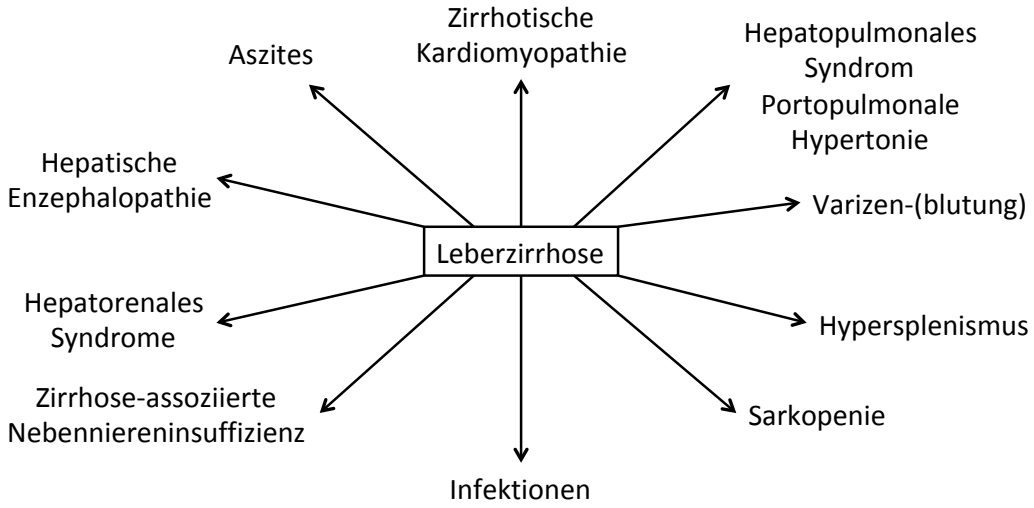

Abbildung 1 : Typische Zirrhose assoziierte Komplikationen.

Zirrhose-assoziierte Komplikationen treten im fortgeschrittenen Stadium der Erkrankung auf und können prinzipiell jedes Organ betreffen.

Das Risiko Zirrhose-assoziierten Komplikationen zu entwickeln wird durch das Ausmaß der portalen Hypertension, welche das Resultat des erhöhten intrahepatischen Widerstandes

und erhöhten Einstrom von Blut in das Splanchnikusgebiet ist, bestimmt (11, 12). Gemessen wird der Pfortaderdruck indirekt durch die invasive Bestimmung des hepatischen portosystemischen Druckgradienten (HVPG). Ab einem HVPG von 6 mmHg besteht eine portale Hypertension, ab 10mmHg ist das Risiko für die Entwicklung von Ösophagusvarizen sowie einer Dekompensation signifikant erhöht, und ab einem Wert von 20mmHg kommt es häufig zu nicht kontrollierbaren Varizenblutungen (13).

1.3.2 Die akute Dekompensation und das akut-auf-chronisches Leberversagen

Unterschiedliche Ereignisse wie bakterielle Infektionen, ein anhaltender Alkoholkonsum oder virale Hepatitiden können eine akute Dekompensation der Zirrhose bis hin zum ACLF (14, 15) auslösen. Die akute Dekompensation ist definiert durch das plötzliche Auftreten von Infektionen, gastrointestinalen Blutungen, einer hepatischen Enzephalopathie und/oder Aszites (14). Kommen weitere extrahepatische Organversagen hinzu, spricht man vom ACLF (14). Entsprechend der EASL-CLIF Kriterien werden die Organversagen über den CLIF-C (organ failure) OF score definiert (Tab. 1).

Tabelle 1: Definition des ACLF über den CLIF-C OF score.

Für den CLIF-C OF score werden entsprechend der individuellen (Dys)funktion von Organsystemen Punktewerte vergeben und anschließend addiert. Der daraus resultierende CLIF-C OF score sagt die Schwere der Lebererkrankung sowie das Sterberisiko von Patienten mit dekompensierter Leberzirrhose voraus. Die rot markierten Bereiche definiteren Organversagen und sind damit Grundlage der ACLF Definition (16).

Organsystem	**1 Punkt**	**2 Punkte**	**3 Punkte**
Leber	Bilirubin <6mg/dl	Bilirubin ≥ 6mg/dl und < 12mg/dl	Bilirubin > 12mg/dl
Niere	Kreatinin <2mg/dl	Kreatinin ≥ 2mg/dl und < 3,5mg/dl	Kreatinin ≥ 3,5 mg/dl oder Dialyse oder Katecholamine*
Hepatische Enzephalopathie	Grad 0	Grad 1-2	Grad 3-4#
Gerinnung	INR<2	INR≥2 bis <2,5	INR≥ 2,5
Kreislauf	MAD≥70 mmHg	MAD<70 mmHg	Katecholamine
Respiration			
PaO_2/FiO_2	>300	≤300 bis >200	≤200
SpO_2/FiO_2	>357	≤357 bis >214	≤214

**Katecholamine müssen zur Therapie einer Niereninsuffizienz/hepatorenales Syndrom gegeben werden*

#Die Intubation aufgrund einer hepatischen Enzephalopathie wird als zerebrales Organversagen (hepatische Enzephalopathie 3 Punkte) gewertet

MAP – Mittlerer arterieller Druck im Blut

PaO2 – Sauerstoffpartialdruck im Blut

FiO2 – Sauerstoffgehalt in der Einatemluft

SpO2 – Sauerstoffsättigung im Blut

INR – Internationalized normalized ratio

Ein ACLF Grad 1 liegt entweder bei einem isolierten Nierenversagen oder bei jedem anderen Organversagen, dann allerdings in Kombination mit einer hepatischen Enzephalopathie Grad 1/2 und/oder Niereninsuffizienz (Serumkreatinin 1,5-2 mg/dL), vor. Bei einem Zwei- oder Mehrorganversagen besteht ein ACLF Grad 2 oder 3 (14, 16, 17).

Generell haben Patienten mit langsam progredienter Leberzirrhose und chronischer Dekompensation, d.h. Persistenz von Zirrhose-assoziierten Komplikationen über das Akutstadium hinaus, ein geringes Regenerationspotential und befinden sich im Endstadium der Erkrankung. Die akute Dekompensation sowie das ACLF können aus jedem Stadium der Lebererkrankung, von der Steatohepatitis bis hin zur fortgeschrittenen Leberzirrhose, entstehen. Einige Patienten zeigen nach Resolution des akuten Ereignisses mitunter persistierende Komplikationen und Organdysfunktionen als Zeichen der eingeschränkten Regeneration (18) (Abb. 2).

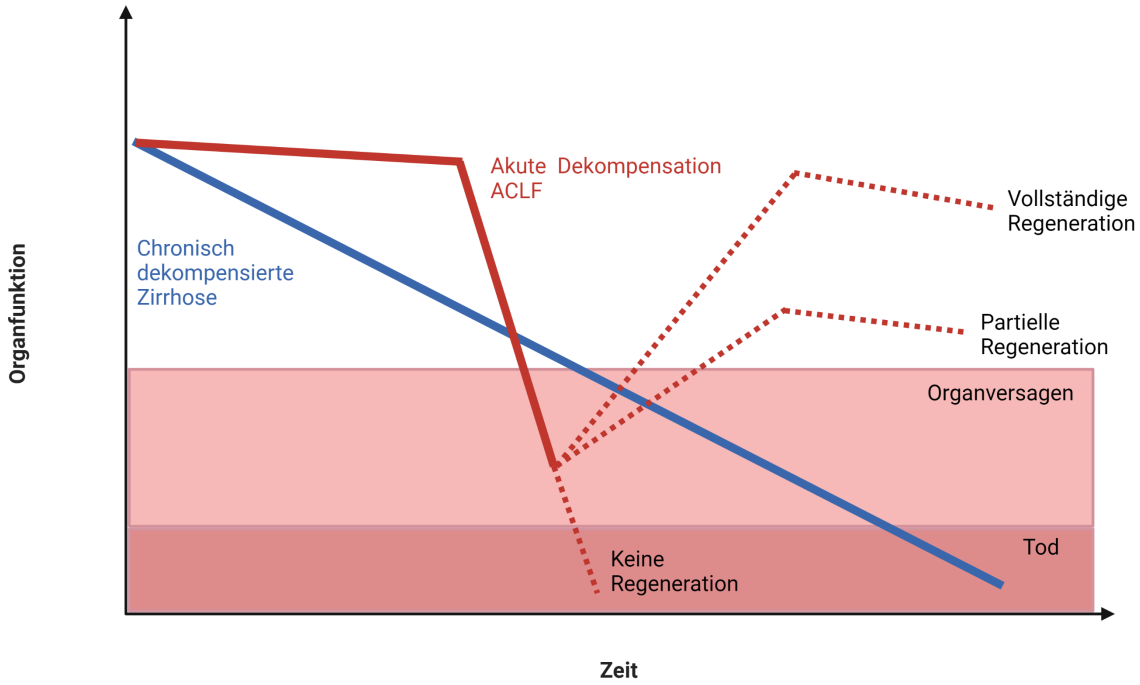

Abbildung 2 : Klinischer Verlauf der fortgeschrittenen Leberzirrhose

Patienten mit chronisch dekompensierter Leberzirrhose zeigen in der Regel einen langsam progredienten Verlauf mit Persistenz von Zirrhose-assoziierten Komplikationen (blaue Linie) die schlussendlich zum Tod führt. Die akute Dekompensation und ACLF können aus allen Stadien der Lebererkrankung heraus entstehen. Prinzipiell ist eine Erholung aus der akuten Phase zum Ausgangszustand möglich. Bei Überlebenden zeigt sich jedoch häufig nur eine partielle Regeneration mit persistierenden residuellen Komplikationen als Zeichen der eingeschränkten Regenerationsfähigkeit [modifiziert nach Jalan 2012 (18)].

1.4 Prognostische Aspekte der Leberzirrhose und ihrer Komplikationen

Patienten im kompensierten Stadium der Zirrhose haben eine mittlere Überlebenszeit von 10-15 Jahren. Mit dem Übergang in ein dekompensiertes Stadium und Entstehung von Zirrhose-assoziierten Komplikationen, wie beispielsweise Aszites oder Varizen, reduziert sich die Überlebenszeit auf 2-4 Jahre (19).

Über ein Klassifikationssystem kann der Einfluss des Auftretens klinischer Komplikationen auf die Überlebenswahrscheinlichkeit abgeschätzt werden. Im kompensierten Stadium erfolgt in Abhängigkeit vom Vorliegen von Varizen und der portalen Hypertension die Einteilung in Stadium 0-2 (20). Die maximale 5-Jahresletalität liegt in diesem Stadium bei 10%. Im Stadium der Dekompensation definiert ein Blutungsereignis das Stadium 3 und ein nicht-Blutungsereignis das Stadium 4, wobei der Aszites die häufigste zu Dekompensation führende Komplikation ist (18-27%). Jedes zweite Dekompensationsereignis definiert das Stadium 5 (20). In diesem Stadium erreicht die 5-Jahresletalität 88%. Mit der weiteren

Progression und Aggravation pathomechanistischer Veränderungen inklusive splanchnischer Vasodilatation, hyperdynamer Kreislaufsituation, bakterieller Translokation und systemischer Inflammation entwickeln Patienten in der Regel eine persistierende Dysfunktion mehrerer Organsysteme (Stadium 6). Typische Zeichen dieses Stadiums sind der refraktäre Aszites, der persistierende Ikterus und/oder hepatische Enzephalopathie sowie das Hepatorenale Syndrom (früher Typ 2). Die Ein-Jahres Letalität liegt in diesem Stadium bei 60-80% (Abb. 3) (20).

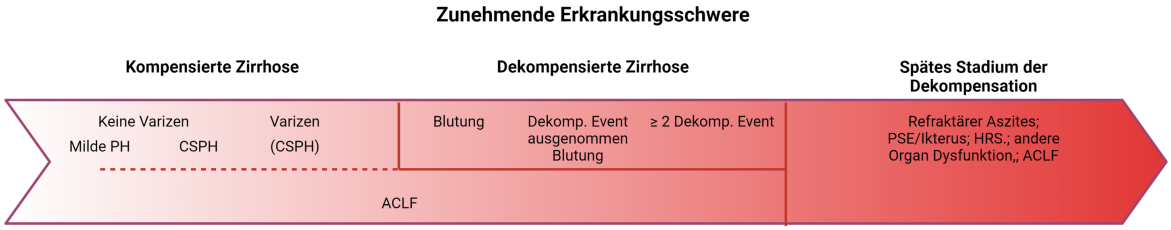

Abbildung 3: Prognose von Patienten mit Leberzirrhose in Abhängigkeit vom Auftreten von Krankheits-assoziierten Komplikationen

Eine zunehmende Zahl an Komplikationen und damit die Progression vom kompensierten zum dekompensierten Stadium erhöht die Letalität [modifiziert nach D'Amico et al (20)].

PH – portale Hypertension

CSPH – klinisch signifikante portale Hypertension (clinical significant portal hypertension)

PSE – portosystemische Encephalopathie (portosystemic encephalopathy)

HRS – Hepatorenales Syndrom (hepatorenal syndrome)

ACLF – Akut-auf-chronisches Leberversagen (acute-on-chronic liver failure)

Andere Prognosescores kombinieren sowohl laborchemische als auch klinische Parameter, die Ausdruck für eine Verschlechterung der Leberfunktion oder Auftreten von Komplikationen sind.

Der Child-Pugh Score war einer der ersten Klassifikationssysteme, der vor über 40 Jahren zur Abschätzung des perioperativen Risikos von Patienten mit Zirrhose entwickelt und wenig später durch Austausch des Ernährungsstatus mit der Prothrombinzeit angepasst wurde (21). Der Score kombiniert die laborchemische Parameter Bilirubin, Kreatinin und INR mit den klinischen Kriterien für Aszites und der hepatischen Enzephalopathie (Tab. 2). Eine Klassifikation in Grad A-C ist über die Einteilung der Punktewerte möglich (Tab. 2) und erlaubt die Vorhersage des Überlebens für Zirrhosepatienten innerhalb eines Jahres. Das mediane 1-Jahresüberleben reduziert sich von 95% im Stadium A, über 80% im Stadium B auf 45% im Stadium C.

Tabelle 2 : Kriterien des Child-Pugh-Scores

Modifiziert nach Durand et al (21)

Kriterium	1 Punkt	2 Punkte	3 Punkte
Albumin	>3,5 g/dl	2,8-3,5 g/dl	<2,8 g/dl
Aszites	Kein oder minimal	Mäßig	Viel
Bilirubin	<34 µmol/l <2 mg/dl	34-51 µmol/l 2-3 mg/dl	>51 µmol/l >3 mg/dl
Quick-Wert (INR)	>70% (<1,7)	40-70% (1,7-2,3)	<40% (>2,3)
Enzephalopathie	Keine	Grad I-II	Grad III-IV

Child-Pugh-Klasse A (5-6 Punkte), Child-Pugh-Klasse B (7-9 Punkte), Child-Pugh-Klasse C (10-15 Punkte). Medianes 1-Jahresüberleben bei Child A, B und C 95%, 80% und 45%

Der Model of Endstage Liver Disease (MELD) Score errechnet sich aus zwei laborchemischen Parametern für die Lebersyntheseleistung (INR, Bilirubin) sowie einem Nierenfunktionsparameter, dem Serum-Kreatinin. Der Score wurde ursprünglich im Jahr 2000 zur 3-Monats Prognose nach Implantation von transjugulären intrahepatischen portosystemischen Stents (TIPS) eingeführt (22). Mittlerweile wird er in unterschiedlichen Ländern, auch in Deutschland, zur Allokation von Spenderorganen bei der Lebertransplantation eingesetzt. Wesentliche Kritikpunkte sind, dass die Laborwerte INR und Serumkreatinin starken Einflüssen unterliegen können (Muskelatrophie – Unterschätzung der Nierenfunktionsstörung, Substitution von Gerinnungsprodukten - Unterschätzung das tatsächlichen INR) und somit intra- und interindividuelle Schwankungen die prognostische Verlässlichkeit einschränken. Anpassungen des MELD Scores, bspw. durch das Einbeziehen vom Serum-Natrium konnten keine signifikante Verbesserung der prognostischen Validität erreichen (23).

Die Entwicklung eines ACLF hat einen erheblichen Einfluss auf das Sterberisiko von Patienten mit Leberzirrhose. Liegt die 28-Tage Sterblichkeit bei der reinen akuten Dekompensation ohne Organversagen noch bei unter 10%, steigt sie bereits mit ACLF Grad 1 auf 40%. Die Letalität mit ACLF Grad 2 liegt bei 55% und beim ACLF Grad 3 bei fast 80% (14). Da die bisher etablierten Prognosescores wie auch der MELD score nicht alle Organsysteme adäquat berücksichtigen, sind sie wenig verlässlich bei der Vorhersage der Überlebenswahrscheinlichkeit (24).

Vom EASL-CLIF Konsortium wurde daher der CLIF-C ACLF score entwickelt, der auf dem CLIF-C OF score (Tab. 1) basiert, diesen jedoch mit der Leukozytenzahl und dem Alter, zwei weiteren wichtigen Prädiktoren für Kurzzeitletalität, kombiniert (16). Der CLIF-C ACLF score konnte die Vorhersagegenauigkeit sowohl für das Kurzzeit- (28 Tage) als auch

Langzeitüberleben (360 Tage) bei ACLF signifikant verbessern (16). Der C-index, welcher ein Maß für die Vorhersagegenauigkeit ist und Werte zwischen 0 und 1 annehmen kann, war mit 0.76 den traditionellen Klassifikationssystem signifikant überlegen (Child-Pugh-Score 0,668, MELD score 0,687, MELD-Natrium 0,684) (16).

1. Problem: Aufgrund der hohen Letalität der dekompensierten Leberzirrhose und des ACLF, insbesondere im ACLF Stadium 3, wurde in der letzten Zeit die Sinnhaftigkeit einer prolongierten intensiven Therapie und Lebertransplantation gegenüber der Palliation kontrovers diskutiert. Um diese Diskussion mit robusten Argumenten zu stützen, ist es notwendig, die Patientengruppen mit ACLF und infauster Prognose von solchen mit Aussicht auf ein spontanes Überleben zu unterscheiden und zu charakterisieren. Dadurch können Strategien sowohl für diagnostische als auch therapeutische Maßnahmen entwickelt werden.

1.5 Rolle inflammatorischer Prozesse in der Progression der Leberzirrhose

Die Progression der Leberzirrhose über die Entwicklung einer akuten Dekompensation und ACLF bis hin zum Tod wird einerseits durch die Grunderkrankung und andererseits ganz wesentlich auch durch die Aktivität des Immunsystems und dessen Antwort auf endogene und exogene Stimuli bestimmt. Hierbei spielen sterile inflammatorische Prozesse, bakterielle Infektionen und eine Immunparalyse eine essentielle Rolle (12).

1.5.1 Systemische Immundysfunktion und deren Folgen

Die Leberzirrhose, insbesondere in Verbindung mit einer portalen Hypertension, ist mit einer zunehmenden Translokation von Bakterien und deren Toxinen aus dem Intestinum über das lokale Lymphsystem und portale Gefäßsystem in die systemische Zirkulation assoziiert. Dieser als intestinale bakterielle Translokation bezeichnete Prozess findet auch bei Gesunden statt. Das Ausmaß ist jedoch signifikant geringer, und die Streuung in die systemische Zirkulation kann durch das lokale Immunsystem kontrolliert werden. Eine mit der Leberzirrhose verbundene qualitative und quantitative intestinale Dysbiose und eine verminderte intestinale Barrierefunktion (“leaky gut”) fördern die Aussaat von Bakterien und Endotoxinen (16, 25, 26). Endotoxine, deren Hauptvertreter das von gram-negativen Bakterien stammende Lipopolysaccharid (LPS) ist, werden durch „Pattern recognition receptors“ (PPR) wie den Toll-like Rezeptoren (TLR) und Nucleotide-binding oligomerization domain Rezeptoren (NODs) erkannt. Die Rezeptoraktivierung induziert durch Rekrutierung einer Reihe von Regulatorgenen (bspw. MyD88, Traf6, Trim) eine pro-inflammatorischen

Signalkaskade, die am Ende in einer Sekretion von inflammatorischen Mediatoren und damit Rekrutierung weiterer Immunzellen mündet (27). Folgerichtig konnten bei Patienten mit akuter Alkoholhepatitis, eine Erkrankung, die auf dem Boden einer chronischen Leberschädigung mit fortgeschrittener Fibrose entsteht, erhöhte zirkulierende LPS Konzentration gemessen werden, die darüber hinaus mit der Überlebenswahrscheinlichkeit assoziiert waren (28). Zudem sind die Konzentrationen zirkulatorischer pro-inflammatorischer Mediatoren wie bspw. IL6, TNFalpha und IL1alpha und anti-inflammatorische Zytokine wie IL10 bei der dekompensierten Leberzirrhose erhöht und steigen bei weiterer Progredienz mit zunehmender Schwere der ACLF Grade weiter an (29). LPS ist über die Bindung an den TLR Subtyp 4 (TLR4) in der Lage eine TNFalpha vermittelte Apoptose auszulösen und somit direkt Gewebs- bzw. Organschäden zu induzieren. Die dadurch freiwerdenden Histone, Nucleosome, humane DNA etc. sind als „Damage associated molecular patterns“ (DAMPs) ebenfalls in der Lage TLRs zu stimulieren (30). Die daraus resultierende sterile und überschießende Immunreaktion ähnelt dem Bild der Sepsis und führt zu hämodynischem Veränderungen mit systemischer Vasodilatation auf der einen Seite und oxidativem Stress und endothelialer Dysfunktion auf der anderen Seite. Die Kombination der beschriebenen Veränderungen führt schlussendlich zu einem Multiorganversagen bzw. schweren ACLF und Tod (16).

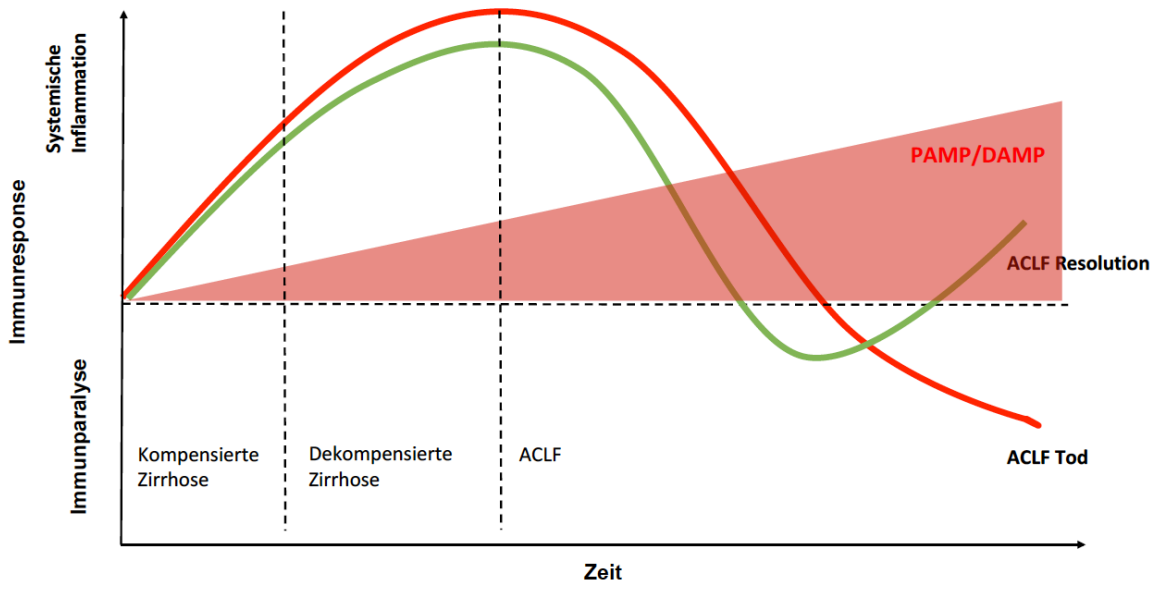

Abbildung 4 : Verlauf der Immundysfunktion bei Leberzirrhose

Durch Stimulation von „Pattern recognition receptors“ (PRR) über Endotoxine (PAMPs) und Zelltod Parameter (DAMPs) wird initial eine überschießende systemische Inflammationsreaktion induziert. Patienten entwickeln Gewebsschäden bis hin zum Multiorganversagen. Im weiteren Verlauf entsteht

auf Grundlage der andauernden Stimulation eine Immunparalyse mit hohem Risiko für sekundäre Infektionen [modifiziert nach Jalan R et al. (18)]

Patienten, die entweder ein ACLF überlebt haben und/oder einen prolongierten klinischen Verlauf zeigen, entwickeln eine Immunzellparalyse, insbesondere des phagozytierenden zirkulierenden Immunsystems (31). Durch die reduzierte bakterielle Clearance steigt die allgemeine Suszeptibilität für sekundäre bakterielle Infektionen. Die Spontan bakterielle Peritonitis (SBP) ist die häufigste Infektion bei Patienten mit Zirrhose. Ob eine ähnliche Dysfunktion der Phagozyten wie im Blut auch in der Peritonealhöhle das Risiko für infektiöse Komplikationen bedingt, ist bisher nicht vollständig geklärt. Peritoneale Makrophagen scheinen eine reduzierte Phagozytoserate aufzuweisen, was Hinweis für eine vergleichbare peritoneale Immundefizienz ist (32).

2. Problem: Es fehlen detaillierte Untersuchen zum Funktionszustand von neutrophilen Granulozytzen, die wesentlich an der bakteriellen Clearance beteiligt sind und Ursache der hohen Suszeptibilität für Infektionen bei Leberzirrhose sein können. Die auffallende Häufung der SBP als infektiöse Komplikation bei Leberzirrhose lässt eine Dysfunktion peritonealer neutrophiler Granulozyten vermuten. Eine genaue Funktionsanalyse sollte Grundlage für die Identifikation von Risikogruppen und potentiellen neuen therapeutischen Angriffspunkten sein.

1.5.2 Das Problem manifester Infektionen

Bakterielle Infektionen sind die häufigsten Zirrhose-assoziierten Komplikationen. Mehr als ein Drittel aller stationären Aufnahmen von Patienten mit Leberzirrhose sind auf infektiöse Komplikationen zurückzuführen (33). Insgesamt 20% aller Patienten mit akuter Dekompensation und 80% aller Patienten mit schwerem ACLF entwickeln eine Infektion während der stationären Behandlung (33). Das Risiko eine bakterielle Infektion zu entwickeln ist gegenüber der Normalbevölkerung um den Faktor 4-5 erhöht (16) und das Sterberisiko liegt nach 30 Tagen bei ca. 40%, fast 4 mal höher ist als bei lebergesunden Vergleichspatienten (34). Patienten mit ACLF, welches durch eine Infektion ausgelöst wurde, haben mit 70% ein vierfach höheres Sterberisiko als Patienten mit ACLF ohne Infektionen als Auslöser (Letalität 30%) (14, 35).

Die SBP und Harnwegsinfektionen machen ca. 50% aller Fälle aus und zählen somit zu den häufigsten infektiösen Komplikationen (36). Hiernach folgen die Bakteriämien, Weichteilinfektionen und Atemwegsinfektionen (36). Bei nosokomialen Infektionen ändert

sich das Spektrum hin zu Harnwegsinfektionen und Atemwegsinfektionen, während die SBP an Bedeutung verliert (36).

