Biomarkers in intensive care patients

Differentiating sepsis from other critical illnesses

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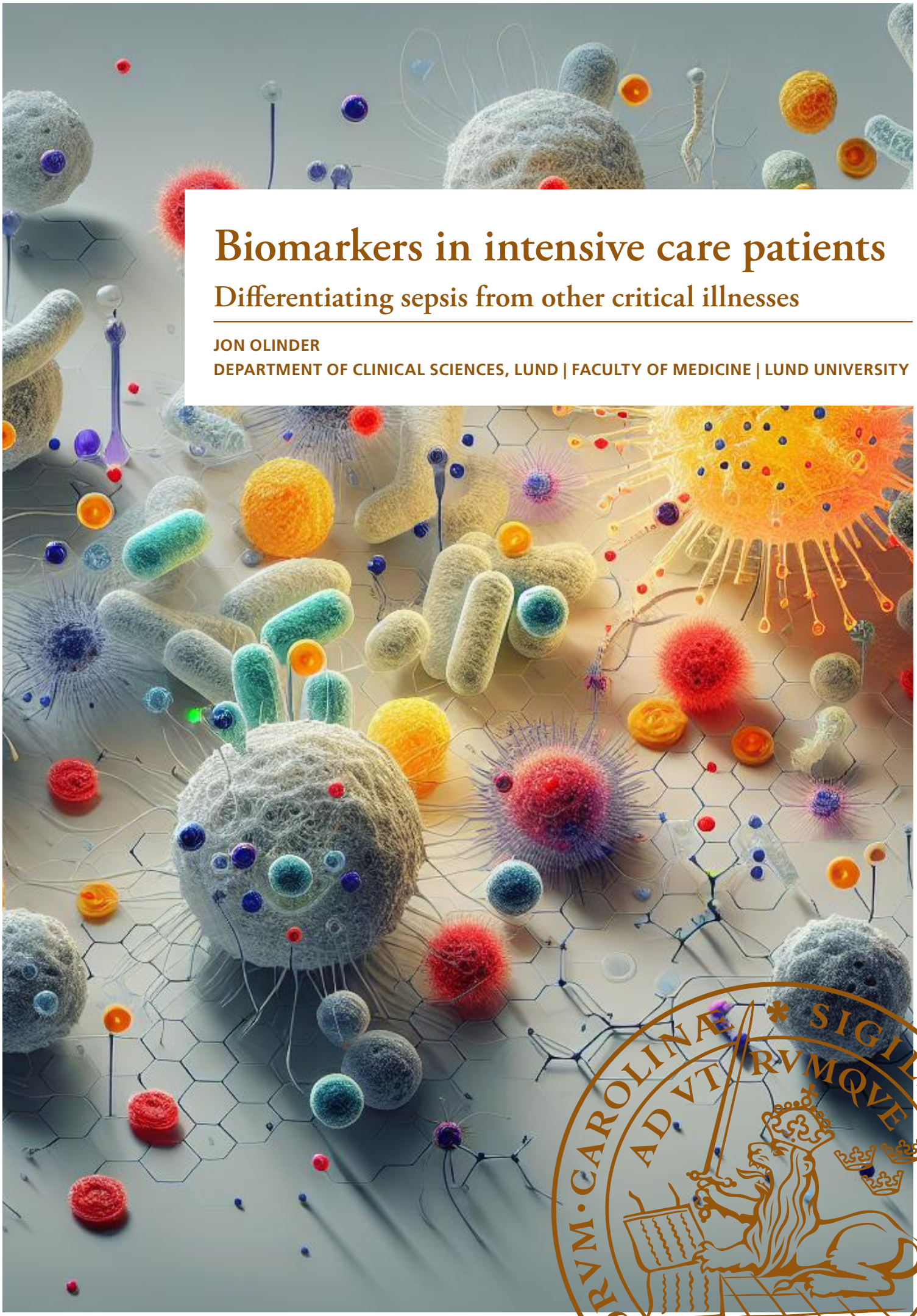

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LUND
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Biomarkers in intensive care patients. Differentiating sepsis from other critical illnesses.

Abstract

Treatment of sepsis and septic shock are challenging illnesses in intensive care units (ICU) together with other critical illness. To identify, predict, monitor, and prognosticate outcome clinicians rely on clinical evaluation, microbial findings and biomarkers.

Initially we performed a pilot study on hepcidin and reticulocyte hemoglobin (Ret-He) involving fifteen patients with community acquired septic shock. Blood samples were collected on seven consecutive days. The abovementioned biomarkers involved in iron metabolism, as well as heparin-binding protein, (HBP), were compared with conventionally used biomarkers, *e.g.* C-reactive protein, procalcitonin (PCT), lactate and with clinical evaluation methods. Response to treatment and complications among the patients were recorded and compared to the dynamics of the biomarkers. Maximal median levels of hepcidin were seen at inclusion, rapidly declining together with lactate and PCT. Correlations were found between hepcidin and CRP, PCT, and white blood count (WBC) performing Pearson correlation analyses. Ret-He correlated negatively with hepcidin. Ret-He decreased upon increase of hepcidin levels with normalization at 96 hours. We also observed that hepcidin was superior to other biomarkers in detecting secondary complications in critically ill patients.

In paper II, we extended the study, enrolling 164 patients with community acquired illness admitted within 24 hours of hospitalization. One hundred patients with sepsis (97/100 fulfilling the septic shock criteria) and 64 critically ill patients with no signs of infection at admission were included. Relevant cultures were found in 69% of sepsis patients. Adequate empiric antibiotics were administered to 98/100 of the septic patients. The study showed that hepcidin and HBP were elevated at inclusion and associated with the diagnosis of sepsis versus non-septic critical illness. Hepcidin declined significantly within the first 24 hours after treatment was started and almost normalized within 72 hours. HBP as well as CRP increased after admission and peaked at 24 hours after inclusion. Hepcidin presented superior sensitivity and specificity compared to HBP. An AUC>0.8 was reached with hepcidin or HBP, respectively, combined with CRP or PCT, respectively. A significant association was noted between low levels of hepcidin and 180-day mortality, whereas high initial concentrations of hepcidin in septic patients were associated with 180-day survival. A negative correlation was noted between hepcidin and SAPS 3 score.

In paper III, we investigated the association between hepcidin and HBP, respectively, and acute kidney injury (AKI) in patients from the same cohort as in study II. Exclusion criterion was known kidney disease prior to enrolment, leaving 140 patients eligible the study, 85 of whom were diagnosed with sepsis and 55 with another critical illness. No significant associations between hepcidin levels at admission and stage 2-3 AKI were observed in the sepsis group. There were neither any significant associations between hepcidin and peak creatinine levels nor the need for renal replacement therapy (RRT) in this patient cohort. Significant associations between HBP levels at admission and stage 2-3 AKI were seen in the whole group, as well as in the sepsis group, and the non-sepsis group separately. A significant association was noted between HBP and the need of RRT as well as with peak creatinine among the sepsis patients.

In paper IV, a collaboration with the Sci-Life Laboratory, and the Department of Biochemistry and Microbiology, Biomedical Center Uppsala University, Uppsala, Sweden was initiated. Thirty patient samples were tested using proteomic technology with a total of 272 mediators of the Olink Proteomics Tool Proseek^{96x96}, employing three different panels categorized as inflammation, immune response, and organ damage. Blood samples were from a subset of patients from study II, collected on day 1, 3, and 7 and the results compared with the biomarkers we have employed in study II. Twenty-five of the Proseek^{96x96} mediators turned out significantly elevated across the first 7 days in patients suffering from sepsis compared to patients suffering from non-septic critical illness.

Key words

Sepsis, hepcidin, biomarkers, acute kidney injury, inflammatory mediators, proteomics

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“because life is a wonderful thing”

To Victor, Alfred and Thilde

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List of scientific papers

1. Olinder J, Ehinger D, Liljenborg E, Herwald H, Rydén C. Plasma Levels of Hepcidin and Reticulocyte Haemoglobin during Septic Shock. J Innate Immun. 2020;12(6):448-460. doi: 10.1159/000508561. Epub 2020 Sep 18.
2. Olinder J, Börjesson A, Norrman J, West T, Carlström J, Gustafsson A, Annborn M, Herwald H, Rydén C. Hepcidin discriminates sepsis from other critical illness at admission to intensive care. Sci Rep. 2022 Sep 1;12(1):14857. doi: 10.1038/s41598-022-18826-0.
3. Olinder J, Stjernqvist MJ, Lindén A, Salomonsson ET, Annborn M, Herwald H, Rydén C. Hepcidin, in contrast to heparin binding protein, does not portend acute kidney injury in patients with community acquired septic shock. PLoS One. 2024 May 2;19(5):e0299257. doi: 10.1371/journal.pone.0299257. eCollection 2024.
4. Olinder J[*], Eliasson L[*], Waserbrot S[*], Herwald H, Rubin K, Rydén C. Temporal changes in profiles of inflammatory mediators in community-acquired septic shock and non-septic critically ill patients. (Unpublished manuscript). 2024.

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Abbreviations

ACCP/SCCM	American College of Chest Physician and the Society of Critical Care Medicine
ADQI	Acute Dialysis Quality Initiative
ADGRG1	Adhesion G protein coupled receptor 1
AI	Anaemia of Inflammation
AKI	Acute Kidney Injury
AKIN	Acute Kidney Injury Network
AMPs	Antimicrobial peptides
APCs	Antigen-Presenting Cells
ATP	Adenosine Triphosphate
AUC	Area Under the Curve
BMP	Bone Morphogenetic Protein
CALCA	Calcitonin Related Polypeptide Alpha
CCL	Cysteine-Cysteine Motif Chemokine (3, 4, 19, 20, and 23)
CFI	Catalytic Free Iron
CLEC6A	C type Lectin Domain Family 6 Member A, also called Dectin 2)
CKD	Chronic Kidney Disease
COX-2	Cyclooxygenase-2
CRP	C-Reactive Protein
CRRT	Continuous Renal Replacement Therapy
CXCL	Cysteine X Cysteine Motif Chemokine (10 and 11)
DAMPs	Danger-Associated Molecular Patterns
DIC	Dissiminated Intravascular Coagulation
ECMO	Extra-Corporeal Membrane Oxygenation
ELISA	Enzyme-Linked Immunosorbent Assay
ERFE	Erythroferrone
Fe-Tf	Iron transferrin
GFR	Glomerular Filtration Rate
HBP	Heparin Binding Protein
HCLS1	Hematopoietic Cell Specific Lyn Substrate 1

HFE	Hemochromatosis protein
HH	Hereditary Hemochromatosis
HJV	Hemojuvelin
IHD	Intermittent Haemodialysis
IL	Interleukin
iNOS	nitric oxide synthase
IV	Intravenous
IRIDA	Iron-refractory Iron Deficiency Anaemia
KDIGO	Kidney Disease Improving Global Outcomes
LAP-TGF-β1	Latency-Associated Peptide-Transforming Growth Factor-beta 1
LC-MS/MS	Liquid Chromatography and Tandem Mass Spectrometry
LILRB4	Leukocyte Ig like Receptor Subfamily B Member 4
LTA	Lipoteichoic acid
LPS	Lipopolysaccharide
MAP	Mean Arterial Pressure
MCP-1	Monocyte Chemotactic Protein
MDRD	Modification of Diet in Renal Diseases
PCT	Procalcitonin
MILR1	Mast Cell Immunoglobulin like Receptor 1
MRSA	Methicillin Resistant *Staphylococcus aureus*
mtDNA	mitochondrial DNA
NET	Neutrophil Extracellular Traps
NIH	National Institutes of Health
NF-κB	Nuclear Factor kappa-light-chain enhancer of activated B-cells
NLRs	Nucleotide Oligomerization Domain-Like Receptors
NO	Nitric Oxide
NOD	Nucleotide Oligomerization Domain
NOS2	Nitric Oxide Synthase 2
NOS3	Nitric Oxide Synthase 3
NPX	Normalized Protein Expression
OPG	Osteoprotegerin

OSM	Oncostatin M
PAMPs	Pathogen-Associated Molecular Patterns
PCT	Procalcitonin
PG	Peptidoglycan
PGE2	Prostaglandin E2
PIK3AP1	Phosphoinositide 3 Kinase Adapter Protein 1
PRRs	Pattern Recognition Receptors
RIFLE	Risk, Injury, Failure, Loss, and End-stage renal disease
ROS	Reactive Oxygen Species
RRT	Renal Replacement Therapy
SAg	Superantigens
S-AKI	Septic Acute Kidney Injury
SAPS3	Simplified Acute Physiology Score 3
SIRS	Systemic Inflammatory Response Syndrome
SIT1	Signaling Threshold Regulating Transmembrane Adapter 1
SOFA	Sequential Organ Failure Assessment
STAT3	Signal Transducer and Activator of Transcription 3
TACO	Transfusion-Associated Cardiac Overload
TFR1+TFR2	Transferrin Receptors 1 and 2
TGF-β	Transforming Growth Factor-β
TLR	Toll-Like Receptors
TNF-α	Tumor Necrosis Factor α
TNF-β	Tumor Necrosis-β
TNFSF14	Tumor Necrosis Factor Ligand Superfamily Member 14
TRALI	Transfusion-Associated Cardiac Overload
TRIM	Transfusion-Related Immunomodulation
RBC	Red Blood Cells
Ret-He	Reticulocyte Hemoglobin
ROC curve	Receiver Operating Characteristics curve
VAP	Ventilator Associated Pneumonia
WBC	White Blood Cells

Introduction

Patients treated in the intensive care unit (ICU) are critically ill due to acute and life-threatening organ dysfunction. A multidisciplinary and holistic approach including advanced monitoring and organ support to reduce morbidity and mortality are fundamental for optimal care of ICU patients. Except for organ support and optimization of the ICU patient´s homeostasis, it is vital to identify and treat the underlying condition and evaluate treatment outcome (1, 2).

