Diploma Thesis

INNATE IMMUNE DYSFUNCTION IN SEPSIS AND ACUTE-ON-CHRONIC LIVER FAILURE

eingereicht von

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Affidavit

Herewith I, Michael Rybczynski, declare that I have written the present diploma thesis fully on my own and without any assistance from third parties. Furthermore, I confirm that no sources have been used in the preparation of the thesis other than those indicated in the thesis itself.

Graz, June 16 Signature

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Abstract

INNATE IMMUNE DYSFUNCTION IN SEPSIS AND ACUTE-ON-CHRONIC LIVER FAILURE

Introduction: The phenomenon of immune paralysis of innate immune cells is well described in sepsis and poses a further challenge to the treatment of this condition. Recently also in acute-on-chronic liver failure (ACLF), sepsis-like immune paralysis of neutrophils and monocytes has been described. Sepsis is a major cause of decompensation of liver cirrhosis and mortality of a patient with sepsis and ACLF is extraordinary high. Therefore we aimed to compare neutrophil function of patients with sepsis and patients with ACLF to identify differences in the immune dysfunction of these two conditions.

Methods: 8 patients with septic shock and 6 patients with ACLF were studied. Neutrophil function was assessed by FACS analysis using the Phagoburst and Phagotest kit. (Orpegen, Heidelberg). Neutrophil function, clinical and biochemical data were compared.

Results: **Patients with ACLF had significantly lower haemoglobin ($p<0.01$), C-reactive protein ($p<0.05$) and sodium ($p<0.05$) levels, but significantly higher bilirubin ($p<0.01$) and albumin ($p<0.05$) levels compared to sepsis. Patients with ACLF had a significantly higher neutrophil resting burst ($p<0.01$) compared to healthy controls and a significantly lower phagocytic capacity compared to patients with sepsis and healthy controls ($p<0.05$ and $p<0.001$ respectively)). Patients with sepsis had lower phagocytic capacity than controls ($p<0.05$) but resting burst was unchanged.**

Conclusion: Neutrophil dysfunction is more pronounced in ACLF with higher resting oxidative burst and lower phagocytic capacity as compared to sepsis, where a low phagocytic capacity without a rise in resting oxidative burst was observed. Since sepsis is very common in ACLF and associated with an extremely high mortality, one can hypothesize that immune paralysis is more pronounced on the background of decompensated liver disease.

Zusammenfassung

NEUTROPHILE GRANULOZYTENFUNKTIONSSTÖRUNG BEI PATIENTEN MIT SEPSIS UND AKUT-AUF-CHRONISCHEM LEBERVERSAGEN

Einleitung: Bei Sepsis wurde eine Immunparalyse beschrieben, die eine große klinische Herausforderung im Management dieser Patienten darstellt. Kürzlich wurde eine ähnliche Immunparalyse der Granulozyten und Monozyten bei Patienten mit akut-auf-chronischem Leberversagen (ACLF) festgestellt. Sepsis ist die Hauptursache für die Dekompensation einer Zirrhose, die Mortalität bei Patienten mit Sepsis und ACLF ist extrem hoch. Wir verglichen die Funktion der neutrophilen Granulozyten von Patienten mit ACLF und Sepsis untereinander um einen Unterschied in der Dysfunktion des angeborenen Immunsystem herauszufinden.

Methodik: In unsere Studie wurden 8 Patienten mit septischem Schock und 6 Patienten mit ACLF aufgenommen. Die Granulozytenfunktion wurde anhand eines Phagoburst und Phagotest kit (Orpegen, Heidelberg) und mit Hilfe der FACS Analyse ausgewertet. Ebenfalls verglichen wir die Neutrophilenfunktion, die klinische Werte, wie auch die Blutwerte untereinander.

Ergebnis: **Patienten mit ACLF hatten, verglichen mit Sepsispatienten, ein signifikant niedrigeres Hämoglobin ($p<0.01$),c-reaktives Protein ($p<0.05$) und Natrium ($p<0.05$), jedoch höhere Bilirubin ($p<0.01$) und Albuminwerte ($p<0.05$).**

Patienten mit ACLF wiesen einen signifikant höheren Resting burst ($p<0.01$) als die Kontrollgruppe auf, die Phagozytoseaktivität war jedoch deutlicher eingeschränkt als bei jenen mit Sepsis oder den gesunden Kontrollen ($p<0.05$ und $p<0.001$).Patienten mit Sepsis hatten eine eingschränktere Phagozytoseaktivität als die Kontrollgruppe ($p<0.05$), der Resting burst war aber unverändert.

Schlussfolgerung: Patienten mit ACLF wiesen eine deutliche Neutrophilendysfunktion mit hohem Resting burst und niedriger Phagozytoseaktivität auf. Sepsispatienten jedoch hatten eine eingeschränkte Phagozytose, der Resting burst war aber unverändert. Sepsis ist sehr häufig der

Auslöser eines ACLF und ist mit einer sehr hohen Mortalität vergesellschaftet. So kann man annehmen, dass die Immunparalyse stärker bei einer dekompensierten Lebererkrankung ausgeprägt ist.

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Abbreviations

ADP- Adenosine di-phosphate

Alb- Albumin

ACLF- Acute- on-chronic- liver failure

ALF- Acute liver failure

ALT- Alanine aminotransferase

AMP- Adenosine monophosphate

AP- Alkaline phosphatase

APASL- Asian pacific Association for the study of the liver

APC- Allophycocyanin

APC- Activated protein C

APTT- Activated partial thromboplastin time

AST- Aspartate aminotransferase

ATP- Adenosine triphosphate

BUN- Blood urea nitrogen

CD- Cluster of Differentiation

Crea- Creatinine

CRP- C- reactive protein

Cys 34 - Cysteine 34

DHR- Dihydrorhodamine

DIC- Disseminated intravascular coagulation

DNA- Deoxyribonucelic acid

ECM- Extracellular matrix

EGF- Epidermal growth factor

FACS- Fluorescence activated cell sorting

FITC- Fluorescein isothiocyanat

Fig- Figure

fMLP- Formyl-Methionyl-Leucyl-Phenylalanine

FSC- Forward scatter channel

GFR- Glomerular filtration rate

GGT- Gamma GT

GM- CSF- Granulocyte- monocyte colony stimulating factor

HAV- Hepatitis A virus

HBV- Hepatitis B virus

Hb-Hemoglobin

HCV- Hepatitis C virus

HE- Hepatic encephalopathy

HEV- Hepatitis E virus

HNA- Human nonmercaptalbumin

HRS- Hepatorenal syndrom

HSC- Hepatic stellate cells

ICU- Intensive care unit

IGF- Insulin like growth factor

IL- Interleukin

INF - Interferon

iNOS- Inducible nitric oxide synthase

IRAK- Interleukin-1 receptor associated kinase

Lys 525- Lysine 525

LBP- Lipopolysaccharide- binding protein

LPS- Lipopolysaccharide

MFI- Mean fluorescence intensity

MOF- Multiple organ failure

Na- Sodium

NADPH- Nicotin amid-adenin-dinukleotid-phosphat

NASH- Nonalcoholic steatohepatitis

NE- Neutrophil elastase

NO- Nitric oxide

PAF- Platelet activating factor

PAMP- Pathogen associated molecular pattern

PBS- Phosphat buffered saline

PE- Phycoerythrin

PKC- Proteinkinase C

PMA- Phorbol 12- myristate 13- acetat

RAAS- Renin Angiotensin Aldosteron System

RES- Reticuloendothelial sytem

RNA- Ribonucleic acid

ROS- Reactive oxygen species

SBP- Spontaneous bacterial peritonitis

1. Introduction

" Septicemia is a state of microbial invasion from a portal of entry into blood stream which causes sign of illness"
Schottmüller 1914

1.1 Epidemiology

Sepsis is one of the leading causes of death in intensive care units in industrialized countries. Due to newer diagnostic methods it is possible for the clinical team diagnose sepsis and confirm infection early. Although a lot of progress has been made in the medical treatment, the incidence of sepsis and mortality increased despite improved treatment during the last decades.(1) Economically, it is a healthcare burden (50.000$/patient).The mortality rates range from 20% to 50%. Male, elder people, patients with immunodeficiency and chronic organ disease are more likely to develop this clinical pattern. Sepsis is more common among men than women, and there are differences between races (more frequent in non- white than in white patients).(2) Some studies described, that even a genetic polymorphism, most frequent the single nucleotide polymorphism (SNP), plays a role in sepsis- induced organ failure and might be important for the bad outcome in some patients.(3)

1.2 Pathogen associated molecular pattern

Bacteria can be divided into a gram- negative and a gram-positive species. Each type of the microbe has different membrane molecules that act as immunostimulants in every mammal's body. These specific structures, called PAMP (pathogen- associated molecular pattern), show a high variety in their molecular construction and are used by the innate immune system as a identifying feature. The most potent PAMP is the lipopolysaccharide (LPS) found in all negative bacteria, where it plays the main role as a stabilizer of their outer membrane.

This lipoglycan consists of three main domains: a lipid, a polysaccharide(divided into an inner and outer core) and the O-Antigen. The Lipid A, a hydrophobic part of the molecule, shows a high endotoxic activity. The O-Antigen, a carbohydrate, is the outermost part of the LPS and thereby the main target of the innate immune and the complement system. (4,5)

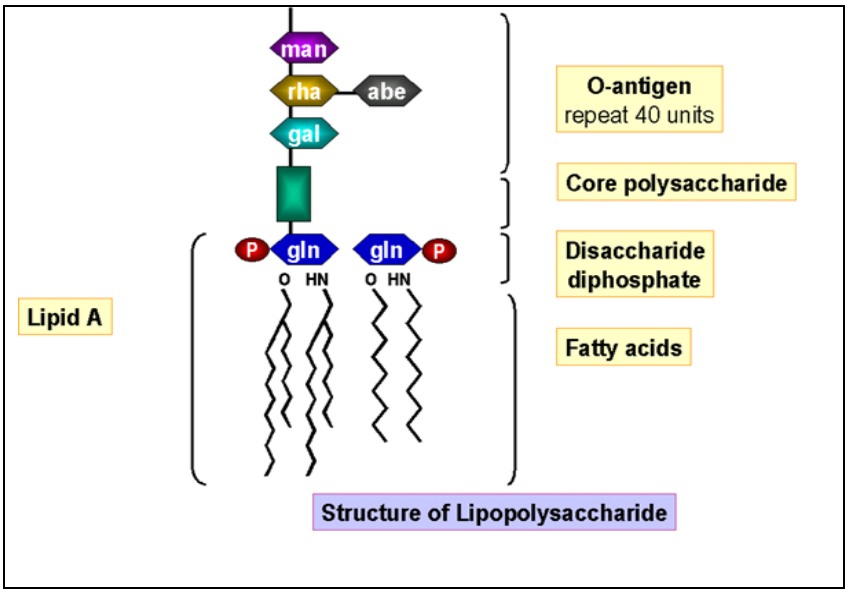

Fig.1:Adapted from Research in the Darveau Lab ,Dept. of Pathology and Microbiology, University of South Carolina.Murray et al. Microbiology. 6th Ed, Chapter 2 and 3
"Structure of the Lipopolysaccharide"

Furthermore, also the chain length of the carbohydrate part of this lipoglycan is a very important factor for activation of the immunity. A long (smooth-type) and a short (rough-type) O chain can be distinguished.(6)

Other relevant molecular pathogens associated with sepsis are the lipoteichonic acid (ampiphile glycolipid, giving the necessary rigidity to gram-positive bacteria) ,peptidoglycan, flagellin and bacterial DNA.

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1.3 Receptors for LPS

1.3.1 Toll like receptors

The activation of the host defence occurs after the interaction between the PAMPs and the toll like receptors (TLRs).(7) TLRs are transmembrane proteins expressed on the cell surfaces of immune cells. The receptors recognize pathogen associated molecular patterns (PAMPs), transduce the signal by recruiting special molecules and initiate the activation of the host defence. (8,9)

This receptors are able to detect bacterial lipoproteins, lipopolysaccharides, lipoteichonic acid, fungal components ,viral RNA and bacterial and viral DNA.