In den meisten Fällen werden Infektionen bei Zirrhose durch gram-negative Erreger (60%), insbesondere *E. coli* und *Klebsiella spp*., hervorgerufen. Allerdings zeigte sich in den letzten Jahren eine zunehmende Rate an gram-positive Erregern, beispielsweise *Enterokokken* und *Staphylokokken* (16). Darüber hinaus werden vermehrt Infektionen mit multiresistenten Erregern, beispielsweise *ESBL* (Extended-Spectrum Beta-Lactamasen) bildende Erreger, *MRSA* (Methicillin resistenter Staphylococcus aureus) oder *Vancomycin-resistenten Enterokokken* (VRE) registriert (16).

Da die systemische Inflammationsreaktion auch ohne Nachweis von bakteriellen Erregern ein wesentliches Charakteristikum der dekompensierten Zirrhose und ACLF ist, kann die Diagnostik zur Unterscheidung zwischen einer Infektion und sterilen Immunreaktionen eine Herausforderung sein. Typische klinische Parameter wie Blutdruck oder Atemfrequenz bzw. der SOFA score sind nur wenig diskriminativ, da sie zur Definition sowohl des ACLF als auch der Sepsis verwendet werden (37). Konventionelle mikrobiologischen Techniken liefern keine verlässlichen Ergebnisse, da sie in rund der Hälfte der Fälle trotz einer Infektion keine Erreger nachweisen können (38). Daher ist es notwendig, sich unverändert auf traditionelle Laborparameter, wie C-reaktives Protein (CRP) oder Procalcitonin (PCT), zu verlassen (39). Das Überschreiten bestimmter Grenzwerte (CRP 24,7 ng/ml und PCT 0,49 µg/L) ist ein starker Prädiktor für das Vorliegen einer Infektion (40) (41, 42). Allerdings bergen diese Parameter durch falsch positive Ergebnisse das Risiko einer Übertherapie und können darüber hinaus keine Erregeridentifikation liefern.

3. Problem: Die hohe Suszeptibilität für bakterielle Infektionen, die häufig schweren Verläufe und die zunehmende Rate an multiresistenten Erreger macht es notwendig, Risikogruppen für Infektionen zu identifizieren und bakterielle Erreger rechtzeitig zu diagnostizieren, um somit frühzeitig eine adäquate Therapie einleiten zu können.

2 Ziel der Arbeit

Ziel der Arbeit war es neue prognostische Parameter zur Identifikation neuer diagnostischer Algorithmen für Patienten mit dekompensierter Leberzirrhose zu evaluieren.

3 Ergebnisse eigener Arbeiten

1. Ein CLIF-C ACLF scores >70 nach maximaler Intensivtherapie identifiziert Patienten mit infauster Prognose
2. Die Funktion neutrophiler Granulozyten aus dem Aszites von Patienten mit Leberzirrhose ist signifikant beeinträchtigt, lässt sich allerdings durch Inkubation mit Patientenplasma wiederherstellen
3. Die Quantität von Micropartikeln im Aszites ist mit der Prognose von Patienten mit Leberzirrhose assoziiert
4. Das Vorliegen eines molekularen Bacteraszites bei dekompensierter Leberzirrhose definiert eine Patientengruppe mit schlechtem Outcome
5. Molekulare Quantifizierung und Differenzierung von *Candida* Spezies im Duodenum von Patienten mit Leberzirrhose sind prognostisch relevant

3.1 Ein CLIF-C ACLF scores >70 nach maximaler Intensivtherapie identifiziert Patienten mit infauster Prognose

Ergebnisse und Interpretation

Bei fortgeschrittenen Lebererkrankungen variiert die 90-Tage Sterblichkeit zwischen 10% im Falle einer dekompensierten Leberzirrhose und 75% bei Patienten mit ACLF Grad 3 (14). Trotz deutlicher Fortschritte beim Verständnis der Pathomechanismen und des klinischen Verlaufs der Dekompensation und ACLF (12, 29, 30) gibt es immer noch keine guten Prädiktoren für die Krankheits-assoziierte Sterblichkeit und ein unzureichendes Wissen über die Faktoren, welche die häufig tödlichen Verläufe bedingen. Gerade bei schwer kranken Patienten mit fortgeschrittenem ACLF und Mehrorganversagen, ist die individuelle Prognose von entscheidender Bedeutung, da häufig rasch über weitere therapeutische Optionen, inklusive kurative Lebertransplantation oder Palliation (best supportive care), entschieden werden muss. Der bisher angewendete MELD score ist für die Patienten mit ACLF ungenau (24) und andere Prädiktoren wie der CLIF-C OF score erlauben es nicht auch in Subgruppen, bspw. Patienten mit ACLF Grad 3, eine weitere prognostische Subklassifikation durchzuführen.

Der CLIF-C ACLF score kann Werte zwischen 0 und 100 annehmen und korreliert besser als konventionelle Scores mit der Sterblichkeit von Patienten mit ACLF (16). Es gab erste Analysen, die gezeigt haben, dass Patienten mit einem CLIF-C ACLF score >65 eine Subgruppe von ACLF Patienten darstellen, die eine nahezu infauste Prognose (>90%) haben (43, 44).

In einer retrospektiven Studie haben wir daher verschiedene Scoringsystem innerhalb einer Kohorte von Patienten mit ausschließlich weit fortgeschrittenem ACLF (ACLF Grad 3) hinsichtlich ihrer prognostischen Genauigkeit getestet.

Abstrakt Zitat Engelmann C et al (45):

„Background: Acute-on-chronic liver failure (ACLF) is a severe complication of cirrhosis and is defined by organ failure and high rates of short-term mortality. Patients with ACLF are managed with multiorgan support in the intensive care unit (ICU). Currently, it is unclear when this supportive care becomes futile, particularly in patients who are not candidates for liver transplant. The aim of this study was to determine whether the currently available prognostic scores can identify patients with ACLF in whom prolonged ICU care is likely to be futile despite maximal treatment efforts.

Methods: Data of 202 consecutive patients with ACLF admitted to the ICU at the Royal Free Hospital London between 2005 and 2012 were retrospectively analyzed. Prognostic scores for chronic liver diseases, such as Child-Pugh, Model for End-Stage Liver Disease (MELD), European Foundation for the study of chronic liver failure (CLIF-C) organ failure (OF), and CLIF-C ACLF, were calculated 48 hours after ICU admission and correlated with patient outcome after 28 days.

Results: The CLIF-C ACLF score, compared with all other scores, most accurately predicted 28-day mortality, with an area under the receiver operator characteristic of 0.8 (CLIF-C OF, 0.75; MELD, 0.68; Child-Pugh, 0.66). A CLIF-C ACLF score cutoff ≥ 70 identified patients with a 100% mortality within 28 days. These patients had elevated inflammatory parameters representing a systemic inflammatory response, most often renal failure, compared with patients below this cutoff.

Conclusions: Patients with ACLF and high CLIF-C ACLF score (≥ 70) after 48 hours of intensive care may reach a threshold of futility for further ongoing intensive support. The best treatment options in this scenario remain to be determined but may include palliative care."

Die entscheidenden Ergebnisse der Studie waren, dass während Patienten mit einem CLIF-C ACLF score von weniger als 70 eine Sterblichkeit von 64% aufwiesen, lag die Letalität oberhalb dieses cut-offs bei 100%. Alle analysierten Patienten wurden vor der Datenerhebung über 48 Stunden intensivmedizinisch betreut und hatten vollen Zugang zu allen organunterstützenden Therapien. Die Ergebnisse berücksichtigen damit auch die Krankheitsdynamik unter maximaler Therapie. Dieser Aspekt wurde in einer weiteren gerade publizierten Studie bestätigt (46) und unterstreicht, dass das generelle Management auch das Ansprechen auf eine Therapie beinhalten muss. Sollte dann im Falle eines hohen CLIF-

C ACLF Scores eine kurative Lebertransplantation nicht rasch zur Verfügung stehen, muss aufgrund der fatalen Prognose die palliative Therapie in Betracht gezogen werden.
Bedeutsam war zudem, dass Patienten mit CLIF-C ACLF score >70 eine significant höhere Leukozytenzahl im Blut sowie positive SIRS Kriterien aufwiesen. Dies stärkt die Theorie, dass inflammatorische und infektiöse Prozesse ganz wesentlich den Verlauf der Erkrankung prägen und daher potentiell als Angriffspunkt für Diagnostik und Therapie sein können.

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Validation of CLIF-C ACLF score to define a threshold for futility of intensive care support for patients with acute-on-chronic liver failure

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Abstract

Background: Acute-on-chronic liver failure (ACLF) is a severe complication of cirrhosis and is defined by organ failure and high rates of short-term mortality. Patients with ACLF are managed with multiorgan support in the intensive care unit (ICU). Currently, it is unclear when this supportive care becomes futile, particularly in patients who are not candidates for liver transplant. The aim of this study was to determine whether the currently available prognostic scores can identify patients with ACLF in whom prolonged ICU care is likely to be futile despite maximal treatment efforts.

Methods: Data of 202 consecutive patients with ACLF admitted to the ICU at the Royal Free Hospital London between 2005 and 2012 were retrospectively analyzed. Prognostic scores for chronic liver diseases, such as Child-Pugh, Model for End-Stage Liver Disease (MELD), European Foundation for the study of chronic liver failure (CLIF-C) organ failure (OF), and CLIF-C ACLF, were calculated 48 hours after ICU admission and correlated with patient outcome after 28 days.

Results: The CLIF-C ACLF score, compared with all other scores, most accurately predicted 28-day mortality, with an area under the receiver operator characteristic of 0.8 (CLIF-C OF, 0.75; MELD, 0.68; Child-Pugh, 0.66). A CLIF-C ACLF score cutoff ≥ 70 identified patients with a 100% mortality within 28 days. These patients had elevated inflammatory parameters representing a systemic inflammatory response, most often renal failure, compared with patients below this cutoff.

Conclusions: Patients with ACLF and high CLIF-C ACLF score (≥ 70) after 48 hours of intensive care may reach a threshold of futility for further ongoing intensive support. The best treatment options in this scenario remain to be determined but may include palliative care.

Keywords: ACLF, Futility, Cirrhosis, Intensive care unit

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Background

Acute-on-chronic liver failure (ACLF) is a syndrome that develops in patients with an acute decompensation of liver cirrhosis and is characterized by development of organ failure and high short-term mortality [1]. The diagnostic criteria for organ failure and subsequent ACLF gradation are based on the European Foundation for the study of chronic liver failure (CLIF) organ failure score (CLIF-OF score), a modified version of the Sequential Organ Failure Assessment (SOFA) score [1, 2]. Depending on the ACLF grade, 28-day mortality ranges from 23.3% in ACLF grade 1 to 75.5% in ACLF grade 3 [1], and most patients require intensive care and organ support [3, 4].

In order to prognosticate mortality in patients with ACLF more accurately, the CLIF consortium derived and validated a new score, the CLIF-C ACLF score [2]. The CLIF-C ACLF score combines CLIF-OF score with patients' age and white blood cell (WBC) count to generate a composite score of 0–100 in a linear range. Validation in an external prospective cohort showed that this score was significantly more accurate than Child-Pugh score, Model for End-Stage Liver Disease (MELD) score, and MELD with serum sodium score in predicting 28-day mortality in ACLF [2]. CLIF-C ACLF score predicted short-term mortality 25% better than all listed scores [2]. The 28-day mortality varied from below 20% in CLIF-C ACLF score < 45 to more than 80% in CLIF-C ACLF score ≥ 65 [2].

The utility of CLIF-C ACLF score in patients with ACLF grade 3, and specifically CLIF-C ACLF score > 64, has been discussed [5, 6] because these patients may still have a poor prognosis in spite of maximal treatment efforts and the associated high costs. Validating the CLIF-C ACLF score on the dataset of the CANONIC (EASL-CLIF Acute-on-Chronic Liver Failure in Cirrhosis) study has shown that in a subset of patients with four or more organ failures and/or CLIF-C ACLF score ≥ 65, 3–7 days after ACLF diagnosis, mortality rates were 100%. Single-center experiences in a small subset of such patients with ACLF ($n = 23$) presented by Cardoso et al. [6] supported this notion, albeit that mortality in this cohort was lower at 86% after 90 days [5]. As a consequence, it has been suggested that intensive care support could be withdrawn in patients with this severity of disease. However, because the available data to support this notion are restricted to the CANONIC cohort and one small, single-center, study, further validation is required before this can be considered for translation into clinical practice.

In this study, we aimed to evaluate the short-term outcome of patients with ACLF and compared the predictive value of the CLIF-C ACLF score against other prognostic scores and clinical variables 48 hours after full intensive care support and regardless of when ACLF was first diagnosed. We also aimed to determine whether the CLIF-C ACLF score could be used to define the futility of ongoing intensive care unit (ICU) support.

Methods

Patients and study design

In this retrospective single-center study, data of 202 consecutive patients with ACLF admitted to the ICU at the Royal Free Hospital London were analyzed. All patients received organ system support, including mechanical ventilation, renal replacement therapy, and vasopressor support as required. All parameters at 48 hours of ICU admission were used to diagnose ACLF and to calculate prognostic scores. The parameters included demographic and biological variables and the number of organs that failed. Data for this study were obtained through archived patient notes, collected between 2005 and 2012 in the hospital, and the follow-up data 28 days after ICU admission were retrieved through a combination of the follow-up clinic notes, patients' general physicians, and direct telephone contact with patients themselves. This database is updated at regular intervals, and some of the patients have previously been analyzed to determine predisposing factors leading to ACLF [7] for use as the validation cohort for the CLIF-C ACLF study [2] and to clarify the role of ammonia, inflammation, and oxygenation in brain dysfunction in ACLF [8].

Diagnostic criteria for ACLF and management

Criteria for the diagnosis of ACLF was made using the CLIF-OF classification, which is a modification of the CLIF-C SOFA score [1, 2]. Organ failures were defined as follows according to the method of Moreau et al. [1]: renal failure as serum creatinine ≥ 2 mg/dl and/or requirement for renal replacement therapy; brain failure as hepatic encephalopathy graded III/IV according to the West Haven Criteria; liver failure, defined as bilirubin ≥ 12 mg/dl; coagulation failure as international normalized ratio (INR) ≥ 2.5; circulation failure, defined as treatment with vasoconstrictors to maintain the arterial blood pressure or to increase the cardiac output; and lung failure as a partial pressure of oxygen/fraction of inspired oxygen ratio ≤ 200 or peripheral capillary oxygen saturation/fraction of inspired oxygen ratio ≤ 214. ACLF grade 1 was defined by the presence of single kidney failure or any other organ failure when in combination with either renal insufficiency (serum creatinine ≥ 1.5 mg/dl) or hepatic encephalopathy grade 1/2. The ACLF grade 2 or 3 was defined by the presence of two or at least three organ failures, respectively.

Prognostic score calculation

The CLIF-C ACLF score was calculated by combining the CLIF-C OF score, age, and WBC count with the

Table 1 Clinical parameters according to survival status after 28 days

Parameter	Alive ($n = 103$)	Dead ($n = 99$)	p Value
Male sex, *n* (%)	70 (68%)	66 (67%)	$p = 0.85$
Age, years	50 ± 12	53 ± 11	$p = 0.19$
MELD score	23 ± 9	30 ± 10	$p < 0.0001$
Child-Pugh score	10.5 ± 1.7	11.9 ± 1.6	$p < 0.0001$
Child-Pugh classification, A/B/C, *n* (%)[a]	2/28/73 (2%/27%/71%)	0/8/90 (0%/8%/92%)	$p = 0.001$
CLIF-OF score	11 (9–12)	13 (11–14)	$p < 0.0001$
CLIF-C ACLF score	50.6 ± 7.3	58.4 ± 9.6	$p < 0.0001$
Number of organ failures, 1–3/4–6, *n* (%)	101/2 (98%/2%)	77/22 (78%/22%)	$p < 0.0001$
Renal replacement, *n* (%)	26 (25%)	41 (41%)	$p = 0.02$
HE classification, 0–2/3–4[b], *n* (%)	91/12 (88%/12%)	78/21 (79%/21%)	$p = 0.07$
Bilirubin, mg/dl; μmol/L	3.4 (1.6–9.1); 58 (27–156)	7.8 (3.5–15.5); 133 (60–265)	$p < 0.001$
INR	1.8 (1.5–2.3)	2.2 (1.9–3.1)	$p < 0.0001$
Albumin, g/dl; g/L	2.6 ± 0.7; 26 ± 7	2.5 ± 0.7; 25 ± 7	$p = 0.23$
Platelet count, 10^9/L	87 (57–137)	80 (53–119)	$p = 0.17$
Sodium, mmol/L	138 ± 9	137 ± 10	$p = 0.20$
Serum creatinine, mg/dl; μmol/L	0.9 (0.7–1.6); 80 (62–142)	1.5 (0.9–2.4); 133 (80–212)	$p = 0.002$
WBC count, 10^9/L	9.4 (6.3–15.1)	10.2 (6.3–16.5)	$p = 0.37$

Abbreviations: ACLF Acute-on-Chronic Liver Failure, *CLIF* European Foundation for the study of chronic liver failure, *HE* Hepatic encephalopathy, *INR* International normalized ratio, *MELD* Model for End-Stage Liver Disease, *OF* Organ failure, *WBC* White blood cell
Categorical variables are displayed in percent and continuous variables as mean ± SD (normally distributed data) or median (IQR) (nonparametric testing)
Ascites grades: 0 = no ascites/slight ascites; 1 = moderate ascites; 2 = severe/refractory ascites
[a]No patient in ACLF 3 was allocated to Child-Pugh class A
[b]Classification according to West Haven Criteria [25]

following formula: CLIF-C ACLF = 10 × (0.33 × CLIF-OFs + 0.04 x Age + 0.63 × ln(WBC count) − 2 [2]. The MELD score and Child-Pugh score were calculated as described previously [9]. The systemic inflammatory response syndrome (SIRS) score expressed the number of SIRS criteria components that were fulfilled.

Statistics

Variables were tested for a normal distribution using quantile-quantile plots and histograms. Differences in normally distributed continuous variables were evaluated by Student's *t* test, whereas variables showing skewed distributions with variance heterogeneity were evaluated by the Mann-Whitney *U* test. Pearson χ^2 test was used to compare categorical variables. The accuracy of the CLIF-C ACLF score in predicting survival was assessed by calculating the area under the receiver operating characteristic (AUROC) curve. A cutoff value was chosen to accurately predict fatalities with a high specificity. Survival analysis was performed according to the CLIF-ACLF cutoff values by using Kaplan-Meier analysis and log-rank test for group comparison. Because only one patient was transplanted in the whole cohort, this event was not considered to be a competing risk. Univariate analysis was carried out to identify the baseline factors associated with occurrence of death (*see* Additional file 1). A multivariate Cox regression model was then fitted individually for each prognostic score with identified potentially confounders of death ($p < 0.2$) in this cohort. All potential confounders that were part of predictive score calculations (MELD, Child-Pugh, CLIF-C ACLF) were not included in the multivariate model. Patients lost to follow-up were censored at the time of last patient

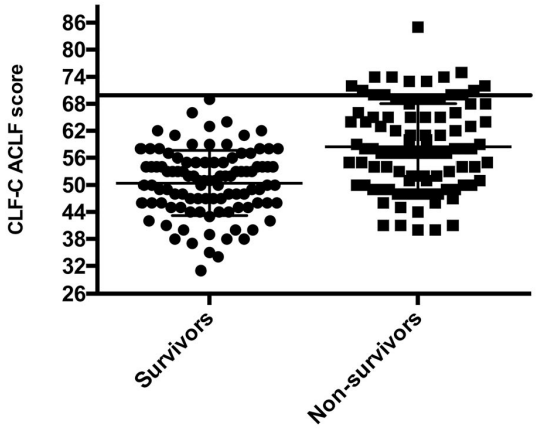

Fig. 1 Individual European Foundation for the study of chronic liver failure (CLIF-C) Acute-on-Chronic Liver Failure (ACLF) scores of survivors and nonsurvivors. The bar represents the CLIF-C ACLF score threshold above which futility of care should be considered

Table 2 Multivariate analysis by Cox regression to adjust prognostic scores with confounders associated with 28-day mortality

MELD score			Child-Pugh score			CLIF-C ACLF score		
Parameter	HR	*p* Value	Parameter	HR	*p* Value	Parameter	HR	*p* Value
MELD score	1.05 (95% CI 1.03–1.07)	< 0.0001	Child-Pugh score	1.35 (95% CI 1.20–1.53)	< 0.0001	CLIF-C ACLF score	1.07 (95% CI 1.05–1.09)	< 0.0001
Age (years)	1.01 (95% CI 1.00–1.03)	0.12	Age (years)	1.02 (95% CI 1.00–1.04)	0.04			
Albumin (g/L)	0.98 (95% CI 0.95–1.01)	0.17				Albumin (g/L)	0.99 (95% CI 0.96–1.02)	0.41
Platelet count (10^9/L)	0.97 (95% CI 0.99–1.00)	0.04	Platelet count (10^9/L)	1.00 (95% CI 0.99–1.00)	0.07	Platelet count (10^9/L)	0.99 (95% CI 0.99–1.00)	0.002
HE (0–2/3–4)	1.31 (95% CI 0.80–2.15)	0.29						

Abbreviations: ACLF Acute-on-Chronic Liver Failure, *CLIF* European Foundation for the study of chronic liver failure, *HE* Hepatic encephalopathy, *MELD* Model for End-Stage Liver Disease

contact. Normally distributed data are presented as mean ± SD, and nonparametric data are presented as median (IQR). A two sided p value < 0.05 was considered statistically significant.

Results

Clinical characteristics of patients

Of the 202 patients included in the study, 99 died within 28 days and 1 was transplanted. In relation to deaths within 48 hours, of the 202 patients included in the study, 9 ACLF grade 3 and 6 ACLF grades 1 + 2 patients died just within this time point, albeit that their retrospective ACLF score classifications were based on the last available data points premortem.

Whereas bilirubin levels (3.4 mg/dl vs. 7.8 mg/dl, $p < 0.001$), INR (1.8 vs. 2.2, $p < 0.0001$), and serum creatinine (0.9 mg/dl vs. 1.5 mg/dl, $p = 0.002$) were higher in patients who died, the sodium level, platelet count, albumin level, and WBC count were not different between survivors and nonsurvivors. Of the patients who survived, 26 (25%) were treated with renal replacement therapy, whereas 41 (41%) ($p = 0.02$) of the nonsurvivors received this therapy. The gender distribution, age, and prevalence of hepatic encephalopathy were similar in both groups. Patients who died more often had a higher number of organ failures than the survivors (4–6 organ failures, survivors 2% vs. nonsurvivors 22%, $p < 0.0001$). The same applied to the CLIF-OF score, which was higher in nonsurvivors (median, 13 [11–14] vs. 11 [9–12]; $p < 0.0001$). All prognostic scores, defining the severity of liver dysfunction, were markedly increased in nonsurvivors. MELD score was 30 ± 10 in nonsurvivors compared with 23 ± 9 in survivors ($p < 0.0001$). In total, 92% of nonsurvivors and 71% of survivors ($p < 0.0001$) had Child-Pugh grade C. The CLIF-C ACLF score of 58.4 ± 9.6 was also statistically

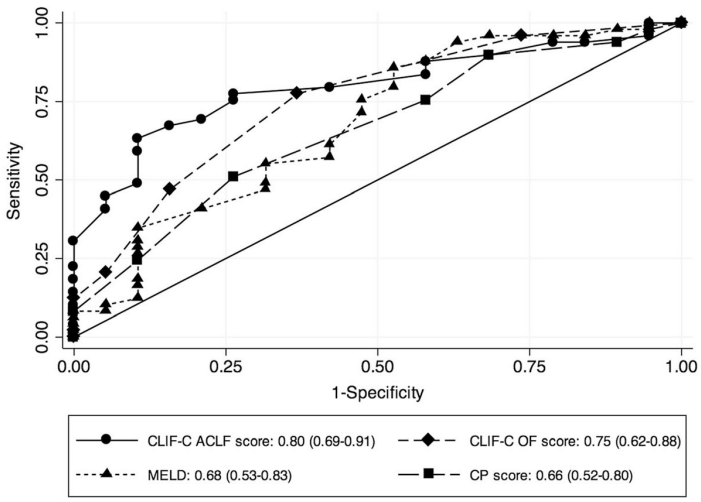

Fig. 2 ROC curves of different prognostic scores in Acute-on-Chronic Liver Failure (ACLF) 3. The European Foundation for the study of chronic liver failure (CLIF-C) ACLF score had the best predictive value for 28-day mortality compared with all other scores. *CP* Child-Pugh, *MELD* Model for End-Stage Liver Disease, *OF* Organ failure

Table 3 Mortality, sensitivity, and specificity for different thresholds of CLIF-C ACLF score

CLIF-C ACLF score	28-Day mortality	Sensitivity	Specificity
≥ 55	80% (95% CI 72–85)	88% (95% CI 75–95)	42% (95% CI 20–67)
≥ 60	88% (95% CI 78–94)	78% (95% CI 63–88)	74% (95% CI 49–91)
≥ 65	94% (95% CI 79–98)	59% (95% CI 44–73)	89% (95% CI 67–99)
≥ 70	100% (95% CI 78–100)	31% (95% CI 18–45)	100% (95% CI 82–100)

Abbreviations: ACLF Acute-on-Chronic Liver Failure, *CLIF* European Foundation for the study of chronic liver failure

higher in patients who died, compared with 50.6 ± 7.3 in survivors ($p < 0.0001$) (Table 1, Fig. 1).

Predictors of 28-day mortality

All prognostic scores were tested individually with significant confounders in a Cox regression analysis to assess and compare their respective predictive abilities. Univariate analysis was carried out to identify potential predictors of 28-day mortality in a univariate Cox regression model (*see* Additional file 1). Variables that were included in the score calculations were not considered for this analysis. Results disclosed CLIF-C ACLF score (HR = 1.07; 95% CI 1.05–1.09; $p < 0.0001$), the Child-Pugh score (HR = 1.35; 95% CI 1.20–1.53; $p < 0.0001$), and the MELD score (HR = 1.05; 95% CI 1.03–1.07; $p < 0.0001$) as the independent predictors of 28-day mortality (Table 2).

Predictors of mortality in ACLF grade 3

Because a previous study [5] has shown that patients with three or more organ failures incur high mortality, further analyses were conducted in patients with ACLF severity grade 3, which is defined by the presence of three or more organ failures [2]. The 28-day mortality in ACLF grade 3 was 72% (49 of 68), and none were transplanted. ROC analysis of all prognostic parameters that were significant upon univariate analysis revealed that the CLIF-C ACLF score had an AUROC of 0.80 (95% CI 0.69–0.91) for predicting 28-day mortality in ACLF grade 3 and was superior to MELD score, CLIF-C OF score, and Child-Pugh score (Fig. 2).

Survival analysis in ACLF grade 3 according to CLIF-C ACLF score

According to the ROC analysis, we depicted different thresholds for the CLIF-C ACLF score to assess their utility in predicting outcome in ACLF 3 patients (Table 3). Applying various thresholds for ACLF score in this population managed in the ICU, it is apparent that 28-day mortality varies from 80% in those with ACLF score ≥ 55 up to 100% in those with ACLF scores ≥ 70. Indeed, the highest specificity for determining 28-day mortality was seen with an ACLF score ≥ 70. Patients with a CLIF-C ACLF score below the threshold of 70 had a mortality of only 64% (34 of 53), which was significantly lower than in patients with ACLF score ≥ 70 ($p = 0.006$) (Fig. 3). The patients with ACLF grade 3 and ACLF score ≥ 70 were significantly older and had a higher number of organ failures. Parameters reflecting an inflammatory response (SIRS, WBC count) were also more elevated compared with patients below this cutoff (Table 4). Patients with CLIF-C ACLF score ≥ 70 incurred more renal failure (93.3% vs. 66%, $p = 0.038$; renal replacement 53% vs. 73%, $p = 0.16$) and a trend toward circulatory failure (87% vs. 62%; $p = 0.07$), whereas all other types of organ failure did not differ from CLIF-C ACLF score < 70 (Table 4).