The characteristics of the intensive care patients are heterogenous and dependent of the primary reason for admission, and preexisting comorbidities, age, and sex. Examples of the most common community acquired causes of ICU care are sepsis/septic shock in need of respiratory and/or vasopressor support, severe trauma, acute cardiovascular conditions, acute respiratory insufficiency, neurological conditions with unconsciousness, and intoxications. In addition to community acquired ICU hospitalization, nosocomial conditions such as surgical complications are commonly seen in the ICU (1, 2).

Despite modern intensive care treatment with its intensive interventions, short-term mortality rates are high, though with a broad spectrum according to geographical area due to *e.g.* economical restrain and microbial antibiotic resistance (1). A commonly seen complication in ICU patients is healthcare-acquired infections due to factors such as increased age, comorbidities, immunosuppression, and the high prevalence of invasive devices and procedures (3).

Among patients that survive ICU treatment, the long term mortality and morbidity are increased, and the quality of life is restricted. Late complications in ICU-patients spans between somatic morbidity such as cognitive impairment, contractures, muscle loss, and psychiatric disorders such as anxiety, depression, post-traumatic stress syndrome, and long-lasting delirium (1).

Community acquired infections in its severest form, sepsis/septic shock accounts for a substantial burden in the ICU.

The theme of this thesis covers an investigation of potential biomarkers in discriminating community acquired sepsis/septic shock from other critical illness at admission to the ICU and the temporal changes during the first week of hospitalization with focus on the key regulator of iron metabolism, hepcidin.

Critical illness in the ICU

Critical illness in the ICU is diverse, utmost complex and often demanding a multifaceted, experienced and interdisciplinary approach with many specialities and different professions involved. The ICU approach is to treat patients who suffer from life-threatening disorders or are at risk of developing them irrespective of the cause. Cornerstones of treating critical illness in an ICU setting include optimization of the patient´s homeostasis, provide multi organ support, and diagnose and treat the underlying disease in collaboration with other specialists. There are though many essential and sometimes not easily met aspects to consider before admitting a patient to the ICU, such as respecting the patients autonomy and will, considering the frailty of patients, does the patient have a realistic and reasonable chance to recover from invasive procedures, is the patient suffering from a reversible condition, and is there a reasonable chance for the patient to recover and return to an acceptable life (2).

Patients are usually categorized into two forms of ICU admissions, the planned admission, where patients need to be physiologically optimized before and/or after surgical interventions, and/or the emergent admissions both from other wards but often directly from the emergency room with severe critical illnesses. Planned surgical interventions demanding ICU care have a lower mortality rate compared to emergency surgical interventions. Acute ICU admissions due to medical conditions are correlated with the highest mortality (2).

Critical diseases requiring ICU care are known to be associated with both short-term hospital mortality, 28-day mortality, and long-term mortality. In a larger Australian retrospective multicentre study of adult patients published in 2022, covering 130.775 patients from 23 ICU centres, the 1-year post-discharge survival was 90% compared to 98% in an age-matched cohort. Survival rates dropped to 73% in the post ICU group compared to 90% in the age-matched cohort after 5 years. In this cohort 48% of the patients were admitted after surgery, 31% from the emergency department, and 8% from other wards. Forty-two % of the patients needed invasive ventilation and 3% needed renal replacement therapy. The most common diagnosis for admission to the ICU in this cohort was cardiac surgery (15%) followed by gastrointestinal surgery (13%), sepsis excluding pneumonia (7,2%), and non-surgical cardiologic conditions (6,8%). Long-term survival was associated with younger age, female sex, and patients with higher socioeconomic status. Long term mortality on the other hand was associated with longer hospital length of stay and

being discharged to another location than home (4). In a recent retrospective multicentre study from Portugal published in 2023 including 37.118 ICU patients, the ICU all-cause mortality was 16.1%. Of patients discharged from the ICU, 9,4% died already during the following hospital stay. At follow-up after 1 year 85% were alive and after 2 years 79.5% (5). In addition to the risk with treatment of an initial life-threatening condition in the ICU the risk of acquiring a hospital associated complication is significant. A substantial amount of ICU complications are due to infections such as ventilator associated pneumonia, central line-associated bloodstream infections, catheter-associated urinary tract infections, and surgical site infections (6). Prevalence surveys in the US suggest that 30% of healthcare-associated infections are acquired in the ICU and are associated with increased mortality, length of stay in the ICU, increased antibiotic consumption, and hospital costs (3).

ICU supportive care includes respiratory support, as well as cardiovascular, renal, central nervous system, gastrointestinal, nutritional, neuromuscular, sleep and delirium support, pain relief support, and infection control including antibiotic stewardship. In addition, ethical aspects such as family engagement, end of life care, and considerations of organ donation are of utmost importance when caring for patients in the ICU (2, 7).

Respiratory support, cardiovascular support with focus on vasopressor support, and renal replacement therapy are treatments usually available and offered in most developed ICU centres. The most common conditions needing these supportive regiments will be discussed below;

Respiratory support includes non-invasive ventilation and invasive ventilation. In addition, at selected and specialized ICU centres extra-corporeal membrane oxygenation, ECMO, is available. Indications of invasive mechanical ventilation with establishment of an endotracheal tube are typically seen in the following patients (8);

1. airway compromised due to trauma, oropharyngeal infection, severe asthmatic bronchospasm or acute exacerbation of chronic pulmonary disease.
2. hypoventilation disorders, *i.e.* impaired ventilation drive due to drug overdose or poisoning, respiratory muscle weakness due to myositis or muscular dystrophy, peripheral nervous system disorders such as Guillain Barré syndrome and restrictive ventilatory defects such as pneumothorax or massive pleural effusion.
3. hypoxemic disorders such as acute respiratory distress syndrome, severe pneumonia, pulmonary oedema, massive pulmonary embolism, and advanced pulmonary fibrosis.

4. increased ventilatory demand caused by severe sepsis, shock, or severe metabolic acidosis.

Circulatory insufficiency with decreased perfusion of vital organs resulting in multiorgan dysfunction are together with respiratory insufficiency the most common indications for admittance to the ICU. Vasopressors and inotropic therapy are vital medications that cause vasoconstriction and improve cardiac contractility. Therapeutic vasopressors are hormones that target and activate different receptors, and include adrenergic, angiotensin II, vasopressin, and dopamine. Norepinephrine is the first drug of choice when it comes to septic shock and vasodilatory shock. The most common disorders that need vasopressor support are the following (9-11);

1. distributive shock *e.g.* due to septic-, neurogenic- or anaphylactic shock.
2. hypovolemic shock caused by *e.g.* post-surgery bleedings, ruptured aneurysm, or major trauma.
3. Cardiogenic shock often due to post-acute myocardial infarction.
4. obstructive shock, *e.g.* massive pulmonary embolism or pneumothorax.
5. post cardiac surgery, transplantation, or extracorporeal circuit leading to the vasoplegic syndrome.

Approximately 5 % of ICU admitted patients need renal replacement therapy (RRT) due to acute kidney failure (AKI). The main purposes of RRT are to normalize fluid balance and to remove toxins. Renal replacement therapy in the ICU is either administrated via intermittent haemodialysis (IHD) or continuous renal replacement therapy (CRRT) (12). Patients who are treated with RRT therapy in an ICU setting are commonly suffering from one of the following conditions, although for some of the conditions the evidence is limited (13-17);

1. Septic shock leading to hypotension and microvascular dysfunction.
2. Cardiac failure caused by decreased cardiac output, thus reduced renal perfusion and subsequently fluid overload.
3. Major trauma and severe burns involving rhabdomyolysis.
4. Overdose with a dialyzable toxin.
5. Severe metabolic acidosis, *e.g.* diabetic ketoacidosis, lactic acidosis, or hyperchloremic acidosis.
6. Major surgery with *e.g.* ischaemia-reperfusion injury.
7. Tumor lysis syndrome after cytotoxic chemotherapy leading to imbalanced serum electrolytes and oliguria.

8. Electrolyte derangements such as hyperkalemia that can be induced by *e.g.* progression of chronic kidney disease and/or AKI, renin-angiotensin-aldosterone system inhibitors, hemolysis, tissue injury or insulin deficit.

Sepsis

The word sepsis derives from the Greek language referring to “the decomposition of animal, or vegetable, or organic matter in the presence of bacteria”. Historically, the first expression of sepsis in a medical context dates back over 2700 years ago from the poems of Homer. Homer used the term “sepsis” derived from the word “sepo”, which means “I rot” (18). The word sepsis was also described by Hippocrates as the dangerous, odiferous, biological decay that could occur in the body. It wasn´t until the nineteenth century the germ theory was introduced as the origin and transmission of infectious diseases including sepsis (18). In 1904, Sir William Osler stated the fundamental role of sepsis; “except on few occasions, the patient seems to die from the body´s response to infection rather from it” (19). Scientifically, in 1914, Hugo Schottmüller defined sepsis as: “Sepsis is a state caused by microbial invasion from a local infectious source into the bloodstream which leads to signs of systemic illness in remote organs” (19, 20). In 1972 Hinshaw and Cox described a severe condition of sepsis, septic shock, as a form of distributive shock caused by loss of vasomotor control leading to arteriolar and venular dilation and - after resuscitation with fluids - characterized by increased cardiac output with decreased systemic vascular resistance (The Fundamental Mechanisms of Shock, Editors Lerner B. Hinshaw, Barbara Cox, Advances in Experimental Medicine and Biology (AEMB, volume 23)). As of today, sepsis was defined by Singer *et al.* in 2016, the so-called sepsis-3, as a life-threatening organ dysfunction caused by a dysregulated response to infection (21).

In spite of the sepsis-3 criteria presented as a consensus document in 2016 the diagnosis is often an initial challenge in critically ill patients (22).

Sepsis definition

Sepsis constitutes multiorgan dysfunction due to a dysregulated host response to infection (21), causing substantial morbidity and mortality in patients and a great impact on hospital resources and health economy world-wide (23). The development and severity of sepsis depend on host factors such as age, comorbidities, and immune status, as well as on pathogen factors including

virulence, microbial species, infectious load, and antimicrobial resistance (21, 24, 25).

The symptoms and pathophysiology of sepsis are complex and multifaceted reflected in the quest to formulate a definition of sepsis over time. During the last four decades there have been several consensus conferences and in August 1991 the Sepsis-1 criteria were agreed upon by the American College of Chest Physician and the Society of Critical Care Medicine (ACCP/SCCM) (26). This first consensus conference introduced the systemic inflammatory response syndrome (SIRS), thereby standardizing the definition of sepsis, allowing multicentre studies. This has resulted in an increased scientific knowledge and better identification of sepsis patients. The SIRS criteria were controversial since the criteria were too sensitive disqualifying its clinical value in the intensive care setting. The sepsis definition was revised in 2001 in a collaboration between the European Society of Intensive and Critical Care Medicine, the ACCP/SCCM, the American Thoracic Society, and the Surgical Infection Society, presented as the Sepsis-2 consensus definition, where the definitions of sepsis and septic shock, including threshold values for organ damage, were clarified (27). However, the SIRS criteria maintained in the sepsis-2 definition. The latest published consensus report, The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3), is the common sepsis definition as of today that was published in 2016 (21). In this consensus report, the SIRS criteria were discarded and replaced by a clinical evaluation tool, Sequential Organ Failure Assessment (SOFA), focusing on organ dysfunction caused by a dysregulated host response to infection (Table 1.). SOFA is an internationally used scoring system based on measurements of failure of the respiratory, cardiovascular, liver, coagulation, renal, and neurological systems (28). According to the Sepsis-3 guidelines, the definition for sepsis is a suspected infection with a SOFA score ≥ 2. Septic shock occurs when a patient has persistant hypotension despite adequate fluid resuscitation and needs vasopressors to maintain a mean arterial pressure (MAP) of ≥ 65 mmHg with a simultanous serum lactate level >2 mmol/L (Figure 1.) (22).