There are 13 TLRs known, but two of them play the major role in sepsis: TLR 4 detecting LPS and TLR 2 sensing lipoteichonic acid, mycoplasma and peptidoglycan.(10)

"Pathogen-associated molecular patterns (PAMPs) and Toll-like receptors"

Bacteria		**Receptors**
Lipopolysaccharide	Gram-negative bacteria	TLR 4
Lipoteichonic acid	Gram- positive bacteria	TLR 2
Peptidoglycan	most bacterias	TLR 2
Flagellin	Flagellated bacteria	TLR 5
Fungus		
Zymosan	Saccharomyces	TLR 2/TLR 6
Phospholipomannan	Candida albicans	TLR 2
Mannan	Candida albicans	TLR 4
O-linked mannosyl residues	Candida albicans	TLR 4
β- glucans	Candida albicans	TLR 2

Tab. 1: Source adapted from: Yearbook of intensive care and emergency medicine (2009)
Hommes T.J, Wiersinga W.J, van der Poll T, The Host Response to Sepsis, 39-50(2009)
"Pathogen-associated molecular patterns (PAMPs) and toll-like receptors (TLRs)"

After binding of the lipopolysaccharide, specifically the lipid-A, to the extracellular LPS- binding protein (LBP-a acute phase protein , 58 kD and synthesized in the liver), the LPS- LBP- complex is transferred to CD14, a cell surface molecule expressed on monocytes, macrophages and neutrophils. CD14 catalysis the insertion of LPS into a receptor structure called TLR-4/ MD-2 complex. (11) MD-2 is a small protein and indispensable for LPS response. One theory says that MD-2 with its affinity to LPS, serves as a holder of the LPS while TLR-4 is needed to mediate the cytokine production. Another one sees the MD-2 as the main inducer of the proinflammatory cytokines. (5)

After the stimulation of TLR, the differentiation factor 88 (MyD88), TIRAP (Toll/interleukin-1-receptor domain-containing adaptor protein), TOLLIP (Toll interacting protein) and IRAKs (IL-1 receptor-associtated kinases) forms a complex and the MAP kinase cascade and the nuclear factor (NF)-kB pathway gets activated.(65) Fig.2.
This finally leads to an expression of inflammatory cytokine genes and the creation of TNF -alpha, IL-6, IL-1ß. (7)

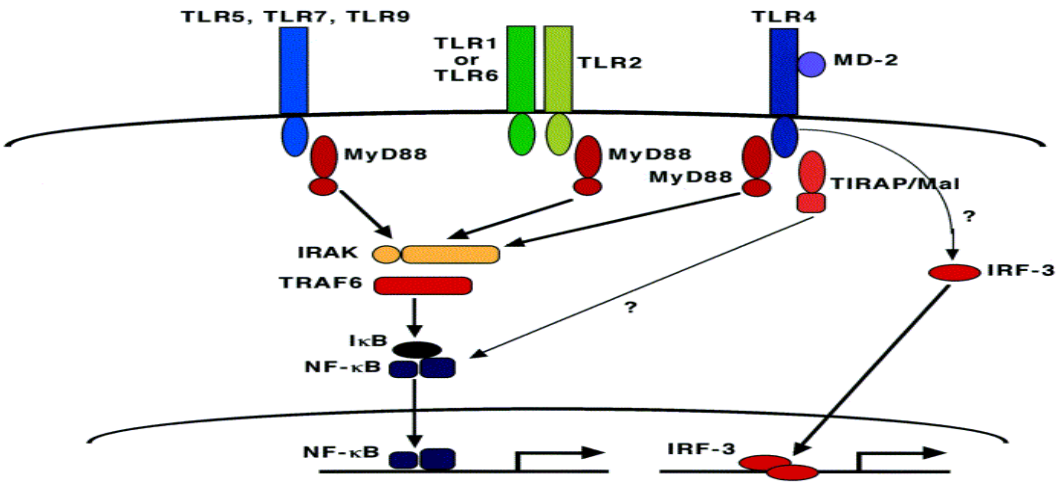


Fig.2: : Akira S. Recognition of pathogen-associated molecular patterns by TLR family. Immunology Letters,Volume 85, Issue 2, 22 January 2003, Pages 85-95

*„**Schematic of the TLR pathway***

1.4 Systemic Inflammatory Response Syndrome (SIRS)

Shortly after the invasion of a microbe in the mammal organisms' bloodstream, the host innate immune system responses as the first line of defence by producing cytokines (TNF-α, IL-6 and IL1ß) in order to express a systemic inflammatory reaction. Cells like endothelial cells, platelets, and mast cells produce inflammation promoters in a large quantity which cause a general reaction of the organism, called the systemic inflammatory response syndrome (SIRS). The cardinal signs of SIRS are fever or hypothermia, tachypnea, tachycardia and leukocytosis or leukopenia. Besides the infection and inflammation, SIRS can be also mediated by ischemia, trauma or a combination of different insults. The reaction of the cellular response can be seen in the patients condition mentioned in the SIRS definition.

The SIRS Definition introduced by American College of Chest Physicians (ACCP) and the Society of Critical Care Medicine (SCCM)

- Fever of more than 38°C or less than 36°C
- Heart rate of more than 90 beats per minute
- Respiratory rate of more than 20 breaths per minute or a $PaCO_2$ level of less than 32 mm Hg
- Abnormal white blood cell count (>12,000/µL or <4,000/µL or >10% bands)

Tab. 2: Source adapted from "American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference. " ***Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis"*** *Crit. Care Med.20 (6): 864–74. 1992*

1.5 Sepsis

In many cases, sepsis is caused by bacteria, mainly by gram-negative or gram-positve bacteria, sometimes by fungi or other microorganisms. Blood cultures prove just in 20% -40 % an infection in cases of severe sepsis and 40-70% in cases of the septic shock.

- **25%** of patients suffer from lower respiratory tract infection caused by *Streptococcus pneumoniae, Klebsiella pneumoniae*, *Staphylococcus aureus* and *Escherichia coli*
- **25%** suffer from urinary tract infections caused by *Escherichia. co*li, *Proteus* species and *Klebsiella* species
- **15%** suffer from soft tissue infections caused by *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus* species
- **15%** suffer from GI infections caused by *Escherichia coli* and *Streptococcus faeciali*

Tab. 3 : Source adapted from “Septic shock”, Sat Sharma Department of Internal Medicine, Divisions of Pulmonary and Critical Care Medicine, University of Manitoba.St Boniface General Hospital.

“**Causes of sepsis”**

There are two reasons for the cause of the septic condition. Firstly, a bacterium has to enter the bloodstream and induce a general host response. Secondly, and that’s the main onset, the organism reacts in this case with an unbalanced immune defence and an excessive inflammation.

This overreaction leads to an excessive production of mediators and furthermore to hypotension and hypovolemia induced by endothelium dysfunction, an uncontrolled activation of the coagulation and anticoagulation, microvascular thrombosis and an altered immune (apoptosis of immune cells) and organ function (multi organ failure). If the patients develop multi organ failure, mortality rates are very high.

International Sepsis Definitions Conference 2001

Definition of sepsis and severe sepsis

Sepsis Definition

- A documented or suspected infection
- Temp > 38°C or <36°C
- Heart rate > 90 beats/min
- Respiratory rate > 20 breaths/ min or <4,3 kPa
- WBC >12.000 cell/ mm^3 or > 10 % immature forms
- Pathologic perfusion parameters

Severe sepsis

- Hypoperfusion
- Hypotension < 90mmHg
- Multi organ dysfunction

Tab. 4 : **International Sepsis Definitions Conference 2001**(SCCM/ESICM/ACCP/ATS/SIS)
"Definition of sepsis and severe sepsis"

1.5.1 Coagulation

After tissue injury, an extravasation of blood into the soft tissue triggers the coagulation by exposing the blood to the tissue factor. This integral membrane protein is a receptor for F VII which is expressed on monocytes, endothelial and nonvascular cells.

The coagulation cascade consists of two pathways: an intrinsinc and an extrinsic

1.5.1.1 The intrinsic pathway includes

- Factor XII, XI, IX, VIII, X, V, prothrombin and fibrinogen

1.5.1.2 The extrinsic pathway gets initiated by

- the Tissue factor, also called factor III that binds to factor VII and Calcium
- The activated Tissue factor and F VII complex triggers the mobilization of

the factors IX and X, which convert to factor IXa and Xa

- Factor Xa and Va modifies Prothrombin to Thrombin (IIa).
- Thrombin binds to Thrombomodulin. This complex activates the protein C (APC-activated protein C).Protein C is a plasma glycoprotein and the defence mechanism against thrombosis.
- APC binds to protein S on the cell surface and thereby inactivates the factor Va
- Thrombin increases its own generation by activating factor V ,VIII and XI and it connects with Fibrin and forms the fibrin clotting factor XIII

Normally, the natural coagulation and anti-coagulation system resides in balance, and thrombosis can be prevented. Though, during an inflammation, receptors on the endothelium recognize bacterial cell wall and LPS, cytokines and chemokines and products of the complement system and respond with an increased swelling, an intensified attachment of platelets and an augmented leukocyte trafficking.TNF-alpha and IL-1ß are the main activators of monocytes and endothelial cells and lead to an expression of tissue factor (TF), platelet activating factor (PAF), nitric oxide and procoagulant molecules. The result is an increased coagulation. Furthermore, these modulators trigger neutrophils to release radicals and neutrophil elastase (NE) which damages the endothelial tissue.(12)

The three major antithrombotic systems TFPI (tissue factor pathway initiator), APC and antithrombin are down regulated in sepsis. The APC down regulation, due to failed thrombin generation, aggravates the cytokine distribution and the vascular injury.(13) This is the beginning of an often seen picture: the disseminated intravascular coagulation (DIC). It is a result of stimulated coagulation, a weak fibrinolytic system and the accompanied thrombocytopenia. (14,15)
The balance between the coagulation and anticoagulation system is disturbed and the forming of clots leads to consumption of factors and platelets. The subsequent vascular injury induces bleeding, clotting of the microvasculature and tissue ischemia. The consequence is the damage of kidney and lungs and often ends with multi organ failure. (15,16)
Another important problem connected with the poor prognosis is the production of nitric oxide (NO) which regulates the relaxation of vessels and acts as an

signalling molecule in neurotransmission and immune response. The inducible nitric oxide synthase (iNOS) generates NO from l-arginine, an α-amino acid. In sepsis, the iNOS and the production of the nitric oxide are often up regulated. This leads to an impaired hemodynamic response with insufficient vasoconstriction and vasodilatation.(17) The vasomotor tone is reduced, the permeability increased. Latter is the cause of leakage of vessels which leads to edema, hypotension and hypoperfusion.

1.6 Liver fibrosis and cirrhosis

1.6.1 Epidemiology

Being the 12th most common cause of death, liver cirrhosis and liver failure is responsible for more than 25,000 deaths in the United States each year, where male are slightly more affected then female.(18) The age of death is usually in the fifth or sixth decade of life.

It is a very malicious disease, because the majority of patients are free from any symptoms for quite a long of time (compensated disease). Normally, the transformation from fibrosis to cirrhosis appears after 15-24 years with symptoms including coagulation problems, hepatic hypertension with variceal bleeding, ascites, hepatorenal syndrome and often hepatic encephalopathy. These clinical complications are in most cases associated with high morbidity and mortality rates. Known risk factors for the cirrhosis are chronic alcohol abuse, HCV infections, cholestasis or nonalcoholic steatohepatitis (NASH).(18)

1.6.2 Pathogenesis

The liver tissue has the unique feature to regenerate as far as the damaging stimulus can be defeated or compensated. One of the compensation occurrences is called fibrosis, a reversible healing ”scaring” process, where the parenchymal cells repair themselves.

Every liver damage causes an inflammation, where chemokines and cytokines like IL-6, IFN-γ, TNF-α, TGF-β1, EGF and IGF are shed by lymphocytes, hepatocytes, biliary or Kupffer cells. (19) This reaction is the beginning of the fibrosing process.

As a result, hepatic stellate cells (HSC) which usually are located in the space of Disse get activated.(20) HSC are the main producers of the extracellular matrix (ECM), a tissue composed of proteins and glycosaminoglycans which accumulates and replaces apoptotic cells. (21, 22)
Other cells initiating the fibrogenic reaction by deposing collagen are myofibroblasts and hepatocytes. (23,24) The latter, responsible for 80% of the liver parenchymal mass, are required to synthesize proteins, carbohydrates and lipids. They also have important secretory and excretory functions.
Their lysis leads to a yielding of the reactive oxygen species (ROS), a defence system containing hydrogen peroxide and superoxide anions. Once ROS starts to act, more immune cells, among others white blood cells, get lured and the inflammation perpetuates. (24)

Once the inflammation stimulus goes beyond a compensation time, the hepatocytes are replaced by fibrillar collagen, a non-resorbable matrix. This kind of tissue leads to a development of irreversible scarring structural abnormal nodules, a defect in the architecture and a dysfunction and insufficiency of the liver. (25) Defined as cirrhosis, numerous and severe complications are the consequence.

