# Dissertation

Lipoproteins and metabolites in sepsis and septic shock
in the intensive care unit

submitted by

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# Declaration

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

Graz, December 2021

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# Abbreviations and Definitions

| | |
|---|---|
| AAA | Aromatic amino acids |
| ABA1 | Apolipoprotein B100 to Apolipoprotein A-I ratio |
| ABCA1 | ATP-binding cassette transporter 1 |
| ABCG1 | ATP-binding cassette sub-family G member 1 |
| ABCG4 | ATP-binding cassette sub-family G member 4 |
| ACAT | Acyl CoA cholesterol acyltransferase |
| AEA | Arylesterase activity |
| AIDS | Acquired Immunodeficiency Syndrome |
| ALF | Acute liver failure |
| AMP | Adenosine monophosphate |
| ApoA1 | Apolipoprotein A-I |
| ApoA2 | Apolipoprotein A-II |
| ApoB | Apolipoprotein B |
| ApoB-48 | Apolipoprotein B-48 |
| ApoB-100 | Apolipoprotein B-100 |
| ApoC | Apolipoprotein C |
| ApoE | Apolipoprotein E |
| ARISE | Australasian Resuscitation in Sepsis Evaluation Study |
| ATP | Adenosine triphosphate |
| a.u. | Arbitrary unit |
| AUROC | Area under the receiver operating characteristic |
| BCAA | Branched-chain amino acids |

| | |
|---|---|
| BCAT | Branched-chain amino acid aminotransferase |
| BCKDC | Branched-chain alpha-keto acid dehydrogenase complex |
| cAMP | Cyclic adenosine monophosphate |
| CD64 | Cluster of Differentiation 64 |
| CE | Cholesteryl ester |
| CEC | Cholesterol efflux capacity |
| CETP | Cholesteryl ester transfer protein |
| CI | Confidence interval |
| CKD | Chronic kidney disease |
| CPMG | Carr–Purcell–Meiboom–Gill pulse sequence |
| CRP | C-reactive protein |
| CVC | Central venous catheter |
| CVD | Cardiovascular disease |
| CVP | Central venous pressure |
| D2O | Deuterium oxide |
| DMEM | Dulbecco's modified Eagle's medium |
| DRKS | Deutsches Register für Klinische Studien = German Clinical Trials Register |
| EGDT | Early goal directed therapy |
| EL | Endothelial lipase |
| eNOS | Endothelial nitric oxide synthase |
| EPIC II | Extended Prevalence of Infection in Intensive Care II study |
| FC | Free cholesterol |
| FiO2 | Fraction of inspired oxygen |

| | |
|---|---|
| GABA | Gamma-Aminobutyric Acid |
| GCS | Glasgow Coma Scale |
| HDL | High-density lipoprotein |
| HDL-C | High-density lipoprotein cholesterol |
| HDL-FC | High-density lipoprotein free cholesterol |
| HDL-P | High-density lipoprotein particles |
| HDL-PL | High-density lipoprotein phospholipids |
| HIV | Human immunodeficiency virus |
| HL | Hepatic lipase |
| ICD | International Classification of Diseases |
| IC | Informed consent |
| ICAM | Intercellular adhesion molecule |
| ICU | Intensive care unit |
| IDL | Intermediate-density lipoprotein |
| IRB | Institutional Review Board |
| JRES | J-resolved pulse sequence |
| LCAT | Lecithin-cholesterol acyltransferase |
| LDL | Low-density lipoprotein |
| LIPOS | Lipid Infusion and Patient Outcomes in Sepsis Study |
| LOS | Length of stay |
| LPS | Lipopolysaccharides |
| LTA | Lipoteichoic acid |
| MAP | Mean arterial pressure |

| | |
|---|---|
| MCP-1 | Monocyte chemoattractant protein 1 |
| mm | Millimeters |
| mmHg | Millimeters of mercury |
| mTOR | Mammalian target of rapamycin |
| N/A | Not applicable |
| NMR | Nuclear magnetic resonance |
| NOESY | Nuclear Overhauser effect spectroscopy pulse sequence |
| NSI | Normalized signal intensity |
| OR | Odds Ratio |
| O-PLS-DA | Orthogonal - Partial Least Squares - Discriminant Analysis |
| paCO2 | Partial pressure of carbon dioxide in arterial blood |
| paO2 | Partial pressure of oxygen in arterial blood |
| paO2/FiO2 | Ratio of paO2 to FiO2, also called P/F-ratio or Horowitz index |
| PC | Principal Component |
| PCA | Principal Component Analysis |
| PCR | Polymerase chain reaction |
| PCT | Procalcitonin |
| PICC | Peripherally inserted central catheter |
| PIRO | Predisposition, Infection, Response, Organ Failure |
| PLS-DA | Partial Least Squares - Discriminant Analysis |
| PLTP | Phospholipid transfer protein |
| PON | Paraoxonase enzyme |
| PROCESS | Protocolized Care for Early Septic Shock Study |

| | |
|---|---|
| PROMISE | Protocolized Management in Sepsis Study |
| PRORATA | Procalcitonin to reduce antibiotic treatments in acutely ill patients trial |
| qSOFA | Quick SOFA |
| rHDL | Reconstituted HDL |
| Rpm | Revolutions per minute |
| S1P3 | Sphingosine-1-phosphate receptor 3 |
| SAA | Serum amyloid A |
| SAPS | Simplified Acute Physiology Score |
| ScvO2 | Central venous oxygen saturation |
| SIRS | Systemic inflammatory response syndrome |
| SOFA | Sequential organ failure assessment |
| SR-B1 | Scavenger receptor B1 |
| SSC | Surviving sepsis campaign |
| STEMI | ST-elevation myocardial infarction |
| sTREM | Soluble triggering receptor expressed on myeloid cells |
| TG | Triglycerides |
| TSP | 3-(trimethylsilyl) propionic acid-2,2,3,3-d4 sodium salt |
| UK | United Kingdom |
| USA | United States of America |
| VCAM | Vascular cell adhesion molecule |
| VLDL | Very low-density lipoprotein |
| V2FC | VLDL-2 free cholesterol |
| V3FC | VLDL-3 free cholesterol |

| | |
|---|---|
| V4FC | VLDL-4 free cholesterol |
| V4TG | VLDL-4 free triglycerides |
| VLPN | VLDL particle number |
| WBC | White blood count |
| WHO | World Health Organization |

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# Zusammenfassung

Hintergrund: Sepsis stellt eine häufige Erkrankung auf Intensivstationen (ICU) dar und ist ein lebensbedrohlicher Zustand mit hoher Mortalität und Morbidität. Lipoproteine, insbesondere das High-Density Lipoprotein (HDL) - Partikel haben eine zentrale Rolle in der angeborenen Immunabwehr. Die Protonen-Kernspin-Resonanz ($^1$H NMR) Spektroskopie stellt eine innovative Technologie zur gezielten und ungezielten Analyse von Metaboliten dar.

Ziel: Die Erforschung quantitativer und qualitativer Veränderungen der Lipoproteine während Sepsis, die Untersuchung der Korrelationen mit Organdysfunktionen und die Assoziation mit ICU- und 28-Tage Mortalität.

Methoden: Erwachsene Patient*Innen mit Sepsis oder septischem Schock wie auch ICU-Kontrollen ohne Sepsis oder Bakteriämie wurden auf der ICU der Universitätsklinik für Innere Medizin, Medizinische Universität Graz eingeschlossen. Sepsis wurde den aktuellen Sepsis-3 Kriterien entsprechend definiert: Vermutete Infektion durch die*den behandelnde*n Ärztin*Arzt und ein Anstieg des "sequential organ failure assessment" (SOFA) Score um ≥2 Punkte. Septischer Schock wurde definiert als Sepsis mit der Notwendigkeit einer Vasopressor-Therapie, um den mittleren arteriellen Druck ≥65mmHg zu halten in Abwesenheit einer Hypovolämie und/oder nach adäquater Flüssigkeitsgabe, sowie ein Laktat von >2mmol/L. Quantitative Lipoprotein-Analysen wurden im Routinelabor durchgeführt. Für qualitative Analysen, wurden sowohl die Arylesterase-Aktivität der HDL-assoziierten Paraoxonase (AEA) wie auch die Cholesterin-Efflux-Kapazität (CEC) in Apolipoprotein-B depletierten Sera untersucht. Zusätzlich wurden gezielte und ungezielte $^1$H NMR Metabolomics-Analysen durchgeführt.

Ergebnisse: Dreiundfünfzig ICU-Patient*Innen mit Sepsis und 25 ICU-Kontrollen wurden prospektiv eingeschlossen. HDL-Cholesterinspiegel <40mg/dL traten häufiger in der Sepsis- als in der Kontrollgruppe auf (85 vs. 52%, p=0,002). Quantitative Lipoprotein-Parameter wie HDL-Cholesterin waren signifikant niedriger in der Sepsis- als in der Kontrollgruppe (14 vs. 39 mg/dL, p<0,0001); zeigten jedoch keine Assoziation mit Mortalitätsendpunkten. Der qualitative HDL-Parameter AEA war signifikant niedriger in der ICU Sepsis- als in der ICU Kontrollgruppe mit 67 vs. 111 mM/min/mL Serum (p<0,0001), während die CEC einen nicht-signifikanten Trend zu niedrigeren Werten in der Sepsis- als in der Kontrollgruppe zeigte (9

vs. 10 %, p=0,091). Die AEA war in der Sepsis-Gruppe in uni- und multivariabler logistischer Regressionsanalyse signifikant mit der ICU- und 28-Tage Mortalität assoziiert. Die Veränderungen der Lipoproteine und Unterschiede zwischen den Gruppen konnte mit gezielter $^{1}$H NMR Metabolomics-Analyse bestätigt werden. Zusätzlich konnten mittels ungezielter $^{1}$H NMR Metabolomics-Analyse Unterschiede für verzweigtkettige Aminosäuren, bestehend aus Valin, Leucin und Isoleucin, in der Sepsis-Gruppe identifiziert werden. Nicht-Überlebende verglichen mit Überlebenden hatten niedrigere Spiegel von Valin (33,0 vs. 55,0 normalized signal intensity units (NSI), p=0,002), Leucin (53,4 vs. 70,8 NSI, p=0,005) und Isoleucin (15,2 vs. 18,1, p=0,012). In uni- und multivariablen Analysen waren verzweigtkettige Aminosäuren signifikant mit ICU- und 28-Tage Mortalität assoziiert.

Konklusion: Die antioxidative und anti-inflammatorische Arylesterase-Aktivität der HDL assoziierten Paraoxonase repräsentiert einen Teil der Funktionalität des HDL-Partikels und war ein starker Prädiktor für ICU- und 28-Tage Mortalität bei ICU Sepsis-Patient*Innen. Mittels Metabolomics-Analyse konnten außerdem erniedrigte Spiegel an verzweigtkettigen Aminosäuren als Parameter für eine erhöhte Mortalität identifiziert werden.

# Abstract

Background: Sepsis is a devastating disease and accounts for high mortality and morbidity in patients treated in the intensive care unit (ICU). Lipoproteins, especially the high-density lipoprotein (HDL) particles, play an important role in the innate immune defense. In addition, proton nuclear magnetic resonance ($^1$H NMR) spectroscopy is an emerging technique for targeted and untargeted analyses of metabolites.

Aim: To investigate quantitative and qualitative changes of lipoproteins during sepsis, investigate correlations with organ dysfunction and associations with ICU- and 28-day mortality.

Methods: Adult patients with sepsis and septic shock, as well as ICU controls without sepsis or bacteremia were enrolled at the ICU of the Department of Internal Medicine at the Medical University of Graz. Sepsis was defined according to the current sepsis-3 criteria with a suspected infection by the treating physician and an increase in the sequential organ failure assessment (SOFA) score by ≥2 points. Septic shock was defined as patients with sepsis, with the need of vasopressor therapy to maintain a mean arterial pressure ≥65mmHg despite adequate fluid resuscitation and/or absence of hypovolemia; and a lactate level of >2 mmol/L. Quantitative assessments of lipoproteins were performed on routine laboratory machines. For qualitative lipoprotein measurements, both the arylesterase activity of the HDL associated paraoxonase (AEA) and cholesterol efflux capacity (CEC) were performed with ApoB-depleted sera. In addition, targeted and untargeted $^1$H NMR metabolomics were studied.

Results: Fifty-three ICU patients with sepsis and 25 ICU controls without sepsis or bacteremia were prospectively enrolled. HDL-C <40mg/dL was more common in sepsis compared to controls (85 vs 52%, p=0.002). Quantitative lipoprotein parameters such as HDL-C were significantly lower in sepsis compared to controls (14 vs 39 mg/dL, p<0.0001), but were not associated with mortality endpoints. The qualitative HDL parameter AEA was significantly lower in sepsis compared to controls at 67 vs 111 mM/min/mL serum (p<0.0001), while the CEC showed a trend towards lower levels in sepsis compared to controls (9 vs 10 %, p=0.091). The AEA was significantly associated with ICU- and 28-day mortality in uni- and multivariable logistic regression analyses in the sepsis cohort. Lipoprotein alterations were confirmed in targeted $^1$H NMR metabolomic analyses. Furthermore, untargeted $^1$H NMR metabolomics identified differences of the branched-chain

amino acids group, consisting of valine, leucine, and isoleucine. Non-survivors compared to survivors had lower levels of valine (33.0 vs 55.0 normalized signal intensity units (NSI), p=0.002), leucine (53.4 vs 70.8 NSI, p=0.005), and isoleucine (15.2 vs 18.1, p=0.012). Branched-chain amino acids were associated with ICU- and 28-day mortality in uni- and multivariable analyses.

Conclusion: Quantity and functionality of lipoproteins are significantly different in ICU patients suffering from sepsis compared to non-sepsis ICU controls. The antioxidative and anti-inflammatory arylesterase activity of the HDL associated paraoxonase, representing parts of the HDL particle functionality, was a strong predictor for ICU- and 28-day mortality in patients with sepsis admitted to the ICU. Furthermore, branched-chain amino acids were additionally identified in metabolomic analyses as significant predictors for mortality endpoints.

# 1. Introduction

Sepsis is a global health threat and one of the leading causes of death worldwide (3-5). It is an important medical emergency and treatment is time critical (6, 7). The incidence of sepsis has been increasing over the last decades. Reasons for this increase include an ageing population with prolonged longevity of chronic conditions, and the widely adopted use of immunosuppressive therapies (8-13). The most common infectious etiology is bacterial, but fungal, viral, and protozoan infections may also occur (14-16). Sepsis is not only a disease of infants, elderly, or patients with comorbidities, but may also affect young healthy individuals (17-19). About 50% of all patients with severe sepsis need intensive care and sepsis accounts for roughly 13% of all intensive care unit (ICU) cases, rendering this disease a major aspect for intensive care medicine (20, 21). Furthermore, from an economical perspective, sepsis is the most expensive disease in hospitals (22, 23).

## 1.1. Sepsis

### 1.1.1. History of sepsis:

The word sepsis is derived from the ancient Greek term σῆψις (sepsis) which can be translated to rotting, i.e. the decomposition in the presence of bacteria and was already mentioned in the Hippocratic collection (4, 24, 25). The signs of inflammation including calor, rubor, tumor, and dolor were introduced by Galen of Pergamon, whereas the fifth sign functio laesa was added by Celsus. Later the scientists Pasteur, Semmelweis, Koch, Lister, and others established the role of pathogens for infections (26, 27). It was then accounted that sepsis is driven by the hosts response to the infection and not only by the infection itself. This concept of a dysregulated host is still the current understanding of this disease (6, 28, 29).

### 1.1.2. Global Epidemiology of sepsis

The true incidence of sepsis is difficult to measure, and different types of techniques are used in epidemiological studies. One easy method is the point prevalence study, where all admitted cases of one single day are counted and analyzed. However, limitations include seasonal variability and that milder cases are more easily accounted for than fulminant severe cases,

which have a high case fatality rate and therefore a shorter ICU stay (8, 30). Retrospective cohort studies have the difficulty of discovering all relevant cases, as often only International Classification of Diseases (ICD) codes are evaluated to collect sepsis cases. This may lead to an underestimation when sepsis was coded differently such as pneumonia or cystitis. On the other hand, sepsis cases may be overestimated, when e.g., systemic inflammatory response syndrome (SIRS) was coded as sepsis even when no infection was present or when sepsis was coded solely because of economic or billing incentives (8, 11, 30, 31). Also, the availability of ICU beds affects the reported severe sepsis incidence because scarce ICU bed availability leads to the presumption of a "natural death" if elderly patients die on the general ward and often the underlying disease, e.g. pneumonia, but not the sepsis is coded as the reason of death (32). An important epidemiological limitation when comparing studies is the changing definitions of sepsis over time. In addition, the noted incidence in all studies is the incidence of treated severe sepsis cases but not the true incidence of all cases (8, 32). Despite all these limitations, the sepsis incidence trend is most likely a true increase over the last years, because of ageing populations with more chronic conditions that are survived longer. More artificial catheters (e.g., central venous catheter (CVC), peripherally inserted central catheter (PICC), Port-a-Cath systems, and others) are implanted, stem-cell transplants and chemotherapy are more widely adopted. Furthermore, antimicrobial-resistant bacteria may cause infections and therefore also affect sepsis outcomes (10-13, 33).

To give a rough estimate for the sepsis incidence, the following paragraph discusses incidence rates assessed in several studies. Martin et al found an increase of sepsis incidence from 1979 with 83 per 100,000 to 240 per 100,000 inhabitants in 2000 (9). Angus et al showed an incidence of 300 per 100,000 population in the United States of America (USA) (34). Esper et al reported similar numbers with an incidence of sepsis that increased from 83 per 100,000 population to 275 per 100,000 during 1979 to 2003 (12). Also in the following years, incidence of severe sepsis increased from 200 per 100,000 in 2003 to 300 per 100,000 in 2007 (35). Higher case numbers were found by Kumar et al with a severe sepsis hospitalization incidence that increased from 143 per 100,000 in 2000 to 343 per 100,000 in 2007 (13). In a meta-analysis analyzing high-income countries during the years 2003-2015, the authors summarized, based on the previous sepsis-2 criteria, an incidence rate for sepsis of 437 per 100,000 people, and for severe sepsis of 270 per 100,000 (3). However, lower numbers of severe sepsis were found in studies in Sweden with roughly 40 per 100,000 in 2005 and in

Spain at 87 per 100,000 during 2006 to 2011 (10, 11). Data from the United Kingdom (UK) and Australia / New Zealand showed 66 and 77 ICU cases per 100,000 adult population, respectively (36, 37). One study evaluated four different epidemiological methods and found highly heterogeneous results with a sepsis incidence ranging from 300 to 1,031 cases per 100,000 population. Nevertheless, the incidence increased, and the case fatality decreased consistently in every applied method (38).

Even more uncertain is the epidemiology in developing countries, though it should be considered that some infections with septic presentation are more common in these regions. Examples include malaria in tropical regions and melioidosis in the Southeast Asian area (39). Furthermore, higher rates of human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS), and other risk factors may be more frequent in low- and middle-income countries (30). Health-care systems are less optimal equipped, medications are not sufficiently universally available, and access to hospitals may be difficult because of lacking infrastructure. Therefore, despite a typically younger population, the rate of sepsis cases is still higher. One study estimated the world-wide number of sepsis cases per year at 19 million, but only used extrapolated data from North American studies (40). Fleischmann et al calculated the "Global Burden of Sepsis" at 31.5 million cases of sepsis and 5.3 million fatalities each year (3).

In summary, despite all limitations of epidemiological studies, the incidence of sepsis and septic shock has been increasing over the last decades in high-income countries. The epidemiology of sepsis on a global scale and especially in developing countries is less clear as precise data are lacking.

### 1.1.3. Epidemiology of sepsis in the ICU

In a prospective longitudinal observational study over four weeks in Germany, 12.6% of all ICU patients had a diagnosis of severe sepsis or septic shock including those who developed sepsis during the ICU stay (21). A cohort study in Australia and New Zealand found that 20.7% of ICU patients had severe sepsis (37). The Extended Prevalence of Infection in Intensive Care (EPIC II) study, a 1-day point prevalence study in ICUs from 75 countries, found that 51% of all patients were considered infected and 71% were on antibiotic treatment. Furthermore, infected patients had a twice as high mortality than non-infected patients (41).

Around 50-69% of all severe sepsis patients need ICU care (20, 34, 42), causing that about one in every ten to twelve patients is at the ICU because of severe sepsis (32, 37). One study even found that 37.4% of patients in European ICUs had sepsis (43). All these studies therefore underline the importance and relevance of this disease for intensive care medicine.

### 1.1.4. Age and gender aspects of sepsis

Elderly patients account for the majority of sepsis patients in western countries, with >60% of all sepsis patients being ≥65 years old (10, 19). The reported age means are varying at around 60-70 years of age in several developed countries worldwide (10, 22, 36, 37, 44, 45). Regarding the sex distribution, the literature is heterogeneous, but overall, most studies report that men have similar to slightly higher rates of sepsis compared to women (9-11, 19, 21, 22, 43, 45-47).

### 1.1.5. Mortality and case fatality rates of sepsis

Over the last decades, independently of the analysis method used and despite increasing sepsis incidence, the case fatality rate has decreased. But due to the higher overall incidence the absolute number of sepsis deaths has risen (8, 20, 36, 38). In general, the mortality represents the number of deaths in a population, typically given in numbers per 100,000 persons, whereas the case fatality rate (=lethality rate) represents the number of deaths by one specific disease, i.e. death divided by total number of sick people. However, some inaccuracy in the wording is found throughout the literature.

The hospital case fatality rate decreased from 45.8% to 37.8% over the years from 1993 to 2003 (19). Similar numbers were reported by Stevenson et al with a decrease in 28-day mortality from 46.9% in 1991-1995 to 29.2% in 2006-2009. This represents a 3% annual decrease during the investigated period (48). Consistently, the ICU sepsis mortality declined from 34.3% to 30.8%, and hospital-mortality from 48.3% to 44.7%, in the years 1996 to 2004 (36). Increasing sepsis severity leads to higher mortality rates with 28-day mortality ranging from 14.9%-47.3% in severe sepsis and up to 34.3%-52.2% in septic shock (22, 44, 45, 49). When applying the sepsis-3 criteria, patients with septic shock also had a high ICU mortality of 38.9-51.9% (46, 50). Septic shock patients from the original sepsis-3 publication had an in-

hospital mortality of 42.3% (6). No specific targeted sepsis therapy was introduced in the last decades. Therefore, the decreasing case fatality rates are likely due to a better general sepsis care and management including earlier recognition, early antibiotic therapy, improved ventilation strategies with low-tidal volume mechanical ventilation, and better intensivist staffing with dedicated allocation of doctors to the ICU (30, 48).
In summary, sepsis is still a devastating disease with case fatality rates as high as 52%. To give a broader perspective, a disease that is widely discussed in media such as acute myocardial infarction has a case-fatality in ST-elevation myocardial infarction (STEMI) patients of "only" 8.1% (6, 51).

### 1.1.6. The development of sepsis definitions

Earlier studies diagnosed sepsis through the presence of shock and positive blood cultures. It was later acknowledged that not all patients who have sepsis show growth on bacterial cultures. The term "septic syndrome" was introduced in 1987 by Balk and Bone using it as the definition in their study. The criteria applied were hypo- or hyperthermia, tachycardia, tachypnea, and evidence of infection with signs of end organ dysfunction (52). Nevertheless, until 1991/1992 there was no consensus regarding the identification of potentially septic patients and studies used heterogeneous definitions and terminology.

#### Sepsis-1

In 1991/1992 the SIRS concept was introduced. It was designed to assess patients with acute injury using clinical and laboratory-chemical parameters and to describe a standard definition for future trials. SIRS was defined as an inflammatory process, independent of the origin including both infectious and non-infectious causes. SIRS criteria were positive if two or more criteria were fulfilled (28) (**Table 1**). Sepsis was defined as SIRS in the context of an infection, and the more severe subgroups are shown in **Table 2**.

**Table 1: Systemic inflammatory response syndrome (SIRS) criteria.**

| SIRS | | | |
|---|---|---|---|
| Temperature | Respiratory rate | Heart rate | White blood count* |
| >38°C<br>OR<br><36°C | >20/min<br>OR<br>paCO2 <32mmHg | >90/min | >12,000<br>OR<br><4,000<br>OR<br>>10% immature band forms |

Adapted from Bone et al 1992 (28). Note that SIRS criteria are fulfilled if ≥2 criteria are positive. * In the absence of other factors causing a derangement, e.g. chemotherapy induced neutropenia.