Discussion

The data presented in this study suggest the CLIF-C ACLF score is the most accurate in predicting short-term (28-day) mortality for patients with ACLF compared with all other tested prognostic scores for chronic liver disease in patients with ACLF, especially for ACLF grade 3. We identified different thresholds of CLIF-C ACLF score to predict short-term mortality, and in order to maximize specificity around a threshold that would inform very high mortality and thereby question the benefit of ongoing ICU supportive care, further analyses were performed using a CLIF-C ACLF score cutoff ≥ 70. Applying a CLIF-C ACLF score cutoff ≥ 70 had 100% specificity for predicting mortality

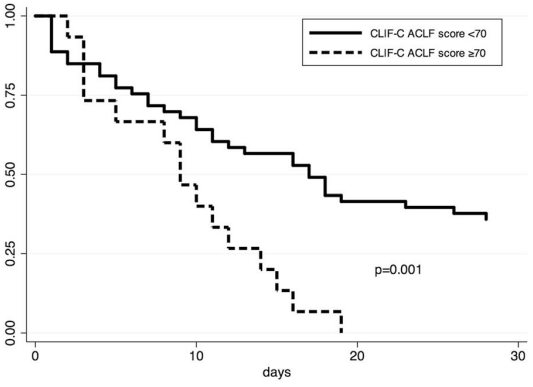

Fig. 3 Twenty-eight-day survival according to the European Foundation for the study of chronic liver failure (CLIF-C) Acute-on-Chronic Liver Failure (ACLF) score in ACLF grade 3. Low 28-day survival is noted in patients with CLIF-C ACLF score ≥ 70, 2 days after receiving full intensive treatment unit supportive therapy

Table 4 Clinical differences in ACLF 3 according to CLIF-C ACLF score

Parameters	CLIF-C ACLF score < 70 *n* = 53	CLIF-C ACLF score ≥ 70 *n* = 15	*p* Value
Age, years	50 ± 11	58 ± 9	$p < 0.001$
Male sex, *n* (%)	33 (62%)	10 (67%)	$p = 0.76$
Etiology of cirrhosis, *n* (%)			$p = 0.52$
ALD	28 (53%)	8 (53%)	
Autoimmune[a]	8 (15%)	1 (7%)	
Viral	5 (9%)	1 (7%)	
Viral + ALD	3 (6%)	1 (7%)	
NASH	1 (2%)	2 (13%)	
Cryptogenic + others	8 (15)	2 (13%)	
Precipitating event (infection/bleeding/both/unknown), *n* (%)	23/12/7/11 (43%/23%/13%/21%)	4/5/1/5 (27%/33%/7%/33%)	$p = 0.46$
CLIF-C OF score	14 (13–15)	16 (15–17)	$p < 0.001$
MELD	31.6 ± 8.7	38.1 ± 10.4	$p = 0.02$
SIRS score	2 (1–3)	3 (2–3)	$p = 0.04$
WBC (10^9/L)	11 (6–17)	21 (15–24)	$p = 0.002$
Bilirubin, mg/dl; μmol/L	10.5 (4.3–15.8); 180 (74–270)	12.5 (6.9–22.8); 214 (118–390)	$p = 0.43$
Creatinine, mg/dl; μmol/L	1.7 (1.1–2.6); 150 (97–230)	2.4 (1.6–4.0); 212.2 (142–354)	$p = 0.15$
Sodium, mmol/L	135 ± 8	136 ± 15	$p = 0.90$
Renal replacement, *n* (%)	28 (53%)	11 (73%)	$p = 0.16$
Median number of organ failures	3 (3–3)	4 (3–5)	$p < 0.001$
Type of organ failure[a]			
Liver failure, *n* (%)	23 (43%)	9 (60%)	$p = 0.255$
Renal failure, *n* (%)	35 (66%)	14 (93%)	$p = 0.038$
Cerebral failure, *n* (%)	16 (30%)	5 (33%)	$p = 0.816$
Coagulation failure, *n* (%)	31 (58%)	11 (73%)	$p = 0.296$
Circulatory failure, *n* (%)	33 (62%)	13 (87%)	$p = 0.074$
Respiratory failure, *n* (%)	35 (66%)	9 (60%)	$p = 0.666$

Abbreviations: ACLF Acute-on-Chronic Liver Failure, *ALD* Alcoholic liver disease, *CLIF* European Foundation for the study of chronic liver failure, *MELD* Model for End-Stage Liver Disease, *NASH* Nonalcoholic steatohepatitis, *OF* Organ failure, *SIRS* Systemic inflammatory response syndrome, *WBC* White blood cell
Categorical variables are displayed in percent and continuous variables as mean ± SD (normally distributed data) and median (IQR) (nonparametric testing)
[a]Organ failures defined according to the CLIF-C OF score [2]

such that all patients above this threshold died within 28 days after ICU admission, despite maximal treatment efforts, including full organ support as per standard of care in our tertiary center. Maximal supportive treatment was provided up to the point that death was thought to be imminent. Despite the relatively limited number of patients with CLIF-C ACLF ≥ 70 ($n = 15$), our data suggest that ongoing intensive care support in these patients, in the absence of liver transplant, may be futile, given no improvement despite full organ support as needed for 48 hours. Our data are in line with previous reports showing a very poor prognosis in similar cohorts of at-risk patients, in whom dynamic assessments of change in CLIF-C ACLF score showed that those with further progression of ACLF grade or failed improvement had high mortality [5, 6]. The best management options in this scenario, given currently available limited therapies, require further evaluation, including the need for palliative care pathways.

The time point at which patients' prognosis is assessed seems to be key. Our data suggest that mortality was relatively low (approximately 35–40%) within the first week after intensive treatment unit (ITU) admission, but beyond this, all remaining patients died quickly (within 2 weeks). This might imply that patients with CLIF-C ACLF ≥ 70 may have limited reserve and regenerative capacity, even if receiving full intervention support for the initial precipitating event. Moreover, the short survival period is an argument that either palliative care or, if eligible, liver transplant [10–12] should be discussed early after assessing the response to intensive care therapy for 48 hours, because the time until death and the window for intervention is very short thereafter.

Liver transplant in so-called high-MELD patients is highly debated because it is associated with significant posttransplant morbidity [13, 14]. Importantly, in our center, transplant selection aims at > 90% one-year survival, which necessitates the exclusion of urgently listing patients with decompensation or ACLF. However, there are data to suggest that overall survival can be in excess of 80% and comparable to patients transplanted without ACLF [12], which is also substantiated in other studies, including studies of living donor liver recipients [10, 15, 16]. By contrast, a retrospective study by Levesque et al. showed in a subgroup of 30 patients with ACLF grade 3 a 12-month survival rate of 43% after cadaveric liver transplant [11]. These studies clearly highlight that although it is worthwhile discussing liver transplant in ACLF grade 3, this must be tempered by assessment of factors that may indicate worse outcome after liver transplant, such as infections, age, and presence of hepatocellular carcinoma, as proposed by Levesque et al. [11]. In addition, patients through debilitation of their advanced liver disease and a continued severe inflammatory state, as seen with ACLF, would be expected to be frail and may not be rescued by liver transplant [17].

To date, interventions such as extracorporeal liver support, such as the trials with MARS (molecular adsorbent recirculation system) [18, 19], have failed to show any clear survival benefit in ACLF 3. When undertaking consideration for such interventions in such an advanced disease cohort, appropriate resource allocation and effectiveness of the intervention must remain major considerations for implementation. Until there are new interventions with proven efficacy, futility of ongoing intensive care support should be discussed early, also taking into consideration that cirrhosis and ACLF represent an increasing health and socioeconomic burden [20]. Such early decision-making processes help facilitate an appropriate and adequate palliative care option in a cohort in whom mortality is high, despite maximal intensive treatment support.

It is important to note that a CLIF-C ACLF score ≥ 70 was associated with distinct clinical features. Notably, the SIRS score and WBC count, which are reflective of an inflammatory response, were significantly higher in those with ACLF score ≥ 70, albeit that infections as specific precipitating events were not overrepresented and patients received antibiotic treatment as part of the standard procedure. This in line with the assertion that increasing disease severity in ACLF is accompanied by a systemic inflammatory response. Claria et al. [21] and others have shown that proinflammatory cytokines increase throughout the different severity grades of ACLF and that such inflammation is associated with higher mortality [22–24]. This may imply that strategies to lower inflammation and thereby risk of new infection, such as gut decontamination, may improve outcomes, but further clinical trials of such interventions are needed. Moreover, it remains to be seen whether these strategies are cost-effective in such sick patients.

There are some limitations of this study that need consideration. First, this study is a retrospective analysis of prospectively gathered data, which may be regarded as a weakness because some potential contributory factors that might influence outcome may not have been assessed at the time of enrollment. Second, a further potential limitation of this study is that the response to supportive therapy in the ICU was evaluated at 48 hours and not beyond. The previously reported outcomes in ACLF grade 3 patients in the CANONIC study by Gustot et al. showed that assessment of CLIF-C ACLF score between days 3 and 7 and a change in score determined longer-term outcome. This supports the idea of repeated assessments to define futility in such patients, in whom a fixed time of assessment may sometimes be difficult [5].

Conclusions

Patients with ACLF who require intensive care supportive treatment should be assessed early after ITU admission using the CLIF-C ACLF score. In patients with ACLF 3 and a CLIF-C ACLF score ≥ 70, who are not suitable for liver transplant, futility of continued currently available intensive supportive therapy should be considered. The best treatment options in this scenario remain to be determined but may include palliative care.

Additional file

Additional file 1: Table S1. All collected parameters were analyzed using univariate Cox regression to identify potential predictors of 28-day mortality. (DOCX 17 kb)

Abbreviations

ACLF: Acute-on-chronic liver failure; AUROC: Area under the receiver operator characteristic; CLIF: European Foundation for the study of chronic liver failure; CLIF-OF: European Foundation for the study of chronic liver failure organ failure score; CP: Child-Pugh; ICU: Intensive care unit; INR: International normalized ratio; ITU: Intensive treatment unit; MELD: Model for End-Stage Liver Disease; NASH: Nonalcoholic steatohepatitis; SIRS: Systemic inflammatory response syndrome; SOFA: Sequential Organ Failure Assessment; WBC: White blood cell

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CE, KLT, and RPM drafted the manuscript. RJ, BA, and RPM contributed to the conception and design of the study. NZ and MS contributed substantially to aspects that were listed for all authors. All authors were involved in the acquisition, analysis, and interpretation of data. All authors revised the

manuscript critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The research and development department at the Royal Free Hospital, where this project was undertaken, considers this study a service evaluation, with no requirement for formal ethics approval and consent to participate. All patient data were fully anonymized and archived according to the local data protection guidelines. The study described conformed to the ethical guidelines of the 1975 Declaration of Helsinki. This study was conducted and reported according to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines.

Consent for publication
Not applicable.

Competing interests
RJ has ongoing research collaboration with Yaqrit and Takeda Pharmaceutical Company Ltd. RJ is also inventor of a drug, L-ornithine phenylacetate (OCR-002), which University College London has licensed to Ocera Therapeutics. RJ is also the founder of University College London spin-off companies Yaqrit Ltd. and CYBERLIVER Ltd. CE has an ongoing research collaboration with Sequana Medical, Merz Pharma, and Novartis. CE has received speaker's fees from Novartis, Gilead Sciences, and Merz Pharma and was a member of the advisory board for Chiesi Farmaceutici. RPM has an ongoing research collaboration with Yaqrit. NZ recieved funding support from Welcome Trust. All other authors declare that they have no competing interests.

Publisher's Note

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3.2 Die Funktion neutrophiler Granulozyten aus dem Aszites von Patienten mit Leberzirrhose ist signifikant beeinträchtigt, lässt sich allerdings durch Inkubation mit Patientenplasma wiederherstellen

Ergebnisse und Interpretation

Bakterielle Infektionen im Allgemeinen und die SBP im Speziellen haben einen großen negativen Einfluss auf die Prognose von Patienten mit Leberzirrhose (47). Die Ursachen der hohen Suszeptibilität für SBP Infektionen bei Patienten mit Leberzirrhose sind bisher unzureichend geklärt. Da das generell hohe Risiko für bakterielle Infektionen mit der Dysfunktion bzw. Paralyse von zirkulierenden phagozytierenden Zellen im Zusammenhang steht (31), ist es naheliegend ähnliche Veränderungen auch bei den peritonealen Phagozyten anzunehmen. Allerdings gibt es nur wenige Untersuchungen, die sich mit der lokalen, abdominellen Immunzellfunktion auseinandersetzen. Erste Ergebnisse haben gezeigt, dass peritoneale Makrophagen eine reduzierte Fähigkeit haben Mikroorganismen zu phagozytieren oder durch oxidativen Burst zu eliminieren (32). Darüber hinaus ist die Opsonierungsfähigkeit im Aszites reduziert, so dass vorhandene Erreger weniger gezielte Immunreaktionen auslösen und dadurch das Risiko zur Entwicklung einer SBP gesteigert ist (32). Es gibt lediglich eine Studie, welche die Funktion von neutrophilen Granulozyten im Aszites untersucht hat. In dieser zeigte sich bei Patienten mit SBP im Vergleich zu Patienten ohne SBP eine signifikante Suppression der oxidativen Burst Rate. Durch eine antibiotische Therapie konnte diese verbessert werden (48). Allerdings fehlte in dieser Untersuchung der Vergleich zur zirkulierenden Neutrophilen, so dass der eigentliche Funktionsverlust peritonealer Neutrophiler unklar blieb.

Abstrakt Zitat Engelmann C et al. (49):

„Systemic immune cell dysfunction is a typical feature of liver diseases and increases the risk of bacterial infection, especially spontaneous bacterial peritonitis. We evaluated functional properties of neutrophil granulocytes in blood and ascites of patients both with and without decompensated cirrhosis. We collected blood and ascites samples from 63 patients with cirrhosis and eight without cirrhosis. Phagocytosis activity (PA) and oxidative burst activity (OBA) were evaluated after ex vivo stimulation with E. coli, while fluorescence signals were measured by flow cytometry. Ascites' neutrophil function tests were repeated after incubation with autologous plasma. Ascites' neutrophils showed an impaired PA and OBA (median blood PA 98.1% (86.8–99.8) vs. ascites' PA 50.5% (0.4–97.3), $p < 0.0001$; median blood OBA 98.7% (27.5–100) vs. ascites' OBA 27.5% (0.3–96.7), $p < 0.0001$). Patients with non-cirrhotic ascites showed higher PA but equally suppressed OBA. Ascites' neutrophil function

could be partially restored after incubation with autologous plasma (median increase PA: 22.5% (−49.7 – +93.2), p = 0.002; OBA: 22.8% (−10.4 – +48.8), p = 0.002). Ascites' neutrophils of patients with cirrhosis are functionally impaired, but could be partially restored after incubation with plasma. Further investigations are needed to identify the factors in ascites that are associated with neutrophils' function."

In dieser Studie konnten wir demnach nachweisen, dass die Phagozytoserate und oxidative Burst Rate von Neutrophilen im Aszites von Patienten mit Zirrhose im Vergleich zu Blutneutrophilen um mehr als die Hälfte reduziert ist. Da es keine Korrelation zwischen der Funktion neutrophiler und zirkulierender Neutrophiler gab, nahmen wir an, dass die Funktion der Immunzellen in beiden Kompartimenten differentiell reguliert wird. Interessanterweise führte die Inkubation der peritonealen Neutrophilen mit autologem Patientenplasma zur signifikanten Funktionssteigerung, was nahelegt, dass supportive Faktoren im Peritoneum defizitär sind.
Die Paralyse lokaler phagozytierender Zellen ist daher ein möglicher Erklärungsansatz für das hohe Risiko von Patienten mit Lebezirrhose eine SBP zu entwickeln. In weiteren Studien sollten nun Faktoren identifiziert werden, welche die Funktion von Phagozyten im Aszites regulieren, um dadurch neue präventive oder therapeutische Ansätze zu finden und somit die Prognose der Patienten zu verbessern.

Engelmann C, Becker C, Boldt A, Herta T, Boehlig A, Splith K, Schmelzle M, Mueller N, Krohn S, Tautenhahn HM, Bartels M, Sack U, Berg T. Ascites' neutrophil function is significantly impaired in patients with decompensated cirrhosis but can be restored by autologous plasma incubation. Sci Rep 2016;6:37926. doi: 10.1038/srep37926.
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Ascites' neutrophil function is significantly impaired in patients with decompensated cirrhosis but can be restored by autologous plasma incubation

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Systemic immune cell dysfunction is a typical feature of liver diseases and increases the risk of bacterial infection, especially spontaneous bacterial peritonitis. We evaluated functional properties of neutrophil granulocytes in blood and ascites of patients both with and without decompensated cirrhosis. We collected blood and ascites samples from 63 patients with cirrhosis and eight without cirrhosis. Phagocytosis activity (PA) and oxidative burst activity (OBA) were evaluated after *ex vivo* stimulation with *E. coli*, while fluorescence signals were measured by flow cytometry. Ascites' neutrophil function tests were repeated after incubation with autologous plasma. Ascites' neutrophils showed an impaired PA and OBA (median blood PA 98.1% (86.8–99.8) vs. ascites' PA 50.5% (0.4–97.3), $p < 0.0001$; median blood OBA 98.7% (27.5–100) vs. ascites' OBA 27.5% (0.3–96.7), $p < 0.0001$). Patients with non-cirrhotic ascites showed higher PA but equally suppressed OBA. Ascites' neutrophil function could be partially restored after incubation with autologous plasma (median increase PA: 22.5% (−49.7 – +93.2), $p = 0.002$; OBA: 22.8% (−10.4 – +48.8), $p = 0.002$). Ascites' neutrophils of patients with cirrhosis are functionally impaired, but could be partially restored after incubation with plasma. Further investigations are needed to identify the factors in ascites that are associated with neutrophils' function.

Patients with end-stage liver diseases typically express features of a dysfunctional immune system that are associated with a suppressed response of peripheral blood neutrophils to invading pathogens[1–3]. This is considered to be part of a general immune exhaustion induced by the continuous intestinal, bacterial, translocation-mediated immune stimulation in cirrhosis[4–8]. It is assumed that there is a strong causal relationship between so-called immune paralysis and the high rate of infectious complications in decompensated liver cirrhosis[9–12]. To date, however, it is unclear why ascites or peritoneal cavities are the predominant site of bacterial infection in patients with decompensated cirrhosis (that is, spontaneous bacterial peritonitis (SBP)), while this type of infection is only rarely seen in patients with malignant ascites[13,14].

Studies that specifically address peritoneal host defence mechanisms in decompensated cirrhosis cases are few and far between. The phagocytosis and oxidative burst capacity of peritoneal macrophages has been found to be severely impaired[15], and the level of opsonic activity in ascites has been linked to the risk of developing SBP[14,15]. Only one study has evaluated functional properties in ascites' neutrophils[16,17], by comparing phagocytosis and

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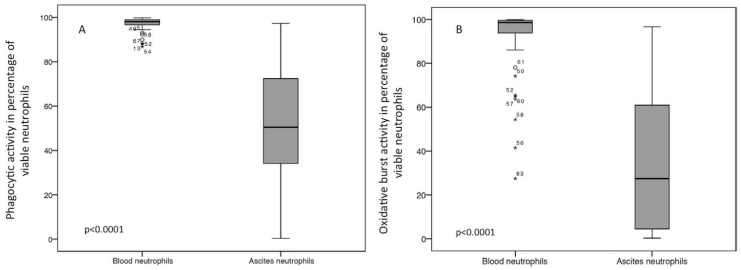

Figure 1. Phagocytic rate (**A**) and oxidative burst rate (**B**) of neutrophils in blood and ascites. Boxplots show that neutrophils' function was significantly diminished in ascites' neutrophils, compared to blood neutrophils. Values are given as the percentage of viable neutrophils.

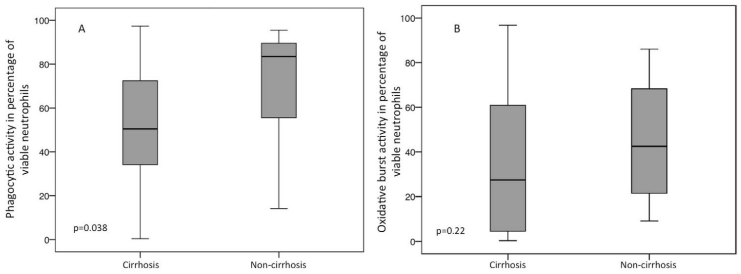

Figure 2. Phagocytic rate (**A**) and oxidative burst rate (**B**) of ascites' neutrophils, presented separately for cirrhosis and non-cirrhosis. Boxplots show that phagocytosis activity, but not oxidative burst activity, in ascites' neutrophils was reduced in cirrhosis compared to non-cirrhotic ascites. Values are given as the percentage of viable neutrophils.

oxidative burst activity in patients with and without SBP. However, the function of the peripheral blood neutrophil counterpart was not studied, so it remains a matter of speculation whether the findings in ascites are simply a reflection of the systemic neutrophil dysfunction that has been observed in patients with liver failure.

Due to this lack, we were interested in whether neutrophils in ascites of patients with decompensated cirrhosis show a higher degree of functional impairment, not only compared to their blood counterparts, but also to ascites' neutrophils derived from patients with non-cirrhotic ascites.

Results

Phagocytic and oxidative burst rate of neutrophils derived from patients with cirrhosis.

Neutrophil function was determined by flow cytometry after stimulation with inactivated and opsonised *E. coli* bacteria. Phagocytic rate and oxidative burst rate were determined as the percentage of active neutrophils in relation to the total number of viable neutrophils. Phagocytosis could be determined in 62 out of 63 blood samples and in 60 out of 63 ascites samples from patients with cirrhosis. Oxidative burst was measurable in all (63/63) blood samples and in 62 out of 63 ascites samples.

The median ascites' phagocytic rate was 50.5% (range 0.4–97.3), compared to 98.1% (range 86.8–99.8; $p < 0.0001$) in blood neutrophils. The median ascites' oxidative burst rate was 27.5% (range 0.3–96.7), compared to 98.7% (range 27.5–100; $p < 0.0001$) in blood (see Fig. 1). The ascites' neutrophil functions were not correlated with the functioning of blood neutrophils (correlation coefficient for phagocytic rate: $r = 0.213$ ($p = 0.102$), and for oxidative burst rate: $r = 0.165$ ($p = 0.2$)). In addition, the ranges of phagocytic and oxidative burst rates were broader in ascitic fluid than in blood neutrophils, ranging from normal to nearly undetectable rates (see Fig. 1), which possibly indicates that additional environmental factors may be involved in the mechanisms of peritoneal neutrophil stimulation.

Neutrophil function in patients with non-cirrhotic ascites. The median phagocytic rate of neutrophils in non-cirrhotic ascites was 83.5% (range 14.1–95.4), 33% higher than in ascites' neutrophils of patients with cirrhosis ($p = 0.038$) (see Fig. 2). The median ascites' neutrophil oxidative burst rate was 42.5% (range 9.1–86). Although the neutrophils increased by about 15% in cirrhotic ascites, they did not reach statistical significance ($p = 0.22$). The ascites' protein level was the major factor differentiating ascitic fluid in cirrhotic and non-cirrhotic

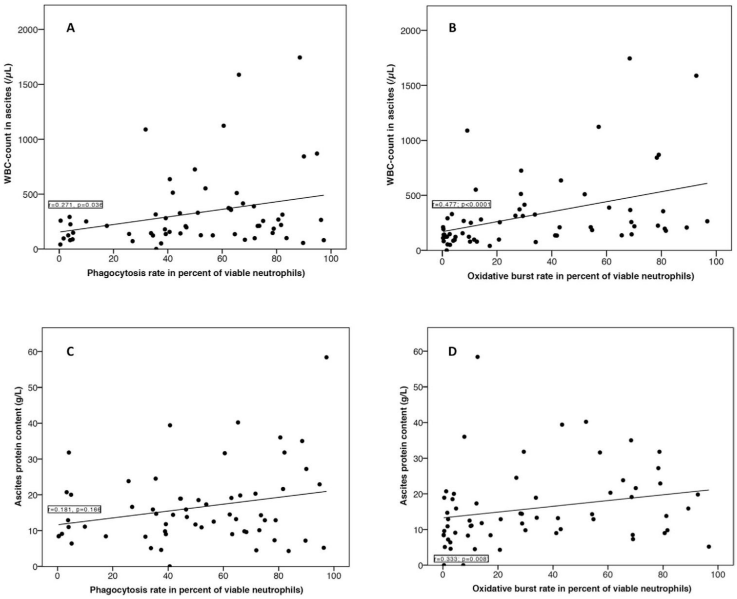

Figure 3. Ascites' neutrophils' functions in patients with cirrhosis in correlation with the ascites' leukocyte count and protein content. Figure (**A**) shows the correlation between the phagocytic rate and WBC count in ascites; figure (**B**) between the oxidative burst rate and the WBC count in ascites' figure (**C**) between the phagocytic rate and ascites' protein content; and figure (**D**) between the oxidative burst rate and ascites' protein content. For correlation analysis, the Spearman-Rho coefficient was calculated and a correlation coefficient of $r > 0.5$ was considered relevant.

patients, being significantly higher in the latter group (median ascites' protein content in cirrhosis was 13.2 g/L (range 0–58.4) vs. 23.55 g/L (range 21.4–46) in non-cirrhosis, $p = 0.001$).

The blood neutrophils of patients without cirrhosis showed a median phagocytosis rate of 99% (97.2–99.8), 1.8% lower than in the cirrhosis group ($p = 0.049$). The median oxidative burst rate, with a median of 98.5% (62.7–100) was not different in blood neutrophils in cirrhosis (−0.2%, $p = 0.792$) compared to patients with cirrhosis.

Factors associated with ascites' neutrophil function in cirrhosis. We further assessed whether patient characteristics, as well as markers of liver disease severity, correlated with neutrophil function in ascites. Although male patients exhibited higher phagocytosis and oxidative burst activity than their female counterparts, the difference did not reach statistical significance (median phagocytic rates in males was 60.5% (range 3.3–96.3) vs. 35.7% (range 0.4–97.3) in females, $p = 0.065$; median oxidative burst rates in males was 28.8% (range 0.3–96.7) vs. 9.7% (range 0.5–89.2) in females, $p = 0.18$). In addition, for all other parameters, including age, body weight, Child-Pugh score, model of end-stage liver disease (MELD) score, white blood cell count (WBC), C-reactive protein and medical treatment, no clear correlation with the level of ascites' neutrophil function could be found (see Supplementary Table 1, Supplementary Figure 3 and Supplementary Figure 4).

The ascites' leukocyte count showed a weak association with the oxidative burst rate (leukocyte count: $r = 0.477$, $p < 0.0001$) but not with the phagocytic rate ($r = 0.271$; $p = 0.036$) in ascites' neutrophils (see Fig. 3). In patients with cirrhosis and SBP, the median oxidative burst rate was significantly higher than in those without SBP (median 48.1% (9.1–92.7) vs. median 19% (0.3–96.7, $p = 0.014$). Concerning the phagocytosis rate, no significant difference in relation to SBP was observed (SBP: median 62.9% (31.6–94.8) vs. no SBP: median 48.9% (0.4–97.3), $p = 0.150$).