1.6.3 Complications

1.6.3.1 Ascites

Ascites is defined as a fluid accumulation in the peritoneal cavity and can be divided into transudate, where the fluid contains less than 2.5 g/dL or the exsudate with a protein concentration greater than 2.5g/dL. Liver cirrhosis accounts for 85% of ascites cases, only 15% show a non-hepatic origin (26,27). According to D'Amico G et al., 50% of patients with the fluid retention pass away in 2 years. (28) Ascites is often initiated by portal hypertension, a pattern usually caused by cirrhosis. The increased resistance to the portal flow and the pressure of the portal vein that is exceeding 10 mm Hg are the main events which lead to an output of nitric oxide, a molecule acting as a vasodilator in the splanchnic arterial and peripheral arterial system. The effect is a general underfilling of the vascular system with a decrease of blood volume and arterial pressure, an increase of cardiac output and the activation of the RAAS system and the sympathetic

nervous system (underfilling theory). These activities and the finally caused sodium and water retention are the main inducers of the accumulation of ascites. (29,30,31) Another reason is an excessive splanchnic lymph production due to the splanchnic vasodilation, followed by its passage into the peritoneal cavity.(32)

1.6.3.2 Spontaneous bacterial peritonitis (SBP)

An impaired gastrointestinal motility with a deranged gut flora, a low function of the innate immune system and the poor filtration of bacterial microorganisms by the cirrhotic liver are responsible for the translocation of the more common gram-negative invaders into the bloodstream and finally into the ascitic fluid.(30,33,34,35) This clinical picture is defined as an accumulation of 250 or more polymorphonuclear leukocytes/ mm^3 in the ascites and it manifests with variable symptoms: temperature above 37.8°C, alterations in the mental status, abdominal pain with an occurring tenderness, paralytic ileus, hypotension and hypothermia. (36)

The SBP is a serious complication appearing in 10 % -30 % in hospitalized patients with ascites. Although there are new promising therapy strategies, mortality rates remain still high, with 20%-40%(37)

1.6.3.3 Hepatorenal Syndrome (HRS)

The already mentioned decrease of blood volume and arterial pressure, the retention of sodium and water and the activation of the blood pressure regulation cascade (RAAS system, sympathetic nervous system) during advanced cirrhosis are the main causes of the underlying diseases.

HRS can be distinguished into a Type 1 which defines the rapid aggravation of the glomerular filtration rate and an increase of creatinine above 2.5mg/dl or a decrease of creatinine clearance below 20ml/minute, and a Type 2 with a slow and moderate deterioration of renal function and a creatinine less than 2.5mg/dl. (38, 39)

Table 1 The main differences between Type1 and Type 2

HRS Type I

- Rapid and severe aggravation of renal function
- 25% of patients with SBP suffer from Type I
- Creatinine > 2.5mg/dl in less than 2 weeks
- SBP+ Type I induces a deterioration of liver function (jaundice, coagulopathy)
- Circulatory system(hypotension , increased renin and norepinephrin levels)

HRS Type II

- Slow and moderate deterioration of renal function
- Creatinine lesser than 2.5 mg/dl
- Liver and circulatory function are impaired
- Refractory ascites with no response to diuretics

Tab. 5: Pathogenesis and treatment of Hepatorenal Syndrome. ArroyoV, Fernandez J, Pere Gines
"The main differences between Type 1 and Type 2"

The mortality rates of the HRS Type I are very high. 50 % of patients who are not treated die within 2 weeks. If the renal function fails, almost all patients fade away in the first 10 weeks.(39)

1.6.3.4 Hepatic encephalopathy

Hepatic encephalopathy is a reversible neurologic symptom often associated with liver dysfunction and the accumulation of toxic substances in the systemic circulation. The initiator is a molecule called ammonia produced in the colon by intestinal bacteria. Usually 90% of it is degraded by the liver in healthy people.
Due to cirrhosis, acute liver failure or a porto-systemic shunt, the organ is not able to detoxify the blood from this compound. This leads, in high concentrations, to absorption of it by astrocytes and further to glia swelling and a pathological accumulation of water, known as a brain edema.(66) These effects are due to the osmotic imbalance caused by glutamine, an amino acid produced as the end product during the metabolization of the neurotoxic ammonia. (40)

Patients demonstrate abnormalities in behavior and consciousness. They often have a delayed reaction time and suffer from movement disorder like asterixis or

flapping tremor. Uremia, pulmonary insufficiency, hyperventilation and barbiturate toxicity are also often diagnosed as a sign of encephalopathy.

According to the West Haven criteria, the HE can be classified into various stages

Stage 0:	Lack of detectable changes in personality or behavior. Asterixis absent.
Stage 1:	Trivial lack of awareness. Shortened attention span. Impaired addition or subtraction. Hypersomnia, insomnia, or inversion of sleep pattern.Euphoria or depression. Asterixis can be detected.
Stage 2:	Lethargy or apathy. Disorientation. Inappropriate behavior. Slurred speech. Obvious asterixis.
Stage 3:	Gross disorientation. Bizarre behavior. Semistupor or stupor. Asterixis generally absent.
Stage 4:	Coma

Tab.6: Andres T. Blei, Juan Cordoba, and the Practice Parameters Committee of the American College of Gastroenterology.Hepatic Encephalopathy. 2001 by Am. Coll. of Gastroenterology. Vol. 96, No. 7.1968-1976

"Stages of hepatic encephalopathy"

1.7 Acute on chronic liver failure (ACLF)

As a relative unrecognized clinical picture a few decades ago, ACLF, according to the study of Bosetti et al, gains nowadays in importance, because his work demonstrates that the organ damage and its consequences will become the 9th most common cause of death in the world. (41)

By definition, it is an acute deterioration of liver function, usually affecting patients with previous compensated cirrhosis which manifests with jaundice, coagulopathy disorders (INR>1.5), ascites, hepatorenal syndrome, variceal bleeding and hepatic encephalopathy at a period within 4 weeks.

ACLF Definitions

- Deterioration of liver function over a period of 2 to 4 weeks
- Coagulopathy INR > 1,5 or a Prothrombin activity < 40%
- Jaundice with a serum bilirubin of 6-20mg/dl
- Hepatic encephalopathy
- Ascites

Tab.7: Source adapted from Shiv Kumar Sarin et al.Acute-on-chronic liver failure: consensus recommendations of the Asian Pacific Association for the study of the liver (APASL).Hepatol Int.
"Definitions of acute-on-chronic-liver failure"

ACLF is in principle, as soon as the initiator is detected and medicated early enough, a reversible condition. The patients overall state and the progress of the underlying disease are important additional factors and at the same time associated with the reversibility.(42) About 60% of cirrhotic patients in the US reach the end-stage liver disease, a stage with high mortalities and only avertable by performing a liver transplantation.(43)
Another study, performed by d'Amico et al. presents a 6 year mortality of 79% in patients with decompensated cirrhosis, showing the severe outcome of the disease. (44)

The deterioration and subsequent conversion from a chronic liver damage into a liver failure is normally induced by an acute insult.
Precipitants causing the acute liver failure vary between the eastern and the western world and are classified into an infectious and a non- infectious etiology. In most cases, alcohol and drugs (mostly acetaminophen) dominates in the West, usually affecting patients with a known or unknown underlying chronic liver damage and causing in most cases an acute liver failure. (45, 46).
Another initiator for a decompensation of a liver disease is the viral hepatitis, causing an ALF, or bleedings, surgery or other infectious agents affecting the liver which lead to ACLF.

In the East (India and Asia), new infections or reactivations of hepatotropic viruses (HAV, HBV, HCV, HEV), parasites and bacteria are among the most common causes of an ACLF.(47,48,49).
Autoimmune liver disease plays a minor role as an etiology, but should be kept clearly in mind.

Oesophageal variceal bleeding is a common complication in end stage liver disease and is associated with mortality rates as high as 25%-50%. (50) It is induced by an increase of the portal pressure (>12mmHg) which is one of the main signs for the decompensation of a stable liver cirrhosis. Controversially discussed for playing a role in ACLF, bleeding is now considered, by the Asian Pacific Association for the study of the liver (APASL), to be an important initiator of ACLF. (51)
Nonhepatic surgery should also be considered as a risk factor for developing ACLF. Del Olmo et al showed in a study that patients with a chronic liver damage undergoing a surgery, face a higher rate of wound related complications. The most common problem was the decompensation of a prior stable liver cirrhosis .Beside this, the risk to die from a surgical intervention was 16,3 % in hepatic injured (Child Pugh B and C) compared with 3,5% in controls.(52)

A much more serious problem is the insufficiency of the immune defence that seems to be a main precipitant of the transition from stable condition to critical illness and that determines the mortality in many cases.
Patients with chronic liver cirrhosis are due to an impairment of the immune system (especially neutrophils), susceptible to infection which often ends in a systemic inflammatory response syndrome or even sepsis.
During this period, increased levels of cytokines, especially TNF α, are released, promoting the injury and apoptosis of hepatocytes. (53) At the same time, these inflammatory modulators inhibit the regeneration activities of the already damaged liver tissue. The result of this inflammation response is the acute on chronic liver failure.

Beside the liver, also the circulatory system is affected. A high portal pressure and increased cardiac output, a low renal perfusion and a general splanchnic

vasodilatation are the effects of either an excessive NO production or a downregulation of vasoconstrictor receptors.(54)

1.8 Neutrophil granulocytes

A neutrophil is a type of microphage that belongs to the innate immune system. Typically 12 µm in diameter and with a lifespan of about 8 hours, it accounts for 60% of all leukocyte cells. A neutrophil plays an essential part of the first line defence due to the fact that it accumulates fast in great quantities, recognises and eliminates many bacterial species before even causing serious harm to the human organism.

Neutrophil granulocytes contain 3 types of granules in their cytoplasm: Primary granula (containing myleperoxidase, lysozym and defensine), secondary granula (with lactoferrin and alkaline phophatase) and tertiary granula (with gelatinase and hydrolases). This digestive enzymes help the microphages, once the microbe is opsonized and engulfed by using the detecting receptors Fc- and C3b, to degrade and eliminate the invader by either producing oxygen radicals or lytic activity.(55)

1.9 Dysfunction of the innate immune system in liver cirrhosis

Over the years, different studies demonstrated a negative influence of the cirrhotic liver on the immune system causing a higher susceptibility for infections and an increased mortality. The incidence of SBP, a serious and common bacterial infection with mortality rates ranging from 17%-50% in patients hospitalized with cirrhosis and ascites lies for example between 7%-23%, infections of the urinary tract appear in 20% and pneumonia, especially community acquired pneumonia, in 15 %.(56,57,58)

It is known that increased TNF-α, IL-1 and IL-6 levels in serum and ascites, a decreased complement system activity and the existence of endotoxin are responsible features for the impaired immunity. (59) Another main reason for this immunodeficiency lies to some extent in the neutrophil granulocytes, a type of the white blood cells which play the key role as the first line defence in the mammals

organism. Normally, neutrophils remain in a resting state. Once they are primed by lipopolysaccharide, the tumor necrosis factor alpha (TNF alpha), platelet-activating factor or granulocyte-monocyte colony stimulating factor (GM-CSF) and NADPH oxidase are upregulated which subsequently lead to a production of superperoxide (O2-). This activated oxygen species and hydrogen peroxide is essential to kill and digest the invader. This response is called oxidative burst.

Whole blood FACS analysis of patients with liver cirrhosis or alcoholic hepatitis showed a picture of impaired neutrophil function which is associated with higher infection and mortality rates.(35) This fact by itself can be explained by the theory that neutrophils from peripheral blood of cirrhotic patients remain in an increased activity without being challenged by bacteria. This permanent activity and production of radicals, called “resting burst”, ends in a loss of energy and finally exhaustion of the neutrophils. The consequence is a paralysis of the innate immune system and the subsequent insufficient response to the invading microorganisms.