Table 1. SOFA-Score

SOFA (Sequential Organ Failure Assessment)					
System					
	SOFA - 0	**SOFA - 1**	**SOFA - 2**	**SOFA - 3**	**SOFA - 4**
Respiration, PaO_2/FiO_2, kPa	**> 53**	**≤ 53**	**≤ 40**	**≤ 27**	**≤ 13**
Coagulation, Platelets 10^9/L	**> 150**	**≤ 150**	**≤ 100**	**≤ 50**	**≤ 20**
Liver, Bilirubin µmol/L	**< 20**	**20-32**	**33-101**	**102-204**	**> 204**
Cardiovascular Hypotension **(Catecholamnine doses are given as µg/kg body weight/minute for at least 1 h)**	**MAP ≥ 70 mmHg**	**MAP < 70 mmHg**	**Dopamine ≤ 5 or dobutamine (any dose)**	**Dopamine 5.1-15 or epinephrine ≤ 0.1 or norepinephrine ≤ 0.1**	**Dopamine > 15 or epinephrine > 0.1 or norepinephrine > 0.1** **Levosimendan (any dose)** **Vasopressin (any dose)**
Central Nervous System (CNS) **Glasgow Coma Scale (GCS)** **RLS**	 **15** **1**	 **13-14** **2**	 **10-12** **3**	 **6-9** **4-5**	 **3-5** **6-8**
Renal **Creatinine µmol/L** **Urine output, mL/day**	 **< 110**	 **110-170**	 **171-299**	 **300-440** **< 500**	 **> 440** **< 200**

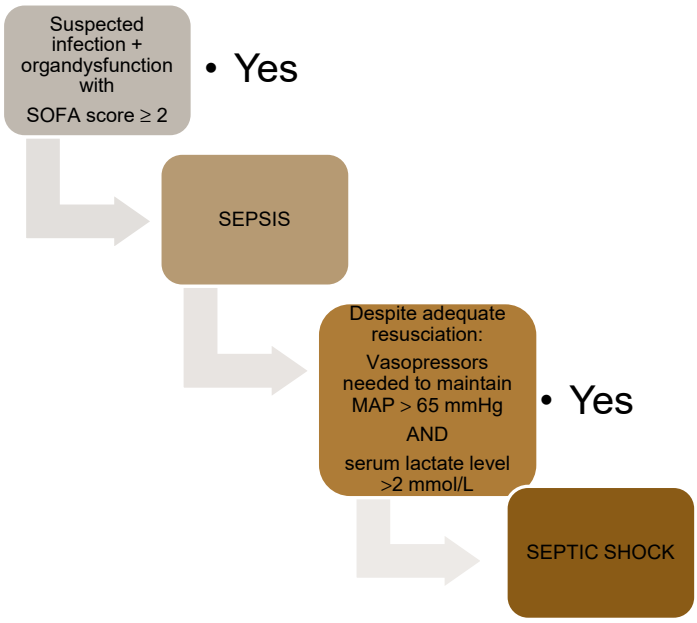

Figure 1. Definition of sepsis and septic shock

Sepsis epidemiology

The global incidence as well as prevalence of sepsis is difficult to interpret from previous published papers due to the fact that the sepsis definitions have changed three times since 1991, and most epidemiological studies have been performed in high income countries (21, 26, 27, 29). The actual global sepsis burden is probably higher due to that low-income countries have a higher infection prevalence and limited health care resources, for prevention and treatment as well as lack of valid registration systems (30). Furthermore, sepsis can be caused by different pathogens, bacteria, virus, fungi, yeast, and parasites, and the syndrome is influenced by factors such as patient age, sex, ethnicity, comorbidities, and immune status that also differ between continents and countries (24). The incidence of sepsis was estimated to be almost 50 million cases worldwide with 11 million sepsis-related deaths in 2017 according to Rudd *et al.* (31). Thus, the global incidence of sepsis was estimated to be 678 per 100 000-person years with a mortality of 148 per 100 000-person years (31). However, from 1990 to 2017 both the age-standardized sepsis incidence and mortality rate have declined, though sepsis still counts for 19.7% of all global deaths reported. A high burden of disease affects especially the population living in areas with a low Socio-demographic Index (31). A study performed in southern Sweden by Mellhammar *et al.* in 2016 showed that the incidence according to the sepsis-3 definition was 780/100 000 in Sweden (32).

There is an uncertainty of the demographic panorama when it comes to understanding the risk of developing sepsis/septic shock. Sepsis disproportionally affects elderly patients (>80 years old) and children (<5 years old). Global incidence of sepsis has been reported to be higher among females compared to males (31), whereas most studies (90%) from high income countries describes the contrary (33, 34). Martin *et al.* reported that the incidence and mortality of sepsis in the U.S. is lower in Caucasians, in patients with fewer comorbidities, and in patients without alcohol disorders (34). Another study from the U.S. found that the incidence of sepsis-related hospitalization was higher in single, widowed, or legally separated patients, regardless of sex (35).

Sepsis awareness and funding

The general understanding of sepsis is disproportionate considering the global mortality burden of sepsis. In a Swedish study published in 2015, 21 % of the interviewed persons had heard of sepsis in comparison to other conditions listed, *e.g.,* stroke (95%), chronic obstructive pulmonary disease, (COPD) 95%, or leukaemia (92%) (36). Among persons in Germany >60 years of age a similar study

was performed in 2018 showing that 88.6 % had heard of sepsis, however 50 % of these could not define sepsis correctly (37).

Every year, the National Institutes of Health (NIH), which is part of the U.S. Department of Health and Human Services, presents and reports research funding for a wide variety of diseases. Data is publicly available on the website https//report.nih.gov/funding. In figure 2. research grant data for 2023 is presented for a few selected diseases.

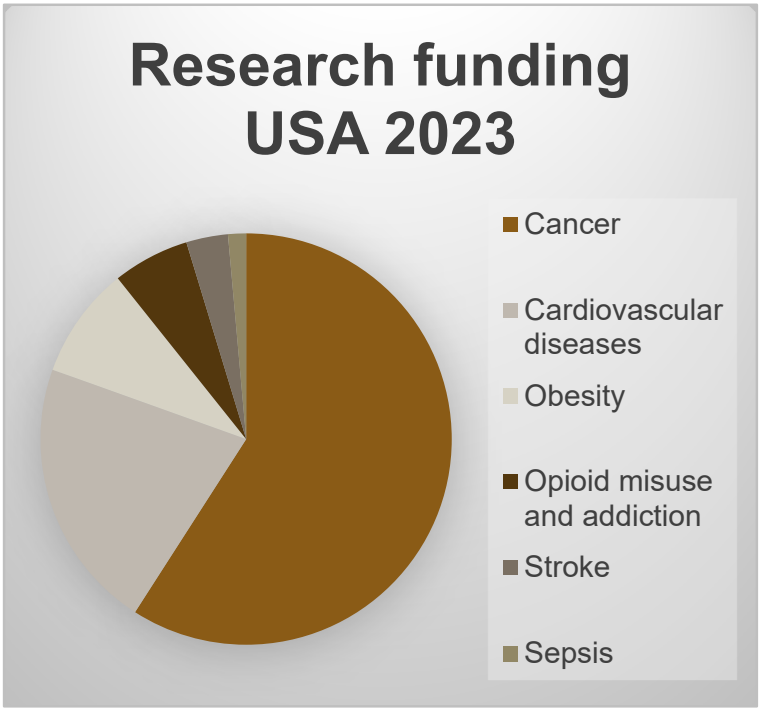

Figure 2. 2023 Research funding, https://report.nih.gov/funding/

Cancer	7,968 million US dollars
Cardiovascular diseases	2,884 million US dollars
Obesity	1,187 million US dollars
Opioid misuse and addiction	812 million US dollars
Stroke	443 million US dollars
Sepsis	191 million US dollars

The latest mortality data from the National Vital Statistics system (NVSS) reported by NIH for some of the diseases are presented in figure 2. Sepsis is a dominating cause of mortality in the US, but when considering the burden of disease in the US and elsewhere there seem to be disproportionate funding, when compared to the global burden of morbidity and mortality due to sepsis.

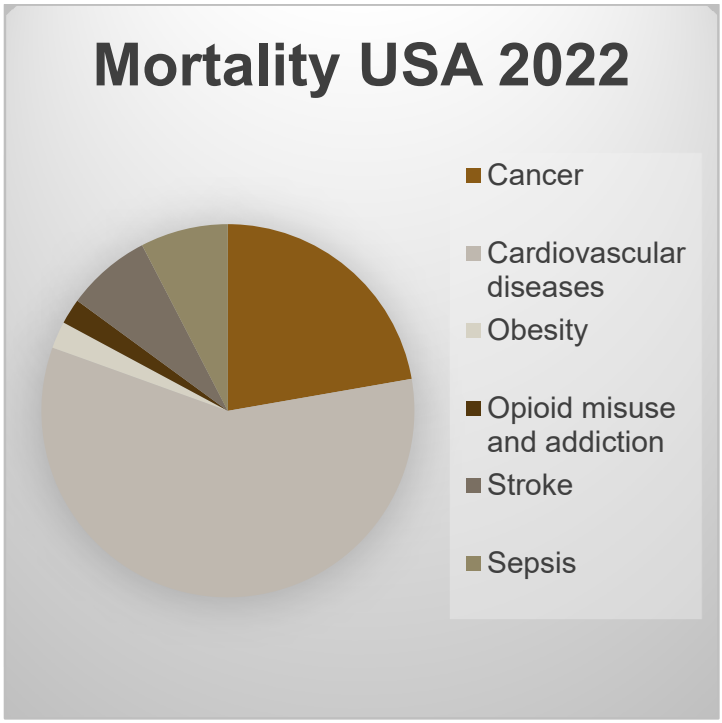

Figure 3. 2022 Mortality USA, https://report.nih.gov/funding/

Cancer	721,871
Cardiovascular diseases	1,839,163
Obesity	74,440
Opioid misuse and addiction	83,473
Stroke	234,363
Sepsis	243,672

Sepsis pathophysiology

The catastrophic state of sepsis is complex and not yet fully understood. The consensus though is that the clinical picture of sepsis is due to the host´s response to an invading pathogen rather than the pathogen itself – as in consensus defined as a multiorgan dysfunction due to a dysregulated host response to infection (21). The primary defence system against infection is the innate immune system, responsible for identification and eradication of foreign pathogens. The innate immune system includes myeloid cells, the complement system, chemical defence mechanisms, as well as epithelial, endothelial, and mucosal structural defence mechanisms. Sepsis involves early activation of pro- and anti-inflammatory response with precipitous alterations of circulating cytokines triggering the systemic acute phase reactants. Accompanied by the pro-inflammatory response, an anti-inflammatory response is triggered, characterized by the release of anti-inflammatory components, *e.g.,* interleukin-4, IL-4, IL-10, IL-11, IL-13 and transforming growth factor beta (TGF-

β) and the balance between these depends on focus of infection, immune status, comorbidities, age, genetic variability, pathogen, virulence factors, and the microbial load. (1, 25, 38-44). A controlled pro- and anti-inflammatory response triggers both the innate and adaptive immune systems as parts of the host´s defense system to reach homeostasis, thus enabling the host to recover from an infection (43). In some individuals though, the pro- and anti-inflammatory responses react excessively in an uncontrollable state with secondary organ injury leading to increased morbidity and mortality. If the individual survives the initial uncontrollable inflammation, the risk of mortality in the long run is increased due to extended immunosuppression related to a prolonged anti-inflammatory state (43).

The link between the innate immune system and the adaptive immune system includes antigen presenting cells (APC), *e.g.* macrophages and dendritic cells, where MHC class I and MHC class II molecules are expressed, activating both $CD8^+$ (cytotoxic) and $CD4^+$(helper) T cells, inducing cell mediated immunity (45). The humoral immunity is activated by $CD4^+$ helper T-cells that activate B-cells enabling the production of antibodies. T- and B-cells both produce a wide variety of cytokines such as IL-2, IL-4, IL-5, IL-10, IL-13, IL-17, IL-25, and IFN-γ. In addition subgroups of IL-17 (IL-17A and IL-17) induce IL-6 and TNF production (45).

Cytokines bind to leukocyte receptors, which leads to leukocyte activation, inducing additional release of cytokines, proliferation, differentiation, and cell migration to the infected tissue. The local inflammation process leads to phagocytosis of microbes and damaged cells, and release of antimicrobial components for extracellular pathogen destruction (46, 47). Furthermore, pro-inflammatory cytokines and chemokines activate neutrophilic leukocytes to produce reactive oxygen species (ROS), inducible nitric oxide synthase (iNOS), and neutrophil extracellular traps (NETs) (48). NETs can physically trap microorganisms, hence locally kill and prevent spreading of pathogens (43, 48). The systemic release of early pro-inflammatory cytokines results in temperature change with fever (49).

The massive generation of pro-inflammatory cytokines, reactive oxygen species, and stress hormones, that occurs during sepsis, alters protein function, stimulates hepatic acute-phase protein production, and initiates a stress response that affects vascular tone and permeability, cardiac output, and coagulation (50). The reactions are part of our homeostatic response, preventing the spread and clear the body from invading pathogens when locally applied. When the immune response becomes dysregulated or incontrollable, a systemic reaction might lead to widespread collateral damage and multi-organ failure, characterized by organ hypoperfusion and tissue oxygen deficit (50).

PAMPs, DAMPs and PRRs

The immune system is triggered by localized recognition, when microbial or damage cell components *i.e.,* Pathogen-associated molecular patterns, PAMPs, and/or Danger-associated molecular pattern, DAMPs are recognized by pattern recognition receptors, PRRs. Distinct classes of PRRs activate different transcriptional programs leading to tailored immune responses (51).

PAMPs are small microbial components, *e.g.* peptidoglycan, toxins, and intracellular DNA components, essential for the survival of the microorganism and are therefore difficult to alter (52). The host innate immune system recognizes several bacterial virulence factors such as toxins and conserved motifs on the pathogen surface via PRRs (52). DAMPs consist of host nuclear or cytoplasmic non-microbial molecules released from host´s cells when these are exposed to damage from microorganisms, leading to host cell lysis and death. Adenosine triphosphate (ATP), histones, and mitochondrial DNA (mtDNA) are examples of endogenous DAMPs that are recognized by PRRs (48, 53).

PRRs are expressed by several cells, *e.g.,* antigen-presenting cells (APCs), phagocytic cells including macrophages, neutrophils, epithelial, and endothelial cells. PRRs include receptors expressed on the cell surfaces, *e.g.,* toll-like receptors (TLRs) as well as intracellular receptors *e.g.,* nucleotide oligomerization domain (NOD) like receptors (NLRs).