Added to this, the ascites' protein content showed neither an association with the phagocytic rate ($r = 0.181$, $p = 0.166$) nor with the oxidative burst rate ($r = 0.333$, $p = 0.008$) (see Fig. 3).

Factors associated with circulating neutrophil function in cirrhosis. There was no clear correlation between functional properties of blood neutrophils and baseline parameters such as age, body weight, Child-Pugh score, serum protein and albumin (Supplementary Table 2). Although oxidative burst rate showed a negative correlation with the MELD score ($r = -0.278$, $p = 0.028$), WBC ($r = -0.27$, $p = 0.032$) and C-reactive protein ($r = -0.34$, $p = 0.022$) the coefficient did not meet our criteria for a valid correlation (i.e. $r > 0.5$). However, in

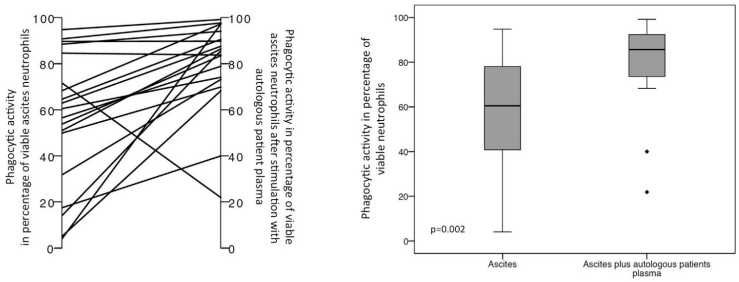

Figure 4. Phagocytic rate of ascites' neutrophils after incubation with autologous plasma. The left plot depicts the individual changes, while the boxplots show the distribution of phagocytic rates of ascites' neutrophils before and after incubation with autologous patients' plasma. Values are given as the percentage of viable neutrophils.

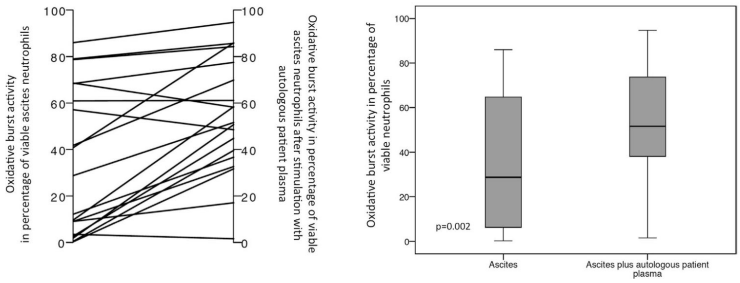

Figure 5. Oxidative burst rate of ascites' neutrophils after incubation with autologous plasma. The left plot portrays the individual changes, while the boxplots present the distribution of oxidative burst rates of ascites' neutrophils before and after incubation with autologous patients' plasma. Values are given as the percentage of viable neutrophils.

patients with more advanced decompensation of liver function defined by a MELD score ≥ 15 or a cirrhosis stage Child-Pugh C the oxidative burst rate was significantly reduced as compared to patients with more preserved liver function (MELD ≥ 15: median oxidative burst rate 19% (range 0.3–89.2) vs. MELD < 15: 43.8% (range 0.3–96.7). $p = 0.023$) or Child-Pugh C category (Child C: median oxidative burst rate 9.7% (range 0.3–80.6) vs. Child B: 41% (range 0.3–96.7) vs. Child A: 38.5% (range 2.8–79) $p = 0.026$).

Ascites' neutrophil function was partially restored after incubation with autologous plasma. We artificially modulated environmental conditions for ascites' neutrophils *ex vivo* via incubation with autologous plasma.

Ascites neutrophils' showed a significant net increase in median phagocytic activity, by 22.5% (range −49.7–93.2) (from 60.5% (range 4.1–94.8) to 85.7% (range 21.9–99.2), $p = 0.002$) (see Fig. 4) after said procedure. However, it did not result in a full restoration of phagocytic activity, which was still lower in ascites' neutrophils than in blood neutrophils' median phagocytic rate in the blood (97.4% (range 86.8–99.8, $p < 0.0001$)). Similarly, although oxidative burst activity did increase after *ex vivo* plasma incubation, with a median net increase of 22.8% (range −10.4–48.8) (median oxidative burst rate 28.8% (range 0.3–86) vs. 51.6% (range 1.6–94.7), $p = 0.002$) (see Fig. 5), it also did not reach the activity levels obtained in blood neutrophils (median oxidative burst rate 96% (range 27.5–100), $p = 0.001$).

A total of five patients did not show any response to plasma incubation, but actually presented a worsening of phagocytosis ($n = 2$) and oxidative burst ($n = 3$); only one of these suffered from non-cirrhotic ascites.

Discussion

Immune paralysis in general, and neutrophil dysfunction in particular, are common phenomena in chronic liver diseases and increase the risk of infectious complications[18]. SBP is by far the most common bacterial infection in this context. This is in contrast to malignant ascites, where SBP is a rare event[14], possibly indicating differences in the response to invading pathogens. Although it has been suggested that the peritoneal cavity in cirrhosis might

be a privileged site with specifically reduced host defence mechanisms[19–22], there have been limited studies evaluating ascites' neutrophil function as a potential contributory factor to the specific susceptibility of the peritoneal cavity to bacterial infections. We therefore evaluated phagocytosis and oxidative burst activity in both ascites and blood neutrophils derived from patients with and without cirrhosis.

In a significant cohort of 63 patients with decompensated cirrhosis, we showed for the first time that, when compared with peripheral blood neutrophils, ascites' neutrophils were severely dysfunctional, showing a reduction of phagocytosis and oxidative burst rates of approximately 50% and 70% respectively. So far, only one study[16] has investigated the functional properties of ascites' neutrophils, looking at 9 and 19 patients with and without SBP. The presence of SBP was associated with lower neutrophil oxidative burst activity, as compared to neutrophils derived from non-infected ascites samples, but activity increased during antibiotic treatment. However, neither the phagocytic rate nor the functional properties of the peripheral blood neutrophils have been studied.

In comparison to previous reports, the functional properties of blood neutrophils in cirrhosis were found to be less depressed in our cohort, which might be, however, partly explained by a relative underrepresentation of patients with heavily impaired liver function[18,23]. Indeed, we could confirm a significantly reduced blood neutrophils oxidative burst rate when only patients with more advanced decompensation of the liver function (i.e. Child C and MELD ≥ 15) were analysed.

An intriguing finding of our study was the high degree of variability of ascites' neutrophil function, ranging from 0.4% to 97.3% for phagocytosis and from 0.3% to 96.7% for oxidative burst. This result was in contrast to the results obtained from the blood-derived neutrophils, in which the activity rates were more homogeneous, ranging from 86.8% to 99.8% (phagocytosis) and from 27.5% to 100% (oxidative burst). This variability of ascites' neutrophil activity could not be explained by certain patient characteristics, such as liver function, inflammatory parameters or age, although it is known that activation, migration and response to bacteria of circulating neutrophils depends on the degree of liver insufficiency, systemic inflammation and age[18,24,25].

We therefore hypothesised that peritoneal cavity- and/or ascite-specific factors must exist, and are responsible for the observed site-specific differences in neutrophil function. Appropriate neutrophil activation is closely regulated by a number of stimulatory, but also inhibitory, factors such as immunoglobulins, complement factors, especially C3b, cytokines (IFN-γ, IL-8, GM-CSF and TNFα) and endotoxin levels[26,27]. At the time of data acquisition and the *ex vivo* experiments, we were not able to directly measure these factors. However, certain results may indicate the presence or absence of such stimulating or inhibiting factors.

We were able to stratify our results according to the ascites' protein content and leucocyte count as well as the cause of ascites (cirrhotic vs. non-cirrhotic), all of which are well-known predictors of SBP risk[14,16,22,28]. We found no association between ascites' neutrophil function and their protein content. However, because both tests – phagocytosis and oxidative burst – were performed using pre-opsonised *E. coli* bacteria, factors associated with the opsonic capacity of ascites fluid could not be studied in our test system. In contrast to the phagocytic rate, the leukocyte count in ascites had a weak, yet still significant, impact on the oxidative burst rate, being lower when the leucocyte count was high, thereby confirming the results of Nieto *et al.*[16]. The oxidative burst capacity of neutrophils derived from non-cirrhotic ascites was identical to that seen in blood neutrophils, but the phagocytosis rate was reduced by approximately 30%. As blood neutrophil function was maintained in our cohort, and neutrophils adapt to their environment, we hypothesised that the functional properties of ascites' neutrophils may improve upon transferral to patient plasma. Indeed, phagocytosis and oxidative burst rates did recover when ascites' neutrophils were incubated with autologous plasma, showing an increase of about 25% in both function tests. This observation is in line with results previously published by Nieto *et al.*[16], which showed that host defence mechanisms are not irreversibly altered in decompensated liver disease, and that treatment as well as active modification of ambient conditions *in vitro* restores neutrophil function. Lebrun *et al.*[29] were the first to show that the functional properties of immune cells can be manipulated by altering their environment. Neutrophils of healthy blood donors were brought into contact with the ascites of patients with cirrhosis ($n = 32$) and those of patients with malignant diseases ($n = 17$). Neutrophil function was assessed by chemiluminescence with pre-opsonised zymosan, a strong stimulating signal, in all samples and in four samples from a phagocytosis test using pre-opsonised *Staphylococcus aureus* cells. All functional neutrophil properties were significantly more favourable in samples derived from malignant ascites, as compared to those obtained from ascites of patients with cirrhosis. In addition, it was effectively demonstrated that a disequilibrium between inhibitory and stimulatory signals most likely contributes to the high variability of ascites' neutrophil function. If the ascites of patients with cirrhosis were diluted with saline, their neutrophil function was partially restored. In contrast, when cirrhotic ascites were diluted with their counterparts derived from malignant ascites, their function was significantly improved. It has to be pointed out, however, that there was a lack of direct evidence for environmental factors affecting neutrophil functions. Our results may, however, stimulate further research that would elucidate the functional mechanisms that are potentially involved in neutrophil activation and migration within the micro-milieu of cirrhotic ascites.

In conclusion, this study was able to show, for the first time, that neutrophil function in ascites is severely impaired. This may explain the high susceptibility to spontaneous bacterial peritonitis in cirrhotic patients. The dysfunction of ascites' neutrophils may be partially restored after incubation with autologous plasma. The high functional variability observed in neutrophils derived from ascites suggests that ascites' neutrophil function is dependent on both stimulatory and inhibitory factors. Further studies are needed to clarify the individual factors involved in ascites' neutrophil activity, as these may become potentially interesting targets for SBP treatment and prevention.

Patients and Methods

Study design. Between August 2014 and May 2015, ascites, fluid and corresponding blood samples were consecutively collected from 63 patients with decompensated cirrhosis at the Section of Hepatology, University

Variable	Cirrhosis (n = 63)	Non-cirrhosis (n = 8)	Level of significance (p)
Age (years), mean ± SD	59.5 ± 10.3	62.9 ± 17.4	0.233
Gender (male/female), n (%)	45/18 (71.4%/28.6%)	5/3 (62.5%/37.5%)	0.053
Etiology of cirrhosis, n (%)		Not applicable	
Alcoholic	45 (71.4%)		
NASH	5 (7.9%)		
Cryptogenic	9 (14.3%)		
Others	4 (6.3%)		
Cause of ascites (non- cirrhosis), n (%)	Not applicable		
Malignant		6 (75%)	
Cardiogenic		1 (12.5%)	
Acute BCS		1 (12.5%)	
Bilirubin (μmol/L), median (range)	29.6 (4–541)	10.4 (3.6–20.9)	0.0001
Albumin (g/L), median (range)	31.9 (13.9–49.5)	33.1 (25.9–54.5)	0.499
INR, median (range)	1.5 (0.9–3.5)	1.1 (0.9–1.53)	0.012
Serum creatinine (μmol/L), median (range)	106 (41–389)	78 (31–107)	0.018
GFR (ml/min), median (range)	60 (10–119)	67 (51–107)	0.483
Thrombocyte count (exp9/L), median (range)	86.5 (29–332)	320 (127–474)	0.001
White blood cell count (exp9/L), median (range)	5.8 (2.9–30.3)	7.05 (2.4–12.6)	0.617
Haemoglobin (mmol/L), median (range)	6.6 (4–9)	6.1 (5.1–7.6)	0.859
C-reactive protein (mg/dL), median (range)	18.9 (1.01–140.31)	16.2 (9.2–125)	0.986
Total protein content blood (g/L), median (range)	63.1 (44.1–78.8)	64 (53.5–64.3)	0.635
Total protein content ascites (g/L), median (range)	13.2 (0–58.4)	23.6 (21.4–46)	0.001
Ascites leukocyte count (/mm^3), median (range)	210 (0–1744)	346.5 (148–2715)	0.036

Table 1. Patient characteristics at baseline paracentesis. Categorical data is displayed as absolute and relative values and metric data as mean ± standard deviation or median (range), as appropriate. NASH = non-alcoholic steatohepatitis. BCS = Budd-Chiari syndrome. GFR = glomerular filtration rate. INR = international normalised ratio. SD = standard deviation.

Hospital Leipzig, for the purpose of evaluating the ascites' neutrophil function by performing *in vitro* tests for phagocytosis and oxidative burst. Patients with ascites but without cirrhosis (n = 8) served as controls. All patients with ascites, who had been admitted to our hospital, were considered for participation in the study. In line with international clinical practice guidelines[29], paracentesis was indicated for new-onset or worsening ascites and in cases where SBP was suspected. Patients with ongoing alcohol abuse, who were receiving immunosuppressive therapy or who were recovering from a liver transplant were excluded from the study. The first paracentesis after enrolment was defined as the baseline for collection of ascites and blood samples. Any subsequent paracenteses were not included in the present analysis. All clinical and laboratory data as obtained exclusively during routine visits not related to the study, was collected retrospectively. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and was approved by the ethics committee of the University of Leipzig (No. 182 – 14 – 02062014). All patients gave written informed consent.

Patient characteristics and data collection. The patients' characteristics are summarised in Table 1. Alcohol abuse was the main cause of cirrhosis (71.4%) and malignancies the main reason for ascites (75%) in the non-cirrhotic group. The median MELD score was 16 points (range 6–38), and median Child-Pugh score 9 points (range 5–14), in patients with cirrhosis. After subsequent Child-Pugh classification, the majority of patients with cirrhosis were assigned to Child-Pugh class B (Class A: n = 11 (17.5%); Class B: n = 30 (47.6%); Class C: n = 22 (34.9%)). SBP was diagnosed on the basis of an elevated leukocyte count in ascites (>500/mm^3) in 11 out of 63 patients (17.5%) with cirrhosis. At baseline, the following clinical and laboratory data was collected: cause of ascites, etiology of liver cirrhosis, drug history, sex, age, liver and renal function test, platelet count, white blood cell count, serum sodium, haemoglobin, C-reactive protein, serum albumin and protein content. Ascites samples were characterised by their total leukocyte count (via automated cell counter) and their protein content was defined according to institutional standards. SBP was diagnosed in patients with cirrhosis if the ascites' leukocyte count was elevated above 500/mm^3 [30].

Ascites and blood sampling. Ascites samples were collected after careful skin disinfection and under ultrasound guidance. For local anaesthesia, 5–10 mL of Xylocaine (1%) was injected, and afterwards, a 6 French paracentesis cannula (Peter Pflugbeil GmbH, Zorneding, Germany) was inserted into the peritoneum. **An** initial fraction of 50 mL **of** ascitic fluid was used for routine laboratory analysis, while 40 mL of ascitic fluid was obtained for tests related to the study. Venous blood samples were collected under standard aseptic conditions, using a 0.8 mm Multifly-Safety needle (Sarstedt AG & Co KG, Nümbrecht, Germany) immediately after paracentesis, and decanted into heparinised tubes (18 mL) and EDTA tubes (5.7 mL). All ascites and blood samples were processed within four hours after sample collection, under pyrogen-free conditions. For antibody staining, 2 mL of ascitic

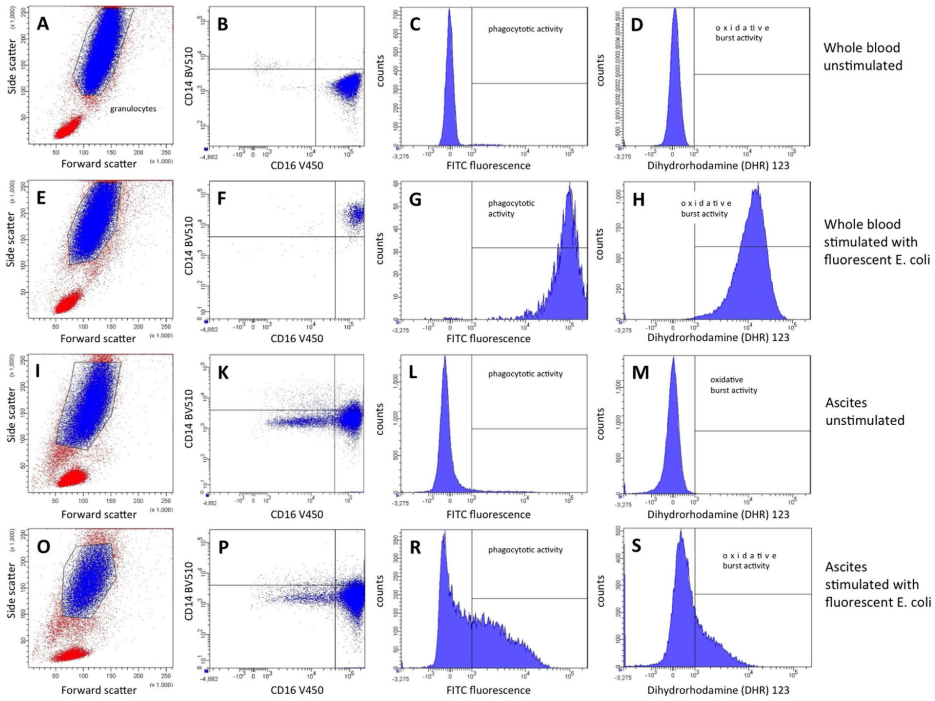

Figure 6. Flow cytometric analyses of phagocytosis and oxidative burst in neutrophils of patients with cirrhosis. Analysis of the neutrophils' granulocyte functions by phagocytosis and oxidative burst in peripheral blood (**A–H**) and ascites (**I–S**). Neutrophils' granulocytes were separated from peripheral blood (**A,E**) and ascites (**I,O**) by FSC vs. SSC and additionally characterised by surface staining with CD14 BV510 and CD16 V450 with (**F,P**) and without (**B,K**) stimulation with fluorescent *E. coli*. In peripheral blood, the expression of CD14 increased after stimulation (**F**) in contrast to neutrophils' granulocytes in ascites (**P**), when both were compared to controls without stimulation (**B,K**). The analysis of phagocytosis and oxidative burst revealed a normal activity in peripheral blood's neutrophils' granulocytes (**G,H**), in contrast to a strongly attenuated activity in ascites' neutrophils' granulocytes (**R,S**), when both were compared to controls without stimulation (**C,D,L,M**). (**A–D**) Whole unstimulated blood. (**E–H**) Whole blood stimulated with fluorescent *E. coli*. (**I–M**) Ascites unstimulated. (**O–S**) Ascites stimulated with fluorescent *E. coli*.

fluid was poured into Eppendorf tubes (1 mL each). In preparation for flow cytometry and neutrophil function tests, the remaining 38 mL of ascitic fluid was centrifuged in a 50 mL tube at 500 × g for five minutes at room temperature. The neutrophil-containing pellet was re-suspended with 1.5 mL of ascites' supernatant and used for the neutrophil function tests. The remaining ascites; supernatant was stored at −80 °C. The heparinised blood was processed for neutrophil function tests without prior preparation. The following steps were the same for blood and ascitic fluid (hereafter referred to as test substances).

Flow cytometry. Each test substance was analysed by flow cytometry using FACS CantoII DiVa software (BD Bioscience, New Jersey, USA), in order to identify neutrophils and perform neutrophil function tests (see Fig. 6). As flow cytometry in ascites is not established, the presence of neutrophils was confirmed by labelling with antibodies against CD14 (BV510; Clone: MfP9; BD Horizon), CD16 (V450; Clone: 3G8; BD Horizon), CD45 (PerCP; Clone: 2D1; BD Bioscience), CD282 (APC; Clone: TL2.1; eBioscience), CD284 (PE-Cy7; Clone: HTA125; eBioscience) and CD62L (APC-Cy7; Clone: DREG-56; BioLegend), antigens that are typically located on neutrophil granulocytes, in the first 26 patients (see Supplementary Figures 1 and 2). In total, 2.5 µL of CD14, 2.5 µL of CD16, 5 µL of CD45, 5 µL of CD282, 5 µL of CD284 and 10 µL of CD62L were added to 100 µL of the test substance and incubated for 15 minutes, while being protected from light, at room temperature. Intact immune cell subtypes were gated and identified by their characteristic forward and sideward scatter. For neutrophil function tests, cell viability could be determined by using propidium iodide, which binds to the DNA of non-viable cells. The latter, as well as artefacts, could be excluded by setting a live gate at the propidium iodide histogram.

Thereafter, flow cytometry for phagocytosis and oxidative burst tests was performed as described in the following section. For the function tests, neutrophils were labelled with CD14 and CD16 antibodies (see Fig. 6).

***In vitro* neutrophil function tests.** *Phagocytosis.* The phagocytosis test was performed using the Phagotest test kit (Orpegen Pharma, Heidelberg, Germany), which contains fluorescein isothiocyanate (FITC)-labelled, opsonised and inactivated *E. coli* bacteria; samples were subsequently analysed by flow cytometry (see Fig. 6). In brief, each test substance was poured into two tubes, one for the phagocytosis test (100 µL) and one for the controls (100 µL), and cooled on ice for 10 minutes. Thereafter, 20 µL of Phagotest reagents (*E. coli* bacteria) were added to the test tubes and incubated in a shaking water bath at 37 °C for 10 minutes. The control tube remained on ice. Phagocytosis was stopped after 10 minutes with 100 µL of quenching suspension. All samples were washed twice in 3 mL of washing solution, before being centrifuged at 500 × g for 5 minutes. The supernatant was discarded. The remaining pellet was then re-suspended in 2 mL of erythrocyte lysis buffer and subsequently incubated in darkness at room temperature for 20 minutes. After centrifugation at 500 × g for 5 minutes, the supernatant containing the lysed erythrocytes was again discarded. In order to identify viable neutrophils, the pellet was re-suspended in 200 µL of DNA staining solution (propidium iodide (PI)). Flow cytometry analysis was performed within one hour. During this period, samples remained on ice in darkness.

Oxidative burst. Phagoburst (Orpegen Pharma, Heidelberg, Germany) contains unlabelled, opsonised *E. coli* bacteria and was used to quantify neutrophil oxidative burst (see Fig. 6). For sample preparation, 100 µL of each test substance was decanted into three tubes for the oxidative burst test, as well as for a negative and positive control. Tubes were cooled on ice for 10 minutes. 20 µL each of unlabelled, opsonised *E. coli* for the oxidative burst test, a washing buffer for the negative control, and PMA solution (phorbol 12-myristate 13-acetate) for the positive control were added to the respective tubes containing 100 µL of the test substance, and incubated at 37 °C in a shaking water bath for 10 minutes. Thereafter, 20 µL of the burst substrate (DHR123) was added to each tube, before they were incubated again at 37 °C in a shaking water bath for 10 minutes. The next steps, consisting of erythrocyte lysis and DNA staining, corresponded to the phagocytosis test and were described previously.

***In vitro* neutrophil function test after incubation with autologous plasma.** We hypothesised that environmental factors play a major role with respect to neutrophil function in ascitic fluid. Accordingly, we modified the environmental conditions *in vitro* and subsequently repeated the neutrophil function tests in the last 19 patients (cirrhotic ascites n = 16, non-cirrhotic ascites n = 3). For this purpose, patients' ascites' neutrophils were incubated with autologous plasma. Heparinised blood was centrifuged at 2000 × g at room temperature for 5 minutes. 300 µL of the resulting plasma (supernatant) was mixed with 300 µL of the re-suspended ascites pellet. Following this, the phagocytosis and oxidative burst tests were performed as previously described.

Statistical analysis

Statistical analysis was performed using SPSS 22 software (SPSS Inc., Chicago, IL). Categorical variables were displayed as percentages or frequencies, and continuous variables as mean ± standard deviation or median and range, as appropriate. A two-sided p-value of < 0.05 was considered statistically significant. Comparison of unpaired samples was performed by Mann-Whitney U test in the case of continuous data and by Chi-square test for discrete data. Paired samples were compared by Wilcoxon signed rank test. For correlation analysis, the Spearman-Rho coefficient was calculated and a correlation coefficient of $r > 0.5$ was considered relevant.

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Author Contributions

Cornelius Engelmann: study concept and design, acquisition of data, analysis and interpretation of data, drafting of manuscript, statistical analysis. Christina Becker: acquisition of data, analysis and interpretation of data, technical support, drafting of manuscript, statistical analysis. Andreas Boldt: acquisition of data, analysis and interpretation of data, technical support. Toni Herta: acquisition of data, analysis and interpretation of data, critical revision of the manuscript for important intellectual content. Albrecht Boehlig: acquisition of data, analysis and interpretation of data, critical revision of the manuscript for important intellectual content. Katrin Splith: analysis and interpretation of data, critical revision of the manuscript for important intellectual content, technical support. Moritz Schmelzle: analysis and interpretation of data, critical revision of the manuscript for important intellectual content, technical support. Niklas Mueller: acquisition of data, analysis and interpretation of data, critical revision of the manuscript for important intellectual content. Sandra Krohn: acquisition of data, analysis and interpretation of data, critical revision of the manuscript for important intellectual content. Hans-Michael Tautenhahn: acquisition of data, analysis and interpretation of data, critical revision of the manuscript for important intellectual content. Michael Bartels: acquisition of data, analysis and interpretation of data, critical revision of the manuscript for important intellectual content. Ulrich Sack: study concept and design, analysis and interpretation of data, critical revision of the manuscript for important intellectual content, administration, study supervision, procurement of funding, material support. Thomas Berg: study concept and design, analysis and interpretation of data, critical revision of the manuscript for important intellectual content, administration, study supervision, procurement of funding, material support. The work presented in this paper was made possible by institutional funding.

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3.3 Die Quantität von Micropartikeln im Aszites ist mit der Prognose von Patienten mit Leberzirrhose assoziiert

Es ist bekannt, dass eine gesteigerte intestinale bakterielle Translokation und Aussaht von Erregern in die Bauchhöhle ein wesentlicher Faktor bei der Entstehung einer SBP ist (26). Die zuvor beschriebene Arbeit hat gezeigt, dass eine reduzierte Funktion von peritonealen Neutrophilen die Manifestation der Infektion weitere begünstigen kann. Der Nachweis der SBP bleibt jedoch unverändert eine Herausforderung, da mehr als die Hälfte aller Kulturproben trotz Infektion negativ bleiben (16). Die Erkrankung wird daher durch eine erhöhte Zahl von neutrophilen Granulozyten (>250 PMN/µl) und somit über die lokale Immunreaktion nachgewiesen (50). Befinden sich Neutrophile und andere Immunzellen im Stadium der Aktivierung beginnen sie Teile ihrer Membran zu sequestrieren (<1µm) und als Micropartikel abzugeben. Die Höhe der zirkulierenden Micropartikel korrelieren generell bei Erkrankungen gut mit dem Ausmaß von Endorganschäden sowie inflammatorischen Reaktionen und kann somit den allgemeinen Verlauf von Erkrankungen vorhersagen (51). Micropartikel enthalten zelluläre Proteine und messenger RNA und können darüber als Mediatoren der interzellulären Kommunikation fungieren (52). Ziel der Arbeit was zu evaluieren, ob Micropartikel im Aszites Marker für die lokale Immunaktivierung sind und Aufschluss über den Verlauf der Leberzirrhose geben können.