**Table 2: Sepsis subgroups (sepsis, severe sepsis, and septic shock) according to sepsis-1.**

| Sepsis | Severe Sepsis | Septic shock |
|---|---|---|
| SIRS + Infection | Sepsis + | Sepsis + |
| | organ dysfunction, hypoperfusion (oliguria, altered mental status, lactic acidosis, …), or hypotension | Severe Sepsis + |
| | | hypotension despite adequate fluid resuscitation (with need of inotropic or vasopressor agents) |

Adapted from Bone et al 1992 (28).

Sepsis-2

The concept of SIRS and the definition of sepsis were re-emphasized in a consensus document published in 2003 and additional criteria were added, but in majority the previous

definition remained untouched (53, 54). SIRS, despite having a low specificity, was left within the definition to provide a common language between clinicians and because of its easy usability at the bedside (55). In the 2003 publication, the term PIRO (Predisposition, Infection, Response, Organ Failure) was introduced, but was never widely adopted or implemented in clinical practice. On the other hand, the sepsis terminology was explained in more detail. Sepsis was defined as SIRS caused by an infection, and it was recommended to use the term severe sepsis for sepsis with organ dysfunction. Septic shock was defined as a state of circulatory failure with persistent sepsis-induced hypotension. Sepsis-induced hypotension was described as a systolic blood pressure <90mmHg, mean arterial pressure (MAP) <60mmHg, or a reduction of systolic blood pressure >40mmHg, each in the absence of other causes such as cardiogenic shock (53, 54).

Over the following years, SIRS was assessed in several studies. In one large study with roughly 270,000 medical and surgical patients admitted to the general ward, 47% had ≥2 SIRS criteria during the stay. The authors concluded that SIRS is unfeasible in the general ward to screen for sepsis and those parameters are extremely non-specific. However, mortality increased with the presence of SIRS on admission, thereof the respiratory criterion having the most impact on mortality rates (56). Like in the general ward, in ICU patients, SIRS was positive with ≥2 points in 93% of patients and therefore very sensitive, but non-specific as it may also be positive in patients not having an infection (57). SIRS criteria were also tested in a large cohort database in Australia and New Zealand. Out of about 1 million patients, 109,663 hat an infection with organ failure, and SIRS criteria were positive in 87.9%. Therefore, 12.1% (1 in 8 patients) were SIRS negative despite having severe sepsis defined by an infection with the presence of organ failure. Thereof, 80% had one positive SIRS criterion and 20% had zero SIRS points (58). Another study also showed that 16% of critically ill patients with infection did not have positive SIRS criteria and found that organ dysfunction was a main driver for mortality (59).

Therefore, SIRS criteria are sensitive but not sensitive enough. Furthermore, the criteria are not specific as the criteria can be positive in other diseases without infection, e.g., subarachnoid hemorrhage, acute liver failure (ALF), pancreatitis, and others (27, 28, 56, 60).

Sepsis-3: The current sepsis definition

After almost unchanged definitions since 1991, in 2016 the sepsis-3 definition was introduced by Singer et al with associated publications from Seymour et al and Shankar-Hari et al (6, 61, 62). The working group called it sepsis-3 and retrospectively named the definition from 1991/1992 as sepsis-1 and the consensus definition from 2003 as sepsis-2. The authors acknowledged that the sepsis definition may change in the future when new scientific knowledge is acquired, which then should be named sepsis-4. Sepsis according to sepsis-3 was re-emphasized as a time critical emergency, where organ and cellular dysfunction are present. Therefore, the term "severe sepsis" was removed for redundancy because sepsis itself is severe and life-threatening (6). The host's response to the infection is a major culprit for tissue and organ damage. Therefore, sepsis was "defined as life-threatening organ dysfunction caused by a dysregulated host response to infection" (6). Organ dysfunction was defined as an increase in the sequential organ failure assessment (SOFA) score by 2 or more points (**Figure 1**). If a patient has no preexisting comorbidities, then the baseline SOFA score should be given zero and the delta-SOFA, i.e. the increase, is calculated from this baseline value (6, 63). Even modest organ dysfunctions with a SOFA score of ≥2 points lead to an in-hospital mortality of >10% (6, 62).

| SOFA score | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| *Respiration* | | | | |
| $PaO_2/FiO_2$, mmHg | <400 | <300 | <200 | <100 |
| | | | —— with respiratory support —— | |
| *Coagulation* | | | | |
| Platelets $\times 10^3/mm^3$ | <150 | <100 | <50 | <20 |
| *Liver* | | | | |
| Bilirubin, mg/dl | 1.2–1.9 | 2.0–5.9 | 6.0–11.9 | >12.0 |
| (μmol/l) | (20–32) | (33–101) | (102–204) | (<204) |
| *Cardiovascular* | | | | |
| Hypotension | MAP < 70 mmHg | Dopamine ≤5 or dobutamine (any dose)[a] | Dopamine >5 or epinephrine ≤0.1 or norepinephrine ≤0.1 | Dopamine >15 or epinephrine >0.1 or norepinephrine >0.1 |
| *Central nervous system* | | | | |
| Glasgow Coma Score | 13–14 | 10–12 | 6–9 | <6 |
| *Renal* | | | | |
| Creatinine, mg/dl | 1.2–1.9 | 2.0–3.4 | 3.5–4.9 | >5.0 |
| (μmol/l) or urine output | (110–170) | (171–299) | (300–440) or <500 ml/day | (>440) or <200 ml/day |

[a] Adrenergic agents administered for at least 1 h (doses given are in μg/kg·min)

**Figure 1: Sequential organ failure assessment (SOFA) score.** From Vincent et al 1996 (64). Reproduced with permission from Springer.

The concept of the SOFA score was implemented, as during the process of the new sepsis definition, the authors investigated several databases, performed an extensive literature review, and had several expert meetings. They found that for prediction of in-hospital mortality of ICU patients, SOFA score had better area under the receiver operating characteristics (AUROC) than SIRS or quick SOFA (qSOFA) at 0.74, 0.64, 0.66, respectively (6, 62). Furthermore, it is logically better to grade organ dysfunction on a scale rather than just note the presence or absence of any organ dysfunction (64).

Septic shock is a subgroup of patients with an increased mortality, and more severe circulatory, metabolic, and cellular abnormalities (**Table 3**). Septic shock according to sepsis-3 is now defined as hypotension requiring vasopressor therapy to maintain a MAP ≥65mmHg and elevated serum lactate >2mmol/L despite adequate fluid resuscitation (6). Hypotension was defined at a MAP <65mmHg because this cutoff was most used in studies, but there is no recommendation regarding the clarification of "adequate fluid resuscitation" as there was highly heterogeneous clinical practice (6, 61). The lactate cutoff at 2 mmol/L had the highest sensitivity and identified patients at higher risk for in-hospital mortality (61).

**Table 3: Sepsis subgroups (sepsis and septic shock) according to sepsis-3.**

| Sepsis | Septic Shock* |
|---|---|
| Suspected infection + | Sepsis + |
| Increase of ≥ 2 points in SOFA score | Vasopressor therapy to maintain a MAP ≥65mmHg |
| | Lactate >2mmol/L |
| | Adequate fluid resuscitation |

Note that the term "severe sepsis" is no longer used. *For septic shock all three additional criteria must be fulfilled. Adapted from Singer et al 2016 (6).

As a side note, outside the ICU, for in-hospital mortality prediction the AUROC of qSOFA at 0.81 was higher than the SOFA score and SIRS at 0.79 and 0.76, respectively. The qSOFA is easier to obtain than the SOFA score and does not require any laboratory results. Therefore, outside the ICU, qSOFA should be used. The three criteria for qSOFA are increased respiratory rate ≥22/min, altered mental state, and systolic blood pressure ≤100mmHg.

Patients with qSOFA ≥2 had a 3- to 14-fold increase for in-hospital mortality, and a score of at least 2 points is therefore considered positive qSOFA. In patients with a qSOFA of 1 point, measuring a lactate of ≥2mmol/L may identify patients with a higher mortality risk (62). However, qSOFA was not developed as a tool for sepsis screening. Nevertheless, a positive qSOFA should prompt clinicians to evaluate the patient for a possible infection and sepsis.

### 1.1.7. The role of definitions for the diagnosis of sepsis

The sepsis definitions even in its most recent version are not meant as screening tools. For instance, qSOFA was tested and analyzed in patients with suspected infection but was not used as a tool to screen for sepsis. Especially the altered mental status criterion with Glasgow Coma Scale (GCS) <15 may identify patients with pre-existing diseases such as dementia (62). SIRS may be helpful in identifying potential sepsis patients, but even when positive, the host response to infection can be adequate (63). In addition, SOFA score calculation is time-consuming, and the SOFA score is used for classification and prognosis, but not for screening purposes. Furthermore, the new definition for sepsis and septic shock may be more restrictive in including patients, therefore leading to a sicker population. This also argues against the use of these tools for screening and early identification (65). Unfortunately, there is no definitive diagnostic test for sepsis and clinical symptoms may be subtle or mimic other diseases (66, 67). Furthermore, a pathogen in blood cultures can only be identified in a fraction of patients and is therefore not included in the definition of sepsis. A positive finding in blood cultures is, depending on the pathogen, called bacteremia, viremia, or fungemia, but may not automatically be considered as sepsis (10, 20, 21, 43, 68). Therefore, taken all these aspects in account, biomarkers are needed to allow for early sepsis recognition and therefore enabling early sepsis therapy and management. However, clinical and public awareness of this disease must also be improved, e.g. by the world sepsis day on 13$^{th}$ of September each year (5).

### 1.1.8. Management of patients with sepsis

Before potential biomarkers are discussed, a short overview about sepsis management and therapy is given in this paragraph. Nevertheless, the whole concept and considerations in treatment strategies would go beyond the scope of this dissertation.

The most important aspect is early recognition allowing for early treatment. Sepsis management is based on general supportive measures including hemodynamic stabilization, ventilation strategies, fluid resuscitation, source control such as drainage of abscesses, microbiological diagnostics, and early empiric broad-spectrum antimicrobial therapy based on severity, host factors and depending on local resistance patterns (14, 15, 30, 69-71). These are the columns and backbone of sepsis therapy. About 20 years ago, an early goal directed therapy (EGDT) was recommended and considered the gold standard in ICU sepsis management with optimization of central venous pressure (CVP), MAP, urine output, and central venous oxygen saturation (ScvO2) (72). However, this EGDT bundle did not prevail as large studies such as Protocolized Care for Early Septic Shock (PROCESS), Protocolized Management in Sepsis (PROMISE), and Australasian Resuscitation in Sepsis Evaluation (ARISE) did not show any mortality benefits. However, general sepsis management has improved over the last decades which may have diluted potential effects. In addition, the most important difference between groups was only in regard to the ScvO2 and CVP targets (73-75). One important keystone of sepsis therapy are antimicrobial substances. Early application of antibiotics is essential and delays in administration are associated with worse outcome, especially in septic shock patients (70, 76-78). Several investigated targeted sepsis therapies unfortunately have failed, and no specific anti-sepsis treatment had withstood the test of time. Furthermore, despite intensive scientific efforts no new drugs ("magic bullets") have been introduced that directly improved outcome of sepsis patients. As an example, the promising substance recombinant activated protein C was part of sepsis therapy for several years but was withdrawn from the market after no benefit was shown in a following study (79-82). Several other studies investigated immune-modulatory substances but failed to show a survival benefit (83, 84). However, inappropriate timing and the inability to achieve therapeutic drug levels may partially explain the negative results of the studies. In addition, the heterogeneity of sepsis patients in large trials may lead to a low signal-to-noise ratio, i.e. a reduced possibility to detect a survival benefit (30). The removal of cytokines using adsorbent filters has, despite initial positive results in case reports and series, been found to be associated with an increased mortality during sepsis (85, 86). Other extracorporeal techniques such as high-volume dialysis have also been investigated but did not improve sepsis outcomes (87). Therefore, the supportive measures in sepsis are essential and time-critical, and early recognition of septic patients is of utmost importance.

### 1.1.9. "Classic" biomarkers for the diagnosis of sepsis

Sepsis leads to severe derangements in biomarkers associated with the inflammatory response. Classical biomarkers include total white blood count (WBC, as used in the SIRS criteria), C-reactive protein (CRP), and procalcitonin (PCT) (63). An optimal sepsis biomarker should have several properties, most importantly, differentiating between infection and inflammation. Furthermore, it should have fast kinetics, high sensitivity and specificity, low turnaround times, and low costs (30). The well-known and widely adopted surviving sepsis campaign (SSC) only mentions, apart from lactate, PCT as a biomarker. PCT may be used for the discontinuation of antibiotics, but the SSC discourages to decide antibiotic initiation based on PCT (88, 89). Also, in the guideline for community-acquired pneumonia by the American Thoracic Society and Infectious Diseases Society of America only PCT as a biomarker is mentioned, including a remark that it cannot rule out bacterial pneumonia (90). The classic biomarkers CRP, PCT and WBC furthermore only partially predict mortality and outcome in sepsis. CRP, detected as early as in 1930, is synthesized in hepatocytes as an acute phase protein and is part of the pentraxin group. It has slow kinetics reaching peak values after 24 to 48 hours and may be increased both from infectious and non-infectious triggers (91-93). Due to the delayed response of CRP and the potential non-infectious activators, CRP is not considered a clinically relevant biomarker in sepsis diagnosis. PCT is a prohormone of calcitonin and mainly secreted by thyroidal C-cells, but also by adipocytes and other tissues. It typically reaches peak values at around 10-24 hours after disease onset (94, 95). A meta-analysis evaluating PCT to differentiate sepsis from non-infectious SIRS in critically ill adults calculated a sensitivity of 77% and specificity of 79% with large heterogeneity in studies. AUROC results were better for surgical compared to medical patients (96). One study even showed increased rates of organ dysfunction with a PCT based escalation strategy (97). In another study, 14.9% of patients with infections *a posteriori* confirmed by infectious disease specialists had a PCT <0.25µg/L and 33.8% of those with a PCT >1µg/L had no infection at all. The overall AUROC for the differentiation between infection and no infection was 0.69. Furthermore, the antibiotic consumption was not reduced in the PCT group (98). However, in the Procalcitonin to reduce antibiotic treatments in acutely ill patients (PRORATA) trial, a PCT guided starting / stopping strategy was non-inferior with regard to mortality but reduced the days on antibiotic therapy (99). Nevertheless, PCT may be elevated not only in patients with infection or sepsis, but also in severe trauma or other conditions with high cytokine load

(95, 100). Over time, several other biomarkers have been tested including soluble triggering receptor expressed on myeloid cells (sTREM) and Cluster of Differentiation 64 (CD64) on activated neutrophils assessed by flow cytometry (101-103). For different reasons, these markers have not found widespread adoption. In a large investigation from Pierrakos and Vincent, 178 biomarkers in sepsis were examined, but most biomarkers failed to carry out a relevant role in clinical routine, especially due to the large overlap between sepsis and non-infectious inflammation (104).

In summary, conventional biomarkers such as CRP and PCT are not able to diagnose sepsis. On the other hand, lipoproteins such as the high-density lipoprotein (HDL) have properties as part of an innate immune response that may be relevant during infections. However, lipoproteins are rarely mentioned in sepsis publications or guidelines.

## 1.2. Lipoproteins

### 1.2.1. The structure and classification of lipoproteins

The five major lipoproteins are chylomicrons, very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL) and HDL, and they can be, as the name describes, separated by density. The density is determined by the lipid to protein ratio. The reported density of HDL is 1.063-1.210 g/mL with HDL2 at 1.063-1.125 and HDL3 at 1.125-1.210 g/mL typically isolated by ultracentrifugation (105-107). However, HDL subfractions can be detected by gel electrophoresis or nuclear magnetic resonance (NMR) (107). Lipoproteins consist of a phospholipid outer layer and a hydrophobic lipid core, which contains triglycerides (TG) and cholesteryl ester. Cholesterol and its storage form, the cholesteryl ester, are water-insoluble, whereas phospholipids are water-soluble. All lipoproteins basically contain the same lipids including phospholipids, TG, and cholesterol, but the concentrations are vastly different. Apolipoproteins stabilize the lipoproteins, can activate or inhibit enzymes, and direct lipoproteins to specific tissues and organs. Apolipoprotein A-I (ApoA1) is the major apolipoprotein of HDL, but other apolipoproteins such as Apolipoprotein A-II (ApoA2) or C (ApoC) can also be found. ApoB-100 is located on VLDL, IDL, and LDL whereas ApoB-48 is found on chylomicrons. Apolipoprotein E (ApoE) is a ligand for LDL-receptor binding and numerous other apolipoproteins exist, of which the

exact function is not entirely understood (105, 108, 109). Several mutations in apolipoproteins have been described. As an example, ApoA1 null alleles lead to a deficiency of HDL, while other mutations such as ApoA1-Milano were thought to lead to an enhanced anti-atherogenic potential of HDL (110-112).

### 1.2.2. The synthesis of HDL, reverse cholesterol transport and associated enzymes

Lipid-free or lipid-poor ApoA1 secreted from liver or small intestine take up phospholipids, unesterified cholesterol and additional apolipoproteins which leads to the formation of discoidal pre-beta1-lipoproteins, the so-called nascent HDL (**Figure 2**) (113, 114). ApoA1 is the essential apolipoprotein for HDL formation (115). Cholesterol efflux to poorly lipidated ApoA1 is mainly mediated by ATP (Adenosine triphosphate)-binding cassette transporter 1 (ABCA1) (114, 116). The unesterified cholesterol is modified to cholesteryl ester by lecithin-cholesterol acyltransferase (LCAT), an HDL-associated enzyme. As cholesteryl ester are more hydrophobic than cholesterol, they move to the inner lipid core. Over time this process causes a structural change from discoidal pre-beta1-lipoprotein particles to spherical alpha-HDL3. These particles further take up cholesterol from the periphery mainly via ATP-binding cassette sub-family G member 1 (ABCG1) and 4 (ABCG4), by spontaneous transfer, and to a lesser extent by scavenger receptor B1 (SR-B1), leading to development of mature alpha-HDL2. Alpha-HDL2 is taken up into the liver or steroidogenic tissues via SR-B1 (106, 114, 117, 118). Cholesteryl ester transfer protein (CETP) transfers TG from ApoB-containing lipoproteins to HDL in exchange for cholesteryl ester. These ApoB-lipoproteins can then be taken up via the LDL receptor in the liver or other tissues (119, 120). On the other hand, phospholipid transfer protein (PLTP) can transfer phospholipids and free cholesterol from TG-rich lipoproteins to HDL providing substrates for LCAT and therefore enhancing conversion from HDL3 to HDL2. The preferred substrate of hepatic lipase (HL) are TG-rich particles. HL promotes hydrolyzation of TG and phospholipids in HDL and IDL. The hydrolyzation of HDL associated TG is further escalated by an increase of TG in HDL through CETP. The TG hydrolyzation then leads to smaller HDL particles (117, 121, 122). Another important plasma enzyme is endothelial lipase (EL), which predominantly splits phospholipids of HDL therefore reducing HDL size, enhancing faster catabolism and consequently leading to decreased HDL levels (123-125). All the above-mentioned associated

enzymes, as well as interactions with other lipoproteins and receptors, lead to a dynamic remodeling and changes in HDL particle content throughout the body.

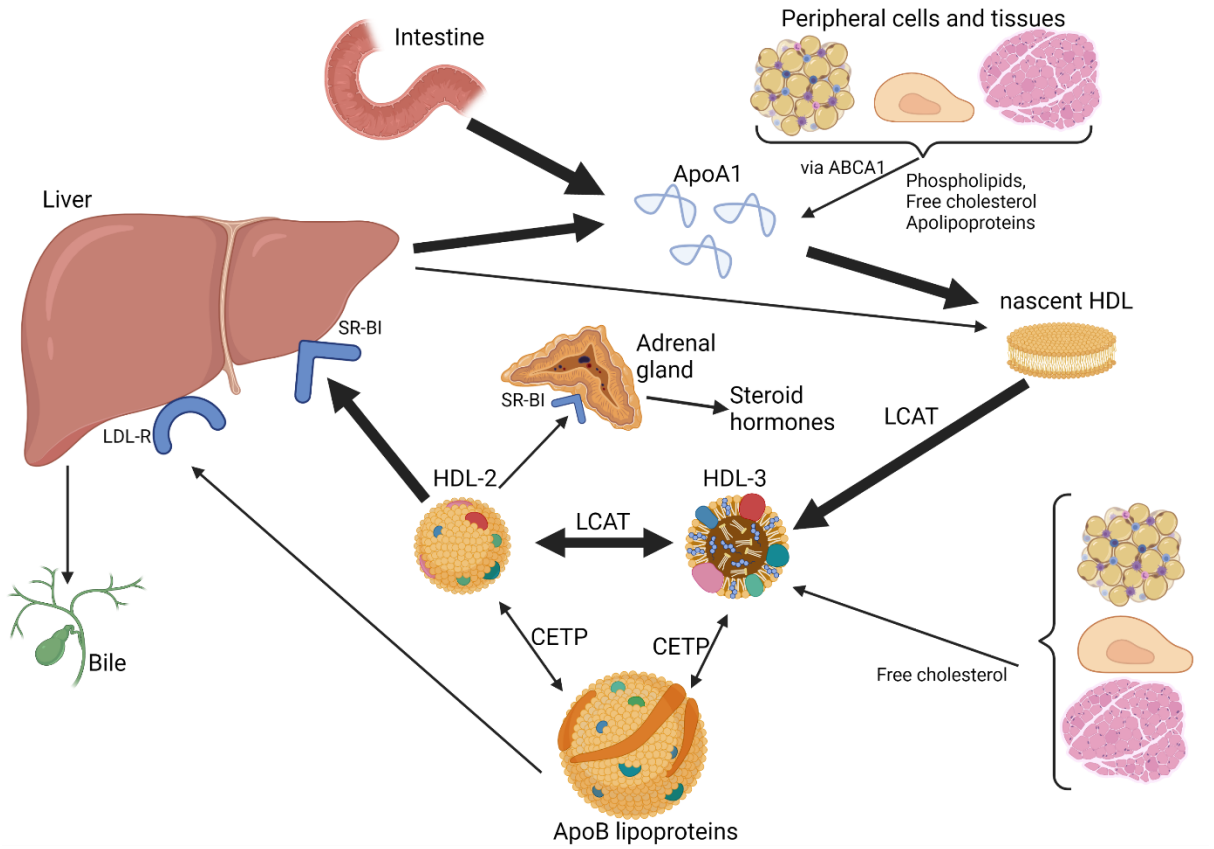


**Figure 2: The HDL cycle.** From lipid-poor ApoA1, to peripheral cholesterol uptake, esterification, and return to the liver. See main text for more details. Own creation by the author of this dissertation. Created with BioRender.com - Permission obtained from Biorender.com.