The PRRs are all unique and are grouped as follows; (52, 54);

- Toll-like receptors detect pathogens either from the microorganism´s cell surface or the lysosome/endosome membranes. There are many different TLRs involved in both Gram-positive and Gram-negative bacterial detection, *e.g.* TLR 2 and TLR 4, including detection of flagellins, TLR 5, bacterial DNA, TLR 9, mycobacteria, TLR 1, TLR 2, TL 4, and TLR 9, yeast and fungi (*e.g. Candida and Aspergillus*), TLR 2 and TLR 4, protozoa (*e.g. Toxoplasma gondii, Leishmania and Plasmodium falciparum*), TLR 2, TLR 4 and TLR 9, and viruses (*e.g.* Herpes simplex 1, and 2, and cytomegalovirus), TLR 9 (52)
- Nucleotide binding Oligomerization Domain (NOD)-like receptors (NLRs), such as NOD1 detects peptidoglycan (PG) from Gram-negative bacteria (52). Activated NLRs induce the formation of the inflammasome, resulting in activation of intracellular signaling pathways, and the expression of several pro-inflammatory cytokines, like tumor necrosis factor α (TNF-α), interleukin (IL) 1β, IL-2, and IL-6, chemokines *e.g.,* interleukin 8 (IL-8), and monocyte chemotactic protein 1 (MCP-1),

enzymes, inducible nitric oxide synthase, several adhesion molecules, and coagulation factors (46, 47, 55, 56)

- NLRP3 inflammasome is activated by mitochondrial malfunction, leading to production of reactive oxygen species (ROS), lysosomal damage and ionic flux, activating Interleukin (IL) IL-1β/IL-18 (54).
- C-type Lectin Receptors (CLRs), *e.g.,* Dectin-1 detects β-glucans from fungal cell walls, activating the cytokines TNF-α, IL-1β, IL-2, IL-8, IL-10, IL-12, and CXCL2. Dectin-1 activates the adaptive immune system by activating $CD8^+$ T cells (51, 57)
- Retinoic acid-inducible gene 1 (Rig1)-Like Receptors (RLRs) are involved in the detection of viral RNA, sensing of foreign nucleic acids in the cytosol, such as RNA derived from evading viruses, *e.g.* SARS-CoV-2 (57, 58)
- Scavenger Receptors (SR) have diverse dynamic and complex interactions with different ligands. Among some of the important features SRs obtain is to induce pathogen elimination by modulating the recruitment and activation of phagocytic cells and thereby regulating the inflammatory response via proinflammatory cytokine production (59).
- Absent in melanoma-2 (AIM2)-like Receptors (ALRs) can recognize intracellular DNA, *e.g.,* double stranded DNA, inducing release of IL-1β and IL-18 (60).

NF-κB pathway

An important signaling pathway that most pattern recognition receptors (PRRs) induce is the activation of the nuclear factor kappa-light-chain enhancer of activated B-cells (NF-κB) pathway (61, 62). NF-κB is a key mediator of the innate immune response, activating an upstream of pro-inflammatory genes including cytokines, chemokines, and adhesion molecules, thus participating in the regulation of the inflammasome (61). NF-κB activates transcription of various genes, thus broadly regulates inflammation, and facilitates the production of inflammatory cytokines, (*e.g.,* IL-1β, IL-2, IL-3, IL-6, IL-8, IL-12, IL-18 and TNF-α), chemokines, (*e.g.,* CXCL1 and CXCL10), and adhesion molecules, but can also indirectly regulate cell proliferation, migration, and apoptosis, thus enabling antigen presenting cells to activate the adaptive immune response via T-cells (61, 62).

Microorganisms like bacteria and bacterial components *e.g.,* lipopolysaccharides (LPS) activate the NF-κB pathway resulting in an expression of an upstream of inflammatory cytokines, that in worst case scenario can cause septic shock.

In summary, the NF-κB pathway induces the following pathophysiological features when septic shock occurs, resulting in multi organ failure with systemic hypoxia, hypotension, hypoperfusion, and a dysregulated coagulation cascade; (62)

1. expression of inflammatory cytokines leading to fever, inflammatory response, and *e.g.,* activation of CRP and hepcidin via IL-6 activation.
2. expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and upregulated production of nitric oxide (NO) and vasodilator prostaglandins that leads to severe hypotension and impaired cardiac contractility.
3. expression of adhesion molecules and chemokines leading to neutrophil-migration, infiltration and activation, causing releasement of reactive oxygen species (ROS) and other proteolytic enzymes that causes endothelial injury and increased permeability resulting in multiple organ failure.
4. expression of tissue factor, factor VIII and plasminogen-activator type 1 that all are involved in the activation of coagulation cascades and impaired fibrinolysis that causes disseminated intravascular coagulation (DIC).
5. expression of enzymes involved in the arachidonic acid metabolism pathway leading to the production of prostaglandins, leukotrienes and thromboxane A_2 that causes tissue injury.

Inflammasomes

Inflammasomes are multimeric protein complexes, part of the innate immunity, present in the cytosol of *e.g.* macrophages. Inflammasomes gather in the cytosol upon activation from certain PRRs *e.g.* abovementioned NLRP3 and AIM2. In general, inflammasomes recruit procaspase-1 which upon activation are cleaved into the active form of caspase-1, which in turn activates the proinflammatory cytokines IL-1β and IL-18, respectively. Caspase-1 can also activate the protein Gasdermin-D that is important for host´s protection against *e.g.* intracellular bacteria and viruses, since Gasdermin-D induces pyroptosis, which is a form of proinflammatory programmed cell death, resulting in cell swelling, membrane rupture and release of cellular contents. The inflammasome can also be activated from *e.g.* Gram-negative bacteria´s lipopolysaccharides (LPS) (63, 64). Abnormal Gasdermin-D activation can cause sustained inflammation in *e.g.* sepsis and Covid-19 infection that can result in a severe cytokine storm the so-called cytokine release syndrome with significantly elevated levels of IL-1β and IL-18 (64).

The endothelial glycocalyx

The glycocalyx of blood vessel endothelium is a key regulator of endothelial and microvascular physiology. The glycocalyx regulates the endothelial permeability, the adhesion and migration of leukocytes, and inhibits intravascular thrombosis (65, 66). Microvascular dysfunction is primarily caused by endothelium and glycocalyx injury leading to hypotension and hypoperfusion. Hypotension is *e.g.,* caused by NOS2-mediated systemic vasodilation with loss of capillary barrier function resulting in fluid outflow to the interstitial space (66). The glycocalyx damage leads to fluid outflow resulting in interstitial oedema which is associated with sepsis-organ failure (65). In addition, the glycocalyx release exposes a pro-thrombotic endothelial membrane that together with the dysregulated immune reaction leads to activation of the coagulation cascade resulting in disseminated intravascular coagulation with dysfunctional microcirculation and widespread endotheliopathy (67-70). The complex pathophysiology and stress of vital organs in sepsis result in hypotension, hypoperfusion, oxygen limitation, and lactate accumulation (67-70). The resulting symptoms in various organs are;

- the lungs where the alveolar-endothelial barrier is damaged causing pulmonary oedema with a ventilation-perfusion mismatch leading to hypoxia and hypercapnia.
- the nervous system where altered endothelial cells weaken the blood-brain barrier enabling toxins, cytokines, and cells to enter leading to cerebral oedema, septic encephalopathy, meningitis, or encephalitis.
- the heart, with septic cardiomyopathy, where increased cardiomyocyte oxidative stress leads to weakened ejection fraction and diminished cardiac output.
- the gastrointestinal tract, with increased risk of microbial translocation due to the increased permeability of the mucosa.
- the liver, where the alterations of the sinusoidal endothelium result in hepatocellular injury and cholestasis as well as to protein production including synthesis and release of C-reactive protein (CRP) and hepcidin.
- the kidneys, where reduced perfusion leads to acute tubular necrosis.

Sepsis related acute kidney injury

Acute kidney injury (AKI) causes extensive morbidity and mortality (71). Although most patients recover from AKI, the condition can cause an irreversibly decreased kidney function (72, 73). Among critically ill patients, sepsis is the most common cause of AKI, and AKI of any origin increases the risk of developing sepsis (74, 75). The development of AKI in patients in intensive care increases mortality six-

to eightfold, with persistent increased mortality also in patients discharged from the ICU (75-77). Among survivors of septic AKI (S-AKI), the risk of progression to chronic kidney disease (CKD) is considerable (75). The pathophysiology of S-AKI is complex and multifaceted, involving insufficient blood flow, oxidative stress, and tubular secretion of cytokines and chemokines (78, 79). Among the many different and sometimes concurrent risk factors for developing S-AKI are septic shock, diabetes mellitus, and hypertension (80).

Classification and diagnosis of AKI

In 2012, the Kidney Disease Improving Global Outcomes (KDIGO) published the three stages of AKI which currently constitute the globally accepted definition (81), (Table 2). The KDIGO-criteria are a consolidation of two previously used AKI staging systems; AKIN (Acute Kidney Injury Network) and RIFLE (Risk, Injury, Failure, Loss, and End-stage renal disease) (82).

The AKI stage 1 criteria are defined either as an increased serum creatinine ≥ 1.5-fold baseline within the last 7 days, or a serum creatinine increase of > 26.5 µmol/L in the last 48 hours, or a urine volume of <0.5 mL/kg/h for at least 6 hours (81). AKI stage 2 is defined as an increased serum creatinine concentration of ≥ 2.0-fold baseline level (81). The most severe form of AKI, stage 3, is defined as serum creatinine ≥ 3-fold compared to baseline, or a baseline level of creatinine of ≥ 353.6 µmol/L, or as instituted renal replacement therapy (81).

Table 2. KDIGO staging of Acute Kidney Injury

Stage	Serum creatinine	Urine output
1	1.5-1.9 times baseline or ≥0.3 mg/dl (≥26.5 µmol/l) increase	<0.5 ml/kg/h for 6-12 h
2	2.0 – 2.9 times baseline	<0.5 ml/kg/h for ≥ 12 h
3	3 times baseline or ≥4.0 mg/dl (≥353.6 µmol/l) increase or initiation of RRT or in patients <18 years a decrease in eGFR <35 ml/min/1.73 m2	<0.3 ml/kg/h for ≥24 h or anuria ≥12 h

The limitation of the abovementioned KDIGO classification is the need of a baseline creatinine before the kidney damage has occurred. The KDIGO group concur that the glomerular filtration rate (GFR) is the ideal standard of measuring kidney function (81, 83). However, the gold standard to determine GFR is measurement of the urinary clearance of inulin as an injected exogenous filtration marker which is a clinically difficult approach, thus an endogenous filtration marker such as creatinine has become a well-established method to estimate GFR. To bypass the limitation of not having a baseline creatinine, there are methods to estimate baseline creatinine levels to enable research in this field (84). One established method is the MDMR-equation, originally formulated to calculate the glomerular filtration rate (GFR), with the assumption of a GFR of 75ml/min/m^2 (85). Creatinine has been evaluated as a biomarker for kidney function and predictor of survival, and the conclusions are that elevated serum creatinine is significantly associated with increased mortality (86-89). Creatinine is a waste product of muscle metabolism and is normally generated at a relatively constant rate (90).

Sepsis-inducing pathogens in the ICU

The understanding on why certain bacteria are frequently found in septic patients is complex and due to different aspects, such as epidemiology and prevalence of certain bacteria, the host´s microbiome, the adaptability of microorganisms, global travel, immune status and co-morbidities of the person as well as to antibiotic resistance (91).

Bacterial sepsis is the most common cause of severe sepsis/septic shock, with an earlier reported overweight of Gram-positive infections in the period of 1979 to 2000. However, in recent studies, including 14.000 ICU patients with positive cultures in 75 countries, 62% of the microbes isolated were Gram-negative bacteria, 47% Gram positive bacteria, and fungi were found in 19% of the patients (42, 92).

In a 24-hour point prevalence worldwide ICU study published in JAMA in 2017, covering 88 countries at 1150 centres, with in total 7936 eligible patients, 54% of the patients had a suspected or proven infection, where up to 70% received at least one antibiotic (either of therapeutic or prophylactic purpose) (93). Among the patients with suspected or proven infection, 65% had at least one positive microbial culture, and of these patients 67% had Gram-negative, and 37% had Gram-positive bacteria, and 16% had a fungal isolate (93). The most common Gram-negative bacteria were *Klebsiella* species (27%), *Escherichia coli* (25%), *Pseudomonas* species (24%), and *Acinetobacter* species. Among the Gram-positive findings *Staphylococcus aureus* including methicillin resistant *Staphylococcus aureus* (MRSA) were most prominent followed by *Enterococcus* species, other *Streptococcus* species, and *Streptococcus pneumoniae* (93).

In a Swedish setting a larger study was performed in the county of Östergötland in the period 2008-2016, where 9,587 microorganisms were isolated from blood cultures. *Escherichia coli* was the most frequently found bacterium, followed by *Staphylococcus aureus, Klebsiella pneumoniae*, *Streptococcus pneumoniae, Enterococcus faecalis,* and *Enterobacter cloacae.* In this study *Streptococcus pyogenes* (Group A) was number 13 out of the most prevalent 17 isolated microorganisms (94). After the Covid-19 pandemic an increased incidence of invasive *Streptococcus pyogenes* (Group A) has been reported both in Sweden and elsewhere (95).