Abstrakt Zitat Engelmann C et al. (53):

"Background: Microparticles (MPs) are small (<1 µm) cell membrane-derived vesicles that are formed in response to cellular activation or early stages of apoptosis. Increased plasma MP levels have been associated with liver disease severity. Here we investigated the clinical impact of ascites MPs in patients with decompensated liver cirrhosis.

Methods: Ascites and blood samples of 163 patients with cirrhosis (ascites n = 163, blood n = 31) were collected between February 2011 and December 2012. MPs were obtained from ascites and from blood by two-step ultracentrifugation and quantified by flow cytometry. Quantitative absolute MP levels were correlated with clinical and laboratory baseline parameters as well as patient outcomes. Ascites microparticles were stained with antibodies against CD66b (neutrophils) and CD3 (lymphocytes) in a subgroup of 60 matched patients.

Results: MPs were detected in all ascites and blood samples. Absolute ascites MP levels correlated with blood levels (r = 0.444, p = 0.011). Low ascites MP levels (<488.4 MP/µL) were associated with a poor 30-day survival probability (<488.4 MP/µL 71.1% vs. >488.4 MP/µL 94.7%, log rank p = 0.001) and such patients had a higher relative amount of ascites microparticles derived from neutrophils and lymphocytes. Low levels of ascites MPs, high MELD score and antibiotic treatment were independent risk factors for death within 30 days.

Conclusions: Ascites MP levels predict short-term survival along with the liver function in patients with decompensated cirrhosis. Further studies which evaluate ascites MPs as disease specific biomarker with a validation cohort and which investigate its underlying mechanisms are needed. Neutrophils and lymphocytes contributed more frequently to the release of microparticles in patients with low ascites levels, possibly indicating an immune activation in this cohort."

Diese Studie zeigte demnach, dass eine Aktivierung von Neutrophilen und Lymphozyten und damit Sequestration von Mikropartikeln typisch für Patienten mit schlechter Prognose auch ohne Nachweis einer SBP ist. Interessanterweise zeigten Patienten mit relativ hohem Anteil an Mikropartikeln von Neutrophilen und Lymphozyten eine signifikant niedrigere Gesamtzahl an Mikropartikeln im Aszites. Bisher gibt es dafür keine gute Erklärung. Es erscheint möglich, dass diese Patienten ein hohes Aszitesvolumen hatten, und somit ein Dilutionseffekt zu den niedrigen Zahlen von Mikropartikeln im Aszites beigetragen hat. Weitere detaillierte Untersuchungen sollten nun klären, ob weitere Subklassen von Mikropartikeln im Besonderen prognostisch für Patienten mit Leberzirrhose sind.

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Absolute quantification of microparticles by flow cytometry in ascites of patients with decompensated cirrhosis: a cohort study

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Abstract

Background: Microparticles (MPs) are small (<1 µm) cell membrane-derived vesicles that are formed in response to cellular activation or early stages of apoptosis. Increased plasma MP levels have been associated with liver disease severity. Here we investigated the clinical impact of ascites MPs in patients with decompensated liver cirrhosis.

Methods: Ascites and blood samples of 163 patients with cirrhosis (ascites n = 163, blood n = 31) were collected between February 2011 and December 2012. MPs were obtained from ascites and from blood by two-step ultracentrifugation and quantified by flow cytometry. Quantitative absolute MP levels were correlated with clinical and laboratory baseline parameters as well as patient outcomes. Ascites microparticles were stained with antibodies against CD66b (neutrophils) and CD3 (lymphocytes) in a subgroup of 60 matched patients.

Results: MPs were detected in all ascites and blood samples. Absolute ascites MP levels correlated with blood levels ($r = 0.444$, $p = 0.011$). Low ascites MP levels (<488.4 MP/µL) were associated with a poor 30-day survival probability (<488.4 MP/µL 71.1% vs. >488.4 MP/µL 94.7%, log rank $p = 0.001$) and such patients had a higher relative amount of ascites microparticles derived from neutrophils and lymphocytes. Low levels of ascites MPs, high MELD score and antibiotic treatment were independent risk factors for death within 30 days.

Conclusions: Ascites MP levels predict short-term survival along with the liver function in patients with decompensated cirrhosis. Further studies which evaluate ascites MPs as disease specific biomarker with a validation cohort and which investigate its underlying mechanisms are needed. Neutrophils and lymphocytes contributed more frequently to the release of microparticles in patients with low ascites levels, possibly indicating an immune activation in this cohort.

Keywords: Cirrhosis, Ascites, Microparticles, MP

Background

Microparticles (MPs) are small membrane-derived extracellular vesicles (<1 µm) that are shed by all cell types after cellular activation and during early stages of apoptosis. As they are formed by outward blebbing of the cell membrane, MPs contain cellular proteins, RNA and miRNA [1–3]. MPs are important messengers in intercellular communication and involved in various pathomechanistic processes. This explains why increasing plasma MP levels generally express disease activity and progression [4–9]. There are several diseases that are accompanied by vascular damage and therefore trigger the release of specific MP subtypes. In diabetes mellitus circulating platelet-derived MPs correlate with the degree of vascular damage, particularly if end-organ damage occurs [10]. MPs released by endothelial cells are involved in

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coronary artery disease [11]. The fact that well established therapies for coronary artery disease that improve patient outcomes, e.g. administration of statins, also reduce MP levels emphasizes the role of MPs as biomarkers of disease activity [12]. Importantly, MPs released in the context of inflammatory reactions or oxidative stress can be both the consequence as well as the active modulator of pathological processes [13, 14].

In liver diseases, plasma MP subtypes correlate well with intrahepatic inflammation in chronic hepatitis C and steatohepatitis [15, 16] as well as severity of liver failure [17]. If chronic liver diseases are not adequately treated, they most often progress to cirrhosis with typical complications such as ascites. Because all cell types shed MPs, they can also be expected to occur in body fluids other than plasma. There are studies demonstrating MPs in synovial fluid [18], pleural fluid [19] and urine [20]. Another source of MP release is the peritoneum. Mrvar-Brecko et al. [21] used electron microscopy to visualize the presence of MPs in a patient with peritonitis and ascites. The largest cohort was investigated by Press et al. [22]. Using flow cytometry, they have nicely shown that tumor-derived MPs are detectable in all ascites samples of 41 patients with ovarian carcinoma and eight patients with benign ovarian neoplasms. However, evidence for the presence of MPs in ascites of patients with cirrhosis is missing.

We therefore investigated whether MPs are detectable in ascites of patients with decompensated cirrhosis and correlated the quantification level with patients' clinical presentation and outcome.

Methods

Study design

In line with the institutional standards of the Section of Hepatology, University Hospital Leipzig, Germany, every patient presenting with ascites between February 2011 and December 2012 was eligible for specimen banking and clinical data collection (n = 180). The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the local ethics committee. All patients gave written informed consent.

All patients with ascites due to decompensated liver cirrhosis were eligible for inclusion in the present analysis. Cirrhosis diagnosis was based on typical laboratory and morphological criteria. Exclusion criteria included immunosuppressive therapy, sepsis at baseline and malignant diseases other than hepatocellular carcinoma (HCC). Baseline was set at the first paracentesis of every patient after study inclusion. All further paracenteses were not considered for study analyses.

At baseline, the following parameters were assessed: etiology of cirrhosis, sex, age, body mass index (BMI), blood pressure, body temperature, drug history and clinical outcome, MELD (Model for End-Stage Liver Disease) score, liver and renal function tests [e.g. glomerular filtration rate (GFR)], international normalized ratio (INR), platelet count, white blood cell (WBC) count, serum sodium, transaminases [aspartate transaminase (AST), alanine transaminase (ALT)], gamma-glutamyltransferase (GGT), C-reactive protein (CrP), serum albumin and in ascites leukocyte count, protein and albumin content. Spontaneous bacterial peritonitis (SBP) was diagnosed according to Wong et al. [23] if the ascites leukocyte count was elevated above 500/mm^3. The ascites polymorphonuclear leukocyte (PMN) count, the gold standard for the diagnosis of SBP, was not available at this institution.

Patient characteristics

The study included 163 patients with liver cirrhosis, of which 21 (12.9%) were diagnosed with hepatocellular carcinoma. The main cause of cirrhosis was alcoholic liver disease (71.8%). All further baseline characteristics are displayed in Table 1.

Table 1 Baseline parameters at index paracentesis

Variable	Value
Age (years)	59 (25–87)
Gender (male/female)	n = 121/n = 42 (74.2%/25.8%)
Etiology	
Alcoholic	n = 117 (71.8%)
NASH	n = 14 (8.6%)
Viral	n = 4 (2.5%)
Others	n = 28 (17.2%)
HCC	n = 21 (12.9%)
Child–Pugh class (A/B/C)[b]	n = 2/n = 60/n = 36 (2%/61.2%/36.7%)
MELD score[a]	16.9 (7–38)
INR	1.4 (1–2.9)
Total bilirubin level (µmol/L)	39.5 (2.9–609.1)
Creatinine level (µmol/L)	108 (38–509)
WBC count (exp9/L)	6.6 (1.2–49.7)
AST (µkat/L)	0.87 (0.1–8.9)
GGT (µkat/L)	1.8 (0.2–22.4)
CrP (mg/L)	25.4 (1.2–186.2)
Serum albumin (g/L)	30.4 (16–47.3)
Ascites protein content (g/L)	11.6 (2.7–50.9)
Ascites leukocyte count (/mm^3)	180 (0–12,300)

Values are given in median (range) for continuous data and in absolute number (%) for discrete data

[a] MELD could not be calculated in 6/163 patients as at least one parameter was not available at paracentesis

[b] Child–Pugh score could not be calculated in 65/163 patients as at least one parameter was not available at paracentesis

Cirrhosis-associated complications were present at the time of index paracentesis with hepatic encephalopathy in 23 out of 163 patients (14.1%), hepatorenal syndrome in 41 out of 163 patients (25.2%) and hepatic hydrothorax in 17 out of 163 patients (10.4%). Twenty-one out of 163 (12.9%) had gastrointestinal bleeding within 4 weeks prior to paracentesis.

Forty-eight of 163 patients (29.4%) were on antibiotic treatment at the time of paracentesis. In total, 60 of 163 patients (36.8%) were treated with non-selective beta-blockers, 82 of 163 patients (50.3%) with proton-pump inhibitors and 82 of 163 patients (50.3%) with lactulose at the time of paracentesis.

SPB was diagnosed by means of high ascites leukocyte counts above 500/mm^3 in 21 out of 163 patients (12.9%). Infections other than SBP occurred in 38 out of 163 patients (23.3%), 18 with urinary tract infections, eight with pneumonia, five with clostridium difficile-associated colitis, three with catheter-associated sepsis, one with soft tissue infection, one with pancreatic abscess and two with infection of unknown origin.

Sampling of ascites and blood

Paracentesis was performed in all patients at baseline (index paracentesis) according to the EASL practice guidelines [24] if SBP was suspected or in patients with new onset or worsening of ascites. Ascites samples were collected under standard aseptic conditions. After skin disinfection, local anesthesia was injected. A 14-gauge cannula was inserted under ultrasound guidance. The first 50 mL of ascitic fluid were discarded to avoid contamination with skin microbiota. The subsequent 50 mL fraction was collected and 4 mL of ascitic fluid was immediately stored at −80 °C. Whole blood samples drawn on the day of paracentesis were available in a subgroup of 31 patients. Blood was centrifuged at 2500*g* and plasma was stored at −80 °C.

Detection of ascites and plasma microparticles

Samples were isolated by two-step ultracentrifugation, as described previously [17]. Shortly, ascites fluid or plasma was thawed and 1 mL was ultracentrifuged at 10,000*g* for 30 min at 5 °C. In a second step the supernatant was ultracentrifuged by 100,000*g* for 90 min at 5 °C. After ascites fluid or plasma was discarded, plasma MPs were resuspended in 300 µL sterile filtered (0.2 µm) PBS, ascites MPs were resuspended in 200 µL sterile filtered (0.2 µm) PBS and all MPs were stored at −80 °C [17]. MPs were identified by flow cytometry. Analysis was performed on a FACS Canto II using DiVa software (BD Bioscience, San Jose, USA). MPs were identified by their characteristic forward and sideward scatter, which were set at logarithmic gain. MPs were simulated using different sizes of standard microbeads (0.5–1.0 µm, Invitrogen), and a microparticle gate (MP gate) was determined using these standards. The MP gate included 1.0 µm beads in its upper and outer corner so that it would contain all microparticles 1.0 µm or less. Events in the MP gate were further assessed with FACSFlow (BD Bioscience, San Jose, USA) and filtered (0.2 µm) PBS alone to distinguish true events from electronic noise. Event numbers of equal sample volumes were counted for 60 s with a flow rate of 120 µL/min. The measurements of all samples were performed on 1 day to avoid day-to-day variability of the flow cytometer. Furthermore, each sample was measured at least twice in random order to further minimize measurement variations. The total number of microparticles in the samples was calculated by multiplying the measured number of events with the ratio of total volume to measured volume. Values were reported as counts per microliter.

Microparticle labeling and detection

In a subgroup of 60 patients ascites MPs were stained with antibodies against surface antigens which allow to allocate their origin to either neutrophils (CD66b) or lymphocytes (CD3). For that purpose 20, non-survivors with low ascites MP levels (<488.4 MP/µL) were matched with two survivors, one with low and one with high ascites MP level, according to the MELD score and age. Respectively, 50 µL MPs were incubated with labeled antibodies FITC-CD3 (UCHT1, lymphocytes, Biolegend, San Diego, USA), APC-CD66b (G10F5; granulocytes, Biolegend, San Diego, USA) for 15 min at RT. 450 µL of cold sterile filtered (0.2 µm) FACS buffer (PBS, 1% BSA, 0.1% NaN_3) and 25 µL counting beads (Biolegend, San Diego, USA) were added. Analysis was performed on a FACS LSRFortessa using DiVa software (BD Bioscience, San Jose, USA) with the same approach and gates as described before. Prior measurements unbound antibody in FACS buffer as well as isotype controls (APC mouse IgM, FITC mouse IgG1; Biolegend, San Diego, USA) were run to exclude background and non-specific binding from real events. The number of positive MP was calculated relative to the number of all gated MPs by FACS (Additional file 1: Figure S1).

Statistical analysis

Continuous variables were displayed as mean ± standard deviation or median with range, as appropriate. Categorical variables have been depicted as frequency and/or percentage. Mann–Whitney U test was used to compare continuous data and Chi square test for discrete data. A two-sided *p* value lower than 0.05 was considered statistically significant. Spearman-Rho correlation coefficient was used to display potential associations between

variables. Survival was evaluated considering all events that occurred from the time of inclusion until 30 days after inclusion. Follow-up data were collected retrospectively by screening data gathered during clinically indicated visits. Patients or their relatives were contacted if follow-up data were not available. Data were censored at transplantation or at last patient contact. For survival analysis, Kaplan–Meier analysis was performed and the log-rank test was used for group comparison. All laboratory and clinical data were considered for Cox regression analysis to identify potential risk factors for short-term death (within 30 days). Factors that were significantly associated in univariate analysis were used for multivariate analysis.

Results

Detection of microparticles

MPs were detected in all ascites (n = 163) and blood samples (n = 31) with a median MP count of 281.5 MP/μL in ascites (range 17.5–32,575.7) and 1469.7 (range 301.8–4926.3; (p = 0.357) in blood. Absolute MP levels in ascites samples correlated to individual MP levels in blood (r = 0.444, p = 0.011) (Fig. 1). A considerable number of patients showed higher MP levels in ascites than in blood samples (n = 11, 35.5%) and vice versa (n = 20, 64.5%), (Additional file 1: Figure S2).

Ascites MP levels correlated weakly with the thrombocyte count (r = 0.295, p = 0.0001) and inversely with the MELD score (r = −0.198, p = 0.013, Fig. 2a, b), but not with age, Child–Pugh score, serum albumin, white blood cell counts, AST, GGT, CRP, GFR, ascites protein content or the ascites leukocyte count. Blood MP levels also showed no significant correlation with the aforementioned parameters (Table 2). Ascites and blood MP levels at index paracentesis were not associated with the following baseline parameters: sex, presence of HCC, antibiotic treatment and non-selective beta-blocker use (Additional file 1: Table S1).

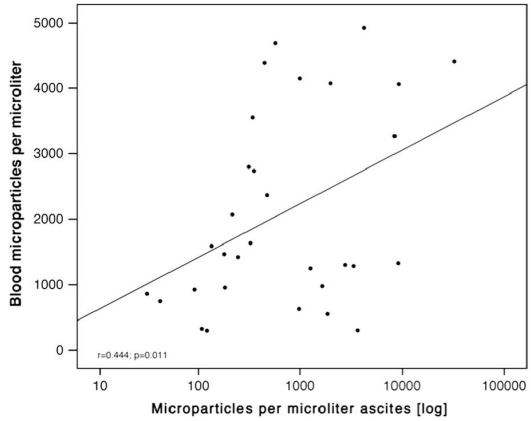

Fig. 1 Correlation of microparticle levels between blood and ascites samples. Analysis was performed by Spearman-Rho. The ascites microparticles axis is scaled logarithmically

Association between microparticles and patient outcomes

Thirty-one of 163 patients (19%) died and two of 163 patients (1.2%) were transplanted within 30 days after study inclusion. Using the Cox regression analysis, ascites MP counts were shown to be significantly associated with the 30-day survival. There was an inverse correlation between risk of death and the ascites MP count (per 100 MP/μL: HR 0.928 (95% CI 0.862–0.999), p = 0.048). In patients who died or were transplanted within 30 days after paracentesis, ascites MP levels were significantly lower [median 180.5 (32.5–2851.6) MP/μL] as compared to patients surviving this period [325.1 (17.5–32,575.1) MP/μL, p = 0.005], (Fig. 3).

After ROC analysis, the best ascites MP quantification cut-off for predicting the 30-day mortality rate was 488.4 MP/μL with a sensitivity of 90.3% and a specificity of 44.7% and an area under the curve (AUROC) of 0.663 (95% CI 0.564–0.763) (p = 0.005).

Kaplan–Meier analysis showed that patients with low levels of MPs in ascites (<488.4 MP/μL, n = 101) had a lower 30-day survival rate of 71.7% when compared to 94.7% in patients with high levels of MP in ascites (>488.4 MP/μL, n = 62), (p = 0.0001) (Fig. 4). In patients with high ascites MP levels the median bilirubin level [28.8 μmol/L (range 5.9–254.2) vs. 44.7 μmol/L (range 2.9–609.1, p = 0.04)] were slightly lower and the median thrombocyte count higher [141 exp9/L (range 34–864) vs. 116 exp9/L (range 16–826, p = 0.001)] than in patients with low ascites MP levels. All other baseline parameters were not different between both groups. Interestingly, blood MP levels were not associated with short-term survival.

Main cause of death was liver failure (Table 3) and sepsis. However, nine of 31 patients were lost to follow up after discharge from the hospital with regard to the cause of death.

Factors associated with patients' outcome

After univariate analysis, five factors were significantly associated with 30-day survival in this study cohort: thrombocyte count, MELD score, antibiotic treatment at paracentesis, ascites leukocyte count and low ascites MP counts (<488.4/μL). After adjustment with these risk factors by using a multivariate cox regression analysis three parameters remained being associated with death: low-level ascites MPs (<488.4/μL), the MELD score and antibiotic treatment at paracentesis (Table 4).

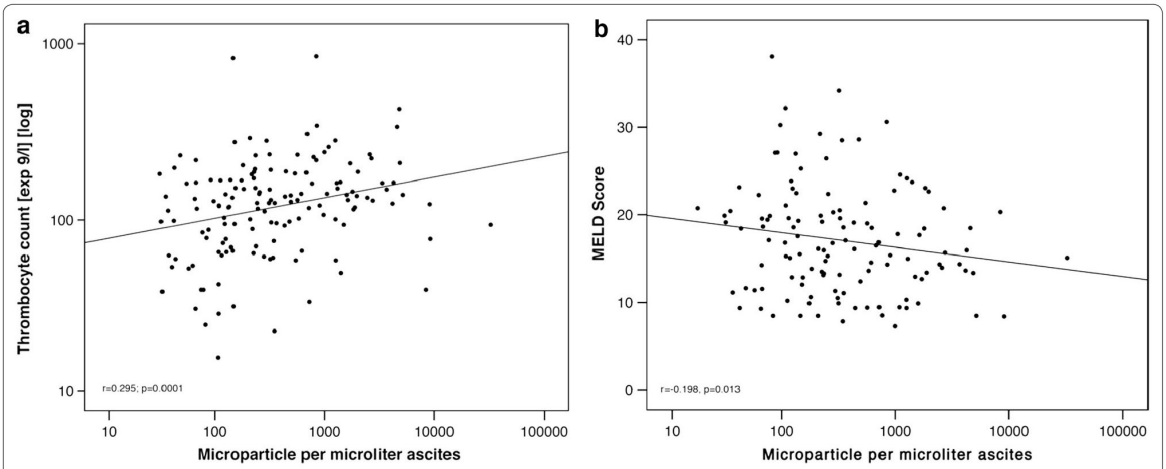

Fig. 2 Correlation between ascites microparticle level and the thrombocyte count (**a**) as well as the MELD score (**b**) in blood. **a** The thrombocyte count in blood was weakly correlated with the level of microparticles per microliter ascites. Chart axis is scaled logarithmically. **b** The MELD score in blood was weakly correlated with the level of microparticles per microliter ascites. MELD axis is scaled logarithmically

Table 2 Correlation between clinical baseline parameters and microparticles in ascites and blood

Variable	Ascites microparticles (n = 163)		Blood microparticles (n = 31)	
	Correlation coefficient (r)	Level of significance (p)	Correlation coefficient (r)	Level of significance (p)
Age (years)	0.042	0.590	0.144	0.440
Child–Pugh score	−0.092	0.390	−0.249	0.263
MELD score	*−0.198*	*0.013*	−0.318	0.990
Serum albumin (g/L)	0.056	0.584	0.286	0.196
Thrombocyte count (exp9/L)	*0.295*	*0.001*	0.140	0.461
WBC count (exp9/L)	0.047	0.570	0.067	0.726
AST (µkat/L)	0.071	0.423	0.011	0.953
GGT (µkat/L)	0.142	0.112	−0.009	0.956
CrP (mg/L)	−0.020	0.818	0.227	0.245
GFR (mL/min)	0.072	0.474	−0.040	0.862
Ascites protein content (g/L)	0.001	0.988	0.366	0.060
Ascites leukocyte count (/mm^3)	0.050	0.951	0.121	0.516

Spearman-Rho correlation (r) was used for analysis

Values highlighted in italics are significant after multivariate analysis

Antibody labelling of ascites microparticles

Ascites MPs were labelled with antibodies capturing surface antigens from either neutrophils (CD66b) or lymphocytes (CD3) in a subpopulation of 60 matched patients. In general, the median relative amount of neutrophil-derived MPs was 32.2% (range 19.3–82.3) whereas the median relative amount of lymphocyte-derived MPs was 12.3% (range 0.6–66.5). There was a good correlation ($r = 0.865$; $p < 0.0001$) between both MP subsets in ascites and a weak correlation between the MELD score and neutrophil-derived MPs (CD66b $r = 0.313$, $p = 0.016$; CD3 0.158, $p = 0.231$). Conventional inflammatory parameters such as the CrP level and white blood cell count (CrP: CD66b $r = 0.89$, $p = 0.526$, CD3 $r = 0.03$, $p = 0.83$; WBC: CD66b $r = -0.035$, $p = 0.79$, CD3 $r = -0.106$, $p = 0.424$) and the leukocyte count in ascites (CD66b $r = -0.052$, $p = 0.698$; CD3 $r = -0.106$, $p = 0.427$) were not associated with the relative amount of immune cell-derived MP subsets. Patients who died with a low ascites MP count (<488.4/µL) had a higher relative amount of both, neutrophil and lymphocyte-derived MPs, than patients

who survived or were transplanted [CD66b: median 43.1% (range 22.7–80) vs. median 29.1% (range 19.3–82.3), p = 0.01; CD3: median 18.1% (range 2.7–66.5) vs. 7.6% (range 0.6–50.8), p = 0.044)]. Moreover, the relative amount of immune cell-derived MPs of median 43.1% (range 25.1–81.3) for CD66b and median 22.4% (range 2.5–50.8) for CD3 in survivors with low ascites MP levels was comparable to non-survivors with low ascites MP levels (CD66b p = 0.578, CD3 p = 0.963) and significantly higher than in survivors with high ascites MP levels (CD66b median 25.6% (range 19.3–65.4), p = 0.006; CD3 median 3.9% (range 0.6–43.9), p = 0.0003) (Fig. 5a–d).