1.9.1 Albumin

Albumin is a globular protein with a molecular weight of 66.000 Dalton. It makes up to 60% of all human serum protein and therefore generates the colloid osmotic pressure. The major binding domians I, II and III which are divided into A and B subdomians, Lys 525 and Cys 34 , are special receptors and essential properties for the transportation of various substances.(60) Albumin binds long chain fatty acids, amino acids, bilirubin, calcium and is able to make complexes with a few metal ions (cooper). Another crucial feature is its interaction and modification of different drugs. (61)

Albumin has also an important role in the interaction and the elimination of endotoxin. It binds the bacterial components, forms complexes and delivers it to cell receptors which subsequently react to the impuls.

Normally, three types of albumin can be found in the human plasma: the most common is the reduced human mercaptalbumin (80%). In 25 % moderately oxidized human nonmercaptalbumin (HNA-1) is generated and a small part is represented by the highly oxidized human nonmecaptalbumin (HNA-2). (61)

Studies showed that during cirrhosis, an oxidation and decreased synthesis of human albumin is taking place which alters its binding capabilities. This leads to an increase of toxic agents, such as bilirubin, a decreased endotoxin binding and inactivating action.(61,62,63)

The bacterial components accumulate in the peripheral blood, prime the innate immune system and lead to energy depletion and a decrease of phagocytosis. This illuminates the influence and negative effect on the immune system and partly explains the advanced infection rates in cirrhotics. For instance, higher amounts of HNA 1 and HNA 2 were detected in liver disease, in ACLF particularly HNA 2 was increased.(63) Another important feature of albumin is its removal of reactive oxygen species and its protective effect on the endothelium.(64)

Latest studies discovered new benefits of albumin. Besides being used as a plasma expander, the application of this protein also improves the condition of cirrhotics suffering from hepatic encephalopathy, spontaneous bacterial peritonitis or hepatorenal syndrome. (63)

2 Aim of the study

Sepsis, with its mortality rates ranging from 20% to 50%, is the second leading cause of death in intensive care units and a great clinical challenge in the patients management.(2) One of its main complications and causes of concern are infections which are supported by the occurring immune paralysis of neutrophils and monocytes. This immunodeficiency leads in many cases to a decompensation of stable cirrhosis and to a highly increased mortality lying in between 80% and 90%. Acute on chronic liver failure (ACLF) is also associated with the paralysis of neutrophils and monocytes and shows a rather poor prognosis.

The aim of our study was to compare the neutrophil function of patients with liver damage in septic shock and ACLF with healthy controls. Therefore we investigated the resting burst, oxidative burst and the phagocytosis of polymorphonuclear leucocytes. Another specific question was the influence of sepsis or ACLF on hemoglobin, serum sodium, albumin and bilirubin.

2.1 Hypothesis

We hypothesize that neutrophils in patients with liver damage suffering from septic shock or ACLF react with a poorer response to invaders than neutrophils from healthy controls. This may result in higher morbidity and mortality rates among the liver patients. We also investigated differences in the biochemistry/blood count.

2.2 Inclusion and Exclusion criteria

Inclusion criteria

- Age above 18 years
- Proven Sepsis (International Sepsis Definitions Conference 2001)
- Proven ACLF (Consensus recommendations of the Asian Pacific Association for the study of the liver)
- Informed consent

Exclusion criteria

- Gastrointestinal bleedings within the last seven days prior the enrolment
- Use of immunomodulating agents
- Presence of malignancy
- History of immunsuppression
- Pregnancy

3 Materials and Methods

3.1 Materials

3.1.1.Devices

Eppendorf Research Pipette®,100-1000µl ,10-100µl,0,5-10µl

Eppendorf ep.T.I.P.S®,10µl,200µl,1000µl

Sarstedt® Transfer Pipette, 3.5 ml

BD Falcon®, 5 ml Polystyrene Round Bottom Tube

BD Falcon®, 50 ml Polypropylene Conical Tube

Pechiney Plastic Packaging®, Parafilm-Laboratory film 4IN. X 125 FT.Roll

Heraeus®, Multifuge 3L-R

GFL®, Waterbath

BD Biosciences®, LSR II flow cytometer

3.1.2 Reagents

Orpegen Pharma®, Phagotest LOT 11625,

Orpegen Pharma ®, Burstest(Phagoburst) LOT 11473

ImmunoTools®, Anti-CD 16 PE. Cat No 21330164

ImmunoTools®, Anti-CD 16 FITC. Cat No 21330163

ImmuniTools®, Anti- CD 14 FITC. Cat No 21279143

Phosphate buffered saline (PBS), Anstaltsapotheke

3.2 Methods

3.2.1 Methods for determing the neutrophil activity

3.2.1.1 The Orpegen Phagoburst®

The Orpegen Phagoburst® kit is an analytical method to investigate the oxidative and resting burst of neutrophils and monocytes. This test allowed us to analyze the phagocytotic activity of microphages after the ingestion of bacteria by determining reactive oxidant and the enzymatic activity.

The Phagoburst-test® contains:

- 1 bottle of **unlabeled opsonized E.coli bacteria** (1 x 10^9 bacteria per ml)
- 1 ampoule of the protein kinase C ligand **phorbol 12- myristate 13- actetat (PMA)** (200 x stock solution, 1,65mM)
- 1 ampoule of the **stimulans N-formyl- Met-Leu-Phe** (fMLP) (200 x stock solution, 1 mM)
- 12 ampoules of **SUBSTRATE DISK (** fluorogenic substrate dihydrorhodamine (DHR) 123)
- 1 bottle of **LYSING SOLUTION (** removes erythrocytes, fixing of leukocytes)
- 1 bottle of **NEUTROPHIL WASHING SOLUTION/ PBS** (Phosphate Buffered Saline Buffer)

To accomplish the test you have to:

- Draw venous blood in 4 ml vacuum tubes
- Label 5 x 5ml Falcon test tubes(Falcon, Becton Dickinson)

  1 Tube: resting burst (20 μl PBS)

  2 Tube: priming with fMLP (20 μl fMLP)

  3 Tube: positiv control with PMA (20 μl PMA)

  4 Tube: opsonized E.coli (20 μl E.coli)

  5 Tube: Phagocytosis of opsonized FITC labelled E.coli (20 μl E.coli FITC)

fMLP: a synthetic peptide that triggers minimal oxidative burst in unstimulates neutrophils
PMA: potent tumor promotor, acitvates the signal transduction enzyme protein kinase C(PKC),initiates neutrophil response

Combine the Substrate disk with 2 ml PBS
Lysing solution: 5 ml of stock solution plus 50ml aqua dest

Add 100 µl of whole blood to each tube and incubate the tubes in the waterbath (37°C) for 20 min. Take the tubes 1-4, add 20 µl of the substrate solution and incubate those again in the waterbath for 20 min.
Pipette 100 µl of the Reagent C (Brillant Blue solution) to tube 5.
In the next step add 3ml of the lysing solution to all tubes and wait until the mixture gets clear.(20 min standing at room temperature)
Centrifuge tubes 1-5 (1800 U/min for 5 min and 4°C). Remove the lysing solution and wash the cells with PBS until the lysis is complete. If the lysing is incomplete, repeat the process once again.
Finally add 3 µl of an ANTI-CD 16 PE antibody to tube 1-5 incubate them for 30 min at 4°C and analyze the samples with FACS.

3.2.1.2 FACS (fluorescens activated cell sorting)

Flow cytometry was developed to measure and sort cells on the basis of their chemical and physical characteristics. Scattered light or the emission of fluorescence is collected by special lenses, giving so information about the structure of the particles. The light is produced by either a laser or an arc lamp and focused on a reading point, a place passed by single cells. The beam hits the particle and scatters in different directions, depending on the size and granularity of the cell. (67)
Lenses that recognize light deviating forward in a 20° angle from the laser beam axis are called forward scatter channels (FSC). They give information about the dimension of the scattering, a situation that correlates with the cell size and is used to distinguish between debris and cells that are still alive. If the light breaks in a 90° angle, it hits the side scatter channel (SSC) giving evidence about cells

granularity. FSC and SSC combined discriminate between cell types and inform so about the population in the tested blood sample.

The laser (light amplification stimulated emission of radiation) emits light of a single wavelength, a so called monochromatic light. The wave is coherent and has a high energy density like a bell shapes curve.(67)

Every **laser light** has its own emission spectrum (nm):

- Argon laser: 488 nm to 515 nm (blue to green)
- Helium Neon laser: 633 nm (red)
- Krypton laser: 568 nm to 647 nm (blue to dark red)
- Diode laser: 635 nm (red)

Arc lamps can be classified into xenon lamps or quicksilver lamps of high pressure. This light source has a wide emission spectrum, a high intensity and has to be air cooled.
Compared to the laser, the lamps are much cheaper but less durable.

3.2.1.2.1 Fluorochrome

Fluorochroms are special dyes used to stain biological substances. Its characteristic is the absorption of shortwaved light in which electrons (particles with a negative electric charge) become activated and change from a resting state into an activated phase. This condition is short lasting (1-10 nanoseconds), the electrons return to a period of rest whereby they emit energy in form of light of a longer wavelength, known as fluorescence. The state of absorption and emission of light is called Excitation and Emission.
Some fluorochromes are already naturally incorporated in cells, others must be extrinsically added. (67)

For this reason they are classified into intrinsic and extrinsic dyes:

Intrinsic fluorescence: Known as autofluorescence. Fluorochromes exist already in cells as **hemoglobin, pyridine, flavine, tryptophan,tyrosin.**

Extrinsic fluorescence: A pigment that attaches to special cell parts (dsDNA and dsRNA)

- **Fluoroscein (FITC)**: Excitation: 495 nm(blue)
  Emission: 525 nm (green)

- **Phycoerythrin(PE)**: Excitation: 488, 565 nm (blue)
  Emission: 600 nm (brown)

- **Allophycocyanin (APC):** Excitation: 650 nm (red)
  Emission: 660nm (black)

- **Tandem fluorochrome**: Stimulation of the first dye, afterwards energy transfer and fluorescenceemission of the second dye

  PE- Cy5: Excitation: 488 nm
  Emission : 674 nm

  APC- Cy 7: Excitation: 650 nm
  Emission: 780 nm

  PerCP: Excitation: 470 nm
  Emission: 680 nm

4 Results

4.1 Data analysis

The main principle of a flow cytometer is the analysis of single cells with the help of emitted fluorescence that is picked up by special lenses. The information (cells) is then processed by a computer and visualized in a diagram (density plot), according to their FSC and SSC. The forward scatter channel depends on the volume of the particle, the side scatter channel informs about the granularity and the size of the cell nucleus. The cell populations may appear in a) different colours, explaining their density: Yellow and green spots stand for a high density population and b) in different regions.

Figure 6 shows a FSC/SSC density plot of a healthy person. Every dot stands for an analyzed cell. The regions R1-R3 represent: R1-Lymphocytes; R2-Monocytes; R3 – Granulocytes.

Density plot

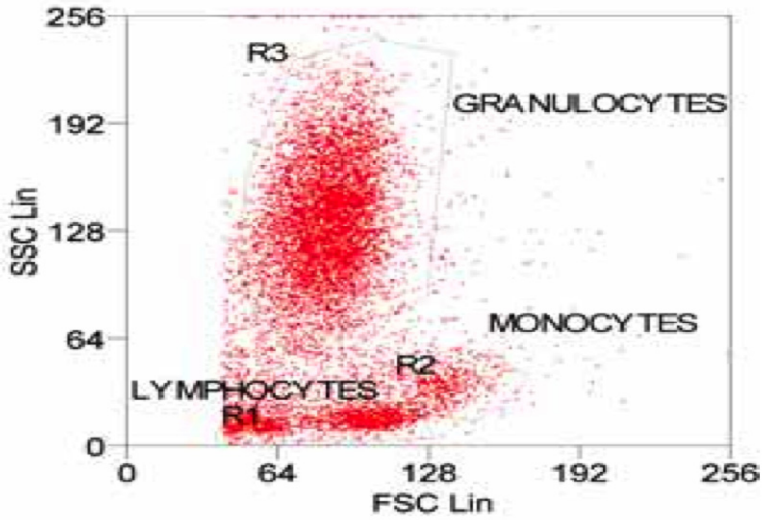


Fig.3. Source: adapted from Misha Rahman,Andy Lane, Angie Swindell, Sarah Bartram-Introduction to flowcytometry.www.ab-direct.com

R1- Lymphocytes are the smallest cells with the lowest amount of granules: SSC and FSC low

R2- Monocytes are large cells, but have barely granules: SSC low, FSC high

R3-Granulocytes are large and possess many granules: SSC and FSC high.