Community-acquired versus hospital-acquired sepsis

A larger retrospective cohort study was performed in the U.S. covering the period 2009-2015, including 136 hospitals, with 2.2 million hospitalizations, where 95.154 of these were reported as being caused by sepsis (96). 83.620 (87.9%) represented community onset (CO)-sepsis and 11.534 as hospital onset (HO)-sepsis, respectively. Patients with HO-sepsis had more severe comorbidities such as heart failure, renal impairment, or cancer, and suffered more often from intra-abdominal infections compared to patients with CO-sepsis. The most common CO-sepsis was due to urinary tract infections (27.3%), followed by pneumonia (25.6%), and skin/soft tissue infections (7.8%), respectively, and these diagnosis were reported as slightly higher among CO-sepsis group versus the HO-sepsis group. *Escherica coli* (22.2%), *Staphylococcus aureus* (20.0%), and Streptococcus species (17.6%) were the three most frequent pathogens isolated in CO-sepsis whereas *Staphylococcus aureus* (23.8%), Enterococcus species (11.2%), and Candida species (11.1%) occurred more often in the HO-sepsis patients (96).

Hospital acquired sepsis was more frequently associated with that patients were admitted to the ICU and also experienced longer hospital lengths-of-stay compared to patients with community acquired sepsis. After adjusting for severity-of-illness, HO-sepsis patients were twice as likely to die compared to CO-sepsis patients, with mortality rates of 33.4% versus 16.8%, respectively (96).

Similar conclusions with both poorer prognosis and longer hospital length-of-stay demanding more resources in HO-sepsis compared CO-sepsis patients were reported in studies from Japan and France (97, 98).

In summary, hospital acquired sepsis seems to strike immunocompromised patients more often, patients who generally suffer from severe comorbidities, and more frequently are found to have complex blood-stream microorganisms isolated e.g. Candida species, as well as Pseudomonas- and Acinetobacter-species (96).

Virulence factors

Bacteria produce and express virulence factors, some to avoid the host innate immune system (99). This includes the ability to penetrate mucosal barriers, disseminate within the host, and replicate in distant organs (99). Virulence factors includes adhesins, toxins, proteases, biofilms, haemolysins, as well as surface molecules such as lipopolysaccharides, lipoprotein, glycoproteins, and capsules (100). In addition, there are intracellular changes in metabolic regulatory networks that are controlled by protein sensors and noncoding regulatory RNAs (100, 101)

Some of the most important and potent bacterial virulence factors that activate the cytokines are toxins, generally divided into two major categories, exotoxins and endotoxins (102). Some bacteria only synthesize one toxin responsible for pathogenicity, *e.g.*, tetanus toxin or botulinum toxin, whereas other bacteria produce multiple toxins that synergistically induce the disease symptoms, *e.g., Staphylococcus aureus* (103).

Superantigens (SAg) are exotoxins, produced by *Staphylococcus aureus* and *Streptococcus pyogenes* that bind to major histocompatibility complex II molecules and T-cell receptor molecules resulting in a massive activation of antigen-presenting cells and T-cells, with subsequent release of high systemic levels of cytokines, such as interleukins IL-1, IL-2, tumor necrosis factor alpha (TNF-α), tumor necrosis beta (TNF-β) and interferon γ (IFN-γ) (102, 104).

Other examples of potent exotoxins include haemolysins that act by forming pores in the host´s membranes alternatively degrading membrane lipids, causing lysis of red blood cells, leukocytes, and/or epithelial cells. The lysis of these cells leads to a release of danger-associated molecular patterns (DAMPs) from dying cells that induces a cytokine response. An example of a haemolysin is the α-toxin from *Staphylococcus aureus* that activates the NF-κB- and caspase-1 pathway that regulates and activates genes that encode the cytokines IL-8, IL-1β, and IL-18 (105).

Components of the Gram-positive bacterial cell wall such as lipoteichoic acid (LTA) and peptidoglycan (PG) also induce innate immunity responses. Peptidoglycan is also part of the Gram-negative bacterial cell-wall (52). Among the pattern recognition receptors (PRR), Toll like receptor 2 (TLR2) is important in detecting LTA and PG. In experimental models TLR2 deficient mice are more susceptible to *Staphylococcus aureus* and *Streptococcus pneumoniae* infection and a polymorphism in human TLR2 gene (seen in a large group of Caucasians) is associated with reduced response to Gram-positive septic shock, especially staphylococcal sepsis (52).

Lipopolysaccharide (LPS) are endotoxins that constitute part of the outer layer of Gram-negative bacteria, *e.g., Escherichia coli* (106). The structure of LPS usually consist of a hydrophobic lipid A region, an oligosaccharide core, and the outermost

O-antigen polysaccharide. Lipid A is recognized by the innate immune system triggering macrophages to produce TNF-α and IL-1β (99). LPS also trigger the adaptive immune system directed against the O-antigen polysaccharide via the recognition and activation of the Toll-like receptor 4 (TLR4) activating for instance NFκB and mitogen-activated protein kinase genes leading to the release of proinflammatory cytokines, such as IL-1, IL-1β, IL-6, IL-8, IL-12, IFN-γ, and TNF-α (99). TNF-α itself is important for the pathophysiology of endotoxic shock causing tissue damage by inducing the expression of proinflammatory cytokines, but is also involved in the production of anti-inflammatory cytokines such as IL-4 and IL-10 (52, 99). Furthermore, TNF-α induces the expression of nitric oxidase synthase (iNOS) and cyclooxygenase (COX-2) that catalyse the production of nitric oxide (NO) and prostaglandin E2 (PGE2). NO and PGE2 are both vasodilators leading to changed vascular permeability, an important part of the pathophysiology of septic shock (99).

Sepsis treatment

The fundamental treatment of sepsis/septic shock in the ICU has not changed much in recent years and is based primarily on four pillars. Optimal strategies of antimicrobial treatment (ideally within one hour of sepsis recognition, including prolonged infusion of beta-lactams, and optimal dosing strategies on accepted pharmacokinetic/pharmacodynamic principles), fluid resuscitation (first-line crystalloids, plus albumin that can be used in patients who have received large volumes of crystalloids), vasoactive support (first-line recommendation is norepinephrine), ventilation support and oxygenation. Furthermore, it is of utmost importance to promptly achieve possible source control by early surgery or drainage if a localized source exists (107-109).

Depending on epidemiological status, the patient's immune status, recent surgical status, and the patient´s previous colonization status the early administration of empiric anti-infective drugs are crucial for survival (107, 109). Improved survival due to early antimicrobial treatment appears to have the strongest correlation with success in patients with septic shock, where several studies support the correlation between time-to-antibiotics and death ranging from an absolute mortality associated with an hour´s delay in antibiotic administration between 1,8-7,6% (110-112). Before initiating empiric anti-infective treatment appropriate routine microbiological cultures should be performed making it possible to target the pathogenic agent and potentially escalate or deescalate antimicrobial treatment at a later stage (109).

Moreover, there are several sepsis goal-oriented strategies dependant on the patient´s status. "Surviving sepsis campaign" has provided extensive treatment

recommendations based on the quality of evidence. Regarding strong recommendations examples include (109);

1. Regulation of stress hyperglycaemia with insulin infusion when the glucose level reaches a level of ≥ 10 mmol/L.
2. Usage of low molecular weight heparin over unfractionated heparin for venous thromboembolism (VTE) prophylaxis, unless any contraindication persists.
3. A restrictive (over liberal) transfusion strategy.

When it comes to restrictive over liberal transfusion strategy there are several potential adverse side effects when giving red blood cell (RBC) transfusions including haemolytic reactions, infections, transfusion-related acute lung injury (TRALI), transfusion-associated cardiac overload (TACO) giving pulmonary oedema due to volume overload, and transfusion-related immunomodulation (TRIM) (113). A propensity score-matched observational study on mortality and morbidity of RBC transfusions in septic patients performed at Skåne University Hospital, Lund showed an association between increased mortality and morbidity with a liberal transfusion setting. The absolute risk increase for death at 180 days for patients in the RBC transfusion group was 11% (95% CI 1.7-19%) (114).

Furthermore, the "Surviving Sepsis Campaign" lists several weak recommendations, such as (109);

1. In adults with sepsis or septic shock and AKI, the recommendation is to use continuous or intermittent renal replacement therapy (RRT).
2. Sepsis patients not reaching the target mean arterial pressure (MAP) despite adequate vasopressor administration should be given IV corticoids.
3. In patients who have risk factors for gastrointestinal bleeding stress ulcer prophylaxis is recommended.

Biomarkers in sepsis

Since most microbial cultures and analyses take several days to analyse, it has been suggested that biomarkers could improve sepsis decision making, through rapid diagnosis and thereby quicker initiation of adequate therapy (115). The definition of a biological marker (biomarker) is: *A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention* (116). In 1780 the Swedish chemist Karl Wilhelm Scheele was the first to report on lactic acid, found in sour milk (117). In 1843, the physician Johann Joseph Scherer reported that lactic acid was detected in human blood under pathological conditions in sepsis in dead patients (117). In 1858 Carl Folwarczny was first to demonstrate lactate in blood of a living patient (117). The acute phase protein, C-reactive protein (CRP) was discovered in 1930 by William S. Tillet and Thomas Francis (118). CRP is a biomarker, widely used in diagnosing infection although the biomarker is also increased in a wide variety of immunological reactions, *e.g.,* in rheumatic diseases, malignancy, trauma, and necrosis (119). Procalcitonin (PCT), identified in 1975 by Deftos *et al*., has shown to be a helpful biomarker in critically ill, septic patients suffering from bacterial infection (69, 120). PCT is today widely used in intensive care units to guide the initiation of antibiotic treatment and studies have shown that PCT is a useful tool in shortening antibiotic use (121). Pierrakos *et al.,* performed an extensive review of sepsis biomarkers in 2020 where they identified 258 published biomarkers. Nine of these were shown to perform better as diagnostic, supportive tools than CRP and/or PCT (122). Ideally, sepsis biomarkers should demonstrate rapid kinetics with high sensitivity and specificity, be costly accessible and yield fast results. In a clinical setting it is also important to have biomarkers to monitor progression of disease, response to therapy, including assessment of the effect of administered antibiotics over time and when appropriate to de-escalate antibiotic treatment, important in itself to avoid increasing antibiotic resistance (7-9).

Iron metabolism and hepcidin

Iron

Iron, a reactive transition metal is vital for all organisms and is an essential component of haemoglobin and enzymes involved in oxidative phosphorylation. Under normal physiologic circumstances extracellular catalytic free iron (CFI) is bound to transferrin, thus free iron ions are almost undetectable in plasma (123). Ferric iron is sequestered into ferritin. There are two subunits of ferritin, L (light)-subunits found in plasma and H (heavy)-subunits found intracellularly where the latter can incorporate iron rapidly. High presence of H-rich ferritin has been shown to be cytoprotective allowing cells to resist injury, thus making the cells more resistant to apoptosis and necroptosis (124). Catalytic free iron is harmful in sense of triggering reactions where oxygen free radicals are generated leading to endothelial injury, erythrocyte injury, mitochondrial damage, DNA damage as well as protein oxidation and lipid peroxidation (125). Emancipation of CFI is described in many acute conditions such as sepsis, myocardial infarction, stroke, acute kidney injury driven by for example metabolic acidosis, increased levels of catecholamine, and tissue injury (124). Exogenous catecholamine such as norepinephrine given to hypotensive patients in intensive care units could theoretically worsen infections through the release of CFI (126). Bacteria, fungi, and parasites depend on iron as a nutrient to thrive and proliferate (127). Patients with iron overload are more susceptible to siderophilic bacteria such as *Escherica coli, Klebsiella pneumoniae, Salmonella enterica, Vibrio vulnificus,* and *Listeria monocytogenes* and iron overload has also been demonstrated to facilitate the development of fungal infections (124, 128, 129).

Hepcidin – discovery and physiological function

The key regulator of iron homeostasis is hepcidin, a cysteine-rich disulphide-bonded 25 amino acid peptide, with a mass of 2700 Dalton, exhibiting antibacterial and antifungal activity (130-132).

Hepcidin was independently discovered by two research groups in the beginning of the 21:century, in plasma by Krause *et al.* in 2000 with the acronym LEAP-1 (Liver

Expressed Antimicrobial Peptide 1), and in urine by Park *et al.* in 2001 (130, 131). Hepcidin is mainly produced by hepatocytes, the synthesis is upregulated at high serum iron levels but also by inflammation stimulated by interleukin-6 (IL-6), oncostatin M, IL-1β, IL-22, IFN-α, activin B and erythropoiesis (133-135), thus hepcidin also acts as an acute phase reactant (136). The bioactive form of hepcidin is the 25 amino acid peptide, but smaller isoforms, such as hepcidin-24, -23, -22 and -20 exist, all of unknown clinical significance (137). Circulating hepcidin occurs mainly in the free form, where <3% is bound to α-2-macroglobulin and albumin (138, 139).

The hepcidin synthesis has been shown to be regulated in three ways *i.e.,* by iron levels, status of erythropoiesis, and via the IL-6 receptor pathway as an inflammatory response (Figure 4) (140).

1. Extracellular iron in the form of holotransferrin (Fe-Tf) is sensed by the two transferrin receptors (TFR1) and (TRF2) that interact with the hemochromatosis protein (HFE). Via a complex route the HFE/TRF2 complex sensitizes the bone morphogenetic protein (BMP) receptor, and once activated the initiation of the SMAD signalling starts which leads to increased hepcidin transcription (Figure 4) (140).
2. Activation of the erythropoiesis results in increased production of erythroferrone (ERFE). ERFE inhibits BMP signalling by binding to BMP2/6 receptor that thereby interrupts the interaction with the BMP receptor. In this way the hepcidin levels decreases and more iron will be available for erythropoiesis (140).
3. The upregulation of hepcidin in humans is rapid. Maximal hepcidin levels were measured in urine at six-hours post-injection of lipopolysaccharide, paralleled by a significant decrease in serum iron. A similar observation was seen after an infusion of IL-6 in humans causing an increase in hepcidin followed by a significant decrease in serum iron and transferrin saturation within a few hours (141, 142). IL-6 acts by binding to the IL-6 receptor on hepatocytes or reticuloendothelial macrophages, which in turn activates the Janus kinase and STAT3 signalling pathway resulting in increased hepcidin expression (Figure 4) (140, 143).