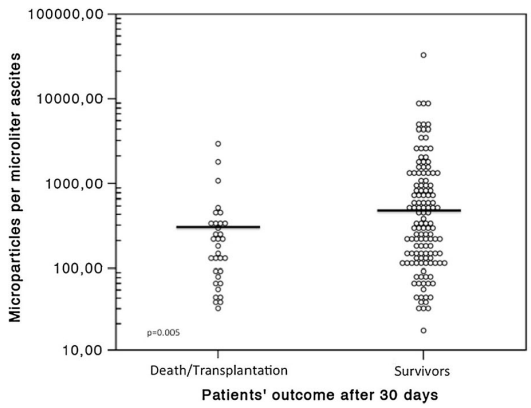

Fig. 3 Total ascites microparticles levels in survivors and in patients who died or were transplanted. Although the range was wide, survivors showed significantly higher MP levels

Table 3 Cause of death (n = 31) taking into account the quantitative level of microparticles in ascites

Ascites MP level	Cause of death				
	Liver failure	Sepsis	Bleeding	Cardiac	Unknown
<488.4/µL (n = 101)	n = 10	n = 6	n = 2	n = 2	n = 8
>488.4/µL (n = 62)	n = 1	n = 1	n = 0	n = 0	n = 1

Liver failure was the main cause of death

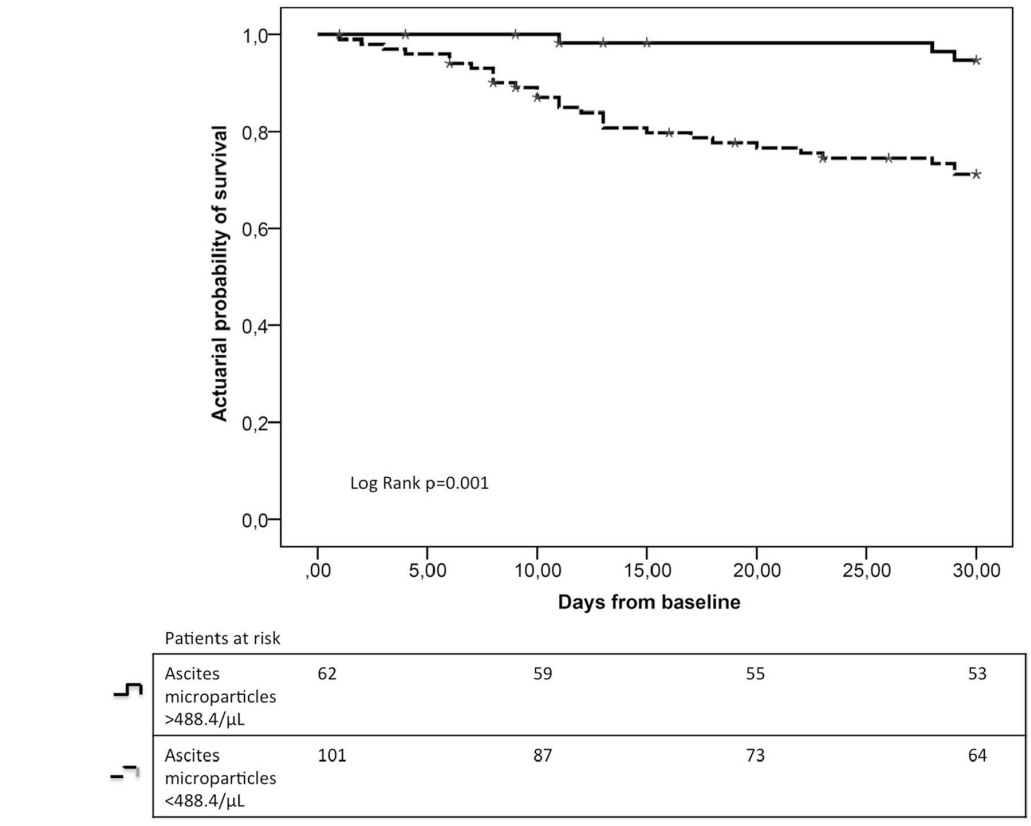

Fig. 4 30-day survival analysis using Kaplan–Meier. Low levels of ascites microparticles were associated with poor short-term survival (30-day)

Table 4 Univariate and multivariate analysis using Cox regression analysis

Variable	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% confidence interval	Level of significance (p)	Hazard ratio	95% confidence interval	Level of significance (p)
Thrombocyte count (exp9/L)	0.992	0.984–0.999	0.024	0.999	0.991–1.007	0.724
MELD score	1.167	1.097–1.241	<0.0001	*1.132*	*1.056–1.214*	*<0.0001*
Antibiotic treatment at paracentesis	3.422	1.518–7.718	0.003	*2.759*	*1.095–6.952*	*0.031*
Ascites leukocyte count (exp9/L)/100	1.019	1.001–1.037	0.039	0.997	0.958–1.038	0.900
Low-level ascites MPs (<488.4/µL)	6.552	1.991–21.561	0.002	*8.723*	*1.148–66.308*	*0.036*

High MELD score, antibiotic treatment at paracentesis and low-level ascites MP (<488.4 MP/µL) were independent risk factors for death after 30 days

Values highlighted in italics are significant after multivariate analysis

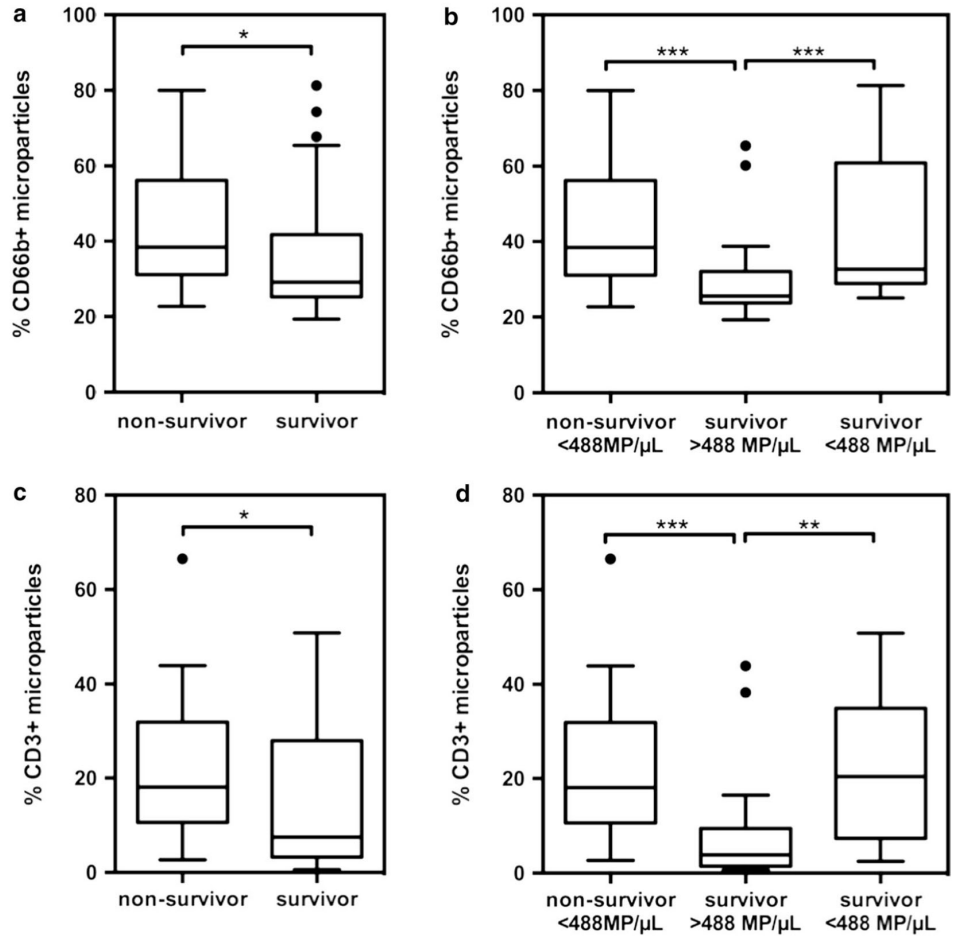

Fig. 5 Relative amount of neutrophil (CD66b) and lymphocyte (CD3)-derived ascites microparticles compared between non-survivors and survivors (**a**, **c**) and according to the ascites MP levels (**b**, **d**). Non-survivors had a higher rate of CD66b positive (**a**) and CD3 positive (**c**) microparticles. **b** Patients with low ascites microparticle levels (<488.4/µL) had a higher relative amount of CD66b positive (**b**) and CD3 positive (**d**) ascites microparticles compared to patients with high ascites microparticle levels irrespective of their survival status. *p < 0.05; **p < 0.01; ***p < 0.001

Discussion

Plasma MPs, in particular cell specific subtypes, have been investigated as novel biomarkers for several pathomechanistic processes. We wanted to determine whether MPs are released into cirrhotic ascites in the first place and, if so, whether overall quantification levels correlate with clinical endpoints in cirrhosis.

This is the first report about ascites MPs in patients with liver cirrhosis. Our study indicates that MPs are ubiquitously present in cirrhotic ascites. This is in line with previous investigations that focused on MP diagnostics in ascites of patients with peritonitis and ovarian neoplasms. Those studies have shown that MPs can be found in peritoneal fluid irrespective of the diagnostic method used (electron microscopy or flow cytometry) and of the cause of ascites [20, 21].

Our survival analysis emphasized the clinical relevance of absolute ascites MP levels in cirrhosis. Patients with a cut-off level below 488.4 MP/μL died more frequently than patients with high levels (30-day survival: <488.4 MP/μL 71.1% vs. >488.4 MP/μL 94.7%, HR 8.571) along with the liver function and other clinical or laboratory parameters at baseline. The high rate of liver failure causing death indicated to some extent that patients with low ascites MP levels are in the final stages of cirrhosis. It is important to highlight that we observed an inverse correlation between ascites MP levels and patients' prognosis whereas in the literature cell-specific plasma MP levels rise with disease progression. This might be explained by a formation of MP-associated immune complexes [25], which is a phenomenon observed in synovial fluid of patients with rheumatoid arthritis by using electron microscopy and a new generation of flow cytometers. Generally, those formations are highly active and proinflammatory and probably express an inflammatory systemic reaction [25]. These complexes would have been missed with the conventional flow cytometers that we used as particle size significantly increases (>1 μm) after complex formation. We gated only particles with a diameter smaller than 1 μm, so that we cannot exclude the presence of high volume complexes.

This theory is supported by the fact that the rate of MPs derived from neutrophils and lymphocytes are much higher in patients with low ascites MP levels and associated with the severity of liver dysfunction, displayed by the MELD score. This observation is well in line with the notion that end stage cirrhosis is accompanied by an inflammatory state [26, 27]. Nevertheless, further prospective studies are needed to substantially clarify the missing correlation with conventional inflammatory parameters as well as the underlying relationship between ascites MPs and patient outcomes. We suggest screening for MP complexes using new generation cytometers on the one hand and screening for further surface antigens in order to identify more cell types, which release MPs in ascites on the other hand. Subsequently performed ex vivo cell-specific stimulation tests to produce MPs can clarify if cell exhaustion is an actual problem in the peritoneal cavity.

Interestingly, the amount of MP production in ascites but also in blood from patients with cirrhosis seems to be highly variable, with MP levels in ascites ranging from 17.5 to 32,575.7 MP/μL, and in blood from 301.8 to 4926.3 MP/μL. This gives rise to at least two questions. The first is which host factors apart from inflammatory processes influence the release of MPs. The second is whether ascites MPs are derived from circulating blood cells or directly produced by autochthonous peritoneal cells. The latter might explain why we not only had a weak correlation between plasma and ascites MP levels but also a considerable number of patients with ascites MP levels higher than those in plasma. In this context it is important to emphasize that blood sample size was rather low potentially reducing its informative value. Nevertheless, the fact that there were no true correlations of overall MP levels, both in ascites and blood, with clinical parameters and immune cell MPs were upregulated in patients with high risk of death suggests that it is worthwhile again to focus on MP subsets in order to clarify the cause of overall ascites MP loss in high risk patients and the high variability. Notably, it is known that specific types of circulating MPs are involved in pathogenic processes such as endothelial dysfunction [4, 11, 23, 28, 29], coagulatory processes [9, 30] and inflammatory reactions [7, 31–33]. In recent years, there has been growing evidence that plasma MPs reflect disease severity in chronic liver diseases. Korneck et al. [16] investigated 67 patients with fatty liver disease and 42 patients with chronic hepatitis C and compared them to healthy individuals in terms of immune cell-derived plasma MP levels. We evaluated stem cell-derived plasma MP levels in acute as well as acute-on-chronic liver failure in rodents and humans (n = 10) [17]. Both studies showed that relative levels of immune cell- and stem cell-derived MPs were increased with heightened disease activity measured by transaminases and liver function tests in individuals with liver insufficiency. To what extend those results and others [34–38] related to circulating MPs can be extrapolated to ascites MPs is speculative at this stage, so that re-examinations are mandatory.

Our study has certain limitations. Due to the retrospective evaluation of all clinical data, some values were missing. Specific information concerning cause of death was not available for 9 of 31 patients who died during the observational period. Although the PMN count in ascitic fluid is the diagnostic gold standard for SBP, we used the

ascites leukocyte count, because the ascites cell count could not be differentiated during the sample collection period. This might be a source of inaccuracy in terms of the relevance of infectious complications. However, the total leukocyte count has also been evaluated as a reliable diagnostic mean for SBP [22]. Moreover, ascites samples have not been centrifuged before freezing. Variation in temperature make cells (erythrocytes and leukocytes) explode and release membranes resembling MPs. However, cells and fragments of cells were removed by the first centrifugation step. If remaining cells had an influence on MP levels in our setting, we should have found a high correlation of the ascites leucocyte count with the MP levels, which is not the case. It is unclear as to whether MP levels correlated with the amount of ascites as a high ascites volume might be associated with low MP concentrations and vice versa. However, this parameters has not been documented. The fact that we were able to detect surface antigens from the original cell emphasizes that true MPs and not debris was captured by flow cytometry in ascitic fluid.

Conclusions

In summary, absolute ascites MP levels represent a novel independent prognostic factor in patients with decompensated liver cirrhosis. Note that the detection of absolute MP levels from ascites samples was shown to be easy, reliable and cost-effective, which is important in the context of potential clinical implementation. Before MPs can be evaluated as biomarkers in cirrhosis, it is essential to clarify the mechanisms behind the association between overall ascites MP levels and clinical endpoints observed in this study. High levels of neutrophils and lymphocyte surface antigens in patients with low level ascites MPs suggests that immune reactions are involved in the pathomechanisms explaining the high risk of death in those patients.

Additional file

Additional file 1: Table S1. Comparison of MP levels in ascites and blood according to baseline parameters at index paracentesis. Values are displayed in median (range) separated according to the presence or absence of parameters. **Figure S1.** Flow cytometry in ascitic fluid: Gating strategy and examples for different antigen expression profiles on ascites microparticles. **Figure S2.** Individual values for microparticles in plasma and ascites.

Abbreviations

MP: microparticles; HCC: hepatocellular carcinoma; BMI: body mass index; MELD: model of end-stage liver disease; GFR: glomerular filtration rate; INR: international normalized ratio; WBC: white blood cell count; AST: aspartate transaminase; ALT: alanine transaminase; GGT: gamma-glutamyl-transferase (GGT); CrP: C-reactive protein; SBP: spontaneous bacterial peritonitis; PMN: polymorphonuclear leukocyte.

Authors' contributions

CE study concept and design, acquisition of data, analysis and interpretation of data, drafting of manuscript, statistical analysis. KS acquisition of data, analysis and interpretation of data, technical support, drafting of the manuscript, statistical analysis. SK acquisition of data, analysis and interpretation of data, technical support. AH, AB acquisition of data, analysis and interpretation of data, critical revision of the manuscript for important intellectual content. SB, JP analysis and interpretation of data, critical revision of the manuscript for important intellectual content, technical support. TB, MS study concept and design, analysis and interpretation of data, critical revision of the manuscript for important intellectual content, administrative, study supervision, obtained funding, material support. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in this published article (and its additional information files).

Consent for publication

Not applicable.

Ethics approval and consent to participate

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the local ethics committee (No: 006-09/No: 356-10-13122010). All patients gave written informed consent.

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3.4 Molekularer Bacteraszites bei dekompensierter Leberzirrhose definiert eine Patientengruppe mit schlechtem Outcome

Ergebnisse und Diskussion

Die rasche Diagnose einer SBP sowie der Nachweis der zugrundeliegenden Erreger ist entscheidend, um das Überleben von Patienten zu verbessern. Es konnte gezeigt werden, dass ähnlich wie bei der Sepsis jede Stunde Verzögerung der Diagnosestellung einer SBP und damit auch des Therapiebeginns das Sterberisiko um 3,3% steigert (54). Darüber hinaus vermindert eine Erreger-inadäquate Behandlung die Überlebenswahrscheinlichkeit, so dass die Speziesdiagnostik ebenfalls eine große Bedeutung hat (33). Die zunehmende Inzidenz von Infektionen mit gram-positiven und resistenten Erregern sowie die unzureichende Spezifität kultureller Nachweismethoden (55, 56) machen es daher notwendig, schnelle und verlässliche diagnostische Techniken zum Nachweis der Erkrankung und der verursachenden Erreger zu entwickeln und diese mit dem klinischen Verlauf der Erkrankung zu korrelieren.

Wir haben daher eine PCR basierte Methode entwickelt, die durch Amplifikation und Sequenzierung des bakteriellen 16S-Gens intakte bakterielle Erreger detektiert, quantifiziert und identifiziert. Ein wesentlicher Vorteil dieser Methode ist, dass trotz hoher Sensitivität durch ultrareine Reagenzien Verunreinigungen nahezu ausgeschlossen werden können (57).

Abstrakt Zitat Engelmann C et al. (58):

"Background: The prognostic relevance of bacterial DNA (bactDNA) detection in ascitic fluid of patients with cirrhosis is still under debate. Using quantitative real-time PCR with broad-range primers targeting the V3 and V4 variable region of the 16S rRNA gene, we measured bactDNA concentrations in patients with and without leukocytic ascites and evaluated the impact on short-term survival.

Patients and methods: Ascites samples from 173 patients with decompensated cirrhosis were consecutively collected between February 2011 and December 2012. BactDNA-positive ascites samples were sequenced and chromatograms were identified using RipSeq. Clinical data collection and survival analyses were carried out retrospectively and correlated with ascites bactDNA levels.

Results: BactDNA was detected qualitatively with a similar frequency in both nonleukocytic and leukocytic ascites [40% (57/144) and 43.5% (10/23), respectively; P=0.724]. However, the median bactDNA level was significantly higher in leukocytic ascites than in nonleukocytic ascites (1.2×10 vs. 5.7×10 copies/ml; P=0.008). Patients' survival was associated significantly with bactDNA level. The 30-day and 180-day survival was reduced if bactDNA was above the quantification limit of 520 copies/ml (84 and 63% vs. 72 and 43%,

respectively; P<0.05) and worst if bactDNA was above 5000 copies/ml. The bacterial spectrum was dominated by Gram-positive strains as shown by direct sequencing.
Conclusion: BactDNA quantification in ascitic fluid samples using culture-independent 16S rRNA gene-based methods seems to be an interesting approach to identify patients at risk of reduced survival. Our study warrants further evaluation of antibiotic treatment in patients with molecular bacterascites."

In dieser Studie konnten wir demnach nachweisen, dass unabhängig vom Vorliegen einer SBP Patienten in ca. 40% der Fälle bactDNA im Aszites haben. Weitaus wichtiger als die bloße Detektion von bactDNA war jedoch die Menge der bactDNA. Einerseits zeigten Patienten mit SBP eine signifikant höhere Kopienzahl und andererseits konnten wir nachweisen, dass unter Berücksichtigung eines Grenzwertes von 520 Kopien/µl Patienten unabhängig vom Vorliegen einer Infektion eine schlechtere Überlebenswahrscheinlichkeit hatten. Inwieweit Patienten mit SBP negativem aber bactDNA positivem Aszites dann tatsächlich eine manifeste SBP entwickeln, konnte in dieser retrospektiven Analyse nicht geklärt werden. Allerdings legt der signifikant höhere Gebrauch von Antibiotika nach der Parazentese trotz gleicher Rate an SBP nahe, dass es sich hierbei um Patienten mit fortgeschrittener Lebererkrankung und Risiko für infektiöse Komplikationen handelt. Inwieweit diese Patienten von einer antibiotischen Therapie bzw. Prophylaxe profitieren, sollte als nächstes in einem prospektiv randomisierten Setting geklärt werden.

Engelmann C, Krohn S, Prywerek D, Hartmann J, Herber A, Boehlig A, Zeller K, Boehm S, Berg T. Detection of molecular bacterascites in decompensated cirrhosis defines a risk with decreased survival. Eur J Gastroenterol Hepatol 2016;28(11):1285-92.
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3.5 Molekulare Quantifikation und Differenzierung von Candida Spezies bei Leberzirrhose

Ergebnisse und Diskussion

Patienten mit Leberzirrhose haben aufgrund des kompromittiertem Immunsystems sowie häufigen antibiotischen Behandlungen ein signifikant erhöhtes Risiko für (invasive) Pilzinfektionen. Ähnlich wie bei der bakteriellen intestinalen Translokation migrieren auch Pilzerreger vom Gastrointestinaltrakt in die systemische Zirkulation. Trotzdem die Inzidenz fungaler Infektionen mit 2,5-9% noch vergleichsweise niedrig ist (59-61), ist das Risiko daran zu versterben mit 50-100% ausgesprochen hoch (62-64). Ein Grund dafür ist, dass adäquate Prädiktoren und diagnostische Techniken unzureichend sind und somit die Therapie häufig zu spät initiiert wird (33, 65). Bisher eingesetzte kulturelle Methoden oder serologische Assays sind zeitaufwendig bzw. weisen sie eine unzureichende Sensitivität auf (66, 67). Wir haben daher eine neue PCR-basierte Methode zur Identifikation und Differenzierung von fungaler DNA entwickelt. Zudem wurde versucht, die Nachteile bisheriger PCR Anwendungen, bspw. Verunreinigung, fehlende Erregeridentifikation, zu überwinden.

Abstrakt Zitat Krohn S et al (68):

"Patients with liver cirrhosis are susceptible to fungal infections. Due to low sensitivity of culture-based methods, we applied a real-time PCR assay targeting the 18S rRNA gene in combination with direct sequencing and terminal-restriction fragment length polymorphism (T-RFLP) in order to establish a novel tool to detect fungal DNA and to quantify and differentiate Candida DNA, also in polyfungal specimens. In total, 281 samples (blood n = 135, ascites n = 92, duodenal fluid n = 54) from 135 patients with liver cirrhosis and 52 samples (blood n = 26, duodenal fluid n = 26) from 26 control patients were collected prospectively. Candida *DNA was quantified in all samples. Standard microbiological culture was performed for comparison. Blood and ascites samples, irrespective of the patient cohort, showed a method-independent low fungal detection rate of approximately 1%, and the Candida DNA content level did not exceed* $3.0x10^{1}$ *copies* ml^{-1} *in any sample. In contrast, in duodenal fluid of patients with liver cirrhosis high fungal detection rates were discovered by using both PCR- and culture-based techniques (81.5% vs. 66.7%; p = 0.123) and the median level of* Candida *DNA was* $3.8x10^{5}$ *copies* ml^{-1} *(*$2.3x10^{2}$*-*$6.3x10^{9}$*). In cirrhosis and controls, fungal positive culture results were confirmed by PCR in 96% and an additional amount of 44% of culture negative duodenal samples were PCR positive. Using T-RFLP analysis in duodenal samples, overall 85% of results from microbial culture were confirmed and in 75% of culture-negative but PCR-positive samples additional Candida species could be identified.*

In conclusion, PCR-based methods and subsequent differentiation of Candida DNA might offer a quick approach to identifying Candida species without prior cultivation"

Mit panfungalen Primern zur Detektion der 18S rRNA Region wurde ein großes Spektrum unterschiedlicher Pilzerreger abgedeckt, und ultrareine Reagenzien minimierten Verunreinigungen. Der Einsatz der direkten Sequenzierung und Bestimmung des terminalen Restriktions Fragment Längen Polymorphismus (T-RFLP) erlaubte die Differenzierung der meisten Candida Spezies und durch zusätzliche Hybridisierungsprimer konnte eine Quantifizierung der fungalen DNA erfolgen. Durch die damit verbundene sichere Detektion, Differenzierung und Quantifikation von Pilzerregern, konnte ein wesentlicher Schritt hin zur adäquaten Pilzerregerdiagnostik nicht nur für die Leberzirrhose sondern auch für andere Erkrankungen gemacht werden (68).
Darüber hinaus konnten wir ein paar wesentliche Ergebnisse zur Bedeutung fungaler Erreger/Infektionen bei Patienten mit Leberzirrhose zeigen. Durch die meist negativen Ergebnisse aus Blut- und Aszitesproben (ca. 1% PCR positiv) ist es wahrscheinlich, dass die Translokation und Zirkulation fungaler Erreger bei Patienten mit Leberzirrhose ohne manifeste klinische Zeichen einer Pilzinfektion selten sind. Wir konnten sowohl bei Patienten mit und ohne Leberzirrhose in 60-80 % Pilzerreger in der Duodenalflüssigkeit nachweisen. Die Detektion von Pilzerregern in der Duodenalflüssigkeit war ohne Krankheitswert. Allerdings zeigten Patienten mit Leberzirrhose und hoher DNA Quantität eine schlechtere Prognose als bei niedriger Quantität.
Als nächster Schritt soll die Methode in Patienten mit fortgeschrittener Lebererkrankung, insbesondere Patienten mit ACLF evaluiert werden. Die Ergebnisse der Studien befinden sich momentan in der Auswertung.

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RESEARCH ARTICLE

Molecular quantification and differentiation of *Candida* species in biological specimens of patients with liver cirrhosis

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Abstract

Patients with liver cirrhosis are susceptible to fungal infections. Due to low sensitivity of culture-based methods, we applied a real-time PCR assay targeting the 18S rRNA gene in combination with direct sequencing and terminal-restriction fragment length polymorphism (T-RFLP) in order to establish a novel tool to detect fungal DNA and to quantify and differentiate *Candida* DNA, also in polyfungal specimens. In total, 281 samples (blood n = 135, ascites n = 92, duodenal fluid n = 54) from 135 patients with liver cirrhosis and 52 samples (blood n = 26, duodenal fluid n = 26) from 26 control patients were collected prospectively. *Candida* DNA was quantified in all samples. Standard microbiological culture was performed for comparison. Blood and ascites samples, irrespective of the patient cohort, showed a method-independent low fungal detection rate of approximately 1%, and the Candida DNA content level did not exceed $3.0x10^1$ copies ml^{-1} in any sample. In contrast, in duodenal fluid of patients with liver cirrhosis high fungal detection rates were discovered by using both PCR- and culture-based techniques (81.5% vs. 66.7%; p = 0.123) and the median level of *Candida* DNA was $3.8x10^5$ copies ml^{-1} ($2.3x10^2$-$6.3x10^9$). In cirrhosis and controls, fungal positive culture results were confirmed by PCR in 96% and an additional amount of 44% of culture negative duodenal samples were PCR positive. Using T-RFLP analysis in duodenal samples, overall 85% of results from microbial culture were confirmed and in 75% of culture-negative but PCR-positive samples additional *Candida* species could be identified. In conclusion, PCR-based methods and subsequent differentiation of *Candida* DNA might offer a quick approach to identifying *Candida* species without prior cultivation.

study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Introduction

The incidence of fungal infections is increasing in patients with end-stage liver diseases and contributes to high fatality rates [1–3]. Fungal infections most often evolve endogenously as fungal pathogens grow uncontrolled in the gastrointestinal tract and migrate into the systemic circulation. This so-called intestinal translocation is promoted and maintained by a cirrhosis-associated impaired intestinal barrier function and a dysfunctional immune system [4–6]. When fungal infections develop, it is of high importance that pathogens are identified rapidly and reliably in order to enable early and adequate antifungal treatment. However, low sensitivity of culture-based standard diagnostic procedures and serological *Candida* antigen assays, as well as time-consuming microbial identification methods [1, 7, 8] have resulted in delayed treatment initiation, which contributes to the high numbers of deaths related to cirrhosis.

Culture-independent PCR-based approaches to rapidly detecting DNA from fungal species have already been developed and evaluated [9, 10]. The amplification of variable sequences of the 18S ribosomal RNA (rRNA) gene or its non-coding internal transcribed spacer region has shown high sensitivities [11, 12]. However, the PCR-based methodology has certain limitations. First, false positive results can be created by not using ultra-clean reagents whereas endogenous PCR inhibitors may cause false-negative results [13]. Second, multispecies identification in fungal PCR followed by subsequent sequencing is difficult due to the lack of a web-based tool for the differentiation of polyfungal amplicons, a platform which already exists for mixed bacterial samples [14]. The latter problem can be addressed using PCR-RFLP techniques, which at least allow *Candida* species to be identified. However, time-consuming prior cultivation of fungal microorganisms is needed and only a small panel of *Candida* species can be distinguished [15–17].

We developed a novel PCR-based method to identify and differentiate fungal DNA. Ultra-clean reagents for DNA isolation and enrichment of fungal DNA were used to overcome the problem of false-positive PCR results. The use of panfungal primers and *Candida*-specific hybridization probes in one PCR setting, enables us to qualitatively detect a broad spectrum of fungal genera and simultaneously quantify DNA from *Candida* species that represent the most prominent fungal pathogens detected in human specimens. To account for the diversity of DNA-positive samples, two different methods were applied to identify fungal species. First, direct sequencing allowed for the quick identification of pathogens that occur frequently in primarily mono-fungal specimens. Second, T-RFLP was used directly with isolated DNA of sample material to differentiate between mixed-*Candida* DNA without prior cultivation using a set of restriction enzymes that distinguishes up to 13 clinically relevant *Candida* species from a single sample at the same time. Therefore, the PCR/T-RFLP method presented here combines for the first time the detection of fungal species, the quantification of the most relevant *Candida* species, and the differentiation of mixed *Candida* strains without any use of conventional cultural techniques. This study evaluates the accuracy of this technique in detecting, quantifying and differentiating *Candida* species in colonized (polyfungal) and non-colonized body specimens. Furthermore, this study assesses the clinical relevance of *Candida* DNA in different body fluids from patients with liver cirrhosis.