### 1.2.3. HDL particles ("HDL-P") compared to HDL cholesterol (HDL-C)

The commonly reported HDL cholesterol (HDL-C) is the amount of total cholesterol within the HDL particles, including both the amount of cholesterol and cholesteryl ester. It was strongly considered that HDL-C concentrations may be causative for cardiovascular disease (CVD) risk (126-128). However, recent knowledge emerged that HDL-C, despite being strongly correlated, is not a causative risk factor for CVD. HDL-C may only act as a surrogate marker for an individual risk. Thus, increasing HDL-C levels to prevent CVD and improve

outcomes has failed in several studies (129-132). Furthermore, Mendelian randomization studies did not show an effect of genetically determined increased HDL-C on CVD risk reduction (133, 134). In addition, some mutations such as the SR-B1 loss-of-function lead to higher HDL-C levels but at the same time to a higher risk of coronary heart disease (135). However, it should be noted that some effect regarding CVD risk reduction with increasing HDL-C was observed in two studies, but this effect may be partially explained by changes in LDL levels and particle numbers (136-138). Furthermore, a large study that investigated niacin to raise HDL-C levels in addition to intensive statin therapy found no beneficial effects with regard to cardiovascular endpoints despite successful increase in HDL-C (139). Another study investigating the addition of niacin to statin therapy found an increase in HDL-C but no effects on major vascular events and even reported an increase in serious adverse events (140). Several studies furthermore found a U-shaped curve regarding HDL-C and all-cause or cardiovascular mortality (141-143). As an example, Madsen et al found the lowest mortality at HDL-C levels of 73 mg/dL in men and 93 mg/dL in women (143). The reason why high HDL-C is not beneficial and even may be harmful is uncertain. Hypotheses include the presence of genetic variants (mutations) that lead to higher HDL-C levels but simultaneously cause other diseases, or the HDL functionality may be altered (130, 143). It is now considered that the functionality of the HDL particle is much more important than the sole quantity of HDL-C, and that cholesterol efflux capacity (CEC) of HDL is a predictor for CVD risk (144, 145). HDL-C is furthermore a poor estimator of HDL particle size and number, and for the composition of HDL particles (146). While in LDL one ApoB is found per particle and thus the ApoB levels are highly correlated with LDL particle number; the evaluation is more difficult for HDL. The proteomic diversity of HDL is much broader, and despite ApoA1 being the main apolipoprotein on HDL, the number of ApoA1 per HDL particle can usually range from one to four copies, but up to ten copies have been found (147, 148). Furthermore, HDL can contain very little lipid content (small lipid-poor HDL particles) or up to 50% of its particle mass (large lipid-rich HDL particles). HDL-C would rely mostly on the lipid-rich particles, and smaller particles such as pre-beta-HDL would be underestimated (147, 149). HDL particle number can be measured directly by NMR or calibrated ion mobility analysis and was found to be a better predictor for CVD than HDL-C (148, 150).

To summarize, HDL-C measurement alone does not adequately represent the multi-faceted functions of HDL particles.

### 1.2.4. The amount of HDL-C and its role during the acute phase

Reduced levels of total cholesterol and HDL-C have been associated with infections and mortality but may also predispose to infection (151-154). In one study, HDL-C on admission was a risk factor for sepsis development; per 1mg/dL HDL-C increase the risk of severe sepsis decreased by 3% (154). Reduced total cholesterol levels also lead to higher rates of surgical site infections and longer hospital stays (153). In one study, 17.5-fold increased mortality odds were found for hospitalized patients with HDL-C < 20mg/dL compared to those with HDL-C >65mg/dL (155). Interestingly, infection rates are increased in people with low HDL-C but are also increased in those with very high HDL-C levels leading to a U-shaped curve (156). A vast number of studies have found that lipoproteins decrease in the acute phase resulting in low cholesterol, low HDL-C, and low apolipoproteins levels (157-161). However, heterogeneous results were found for the association of disease severity, e.g. the rate of organ dysfunction or ICU length of stay (LOS), with HDL-C, total cholesterol, or apolipoprotein levels. Furthermore, varying results were found for the association of decreased (apo-) lipoproteins with increased mortality during sepsis (162-166). For instance, Chien et al found an increased rate of death in association with reduced HDL-C levels, while Lee et al were not able to demonstrate this effect (162, 165). Explanations for decreased HDL-C levels during the acute phase include acute consumption, decreased liver synthesis, elevated levels of EL and secretory phospholipases A2, decreased LCAT activity, and increased removal via the SR-B1 receptor in the liver and adrenal gland (164, 167). Furthermore, the HDL composition dramatically changes during inflammation. Serum amyloid A (SAA) levels increase and ApoA1, usually the most abundant apolipoprotein on HDL, can partially be replaced by SAA. ApoA1 is the key activator of LCAT and decreased ApoA1 therefore may contribute to reduced HDL-C levels during sepsis (161, 168, 169).

### 1.2.5. The function of HDL

HDL-C and HDL are best known for their role in reverse cholesterol transport, and their role in atherosclerosis commonly found in people consuming western diet (170). However, from an evolutionary biological perspective, HDL did not solely emerge to compensate for excessive amounts of cholesterol but was most likely also an important part of the innate immune response. HDL particles have pleiotropic effects for the body's defense system

(**Figure 3**). HDL can bind and neutralize lipopolysaccharides (LPS) found on gram-negative bacterial cell walls. While LPS binding is performed by all lipoproteins, the strongest effect was found for HDL (171). Furthermore, the cytokine production caused by endotoxins is mitigated (106, 172-174). Similar to gram-negative bacteria, lipoteichoic acid (LTA) from gram-positive bacteria can be neutralized by HDL (175). In addition, HDL stabilizes the barrier by inhibiting endothelial cell apoptosis. HDL-associated sphingosine-1-phosphate activates the endothelial nitric oxide synthase (eNOS) via sphingosine-1-phosphate (S1P3) receptor 3 and SR-B1 in endothelial cells. Moreover, HDL promotes prostacyclin formation leading to reduced vascular tension. HDL reduces platelet activation, adhesion molecule expression such as vascular cell adhesion molecule (VCAM), E-selectin, and intercellular adhesion molecule (ICAM), and suppresses chemokines (105, 176-180). Furthermore, antioxidant and anti-inflammatory properties are also modulated by HDL, and these effects include the prevention of HDL or LDL oxidation. Oxidized lipoproteins are less effective in mediating the above-mentioned effects and have lower anti-inflammatory properties. The antioxidant and anti-inflammatory effects of HDL are in part driven by the HDL associated enzyme paraoxonase (PON) 1 (181, 182). For instance, in mice that are PON1 deficient, HDL is unable to prevent LDL oxidation (181). PON1 is synthesized in the liver and is associated with the HDL particle in circulation (183). It can hydrolyze lipid peroxides including oxidized phospholipids (179, 184). However, the HDL is a highly dynamic particle, and the local environment is essential to establish strong effects of PON1. Factors influencing the effectivity include other associated apolipoproteins and enzymes such as ApoA1 and LCAT (183). Overall, almost 100 proteins have been found to be associated with the HDL particle, although, most of those proteins are found with only one or less copies per HDL particle (107). Apart from the bacterial defensive role, HDL can bring cholesterol to the adrenal gland for steroid synthesis. In mice, uptake into the adrenal gland is mediated via the SR-B1 receptor and may be increased during times of stress. In humans, both LDL and HDL contribute to cholesterol delivery to the adrenal gland (185-187).

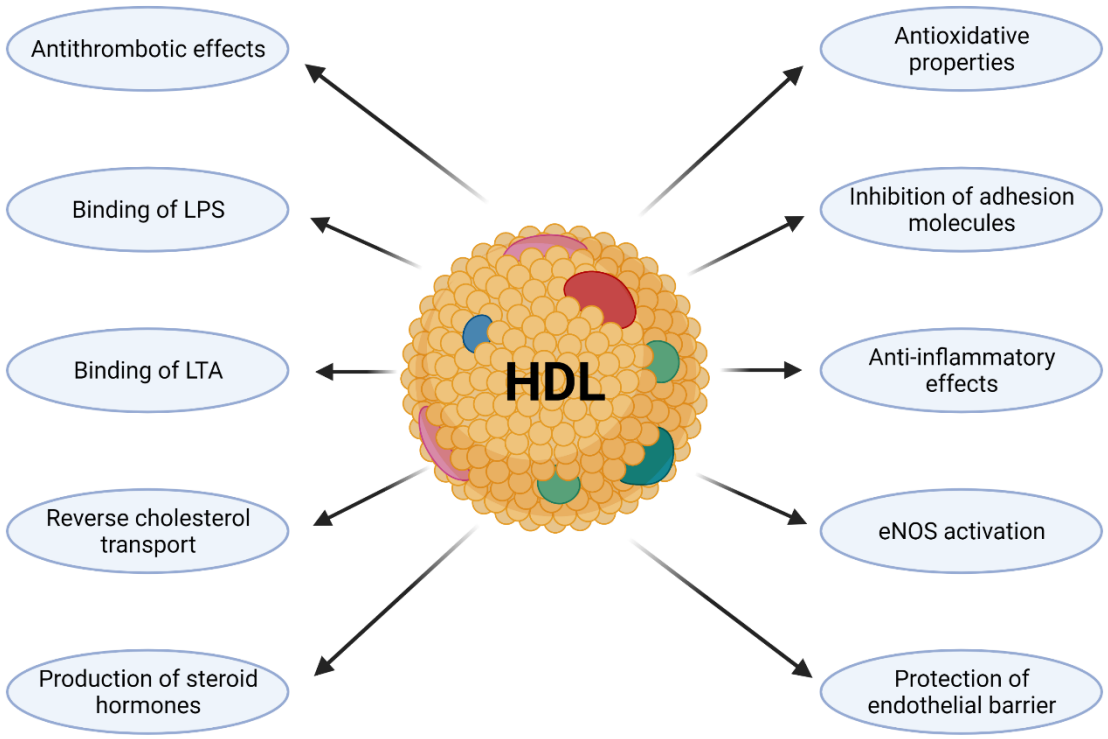


**Figure 3: Functions of HDL.** See main text for more details. Own creation by the author of this dissertation. Created with BioRender.com - Permission obtained from Biorender.com.

## 1.3. Metabolomics

Proton nuclear magnetic resonance ($^1$H NMR) spectroscopy is an emerging technique in medical studies. A sample is exposed to an external magnetic field ($B_0$) in a super conducting magnet. This results in the nuclei to align along the magnetic field and precess around this axis. The hydrogen nucleus is present in almost all biological samples and is usually just called the "proton". The sample is stimulated with radiofrequency pulses to generate coherence between spins and to allow for a precession out of the direction of the main magnetic field to occur. The frequency must match the Larmor frequency – therefore the name "resonance" in NMR. In addition, at resonance frequency a spin inversion of nuclei happens. After stimulation, nuclei relaxation occurs and the energy, which was adsorbed during the resonance stimulation, is released during return to the original energy level.

However, most importantly, the precession of the nuclei leads to an induction of a current in a detection coil. The return of the nuclei to re-align with the main magnetic field $B_0$ leads to an oscillating wave called free-induction decay. This predictable unique spectroscopic pattern is then processed by Fourier transformation and is used to identify the molecule by the association of the detected resonance frequencies and its chemical structure (188, 189). Individual protons within a molecule show different chemical shifts / resonance frequencies depending on their chemical environment, i.e. the number and distribution of electrons around these protons, which enables the identification and quantification of individual metabolites within complex mixtures ("finger-printing"). With $^1$H NMR numerous metabolites may be measured at the same time, which are called metabolomic profile or metabolome (190). The genome, transcriptome, and proteome are displayed within the metabolome. The metabolome also represents influencing factors from the environment allowing to obtain broad information regarding the patient's metabolic status at a given timepoint (190). $^1$H NMR can be used to measure and quantify lipoproteins and metabolites with high reliability. Metabolomics can be used for targeted analyses of specific metabolites or can be applied in an untargeted fashion, the so-called global metabolomics approach, to identify previously unknown relevant metabolites. Hundreds to thousands of metabolites are detected in these unbiased methods, and differences between two groups, e.g. survivors and non-survivors can be investigated (191). NMR metabolomics has little sample preparation effort, and the robustness and reproducibility are very high (188, 190). NMR is non-destructive to the sample and quantitative results are provided; however, sensitivity is lower than other methods such as gas chromatography mass spectrometry (191). Good communication and interaction between laboratory NMR specialists and clinicians are necessary to choose the optimal protocol including magnetic field strength, acquisition temperature, pulse sequences, and to provide correct processing of obtained spectra.

## 2. Rationale & Fields of scientific uncertainty

Despite innumerable publications on the role of HDL-C in atherosclerosis and cardiovascular disease, data are still sparse in ICU patients. Furthermore, the role of HDL still needs to be investigated during times of infection and sepsis, especially in critically ill patients admitted to the ICU. The protective value of HDL as part of the innate immune system may not only depend on the amount of HDL-C but lay more on the functionality of HDL particles, particularly regarding its anti-inflammatory or antioxidative properties. Furthermore, detailed pathophysiological investigations in sepsis patients may offer the potential to identify lipoproteins and associated proteins as well as metabolites that are linked with prognosis of ICU sepsis patients and may be suitable as a therapeutic target in the future.

## 3. Aims of the study

The aim of the study was to investigate quantitative and qualitative lipid parameters in patients admitted to the ICU for sepsis and septic shock, and to examine differences between ICU sepsis and ICU non-sepsis patients. Lipoproteins are explored for the association with the SOFA score, and for the prognostic potential regarding ICU- and 28-day mortality.

# 4. Material & Methods

Parts of these methods were previously published (1, 2).

## 4.1. Study population, Inclusion and Exclusion criteria, Definitions

In this study, we prospectively recruited adult patients with sepsis and septic shock who were admitted to the medical ICU at the Department of Internal Medicine at the Medical University of Graz, Austria. Exclusion criteria were age above 100 years, patients in palliative or comfort terminal care only, pregnancy, and/or a diagnosis of AIDS.

For sepsis definition the description presented in the currently valid sepsis-3 publication were used. Therefore, sepsis was recognized as a suspected infection by the treating physician and an increase in the SOFA score by 2 points or more (6). The difference in SOFA score was calculated from the baseline SOFA, which was inferred from previous hospital electronic medical records. A SOFA score of zero was applied if no pre-existing conditions were present. In addition, the definition according to sepsis-3 for the subgroup of septic shock was used. Patients had to fulfill criteria for sepsis and additionally needed to have an elevated lactate level above 2 mmol/L, and a necessity for vasopressor therapy to maintain a MAP equal or above 65 mmHg, despite adequate fluid resuscitation and/or the absence of hypovolemia. In addition, we recruited a cohort of consecutive adult patients who were admitted to the medical ICU but did not have sepsis or bacteremia at the time of sample procurement.

## 4.2. Ethical considerations

The study protocol and amendments were approved by the local Institutional Review Board (IRB), the Ethics committee of the Medical University of Graz, Austria (study protocol number 30-258 ex 17/18). The study was registered in a World Health Organization (WHO) approved Trials Registry, the German Clinical Trials Register (DRKS-ID # DRKS00015315). The study complied with the Declaration of Helsinki. From all conscious patients, written

informed consent (IC) was acquired. In patients comatose on admission, IC was obtained when regaining consciousness.

## 4.3. Pre-specified analyses

The primary objective of the study was to assess the rate of dyslipidemia (definition used in this study: HDL-C < 40mg/L). Further endpoints were the correlations between lipoproteins and SOFA score, associations of lipoproteins and other lipid parameters with ICU- and 28-day mortality, and the differences of lipoproteins and other lipid parameters between patients in the ICU sepsis and those in the ICU control cohort.

## 4.4. Laboratory measurements

Routine laboratory parameters including complete blood cell count, levels of creatinine, bilirubin, CRP, PCT, albumin, total cholesterol, HDL-C, TG, ApoA1, and ApoB were measured in the clinical laboratory of the Medical University of Graz on a Sysmex (Sysmex Austria GmbH, Vienna, Austria), Cobas (Roche Diagnostics, Mannheim, Germany), or BN II analyzer (Siemens Healthcare Diagnostics, Vienna, Austria), as applicable. For additional analyses, blood samples were centrifuged for 10 minutes at 3000 rpm, and stored in aliquots at -80°C.

## 4.5. Lipoprotein measurements

### 4.5.1. ApoB-depleted serum

To create the ApoB-depleted serum, 40µL polyethylene glycol (20% in 200 mmol/L glycine buffer) was added to 100µL of serum, leading to precipitation of ApoB-containing lipoproteins. After 20 minutes room-temperature incubation and subsequent centrifugation with 9,703g for 20 minutes at 4°C, the HDL-containing supernatants, which correspond to the ApoB-depleted serum, were collected. In these ApoB-depleted sera, qualitative HDL metabolism parameters were measured in batch.

### 4.5.2. Measurement of Arylesterase activity (AEA)

To measure Ca2+-dependent AEA of PON, a photometric assay with phenylacetate as a substrate was used. Details have been described previously (192). 200µL of buffer (100mmol/L Tris, 2mmol/L CaCl2 (pH 8.0), 1 mmol/L phenylacetate) were mixed with ApoB-depleted serum. In parallel, blanks containing buffer without ApoB-depleted serum were measured for baseline acquisition. All samples were measured as duplicates. The rate of phenylacetate hydrolysis was observed at a wavelength of 270nm in a photometer with measurements every 15 seconds to create a kinetic plot. Activity was then calculated through the molar extinction coefficient of 1,310 L $mol^{-1}$ $cm^{-1}$ according to the Beer–Lambert law.

### 4.5.3. Measurement of Cholesterol efflux capacity (CEC)

The CEC was measured using a previously published method (144, 192). J774 cells, which are a murine macrophage cell line, were plated on a well and labeled with 1 µCi/mL [$^3$H]cholesterol (Perkin Elmer, Boston, MA, USA) for 24 hours. All measurements were performed in the presence of an acyl CoA cholesterol acyltransferase (ACAT) inhibitor, to avoid cholesterol uptake into cell walls. A concentration of 2µg/mL of the ACAT inhibitor Sandoz 58-035 (Sigma, Darmstadt, Germany) was used. As the J774 cells have low ABCA1-levels, but ABCA1 is particularly necessary for cholesterol efflux from macrophages, cyclic adenosine monophosphate (cAMP) was used to upregulate ABCA1. Serum-free Dulbecco's modified Eagle's medium (DMEM) containing 0.3 mmol/L 8-(4-chlorophenylthio)-cAMP (Sigma, Darmstadt, Germany) was applied for 6 hours. The cAMP addition leads to an increase of ABCA1-dependent cholesterol efflux to about 40%, which is an about 3-fold increase. In addition, a cholesterol efflux via SR-B1 accounts for 10% and passive diffusion for about 50% of CEC (193). After the labeling time, cells were rinsed three times to remove non-cell associated [$^3$H]cholesterol. The cells were then incubated with 2.8% ApoB-depleted serum for 4 hours as published by Khera et al (144) to allow for [$^3$H]cholesterol efflux measurement. For that purpose, 100µL from the cell supernatant was removed and the effluxed [$^3$H]cholesterol was determined using liquid scintillation counting. A serum control was measured on each plate to correct for the possibility of an interassay variation between plates. Values of sera were normalized to this value. All samples were measured in duplicates. To assess the percentage of cholesterol effluxed from cells and to determine total cell

associated [$^3$H]cholesterol, cells not exposed to serum were lyzed using isopropanol to extract the radioactive cholesterol from within the cells.

## 4.6. Metabolomic measurements

All metabolomic analyses including a targeted and untargeted approach were performed in batch after the recruitment period.

### 4.6.1. Reagents for metabolomic analyses

Methods published in Reisinger et al 2021 (2), reproduced with permission from Nutrients: "Sodium phosphate, dibasic ($Na_2HPO_4$), sodium hydroxide (NaOH), hydrochloric acid (HCl, 32% m/v), and sodium azide ($NaN_3$) were obtained from VWR International (Darmstadt, Germany). 3(trimethylsilyl) propionic acid-2,2,3,3-$d_4$ sodium salt (TSP) was obtained from Alfa Aesar (Karlsruhe, Germany). Deuterium oxide ($D_2O$) was obtained from Cambridge Isotopes laboratories (Tewksbury, MA, USA). Deionized water was purified using an in-house Milli-Q Advantage Water Purification System from Millipore (Schwalbach, Germany). All chemicals were used without further purification. The phosphate NMR buffer solution was prepared by dissolving 5.56 g of anhydrous $Na_2HPO_4$, 0.4 g of TSP, and 0.2 g $NaN_3$, in 400 mL of $D_2O$ and adjusted to pH 7.4 with 1M NaOH and HCl. Upon addition of $D_2O$ to a final volume of 500 mL the pH was re-adjusted to pH 7.4 with 1M NaOH and HCl."

### 4.6.2. Lipoprotein quantification using NMR

Methods published in Reisinger et al 2021 (2), reproduced with permission from Nutrients: "Blood serum lipoproteins were analyzed on a Bruker 600 MHz Avance Neo NMR spectrometer using the Bruker IVDr lipoprotein subclass analysis protocol. Serum samples were thawed, and 330 µL of each sample mixed with 330 µL of Bruker serum buffer (Bruker, Rheinstetten, Germany). The samples were mixed gently, and 600 µL of the mixed sample were transferred into a 5 mm SampleJet rack tube (Bruker, Rheinstetten, Germany). Proton spectra were obtained at a constant temperature of 310 K using a standard nuclear Overhauser effect

spectroscopy (NOESY) pulse sequence (Bruker: noesygppr1d), a Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence with presaturation during the relaxation delay (Bruker: cpmgpr1d) to achieve water suppression, and a standard 2D J-resolved (JRES) pulse sequence (Bruker: jresgpprqf) (190). Data analysis was carried out using the Bruker IVDr Lipoprotein Subclass Analysis (B.I.LISA$^{TM}$) method."

### 4.6.3. Metabolic quantification using NMR

Methods published in Reisinger et al 2021 (2), reproduced with permission from Nutrients: "To remove proteins and to quench enzymatic reactions in the samples, 200 µL serum was mixed with 400 µL methanol and stored at -20°C for 1 hour until further processing. Afterwards the samples were spun at 17,949 rcf at 4°C for 30 minutes. Supernatants were lyophilized and 500 µl of NMR buffer in $D_2O$ were added to the samples and transferred to 5 mm NMR tubes. All NMR experiments were performed at 310 K on an AVANCE™ Neo Bruker Ultrashield 600 MHz spectrometer equipped with a TXI probe head and processed as described previously (194). The 1D CPMG (Carr-Purcell-Meiboom-Gill) pulse sequence (cpmgpr1d, 512 scans, 73,728 points in F1, 11,904.76 HZ spectral width, 512 transients, recycle delays 4 s) with water suppression using pre-saturation, was used for $^1H$ 1D NMR experiments. Bruker Topspin version 4.0.2 was used for NMR data acquisition. The spectra for all samples were automatically processed (exponential line broadening of 0.3 Hz), phased, and referenced using TSP at 0.0 ppm using Bruker Topspin 4.0.2 software (Bruker GmbH, Rheinstetten, Germany). Spectra pre-processing and data analysis have been carried out using the state-of-the-art data analysis pipeline proposed by the group of Prof. Jeremy Nicholson at Imperials College London using Matlab® scripts and MetaboAnalyst 4.0 (195). NMR data were imported to Matlab® vR2014a (MathWorks, Natick, MA, USA), regions around the water, TSP, and remaining methanol signals excluded, and probabilistic quotient normalization (196) was performed to correct for sample metabolite dilution; and reported concentrations correspond to normalized concentrations."

## 4.7. Statistical analyses

All conventional statistical analyses were completed with GraphPad Prism 8.0 (GraphPad Software, San Diego, California, USA), Stata 15.0 (Stata Corp., Houston, Texas, USA) and SPSS 26 (SPSS Inc, Chicago, IL, USA). Metabolomic statistical analyses were performed using Matlab® vR2014a (MathWorks, Natick, Massachusetts, USA) and graphically depicted with MetaboAnalyst (195). For continuous variables results were summarized using median and $25^{th}$-$75^{th}$ percentile. In categorical variables absolute values and percentages were described. The associations between groups were investigated with crosstabulations, Mann-Whitey-U-Tests, $\chi^2$-Tests, and Fisher's exact tests, as appropriate. Correlations were calculated using the Spearman rank correlation coefficient. All prognostic associations (ICU- and 28-day mortality) with lipoprotein parameters, SOFA score, and other variables, were calculated with univariable logistic regression. In multivariable analyses, the SOFA score as a variable and in addition each variable that was significantly ($p \leq 0.05$) associated with the outcome in univariable analysis were used. Kaplan-Meier functions estimators and log-rank tests were used for survival analysis. For metabolomic analyses, multivariate statistical methods were used. Principal Component Analysis (PCA), Partial Least Squares - Discriminant Analysis (PLS-DA), Orthogonal-Partial Least Squares - Discriminant Analysis (O-PLS-DA) (197) (198) were performed with associated data consistency checks and 7-fold cross-validation, expressed by $Q^2$. Formal adjustment for multiple testing in NMR analysis was performed with the Sidak correction method. Otherwise, the significance level was defined at 0.05.