We investigated the resting burst and phagocytosis of neutrophils in healthy controls and cirrhotic patients treated with albumin. For this purpose, we used the Phagoburst kit (Orpegen Pharma, Heidelberg, Germany). This test enables it to define the phagocytotic activity of stimulated and unstimulated cells. (35)

4.1.1 FACS analysis of healthy controls

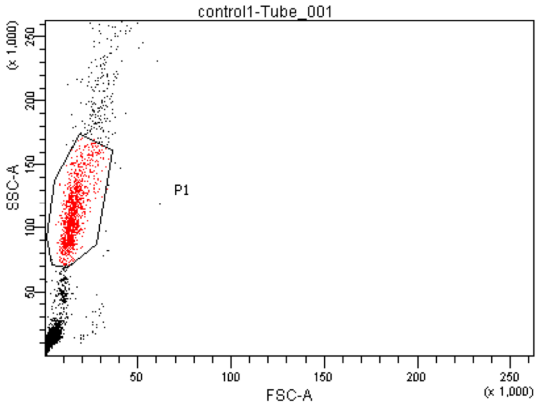
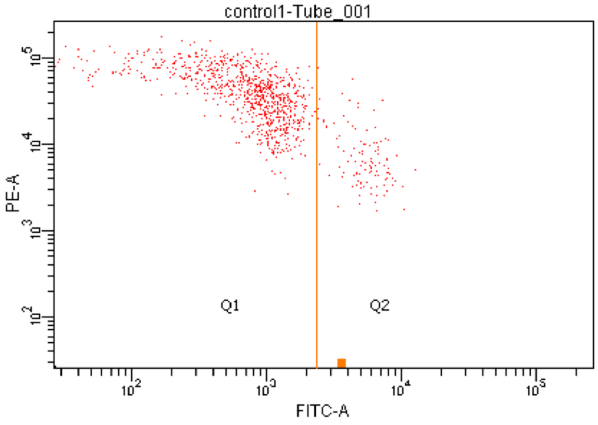
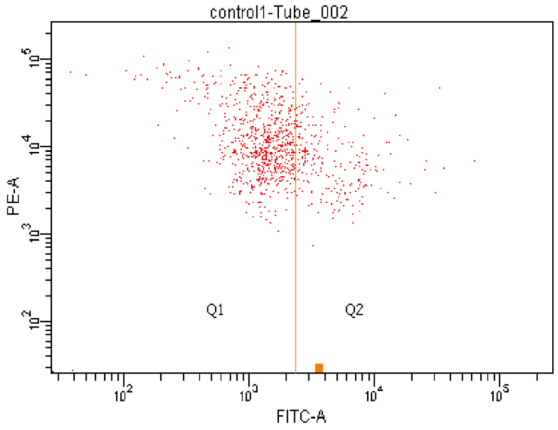


Fig.4. Neutrophil reaction to PBS



Experiment Name:	Burst+Phago	
Specimen Name:	control1	
Tube Name:	Tube_001	
Record Date:	Apr 3, 2008 4:08:21 PM	
Population	%Parent	FITC-A Mean
☒ Q2	9.2	5,659
☒ P2	####	3,585

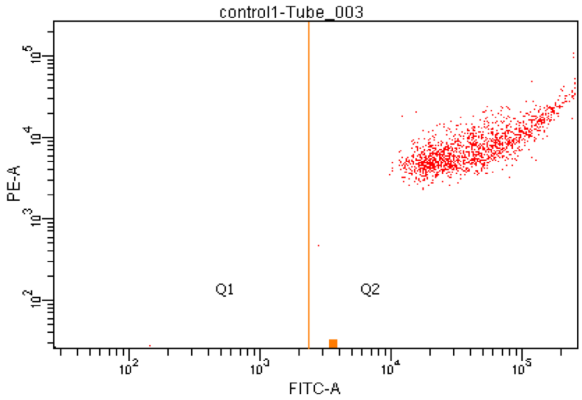
Fig.5. Neutrophil reaction to PBS



Experiment Name: Burst+Phago
Specimen Name: control1
Tube Name: Tube_002
Record Date: Apr 3, 2008 4:08:56 PM

Population	%Parent	FITC-A Mean
☒ Q2	25.1	5,772
☒ P2	####	3,615

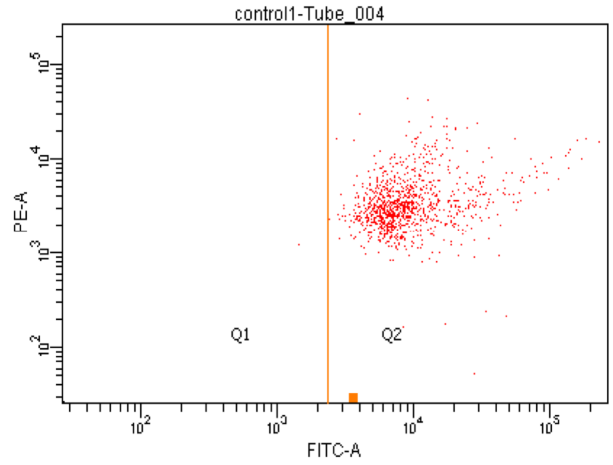
Fig.6. Neutrophil reaction to fMLP



Experiment Name: Burst+Phago
Specimen Name: control1
Tube Name: Tube_003
Record Date: Apr 3, 2008 4:09:20 PM

Population	%Parent	FITC-A Mean
☒ Q2	99.9	58,140
☒ P2	####	56,006

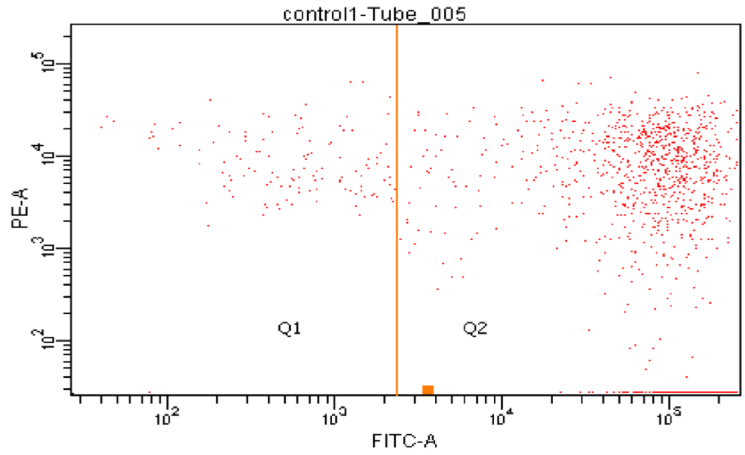
Fig.7. Neutrophil reaction to PMA



Experiment Name:	Burst+Phago
Specimen Name:	control1
Tube Name:	Tube_004
Record Date:	Apr 3, 2008 4:09:38 PM

Population	%Parent	FITC-A Mean
☒ Q2	99.9	13,285
☒ P2	####	13,274

Fig.8. Neutrophil reaction to opsonized *E.coli*



Experiment Name:	Burst+Phago
Specimen Name:	control1
Tube Name:	Tube_005
Record Date:	Apr 3, 2008 4:09:57 PM

Population	%Parent	FITC-A Mean
☒ Q2	83.0	101,450
☒ P2	####	99,656

Fig.9. Neutrophil reaction to FITC opsonized *E.coli*

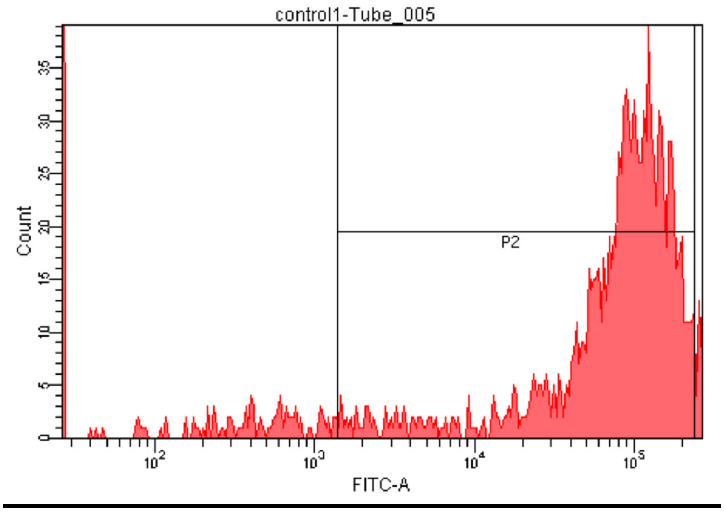


Fig.10. Diagram shows response to FITC opsonized *E.coli*

4.1.2 Explanation of figures 4-10

The first figure shows the gated neutrophil population (red cells). Dead cells and debris lie below and are visible as black dots. (Fig 4)

In figure 5, the resting burst of unstimulated neutrophils (Q1) was measured. The main population lies inside Q1, showing that the neutrophils remain in a resting state (burst activity). Even after adding fMLP which triggers the oxidative burst when cells are primed, the neutrophils show a very weak reaction (main population still inside Q1). (Fig 6)

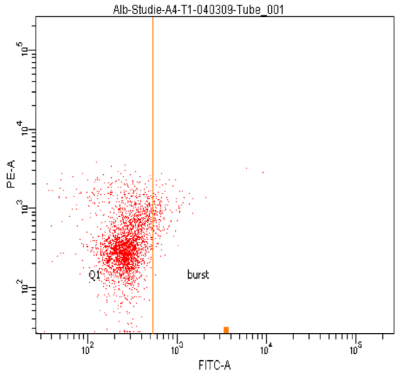
The picture changes after adding PMA which is the positive control Nearly all cells are activated and show a high oxidative burst. (Fig 7).

The reaction to a physiological stimulus is finally visible in figure 8, where the majority of cells stimulated with opsonized *E.coli* react with a production of free oxygen radicals.

Figure 9 and 10 demonstrates the phagocytosis of neutrophils primed with opsonized FITC labelled *E.coli*.

- Fig 9: The cells are scattered and show a high phagocytotic activity. 99.656 is the mean fluorescence intensity (MFI), a number for cells that phagocytized at least one bacterium
- . Fig 10: The diagram shows the strong response (phagocytosis) of the innate cells to opsonized FITC labelled *E.coli*.

4.1.3 FACS analysis of patients with liver cirrhosis treated with albumin



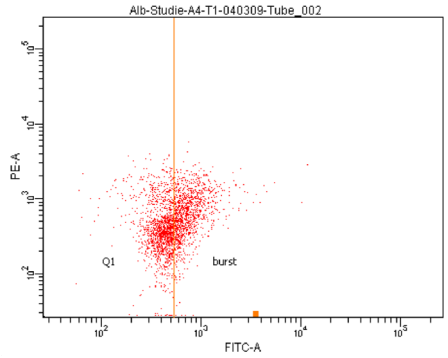
Experiment Name: Burst+Phago new
Specimen Name: Alb-Studie-A4-T1-040309
Tube Name: Tube_001
Record Date: Mar 4, 2009 4:10:12 PM

Population	%Parent	FITC-A Mean
burst	6.1	788
phagocytosis	####	5,801

Tube: Tube_001

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
neutrophils	2,797	28.0	28.0
Q1	2,625	93.9	26.2
burst	172	6.1	1.7
Q3	0	0.0	0.0
Q4	0	0.0	0.0
phagocytosis	3	0.1	0.0

Fig.11.Neutrophil reaction to PBS



Experiment Name: Burst+Phago new
Specimen Name: Alb-Studie-A4-T1-040309
Tube Name: Tube_002
Record Date: Mar 4, 2009 4:10:28 PM

Population	%Parent	FITC-A Mean
burst	45.6	832
phagocytosis	####	3,258

Tube: Tube_002

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
neutrophils	2,540	25.4	25.4
Q1	1,382	54.4	13.8
burst	1,158	45.6	11.6
Q3	0	0.0	0.0
Q4	0	0.0	0.0
phagocytosis	30	1.2	0.3