Results

Patient characteristics

The following clinical and laboratory parameters were assessed at baseline (date of paracentesis and/or endoscopy): etiology of cirrhosis, sex, age, MELD score, Child-Pugh score, liver-function tests, white blood cell count (WBC), C-reactive protein (CrP), leukocyte count in ascitic

Table 1. Patient characteristics at baseline.

Variable	Cirrhosis (n = 135)	Non-cirrhosis (n = 26)	Level of significance (p)
Age (years), median (range)	59 (25–87)	53 (22–88)	0.348
Gender (male/female), n (%)	**104/31 (77.0%/23.0%)**	**13/13 (50.0%/50.0%)**	**0.008**
Aetiology of cirrhosis, n (%) (n = 119)		Not applicable	
Alcoholic	97 (71.9)		
NASH	7 (5.2)		
Viral	8 (5.9)		
Cryptogenic	16 (11.9)		
Others	7 (5.2)		
Type of previous decompensation, n (%)			
(n = 135)		Not applicable	
Ascites	119 (88.1)		
Hepatic encephalopathy	46 (34.1)		
Bacterial infection	74 (54.8)		
Gastrointestinal hemorrhage	44 (32.6)		
Reason of hospital admission, n (%); (n = 135)		Not applicable	
Ascites	104 (77.0)		
Hepatic encephalopathy	25 (18.5)		
Bacterial infection	71 (52.6)		
Gastrointestinal hemorrhage	24 (17.8)		
Previous endoscopies, n (%)	**50 (37.0)**	**5 (19.2)**	**< 0.0001**
MELD score, median (range)	**14 (5–40)**	**Not applicable**	
Bilirubin (μmol/l), median (range)	**34.5 (3–643)**	**8.5 (6–25)**	**<0.0001**
Albumin (g/l), median (range)	**33.9 (16–47)**	**42.4 (32–49)**	**0.006**
INR, median (range)	**1.4 (1–3)**	**1 (1–1)**	**0.0001**
Serum creatinine (μmol/l), median (range)	90 (29–591)	72 (50–132)	0.059
GFR (ml/min), median (range)	72.8 (8–150)	87.6 (41–112)	0.313
ALAT (μkat/l), median (range)	0.4 (0–3)	0.34 (0–1)	0.787
GGT (μkat/l); median (range)	**1.6 (0–25)**	**0.64 (0–17)**	**0.04**
Thrombocyte count (exp9/l), median (range)	**124.5 (29–826)**	**233 (99–354)**	**<0.0001**
White blood cell count (exp9/l), median (range)	6.2 (0–33)	6.5 (2–15)	0.882
Hemoglobin (mmol/l), median (range)	**6.4 (4–10)**	**8.6 (5–10)**	**<0.0001**
C-reactive protein (mg/dl), median (range)	**21.7 (1–146)**	**1.8 (0–23)**	**<0.0001**
Antibiotic treatment, n (%)			
At Baseline	68 (50.4)	0 (0)	
3 months prior sampling	29 (21.5)	1 (3.8)	0.05
Proton pump inhibitors (n, %)	84 (62.2)	12 (46.2)	0.134

Categorical data are displayed as absolute and relative values and metric data as mean ± standard deviation or median (range), as appropriate. NASH = non-alcoholic steatohepatitis; GFR = glomerular filtration rate; INR = international normalized ratio; ALAT = Aspartat-Amino-Transferase; GGT = Gamma-glutamyltransferase.

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fluid, and drug history. Patients were followed-up until October 2014 in order to capture fatalities and/or liver transplantations.

There were some significant differences between patients with and without cirrhosis at baseline. Parameters representing the liver function, such as bilirubin, albumin, Gamma-glutamyl-transferase (GGT), and INR, as well as the thrombocyte count, were significantly different in the cirrhosis group (Table 1). The C-reactive protein (CrP) level, which is a global marker for inflammation, was higher in individuals with cirrhosis, and the hemoglobin level was

significantly better in patients without cirrhosis (Table 1). Patients with liver cirrhosis received more often an antibiotic treatment (21.5% vs 3.8%, p = 0.05) and had a higher number of endoscopic procedures (37.0% vs 19.2%, p<0.0001) in comparison to controls three months before duodenal fluid sampling. The use of proton pump inhibitors was not significantly different between both cohorts (Table 1).

Detection and quantification of Candida DNA in biological specimens

We developed a real-time PCR for the detection and quantification of Candida DNA. DNase treatment was applied prior DNA isolation to ensure that the assay only captures DNA from intact fungal cells. Analytical sensitivity (limit of quantification) of the PCR was 370 copies ml^{-1} whereas clinical sensitivity was 96.1% compared to 71.0% using microbial culture as gold standard. Clinical specificity was 93.2% compared to 99.3% in culture methods. The predictive capacity to detect fungal pathogens proven by cultural techniques was calculated using the receiver operating characteristic curve (ROC curve, Fig 1). The area under the curve (AUROC) of 0.946 (95% CI 0.911–0.982; p<0.0001) and the Cohens kappa coefficient of κ = 0.779 (p<0.0001) confirmed the high concordance with conventional methods, and moreover, suggested the here described molecular methods as an effective and suitable tool for the detection and quantification of Candida DNA.

Comparable fungal pathogen detection rates by using cultural and molecular methods

Microbial culture and PCR analyses in patients with cirrhosis showed low fungal detection rates in blood (culture: n = 1/135; 0.7%; PCR: n = 1/135; 0.7%) and ascites (culture: n = 0/92;

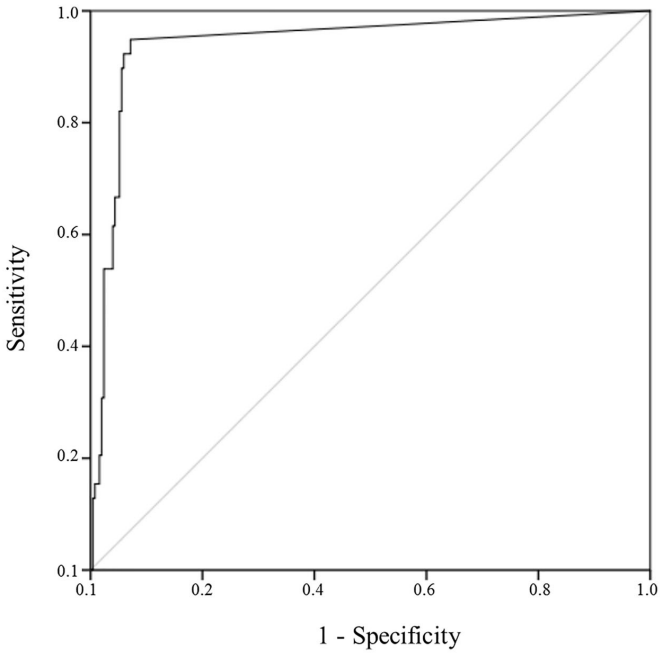

Fig 1. Receiver operating characteristic curve (ROC) of 18S rRNA gene based quantitative PCR from biological samples of patients with liver cirrhosis and controls.

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Table 2. Fungal pathogen and *Candida* DNA detection rates from microbial culture, real-time PCR analysis, and its statistical significance.

Patients with cirrhosis (n = 135)					Control patients without cirrhosis (n = 26)				
Sample material	**No. of samples**	**Culture positive (%)**	**PCR positive (%), median copies/ml (range)**	***P* value culture vs. PCR**	**No. of samples**	**Culture positive (%)**	**PCR positive (%), median copies/ml (range)**	***P* value culture vs. PCR**	***P* value $PCR_{cirrhosis}$ vs. $PCR_{control}$**
Blood	n = 135	n = 1 (0.7%)	n = 1[a] (0.7%), $3.0x10^1$		n = 26	n = 0 (0%)	n = 0 (0%)		
Ascites	n = 92	n = 0 (0%)	n = 1 (1.1%), $1.3x10^1$						
Duodenal fluid	n = 54	n = 36 (66.7%)	n = 44[b] (81.5%), $3.8x10^5$	0.123	n = 26	n = 12 (46.2%)	n = 16[c] (61.5%) $1.2x10^5$	0.404	0.096
			($2.3x10^2$-$6.3x10^9$)				($2.5x10^2$-$2.4x10^7$)		

[a] 1/1

[b] 34/36

[c] 12/12 positive PCR results confirming microbial culture

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0%; n = 1/92; 1.1%). The level of *Candida* DNA was below 10^2 copies/ml (blood: $3.0x10^1$; ascites: $1.3x10^1$) in both fungal DNA-positive samples (Table 2). In contrast, duodenal fluid of patients with cirrhosis was significantly colonized with fungal microorganisms. The culture-based detection rate of 66.7% was comparable to the PCR-based detection rate of 81.5% (p = 0.123). The PCR-based approach failed to detect fungal DNA in 5.6% (2 out of 36) of culture-positive duodenal fluids in cirrhosis but delivered additional positive results in 10 out of 18 (55.6%) culture-negative samples. In control patients, the frequency of fungal DNA detection (61.5% in controls vs. 81.5% in cirrhosis; p = 0.096) and culture positive results (46.2% in controls vs. 66.7% in cirrhosis; p = 0.093) in the duodenal samples was numerically but not statistically lower compared to patients with liver cirrhosis (Table 2). The median amount of *Candida* DNA in the duodenal fluid was $3.8x10^5$ 18S rRNA gene copies ml^{-1} in patients with cirrhosis ($2.3x10^2$-$6.3x10^9$) and therefore not different to $1.2x10^5$ 18S rRNA gene copies ml^{-1} in patients without cirrhosis ($2.5x10^2$-$2.4x10^7$, p = 0.618).

Antibacterial treatment enhances the duodenal Candida DNA level in patients with cirrhosis

Clinical factors that could potentially affect fungal DNA detection and *Candida* DNA quantification in cirrhosis were identified using a cross-sectional analysis at baseline. The PCR results for blood and ascites samples were not considered for this type of analysis because of their low detection rates.

At baseline, four out of 135 patients (3.0%) were treated with amphotericin B due to fungal esophagitis, and two patients (1.5%) were treated systemically with fluconazole due to suspected candidiasis. Moreover, 66 out of 135 patients (48.9%) were on antibiotic therapy, another 66 (48.9%) were receiving non-selective beta-blockers, and 82 (60.7%) were taking proton pump inhibitors. Antibacterial treatment did not affect the rate of fungal DNA detection in duodenal fluid but increased the amount of *Candida* DNA significantly by approximately two log levels (antibiotic treatment: $2.8x10^6$ [$2.2x10^3$–$4.4x10^8$] vs. no antibiotic treatment: $3.2 x 10^4$ [$2.3x10^2$–$6.3x10^9$]; p = 0.001). None of the other drugs were significantly associated with the presence or quantity of duodenal fungal DNA (S3 Table).

Neither gender, Child-Pugh score, MELD, etiology of liver disease, nor additional baseline parameters, as depicted in S1 and S2 Tables, were significantly associated with the presence or quantification of fungal DNA in duodenal fluid.

Association of Candida DNA levels with survival of patients with cirrhosis

Although the survival curves of patients with and without Candida DNA diverged within 30 days after baseline showing a benefit for DNA negative patients, this result was statistically not significant (actuarial 30-day survival rate: DNA negative 100% vs. DNA positive 88.6%; p = 0.254; S1 Fig). The estimated overall survival of 517 days (95% CI 330–704) in patients with PCR-negative results was not different to 555 days (95% CI 447–662, p = 0.777) in patients with PCR-positive results. The survival rates were not associated by the cultural detection of fungal pathogens, whereas the *Candida* DNA quantification level was significantly associated with patients' outcome. Increasing *Candida* DNA levels enhanced the risk for short-term death (30 day survival: HR 1.003 [1.001–1.005], p = 0.007.

Fungal pathogen differentiation using cultural and molecular methods in non-colonized material

While no fungal pathogens were detected in the blood samples of the control patients, there was one positive blood result in a patient with cirrhosis that showed a monofungal infection with *Candida albicans* detected by microbial culture (Fig 2). This result was confirmed through direct sequencing of the PCR product. Although there were no culture-positive ascites samples, a PCR and subsequent sequence analysis identified *Saccharomyces cerevisiae* in one ascites sample (Table 2).

Fungal pathogen differentiation using cultural and molecular methods in colonized material

Candida albicans was the predominant species detected by microbial culture methods in the duodenum of both patient groups, followed by *C. glabrata* (Fig 2). Less frequent *Candida* species, such as *C. guilliermondii*, *C. lusitaniae*, *C. tropicalis*, *C. kefyr*, *C. krusei* and *C. rugosa* were only captured in patients with liver cirrhosis. Non-*Candida* genera were not detected. Further

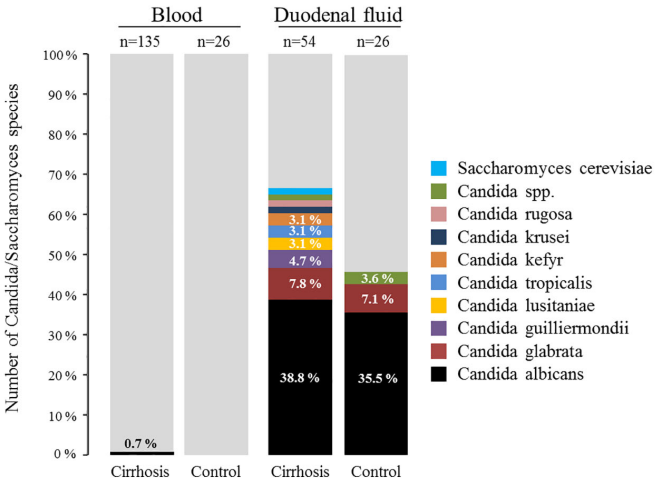

Fig 2. Detection of fungal species in blood and duodenal fluid of patients with liver cirrhosis and in control patients by using microbial culture techniques. The number of each sample group equals 100%. Stacked bars without percentage value correspond to 1.6%.

https://doi.org/10.1371/journal.pone.0197319.g002

microbial culture analyses revealed that seven out of 36 (19.4%) duodenal samples in cirrhosis and one out of 12 (8.3%) duodenal samples in controls (p = 0.659) were polyfungal, containing more than one *Candida* species.

Due to the high diversity in polyfungal samples, we established a T-RFLP analysis directly from isolated bacterial DNA to differentiate fungal pathogens to the species level and compared these data to results obtained through conventional cultural methods (Fig 3). In duodenal samples with positive fungal culture and valid PCR/T-RFLP results (n = 40), 92.3% (48/52) of *Candida* species (highlighted as bars in Fig 3) could be confirmed using T-RFLP. In these duodenal fluids, additional *Candida* species (n = 12, highlighted with stars) were detected in 27.5% samples (n = 11; Fig 3A).

The DNA quantity was adequate for the T-RFLP analysis in nine of 26 duodenal samples (34.6%) in which *Candida* DNA was identified, although the microbial culture results were negative (Fig 3B). These data contained sequences of *C. albicans*, *C. glabrata*, *C. guilliermondii*, and *C. kefyr*. The T-RFLP analysis could not be performed on seven out of 45 culture-positive samples (15.5%), as either the PCR was negative (two out of 45; Fig 3C) or the PCR product was quantitatively inadequate (five out of 45; Fig 3D).

Discussion

Patients with end-stage liver diseases have an increased risk for infections with fungal pathogens resulting in devastating complications. Reliable diagnostic tests could facilitate early and adequate antifungal treatment, a potential lifesaving measure in this cohort [3, 18, 19]. Since cultural methods have a poor diagnostic quality and lack of providing reliable pathogen quantification levels, we established PCR-based techniques that allow us to detect panfungal 18S rRNA genes, and quantify and differentiate *Candida* DNA quickly.

These techniques were able to detect fungal pathogens accurately with a high concordance of about 96% compared to cultural techniques. Fungal DNA was amplified in additional 44%

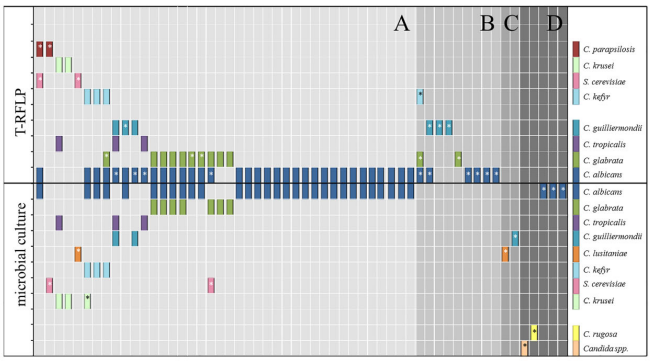

Fig 3. Fungal species analysis using either the T-RFLP or conventional culture methods on duodenal fluid samples from patients with liver cirrhosis and controls (n = 56). Each bar represents a single species. A star indicates that a fungal species could not be confirmed by the alternative method. The x-axis presents single duodenal samples so that multiple culture or T-RFLP-identified pathogens per sample are stacked in columns. Samples with culture-positive and PCR/T-RFLP-positive samples are compared in A (n = 40). Culture-negative but PCR/T-RFLP-positive samples are displayed in B (n = 9) and culture-positive and PCR/T-RFLP-negative samples are presented in C (n = 2). The duodenal samples that were culture-positive and PCR-positive but T-RFLP-negative are represented in area D (n = 5). Additional duodenal samples that were culture negative but PCR-positive with DNA not being sufficient for T-RFLP analysis (n = 3) have been left out in analysis.

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of culture-negative duodenal samples. This and the fact that overall fungal detection rate in duodenal fluid was higher with PCR (75%) than with conventional techniques (60%) clearly show that molecular tests can serve as an additional tool to capture fungal pathogens. However, it remains an open issue as to whether culture-negative but PCR-positive results have an actual clinical impact. The importance of PCR positive results is further questioned as we could not show an association between the mere fungal pathogen detection and patients' clinical course. It is unlikely that the test captures contaminations leading to false-positive results, as blood and ascites samples were in almost all cases negative. Additionally, we have tested endoscopic channels regarding fungal impurities by analyzing the irrigation fluid after endoscope cleansing. Out of 24 samples only three were positive for fungal DNA and moreover had a very low quantification of $1.0x10^3$ copies ml^{-1}. The fact that 97% of duodenal samples were above that quantification range speaks against a relevant test contamination.

However, it is potentially the patients' selection which causes the lack of clinical association as the severity of the liver disease has been already identified as an important factor determining the risk for fungal infection [3]. Since patients with cirrhosis in this study had comparably low MELD scores of approximately 14, the risk for spontaneous invasive mycosis was presumably low and might explain the low fungal DNA detection rate in those primarily sterile body specimens. Recent studies have diagnosed *Candida*-specific bloodstream infections in 9% and 16% of patients already with moderately impaired liver function (MELD $>$ 16) [18, 20]. Other fungal infections with *Aspergillus* or *Cryptococcus* species that were not detected in any of our samples have been found to be predominant in severely immunosuppressed patients, notably after liver transplantation [21, 22] or in patients with severe liver diseases [3] and a MELD score of 26. Therefore, performing a study in patients with higher risk for fungal infections, such as acute decompensated cirrhosis or acute-on-chronic liver failure, might be more appropriate to identify patients at risk for fungal infectious complications.

However, one significant strength of the here presented 18S rRNA gene-based PCR method is the ability to measure the *Candida* DNA quantitatively. The Candida DNA quantification level in the duodenal fluid was a potential predictor for patients' short-term survival. The number of patients and events (n = 5) was inadequate to perform a multivariate Cox-regression analysis to adjust results to potential confounders, leaving the reliability of these results open so far. Besides, it is unclear whether fungal duodenal DNA directly leads to invasive infection and by that to fatalities in cirrhosis. At least, there is data suggesting that intestinal fungi might be the source for systemic spreading and infections via translocation [23]. The relevance of this mechanism is still not well explored in cirrhosis and the results shown here do not preclude that duodenal fungal DNA is just a surrogate marker for previous bacterial therapy. Therefore, further investigations are necessary to explore the mechanisms behind the association between fungal duodenal DNA and death. Yang and Schnabl have nicely shown that alcohol has been proven to induce a reduced intestinal fungal diversity, a Candida overgrowth and a translocation of fungal beta-glucan into the systemic circulation. Importantly, antifungal treatment decreased the fungal translocation and improved the liver damaging effect of alcohol [24]. Nevertheless, we did not find a significant association between alcoholic liver disease and presence or quantification of Candida DNA in duodenal fluid in our cohort (data not shown).

A pre-existing antibiotic treatment further increased the duodenal *Candida* DNA quantification level in cirrhosis. This fits to previous data showing an elevated fungal stool colonization in cancer patients after broad-spectrum antibiotic therapy [25, 26]. In this regard, Bajaj et al. reported, that patients with cirrhosis admitted due to bacterial infectious more likely developed fungal complications with a significant mortality rate of more than 50% [27]. As a consequence clinicians should closely review the indication for antibacterial treatments in patients with liver cirrhosis.

A further advantage of the PCR/T-RFLP method described here is the capacity to identify *Candida* species without the need for expertise in both handling and interpretation of fungal strains, which is necessary for culture techniques. PCR/T-RFLP overall confirmed 85% of strains identified by conventional methods and in 75% of culture-negative duodenal samples with positive PCR result additional species were identified. Thus, the application of this type of fungal pathogen identification and quantification is of high interest particularly in frequently colonized and polyfungal specimens where conventional methods certainly have a weakness.

It is important to mention that all analyses confirmed *Candida* species, in particular *Candida albicans*, as the predominant fungal pathogen. Van Thiel et al. [28] and Low and Rotstein [29] reported similar data, although non-albicans species, such as *C. glabrata*, *C. tropicalis*, and *C. parapsilosis*, obviously gain importance in our analysis.

The here presented method can have a weakness if the number of pathogens is low and only a small sample volume is screened. This could explain why the PCR failed to detect fungal DNA in two cases of culture-positive duodenal samples. Using larger volumes of sample material in DNA isolation and PCR analysis might overcome this problem.

Moreover, the fact that almost all blood and ascites samples were PCR negative might indicate that an intestinal translocation of fungal species as a source for blood stream infections might be overestimated. However, it should be considered that the maximum time span between ascites and duodenal sampling and blood sampling was five days. Since fungal pathogens probably occur temporarily, it might partially explain the low incidence of blood fungal DNA when measured in a single sample obtained from one time point.

In conclusion, the here presented PCR-based method reliably detects fungal DNA and is able to quantify and differentiate *Candida* species without any use of microbial culture methods. These methods are equally effective and quicker than conventional techniques and, therefore, could potentially serve as an additional diagnostic mean. Future prospective studies must address whether positive results in duodenal fluid could change the clinical management of patients with cirrhosis.

Materials and methods

Study design

This study included patients (n = 135) admitted with liver cirrhosis that were either decompensated with ascites or scheduled for an elective endoscopic procedure. The exclusion criteria included prior liver transplantation, malignancies other than hepatocellular carcinoma, and immunosuppressive therapy. Patients without liver diseases and infections who underwent endoscopy and did not receive antibiotic therapy at study enrolment served as the control group. Between May 2012 and March 2013, ascites samples (n = 92) and duodenal fluid samples (n = 73) were collected from 135 patients with cirrhosis and 26 non-liver disease patients at the University Hospital Leipzig. Corresponding blood samples were collected from each patient (n = 161) within five days. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committee of the University of Leipzig (No. 356-10-13122010). All of the patients gave their written informed consent.

Sample preparation

All samples were obtained during medically indicated procedure and were not part of the study protocol. In total, 3 ml blood and 50 ml ascites fluid were used for the analysis. In 30 out of 80 (37.5%) endoscopic procedures, an injection of 10 ml water was required to collect 3 ml of the duodenal fluid samples.

Blood, ascites, and duodenal fluids were analyzed using conventional microbiological culture methods to measure the presence of fungal pathogens. Ten milliliters of blood or ascites were inoculated in an aerobic and an anaerobic BacT/Alert blood culture bottle (bioMérieux, Marcy l'Étoile, France). A volume of 10μl of duodenal fluid was grown on Brucella-, Columbia blood-, and Columbia CAP selective agar, as well as on Endo-, Sabouraud-, and *Candida* ID agar. Additionally, 100 μl duodenal fluid or 300 μl in case samples were flushed were inoculated in Sabouraud bouillon to ensure fungal growth in samples with a low pathogen content. Isolated strains were identified using the Vitek 2 system (bioMérieux).

For the culture-independent analysis, 50 ml of ascites were centrifuged at 4500 rpm for 25 min in order to increase the number of fungal cells for DNA isolation and the pellet was resuspended in 3 ml supernatant. One ml of ascites pellet was used for DNA isolation which is comparable to 20 ml of ascites fluid for microbial cultural. Ascites pellets, fluid samples from duodenum, and whole-blood samples were stored with glycerin (final concentration of 20%) at -20˚C until further sample processing.

DNA-extraction

DNA was isolated from 1 ml resuspended ascites cell pellet, 1 ml whole blood, and 200 μl duodenal fluid using the MolYsis Complete5 (Molzym, Bremen, Germany) DNA isolation kit, as described previously [30] , and subsequently analyzed using PCR. By using a DNase which is included in the DNA isolation kit we destroyed free circulating DNA and exclusively extracted DNA from intact fungal cells. The DNA isolation was performed in a HEPA-filtered hood with daily UV-radiation. A negative control using fetal calf serum instead of the sample material was included in each isolation series.

Quantification of fungal DNA and sequence analysis

The Bioline SensiFast No Rox PCR Mastermix (Bioline, Luckenwalde, Germany) and the panfungal oligonucleotide primer pair `5′-ATT GGA GGG CAA GTC TGG TG-3′` and `5′-C CG ATC CCT AGT CGG CAT AG-3′` [11] covering the V4 variable region of the 18S rRNA gene were used to amplify the fungal DNA. In the same PCR approach, the hybridization probes, `5'-LC Red640-CGA AAG TTA GGG GAT CGA AGA TG-3′` and `5′-CCA AGG ACG TTT TCA TTA ATC AAG A-Fl-3′`, were used to quantify the *Candida* DNA [12] with a quantification limit of 370 copies ml^{-1} (experimentally measured and confirmed using probit analysis). The combination of using panfungal primers with *Candida*-specific probes in one PCR setting has two advantages. First, it enables the quantification of 18S rRNA gene copies of *Candida* species based on the determination of crossing points (Cp values) generated in quantitative PCR. Second, even if the sample does not contain *Candida*-derived DNA, non-*Candida* genera, such as *Aspergillus*, *Cryptococcus*, or *Fusarium*, can be qualitatively detected using the panfungal oligonucleotide pair and which are visible as a band after gel electrophoresis. Both amplicons, either from *Candida* or non-*Candida* genera, can be further analyzed using direct sequencing or T-RFLP. Each 20-μl-PCR reaction contained 10 μl of Mastermix, 1 μl of each primer (10 μM), 1 μl of each probe (5μM), 2.2 μl of PCR-grade water, and 5 μl of template DNA. Real-time PCR was performed on a LightCycler 480II instrument (Roche, Mannheim, Germany) with the following amplification steps: initial denaturation at 95˚C for 10 min, 45 cycles of 15 sec at 95˚C, 10 sec at 58˚C, and 20 sec of 72˚C with a fluorescence acquisition at the end of each 58˚C step. A control using no template and a PCR-positive control containing *Candida albicans* DNA were included in each PCR run. All of the PCR reactions were subsequently subjected to agarose gel electrophoresis to obtain the correct sample size (approximately 500 bp) for each PCR product.