# 5. Results

## 5.1. Baseline characteristics and study population

In this study, 53 sepsis patients (49% were in the more severe subgroup of septic shock) and 25 controls without sepsis or bacteremia at the time of sample acquisition were prospectively recruited (**Table 4**). These control ICU patients, e.g., patients with acute myocardial infarction, intoxications, hemorrhagic shock, cardiac arrest, acute kidney injury, and other diseases, suffered from varying degrees of inflammation without sepsis. Of the initially 27 recruited patients, two patients had to be excluded retrospectively because microbiological results showed signs of infection at the sampling time point (one patient with positive *Haemophilus influenza* polymerase-chain reaction (PCR) in broncho-alveolar fluid and lung infiltrates, and one patient with admission for non-STEMI but severe *Enterococcus faecalis* bacteremia within the same day), resulting in 25 control patients being investigated.

The sepsis group had a median age of 66 [50-75] years compared to 72 [65-79] years in the control cohort (p=0.012). Forty percent were female in the sepsis and 60% in the control cohort (p=0.144). The rates of pre-existing diabetes or liver disease, as well as intake of lipid-lowering or antidiabetic drugs were similar between the two groups. The majority of sepsis patients (85%) required catecholamine therapy with a median duration of treatment of 2 [1-7] days. The ICU and hospital LOS were 6 [3-10] and 16 [7-26] days in the sepsis cohort, respectively. In the ICU control cohort, the median ICU LOS was 3 [2-6] days whereas the hospital LOS was 15 [8-27] days. In this group 52% of patients required catecholamine therapy with a median duration of treatment of 1 [0-2] day.

In sepsis patients, the median SOFA score was 9 [7-13] points and median Simplified Acute Physiology Score III (SAPS III) was 65 [56-75] points. Most infections were community-acquired (91.6%) with only 9.4% being hospital-acquired, i.e. nosocomial. Growth of bacteria was detected in 52% of obtained blood cultures, with thereof being 30% gram-positive, 18% gram-negative, 2% mixed, 2% fungi. The main septic focus was the lungs followed by the abdomen (**Table 5**).

**Table 4: Baseline characteristics and outcomes of the study population**.

| Variable | Sepsis cohort (n=53) | Control cohort (n=25) | p-value |
|---|---|---|---|
| **Demographics & Premedication** | | | |
| Age (years) | 66 [50-75] | 72 [65-79] | 0.012 |
| Body mass index (kg/m$^2$) | 25.8 [23.4-29.8] | 27.8 [23.9-30.2] | 0.483 |
| Female sex | 21 (40%) | 15 (60%) | 0.144 |
| Anti-diabetic therapy | 12 (23%) | 8 (32%) | 0.413 |
| Statin therapy | 15 (28%) | 7 (28%) | 1.000 |
| Diabetes | 15 (28%) | 8 (32%) | 0.793 |
| Liver disease | 3 (6%) | 2 (8%) | 0.653 |
| Propofol therapy before sample acquisition | 3 (6%) | 7 (28%) | 0.010 |
| Enteral/parenteral nutrition before sample acquisition | 5 (9%) | 1 (4%) | 0.658 |
| Mechanical ventilation at sample acquisition | 22 (42%) | 9 (36%) | 0.805 |
| | | | |
| **Quantitative lipid parameters** | | | |
| HDL cholesterol (mg/dL) | 14 [7-33] | 39 [33-55] | <0.0001 |
| Triglycerides (mg/dL) | 162 [105-274] | 115 [80-145] | 0.006 |
| Total cholesterol (mg/dL) | 106 [84-130] | 114 [96-156] | 0.193 |
| LDL cholesterol (mg/dL)[a] | 57 [28-74][b] | 51 [36-77] | 0.793 |
| Apolipoprotein A-I (mg/dL) | 60 [31-90] | 103 [85-130] | <0.0001 |
| Apolipoprotein B (mg/dL) | 67 [48-84] | 66 [51-77] | 0.991 |
| | | | |
| **Qualitative lipid parameters** | | | |
| Arylesterase activity (AEA) (mM/min/mL serum) | 66.5 [40.9-89.5] | 111.2 [80.4-152.7] | <0.0001 |
| Cholesterol efflux capacity (%) | 9.2 [7.6-11.0] | 9.9 [9.1-12.7] | 0.091 |
| | | | |
| **Laboratory covariables** | | | |
| White blood count (G/L) | 14.9 [9.1-26.5] | 9.1 [6.6-13.5] | 0.011 |
| Hemoglobin (g/dL) | 10.8 [8.7-13.0] | 10.8 [8.6-13.3] | 0.672 |
| Platelets (G/L) | 164 [86-267] | 180 [133-243] | 0.312 |
| C-reactive protein (mg/dL) | 213 [119-309] | 12 [4-31] | <0.0001 |
| Procalcitonin (ng/mL) | 8.8 [1.2-35.1] | 0.2 [0.1-0.3] | <0.0001 |
| Serum bilirubin (mg/dL) | 0.9 [0.5-2.3] | 0.4 [0.3-0.8] | 0.002 |
| Serum creatinine (mg/dL) | 2.3 [1.6-4.0] | 1.2 [0.9-2.3] | 0.003 |
| Serum albumin (g/dL) | 2.9 [2.4-3.2] | 3.7 [2.4-3.2] | <0.0001 |
| | | | |
| **Illness severity and outcomes** | | | |
| SOFA score (points) | 9 [7-13] | 5 [3-9] | <0.0001 |
| Catecholamine therapy | 45 (85%) | 13 (52%) | 0.004 |
| ICU length of stay (days) | 6 [3-10] | 3 [2-6] | 0.031 |
| Hospital length of stay (days) | 16 [7-26] | 15 [8-27] | 0.940 |
| 28-day mortality | 25 (47%) | 4 (16%) | 0.011 |
| ICU mortality | 19 (36%) | 4 (16%) | 0.110 |

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Table 4 Legend (previous page): Data are reported as medians [25th-75th percentile], or absolute values and frequencies (%). [a]LDL cholesterol calculated according to the Friedewald formula. [b]49 values.

**Table 5: Distribution of the septic focus**

| Focus | n (%) |
|---|---|
| Pulmonal / Lung | 22 (41.5%) |
| Abdominal (non-genitourinary) | 9 (17.0%) |
| Urogenital tract infection / Urosepsis | 6 (11.3%) |
| Unknown / no clear focus identified | 6 (11.3%) |
| Skin / Soft tissue | 4 (7.5%) |
| Osteomyelitis and spondylodiscitis | 3 (5.7%) |
| Catheter related infection | 2 (3.8%) |
| Other | 1 (1.9%) |

The age of ICU sepsis survivors compared to ICU sepsis non-survivors was statistically not significant different (63 [46-76] vs 66 [53-73]; p=0.860). The baseline characteristics of sepsis survivors and non-survivors were mostly similar. However, non-survivors compared to survivors had higher rates of mechanical ventilation (68% vs 26%; p=0.007), higher SOFA score (13 vs 8; p=0.001), and higher rates of catecholamine therapy (100% vs 76%; p=0.040) (**Table 6**).

On the other hand, patients with septic shock compared to those sepsis patients without shock had higher SOFA score (13 vs 8; p=0.003) as representation of more severe organ dysfunction and higher rates of catecholamine therapy (100% vs 70%; p=0.004; **Table 7**). Levels of creatinine (3.1 vs 1.9 mg/dL; p=0.016) and procalcitonin (18.8 vs 2.6 ng/mL; p=0.011) were higher in patients with septic shock compared to those without shock. Patients with pre-existing diabetes mellitus were more common in those with septic shock compared to those with sepsis but not suffering from shock (42% vs 15%; p=0.035).

**Table 6: Differences between sepsis ICU survivors and non-survivors.**

| Variable | Survivors (N=34) | Non-survivors (N=19) | p-value |
|---|---|---|---|
| **Demographics & Premedication** | | | |
| Age (years) | 63 [46-76] | 66 [53-73] | 0.860 |
| Body mass index (kg/m$^2$) | 27.4 [23.5-30.6] | 24.7 [22.4-28.7] | 0.159 |
| Female sex | 12 (35%) | 9 (47%) | 0.569 |
| Anti-diabetic therapy | 8 (24%) | 4 (21%) | 1.000 |
| Statin therapy | 11 (32%) | 4 (21%) | 0.528 |
| Diabetes | 11 (32%) | 4 (21%) | 0.528 |
| Liver disease | 1 (3%) | 2 (11%) | 0.290 |
| Propofol therapy before sample acquisition | 2 (6%) | 1 (5%) | 1.000 |
| Enteral/parenteral nutrition before sample acquisition | 2 (6%) | 3 (16%) | 0.336 |
| Mechanical ventilation at sample acquisition | 9 (26%) | 13 (68%) | 0.007 |
| | | | |
| **Quantitative lipid parameters** | | | |
| HDL cholesterol (mg/dL) | 14 [8-35] | 14 [5-24] | 0.458 |
| Triglycerides (mg/dL) | 167 [112-277] | 150 [101-253] | 0.617 |
| Total cholesterol (mg/dL) | 108 [94-129] | 97 [60-147] | 0.204 |
| LDL cholesterol (mg/dL)[a,b] | 53 [32-70] | 65 [27-85] | 0.573 |
| Apolipoprotein A-I (mg/dL) | 71 [32-92] | 43 [20-80] | 0.099 |
| Apolipoprotein B (mg/dL) | 69 [55-84] | 59 [42-80] | 0.447 |
| | | | |
| **Qualitative lipid parameters** | | | |
| Arylesterase activity (AEA) (mM/min/mL serum) | 79.6 [57.7-96.7] | 49.2 [29.6-70.2] | 0.002 |
| Cholesterol efflux capacity (%) | 9.6 [8.7-11.3] | 8.4 [6.3-10.9] | 0.051 |
| | | | |
| **Laboratory covariables** | | | |
| White blood count (G/L) | 14.3 [9.4-24.6] | 15.3 [6.0-27.2] | 0.753 |
| Hemoglobin (g/dL) | 10.8 [8.8-12.4] | 10.9 [8.5-13.6] | 0.704 |
| Platelets (G/L) | 165 [75-261] | 164 [96-294] | 0.718 |
| C-reactive protein (mg/dL) | 197 [84-292] | 272 [148-384] | 0.170 |
| Procalcitonin (ng/mL) | 7.7 [1.2-36.4] | 16.4 [0.8-35.3] | 0.388 |
| Serum bilirubin (mg/dL) | 1.0 [0.5-2.5] | 0.7 [0.4-2.1] | 0.553 |
| Serum creatinine (mg/dL) | 2.1 [1.6-3.5] | 3.3 [1.6-5.8] | 0.211 |
| Serum albumin (g/dL) | 3.0 [2.5-3.3] | 2.7 [2.2-3.2] | 0.221 |
| | | | |
| **Illness severity and outcomes** | | | |
| SOFA score (points) | 8 [6-10] | 13 [11-14] | 0.001 |
| Catecholamine therapy | 26 (76%) | 19 (100%) | 0.040 |
| ICU length of stay (days) | 6 [3-10] | 5 [2-12] | 0.830 |
| Hospital length of stay (days) | 23 [15-29] | 5 [2-12] | <0.0001 |
| 28-day mortality | 6 (18%) | 19 (100%) | <0.0001 |
| ICU mortality | 0 (0%) | 19 (100%) | N/A |

See Table 4 Legend for details.

**Table 7: Differences between sepsis patients with and without septic shock.**

| Variable | Sepsis (N=27) | Septic shock (N=26) | p-value |
|---|---|---|---|
| **Demographics & Premedication** | | | |
| Age (years) | 66 [48-74] | 61 [53-75] | 0.708 |
| Body mass index (kg/m$^2$) | 25.3 [22.9-29.7] | 26.8 [23.4-30.3] | 0.587 |
| Female sex | 8 (30%) | 13 (50%) | 0.130 |
| Anti-diabetic therapy | 3 (11%) | 9 (35%) | 0.054 |
| Statin therapy | 8 (30%) | 7 (27%) | 0.827 |
| Diabetes | 4 (15%) | 11 (42%) | 0.035 |
| Liver disease | 0 (0%) | 3 (12%) | 0.111 |
| Propofol therapy before sample acquisition | 2 (7%) | 1 (4%) | 1.000 |
| Enteral/parenteral nutrition before sample acquisition | 3 (11%) | 2 (8%) | 1.000 |
| Mechanical ventilation at sample acquisition | 11 (41%) | 11 (42%) | 0.908 |
| | | | |
| **Quantitative lipid parameters** | | | |
| HDL cholesterol (mg/dL) | 27 [10-35] | 10 [5-21] | 0.020 |
| Triglycerides (mg/dL) | 130 [101-219] | 188 [121-288] | 0.223 |
| Total cholesterol (mg/dL) | 114 [84-160] | 103 [73-127] | 0.168 |
| LDL cholesterol (mg/dL)[a,b] | 57 [29-85] | 58 [24-69] | 0.433 |
| Apolipoprotein A-I (mg/dL) | 80 [35-96] | 43 [27-77] | 0.060 |
| Apolipoprotein B (mg/dL) | 71 [48-83] | 62 [49-87] | 0.413 |
| | | | |
| **Qualitative lipid parameters** | | | |
| Arylesterase activity (AEA) (mM/min/mL serum) | 69.2 [49.2-88.7] | 63.5 [37.5-90.9] | 0.466 |
| Cholesterol efflux capacity (%) | 9.5 [8.7-11.0] | 9.1 [6.9-10.9] | 0.219 |
| | | | |
| **Laboratory covariables** | | | |
| White blood count (G/L) | 12.8 [7.1-23.9] | 17.8 [11.2-33.1] | 0.176 |
| Hemoglobin (g/dL) | 10.8 [9.0-11.8] | 10.5 [8.5-13.7] | 0.769 |
| Platelets (G/L) | 204 [96-285] | 145 [37-198] | 0.064 |
| C-reactive protein (mg/dL) | 221 [155-309] | 207 [77-311] | 0.545 |
| Procalcitonin (ng/mL) | 2.6 [0.4-34.2] | 18.8 [7.0-66.5] | 0.011 |
| Serum bilirubin (mg/dL) | 0.7 [0.9-3.4] | 1.0 [0.5-3.3] | 0.294 |
| Serum creatinine (mg/dL) | 1.9 [0.9-3.4] | 3.1 [1.9-5.2] | 0.016 |
| Serum albumin (g/dL) | 3.0 [2.5-3.4] | 2.7 [2.4-3.1] | 0.207 |
| | | | |
| **Illness severity and outcomes** | | | |
| SOFA score (points) | 8 [5-11] | 13 [8-14] | 0.003 |
| Catecholamine therapy | 19 (70%) | 26 (100%) | 0.004 |
| ICU length of stay (days) | 6 [2-11] | 6 [3-9] | 1.000 |
| Hospital length of stay (days) | 16 [8-25] | 17 [4-26] | 0.563 |
| 28-day mortality | 11 (41%) | 14 (54%) | 0.339 |
| ICU mortality | 7 (26%) | 12 (46%) | 0.125 |

See Table 4 Legend for details.

## 5.2. Laboratory results

Hemoglobin levels and platelets were similar, while the inflammatory parameters were higher in sepsis patients than controls (**Table 4**). White blood count was 14.9 G/L in sepsis patients compared to 9.1 G/L in control patients (p=0.011). Similarly, CRP and PCT were higher in the sepsis group compared to controls at 213 vs 12 mg/l ($p < 0.0001$) and 8.8 vs 0.2 ng/mL ($p<0.0001$), respectively.

## 5.3. Prevalence of dyslipidemia in the sepsis and control cohort

Dyslipidemia was defined in this study as a serum HDL-C level lower than 40 mg/dL. When considering this cutoff, the corresponding prevalence of dyslipidemia was 85% [95% CI: 72-93] in the sepsis group and 52% [95%CI: 31-72] in the control cohort (p=0.002).

## 5.4. Qualitative and quantitative changes of lipoproteins

The levels of total cholesterol were similar in the sepsis compared to the control group at 106 [84-130] and 114 [96-156] mg/dL (p=0.193), respectively (**Table 4, Figure 4**). However, there was a significant difference in HDL-C levels between sepsis and controls measuring 14 [7-33] and 39 [33-55] mg/dL ($p<0.0001$), respectively (**Table 4, Figure 4**). Apart from quantitative lipoprotein differences, AEA and CEC were measured reflecting qualitative lipoprotein capabilities. The AEA was significantly lower in the sepsis compared to the control cohort (67 vs 111 mM/min/mL serum, $p<0.0001$), while the CEC was decreased but not significantly different between groups (9% vs 10%, p=0.091, **Table 4, Figure 4**).

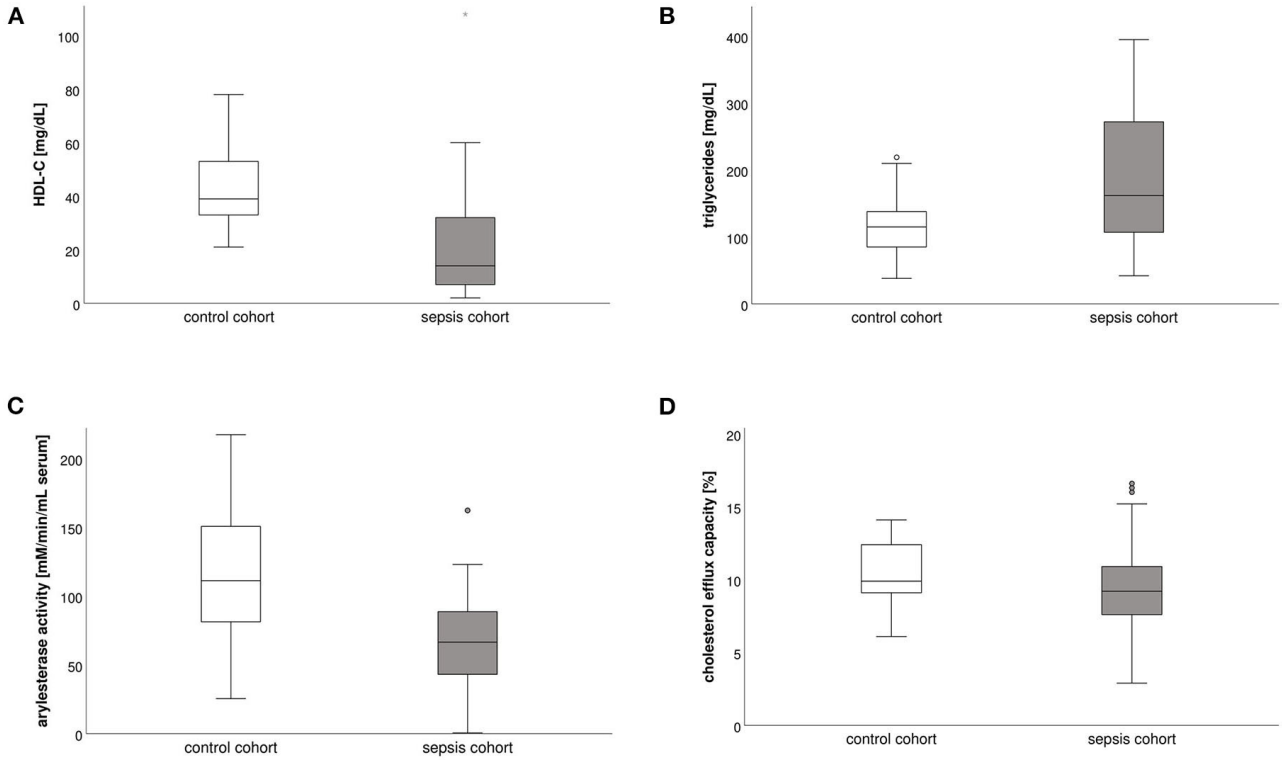


**Figure 4: Boxplots of lipoprotein parameters**. Sepsis cohort (n=53, grey box plots) and control cohort (n=25, white box plots). The p-values were <0.0001, 0.006, <0.0001 and 0.091 for HDL-C, triglycerides, arylesterase activity, and cholesterol efflux capacity, respectively. Reproduced from (1) with permission from Frontiers in medicine.

### 5.4.1. Correlations

Increasing disease severity in the sepsis cohort as measured by an increased SOFA score was correlated with decreasing HDL-C levels (r=-0.31, p=0.03) and AEA (r=-0.32, p=0.02; **Table 8**). In addition, lower AEA correlated with lower HDL-C levels (r=0.60, p<0.001) in the sepsis cohort. In the control cohort, the SOFA score was not correlated with HDL-C (r=-0.17, p=0.41) nor AEA (r=-0.23, p=0.28).

**Table 8: Correlation matrix of lipoprotein parameters and selected covariables in the sepsis cohort.**

| | ApoA1 | TG | AEA | CEC | SOFA | Bilirubin | CRP | Creatinine | Albumin |
|---|---|---|---|---|---|---|---|---|---|
| HDL-C | 0.803*** | -0.491*** | 0.600*** | 0.537*** | -0.308* | -0.345* | -0.168 | -0.250 | 0.593*** |
| Apolipoprotein A-I | | -0.299* | 0.717*** | 0.688*** | -0.367** | -0.365** | -0.113 | -0.161 | 0.650*** |
| Triglycerides | | | -0.071 | 0.050 | 0.114 | 0.078 | 0.385** | -0.008 | -0.425** |
| Arylesterase activity | | | | 0.716*** | -0.324* | -0.185 | -0.033 | -0.183 | 0.612*** |
| Cholesterol efflux capacity | | | | | -0.221 | -0.180 | 0.105 | -0.125 | 0.581*** |
| SOFA score | | | | | | 0.429** | 0.097 | 0.310* | -0.159 |
| Bilirubin | | | | | | | -0.109 | 0.098 | -0.200 |
| C-reactive protein | | | | | | | | 0.099 | -0.281* |
| Creatinine | | | | | | | | | -0.239 |

*p<0.05 **p<0.01 ***p<0.001

Data are Spearman's rank-based correlation coefficients.

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### 5.4.2. Sepsis 28-day mortality

Twenty-five patients succumbed within 28-days after ICU admission resulting in a 28-day mortality of 47%. Older age (OR per 5 years increase = 1.23, 95%CI: 1.02-1.50; p=0.033), lower albumin (OR per 1 g/dL increase = 0.36, 95%CI: 0.14-0.93; p=0.034), and higher CRP (OR per 100 mg/L increase = 1.72, 95%CI: 1.07-2.77; p=0.025) were significantly associated with higher 28-day mortality in univariable logistic regression analyses in the sepsis group (**Table 9**).

Quantitative lipoprotein parameters such as HDL-C (OR per 10mg/dL increase = 0.88, 95%CI: 0.66-1.18; p=0.381), total cholesterol (OR per 10mg/dL increase = 0.90, 95%CI: 0.79-1.02; p=0.099), and TG (OR per 10mg/dL increase = 0.98, 95%CI: 0.93-1.04; p=0.501) were not associated with outcome in univariable logistic regression analyses. However, higher AEA was clearly associated with lower risk of 28-day mortality (OR per 10mM/min/mL serum increase = 0.76, 95%CI: 0.61-0.94; p=0.010), while the CEC (OR per 10% increase = 0.20, 95% CI: 0.03–1.45; p = 0.111) was not associated with 28-day mortality.

For AEA, in patients with an empirical cutoff below the 25$^{th}$ percentile of the AEA distributional range, the 28-day survival estimate was 31% [10-55%], while in patients above this cutoff the 28-day survival estimate was 60% [43-73] (log-rank p=0.0035, **Figure 5**).