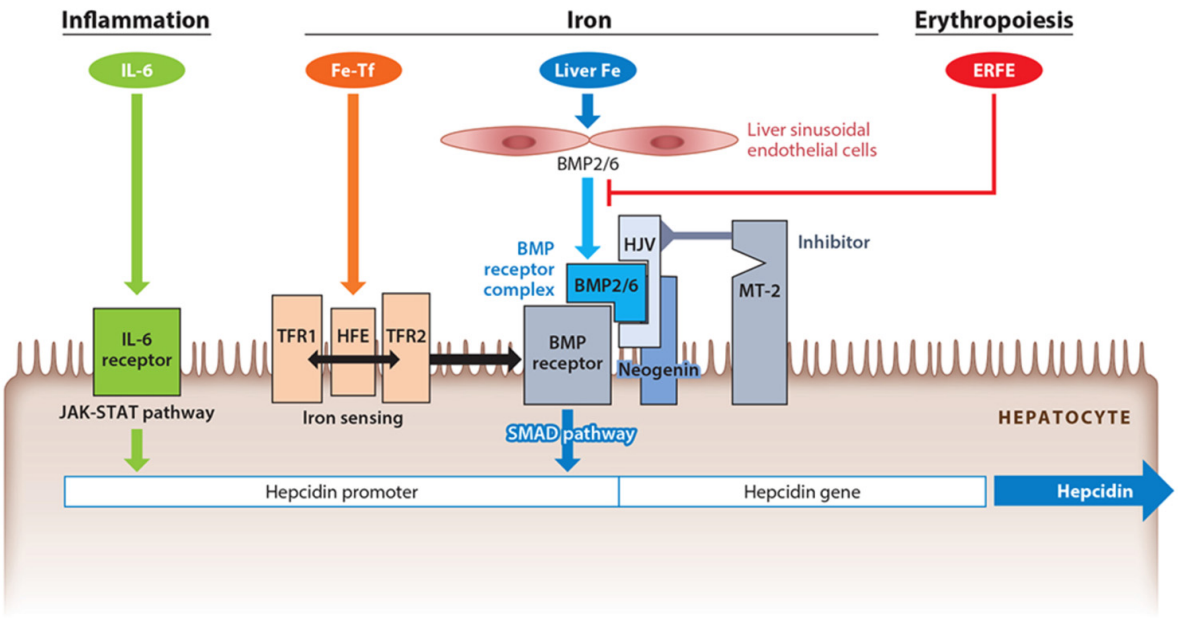

Figure 4. Molecular mechanisms of hepcidin regulation. *Center:* Iron regulates hepcidin through two distinct mechanisms. Extracellular iron in the form of iron transferrin (Fe-Tf) is sensed by the two transferrin receptors (TFR1 and TRF2). Binding of Fe-Tf to its receptors promotes hemochromatosis protein (HF) interaction with TFR2 instead og TFR1, and the HFE/TFR2 complex then sensitizes the bone morphogenetic protein (BMP) receptor to its ligands BMP2 and -6 or the BMP2/6 heterodimer. HFE may also directly stabilize the BMP receptor by preventing its ubiquitination. Hemojuvelin (HJV), a membrane linked BMP coreceptor, potentiates the BMP receptor activation. Once activated, BMP receptors initiate SMAD signaling which increases hepcidin transcription. Increased intracellular iron in the liver enhances BMP6 and -2 production by liver sinusoidal endothelial cells, eventually leading to activation of the BMP receptor on hepatocytes. Under low-iron conditions, low Fe-Tf concentration and low intracellular iron both lead to decreased BMP pathway signaling and decreased hepcidin m-RNA expression. Furthermore, matriptase-2 (MT-2) protease Is stabilized in low-iron conditions and inhibits and cleaves HJV and other molecules of the BMP pathway., thus further decreasing the SMAD signaling. *Left:* Inflammation stimulates hepcidin production by increasing the transcription of hepcidin through the interleukin (IL)-6-JAK-STAT pathway. *Right: Erythropoiesis activation leads to increased production of erythroferrone (ERFE) by erythroblasts. ERFE then inhibits BMP signaling by binding to BMP2/6 and interfering with its interaction with the BMP receptor, thereby lowering hepcidin and making more iron available for erythropoiesis.* Reprinted with permission by Elizabeth Nemeth (140).

Hepcidin acts by altering the distribution of the receptor ferroportin, present on hepatocytes, macrophages, and duodenal enterocytes but can also be found on erythrocytes (140). Increased serum iron as well as inflammation results in transition of ferroportin from the cell membrane to intracellular lysosomes. Ferroportin functions as the iron exporter responsible for iron transfer to plasma and is known to be regulated by hepcidin only. Ferroportin degradation prevents iron efflux resulting in higher intracellular iron concentrations (144). Ferroportin is evolutionary highly conserved, built up of a 12-transmembrane domain and found also in plants and invertebrates (140).

Hepcidin, regulates ferroportin by directly binding and occluding ferroportin, and also by inducing a conformational change resulting in endocytosis of the hepcidin-ferroportin complex and lysosomal degradation (140). Hepcidin acts as the key

regulator of the iron flow that enters the plasma compartment mainly via three routes; Firstly, hepcidin regulates the uptake of dietary iron in the duodenum via the enterocytes, secondly hepcidin regulates the release of recycled (old red blood cells) iron from macrophages, and thirdly hepcidin regulates the release of stored iron from hepatocytes (140, 145). Iron and hepcidin regulate each other via an endocrine feedback loop (140). When there is an excess of iron, more hepcidin is synthesized by the hepatocytes, limiting further release from iron stores (hepatocytes and macrophages) and further iron absorption (enterocytes in duodenum) (Figure 5a.). When there is an iron deficiency, less hepcidin is synthesized from hepatocytes, making more iron available in plasma (Figure 5b.).

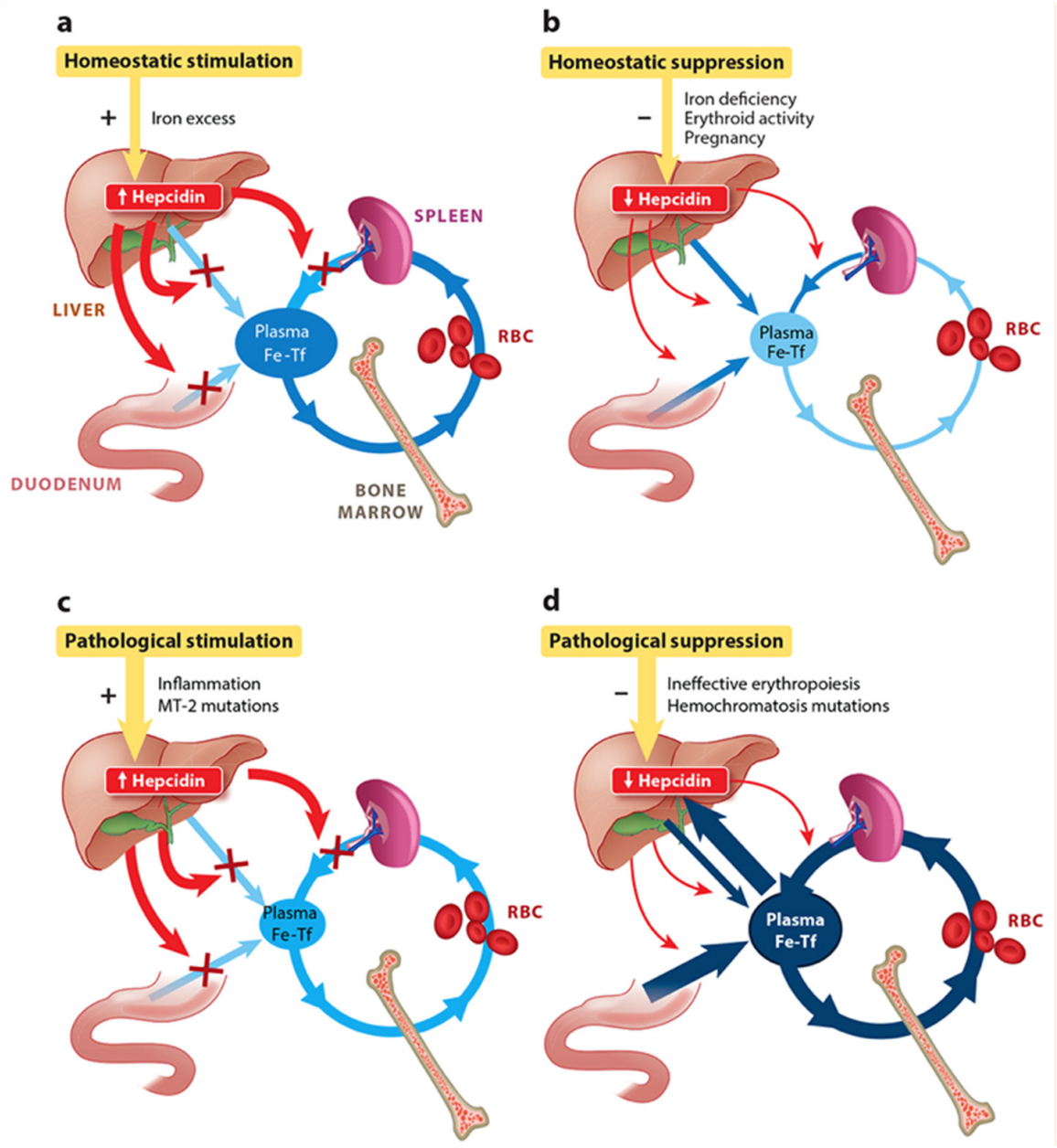

Figure 5. The homeostatic role of hepcidin, (a) iron excess, (b) iron deficiency. Pathological hepcidin stimulation (c) and pathological suppression i.e. iron-overload disorders (d).

Iron flows are shown in shades of blue indicative of intensity. Hepcidin and its effects are shown in red: normal and pathological hepcidin modulators are shown in yellow. Fe-Tf (iron transferrin), MT-2 (matriptase-2), RBC (red blood cell). Reprinted with permission by Elizabeth Nemeth (140).

Hepcidin evolution

The name hepcidin originates from its production site in hepatocytes, “hep”, and “cidin” for its bactericidal attributes (146). Recently hepcidin has been shown also in bone marrow of both mice and humans (147).

In humans, hepcidin is encoded by the single HAMP gene (Hepcidin Anti-Microbial Peptide), located on chromosome 19 at position q13.12. The HAMP gene is translated to an 84 amino acid pre-propeptide presenting a furin cleavage site. Hepcidin is processed primarily in the liver and thereafter distributed to plasma and finally eliminated via urine. In urine the dominant hepcidin form is hepcidin-25 consisting of 25 amino acid and two shorter peptides hepcidin-20 and hepcidin-22 (131, 148). Hepcidin belongs to the β-defensin family, in addition to the function as the key regulator of iron homeostasis, hepcidin also exhibits direct antimicrobial activities as an antimicrobial peptide (AMP) (146). Antimicrobial peptides (AMPs) are phylogenetically ancient molecules that are essential in multiple aspects of host defence. AMPs are rapidly mobilized in the first line of defence as part of the innate immune system, and play a role as signalling molecules for the innate immune system and the adaptive immunity (149). AMPs act by permeabilizing the cell membranes of microorganisms which results in the efflux of solutes (106). Hepcidin has been isolated in several animal species such as mammals, birds, reptiles, amphibians, and fish (146, 149). Segat *et al*., performed a systematic analysis of the evolution of hepcidin and concluded that hepcidin is conserved in all primate species. This result suggests that hepcidin´s main role is to act as an iron regulating hormone which requires close interaction with the conserved iron transporter ferroportin (149).

Hepcidin and sepsis – overview of clinical studies

Elevated serum hepcidin levels have previously been reported in children with severe infection, as well as in adults suffering from pneumonia and sepsis (150-152). Our pilot-study published 2020 showed that hepcidin increased fast and declined promptly as patients improved during the first 24 hours of treatment in the ICU, similarly to procalcitonin in patients with septic shock receiving adequate empiric antibiotic treatment (153). In a Chinese report published in 2018, 183 patients were enrolled divided in three categories by diagnosis; non-sepsis (n=93), sepsis (n=48), and septic shock (n=42), a correlation was shown between hepcidin

and the severity of sepsis and combining levels of hepcidin and procalcitonin could ameliorate the accuracy in diagnosing sepsis (abstract only, in English, article published in Chinese) (154). In a recently published meta-analysis on the diagnostic value of hepcidin in sepsis, 8 studies were incorporated, covering in total 1047 patients (625 patients with sepsis and 422 non septic patients) (155). Hepcidin showed a pooled sensitivity of 0.88 (95% confidence interval [CI]: 0.76-0.94) and specificity of 0.91 (95% [CI]: 0.76-0.97) for sepsis diagnosis and the ROC curve revealed an AUC of 0.95. The study showed absence of publication bias but two of the included studies were written in Chinese, and one publication is not available on PubMed.