Fig.12.Neutrophil reaction to fMLP

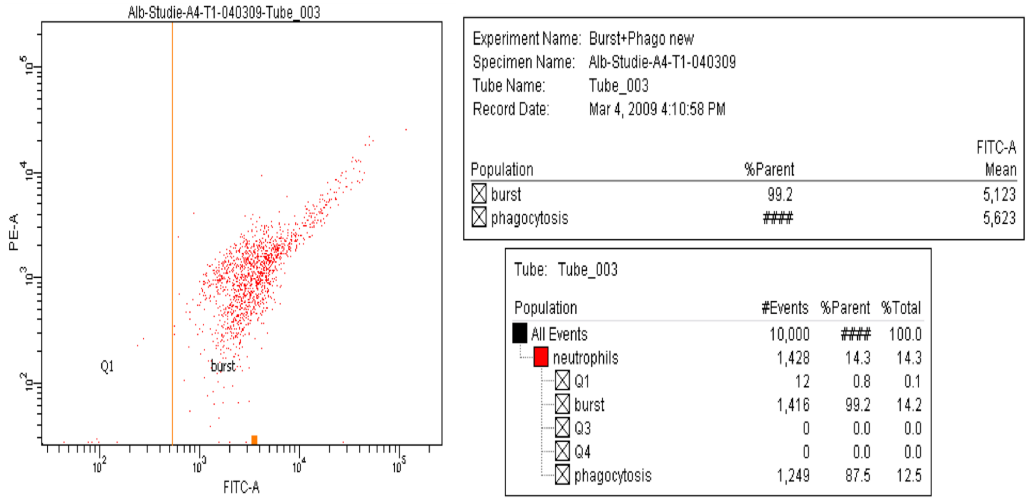


Fig.13. Neutrophil reaction to PMA

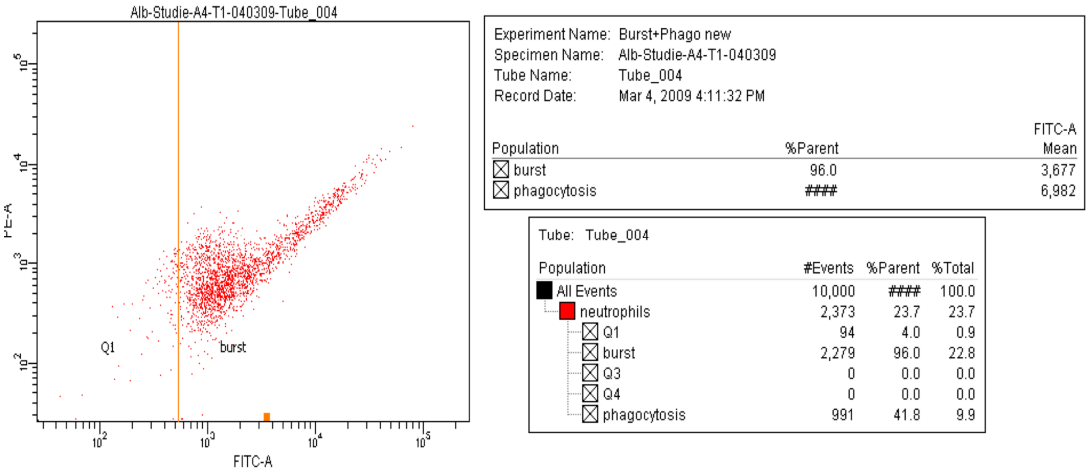


Fig.14.Neutrophil reaction to opsonized *E.coli*

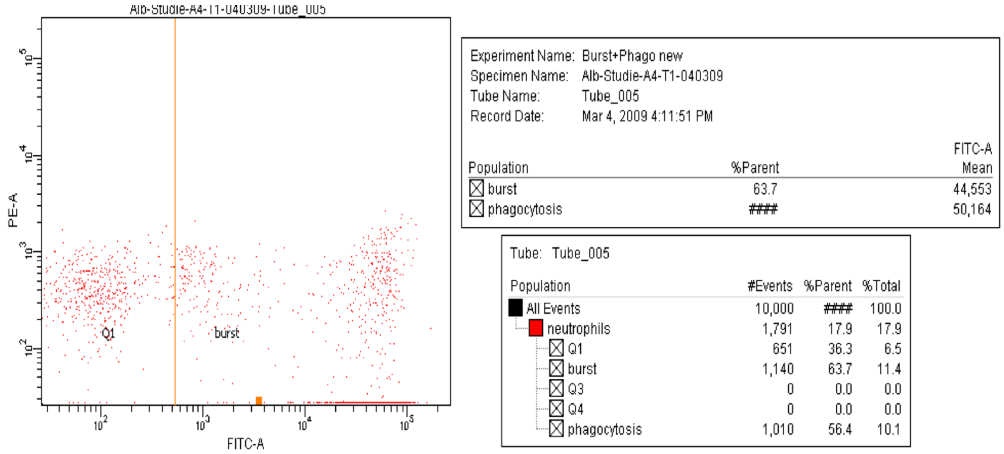


Fig.15. Neutrophil reaction to FITC opsonized *E.coli*

4.1.4 Explanation of figures 11-15

Figure 11 shows the neutrophil reaction after adding PBS. The resting burst is not increased (population inside Q1 6.1%), the cells remain in a resting state.

However, after adding fMLP, the oxidative burst raises to 45,6%. (Fig.12.) Compared to the healthy controls, the neutrophils from the cirrhotic patient react with more activity (priming). (Fig.7) This is a sign for the already occurred mobilization in the peripheral blood that can be triggered by endotoxin.

The positiv control with PMA primes 99.2% of the investigated innate immune cells and shows no difference to figure 7. (Fig. 13)

Opsonized *E.coli* which are added to investigate the oxidative burst, initiate a higher response in the liver patient as in the control, also an indication for the occurred activation of neutrophils in peripheral blood.(Fig. 14)

The analysis of the diagram displaying the phagocytosis of opsonized FITC labelled *E.coli* gives evidence about a weaker triggering of neutrophils compared with the healthy control. (Fig.15) Recalling the results from figure 9, the mean fluorescence intensity (MFI) in a healthy control was 99,656, however, in the albumin treated patient the MFI is 50,165, showing the poorer response of neutrophils to FITC labelled *E.coli*.

4.2 Patient characteristics

14 patients were enrolled and classified according to the ACLF (Consensus recommendations of the Asian Pacific Association for the study of the liver (APASL)) or Sepsis (SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference 2001) criteria in the appropriate groups. From the 14 patients 13 were male (7 sepsis, 6 ACLF). The reason for that might lie in the fact that men are more prone to develop sepsis and liver cirrhosis, however, this has not been further investigated in this study.

	All patients (n14)	**Septic shock (n8)**	**ACLF (n6)**
Age (years)	51,55	61,2±6,7	41,2 ±2,9
Blood count			
Hb	10,88	11,51	9,2
WBC	16,45	15,1	20,07
Platelets	140,2	144,87	112,21
CRP	164,5	207,8	107
Quick	66,9	77	54
APTT	62,8	55	74
Crea	2,8	3	3
BUN	139,85	144,25	134
Bilirubin	13,1	4	23
ALT	277	421	86
AST	456,7	684	153
AP	169	150	191,4
GGT	195,5	133,1	278,6
Alb	2,7	2,5	3
Na	144	148	137,6

Tab.8: Age and biochemistry of investigated patients. **Septic shock**: 8 patients suffered from an septic shock. The average age was 61,9 ± 6,7 years. 7 men and 1 woman were affected

ACLF: 6 patients suffered from ACLF. The average age was 41.2 years. Only men were affected

A main difference between the two investigated groups (septic shock vs. ACLF) was the patients' average age. Patients with septic shock were approximately 62 years old (61.9±6.7), those who suffered from ACLF had in average 41 years (41.2±2.9). ALCF patients were significantly younger than those with septic shock ($p<0.01$).

4.3 Neutrophil acitivity

4.3.1 Resting burst

Patients with ACLF had a significantly higher resting burst compared with the healthy controls ($p<0.01$). Neutrophils in ACLF showed a burst activity of almost 60%, in the control group only 10%. In sepsis, the resting burst was also increased (27% resting burst) compared to the healthy population, but this was statistically not significant.

The increased resting burst in ACLF implicates that neutrophils are already activated without stimulation in the patients' peripheral blood.

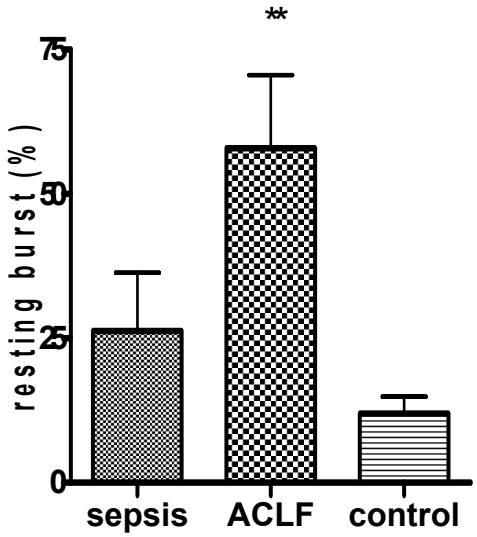


Fig .16: Resting burst of neutrophils in septic patients, ACLF patients and healthy controls

4.3.2 Priming

Neutrophils of control and septic patients primed by fMLP showed an activity of 20% - 25% (sepsis: 25%, control: 20%). Compared with these 2 groups, the patients with ACLF had significantly higher priming (75 %).

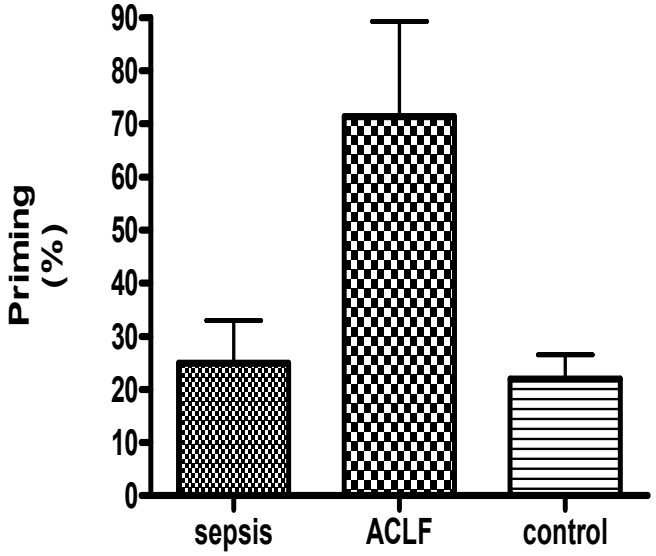


Fig. 17 : Priming reaction of neutrophils in septic patients, ACLF patients and healthy controls

4.3.3 Phagocytosis

The phagocytosis of neutrophils in patients suffering from acute-on-chronic-liver failure is significantly decreased, showing only an activity of 30 - 40% ($p<0.001$). Compared with the healthy controls which react with 100% phagocytosis, there is a discernible difference demonstrating the impairment of the innate immune system during ACLF. In sepsis, neutrophils are also affected and show a reduced response of 70%.

ACLF has a serious effect on neutrophils. Due to the increased resting burst, the cells become energy depleted and lose their ability to respond to a stimulus, in our case *E.coli*.

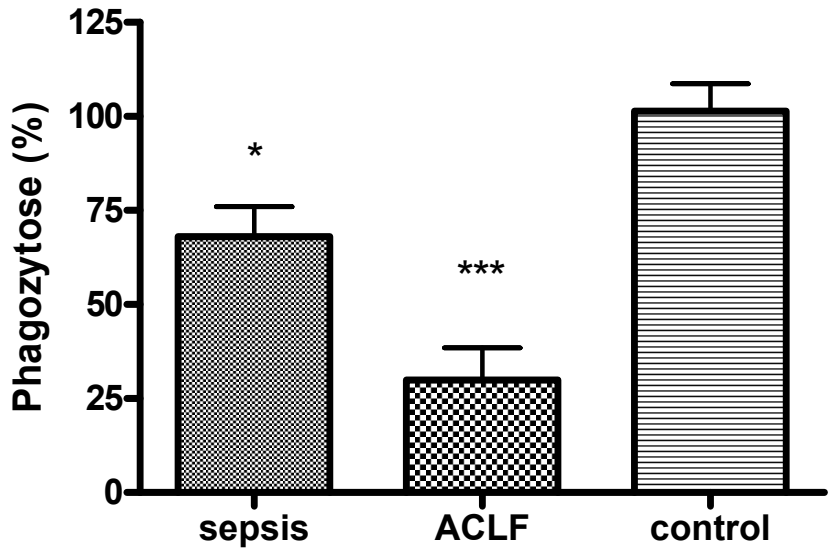


Fig. 18: Phagocytosis in septic patients, ACLF patients and healthy controls

4.4 Blood values

4.4.1 Serum Sodium

The normal range for serum sodium (Na) in adults is 135- 145 mmol/l. In both groups(septic shock and ALCF) the sodium is in the healthy region. Patients with ACLF have lower sodium levels (138 mmol/) than those with a septic shock (148mmol/l). ($p<0.05$)

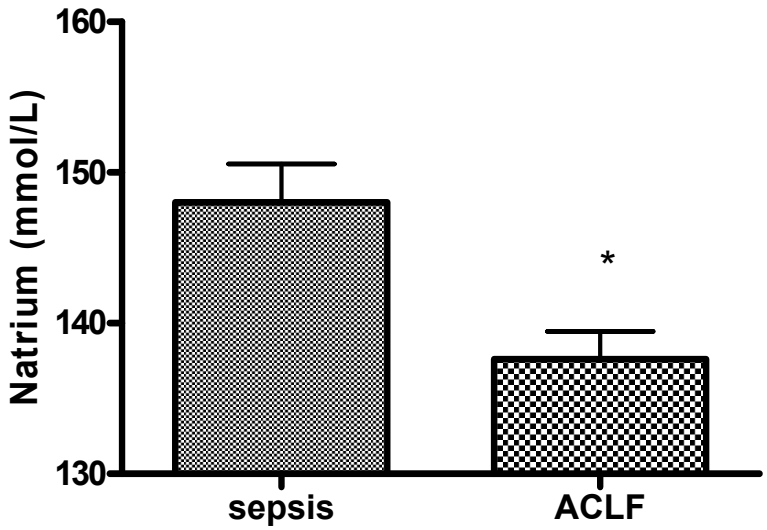


Fig. 19: Serum sodium in patients with sepsis or ACLF

4.4.2 Albumin

The normal serum albumin concentration ranges from 3,5 to 4,5 g/dl.