All PCR-positive blood and ascites samples with the appropriate size were purified and directly sequenced (GATC, Constance, Germany). Chromatograms were identified up to the genus level with a sequence identity higher than 98% using the BLAST tool [31].

For analysis of a possible contamination with Candida DNA from intact cells in the endoscopic working channel we analyzed the working and the auxiliary-water channel of three properly cleaned endoscopes. All samples were tested negative for fungal growth. Fungal DNA was isolated in quadruplicates from each sample and quantitative 18S rRNA gene based PCR revealed 3 out of 24 (12.5%) positive results for Candida DNA with a maximum of $1.0x10^3$ copies ml^{-1} deriving from *Candida guilliermondii* (using T-RFLP analysis).

T-RFLP analysis for the differentiation of Candida DNA

For the selection of the restriction enzyme set in polyfungal samples, we used the most clinically relevant standard fungal strains obtained from the Institute for Laboratory Medicine, Clinical Chemistry and Molecular Diagnostics of University Hospital Leipzig: *C. albicans* (ATCC10231, ATCC14053, ZL13, ZL18, ZL23, ZL8b), *C. krusei* (ATCC6258, RV54, ZL11, ZL16, ZL20), *C. tropicalis* (ATCC13803, RV101, ZL9, ZL22), *C. glabrata* (ATCC2001, ZL12), and *C. parapsilosis* (ATCC22019), as well as sequenced strains from *C. haemulonii* (RV22), *C. dubliniensis* (RV30), *C. norvegensis* (RV31), *C. kefyr* (ZL17, ZL21), *C. guilliermondii* (ZL10), *C. sphaerica* (RV208), *C. lusitaniae* (ZL24), and *S. cerevisiae* (RV20, ZL14).

In order to show the phylogenetic relationship between these fungal strains, parsimony trees were calculated using the program package ARB [32] and added to the Silva small subunit ribosomal RNA reference database, version 108 [33] (S2 Fig).

The DNA from the standard strains, duodenal samples of patients with liver cirrhosis, and control patients that were PCR-positive, were amplified with the panfungal 18S rRNA-based oligonucleotide primer pair that was already used in the quantitative PCR, excluding an additional 5'-FAM-(6-carboxyfluorescein)-labeling of the forward primer. PCR was performed in a total volume of 25 μl containing 12.5 μl MyTaq HS Mix (Bioline), 1 μl of each primer (10 μM), 5.5 μl of PCR-grade water, and 5 μl of template DNA using a Veriti Thermal Cycler (Thermo Fisher Scientific, Waltham, USA). The amplification steps were as follows: initial denaturation at 95˚C for 1 min, 40 cycles of 15 sec at 95˚C, 15 sec at 55˚C and 10 sec at 72˚C, followed by a final elongation for 10 min at 72˚C. The purification of PCR products was performed, as described above, and the DNA content of each PCR product was quantified using the Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific).

After *in silico* enzyme digestion of all the standard strains using REPK Version 1.3 [34], a combination of five enzymes led to the best possible differentiation of the above pathogens: Hpy188I, BfaI, HaeIII, BglI and SmaI (New England Biolabs, Frankfurt, Germany).

For T-RFLP profiling, the PCR product from the standard strains (10 ng) and from the patient samples (40 ng) were separately digested for 3 h with 2 U of each restriction endonuclease at 37˚C (Hpy188I, BfaI, HaeIII, BglI) or 25˚C (SmaI). The cleaned DNA pellets of each sample were suspended in HiDi Formamide containing 1.5% MapMarker-1000 Rox standard (Applied Biosystems, Foster City, USA). The samples were denatured at 95˚C for 10 min and subsequently chilled on ice. Using the ABI Prism 3100 genetic analyzer (Applied Biosystems), terminally labeled 18S rRNA gene fragments were separated using capillary electrophoresis. The lengths of the labeled terminal restriction fragments (T-RF) within the range of 50–510 bp were determined using Genemapper V3.7 software (Applied Biosystems). The *Candida* species were only identified successfully if the T-RF from all five enzymes showed the correct base-pair length (within the range of two base pairs), which corresponded to the reference strain presented in supplement material (S4 Table).

Statistical analysis

Statistical analysis was performed by using SPSS 20 (SPSS, Illinois, USA). Categorical variables were displayed as percentages or frequencies, and the continuous variables were displayed as the mean ± standard deviation or the median and range, as appropriate. A two-sided p-value of < 0.05 was considered statistically significant. The comparison of unpaired samples was performed using a Mann-Whitney U test in the case of continuous data and a Chi-square test in the case of discrete data. For the correlation analysis, the Spearman-Rho coefficient was calculated and a correlation coefficient of $r > 0.5$ was considered relevant. A survival analysis was performed using Kaplan-Meier analysis, as appropriate, and compared using a Log-Rank test. Receiver-operator characteristic (ROC) curves were plotted and AUROC (area under the ROC curve) as well as κ coefficient were calculated.

Supporting information

S1 Table. Baseline parameters of patients with liver cirrhosis compared with *Candida* DNA-positive and -negative duodenal samples.
(PDF)

S2 Table. Correlation analysis using the Spearman Rho method between baseline parameters and Candida DNA quantification levels in duodenal samples.
(PDF)

S3 Table. Association between drug therapy and the presence and quantification of Candida DNA in duodenal fluid.
(PDF)

S4 Table. *In silico* and measured T-RF in reference to *Candida* strains.
(PDF)

S1 Fig. Thirty-day survival analysis using the Kaplan-Meier method.
(TIF)

S2 Fig. Parsimony tree depicting the relationship and affiliation between 18S rRNA gene sequences from *Candida* and *Saccharomyces* reference strains. The final position within the tree and bootstrap values were calculated using the ARB Parsimony Interactive tool (bootstrap values above 50% are shown; scale bar indicates 10% of estimated sequence divergence). The Genbank accession numbers for the reference strains were included and generated as part of this study.
(TIF)

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4 Allgemeine Diskussion und Ausblick

Die dekompensierte Leberzirrhose und das ACLF sind Erkrankungen, die seit mehr als 20 Jahren Gegenstand intensiver Forschung sind (14). Bisherige Ergebnisse legen nahe, dass eine systemische Inflammationsreaktion und bakterielle Infektionen sowohl in der Phase der Dekompensation als auch beim Fortschreiten hin zu Organversagen und ACLF eine übergeordnete pathophysiologische Rolle spielen (12).

Mit den hier gezeigten Arbeiten konnten wir Patientengruppen mit hohem Risiko für fatale Verläufe identifizieren. Wir konnten mechanistische Erklärungsansätze in peritonealen Neutrophilen für die hohe Rate an infektiösen Komplikationen im Peritoneum (SBP) liefern. Und schlussendlich war es uns möglich, neue diagnostische Methoden zur Detektion, Klassifikation und Quantifizierung von bakteriellen und fungalen Erregern zu liefern, um somit eine Grundlage zur Etablierung eines adäquaten Managements von Patienten mit Leberzirrhose im Endstadium zu ermöglichen.

Zukünftige Projekte sollten klären, über welche Faktoren die Funktion von phagozytierenden Zellen verbessert werden kann, ob der molekulare Nachweis von Erregern eine Indikation zur antimikrobiellen Therapie darstellt und ob die frühzeitige Identifikation von Erregern eine Anpassung der antimikrobiellen Therapie möglich macht. Die gezielte und erregergerechte Therapie wird in Zukunft auch aufgrund der zunehmenden Antibiotikaresistenzen gerade in Patientengruppen mit hohen Antibiotikagebrauch, wie bspw. Leberzirrhose (33), als auch durch die unzureichende Neuentwicklung von antibiotischen Substanzen deutlich an Stellenwert gewinnen. Eine große retrospektive, europäische Studie an 455 Patienten mit Leberzirrhose im Endstadium konnte zeigen, dass die allgemeine Rate an Infektionen mit 40% sehr hoch ist und diese in einem Drittel durch multiresistente Bakterien und/oder Pilze hervorgerufen wurden. Diese waren mit einem erhöhtem Sterberisiko assoziiert und traten bei nosokomialen Infektionen dreimal häufiger auf (odds ratio 2,74) (33). Eine weitere Studie konnte zeigen, dass ungefähr die Hälfte aller nosokomialen Infektionen nach der Behandlung einer anderen Infektion auftraten (69).

Gerade für den gezielten Einsatz einer kalkulierten oder prä-emptiven Antibiotikatherapie wird es notwendig sein, bei Patienten mit dekompensierter Leberzirrhose/ACLF zwischen einer sterilen und Infekt-bedingten systemischen Inflammation zu unterscheiden. Klinisch ist dies eine große Herausforderung, da die Definitionen sowohl für Sepsis als auch für ACLF auf dem SOFA score basieren und damit eine Differenzierung zwischen beiden Erscheinungsformen kaum möglich ist. In einer retrospektiven Studie bestehend aus 259 Patienten mit Zirrhose und bakteriellen Infektionen zeigten der konventionelle Sepsis-3 und

quick SOFA Score eine gute Vorhersagegenauigkeit für die Krankenhaussterblichkeit (AUROC 0.784 und 0.732) (37). Allerdings erlaubt auch diese Arbeit keine Rückschlüsse darauf, ob diese Scores in der Lage sind, infizierte Patienten von nicht infizierten Patienten zu unterscheiden. Im Rahmen des europäischen internationalen Leberkongresses (ILC 2021) wurde zwei Arbeiten vorgestellt, die zumindest das Potential haben, die Prädiktion von Infektionen und Unterscheidung zwischen steriler und Infekt-getriggerter Inflammation bei Leberzirrhose zu verbessern. Eine Arbeitsgruppe aus Großbritannien präsentierte eine Subanalyse einer großen interventionellen Studie, welche den Effekt von Humanalbumin bei Patienten mit fortgeschrittener Leberzirrhose untersuchte. In dieser Analyse wurden serologische Biomarker hinsichtlich ihre prädiktiven Genauigkeit zur Vorhersage von nosokomialen Infektionen evaluiert. Während Procalcitonin, Plasma Calprotektin, Lipopolysaccharide Binding Protein, CRP, CD163 und CCL8 keine Zugewinn erbrachten, waren sowohl die Leukozytenzahl also auch die lösliche Form von CD14 bei Patienten mit späteren Infektionen signifikant erhöht (70). Eine weitere Gruppe aus Indien zeigte Ergebnisse einer prospektiven Studie welche sowohl Plasmamarker als auch zirkulierende Immunzellen untersuchte. Hier zeigte sich, dass IL-1RA, IL-18 und TREM1 sowie TIM3+ Monozyten in der Lage waren zwischen einer Sepsis und ACLF assoziierten SIRS zu unterscheiden (71).

Es ist bisher noch vollkommen unklar, ob Mikropartikel bzw. Mikrovesikel hilfreich sein werden, diese diffizile aber grundlegende Frage zu beantworten. Die in unserer Arbeit gezeigten Ergebnisse legen nahe, dass Mikropartikel generell das Ausmaß der Immunaktivierung anzeigen (53). Verschiedene physiologische und pathophysiologische Prozesse, welche im Rahmen von Lebererkrankungen entstehen können Ausgangspunkt von Mikropartikeln sein. Hierzu gehören die Apoptose von Hepatozyten, Aktivierung nicht nur von Immunzellen sondern auch Stellatzellen und Dysfunktion, endothelialer Stress, Firbosierungsprozesse und vieles mehr (72). Es ist daher notwendig, Mikropartikel genau zu charakterisieren, um mehr Informationen hinsichtlich Ausgangspunkt und Krankheitsprozess liefern zu können. Dafür ist es hilfreich, dass sequestrierte Mikropartikel sowohl phenotypische als auch funktionale Information der Herkunftszellen enthalten (72). Dementsprechend konnten wir zeigen, dass der Nachweis von Mirkopartikeln von neutrophilen Granulozyten und Monozyten ein prognostisch ungünstiger Faktor ist (53). Andere Studien berichteten, dass zirkulierende Mikropartikel von Hepatozyten (CK18) (73) oder Endothelzellen (CD31+/CD41-) (74) mit einer deutlich schlechteren Prognose verbunden sind. Mikropartikel enthalten jedoch auch molekulare Information, bspw. miRNA, die wahrscheinlich an der interzellulären Kommunikation beteiligt sind (72). Es bleibt daher abzuwarten, ob hierüber eine Modulation von Krankheitsprozessen möglich sein wird.

Unsere Ergebnisse zur bactDNA und fungalen DNA geben Hinweise darauf, dass molekulare PCR basierte Methoden, Patienten mit Infektion verlässlich identifizieren und daher in Zukunft zur Differenzierung zwischen steriler und infektiöser Immunreaktion herangezogen werden könnten. Der Vorteil dieser PCR basierten Methoden gegenüber den oben genannten „inflammatorischen" Markern ist der direkte kulturunabhängige Erregernachweis, über den eine umgehende Anpassung der antibiotischen Therapie möglich ist. Auch wenn Antibiotikaresistenzen nicht erfasst werden, wäre es ein großer Schritt in Richtung gezielter antimikrobieller Therapie als Grundlage einer Verbesserung des Managements von Patienten mit fortgeschrittener Leberzirrhose. Es wird notwendig sein, die Relevanz dieser diagnostischen Ergebnisse anhand prospektiver interventioneller Studien zur evaluieren und einen Effekt auf die Prognose der Patienten bei entsprechender Therapie nachzuweisen.

Trotzdem wäre es entscheidenden, bereits die Entstehung von Infektionen, bspw. durch Modulation der Immunzellfunktion und Verbesserung der antimikrobiellen Funktion zu verhinden. Eine Studiengruppe aus Spanien untersuchte den Effekt von Humanalbumin auf die Funktion von Leukozyten bei Patienten mit akut dekompensierter Leberzirrhose. Die Albumingabe sowohl in vivo als auch ex vivo führte zu einer geringeren Expression von pro-inflammatorischen Zytokinen IL-6, TNFa und IL-1b in PMNs (polymorphnuclear leukocytes) und PBMCs (peripheral blood mononulcear cells) sowie Reduktion der zirkulierenden Zytokinlevel (75). Die Inkubation von isolierten Leukozyten *ex vivo* mit CpG-DNA, ein Endotoxin bzw. bakterielle Komponenten, und anschließend Humanalbumin zeigte, dass der massive immunstimulierende Effekt von CpG-DNA mit Aktivierung von Genen für DNA Sensing, Neutrophilen Rekruitment, Aktivierung von myeloiden Zellen sowie Zytokinproduktion signifikant durch Albumin reduziert werden konnte. Weitere Experiment konnten zusätzlich zeigen, dass Albumin in die Zellen aufgenommen wird und im Bereich von frühen Endosomen seine Funktion insbesondere durch Reduktion der Signaltransduktion von Toll-like Rezeptor 9 (TLR9) und andere endosomale TLRs entfaltet (75). Zudem untersuchten die Autoren den Effekt von Humanalbumin auf die Funktion von Leukozyten, insbesondere Phagozytose und Efferocytose, welche essentielle Mechanismen zur Clearance von Pathogenen darstellen. Hierbei viel auf, dass die Gabe von Humanalbumin die potentielle Clearance von Erregern verbessern kann. Allerdings blieb unklar, ob die Verbesserung der Immunfunktion zu einer tatsächlichen Reduktion Infektionen beitragen kann (75)

Dieser Ergebnisse liefern einen potentiellen Erklärungsansatz, warum peritoneale Neutrophile eine signifikant reduzierte Phagozytose und Oxidative Burst Rate haben. Albumin scheint für Aufrechterhaltung der antibakterielle Clearance Funktion von Leukozyten essentiell zu sein (49).

In der klinischen Praxis wird Albumin hauptsächlich aufgrund seiner kolloidosmotischen Eigenschaften eingesetzt. Zu den Indikationen zählen die Plasmaexpansion bei hepatorenalem Syndrom, nach großvolumiger Parazentese zur Verhinderung eine kardiozirkulatorischen Dysfunktion sowie nach Spontan bakterieller Peritonitis (SBP) zur Reduktion des Risikos eines Nierenfunktionsstörung (50). Inwieweit eine Stabilisierung der Immunfunktion mit Reduktion sekundärer Infektionen oder auch eine Minimierung der systemischen Inflammationsreaktion eine tragende Rolle spielen, ist bisher nicht vollständig geklärt. In der kürzlich publizierten ANSWER Studie wurde 431 Patienten randomisiert entweder mit Standardtherapie oder Langzeit Albuminsubstitution (40g 2 x wöchentlich für 2 Wochen und danach 40g wöchentlich) für 18 Monate behandelt. Im Albuminarm konnte die 18 Monate Überlebenswahrscheinlichkeit von 66% auf 77% gesteigert werden. Zudem konnte Albumin die Rate an Zirrhose-assoziierten Komplikationen wie SBP, Aszites und Hepatorenales Syndrom senken (76). Zwei Pilotstudien konnten hiernach zeigen, dass die Verbesserung der kardiozirkulatorischen Dysfunktion auch mit einer Reduktion der systemischen Inflammation verbunden war (77). Allerdings ist die ungezielte Substitution von Humanalbumin in Patienten mit Leberzirrhose nicht unkritisch und hat möglicherweise einige Limitationen. Die gerade im New England Journal of Medicine publizierte randomisierte ATTIRE Studie zeigte, dass die Substitution von Albumin bei dekompensierter Leberzirrhose mit dem Ziel die Blutalbuminkonzentration auf >30g/L anzuheben nicht das Überleben verbessert und zu vermehrten „Severe Adverse Events“ insbesondere Lungenöde führt (78). Es gilt daher, in Zukunft die Zielgruppen, welche von einer Substitution mit Humanalbumin profitieren, besser zu charakterisieren.

Alternative Versuche, durch Immunmodulation eine Verbesserung der antimikrobiellen Funktion von Immunzellreihen zu induzieren, erbrachten ebenfalls vielversprechende Ergebnisse. So konnte gezeigt werden, dass eine myeloider immunsuppressiver Subtyp (CD14+CD15-CD11b+HLA-DR-) die Funktion von T-Zellen signifikant supprimiert. Die Behandlung mit einer TLR-3 Agonisten konnte die Immunzellfunktion wiederherstellen und somit potentielle die Entstehung von sekundären Infektionen verhindern (79)

Weitere neue Therapien für die Modulation der sterilen Inflammationsreaktion bei dekompensierter Leberzirrhose werden bereits in klinischen Studien getestet. Die Aktivierung des Immunsystems erfolgt in großem Maße durch die Endotoxin – Toll-like Rezeptor 4 (TLR4) Achse. Die Ursache dafür liegt einerseits in einer vermehrten Translokation von

Endotoxinen aus dem Intestinum in die systemische Zirkulation und andererseits in einer Zirrhose bedingten TLR4 Überexpression in der Leber (80). Vorklinische Studien an Ratten (Gallengangsligatur + Endotoxin) und Maus (Tetrachlorkohlenstoff + Endotoxin) Modellen für ACLF haben gezeigt, dass sowohl die Inaktivierung von Endotoxinen durch die rekombinante alkalische Phosphatase (80) als auch die Inhibition der TLR4 Signaltransduktion über TAK-242 (81) zu einer deutlichen Reduktion der Inflammation und Organschaden führen kann. Die erste klinische Phase 2 Studie zur Testung von TAK-242 bei ACLF ist bereits in Vorbereitung. Zudem ist eine weitere EU geförderte Studie in Vorbereitung, bei der TAK-242 als Kombinationspartner für den Granulocyte-colony stimulating factor (G-CSF) verwendet werden soll. Die erste multizentrische Studie konnte zeigen, dass im Gegensatz zu vorherigen Ergebnissen G-CSF allein bei ACLF keinen positiven Effekt hat (82). In präklinischen Studien mit ACLF Mausmodellen induzierte G-CSF sogar eine überschießende Immunantwort und führte zu einer erhöhten Letalität (82). Die Kombination aus G-CSF und TAK-242 führte zu einer signifikanten Verbesserung des Überlebens, der systemische Inflammation und des Organschadens und induzierte darüber hinaus eine verstärkte Regenerationsantwort (83). Diese Ergebnisse passen zu den Resultaten einer amerikanischen Studie, bei der die Rolle des IL22-Signaltranduktionsweges beim ACLF evaluiert wurde. Unter anderem untersuchte die Gruppe auch, welchen Effekt die Erkrankung an sich auf die Regeneration der Leber hat. Die chronische Schädigung der Leber bzw. die Leberzirrhose zog eine leichte Reduktion der Regenerationsfähigkeit der Hepatozyten mit sich. Bakterielle Infektionen mit *Klebsiella pneumoniae* führten allerdings dazu, dass Hepatozyten ihre Teilungsfähigkeit vollständig verloren. Diese Ergebnisse unterstreichen noch einmal die Relevanz bakterieller Infektionen bei Leberzirrhose und verdeutlich, dass bereits das Auftreten infektiöser Komplikationen vermieden werden muss. Jedoch wird es gleichermaßen essentiell sein, durch das richtige Maß an therapeutischen Interventionen, Behandlungs-assoziierte Komplikationen zu vermeiden.

Ergebnisse anderer Studien, in welchen vielversprechende Ergebnisse aus vorklinischen Untersuchungen nicht im Menschen bestätigt werden konnten (84), zeigen allerdings auch, dass die Erkrankung in Ihrer klinischen Erscheinung und hinsichtlich der zugrundeliegenden Pathomechanismen sehr heterogen und komplex ist (12). Ein fundiertes Wissen der zugrundeliegenden Pathogenese ist daher entscheidend, um einerseits neue therapeutische Targets zu entdecken und anderseits Subgruppen zu identifizieren, die von bestimmten Behandlungen profitieren können (12).
Es ist naheliegend, dass es aufgrund der Komplexität und Heterogenität der Erkrankung in Zukunft notwendig sein wird, Kombinationstherapien bestehend aus unterschiedlichen Agenzien zu verwenden. Ein erster Schritt in die Richtung ist das A-TANGO Projekt, welches

mit Unterstützung der EU (Horizon 2020) die Kombination aus dem immunmodulatorisch wirkenden G-CSF und TAK-242 bei Patienten mit ACLF testen wird. Die Charité Universitätsmedizin Berlin wird sich als führendes Deutsches Zentrum an dieser internationalen Studie beteiligen.

Die oben beschriebene Komplexität und Heterogenität wird es zudem notwendig machen, die Erkrankung besser zu definieren und phänotypische Variationen mit pathomechanistischen Veränderungen zur verbinden. Patienten können verschiedene Organversagen in unterschiedlicher Kombination entwickeln (17). Die Krankheitsdynamik variiert zwischen rascher Resolution innerhalb weniger Tage bis zum rapid progressiven Verlauf mit Multiorganversagen und Tod in kurzer Zeit (43). Unsere bisherigen Mittel sind bisher nicht ausreichend, um uns den Verlauf der Erkrankung verlässlich vorherzusagen. Einerseits können die Resultate unserer oben beschriebenen Studien dabei helfen, die Mechanismen der dekompensierten Leberzirrhose und des ACLF besser zu verstehen. Auf der anderen Seite sind die Kriterien zur Definition des ACLF unverändert unscharf. Die hepatische Enzephalopathie bzw. das zerebrale Versagen werden über die West-Haven Kriterien definiert. Diese basieren auf einer rein subjektiven Einschätzung der klinischen Symptomatik. Während höhere Stadien der Enzephalopathie zweifelsfrei zugeordnet werden können, ist dies bei der leichten Enzephalopathie schwierig und kann zu falsch positiven und falsch negativen Ergebnissen führen. Die Beurteilung der zirkulatorischen Funktion über die Einmalmessung des mittleren arteriellen Blutdrucks ist fehleranfällig, da Maßnahmen wie Infusionstherapie, Sedierung oder Terlipressintherapie rasch zu einer artifiziellen Änderung des Wertes führen können. Da in Zukunft weitere klinische Studien zur Etablierung neuer Therapieformen kommen werden, ist es notwendig, die Definition so zu modifizieren, dass Sie eine klare Selektion von Patienten ermöglicht und zudem möglichen Zulassungsprozessen nationaler und internationaler Behörden standhält.

Aktuell laufende EU geförderte Studien wie DECISION und MICROPREDICT werden dazu führen, dass die Pathogenese der Erkrankung besser verstanden wird, neue therapeutische Targets gefunden werden und somit in den nächsten Jahren weitere klinische Studien in der Patientengruppe mit fortgeschrittener Leberzirrhose vorbereitet werden.

Das Ziel des DECISION Projektes ist es die Pathophysiologie der dekompensierten Leberzirrhose und insbesondere die Ursachen der Transition zum ACLF zu verstehen. Neue Kombinationstherapien vom Tiermodell in humane Studien zu bringen. Tiermodelle für die dekompensierte Leberzirrhose und ACLF neu zu entwickeln bzw. zu verbessern sowie

Marker für Krankheitsprozesse zu evaluieren [https://decision-for-liver.eu/for-scientists/objectives/ (Stand am: 28.09.2021)].
Im MICROPREDICT Projekt werde Crosstalk Mechanismen zwischen dem humanen Mikrobiom und anderen Organ im Rahmen der Leberzirrhose untersucht [https://microb-predict.eu/for-scientists/objectives/ (Stand am: 28.09.2021)]. Der Zusammenhang zwischen einer intestinalen Dysbiose und dem Verlauf von Lebererkrankungen sowie Auftreten von Zirrhose-assoziierte Komplikationen wie Varizenblutungen oder SBP ist schon lange bekannt (85). Dieses Projekt soll nun Möglichkeiten zur Intervention und Modulation des Mikrobioms untersuchen.

Auch im oben beschriebenen A-TANGO Projekt werden zahlreiche Substudien die Möglichkeit bieten, die Pathogenese der fortgeschrittenen Leberzirrhose substantiell zu untersuchen und neue Angriffspunkte für Therapien zu entdecken [https://www.atango.eu/#objectives (Stand am: 28.09.2021)].

Diese neue Bestrebungen zeigen deutlich, dass wir uns bei der fortgeschrittenen Leberzirrhose mittlerweile mehr und mehr von der rein symptomatischen Therapie mit Kontrolle der Komplikationen und Elemination von auslösenden Faktoren einer Dekompensation hin zu Behandlungsoptionen, welche den Krankheitsverlauf modifizieren („disease modifying agents“), bewegen (86).

Das Ziel aller wissenschaftlicher Bestrebungen sollte es dabei sein, durch diagnostische und therapeutische Maßnahmen die Prognose und Lebensqualität von Patienten mit fortgeschrittenen Lebererkrankungen zu verbessern.

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Erklärung

§ 4 Abs. 3 (k) der HabOMed der Charité

Hiermit erkläre ich, dass

- weder früher noch gleichzeitig ein Habilitationsverfahren durchgeführt oder angemeldet wurde,
- die vorgelegte Habilitationsschrift ohne fremde Hilfe verfasst, die beschriebenen Ergebnisse selbst gewonnen sowie die verwendeten Hilfsmittel, die Zusammenarbeit mit anderen Wissenschaftlern/Wissenschaftlerinnen und mit technischen Hilfskräften sowie die verwendete Literatur vollständig in der Habilitationsschrift angegeben wurden,
- mir die geltende Habilitationsordnung bekannt ist.

Ich erkläre ferner, dass mir die Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis bekannt ist und ich mich zur Einhaltung dieser Satzung verpflichte.

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Datum Unterschrift