Overview of clinical studies on hepcidin and sepsis

Table 3. Non-systematic overview of clinical studies on hepcidin and sepsis

Year	Authors	n_{pat}	Sites	Population	Sampling from and method	Comparison	Outcome measure
2011	Van Eijk et al. (150)	92	1 (Netherlands)	Adults	Serum hepcidin Mass Spectrometry	Sepsis patients emergency ward Correlation with IL-6	Hepcidin correlated with IL-6 R=28, p=0.0005
2013	Wu et al. (151)	65	1 (USA)	Premature infants	Serum hepcidin ELISA	Premature infants versus term infants	AUC 0.93
2016	Tacke et al. (156)	311	1 (Germany)	Adults	Serum hepcidin ELISA	Septic and nonseptic ICU and healthy blood donors	Hepcidin levels 50.3 ng/mL in septic patients versus 8.0 ng/mL in healthy control P<0.001
2018	Yesilbas et al. (157)	89	1 (Turkey)	Pediatric patients	Serum hepcidin ELISA	Sepsis/septic shock versus healthy control group and pediatric intensive care unit control group	AUC Sepsis+shock compared to healthy control group AUC 1.0
2018	Qui et al. (154)	183	1 (China)	Adults	Uknown	Sepsis/Non-sepsis, ICU	Hepcidin AUC 0.865
2019	Wakakuri et al. (158)	113	1 (Japan)	Adults	Serum hepcidin ELISA	Hepcidin levels in SIRS patients with bacteremia or culture	Hepcidin mean Bacterial pat, 209 ng/mL

						negative infections vs. non-bacterial infections	Culture neg patients, 168 ng/mL Non bacterial infections, 142 ng/mL P=0.001
2019	Jiang et al. (159)	218	1 (China)	Adults	Serum hepcidin ELISA	Sepsis divided into survival and non-survival group versus control group compared to 28-day mortality	Hepcidin AUC 0.808
2021	Hagag et al. (160)	80	1 (Egypt)	Neonates	Serum hepcidin ELISA	Late onset sepsis versus control group	Hepcidin: Sensitivity 95% Specificity 90%
2022	Sherbiny et al. (161)	123	1 (Egypt)	Premature infants. Suspected late on set sepis	Serum- and urine hepcidin ELISA	Late onset sepsis versus non-septic control	AUC Serum hepcidin 0.935 Urine hepcidin 0.878
2023	Hortová-Kohoutková et al. (162)	86	1 (Czech Republic)	Adults	Serum hepcidin ELISA	Septic shock + SARS-CoV-2 in ICU versus non-septic control	AUC Septic shock 0.79 p=0.03
2023	Czempik et al. (163)	90	1 (Poland)	Adults	Serum hepcidin ELISA	Association between standard iron biomarkers in septic patients	No significant correlations determined between hepcidin and standard iron biomarkers p=0.13
2024	Zhang et al. (155)	1047	8 (China (3), Egypt (2), Turkey, USA, Sweden	Adults and pediatric patients	7 used Serum hepcidin ELISA 1 used serum hepcidin Mass Spectrometry	Metaanalysis Hepcidin as diagnistic tool differentiating sepsis from non-sepsis	Hepcidin: Sensitivity 0.88 (95% CI 0.76-0.94) Specificity 0.91 (95% CI 0.76-0.97) AUC 0.95

Hepcidin Disorders

Several disorders with over- and under-expression of hepcidin have been identified, (Table 4) (140).

Hepcidin deficiency is noted in patients with hereditary hemochromatosis (HH), in iron-loading anaemias where the erythropoiesis is dysfunctional, and in chronic liver diseases, the latter since that the synthesis of hepcidin mainly occurs in hepatocytes (140).

Hepcidin overexpression

Overexpression of hepcidin occurs in patients with anaemia with iron-restricted erythropoiesis such as anaemia of inflammation (AI) due to chronic disease, iron-refractory iron deficiency anaemia (IRIDA), and Castleman disease. Hepcidin overproduction has been found in local cancer cells implied to secure iron locally, for the cancer cells to thrive and proliferate (140, 164).

Anaemia of inflammation commonly seen in clinical settings, is most often normochromic and normocytic (164). The disorders that cause AI are diverse, *e.g.,* secondary to infection, autoimmune disorders such as rheumatic disease, and inflammatory bowel syndrome, chronic kidney disease (CKD), cancer, obesity, but also in the elderly (165). In CKD overexpression of hepcidin is due to ineffective renal clearance (140)

IRIDA is an autosomal recessive disease with overexpression of hepcidin caused by mutations in the hepcidin suppressor matriptase-2, resulting in microcytic anaemia, low transferrin saturation, and hypoferremia. Patients with IRIDA are unresponsive to oral iron therapy (166).

Castleman disease, characterized by hypochromic microcytic anaemia, is a lymphoproliferative disease with IL-6 overexpression with lymphadenopathy and multiple organ involvement (167).

Hepcidin under expression

Hereditary hemochromatosis (HH) is a group of genetic disorders where inadequate production of hepcidin results in excessive uptake of dietary iron from the duodenum. The accumulation of iron, in tissues affecting liver, skin, joints, heart, and pancreas causes iron-mediated injury resulting in organ dysfunction and failure (168). Hemochromatosis is treated by phlebotomy with a depletion of 200-250 mg of iron per unit of whole blood extracted (140).

Inherited iron-loading anaemias include thalassemia's, congenital dyserythropoietic anaemias, and sideroblastic anaemias. Myelodysplastic syndromes constitute acquired iron-loading anaemia. These conditions cause bone marrow hyperplasia and insufficient erythropoiesis. Patients with iron-loading anaemias are often treated with blood transfusions leading to secondary iron overload and hepcidin suppression (164).

The expression of hepcidin decreases substantially in advanced liver diseases of any aetiology, including alcoholic liver disease, chronic hepatitis C, and non-alcoholic fatty liver disease due to the dysfunction of hepatocytes (140, 164).

Table 4. Disorders resulting in hepcidin over- or under expression

Disease	Pathophysiology	Serum/plasma hepcidin
Infections	Inflammation → elevated hepcidin → iron restriction	↑
Autoimmune diseases, *i.e.* rheumatological disease, inflammatory bowel disease	Inflammation → elevated hepcidin → iron restriction	↑
Cancer	Inflammation → elevated hepcidin → iron restriction	↑
Chronic kidney disease	Inflammation + decreased renal clearance → hepcidin excess → iron restriction	↑
IRIDA	Genetic overproduction of hepcidin → iron restriction	↑
Iron overload	Transfusions, iron therapy	↑
Castlemans disease	IL-6 overexpression → elevated hepcidin	↑
Hereditary hemochromatosis (SLC40A1 mutation)	Ferroportin resistance to hepcidin → iron overload	↑
Non-transfusion-dependant thalassemia	Ineffective erythropoiesis → stimulation of erythroferrone → suppression of hepcidin → iron overload	↓
Transfusion-dependant thalassemia	Insufficient erythropoiesis → hepcidin suppression → iron overload Transfusion → elevated hepcidin → iron overload	↓↑
Hepatitis B and C	Suppression of hepcidin by virus → loss of hepatocytes → iron overload	↓
Alcoholic liver disease	Suppression of hepcidin by alcohol → loss of hepatocytes → iron overload	↓
Non-alcoholic fatty liver disease	Suppression of hepcidin by fat overload → loss of hepatocytes → iron overload	↓
Iron deficiency	Malnutrition. Blood loss	↓
Hereditary hemochromatosis (HFE, TFR2, HJV, HAMP mutations)	Genetic hepcidin deficiency → iron overload	↓

Therapeutic possibilities

There are several studies suggesting a therapeutic beneficial potential to approach the hepcidin-ferroportin axis, and thereby potentially replace current therapies of hepcidin disorders to decrease the burden of disease (128, 164).

Hepcidin has been considered to have a preventive role since preclinical studies have shown that hepcidin induces intracellular ferritin (H-rich ferritin). The presence of H-rich ferritin has been shown to be cytoprotective by increasing cellular resistance to apoptosis and necroptosis (169, 170).

A phase 1 placebo-controlled study showed that parenteral administration of hepcidin resulted in a rapid decrease of serum iron and transferrin saturation with a nadir at 8-12 hours, thus theoretically restricting free iron as a nutrient for invading microbes (171). Hepcidin agonists have been suggested as effective treatment in patients with siderophilic infection in patients with chronic liver disease or iron overload (128).

Hepcidin replacement therapy to limit iron absorption could potentially be a therapeutic option in patients with hereditary hemochromatosis (HH), polycythemia vera, and β-thalassemia (128, 164). Experiments in murine models with HH and β-thalassemia have shown that the iron overload was corrected by hepcidin (172-174). The erythropoiesis improved in mice with β-thalassemia upon hepcidin administration (172-174). Furthermore, there are experimental models suggesting that administration of exogenous hepcidin may be therapeutically effective in treating liver fibrosis and obesity (175, 176). Rusfertide (PTG-300), a pepticidic mimetic of hepcidin has shown efficacy in reducing the number of phlebotomies in patients with polycythemia vera and maintained a haematocrit of less than 45% in a phase 2 study (177, 178). A phase 3, global, multicentre study, the VERIFY trial (NCT05210790), is ongoing with the drug Rusfertide in humans for the treatment of polycythemia vera (177, 178). Rusfertide has also shown promising results in patients with HH (179).

Hepcidin agonists have also shown to be effective in reducing mortality in mice with siderophilic infections caused by *Vibrio vulnificus, Yersinia enterocolitica,* and *Klebsiella pneumoniae* and reducing abscess formation in iron-loaded mice infected with *Yersinia enterocolitica* (180-183).

Hepcidin antagonists *e.g.,* hepcidin-neutralizing antibodies can be potential therapeutic options in patients suffering from anaemia of inflammation secondary to arthritis, inflammatory bowel diseases, genetic IRIDA, chronic infections, malignancies, and in patients with CKD (140, 184, 185).

Hepcidin - Laboratory testing

Hepcidin Assays

There are currently two different assays for analysis of hepcidin, by immunoassay or mass spectrometry (186, 187).

ELISA – Immunoassay (IA)

Enzyme-linked immunosorbent assay (ELISA) is widely used based on an antigen binding to its specific antibody, enabling detection of small quantities of antigens such as proteins, peptides, and other biological molecules (188). ELISA utilizes enzyme-labelled antigens and antibodies to detect those biological molecules, where the most commonly used enzymes are alkaline phosphatase and glucose oxidase (188). It has though proved to be challenging to develop assays to quantify hepcidin in biological samples, due to the small evolutionary size of hepcidin and hepcidin´s tendency to aggregate and stick to laboratory plastics (137). Another challenge is that the immunoassay lack specificity to hepcidin-25 but rather measure all isoforms of hepcidin *i.e.,* hepcidin-20, -22 and -24 that are formed following N-terminal degradation of hepcidin, thereby overestimating hepcidin-25 concentration due to this cross-reactivity (137, 186, 189).

ELISA-assays to measure hepcidin serum concentration using a recombinant hepcidin25-His peptide and a polyclonal antibody against this peptide have been developed (190). Through this method it is possible to identify native hepcidin. The detection interval ranges from 10-1500 µg/L (190).

Liquid chromatography and tandem mass spectrometry (LC-MS/MS)

Hepcidin measurement by mass-spectrometric assay is more expensive and resource demanding but has the advantage of being more precise in detecting the correct isoform of hepcidin, hepcidin-25 (137). Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) can identify and quantify a wide range of potential biological analytes such as small molecules, peptides, and proteins with high sensitivity and specificity (191) and can be summarized as follows (192-194);

The liquid chromatography step separates biological molecules in columns *e.g.,* solid phase extraction (SPE) columns, where each column has a stationary phase and a mobile phase. Based on the affinity due to size, charge or level or hydrophobicity biological molecules exhibit the mobile phase and passes through a detector *e.g.,* ultraviolet light absorption or refractive index that provides a signal. The signal unfortunately does not yield a reliable result at low concentrations, thus the process has limited specificity.

Biological molecules exiting the column from the liquid chromatography are either heated or injected into a nebulizer and thereby evaporated and ionized. The interface between the liquid chromatography (LC) and mass spectrometry (MS) is the electrospray ionisation which is a crucial step since MS only can measure gas phase ions.

The gas phase ions are then exposed to electro fields or electromagnetic fields. The flight paths of the ions are altered by varying the applied fields which ensures the ions separation from one another on the basis of their mass-to-charge (m/z) values.

Unique with the tandem mass spectrometry LC MS/MS is the tandem quadrupole mass filter divided into mass spectrometry (MS) 1 and MS2 with a collision cell between MS1 and MS2. A quadrupole consist of four parallel rods that create a fluctuation field with a voltage between the rods, thus controlling the trajectory of ions through the mass spectrometer. The quadrupole can be programmed by varying the voltages with time so that a specific mass-to charge (m/z) value is stable down the trajectory of the quadrupole. Ions that are too large or small will have an unstable flight path and will therefore strike the rods in the quadrupole. The stable ions that pass the trajectory are then detected usually via an electron multiplier with high specificity. The abundance of ions is then plotted into a total chromatogram.

For hepcidin-25, endogenous and isoptin-marked hepcidin-25 are retarded to solid phase extraction (SPE) columns, where the main part of the other endogenous material is washed out afterwards (195). This clean-up step is important for establishing the stability of hepcidin-25 where an appropriate buffer pH is crucial for a stable step of hepcidin-25 (189).

Mass-spectrometric assays detect the mass of the biological active 25-amino-acid hepcidin, quantifying the peak value relative to an internal standard value (137). The cleaned-up sample is then analysed using liquid chromatography and tandem mass spectrometry (LC-MS/MS) (195). The quota of hepcidin/isoptin-marked hepcidin is then calculated and the concentration is decided by calibrators with known concentrations (195). The reference interval for serum hepcidin LC-MS/MS is set to 1-12 nmol/L (196, 197).