Figure 20 shows Albumin levels of patients with septic shock or ACLF. In both groups, the protein concentration is slightly decreased. Patients with ACLF had a higher Albumin level (3 g/dl) than patients with Sepsis (2.5 g/dl).$p<0.05$

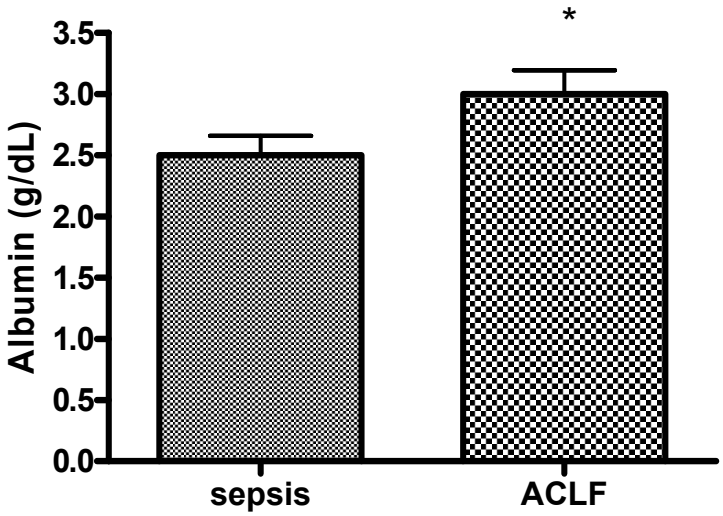


Fig. 20: Albumin in patients with sepsis or ACLF

4.4.3 Hemoglobin

The normal hemoglobin (Hb) levels vary between men and women. For men it is 14- 18 g/dl, for women 12- 16 g/dl.

Patients affected by sepsis or ACLF have reduced hemoglobin levels. In our study, patients with ACLFhad a more severe drop in hemoglobin (9g/dl) than patients with sepsis (12g/dl).$p<0.01$

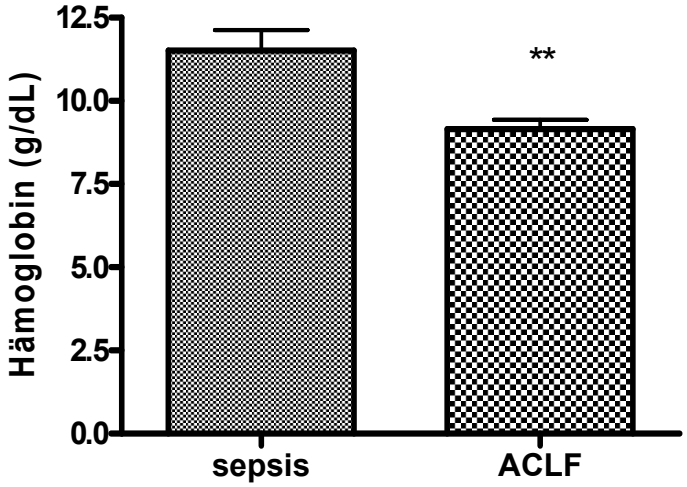


Fig. 21: Hemoglobin in patients with sepsis or ACLF

4.4.4 Bilirubin

The normal results for total bilirubin are 0.3-1.9 mg/dl. The analysis of blood samples in our study showed a pronounced hyperbilirubinemia in patients suffering from ACLF, where the concentration exceeded 23mg/dl. During sepsis, bilirubin increases as well (3mg/dl),but not as much as in ACLF. $p<0.01$

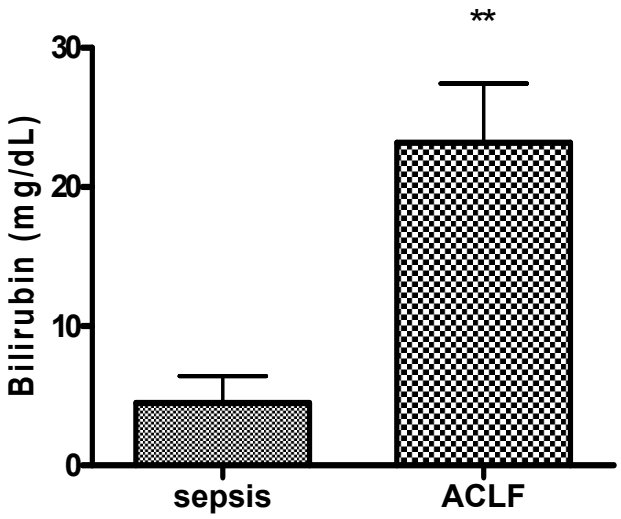


Fig. 22: Bilirubin in patients with sepsis and ACLF

5 Discussion

The aim of our study was to assess the effect of immune paralysis on mortality in patients with septic shock or ACLF. Sepsis is a well known and an often described phenomenon in intensive care. ACLF in contrast was firstly mentioned 15 years ago and defined as an acute deterioration of a preexisting chronic liver disease in 2 to 4 weeks. The acute decompensation of the liver is often triggered by complications of cirrhosis including infections, gastrointestinal bleedings or excacerbations of the underlying disease. According to the latest studies, infections are very common and main causes for the conversion from a chronic liver damage into an acute liver failure which is often associated with multiple organ failure and high mortality rates. (68)
The susceptibility to infections of patients with cirrhosis is mainly caused by a malfunction of the innate immunity, especially neutrophils and monocytes. A defective gut mucosa and increased bowel permeability, a decreased Kupffer cells function and the synthesis of HNA-1 and HNA-2 lead to an abnormal accumulation of bacterial products in the peripheral blood which are responsible for the weak immune response. (62, 69, 70,)

Due to this immunosuppression, the patients suffer from severe infections commonly ending in sepsis. Both clinical pictures are frequent medical emergencies at the ICU. They are clinical challenges and an economic burden for the health care system. In Germany, 75.000 people suffer from severe sepsis or a septic shock per year and 79.000 from sepsis. Mortality rates in intensive care vary between sepsis, severe sepsis and septic shock. 5%-36% of patients die during sepsis whereas 40%-56 % pass away due to severe sepsis or septic shock.(71) A day of care of a patient costs about 1200 Euro, the entire therapy of septic patients consumes in average 23.000- 29.000 Euro. The work of the clinical team on ICU accounts for 40%-60% of the whole intensive care budget. (72)

Our special interest was directed towards the function measurement of neutrophils (resting burst and phagocytosis).
The reason for our investigations of polymorphonuclear cells are the latest findings about their dysfunction and the influence of neutrophil function on prognosis in sepsis or acute on chronic liver failure. Especially in patients with an underlying liver disease, the innate immune cells show a poor response to invaders. A high resting burst and low phagocytosis, a probable consequence of elevated endotoxin levels in serum, leads to increased infections with sepsis, organ failure or even mortality. (35)

In liver disease, the oxidation of albumin and the elevation of endotoxin concentrations in the patients' serum play a major role in the development of the paralysed immune defence.
Oxidized albumin is characterized by an impaired structure and a defective binding and forming of proper complexes with bacterial products. Normally, the lipopolysaccharide- protein complex is delivered to cell receptors, usually CD 14 and TLR 4 which get stimulated and react with the production of proinflammatory cytokines. (62, 73)
In liver cirrhosis, the circle of recognizing, binding and inactivating of endotoxin is interrupted due to the deteriorated synthesis of the protein. This, the increased bowel permeability and a decreased functional efficiency of the reticuloendothelial system constitute to the accumulation of endotoxin in the peripheral blood. This leads to an energy depletion of neutrophils and a phagocytosis defect. (62,69)

Endotoxin itselfs has also an effect on destructive processes of the liver. On the one hand it impairs the microcirculation of the liver. On the other it stimulates the pro-inflammatory cytokine production which deteriorates the liver function by damaging the tissue. (74)

We studied a patients' collective with either a sepsis or acute on chronic liver failure. All patients were treated according to the standard protocols of the medical intensive care unit in the Medical University Hospital Graz.

The average age was 51.6 years, patients with septic shock were significantly older (61,2 years) than patients with ACLF (41,2 years).
In terms of sex differences, more men than women were affected by either a sepsis shock or ACLF. The mortality rates were very high. 62.5% of patients died in the sepsis group and in the ACLF group all patients died (100%).
Sepsis must be seen as a complex reaction of the whole organism. Every functional unit of the body is affected and reacts with a strong response which subsequently ends in an overreaction and decompensation. A major role plays the innate immune system as it initiates the reaction to an infection and combats the invader.

We observed high mortality rates in both investigated groups and assume the innate immune system as an important factor for the bad outcome.
The reason behind our expectations is the data analysis of the resting burst, the priming activity and the decreased phagocytosis of neutrophils from septic or liver failure patients. The origin of the low phagocytosis lies in the endotoxin and its priming effect on the neutrophil resting burst.
The resting burst in patients with ACLF was significantly elevated. The neutrophils were definitely primed and showed a burst activity of 60%. Compared to the healthy controls and sepsis patients, whose cells possessed 10% and 27% activity, this is a significant increase and indicates that the liver has an important effect on the immunity. As a main detoxification organ for endotoxin, it keeps the endotoxin concentration in serum low. During ACLF, the liver loses its detoxification capacity. The reason is a malfunction of the reticulo-endothelial system (RES), especially the Kupffer cells which normally absorb free endotoxin, and a decreased and defective albumin synthesis which normally binds endotoxin.
Increased levels of endotoxin or other bacterial products, such as bacterial DNA, are likely to be the cause for the inappropriate activation of the innate immunity.
Due to the TLR 2 and TLR 4 receptors which recognize bacterial wall elements, such as endotoxins, the cells are activated and this activation utilises a lot of energy of the cells. (75) Subsequently, the neutrophils are not able to respond properly to a stimulus. The consequence is, that even a minimal concentration of bacteria in the patients' organism overstrains the already weakened innate immune system. In our study we investigated the activation of neutrophils in the

peripheral blood in order to study immune paralysis.

Unstimulated neutrophils, like those in non liver disease patients, react to the trigger (fMLP) with a low oxidative burst activity. However, the neutrophils from liver disease patients show a higher response to fMLP which is a sign for the already occurred priming of the cells in peripheral blood. (35) The data analysis shows a significant increase of the activity in patients with ACLF (Priming: ~75 %). In contrast to ACLF, the stimulation of the neutrophils in the control and septic group was evidently lower with 20% in the controls and 25% in the septic patients. The high oxidative resting burst, which subsequently leads to the energy depletion of the neutrophils, has a devastating effect on the immunity.
In septic patients, the neutrophils react with 70% phagocytosis, in ACLF only with 30% -40 %. These results manifest the theory of immunity defect in patients with an ongoing bacteremia or liver failure. The difference in phagocytosis between the two clinical pictures is dependent on the character and duration of the condition. ACLF has a chronic component, in the majority of cases a liver cirrhosis that has an immunsuppressive effect on neutrophils and monocytes due to increased LPS levels. The low phagocytosis in ACLF is explainable by the depletion of energy during the high resting burst. In the true sense of word, the neutrophils burn out themselves.