**Table 9: Univariable logistic regression models for 28-day and ICU mortality in the sepsis cohort.**

| Outcome variable | 28-day mortality | | | | ICU mortality | | |
|---|---|---|---|---|---|---|---|
| **Variable** | **Odds ratio** | **95% confidence interval** | **p** | | **Odds ratio** | **95% confidence interval** | **p** |
| | | | | | | | |
| **Demographics & Premedication** | | | | | | | |
| Age (per 5 years increase) | 1.23 | 1.02-1.50 | 0.033 | | 1.06 | 0.89-1.27 | 0.511 |
| Body mass index (per 5 kg/m$^2$ increase) | 0.72 | 0.41-1.25 | 0.245 | | 0.57 | 0.30-1.08 | 0.085 |
| Female sex | 2.71 | 0.87-8.42 | 0.085 | | 1.65 | 0.53-5.17 | 0.390 |
| Anti-diabetic therapy | 0.75 | 0.20-2.75 | 0.665 | | 0.87 | 0.22-3.37 | 0.836 |
| Statin therapy | 0.45 | 0.13-1.57 | 0.210 | | 0.56 | 0.15-2.08 | 0.384 |
| | | | | | | | |
| **Quantitative lipid parameters** | | | | | | | |
| HDL cholesterol (per 10mg/dL increase) | 0.88 | 0.66-1.18 | 0.381 | | 0.84 | 0.61-1.17 | 0.312 |
| Triglycerides (per 10mg/dL increase) | 0.98 | 0.93-1.04 | 0.501 | | 0.99 | 0.93-1.05 | 0.715 |
| Total cholesterol (per 10mg/dL increase) | 0.90 | 0.79-1.02 | 0.088 | | 0.89 | 0.78-1.02 | 0.099 |
| ApoA1 (per 10mg/dL increase) | 0.92 | 0.80-1.07 | 0.294 | | 0.87 | 0.73-1.03 | 0.099 |
| | | | | | | | |
| **Qualitative lipid parameters** | | | | | | | |
| Arylesterase activity (AEA) (per 10mM/min/mL serum increase) | 0.76 | 0.61-0.94 | 0.010 | | 0.71 | 0.56-0.90 | 0.004 |
| Cholesterol efflux capacity (per 10% increase) | 0.20 | 0.03-1.45 | 0.111 | | 0.11 | 0.01-1.03 | 0.053 |
| | | | | | | | |
| **Laboratory covariables** | | | | | | | |
| White blood count (per 1G/L increase) | 1.02 | 0.98-1.07 | 0.357 | | 1.00 | 0.96-1.05 | 0.960 |
| Hemoglobin (per 1g/dL increase) | 0.94 | 0.77-1.15 | 0.574 | | 1.03 | 0.84-1.25 | 0.801 |
| Platelets (per 100 G/L increase) | 1.11 | 0.71-1.75 | 0.640 | | 1.14 | 0.71-1.81 | 0.593 |
| C-reactive protein (per 100mg/dL increase) | 1.72 | 1.07-2.77 | 0.025 | | 1.40 | 0.90-2.18 | 0.136 |
| Serum bilirubin (per 1mg/dL increase) | 0.89 | 0.74-1.08 | 0.245 | | 0.94 | 0.80-1.11 | 0.484 |
| Serum creatinine (per 1mg/dL increase) | 1.01 | 0.85-1.19 | 0.905 | | 1.04 | 0.88-1.23 | 0.653 |
| Serum albumin (per 1g/dL increase) | 0.36 | 0.14-0.93 | 0.034 | | 0.54 | 0.22-1.31 | 0.171 |
| | | | | | | | |
| **Sepsis severity** | | | | | | | |
| SOFA score (per 1 point increase) | 1.13 | 0.97-1.31 | 0.113 | | 1.36 | 1.12-1.65 | 0.002 |

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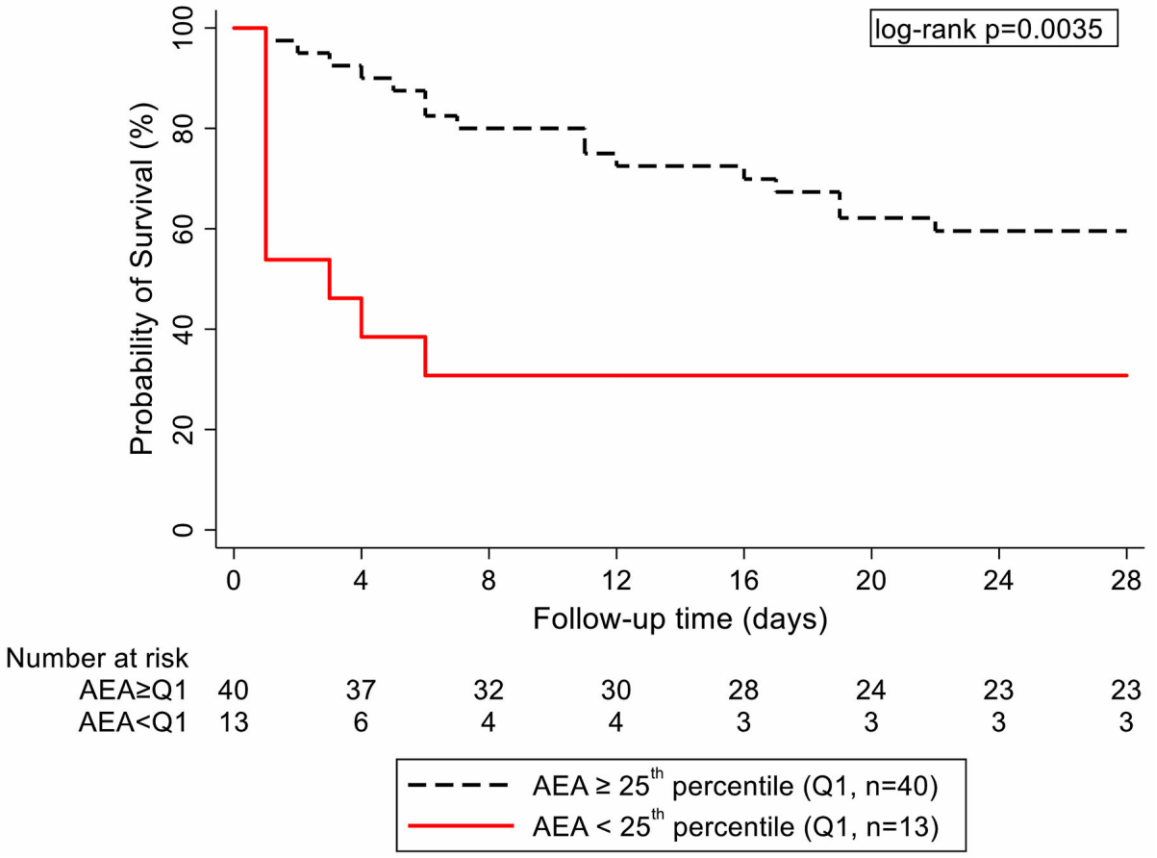


**Figure 5: Kaplan-Meier survival cures for arylesterase activity**. Sepsis patients with arylesterase activity below (solid red curve) or above (dashed black curve) the 25$^{th}$ percentile. Reproduced from (1) with permission from Frontiers in medicine.

Then a multivariable analysis regarding 28-day mortality was performed (**Table 10**). Due to the importance of the SOFA score as a marker for organ failure or dysfunction, we always considered this variable in the model. Furthermore, all variables associated with the endpoint with a $p \leq 0.05$ were considered. Albumin had a strong collinearity with CRP and was therefore not included in the model. In the final model for 28-day mortality, we found that AEA (OR per 10 mM/min/mL serum increase = 0.76, 95%CI: 0.59-0.98; p=0.032) prevailed in multivariable analysis, while the SOFA score (OR per 1 point increase = 1.11, 95%CI: 0.91-1.35; p=0.292), age (OR per 5 years increase = 1.27, 95%CI: 0.99-1.64; p=0.064), and CRP (OR per 100mg/L increase = 1.71, 95%CI: 0.99-2.94; p=0.053) were not associated with 28-day outcomes with the sample size of this study.

**Table 10: Multivariable models for 28-day and ICU mortality in the sepsis cohort.**

| **Multivariable Model #1: 28-day mortality** | **Odds Ratio** | **95% CI** | **p** |
|---|---|---|---|
| Arylesterase activity (AEA) (per 10mM/min/mL serum increase) | 0.76 | 0.59-0.98 | 0.032 |
| SOFA score (per 1 point increase) | 1.11 | 0.91-1.35 | 0.292 |
| Age (per 5 years increase) | 1.27 | 0.99-1.64 | 0.064 |
| C-reactive protein (per 100mg/dL increase) | 1.71 | 0.99-2.94 | 0.053 |
| | | | |
| **Multivariable Model #2: ICU mortality** | **Odds Ratio** | **95% CI** | **p** |
| Arylesterase activity (AEA) (per 10mM/min/mL serum increase) | 0.74 | 0.57-0.96 | 0.026 |
| SOFA score (per 1 point increase) | 1.30 | 1.06-1.59 | 0.010 |

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### 5.4.3. Sepsis ICU mortality

Several factors, which are not directly related or affected by ICU care, can influence the 28-day mortality. These factors can, to some extent, lead to a dilution of the associations, especially in smaller sample sizes. Therefore, additionally ICU mortality as a more direct representative of patient outcomes was investigated.

The ICU mortality in this study was 36% in the sepsis cohort. In univariable logistic regression, the associations with outcome were mostly similar to the associations with 28-day mortality. Associations with ICU mortality were significant for SOFA score and AEA (**Table 9**). In detail, higher AEA (OR per 10 mM/min/mL serum increase = 0.71, 95%CI: 0.56– 0.90; p=0.004) was significantly associated with lower ICU mortality, while the CEC was borderline but not statistically significant associated (OR per 10% increase = 0.11, 95%CI: 0.01–1.03; p=0.053). Furthermore, higher SOFA score was associated with increased ICU mortality (OR per 1 point increase = 1.36, 95%CI: 1.12-1.65; p=0.002). In multivariable analysis, AEA (OR per 10 mM/min/mL serum increase = 0.74, 95%CI: 0.57-0.96; p=0.026) and SOFA score (OR per 1 point increase = 1.30, 95%CI: 1.06-1.59; p=0.010) remained as independent risk factors for ICU mortality (**Table 10**).

### 5.4.4. AUROC

For 28-day mortality the discriminatory potential of the SOFA score was poor (AUROC = 0.62, 95%CI: 0.46-0.78). However, the 28-day discriminatory ability was strong for AEA (AUROC = 0.71, 95%CI: 0.57-0.85) in the sepsis cohort. For discrimination between ICU survivors and non-survivors, both the SOFA score (AUROC = 0.78, 95%CI: 0.65-0.91) and AEA (AUROC = 0.76, 95%CI: 0.63-0.89) were strong discriminators.

## 5.5. Metabolomics

In the first part of the study, we found significant differences between quantitative and qualitative lipoprotein parameters. However, a limitation was the targeted analyses of selected lipoproteins and variables as pre-specified parameters of interest.

The second part of the study consisted of two steps. To further investigate any potential lipoprotein differences between ICU sepsis and ICU control patients a targeted metabolomic analysis was performed. In sepsis patients, an untargeted metabolomic analysis was additionally done, allowing for the detection of previously unknown metabolites that might play a role in sepsis ICU trajectory and outcome.

### 5.5.1. Step One: Targeted lipoprotein $^{1}$H NMR spectroscopy analysis

Initially, a targeted $^{1}$H NMR spectroscopy metabolomic analysis of lipoproteins in both cohorts was performed. This was done to investigate the differences between the two groups using an independent method and therefore allowing for confirmation or rejection of the previous findings. For this analysis, one patient in the sepsis cohort had to be excluded before measurements were performed because of insufficient sample quantity in the corresponding aliquot.

The principal component analysis can detect differences and similarities between samples, therefore enabling clustering. Allocation to a specific group is not given to the algorithm, i.e. it is called unsupervised. In PCA visual differences between sepsis and control patients with a principal component 1 (PC 1) of 76.8% and principal component 2 (PC 2) of 11% (**Figure 6A**) were found. We then performed an O-PLS-DA, which is a robust method to detect the difference between clusters when group allocation is given to the algorithm. This clustering is then tested with a cross-validation within the data set to check the robustness of the model. In this study, there was a separate clustering for sepsis and control patients with strong-moderate goodness of fit (correlation coefficient $R^2Y = 0.405$) and a cross-validation score $Q^2$ of 0.292 ($p<0.01$; **Figure 6A**). Furthermore, in this targeted metabolomic analyses differences of lipoproteins between the two groups were investigated (**Figure 6B and 5C, Table 11 and**

**12**). Levels of HDL free cholesterol (HDL-FC), HDL-C, HDL phospholipids (HDL-PL), and ApoA1 were significantly lower in patients with sepsis compared to controls. VLDL variables were significantly higher, while LDL parameters were not significantly affected.

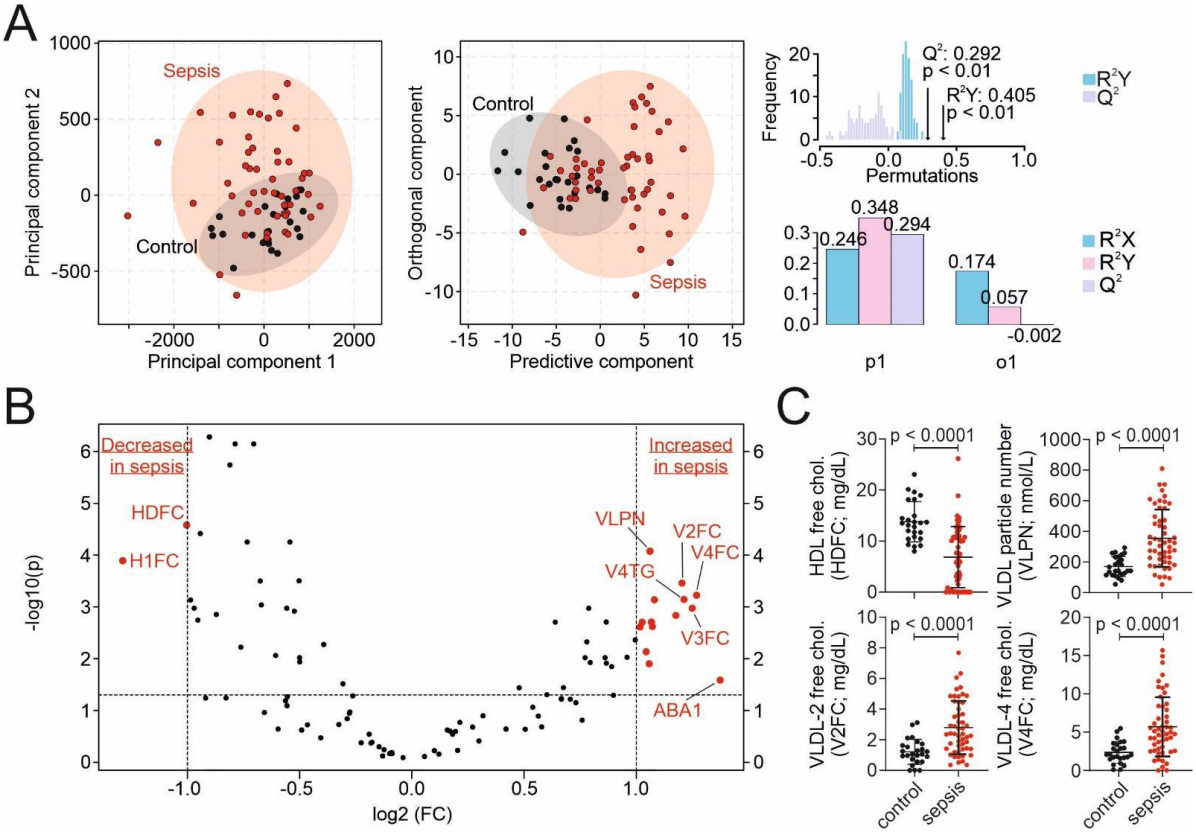


**Figure 6: Targeted metabolomic assessment of lipoproteins.** Reproduced from (2) with permission from Nutrients. A: Multivariate analyses with PCA and O-PLS-DA for sepsis (red) and controls (black). B: Volcano plot for differences between groups (logarithmic scale). Vertical dashed lines represent changes by the factor 2. Horizontal dashed line represents a significance of 5%. C: Boxplots for most changed and most significant lipoproteins.

**Table 11: Targeted metabolomic analyses of lipoproteins.**

| Variables | Sepsis patients (N=52) | Controls (N=25) | p-Value | Below Sidak-treshold |
|---|---|---|---|---|
| **Main classes** | | | | |
| Triglycerides (mg/dL) | 185 [129-310] | 101 [87-157] | <0.001 | yes |
| Total cholesterol (mg/dL) | 117 [106-148] | 143 [119-194] | 0.011 | no |
| LDL cholesterol (mg/dL) | 57 [39-76] | 77 [53-106] | 0.012 | no |
| HDL cholesterol (mg/dL) | 20 [13-30] | 41 [32-51] | <0.001 | yes |
| Total ApoA1 (mg/dL) | 72 [52-98] | 120 [98-139] | <0.001 | yes |
| Total ApoA2 (mg/dL) | 19 [15-23] | 24 [20-27] | <0.001 | no |
| Total ApoB100 (mg/dL) | 82 [63-103] | 74 [59-87] | 0.171 | no |
| LDL to HDL ratio | 2.6 [1.7-3.7] | 1.9 [1.5-2.5] | 0.009 | no |
| ApoB100 to ApoA1 ratio | 1.3 [0.7-1.7] | 0.6 [0.5-0.8] | <0.001 | yes |
| | | | | |
| **Particles** | | | | |
| Total particle number (nmol/L) | 1494 [1149-1877] | 1338 [1063-1588] | 0.171 | no |
| VLDL particle number (nmol/L) | 324 [205-490] | 142 [118-233] | <0.001 | yes |
| IDL particle number (nmol/L) | 157 [79-300] | 87 [61-137] | 0.002 | no |
| LDL particle number (nmol/L) | 930 [737-1225] | 1028 [720-1254] | 0.640 | no |
| | | | | |
| **Triglycerides in subclasses** | | | | |
| VLDL (mg/dL) | 94 [63-199] | 50 [43-111] | 0.010 | no |
| IDL (mg/dL) | 13 [7-31] | 6 [3-15] | 0.007 | no |
| LDL (mg/dL) | 32 [19-58] | 22 [17-30] | 0.006 | no |
| HDL (mg/dL) | 15 [10-19] | 13 [10-16] | 0.124 | no |
| | | | | |
| **Cholesterol in subclasses** | | | | |
| VLDL (mg/dL) | 28 [20-41] | 17 [12-24] | <0.001 | no |
| IDL (mg/dL) | 21 [11-37] | 11 [7-16] | 0.002 | no |
| LDL (mg/dL) | 57 [39-76] | 77 [53-106] | 0.012 | no |
| HDL (mg/dL) | 20 [13-30] | 41 [32-51] | <0.001 | yes |
| | | | | |
| **Free cholesterol in subclasses** | | | | |
| VLDL (mg/dL) | 13 [10-20] | 8 [6-13] | 0.002 | no |
| IDL (mg/dL) | 6 [3-11] | 3 [2-4] | <0.001 | no |
| LDL (mg/dL) | 24 [19-34] | 29 [21-39] | 0.107 | no |
| HDL (mg/dL) | 6 [1-11] | 14 [11-17] | <0.001 | yes |
| | | | | |
| **Phospholipids in subclasses** | | | | |
| VLDL (mg/dL) | 22 [15-41] | 15 [11-28] | 0.039 | no |
| IDL (mg/dL) | 5 [3-11] | 4 [2-6] | 0.133 | no |
| LDL (mg/dL) | 40 [28-55] | 51 [34-62] | 0.095 | no |
| HDL (mg/dL) | 36 [18-51] | 62 [49-73] | <0.001 | yes |
| | | | | |
| **Apolipoproteins in subclasses** | | | | |
| ApoA1 in HDL (mg/dL) | 67 [46-98] | 119 [98-138] | <0.001 | yes |
| ApoA2 in HDL (mg/dL) | 20 [17-25] | 25 [21-27] | 0.006 | no |
| ApoB in VLDL (mg/dL) | 18 [11-27] | 8 [7-13] | <0.001 | yes |
| ApoB in IDL (mg/dL) | 9 [4-17] | 5 [3-8] | 0.002 | no |
| ApoB in LDL (mg/dL) | 51 [41-67] | 57 [40-69] | 0.640 | no |

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**Table 12: Targeted metabolomic analyses - lipoprotein subgroups.**

| Variables | Sepsis patients (N=52) | Controls (N=25) | p-Value | Below Sidak-treshold |
|---|---|---|---|---|
| **VLDL subfraction - triglycerides** | | | | |
| VLDL 1 triglycerides (mg/dL) | 36.1 [14.4-88.1] | 26.9 [14.3-54.8] | 0.184 | no |
| VLDL 2 triglycerides (mg/dL) | 17.8 [7.3-28.7] | 11.2 [6.9-16.8] | 0.069 | no |
| VLDL 3 triglycerides (mg/dL) | 19.0 [10.5-28.9] | 10.9 [6.9-16.1] | 0.001 | no |
| VLDL 4 triglycerides (mg/dL) | 19.7 [13.0-29.3] | 10.2 [6.5-14.3] | <0.001 | yes |
| VLDL 5 triglycerides (mg/dL) | 5.3 [3.9-6.6] | 3.6 [2.8-4.5] | <0.001 | yes |
| **VLDL subfraction - cholesterol** | | | | |
| VLDL 1 cholesterol (mg/dL) | 7.0 [3.7-11.5] | 4.4 [2.9-8.3] | 0.082 | no |
| VLDL 2 cholesterol (mg/dL) | 3.3 [1.4-5.3] | 2.2 [1.1-3.3] | 0.061 | no |
| VLDL 3 cholesterol (mg/dL) | 4.9 [3.0-8.2] | 3.3 [1.5-4.2] | 0.004 | no |
| VLDL 4 cholesterol (mg/dL) | 9.5 [4.0-14.2] | 5.1 [2.7-6.2] | <0.001 | no |
| VLDL 5 cholesterol (mg/dL) | 2.0 [1.2-3.4] | 1.3 [0.7-2.0] | 0.003 | no |
| **VLDL subfraction – free cholesterol** | | | | |
| VLDL 1 free cholesterol (mg/dL) | 1.0 [0.0-4.0] | 0.9 [0.0-2.6] | 0.300 | no |
| VLDL 2 free cholesterol (mg/dL) | 2.4 [1.4-4.3] | 1.1 [0.6-1.7] | <0.001 | yes |
| VLDL 3 free cholesterol (mg/dL) | 3.1 [1.7-4.4] | 1.4 [0.8-2.3] | <0.001 | yes |
| VLDL 4 free cholesterol (mg/dL) | 4.9 [2.7-8.2] | 2.1 [1.5-3.5] | <0.001 | yes |
| VLDL 5 free cholesterol (mg/dL) | 1.0 [0.6-1.8] | 0.7 [0.2-1.1] | 0.007 | no |
| **VLDL subfraction – phospholipids** | | | | |
| VLDL 1 phospholipids (mg/dL) | 4.7 [1.4-10.2] | 3.9 [1.6-7.5] | 0.601 | no |
| VLDL 2 phospholipids (mg/dL) | 3.6 [1.0-6.1] | 2.9 [1.3-3.6] | 0.149 | no |
| VLDL 3 phospholipids (mg/dL) | 5.3 [3.2-8.6] | 3.0 [1.5-4.6] | 0.003 | no |
| VLDL 4 phospholipids (mg/dL) | 9.1 [5.8-13.9] | 4.7 [3.4-6.6] | <0.001 | yes |
| VLDL 5 phospholipids (mg/dL) | 2.5 [1.8-3.6] | 2.0 [1.2-2.5] | 0.023 | no |
| | | | | |
| **Continued on next page** | | | | |

| continued | Sepsis patients (N=52) | Controls (N=25) | p-Value | Below Sidak-treshold |
|---|---|---|---|---|
| **LDL subfractions** | | | | |
| LDL 1 particle number (nmol/L) | 260 [118-439] | 209 [137-317] | 0.453 | no |
| LDL 2 particle number (nmol/L) | 193 [151-248] | 170 [116-255] | 0.507 | no |
| LDL 3 particle number (nmol/L) | 182 [148-254] | 207 [99-253] | 0.939 | no |
| LDL 4 particle number (nmol/L) | 104 [36-156] | 119 [53-233] | 0.281 | no |
| LDL 5 particle number (nmol/L) | 46 [0-124] | 119 [60-162] | 0.005 | no |
| LDL 6 particle number (nmol/L) | 146 [11-240] | 186 [116-253] | 0.187 | no |
| **LDL subfraction - triglycerides** | | | | |
| LDL 1 triglycerides (mg/dL) | 13.4 [8.7-23.6] | 7.8 [5.8-10.8] | <0.001 | yes |
| LDL 2 triglycerides (mg/dL) | 4.0 [2.6-7.4] | 2.7 [1.7-4.0] | 0.005 | no |
| LDL 3 triglycerides (mg/dL) | 3.5 [2.9-5.6] | 3.1 [2.2-4.1] | 0.030 | no |
| LDL 4 triglycerides (mg/dL) | 3.4 [1.7-7.5] | 3.1 [1.4-3.7] | 0.093 | no |
| LDL 5 triglycerides (mg/dL) | 2.2 [0.7-4.2] | 1.7 [1.1-2.5] | 0.824 | no |
| LDL 6 triglycerides (mg/dL) | 4.0 [3.0-6.0] | 3.7 [2.8-4.4] | 0.279 | no |
| **LDL subfraction - cholesterol** | | | | |
| LDL 1 cholesterol (mg/dL) | 19.1 [6.3-31.4] | 16.7 [10.2-26.9] | 0.983 | no |
| LDL 2 cholesterol (mg/dL) | 14.2 [10.1-18.1] | 15.6 [9.7-20.6] | 0.453 | no |
| LDL 3 cholesterol (mg/dL) | 14.2 [9.7-18.0] | 17.1 [7.3-21.9] | 0.415 | no |
| LDL 4 cholesterol (mg/dL) | 4.9 [0.0-9.9] | 9.2 [3.0-18.6] | 0.020 | no |
| LDL 5 cholesterol (mg/dL) | 0.4 [0.0-4.6] | 7.7 [2.6-11.6] | 0.001 | no |
| LDL 6 cholesterol (mg/dL) | 6.6 [0.0-11.7] | 10.6 [6.1-14.8] | 0.019 | no |
| **LDL subfraction – free cholesterol** | | | | |
| LDL 1 free cholesterol (mg/dL) | 6.2 [2.9-10.2] | 6.0 [3.7-8.6] | 0.909 | no |
| LDL 2 free cholesterol (mg/dL) | 5.0 [3.8-6.6] | 5.7 [3.4-7.5] | 0.405 | no |
| LDL 3 free cholesterol (mg/dL) | 4.8 [2.8-6.8] | 5.1 [3.1-7.5] | 0.483 | no |
| LDL 4 free cholesterol (mg/dL) | 2.5 [0.7-3.9] | 3.3 [2.0-6.4] | 0.034 | no |
| LDL 5 free cholesterol (mg/dL) | 1.3 [0.0-2.7] | 3.2 [1.4-4.4] | <0.001 | no |
| LDL 6 free cholesterol (mg/dL) | 2.0 [0.0-3.6] | 3.6 [2.8-4.9] | <0.001 | no |
| **LDL subfraction – phospholipids** | | | | |
| LDL 1 phospholipids (mg/dL) | 13.0 [5.4-20.8] | 11.1 [7.1-16.3] | 0.761 | no |
| LDL 2 phospholipids (mg/dL) | 8.3 [6.6-10.8] | 9.2 [5.8-12.3] | 0.624 | no |
| LDL 3 phospholipids (mg/dL) | 8.0 [5.8-11.0] | 10.3 [4.6-13.0] | 0.466 | no |
| LDL 4 phospholipids (mg/dL) | 3.3 [0.7-6.2] | 5.5 [2.4-10.5] | 0.042 | no |
| LDL 5 phospholipids (mg/dL) | 0.9 [0.0-3.9] | 4.9 [2.8-6.8] | <0.001 | no |
| LDL 6 phospholipids (mg/dL) | 4.2 [0.1-6.8] | 6.7 [4.7-9.6] | 0.014 | no |
| **LDL subfraction – ApoB** | | | | |
| LDL 1 ApoB (mg/dL) | 14.3 [6.5-24.2] | 11.5 [7.5-17.5] | 0.453 | no |
| LDL 2 ApoB (mg/dL) | 10.6 [8.3-13.7] | 9.4 [6.4-14.0] | 0.507 | no |
| LDL 3 ApoB (mg/dL) | 10.0 [8.1-14.0] | 11.4 [5.5-13.9] | 0.939 | no |
| LDL 4 ApoB (mg/dL) | 5.7 [2.0-8.6] | 6.5 [2.9-12.8] | 0.281 | no |
| LDL 5 ApoB (mg/dL) | 2.5 [0.0-6.8] | 6.5 [3.3-8.9] | 0.006 | no |
| LDL 6 ApoB (mg/dL) | 8.0 [0.6-13.2] | 10.2 [6.4-13.9] | 0.187 | no |
| | | | | |
| **Continued on next page** | | | | |

| continued | Sepsis patients (N=52) | Controls (N=25) | p-Value | Below Sidak-treshold |
|---|---|---|---|---|
| **HDL subfraction – triglycerides** | | | | |
| HDL 1 triglycerides (mg/dL) | 5.4 [3.5-7.7] | 4.1 [2.6-5.3] | 0.104 | no |
| HDL 2 triglycerides (mg/dL) | 2.5 [1.8-3.2] | 2.1 [1.5-2.9] | 0.167 | no |
| HDL 3 triglycerides (mg/dL) | 2.6 [1.7-3.2] | 2.2 [1.7-2.7] | 0.236 | no |
| HDL 4 triglycerides (mg/dL) | 4.0 [3.1-4.8] | 3.6 [2.7-4.3] | 0.161 | no |
| **HDL subfraction – cholesterol** | | | | |
| HDL 1 cholesterol (mg/dL) | 6.6 [0.0-12.3] | 11.3 [8.2-17.8] | <0.001 | yes |
| HDL 2 cholesterol (mg/dL) | 3.2 [0.9-4.9] | 5.8 [4.4-6.8] | <0.001 | yes |
| HDL 3 cholesterol (mg/dL) | 4.3 [2.2-7.0] | 7.4 [5.9-8.1] | <0.001 | yes |
| HDL 4 cholesterol (mg/dL) | 9.2 [5.8-13.7] | 14.3 [9.8-20.2] | 0.003 | no |
| **HDL subfraction – free cholesterol** | | | | |
| HDL 1 free cholesterol (mg/dL) | 0.4 [0.0-3.2] | 4.1 [2.5-5.4] | <0.001 | yes |
| HDL 2 free cholesterol (mg/dL) | 1.2 [0.6-1.7] | 1.8 [1.4-2.4] | <0.001 | yes |
| HDL 3 free cholesterol (mg/dL) | 0.7 [0.2-1.6] | 1.8 [1.5-2.3] | <0.001 | yes |
| HDL 4 free cholesterol (mg/dL) | 2.8 [1.4-4.4] | 3.5 [2.7-5.0] | 0.026 | no |
| **HDL subfraction – phospholipids** | | | | |
| HDL 1 phospholipids (mg/dL) | 8.8 [0.1-17.6] | 16.0 [10.7-23.0] | <0.001 | no |
| HDL 2 phospholipids (mg/dL) | 6.6 [3.3-8.9] | 9.9 [7.5-13.3] | <0.001 | yes |
| HDL 3 phospholipids (mg/dL) | 7.8 [5.3-11.2] | 12.6 [9.3-14.1] | <0.001 | yes |
| HDL 4 phospholipids (mg/dL) | 14.1 [10.3-18.1] | 20.4 [16.7-26.2] | <0.001 | yes |
| **HDL subfraction – ApoA1** | | | | |
| HDL 1 ApoA1 (mg/dL) | 8.7 [0.0-20.6] | 21.9 [12.1-27.2] | <0.001 | yes |
| HDL 2 ApoA1 (mg/dL) | 9.2 [4.9-12.9] | 14.6 [11.8-18.7] | <0.001 | yes |
| HDL 3 ApoA1 (mg/dL) | 14.7 [10.5-21.0] | 22.2 [18.3-24.3] | <0.001 | yes |
| HDL 4 ApoA1 (mg/dL) | 40.8 [29.1-51.8] | 56.1 [47.6-79.2] | <0.001 | yes |
| **HDL subfraction – ApoA2** | | | | |
| HDL 1 ApoA2 (mg/dL) | 1.4 [0.1-2.1] | 2.0 [0.9-3.1] | 0.032 | no |
| HDL 2 ApoA2 (mg/dL) | 2.1 [1.6-2.8] | 2.2 [1.5-3.2] | 0.539 | no |
| HDL 3 ApoA2 (mg/dL) | 4.0 [2.7-5.3] | 4.4 [3.9-5.2] | 0.221 | no |
| HDL 4 ApoA2 (mg/dL) | 11.4 [6.2-15.3] | 14.3 [11.4-18.6] | 0.014 | no |

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In this investigation, the concentrations of lipoprotein parameters were obtained using an independent method. To validate or discard the previous findings, univariable logistic regression analyses was performed again with the variables identified in metabolomic analysis. Similar to the previous investigation, sole quantitative variables were not significantly associated with mortality outcomes regarding ICU- and 28-day mortality in control and sepsis cohort (**Table 13**).

**Table 13: Metabolomics - univariable logistic regression for lipoproteins in sepsis cohort.**

| Outcome variable | 28-day mortality | | | | ICU mortality | | |
|---|---|---|---|---|---|---|---|
| **Variable** | **Odds ratio** | **95% confidence interval** | **p** | | **Odds ratio** | **95% confidence interval** | **p** |
| **Main classes** | | | | | | | |
| Triglycerides (mg/dL) | 0.78 | 0.37-1.62 | 0.501 | | 0.90 | 0.42-1.93 | 0.789 |
| Total cholesterol (mg/dL) | 0.44 | 0.15-1.25 | 0.121 | | 0.43 | 0.15-1.21 | 0.108 |
| LDL cholesterol (mg/dL) | 0.90 | 0.59-1.37 | 0.629 | | 0.85 | 0.56-1.30 | 0.456 |
| HDL cholesterol (mg/dL) | 0.92 | 0.53-1.58 | 0.756 | | 0.85 | 0.49-1.49 | 0.576 |
| Total ApoA1 (mg/dL) | 0.92 | 0.46-1.85 | 0.824 | | 0.74 | 0.36-1.52 | 0.417 |
| Total ApoA2 (mg/dL) | 0.23 | 0.07-0.78 | 0.018 | | 0.37 | 0.14-0.99 | 0.047 |
| Total ApoB100 (mg/dL) | 0.60 | 0.20-1.78 | 0.357 | | 0.67 | 0.22-2.06 | 0.485 |
| **Particles** | | | | | | | |
| Total particle number (nmol/L) | 0.60 | 0.21-1.76 | 0.356 | | 0.67 | 0.22-2.03 | 0.482 |
| VLDL particle number (nmol/L) | 0.91 | 0.48-1.72 | 0.765 | | 1.02 | 0.52-2.00 | 0.947 |
| IDL particle number (nmol/L) | 0.71 | 0.45-1.12 | 0.144 | | 0.77 | 0.51-1.17 | 0.223 |
| LDL particle number (nmol/L) | 0.75 | 0.35-1.63 | 0.473 | | 0.78 | 0.35-1.74 | 0.550 |
| **Triglycerides in subclasses** | | | | | | | |
| VLDL (mg/dL) | 0.94 | 0.57-1.58 | 0.826 | | 0.95 | 0.56-1.63 | 0.857 |
| IDL (mg/dL) | 0.87 | 0.59-1.28 | 0.475 | | 0.94 | 0.62-1.40 | 0.749 |
| LDL (mg/dL) | 0.73 | 0.38-1.40 | 0.339 | | 0.88 | 0.45-1.71 | 0.697 |
| HDL (mg/dL) | 1.03 | 0.49-2.17 | 0.928 | | 1.09 | 0.49-2.41 | 0.838 |
| **Cholesterol in subclasses** | | | | | | | |
| VLDL (mg/dL) | 0.69 | 0.36-1.32 | 0.259 | | 0.72 | 0.37-1.42 | 0.347 |
| IDL (mg/dL) | 0.73 | 0.45-1.16 | 0.184 | | 0.78 | 0.49-1.23 | 0.826 |
| LDL (mg/dL) | 0.90 | 0.60-1.37 | 0.629 | | 0.85 | 0.56-1.30 | 0.456 |
| HDL (mg/dL) | 0.92 | 0.53-1.58 | 0.756 | | 0.85 | 0.49-1.49 | 0.576 |
| **Free cholesterol in subclasses** | | | | | | | |
| VLDL (mg/dL) | 0.80 | 0.42-1.52 | 0.499 | | 0.79 | 0.41-1.54 | 0.495 |
| IDL (mg/dL) | 0.73 | 0.43-1.25 | 0.257 | | 0.81 | 0.47-1.40 | 0.457 |
| LDL (mg/dL) | 1.01 | 0.55-1.85 | 0.985 | | 1.02 | 0.54-1.94 | 0.955 |
| HDL (mg/dL) | 1.03 | 0.72-1.45 | 0.861 | | 0.90 | 0.62-1.31 | 0.594 |
| **Phospholipids in subclasses** | | | | | | | |
| VLDL (mg/dL) | 1.05 | 0.71-1.55 | 0.811 | | 1.01 | 0.67-1.52 | 0.962 |
| IDL (mg/dL) | 0.77 | 0.49-1.20 | 0.244 | | 0.87 | 0.55-1.37 | 0.551 |
| LDL (mg/dL) | 0.87 | 0.40-1.90 | 0.731 | | 0.89 | 0.40-1.99 | 0.773 |
| HDL (mg/dL) | 1.07 | 0.66-1.74 | 0.777 | | 1.02 | 0.62-1.69 | 0.942 |
| **Apolipoproteins in subclasses** | | | | | | | |
| ApoA1 in HDL (mg/dL) | 1.09 | 0.78-1.54 | 0.613 | | 1.00 | 0.71-1.42 | 0.991 |
| ApoA2 in HDL (mg/dL) | 0.20 | 0.05-0.74 | 0.016 | | 0.34 | 0.12-1.00 | 0.049 |
| ApoB in VLDL (mg/dL) | 0.89 | 0.44-1.77 | 0.732 | | 1.01 | 0.49-2.08 | 0.979 |
| ApoB in IDL (mg/dL) | 0.66 | 0.38-1.15 | 0.147 | | 0.75 | 0.43-1.30 | 0.310 |
| ApoB in LDL (mg/dL) | 0.75 | 0.34-1.65 | 0.475 | | 0.78 | 0.34-1.77 | 0.556 |

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### 5.5.2. Step Two: Untargeted $^{1}$H NMR spectroscopy metabolomics analysis

$^{1}$H NMR spectroscopy enables the possibility to perform an untargeted metabolomic analysis. All metabolites present, at a given timepoint, are assessed and differences between groups are explored. As thousands of variables are investigated, only highly significant differences between groups are examined. We extracted metabolites from serum, thus removing lipoproteins which otherwise cloak metabolite signals, thereby enhancing signal quality. Differences between survivors and non-survivors in the sepsis group, as well as differences between patients with or without septic shock were investigated.

Using PCA, we found visual differences between survivors/non-survivors in both, the sepsis group without shock and in the septic shock group, with a PC1 and PC2 of 55% and 14.3%, respectively (**Figure 7A**). The PLS-DA resulted in a Component 1 and 2 of 50.4% and 14.6%, respectively. In O-PLS-DA for the septic shock group, survivors and non-survivors had an acceptable clustering goodness of fit with a $R^2Y$ of 0.965 and a cross-validation score $Q^2$ of 0.346 ($p<0.01$; **Figure 7B middle-left image**). Similarly, between survivors of the shock and no-shock group, there was acceptable goodness of fit with $R^2Y$ of 0.966 ($Q^2$ = 0.438, $p<0.01$, **Figure 7B middle-right image**). On the other hand, no significant clustering for survivors versus non-survivors in the no-shock group (**Figure 7B left image**), or for non-survivors between the no-shock and shock group (**Figure 7B right image**) were found (p values 0.93 and 0.62, respectively).

Several metabolites were different between sepsis and septic shock, as well as survivors and non-survivors. Lactate was significantly higher in patients with septic shock compared to patients without shock. Lactate was therefore the internal validation metabolite, as this finding is not unexpected, because elevated lactate is a parameter found in the definition of septic shock (**Figure 7E far-left boxplot**).

The branched-chain amino acids (BCAA) were significantly different between sepsis survivors and non-survivors (**Figure 7C**). In detail, levels of valine (55.0 vs 33.0 normalized signal intensity (NSI) units, p=0.002), leucine (70.8 vs 53.4 NSI, p=0.005) and isoleucine (18.1 vs 15.2 NSI, p=0.012) were higher in ICU survivors compared to non-survivors (**Figure 7C**). In the investigation of differences between septic shock and no-shock patients, lower levels of BCAA in the former group were found. In detail, valine at 43.3 vs 64.3 (p=0.005),

leucine at 57.0 vs 73.0 (p=0.034), and isoleucine at 15.2 vs 17.9 (p=0.048) were lower in patients with septic shock compared to those without septic shock (**Figure 7E**).

To further stratify patients and identify possible biomarkers in the more severely ill patients with septic shock, we investigated differences between survivors and non-survivors, and found that BCAAs and 3-hydroxybutyrate were lower in non-survivors, while other unassignable metabolites were higher in non-survivors (**Figure 7D**).

Among other metabolites, the most prominent metabolic differences between groups were found for phenylalanine and tyrosine, however, these changes did not reach statistical significance.

Figure 7 Legend (next page): A: PCA and PLS-DA for separation of survivors and non-survivors in the sepsis cohort in patients with or without septic shock. B: O-PLS-DA for the four groups of survivors and non-survivors in the shock or no-shock group (see main text for more details). C: Volcano plot (logarithmic scale) for metabolomic differences between groups (sepsis survivors vs non-survivors). Horizontal dashed line represents a significance of 5%. Boxplots for most changed and most significant metabolites. D: Volcano plot and boxplots for differences of septic shock survivors vs non-survivors. E: Boxplots for the most changed and most significant metabolites in sepsis patients with or without septic shock.

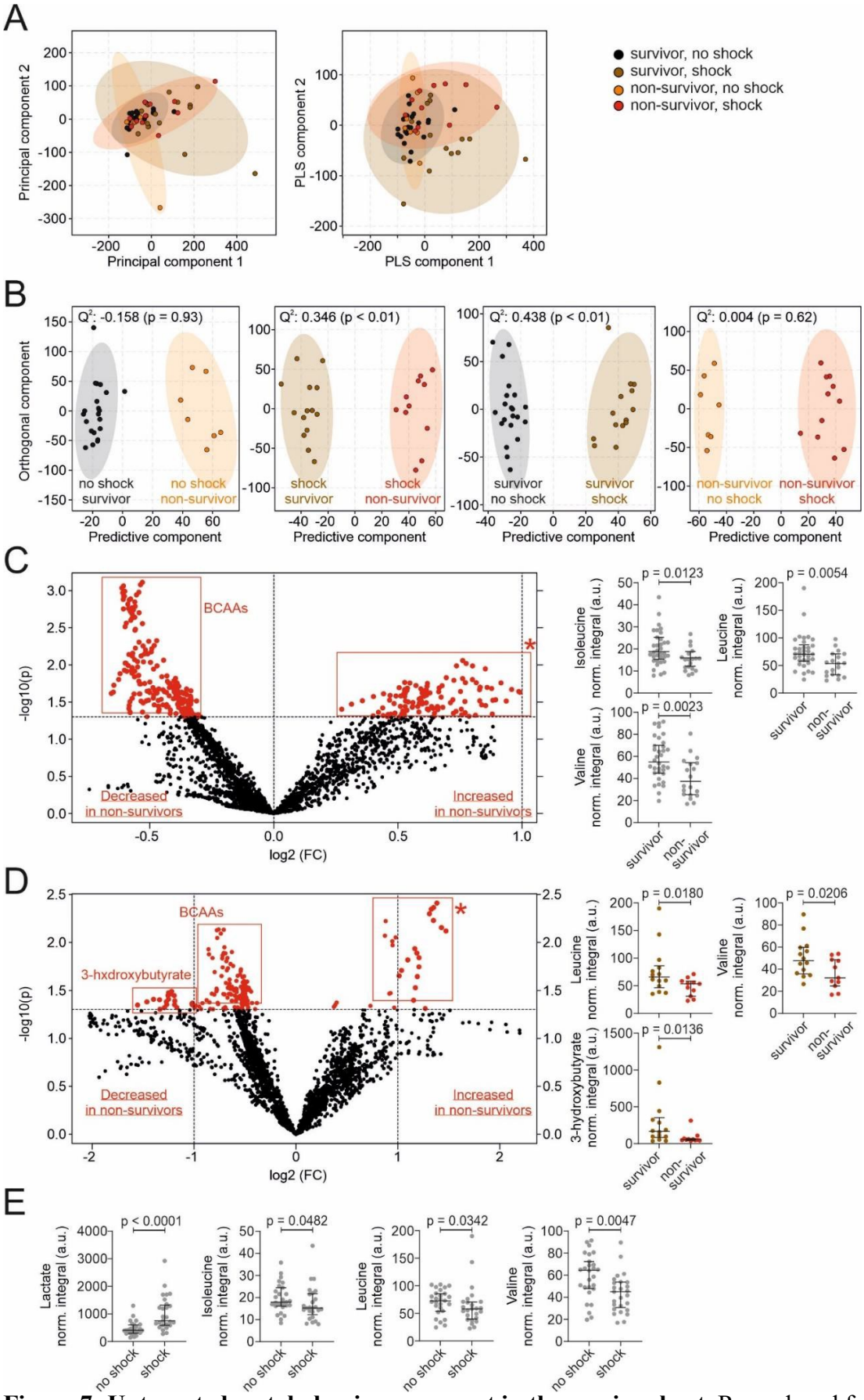


**Figure 7: Untargeted metabolomic assessment in the sepsis cohort.** Reproduced from (2) with permission from Nutrients. * = unassigned metabolites.

### 5.5.3. Correlations:

In the investigation of relationships between the metabolites, BCAA partially correlated with the SOFA score. Valine (-0.338, p=0.01) and isoleucine (-0.284, p=0.04) inversely correlated with the SOFA score, while leucine (-0.220, p=0.114) did not. Also, the BCAA correlated with each other. In detail, valine correlated with leucine (0.860, p<0.0001) and isoleucine (0.833, p<0.0001), and leucine with isoleucine (0.798, p<0.0001). Among the inflammatory markers, CRP inversely correlated with valine (-0.271, p=0.05) but not leucine (-0.223, p=0.109) or isoleucine (-0.208, p=0.136), while PCT inversely correlated with all three BCAA, valine (-0.401, p=0.003), leucine (-0.396, p=0.003) and isoleucine (-0.408, p=0.002). No correlations for age or BMI with SOFA score, lactate, 3-hydroxybutyrate, inflammatory markers, or BCAA were found.

### 5.5.4. Sepsis ICU- and 28-day mortality investigated with metabolomic results

In univariable logistic regression, several variables were associated with 28-day mortality as outcome of interest in the sepsis cohort. Higher levels of the BCAA, valine (OR per doubling = 0.18, 95%CI: 0.06-0.56; p=0.003), leucine (OR per doubling = 0.19, 95%CI: 0.06-0.59; p=0.004), and isoleucine (OR per doubling = 0.29, 95%CI: 0.09-0.93; p=0.038) were associated with decreased 28-day mortality (**Table 14**). Furthermore, older age and higher CRP were associated with increased risk of 28-day mortality as shown above (**Table 9**).

Regarding ICU mortality as outcome of interest in univariable logistic regression solely SOFA score and BCAA levels were significantly associated (**Table 9, Table 14**). In detail, higher valine (OR per doubling = 0.19, 95%CI: 0.06-0.58; p=0.004), higher leucine (OR per doubling = 0.22, 95%CI: 0.07-0.66; p=0.007), and higher isoleucine (OR per doubling = 0.23, 95%CI: 0.07-0.81; p=0.023), as well as lower SOFA score (OR per 1 point increase = 1.36, 95%CI: 1.12-1.64; p=0.002) were associated with a lower risk of ICU mortality.