The increased oxidative burst of neutrophils in peripheral blood results partly from the activation of toll like receptors (TLR) by pathogen associated molecular patterns (PAMPs). These receptors are transmembrane proteins which normally initiate the activation of the host defence and regulate the secretion of reactive oxygen species and proinflammatory cytokines. 13 types of TLR are known, whereas TLR 2, 4 and 9 are responsible for the recognizing of bacterial products and are mainly expressed in infection. During endotoxinemia, the cells present an overexpression of TLRs leading to a neutrophil dysfunction and thereby to a higher susceptibility to infections, mainly in patients with alcoholic hepatitis. (35, 75)

Energy metabolism of neutrophils

Polymorphonuclear cells gain their energy from glycolysis, a metabolic pathway that produces ATP. The main concentration of ATP in this cells is about 1,9 fmol. During phagocytosis, the concentration of this nucleotide falls to 0.8 fmol, where it resides in a steady state. However, the energy metabolism increases due to an elevation of ATP production. A deficiency of glucose or intracellular ATP has a negative influence on phagocytosis, increases the oxygen consumption and leads to an overproduction of hydrogen peroxide and neutrophil elastase. (76)
The inhibition of the oxidative resting burst in neutrophils is partially controlled by the nucleotides Adenosine, AMP and ADP.
Neutrophils have the ability, to control their own resting burst. Therefore they produce adenosine in a large quantity which accumulates extracellular and so inhibits the burst activity. (77)

Studies show that hospitalised patients with cirrhosis have a 32%- 34 % chance to suffer from an bacterial infection and are in 5% -7% more prone to infections than the normal population. (78) The 1- year mortality in cirrhotic patients depends on the stage of the liver disease. It varies between 10% and 82%, whereas the decompensated cirrhosis is responsible for a 6- year mortality of 79%. (79,28) This high mortality rates occur in a large percentage in patients with multiple organ failure (MOF) which is a common complication in the intensive care of cirrhotics. MOF is a sign for the acute deterioration of stable cirrhosis (ACLF) requiering a close meshed intensive care and where the probability of death in the early stages of the therapy lies in between 46% to 89%. (63)

The high infection rates in cirrhosis and ALCF have many reasons and are to some extent explainable by the theory of immune paralysis.
As already mentioned, the immune dysfunction affects Kupffer cells, neutrophils and monocytes. Kupffer cells play a major as a part of the reticuloendothelial system, as they recognize endotoxin which is usually increased in patients with alcoholic hepatits. (80) Once they identify the bacterial component, the release of proinflammatory cytokines and radicals induces a systemic inflammatory response

in the liver, worsening the stage of fibrosis. (81)

In cirrhosis, the development of hepatic shunts causes a diminishment of Kupffer cells or a decrease of their activity. The consequence is a reduction in the effectiveness of the RES and thereby an increase of infection rates in cirrhotics.

Neutrophils are the first line defence and an essential part of the innate immunity. Their defence function is significantly reduced in patients with liver cirrhosis or ACLF. The reason for the impaired immunity is the priming effect of endotoxin in peripheral blood which leads to the decreased phagocytosis due to energy depletion. Besides neutrophils, also monocytes become paralysed by endotoxin in patients with liver disease and lead to a further impairment of the immunity. The pathomechanism for the elevated endotoxin concentration in serum of liver disease patients is partially a consequence of increased gut permeability due to an altered gut flora and defective barrier, where higher amounts of bacteria gain access to the hepatic portal system. There is also a relation between portal hypertension which causes an accumulation of edema in the bowel wall and thereby an impairment of the gut barrier and the amount of bacteria transmitted through the bowel mucosa. (63)

Normally, the Kupffer cells filter out the endotoxin from the blood stream that further gets deactivated by hepatocytes through deacytelation and then transported away by bile acids. In cirrhosis, the endotoxin removal by the liver is significantly diminished due to a low activity and number of Kupffer cells and hepatocytes and the development of portosystemic shunts. The latter enables the blood to bypass the liver without being cleansed from the bacterial components. The endotoxin remains in the serum and accumulates in the systemic circulation, where it primes the innate immune system and contributes to the immune paralysis.(63)

In summary, these data shows that due to cirrhosis and its effect on the immunity, the patients suffer more frequently from sepsis which is a main cause for the decompensation of stable cirrhosis and the precipitant factor for the acute on chronic liver failure.

Sepsis and ACLF also causes alterations in many other laboratory findings.

Sodium, for instance, is essential for the balance of body water. The organism has the ability to compensate certain Na fluctuations, larger variations lead to severe complications and can be associated with a poorer prognosis. (82)
Sodium seems to be of particular importance in cirrhosis and ACLF because a low sodium deteriorates the condition of critical ill cirrhotics and often leads to complications and higher mortality rates.(93) Radha Krishna Y et al showed in a study that beside encephalopathy grade 3 and 4 and renal failure, hyponatremia is also a predictor for a doubtful outcome in ACLF. (83)
In our patients, sodium disturbances could not be detected. All measured sodium levels were within the normal range (135-150mmol/l) which is most likely due to the rapid application of standardized protocols in sepsis and ACLF including the early administration of fluid.

Albumin is another important molecule in ACLF. ACLF is often associated with the hepatorenal syndrom (HRS) that worsens the anyway bad prognosis. In order to oppose the complications of the HRS (renal dysfunction, splanchnic vasodilation, ascites), albumin infusions are administered.To achieve better results, the albumin therapy is combined with terlipressin, a vasoconstrictor that interrupts the vasodilation of the splanchnic vessels and increases the glomerular filtration rate (GFR). (38)
Another rationale for the use of albumin therapy is the knowledge about the existence of highly oxidized human nonmecaptalbumin (HNA 2) in ACLF, a protein with reduced binding characteristics and detoxification functions. The idea of the albumin infusion was to substitute the inferior HNA and so increase the effects of the binding and detoxification factor.
In our study, only patients with ACLF received the albumin therapy which explains that their values were significantly higher as in our sepsis group.
In sepsis, hypoproteinemia (<3.5 g/dl) is also a common condition, accompanied by a poor prognosis and higher mortality rates. Pulmonary edema and fluid dysbalances are complications connected to a low oncotic pressure, a consequence of low albumin concentrations. Veneman et al studied the effect of albumin infusions in critical ill patients. The group demonstrated that there is no positive effect of albumin therapy in intensive care patients.(84)

In this case, the administration of albumin, like in ACLF, did not have any promising effects on improving the outcome and is therefore not recommended as a standard therapy.

Beside hypoproteinemia, also anemia could be found in both groups. Patients with sepsis had a slightly decreased hemoglobin average value of 11.5g/dl (8.7-13.5g/dl) whereas in ACLF, the anemia was more pronounced, with a mean concentration of 9g/dl (8.2-10.1g/dl). Low hemoglobin levels are a common problem in cirrhotics and can be a complication of an esophageal or other inner bleeding, bone marrow suppression and hypersplenism.(85) Esophageal bleedings are caused by enlarged blood vessels in the esophagus that burst, after the portal pressure reaches a certain level. Other bleedings are consequences of an insufficient production of coagulation factors which are normally manufactured in a healthy liver. Another reason for a failed coagulation might be a DIC (disseminated intravascular coagulation) that commonly occurs in patients with a septic component. (14)
The blood clotting parameters in the ACLF patients were all altered. The results from the quick test (mean percentage of 54%) showed a decrease of extrinsic factors, the activated partial thromboplastin time (aPTT) was prolonged, with 74 seconds in average, a sign for a reduction of intrinsic factors. (86)
In sepsis, the anemia is partially explainable by the depression of the erythropoietic system due to whether an inappropriate production of erythropoetin which might be a result of a beginning renal insufficiency, or an iron deficiency. (85) Another plausible cause for anemia in sepsis was described by Piagnerelli, who said, that an excessive blood sampling on the ICU also leads to low hemoglobin levels. (87) However, our patients suffered from a mild anemia and so remained untreated.

Another indicator for the patients' severe condition and the upcoming liver decompensation is an increase of total bilirubin. In patients with ACLF, the hyperbilirubinemia varied between 14,5mg/dl - 41mg/dl. Each value was excessively increased and indicated the acute decompansation of the underlying chronic liver disease.

Also the significant elevation of the liver transaminases (AST, ALT), enzymes that are associated with the liver tissue, and the gamma- glutamyltransferase (GGT) were used as indirect measures for ACLF.
In sepsis in contrast, the rise of the decomposition product of hemoglobin is in fact in the pathological range (~ 4mg/dl), compared to ACLF the increase is minimal. Anyway, Zhai et al. investigated the influence of bilirubin in sepsis and discovered a negative effect of it on the development of ARDS and the subsequent mortality rates in ICU.(88)

A decompensation of the liver leads to an increase of toxic substances (ammonia, bilirubin, benzodiazepine and tryptophan) and further to jaundice and hepatic encephalopathy. To prevent and avoid these complications which are associated with the accumulation of these substances, a liver dialysis can be performed. One therapeutic option is the molecular adsorbent recirculating system (MARS®), an extracorporeal dialyzer that uses a 20% human albumin solution which filters out the toxins of the venous blood. Due to a semipermeable membrane separating the patients' blood from the protein solution, the toxin passes through and gets bound to the albumin in the extracorporeal circuit. The loaded albumin solution flows afterwards to a low- flux dialyzer, gets cleansed from the wastes and recirculates to the semipermeable membrane where it is ready for another removal. The extraordinary advantage of the dialyzing kit is that the filters are permeable for water-soluble and albumin bound toxins.
The MARS® dialyzer is an essential part of the ongoing therapy and can be used for acute on chronic liver failure, acute liver failure and intractable pruritus. (89)

Another important component of the MARS® therapy is the removal of proinflammatory cytokines and bacterial products. This immunmodulating agents can accumulate in a large amount in the patients serum during cirrhosis or liver failure and so deteriorate the function of the innate immunity. Stadlbauer et al. investigated the effect of MARS® on the inflammatory status in patients with cirrhosis and superimposed alcoholic hepatitis and found a decrease of the oxidative resting burst in neutrophils.(90)

As above mentioned, renal insufficiency or even failure accompanies sepsis and ACLF in nearly all our study cases and is thereby an important predictor for the outcome. The statistical analysis of our patients' renal function identifies in the majority of cases a loss of renal function. In average, the creatinine was in both groups elevated to 3 mg/dl, signalling a deterioration of the glomerular filtration rate (GFR). The main cause of acute renal failure in ACLF is the hepatorenal syndrome, whereas in sepsis, damaged or necrotized renal tubules lead to the malfunction of the kidneys.

Another sensitive parameter for a renal decompensation, the blood urea nitrogen (BUN), was also heavily increased in sepsis and ACLF. The patients contained BUN levels of about 138,8 mg/dl in average in blood. The increase of creatinine and blood urea nitrogen indicates the insufficiency and demands an appropriate therapy. Fluid management with 0.9% saline infusions and the assessment of urine output plays a pivotal role, as far as the patient suffers from hypovolaemia and a low renal blood flow. If interstitial fluid accumulates in lungs or the peritoneal cavity, or if the patient is oliguric, usually loop diuretics, mannitol or vasoconstrictors like low dose dopamine are given.

The effects of the mentioned medication are still questionable, because studies showed, that there is no prevention or better outcome after the use of it in acute renal failure. Only in portal pressure and ascites, an intensive diuretic therapy showed an improvement of the situation. (91, 92)

6 Conclusion

Patients with sepsis or acute on chronic liver failure suffer from potentially lethal complications and require a special intensive care. The physicians are often confronted with patients in a very bad condition, severe symptoms and a following difficult treatment. The last decade brought a lot of progress in the treatment of both clinical pictures, nevertheless it is still a great clinical challenge associated with high mortality rates. The results of our study demonstrate the severity of both medical conditions and highlight once more the importance of the innate immune system and the effect of its paralysis on the patients' prognosis.

A possible causal relationship between neutrophil function and potential outcome is particularly noticeable in ACLF patients, where severe defects in innate immune function (oxidative burst and phagocytosis) were associated with 100% mortality.

In summary it can be said, that ACLF has a more severe progress and a worse prognosis than sepsis. Therefore, patients with ACLF require an optimal management and an early goal- directed therapy

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