**Table 14: Metabolomics - univariable logistic regression for metabolites in sepsis cohort.**

| Outcome variable | 28-day mortality | | | | ICU mortality | | |
|---|---|---|---|---|---|---|---|
| **Metabolites** | **Odds ratio** | **95% confidence interval** | **p** | | **Odds ratio** | **95% confidence interval** | **p** |
| | | | | | | | |
| Valine (per doubling) | 0.18 | 0.06-0.56 | 0.003 | | 0.19 | 0.06-0.58 | 0.004 |
| Leucine (per doubling) | 0.19 | 0.06-0.59 | 0.004 | | 0.22 | 0.07-0.66 | 0.007 |
| Isoleucine (per doubling) | 0.29 | 0.09-0.93 | 0.038 | | 0.23 | 0.07-0.81 | 0.023 |
| Acetate (per doubling) | 1.26 | 0.57-2.80 | 0.572 | | 1.24 | 0.54-2.85 | 0.609 |
| 3-Hydroxybutyrate (per doubling) | 0.91 | 0.61-1.38 | 0.668 | | 0.79 | 0.50-1.26 | 0.326 |
| Phenylalanine (per doubling) | 1.77 | 0.75-4.19 | 0.194 | | 1.23 | 0.53-2.88 | 0.631 |
| Tyrosine (per doubling) | 0.83 | 0.35-1.95 | 0.665 | | 0.82 | 0.33-2.04 | 0.675 |
| Lactate (per doubling) | 1.15 | 0.65-2.04 | 0.632 | | 1.00 | 0.55-1.81 | 0.996 |
| Citrate (per doubling) | 1.41 | 0.44-4.54 | 0.563 | | 0.90 | 0.27-3.03 | 0.865 |

Reproduced and adapted from (2) with permission from Nutrients. Most prominent changed metabolites between sepsis survivors and non-survivors identified with untargeted metabolomic analysis. Odds ratio per doubling of the predictor variable were obtained by a log2(x+1) transformation of the variable.

In multivariable logistic regression analyses, valine prevailed as a significant predictor both for 28-day and ICU mortality (**Table 15**). Likewise, the other BCAA also persisted as significant predictors of outcome in multivariable analyses for 28-day mortality. For ICU mortality valine and leucine, but not isoleucine prevailed in multivariable models (**Table 15 and 16**).

**Table 15: Metabolomics - multivariable logistic regression models for metabolites for ICU mortality in sepsis patients.**

| **Multivariable model 1: ICU mortality** | | **Odds ratio** | **95% confidence interval** | **p** |
|---|---|---|---|---|
| SOFA score (per 1 point increase) | | 1.29 | 1.06-1.57 | 0.012 |
| Valine (per doubling) | | 0.26 | 0.08-0.85 | 0.026 |
| | | | | |
| **Multivariable model 2: ICU mortality** | | **Odds ratio** | **95% confidence interval** | **p** |
| SOFA score (per 1 point increase) | | 1.33 | 1.09-1.62 | 0.005 |
| Leucine (per doubling) | | 0.27 | 0.09-0.83 | 0.022 |
| | | | | |
| **Multivariable model 3: ICU mortality** | | **Odds ratio** | **95% confidence interval** | **p** |
| SOFA score (per 1 point increase) | | 1.31 | 1.08-1.59 | 0.006 |
| Isoleucine (per doubling) | | 0.35 | 0.09-1.27 | 0.111 |

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**Table 16: Metabolomics - multivariable logistic regression models for metabolites for 28-day mortality in sepsis patients.**

| **Multivariable model 1: 28-day mortality** | | **Odds ratio** | **95% confidence interval** | **p** |
|---|---|---|---|---|
| Age (per 5-year increase) | | 1.25 | 1.00-1.56 | 0.048 |
| C-reactive protein (per 100 mg/L increase) | | 1.37 | 0.80-2.35 | 0.257 |
| Valine (per doubling) | | 0.19 | 0.05-0.66 | 0.009 |
| | | | | |
| **Multivariable model 2: 28-day mortality** | | **Odds ratio** | **95% confidence interval** | **p** |
| Age (per 5-year increase) | | 1.24 | 1.00-1.55 | 0.049 |
| C-reactive protein (per 100 mg/dL increase) | | 1.39 | 0.80-2.40 | 0.240 |
| Leucine (per doubling) | | 0.20 | 0.06-0.69 | 0.011 |
| | | | | |
| **Multivariable model 3: 28-day mortality** | | **Odds ratio** | **95% confidence interval** | **p** |
| Age (per 5-year increase) | | 1.27 | 1.02-1.58 | 0.034 |
| C-reactive protein (per 100 mg/dL increase) | | 1.50 | 0.90-2.51 | 0.122 |
| Isoleucine (per doubling) | | 0.26 | 0.07-0.98 | 0.047 |

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To further investigate the 28-day mortality in the sepsis cohort, the SOFA score was also included into exploratory models, as this variable was not statistically significant associated with the endpoint in univariable logistic regression analysis with the sample size of this study. The additional models revealed that both valine and isoleucine prevailed in multivariable logistic regression analyses (**Table 17**).

**Table 17: Metabolomics - additional multivariable logistic regression models for 28-day mortality in sepsis patients (including the SOFA score).**

| **Multivariable model 1: 28-day mortality** | | **Odds ratio** | **95% confidence interval** | **p** |
|---|---|---|---|---|
| Age (per 5-year increase) | | 1.33 | 1.03-1.71 | 0.028 |
| C-reactive protein (per 100 mg/dL increase) | | 1.36 | 0.78-2.36 | 0.272 |
| Valine (per doubling) | | 0.21 | 0.06-0.78 | 0.019 |
| SOFA score (per 1 point increase) | | 1.14 | 0.94-1.38 | 0.185 |
| | | | | |
| **Multivariable model 2: 28-day mortality** | | **Odds ratio** | **95% confidence interval** | **p** |
| Age (per 5-year increase) | | 1.34 | 1.04-1.73 | 0.024 |
| C-reactive protein (per 100 mg/dL increase) | | 1.32 | 0.76-2.32 | 0.326 |
| Leucine (per doubling) | | 0.21 | 0.06-0.75 | 0.016 |
| SOFA score (per 1 point increase) | | 1.18 | 0.97-1.43 | 0.100 |
| | | | | |
| **Multivariable model 3: 28-day mortality** | | **Odds ratio** | **95% confidence interval** | **p** |
| Age (per 5-year increase) | | 1.36 | 1.05-1.75 | 0.019 |
| C-reactive protein (per 100 mg/dL increase) | | 1.47 | 0.86-2.49 | 0.156 |
| Isoleucine (per doubling) | | 0.29 | 0.08-1.15 | 0.078 |
| SOFA score (per 1 point increase) | | 1.17 | 0.97-1.42 | 0.099 |

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### 5.5.5. AUROC investigated with metabolomic results

For 28-day mortality, valine (AUROC=0.75, 95%CI: 0.62-0.89), leucine (AUROC=0.75, 95%CI: 0.62-0.88), and isoleucine (AUROC=0.69, 95%CI: 0.54-0.83) were strong discriminators for the outcome. Also, regarding ICU mortality, valine (AUROC=0.75, 95%CI: 0.62-0.89), leucine (AUROC=0.73, 95%CI: 0.59-0.88), and isoleucine (AUROC=0.71, 95%CI: 0.57-0.85) were strong markers to differentiate survivors from non-survivors (**Figure 8 upper-panel)**.

The AUROC for the SOFA score in regard to ICU- and 28-day mortality was shown above. We investigated exploratorily, if a ratio of the SOFA score to a sum of BCAA (valine plus leucine plus isoleucine) may be a good discriminator and found a higher AUROC of 0.74 (95%CI: 0.60-0.88) and 0.85 (95%CI: 0.73-0.96) for 28-day and ICU mortality, respectively (**Figure 8 lower-panel**).

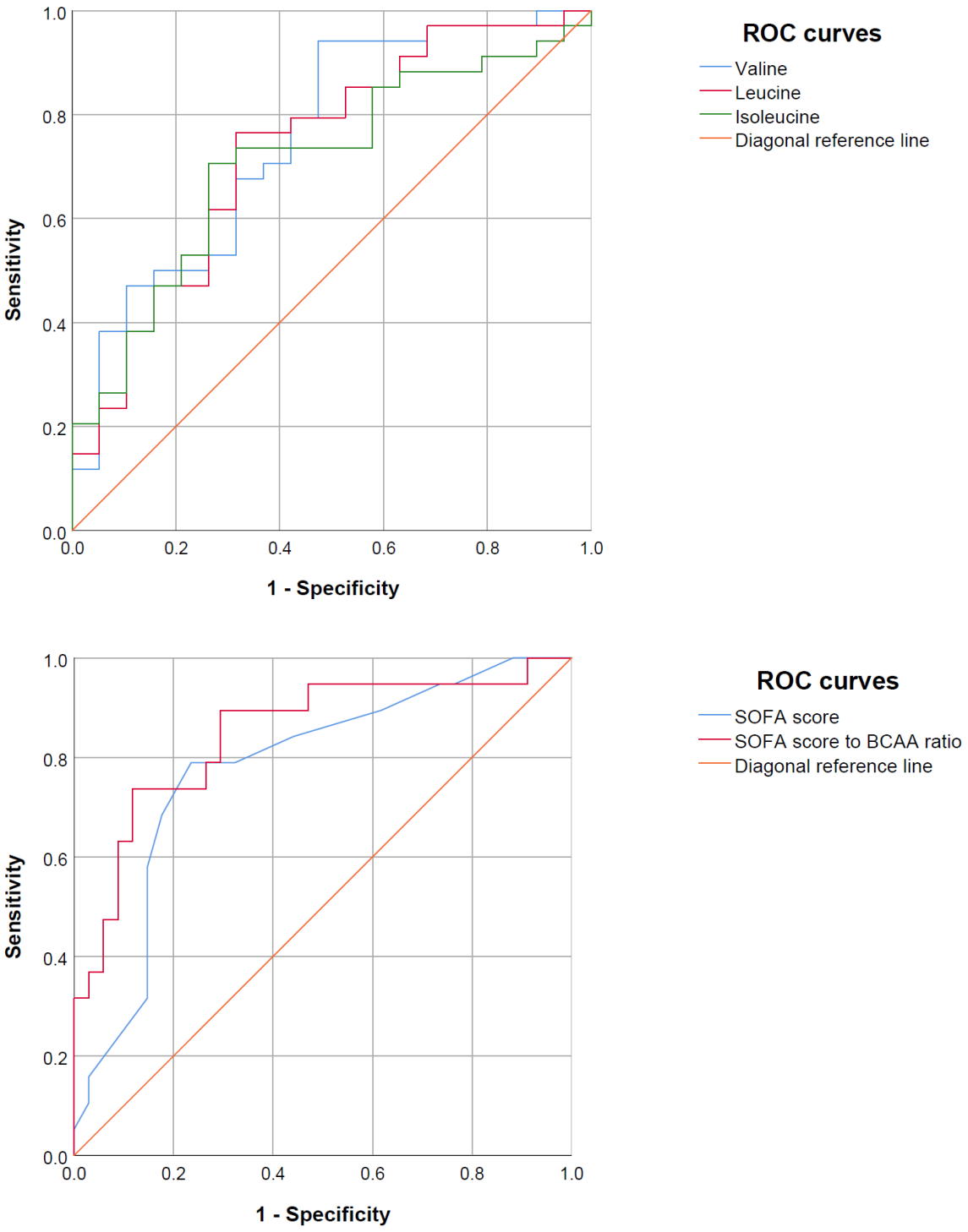


**Figure 8: AUROC of branched-chain amino acids for ICU mortality.** Reproduced and adapted from (2) with permission from Nutrients.

Upper panel: Valine (blue line), Leucine (red line), Isoleucine (green line).

Lower panel: SOFA score (blue line), ratio of SOFA score to BCAA (red line).

The orange line is the diagonal reference line representing a 50% chance.

### 5.5.6. Survival analyses

We performed 28-day Kaplan-Meier survival-analyses and compared groups using a log-rank test. Groups were defined as those above and those below the 25$^{th}$ percentile of the respective BCAA, which was 33.3, 44.1, and 13.7 NSI for valine, leucine, and isoleucine, respectively. The survival estimates for valine were 66% vs 14% (log-rank p=0.0001), for leucine 64% vs 21% (log-rank p=0.003), and for isoleucine 61% vs 29% (log-rank p=0.003) in those patients above compared to those below the 25$^{th}$ percentile (**Figure 9**).

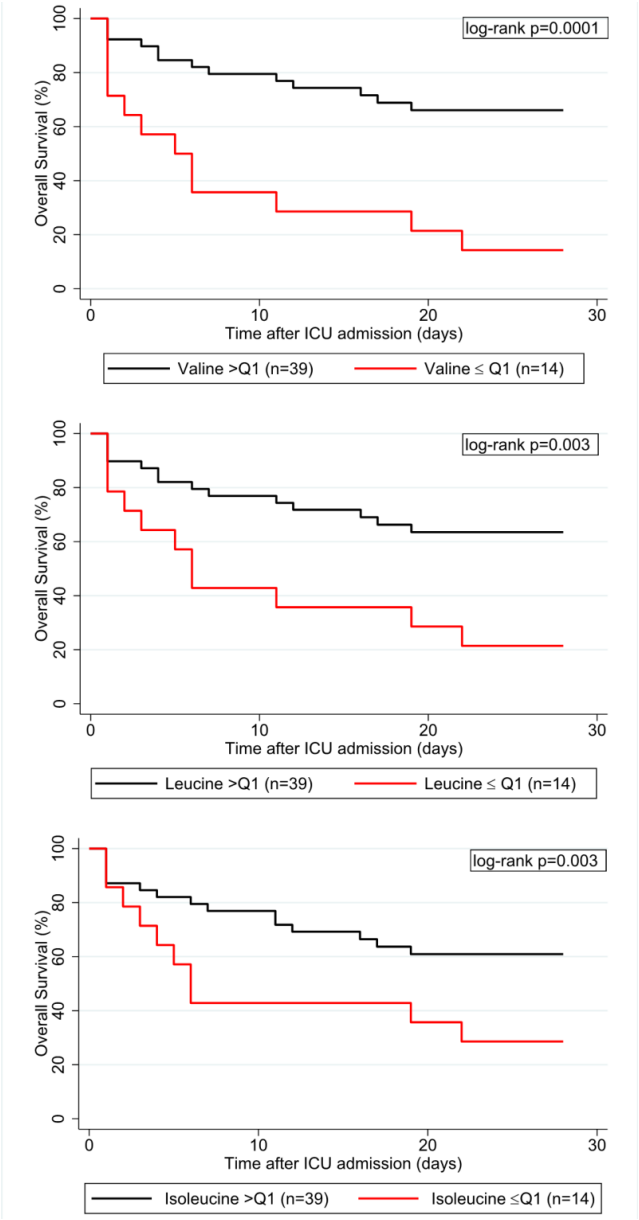


**Figure 9: Kaplan-Meier survival curves for branched-chain amino acids.** Reproduced and adapted from (2) with permission from Nutrients.

# 6. Discussion

In my thesis, we investigated quantitative and qualitative alterations of lipoproteins in patients with sepsis and septic shock who were admitted to the ICU. We found that the functionality parameter arylesterase activity (AEA) of the HDL associated paraoxonase (PON) was a robust predictor of ICU- and 28-day mortality. Furthermore, we investigated metabolites of sepsis patients to validate our findings, to identify novel biomarkers allowing for early sepsis detection, and to discover potential new therapeutic targets. We were able to reveal significantly lower levels of the branched-chain amino acids (BCAA) group consisting of valine, leucine, and isoleucine in sepsis non-survivors compared to survivors.

Sepsis is still a major contributor to mortality and morbidity in ICUs worldwide. The general management of sepsis patients has improved over recent years, leading to a reduction in case fatality rates. It is of utmost importance to detect sepsis early, allowing for immediate ICU admission, monitoring, and treatment using established and proven therapies. This early recognition of sepsis is necessary to allow prompt source control, to warrant aggressive resuscitation measures including calculated administration of intravenous fluids and vasopressors, as well as early antimicrobial therapy (88, 89). Several biomarkers have been investigated as diagnostic and therapeutic options but were not consistently able to demonstrate their value in sepsis patients. HDL has an important role in the reverse cholesterol transport, especially in a modern world where a high caloric intake and obesity are pandemically-like present. However, HDL is a complex particle with a vast number of associated proteins and has prevailed over evolutionary-biological processes likely because it influences several other relevant body functions including infection defense mechanisms.

In this study, we prospectively recruited 53 patients with sepsis and 25 controls without sepsis or bacteremia at the time of sampling. The median age of the sepsis cohort was 66 years, which is similar to other studies investigating ICU sepsis patients. Exemplarily, a study in Dutch ICUs found a mean age of 64. Similarly, in a severe sepsis study assessing 198 ICUs in Europe the median age was 65 years (43, 199). The age of admitted patients has been increasing over the years and an analysis in the UK found that from 1996 to 2004 the mean age increased from 59.5 to 62.2 years (36). In our study, the median time from ICU admission until discharge from ICU was 6 days, while the hospital LOS was 16 days. In a study from 2001, the hospital LOS of ICU sepsis patients was 23.3 days (34). Mean hospital LOS

significantly decreased from 17.3 in 2000 to 14.9 in 2007 (13). A later study found even shorter hospital LOS at 11.1 days (45). On the other hand, a cohort study of severe sepsis patients found a LOS similar to our study, with median ICU- and hospital LOS of 6 and 18 days, respectively (37). However, the reported ICU LOS throughout several studies had a wide range, stretching from 10.3 to 16 days (21, 41, 200). This may be driven by ICU bed capacities at the respective centers. In our study, the septic focus was localized in 42% in the lungs. In 17% an abdominal focus was present, followed by a urogenital focus in 11% of cases. These results are comparable to several other studies that also found the lungs and the abdomen to be the predominant foci (21, 43, 44, 199).

Sepsis necessitating ICU care represents a disease that has extraordinarily high mortality rates. In our study, the ICU- and 28-day mortality were 36% and 47%, respectively. In a cohort study from several ICUs in Australia and New Zealand, severe sepsis ICU- and 28-day mortality were 26.5% and 32.4%, respectively. However, compared to our study the patients were younger and about a quarter were surgical patients (37). Another study found that the mortality was higher in patients with older age, higher severity of comorbidities, medical patients, absence of an identifiable pathogen, number of organs failed, and in women (10). Sepsis incidence has been rising over the last decades, whereas case-fatality decreased. This is possibly attributed to better overall supportive ICU care but may also be driven by a dilution effect because less sick patients are admitted to ICU (30). In addition, in the USA more patients admitted to the ICU had single organ failure while in Europe patients had higher rates of multiple organ failure. Therefore, raw hospital mortality was higher in Europe with an absolute difference of 12.8%. After adjustment for sepsis origin, disease severity and other variables, the odds of mortality were not significantly different between Europe and the USA (49). The ICU- and 28-day mortality were therefore, at first glance, higher in our cohort compared to the above-mentioned study. This fact is likely due to a negative selection bias as only more severely ill patients are admitted to our ICU as a tertiary center. Furthermore, more stringent admission criteria are applied as medical ICU bed capacities are highly limited in our region. This negative influencing factor has been noted before; case-fatality rises when there is limited ICU bed capacity (30, 50). Other factors may also explain the differences between the studies. Earlier studies used the previous sepsis-2 definition, which included a less sick cohort than the currently valid sepsis-3 definition, which is much more restrictive. In our study, only patients fulfilling sepsis-3 criteria were included. In a meta-analysis

investigating studies from 2005 to 2018, septic shock was the cause for ICU admission in 10.4%, but only in 6.5% when applying sepsis 3-criteria (50). In the same paper, the study authors found an ICU mortality of 37.3%, hospital mortality of 39.0% and 28/30-day mortality of 36.7% when applying the "old" criteria. However, when using the sepsis-3 criteria for septic shock the ICU- and hospital mortality increased to 51.9% and 52.1%, respectively (50). Furthermore, in a German study an ICU- and hospital mortality of 44.3% and 50.9% for septic shock patients were found (21). Furthermore, we included only medical patients who have higher odds for mortality compared to surgical patients (10, 43). Therefore, considering all the mentioned factors, the mortality rates in our study are comparable to the literature (44, 46, 201). Some factors must additionally be considered when investigating sepsis patients. Balanced crystalloids and no colloidal infusions, except for the application of albumin, were used in our patients, which is in accordance with current guidelines and studies (88, 89, 202, 203). Noradrenalin/norepinephrine is the recommended first line vasopressor and was used in all patients that needed catecholamine therapy. If necessary, vasopressin and rarely dobutamine were used as needed (204-206).

## Lipoproteins

Lipids are water-insoluble structures and are therefore either bound to transport proteins or packed together with proteins, leading to the formation of lipoproteins. In the reverse cholesterol transport, HDL particles take up cholesterol from peripheral tissues and transport cholesterol to the liver. There, cholesterol uptake occurs via the SR-B1 pathway, or cholesterol is transferred to ApoB-lipoproteins and is then taken up via the LDL receptor into the liver. Excess cholesterol can then be removed from the body by excretion into the bile (207, 208). Despite a suggested beneficial effect of high HDL-C levels in large scale epidemiological studies that investigated cardiovascular risk factors, the causative role was not established. In contrary, it was even found that the HDL-C levels had a U-shaped form for cardiovascular risk (143). The capacity for reverse cholesterol transport is depending on the functionality of HDL particles. For example, an inverse association of carotid intima-media thickness was found with CEC but not with HDL-C (144). Apart from the important role of HDL in reverse cholesterol transport, the HDL particle is also an essential part in immune-defense and inflammatory response. HDL may bind and neutralize LPS and LTA of bacterial

cell walls, inhibit adhesion molecules and platelet activation, modulate eNOS, and exhibit antioxidant properties (173, 175, 209). HDL also regulates endothelial function, which is especially important during sepsis where microcirculation disturbances are commonly present (176, 210). In a network analysis of protein alterations, it was identified that HDL is the central factor in sepsis patients (211).

In this thesis, we found that the quantitative HDL-C levels were significantly lower in ICU sepsis at 14 mg/dL compared to ICU control patients without sepsis or bacteremia at 39 mg/dL. However, in sepsis patients, levels between survivors and non-survivors were not statistically significant different. Similarly, in a study by Tanaka et al sepsis patients had much lower HDL-C and total cholesterol levels than trauma patients, despite adjustment for age and sex (164). Therefore, trauma which also results in severe stress and inflammation did not cause lipid levels as low as in sepsis. However, different to our study, Tanaka et al investigated surgical ICU patients with the primary sepsis foci being peritonitis. The same group found in another study, that HDL-C was significantly lower in twenty septic shock patients compared to twenty ICU controls without septic shock. Sepsis patients furthermore showed a shift to larger HDL particles (157). Another study also has shown that HDL is remodeled during the acute phase, and that the number of large particles increases (212). The lower levels of HDL-C during the acute phase of sepsis may be a marker of disease severity and are most likely caused by an acute decline. Nevertheless, it may be possible that lower levels of HDL-C pave the way for a higher chance of acquiring sepsis. In the study by Grion et al per 1mg/dL increase in HDL-C on admission the risk of developing sepsis decreased by 3% (154). On the other hand, in a study assessing baseline lipid levels in about 30,000 individuals, sepsis events occurred in 1,845 cases. The authors found that serum HDL-C had no association with the occurrence of sepsis (213). Various studies have shown that the decreased levels of lipoproteins including HDL-C develop during an acute phase and that sepsis patients have lower levels than healthy individuals (158, 159, 161, 214). Several explanations for decreased HDL-C levels are possible: acute consumption of HDL particles for immune-defense, reduced liver synthesis of ApoA1 and therefore decreased formation of HDL particles, and increased removal via the SR-B1 receptor. Furthermore, increased EL and decreased LCAT activity may also contribute to the reduced HDL-C levels (164, 167, 215, 216). In addition to the non-significant association of the quantitative HDL-C with outcomes as seen in our study; HDL-C is furthermore not a good marker for HDL composition (146).

We found a strong inverse correlation between higher SOFA score as an established parameter for organ dysfunction and HDL-C levels. Similarly, in surgical patients, SOFA score was correlated with HDL-C. HDL-C levels were lower in those patients that were mechanically ventilated (164). Cirstea et al found that decreased levels of HDL-C were associated with higher rates of organ dysfunction in suspected sepsis (163). Likewise, Lekkou et al found a correlation of HDL-C with inflammatory markers and with the SAPS II score, which is another disease severity score previously used in intensive care medicine (217, 218). Despite strong correlations with severity, as well as significant differences between ICU controls and sepsis patients, we found no significant association of HDL-C levels with ICU- and 28-day mortality in logistic regression analyses. Animal models have shown that HDL-C deficient mice are more likely to die from septic shock (219), but murine and human lipid metabolism are not directly comparable (220). In humans, studies found heterogeneous results, partially explained by different cohorts. However, ambiguous results were also found when only medical ICU patients were included. Chien et al found that HDL-C on day one was associated with sepsis 30-day mortality (162). In ALF and acute on chronic liver failure, HDL-C levels were significantly lower in non-survivors compared to survivors but were not a superior prognostic marker compared to conventional biomarkers such as bilirubin and international normalized ratio (221). The before-mentioned group of Lekkou et al was able to show that survivors of community-acquired severe sepsis had higher HDL-C levels, and that all patients with HDL-C >25mg/dL survived (217). In contrast, in a study by Lee et al, HDL-C levels were not significantly different between survivors and non-survivors. Furthermore, only TG and SOFA score were associated with mortality, while HDL-C was not (165). A study by Shor et al showed that 1 mg/dL increase in HDL-C lead to a 11% relative decrease in the odds of sepsis. The authors also found that HDL-C levels <20 mg/dL compared to >65mg/dL were associated with a 17.5-fold increase in odds for death in hospitalized patients (155). However, the group investigated lipoprotein levels by chart review in hospitalized patients not necessarily directly on admission and not only in ICU patients. Van Leeuwen et al also did not find significant differences in lipoprotein concentrations between survivors and non-survivors (161). Therefore, our results are in line with several of these studies as we did not find a significant difference of HDL-C in survivors and non-survivors, nor an association of HDL-C with ICU or 28-day mortality in sepsis patients. Our findings and the current literature do not provide a clear causal relationship between HDL-C levels and mortality

endpoints. This further underscores the importance of the quality of HDL compared to pure quantity of HDL-C. In a study by Guirgis et al, the HDL inflammatory index, i.e. the ability of HDL to protect LDL from oxidation, at emergency department enrollment was not significantly different between patients with or without unfavorable outcomes. Nevertheless, an increase of the HDL inflammatory index during the first 48 hours was found to be associated with adverse outcome (222). However, the study did not correlate the obtained results with severity of disease such as the SOFA score, had a small sample size, and not all patients were admitted to the ICU. Furthermore, longitudinal changes over two days do not allow for early outcome prediction or intervention. The same study group performed a subgroup analysis in patients >65years and compared results to elderly healthy volunteers. The HDL inflammatory index was higher, e.g. more dysfunctional HDL was present, in patients with sepsis compared to healthy individuals (223). Despite that the studies did not solely investigate ICU patients; the results nevertheless underline that the functionality of HDL is relevant in sepsis. Apart from sepsis, similar results were found in other cohorts as well. The absolute number of HDL-C is not associated with reduced cardiovascular risk in patients with chronic kidney disease (CKD). It was found that dysfunctional HDL is present in dialysis patients, which has less effective antioxidative mechanisms and reduced CEC (224). However, further studies are still needed, as one monocentric investigation found no association of CEC with cardiovascular risk in CKD G2-G4 patients (225). On the other hand, clinical studies in the general population have shown a correlation between CEC and the occurrence of CVD (144, 226). For CEC we were able to demonstrate a trend towards decreased CEC in non-survivors compared to survivors at 8.4% and 9.6% (p=0.051), respectively. The CEC as a functionality marker was not statistically significant associated with ICU or 28-day mortality, but the CI suggests an association of decreased CEC with increased ICU mortality in sepsis patients, which may have become significant with a larger sample size.

The major finding of our study was that HDL-C levels, i.e. the quantitative amount, were not associated with mortality outcomes, whereas the qualitative marker AEA of the HDL associated PON was associated with ICU- and 28-day mortality in uni- and multivariable analyses. PON represents parts of the functionality of the HDL particle, especially its anti-inflammatory and antioxidative properties. These effects are especially relevant during acute critical illness and sepsis (227, 228). PON1 furthermore inhibits the formation of oxidized

LDL in the presence of reactive oxygen species and reduces monocyte chemoattractant protein 1 (MCP-1) production and release (181, 184, 229). In addition, it may be concluded that lower HDL-C is a marker of disease severity, while the PON1 reflects the capability of the defense mechanisms and determines outcomes. PON1 can hydrolyze oxidized phospholipids and acts as a lactonase leading to neutralization of homoserine lactones, e.g. from pseudomonas bacteria (183, 230-233). Several enzymes such as platelet-activating factor acetylhydrolase and PON1 are more prominent in small dense HDL and these smaller HDL particles have stronger antioxidative effects (234). In sepsis, however, there is a shift towards larger HDL particles (212). Our study shows a significant lower AEA in sepsis patients compared to controls (67 vs 111 mM/min/mL serum, $p<0.0001$). We were able to show that AEA was associated with ICU- and 28-day mortality in univariable analysis. Furthermore, the significant association with mortality endpoints prevailed in multivariable analyses, even when adding the SOFA score into the model. This underlines the importance of the functionality of HDL particles during sepsis. The CEC in our study was also lower in sepsis compared to control patients, but this difference did not reach statistical significance. Apart from our study, only few and small studies investigated qualitative HDL properties such as AEA and CEC during sepsis. In a study with only fifteen septic patients, the authors found no significant correlation between SOFA score and PON on admission (229). In our study, we found significant inverse correlations of increasing SOFA score with reduced AEA, but no correlation with CEC. Another small study assessed the PON1 in ten sepsis and ten controls and found similar results between the groups (223). One study group suggested that PON1 monitoring may be used as a marker of the sepsis trajectory during the ICU stay (227). Sharma et al found in a proteomic analysis, that patients with hospital acquired pneumonia had lower levels of PON1 in comparison to healthy volunteers (211). Draganov et al found that PON1 levels were lower in sepsis non-survivors compared to survivors (235). The group of Bojic et al found that surgical sepsis patients had lower PON1 activity compared to healthy controls and that PON1 was inversely associated with the risk of death (236). Therefore, the absence of differences between sepsis and control groups in some studies may be due to the small sample size. However, the findings of our study are in line with the few larger studies that have been reported and showed lower PON1 in sepsis compared to non-sepsis patients. Nevertheless, data on the functionality of HDL particles during sepsis are still sparse and further investigations are necessary. Paraoxon as a substrate for PON1 depends on the

polymorphism of two allozymes. In contrast, phenylacetate is non-discriminating and reflects both allozymes. In addition, several other genetic polymorphisms for PON1 have been identified (231, 237). Therefore, we chose AEA with phenylacetate as a substrate to assess PON1 effectiveness as it is more representative of the enzyme and is not altered by PON1 genetic polymorphisms. Furthermore, paraoxon is highly toxic, while phenyl acetate used in AEA is much safer to handle (233, 238). As noted, activity of PON is decreased during sepsis. On the other hand, levels of SAA rise during the acute phase and these proteins associate with HDL. ApoA1, usually the most abundant protein of HDL, is displaced by SAA. In addition, other proteins including PON are also dislocated from HDL particles (154, 161, 227, 233, 239, 240). We also found that SAA levels are significantly elevated in sepsis patients compared to ICU control patients (own unpublished data).

To improve sepsis outcomes and because of the pleiotropic effects of the HDL particle, several different lipid-based therapies have been investigated. Reconstituted HDL (rHDL) and ApoA1 mimetic peptides such peptide 4F may be therapeutic options during sepsis. ApoA1 mimetic peptides bind phospholipids and can associate with HDL (241, 242). For rHDL different formulations have been used with 1:50 to 1:150 (up to 1:200) ratios of ApoA1 to soybean phosphatidylcholine (243, 244). rHDL consisting of either phospholipid plus HDL apoprotein or phospholipid plus synthesized 18-amino acid peptide (mimicking ApoA1) were effective in LPS binding, while sole HDL apolipoprotein was not (174). In an animal model in rats, ApoA1 mimetic peptides stabilized hemodynamics after induced endotoxemia (245). Another animal study found that a reconstituted lipoprotein, containing human ApoA1 and phosphatidylcholine (1:200 ratio) protected rabbits from endotoxemia. In addition, the authors found that phosphatidylcholine without ApoA1 was significantly less effective (246). In three different experimental sepsis models in mice, rHDL significantly improved survival (247). In one study, rHDL was able to increase HDL levels and to restore endothelial vascular function in ABCA1 heterozygote humans (248). rHDL was also able to reduce cytokines and flu-like symptoms in healthy males exposed to *Escherichia coli* endotoxin (249). Likewise, in another study, healthy volunteers were exposed to *Escherichia coli* endotoxin and received either a soy-based phospholipid-rich solution (92.5% phosphatidylcholine and 7.5% triglycerides emulsified in sodium cholate) or placebo. Patients in the former group had lower inflammatory markers, lower temperature, and less clinical symptoms (250). A multicenter phase II randomized placebo-controlled trial (Lipid Infusion and Patient Outcomes in Sepsis -

LIPOS study) tested a phospholipid emulsion in 1,379 patients with suspected or confirmed gram-negative severe sepsis. There were no significant differences between 28-day mortality or new-onset organ failure between the phospholipid or placebo group (251). Limitations include, however, that recruitment was performed in 31 countries leading to large heterogeneity in patient care. In a secondary analysis of the LIPOS study, the subgroups of patients with serum albumin ≥1.5g/dL plus either cholesterol ≥1mM (40mg/dL) or HDL≥0.5mM (20mg/dL) had improved survival when treated with the study drug and it was concluded that the negative results of the LIPOS trial may have been affected by the rate of intravenous corticosteroid application (252). A rHDL (CSL-111 ®) was investigated for coronary artery disease and failed to provide significant differences between groups in atheroma or plaque volume but improved coronary score on angiography (253). Therefore, a large multicenter trial of CSL-112 ®, the successor of the above mentioned rHDL, in cardiovascular patients is currently performed. A search in PubMed for CSL-111/112 and sepsis only provided the study from Tanaka et al (247), but no additional results were found. In the Clinical Trial Registry (clinicaltrials.gov) currently no study for CSL-111/112 or other rHDL in sepsis patients is listed. However, an intervention study using a lipid emulsion (Smoflipid ®) is currently recruiting sepsis patients. Despite the negative results of the LIPOS trial, further studies, with a more homogenous protocolized patient care and with consideration of corticosteroid application, are necessary.

## Metabolomics and BCAA

In this study, we performed a targeted proton NMR spectroscopy for lipoprotein-derived parameters to validate and extend the previous findings. With this independent method we were able to show that levels of several lipoproteins were significantly altered. Confirming our previous data, we found that HDL-C, HDL-FC, ApoA1, and other parameters were significantly lower, while TG were higher in sepsis patients than in controls. These findings were highly significant as the corresponding p-values were below our calculated Sidak limit to correct for multiple testing. In multivariate analysis using PCA there were strong visual differences between ICU sepsis and ICU control patients. In O-PLS-DA, an algorithm where group assignment is provided, the clustering was strong for sepsis and controls. However, similar to the previous investigation, the quantitative lipoproteins were not associated with

ICU- or 28-day mortality in both cohorts. These data therefore confirm the previous findings that qualitative changes of lipoproteins are more relevant during sepsis than sole quantitative alterations.

In addition to targeted investigations, $^{1}$H NMR allows for untargeted metabolomics ("global metabolomics") and provides results with strong robustness and allows for reproduceable quantification. Within the metabolome, where a multitude of metabolites are present, effects of the genome, transcriptome, the proteome, and environmental factors are embedded (254). The metabolome gives a snapshot of all molecules present and indicates any physiological or pathological functions of pathways within the human body. Usually during classic investigations, only known biomarkers that are presumed to be relevant are examined. Other unknown but highly relevant metabolites that play a role in a given disease or condition may be missed. Therefore, to extend previous findings and to identify potential biomarkers and options for future therapeutic interventions, we used this untargeted analysis in the sepsis cohort. To enhance signal quality, we extracted samples and precipitated lipoproteins as these large structures may mask small metabolite differences. Thus, the signal-to-noise ratio is improved allowing small differences to be detected (255).

We were able to show that the levels of BCAA, which consist of valine, leucine, and isoleucine were significantly lower in sepsis non-survivors compared to ICU sepsis survivors. BCAA must be consumed by dietary intake of proteins, as this group is part of the essential amino acids that cannot be synthesized by the body itself (256). BCAA are necessary for protein synthesis and for improvement of muscle function and strength. In contrast to other amino acids, BCAA are not primarily metabolized in the liver but through muscle tissue (257). It is well known that patients with diabetes mellitus and insulin resistance have higher levels of BCAA (258). Insulin resistance and hyperglycemia are also common features during acute critical illness with a great degree of inflammation such as sepsis and studies have investigated glucose targets in those patients (259, 260). Critical illness neuropathy and myopathy with the degradation of muscle tissue and decreased muscle strength are also common findings in sepsis patients (261). We found that BCAA levels were significantly lower in sepsis survivors than non-survivors, and lower in patients with septic shock compared to those without shock. This leads to the assumption, that the breakdown, i.e. the catabolism of valine, leucine and isoleucine must be even higher than the release of BCAA from muscle protein degradation with increasing sepsis severity. BCAA can be degraded

through a reversible transamination modulated by branched-chain amino acid aminotransferase (BCAT) followed by an irreversible oxidative decarboxylation which is performed by the branched-chain alpha-keto acid dehydrogenase complex (BCKDC). The BCAT is found in various tissues but barely in the liver, while the BCKDC, which is an enzyme that is found on the inner mitochondrial membrane, is present throughout the body including the liver (256, 262, 263). As BCAA must be consumed dietary to maintain levels in the human body and cannot be synthesized, it might also be possible that the lower levels of BCAA found in sepsis non-survivors have not occurred after sepsis but beforehand. On this remark, four theories are possible. First, after an initial infection when sepsis has not yet developed, levels of BCAA are decreased because of a reduced appetite leading to a decreased oral intake while the body is fighting the infection (264, 265). Second, it might be possible that low BCAA are present even before the infection took place. Hence, low BCAA levels may predispose to sepsis and lead to worse trajectories when sepsis occurs. Reduced levels of BCAA may therefore be a representation of reduced muscle mass leading to less reserves during critical illness necessitating ICU care (266). Third, reduced BCAA may also be a surrogate marker for distorted gastrointestinal function during critical illness with permeability and uptake of nutrients being altered (267). Fourth, increased BCAA catabolism leads to increased glutamine synthesis and glutamine is a substrate used by macrophages and other inflammatory cells, therefore representing an immune response to infection (262). To elucidate on this important topic, widespread population analysis of BCAA levels need to be performed and cohorts then must be followed to investigate the rate of sepsis occurrence. Therefore, the current knowledge is still limited, but animal studies suggest that the BCAA decrease early after endotoxin application (268). Nevertheless, independently of the underlying reason for reduced BCAA levels, measurement of BCAA on admission is a strong predictor of mortality. We found that BCAA levels were associated with mortality outcome in uni- and multivariable regression analyses. Therefore, in sepsis patients admitted to the ICU, obtaining a BCAA level may serve as a prognostic marker, but could also be a target for therapeutic intervention. Huang et al investigated patients with severe infection and found that two types of metabolic changes were associated with higher chance of death – phenylalanine >84µM and phenylalanine <84µM with leucine <93µM (269). Similarly, in our study we found reduced levels of leucine in non-survivors, while the differences of phenylalanine did not reach statistical significance. Other studies found in small cohorts that BCAA levels are

reduced in sepsis patients compared to healthy individuals (270, 271). Also, a study group found that levels of BCAA in sepsis patients admitted to ICU were reduced compared to ICU patients without sepsis (272). The group of Liu et al investigated metabolites in ICU septic shock survivors and non-survivors. They found significant differences of several metabolites, notably, an increase of alanine, glutamate, glutamine, methionine, aromatic amino acids (AAA) as well as lactate and citrate in non-survivors (273). Similarly, Freund et al found that levels of AAA and sulfur-containing amino acids were higher, while levels of BCAA and alanine were lower in sepsis non-survivors compared to survivors (274). Sepsis patients are classified in those with and those without septic shock. Currently, the sepsis-3 criteria are used, and the septic shock definition consists of a necessity of vasopressor therapy to maintain a MAP above 65 mmHg despite adequate fluid resuscitation, and one biomarker, lactate, which must be above 2 mmol/L (6). Therefore, patients classified as septic shock must have had elevated lactate levels. In our study, we also found that lactate was significantly higher in septic shock patients compared to sepsis patients without septic shock and therefore provided an internal validation of our data. Interestingly, however, it was the most prominent marker to differentiate between those groups, and no other biomarker provided better differentiation in patients classified according to the current definition. The group of Puskarich et al found that BCAA on admission were predictors for resolution of septic shock within 48 hours (275). Therefore, BCAA may not only be predictors of outcome, but also correlate with disease severity. In our study, we were able to show that BCAA were inversely correlated with SOFA score representing the severity of organ dysfunction. In the septic shock subgroup, in addition to lower BCAA, levels of 3-hydroxybutyrate were also significantly decreased in non-survivors compared to survivors. BCAA additionally came into spotlight in liver cirrhosis patients and were especially investigated in the role during hepatic encephalopathy. BCAA were found to improve hepatic encephalopathy and cirrhotic patients profit from a supplementation with BCAA (276, 277). The mechanistic background for this positive effect is that BCAA increase the detoxification of ammonia in muscle tissue and ammonia is a key factor of hepatic encephalopathy (276, 278, 279). In one study, significant differences were found between patients with septic shock and encephalopathy compared to infected patients without encephalopathy. The former had lower levels of cysteine, isoleucine, glutamine, and arginine but increased levels of gamma-aminobutyric acid (GABA), tryptophan, phenylalanine, urea and ammonia (280). BCAA compete with the AAA, tryptophan, tyrosine,

and phenylalanine, for transportation across the blood-brain-barrier. BCAA therefore reduce cerebral tryptophan levels, a precursor for serotonin, which is a key substance in central fatigue (281). In addition, one can calculate the ratio between BCAA and the AAA phenylalanine and tyrosine, i.e. (valine plus leucine plus isoleucine) divided by (phenylalanine plus tyrosine), sometimes called Fischer's ratio – the normal ratio being around three (282). One study found that the Fischer's ratio was higher in healthy controls compared to SIRS or sepsis patients (271). Furthermore, surgical patients without septic encephalopathy had higher Fischer's ratio than sepsis patients suffering from encephalopathy (283). Some studies suggest beneficial effects of nutritional therapy that is enriched in BCAA in critically ill patients. For example, in both human and animal studies, high BCAA content was associated with increased BCAA/AAA ratio which improves encephalopathy and was associated with an improved nitrogen balance representing the net effect of muscle anabolism and catabolism (284-287). One randomized study found that BCAA supplementation to parenteral nutrition improved survival in sepsis patients (288). Furthermore, in liver transplant recipients the rate of postoperative bacteremia was significantly reduced in those receiving oral BCAA (289). Nevertheless, an earlier study showed no benefit of BCAA supplementation, however, limitations include that not all patients were treated in the ICU and were predominantly surgical patients (290). BCAA inhibit proteolysis, improve immune function, and activate mammalian target of rapamycin (mTOR) pathways. Furthermore, BCAA support lymphocyte growth and natural killer cell activity, which may be especially beneficial during sepsis (263, 291-293). A small study in post-surgical patients also found that BCAA increased lymphocyte levels (294). Notably, sarcopenia is a risk factor for an inferior outcome in critically ill patients with sepsis (295, 296). BCAA can enhance muscle mass and counteract muscle degradation (297-299). Therefore, BCAA may have the highest effectiveness in elderly, sarcopenic, and cirrhotic critically ill patients suffering from sepsis.

# 7. Strengths and Limitations

In this work, we investigated patients with sepsis admitted to the ICU as well as ICU controls without sepsis or bacteremia. A major strength is the exploration of lipoprotein parameters and metabolomics in patients under real-world non-experimental conditions. In comparison, mouse models have the limitation that the murine immune system is different to humans. Furthermore, LPS mouse models often do not represent the clinical status observed in humans and LPS may be used for endotoxic shock simulation but not for sepsis models. Often sudden endotoxemia or high loads of bacteria are used in models, whereas in reality only few sepsis patients present that fulminant. In contrast, sepsis may progress more slowly, and symptoms can be very subtle. Therefore, those animal models have only limited value in the investigation of sepsis prognostic markers and therapeutic options. Furthermore, mice have different lipoprotein metabolism and are naturally CETP deficient, leading to cholesterol transportation occurring mainly with HDL rather than LDL.

Another strength is the robustness of our data as we were able to study samples using two independent methods which provided us with congruent results. Lactate was found to be significantly different between sepsis and septic shock, which was expected due to current sepsis-3 criteria. However, this finding underlines the strength and robustness of our data and the good internal validity.

One more strength of our study is, that we only included medical ICU patients, which represent a more homogeneous critically ill population. Other studies have included patients from emergency departments, general wards, and ICU and therefore leading to a dilution effect and less comparability. We were furthermore able to obtain samples early, in median 3.3 hours, after ICU admission thus providing an immediate snapshot of the biochemical picture.

In addition, the appliance of an untargeted metabolomics approach enabled us to identify altered BCAA in sepsis patients with significantly lower levels in non-survivors compared to sepsis survivors. This underlines the value of specialized laboratory methods to identify potential new markers, but also emphasizes the necessity of a good cooperation between clinicians at the bedside and specialists in the laboratory.

Several limitations of our study must be considered. First, we only included medical ICU patients and performed a monocentric investigation. Therefore, despite very good internal validity, the external validity may be limited. Results may be applicable for other ICUs in Western countries but may not be transferable to all sepsis patients worldwide. According to a WHO letter six million deaths per year occur due to sepsis worldwide. Prevention strategies such has vaccines, good sanitation, access to save childbirth facilities, and access to clean water can reduce the number of sepsis cases per year. As we included patients only at ICU admission, leading to the inclusion of only the most critically ill patients and therefore more homogeneity, we cannot rule out that patients presented at different times within the disease. Some patients may have had a fulminant rapid trajectory, while others have subtle symptoms and only present to the hospital and ICU after a few days. This fact is a problem of all sepsis studies as patients may present at different time points of the disease. However, we were able to show that BCAA were significantly lower in sepsis non-survivors, and that BCAA levels were associated with mortality endpoints. Therefore, despite the unknown timepoint of presentation, BCAA may represent an integral of anabolism and catabolism over the last days prior to hospital admission. In this work, we only investigated whether patients survived or succumbed during the ICU stay or within 28 days after admission. However, sepsis survivors also have high morbidity such as neurocognitive disabilities, nutritional disorders, and physical limitation after the ICU stay. Therefore, in future studies differences in morbidity and long-term sequelae should also be investigated. A large limitation of our study is sample size, as a larger cohort may have enabled us to detect even smaller differences between groups and would have narrowed confidence intervals.

# 8. Conclusion and Outlook

We were able to provide novel insights regarding the role of lipoproteins in medical ICU patients during sepsis. Furthermore, we were able to show that septic dyslipidemia consists of quantitative changes of HDL-C and even more relevant, involves qualitative HDL particle changes. Most importantly, the anti-inflammatory and antioxidative arylesterase activity of the HDL associated PON was a strong predictor for ICU- and 28-day mortality. This effect even prevailed in multivariable analysis despite addition of the SOFA score, which is a broad ICU scoring system based on the severity of organ dysfunction. Furthermore, we validated lipoprotein findings using $^{1}$H NMR spectroscopy as an independent method and therefore provide robust results with strong internal validity. In an additional $^{1}$H NMR untargeted global metabolomics approach, we found that, apart from lipoproteins, branched-chain amino acids were significantly lower in sepsis non-survivors compared to survivors. The branched-chain amino acids were furthermore significantly associated with ICU- and 28-day mortality.

Future investigations should focus on examining arylesterase activity in a larger cohort of sepsis as well as other critically ill patients. This is especially urgent as many previous sepsis therapies have failed. The HDL particle has pleiotropic effects and may offer a wide potential to mitigate the deleterious outcome of sepsis patients.

More insights also need to be generated regarding the role of plasma platelet activating factor acetyl hydrolase, which is another anti-oxidative enzyme. In addition, phospholipase A2 cleaves phospholipids leading to the generation of lysophophatidylcholine, which also exhibit anti-inflammatory effects.

In our cohort, as a next step, we plan on investigating HDL metabolism associated enzymes such as LCAT, CETP, and PLTP in sepsis and control patients. Furthermore, endothelial lipase and serum amyloid A, which also influence HDL particles, will be examined.

Ongoing investigations are particularly important as sepsis rates are increasing. The case-fatality rate of sepsis is even higher than cardiovascular diseases, which has been in the spotlight, while sepsis did not receive widespread public attention over the last years.

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