When applied to samples from patients with community-acquired pneumonia Oppen *et al.,* reported that hepcidin concentration measurements turned out to be higher when using immunoassay compared to LC-MS/MS (186). Furthermore, they

reported a difference in values of hepcidin-25 in serum concentrations compared to plasma, with hepcidin levels on average being 22 % lower in serum compared to plasma (186).

Heparin-binding protein (HBP).

Heparin binding protein is also known as azurocidin or cationic antimicrobial protein of 37000 Dalton (198, 199). HBP is a antimicrobial inflammatory mediator produced by neutrophils and stored prefabricated in both primary and secretory vesicles (199). Neutrophils activated by IL-8 as well as directly by bacterial proteins release HBP (200, 201). HBP has multiple functions, *e.g.,* bactericidal activity, increasing the vascular permeability by inducing vascular leakage, acts as a chemoattractant for cells, and contributes to bacterial opsonization (199, 202). HBP has been described as a promising early marker of sepsis, and a systematic review of 26 studies, the majority of which were performed in China, reported a pooled specificity of 0.91 (95% CI, 0.82-0.96) for HBP as a biomarker of sepsis (203-205).

Routine biomarkers in the ICU

C-reactive protein (CRP).

The most widely used biomarker for measuring inflammation is CRP, a protein mainly synthesized in the liver as a response to inflammatory activity induced mainly by IL-6 and TNF-α (119, 206). Thus, induction of CRP is not specific for bacterial infections but is also elevated in various form of inflammatory conditions such as rheumatic disorders, chronic inflammatory disorders, tissue injury, malignancies, and thromboembolism (207, 208). Limitations of CRP for clinical decisions include the long half-life of around 18-20 hours, and the 24-hour delay in change following tissue damage, *i.e.,* CRP levels reflect the previous day´s inflammatory activity (119, 209, 210).

Procalcitonin (PCT).

A precursor to the hormone calcitonin, procalcitonin (PCT), is released primarily from thyroid C-cells. During bacterial sepsis in animals, gene expression for PCT is also found in *e.g.,* lungs, liver, and adipose tissue (211, 212). Experiments in humans show that PCT rises 4 hours after endotoxin injection, reaching peak values after 8 hours in healthy individuals (213). In the early stage of sepsis PCT has shown higher specificity than CRP (214, 215). A meta-analysis based on 30 studies showed a mean sensitivity of PCT of 77% and a mean specificity of 79% for sepsis (69). Normal serum levels of PCT are almost undetectable but increase especially during

bacterial infections (216, 217). In addition, PCT levels correlate with the severity of sepsis, thus suggesting that PCT is a useful tool to differentiate sepsis from non-septic conditions, thereby reducing unnecessary use of antibiotics and antibiotic-related side effects without increasing mortality rates (216, 218-221).

Proseek® Multiplex^{96x96} Immunoassay

Sci-Life Laboratory Uppsala has established a platform for supporting scientists in studies utilising the Proseek® Multiplex^{96x96} Proteomics Tool developed in Uppsala (Olink, Uppsala, Sweden) providing a multiplicity of biomarkers that can be analysed for various purposes such as inflammation, autoimmunity, and cancer. In our study we analysed 272 different biomarkers from three separate panels, the Immune Response, Inflammation, and Organ Damage panels. Biomarkers in these panels include several pro- and anti-inflammatory cytokines (*e.g.,* several different interleukins (IL) and oncostatin M), chemokines (*e.g.,* C-X-C motif chemokine ligand 10), signalling adaptor proteins (*e.g.,* Phosphoinositide-3-Kinase Adaptor Protein 1), cell surface receptors (*e.g.* Triggering Receptor Expressed on Myeloid Cells 1), and pattern recognition receptors (*e.g.,* C-type lectin domain family 4), just mentioning a few. The analysis is based on Proximity Extension Assay (PEA) technology. The 96-plex immunoassay is performed in three steps, first, the incubation step, where 96 pairs of antibodies labelled with unique complementary oligonucleotide sequences are added. Each antibody pair specifically binds to one of the biomarkers, and the oligonucleotide sequences hybridize pairwise in a proximity-dependant reaction. In the event of antibody cross-reactivity, the mismatched oligonucleotide sequences are unable to hybridize, thus no further readout occurs. In the next step hybridized sequences extend and pre-amplify. In the third detection step amplification and eventually detection by real-time polymerase reaction occurs.

The results are generated in quantification cycle values consisting of the analysed biomarkers and controls for each patient sample. These values are hereafter transferred to a software where quality controlling, normalization, and conversion of quantification cycle values are performed into normalized protein expression (NPX) values. NPX values are arbitrary units on the Log2 scale inverted compared to the quantification cycle scale. High NPX values correlate to a high protein concentration and vice versa, but only relative quantifications, meaning that no absolute values are received (222).

The present investigation – overall aim and rationale of the thesis

Overall aim

The overall aim of the thesis has been to investigate biomarker changes in patients with community acquired critical illness admitted to the ICU especially the added value of hepcidin in comparison to other biomarkers *e.g.,* Ret-He, HBP, CRP, PCT, lactate, and white blood count (WBC), for identification, prediction, monitoring, and outcome comparing patients with sepsis and patients with non-septic critical illness treated in intensive care.

Specific aims

Paper 1

- To investigate if and how the dynamics of hepcidin and reticulocyte haemoglobin (Ret-He) could serve as biomarkers for septic shock in patients admitted to the Intensive Care Unit, at the tertiary hospital, of Helsingborg, Sweden. We investigated reticulocyte hemoglobin (Ret-He), hepcidin, heparin-binding protein (HBP), C-reactive protein (CRP), procalcitonin (PCT), white blood count (WBC), lactate, and results of clinical evaluation tools in patients with severe sepsis/septic shock in need of intensive care. In addition, complications among the patients were compared to the dynamics of the biomarkers.

Paper 2

- Our aim was to evaluate whether hepcidin levels in serum from acutely admitted ICU patients with community acquired critical illness could discriminate severe sepsis/septic shock from critical illness due to non-septic conditions. We investigated the potential value of hepcidin levels compared to HBP levels and the commonly used biomarkers for sepsis *i.e.,* WBC, CRP, lactate, and PCT as prognostic markers of outcome of disease.

Paper 3

- The aim of this study was to elucidate associations between hepcidin and HBP levels in serum and renal failure, assessed by the level of acute kidney failure (AKI), among critically ill patients with sepsis or non-septic conditions occurred during the study period, and any association with mortality. We evaluated the possible associations between hepcidin and HBP, respectively, and peak creatinine concentrations, as well as the need for renal replacement therapy among the two patient groups.

Paper 4

- Using proteomic measurements of infection, inflammation, and organ damage tools provided by Olink, we aimed to investigate associations and time course comparisons between cytokines, chemokines, and inflammatory proteins (for simplicity referred to as "inflammatory mediators"), with hepcidin, HBP, and routinely used biomarkers, *i.e.,* CRP, PCT, lactate, and WBC in patients during the first week of intensive care (day 1, 3, and 7).

Overall rationale

Sepsis and septic shock are volatile conditions where the clinical picture differs due to host factors, such as age, immune status and comorbidities, and factors influenced by microbial species, infectious load, and virulence (21, 24, 25). Except for clinical evaluation and microbial findings, clinicians rely on biomarkers to identify, predict, monitor and prognosticate the outcome of sepsis and septic shock. Hepcidin, discovered in year 2000, is an important regulator of iron metabolism and an acute phase reactant, exhibiting antibacterial and antifungal activity, and has been suggested to be a potential biomarker for infections (130, 131, 158). The clinical Chemistry Laboratory in Helsingborg, Lund University, was one of the first laboratories in the Nordic countries to introduce hepcidin as part of anaemia investigation, making the analysis easily accessible for any research project. In addition, access and great collaboration with the intensive care unit in Helsingborg made it possible to investigate hepcidin in the most severe community acquired illnesses in patients urgently admitted to the ICU.

Specific rationale

Paper 1

In paper 1 we wanted to explore how hepcidin and Ret-He change in septic patients treated in the ICU shortly after being admitted to the hospital. The study consisted of 15 patients suffering from septic shock performed to investigate whether a larger study would be of interest. Complications among the patients were recorded during the first week of ICU care and compared with the dynamics of the commonly used biomarkers.

Paper 2

The results on hepcidin in paper 1 convinced us to expand the study including all critically ill patients admitted with community acquired illness to the ICU. The study was performed as a single-centre, observational study to investigate whether hepcidin levels in serum could discriminate sepsis/septic shock from critical illnesses due to non-septic conditions. We investigated the potential prognostic value of hepcidin at arrival and the dynamics during the first 7 days of care in the ICU and compared this with HBP, earlier reported from Lund (203, 205), as well as with the commonly used biomarkers and the clinical evaluation tools applied to evaluate patients in the ICU such as SAPS3 and SOFA-score.

Paper 3

A protective effect of hepcidin on haemoglobin-mediated kidney injury has been reported (223, 224). The focus of the third report was therefore to investigate if there was any correlation between hepcidin and AKI in the well-defined cohort from paper 2 of ICU patients with community acquired critical illness. HBP was included in the calculations, since previous studies showed a significant association between HBP and the development of sepsis-induced AKI (225-227).

Paper 4

In collaboration with the Sci Life Laboratory, and the University of Uppsala, Sweden, we performed an exploratory study on thirty of the patients from our cohort using the Olink Proteomics Tool, Proseek® Multiplex^{96x96}. The rationale of the study was to investigate if there are associations between cytokines, chemokines, and organ damage proteins and compare these with the previously noted levels of hepcidin, HBP, and conventionally used biomarkers in a selection of the well-defined patients during the first week of intensive care treatment, and any possible prognostic value in patients with critical illness.

Thesis methods

Overall methods

Patient selection and data collection

All four papers were conducted with a population of patients as a single centre observational study at the ICU at the tertiary hospital of Helsingborg, Sweden. Clinical data and blood samples were from patients ≥ 18 years of age, admitted to the ICU within 24 hours of arrival to the hospital, and analysed during the morning hours on the first seven consecutive days in the ICU. All patient data including microbial data were collected from medical journals. Patients with a suspected sepsis/septic shock received broad-spectrum antibiotics according to local treatment guidelines. Care and treatment were performed by the regular staff at the ICU and not by anyone involved in the study. Exclusion criteria included blood transfusion or surgery within 7 days before inclusion in the study, and pregnancy and age <18 years.

Ethics

The studies were approved by the local Ethics Committee in Lund (Dnr. 2014/4, 2014/195, 2015/467 and 2019/04558). Oral and written informed consent was collected from the patients and if unable at inclusion, their next-of-kin approved, thus delayed consent from the patient was accepted by the Ethics Committee.

Biomarker analysis

Blood samples were centrifuged within 30 minutes after sampling and delivery to the Clinical Chemistry Laboratory, Helsingborg. Blood samples for analysis of hepcidin, HBP, and the Proseek® multiplex biomarkers were stored at -80°C prior to analysis. Analysis of Reticulocyte haemoglobin (Ret-He), reticulocytes, thrombocytes, haemoglobin, white blood count, creatinine, CRP, procalcitonin, and lactate were immediately performed at the Clinical Chemistry Laboratory, Helsingborg Hospital. Hepcidin was analysed using mass spectrometry with a 6500 QTrap® (Sciex, Washington, DC, USA) at the Clinical Chemistry Laboratory initially in Helsingborg and then at Lund University Hospital due to transfer of the Mass-spectrometer. HBP analyses were performed with an in-house sandwich ELISA at the Biomedical Centre in Lund. The Proseek® Multiplex^{96x96} Immunoassays were performed at the Sci Life laboratory in collaboration with the

Department of Medical Biochemistry and Microbiology at the Biomedical Centre, Uppsala University, Sweden.

Definitions
Sepsis-3 was defined according to the 2016 consensus publication (22). AKI was defined according to the Kidney Disease Improving Global Outcomes (KDIGO) published in 2012 (81).

Specific methods

Paper 1

The study was an explorative observational, single centre, pilot study investigating the iron metabolism biomarkers hepcidin and reticulocyte haemoglobin (Ret-He) in patients ≥ 18 years diagnosed with septic shock and transferred to the ICU within 24 hours after hospital admission. All patients had to fulfil the sepsis-3 definition (suspected infection and organ dysfunction with SOFA score ≥ 2) and the definition of septic shock meaning vasopressor support to maintain MAP > 65 mmHg and a lactate level above 2.0 mmol/L.

Biomarker samples were obtained at the time of inclusion at arrival to the ICU (within <24 hours of arrival to the hospital) and every morning during seven consecutive days. Relevant microbial investigations were performed, and clinical evaluation was obtained by daily SOFA score evaluations. Demographics, microbial findings, possible source of infection, co-morbidities, antibiotics, microbial findings, length of stay, clinical complications, and 28 days mortality were collected.

Focus of the study was to investigate if hepcidin and Ret-He correlated with conventionally used biomarkers (CRP, PCT, lactate, white blood cells (WBC), and haemoglobin), or with HBP in patients with septic shock. In addition, we focused on the biomarker kinetics over time, the possible correlation with SOFA score, and any complications that could occur during the seven-day investigation.

Data were presented descriptively, and Box plots were performed for each biomarker for the seven days of the study. Pearson correlation analyses were performed to evaluate correlations between the biomarkers. Linear mixed models were utilized to evaluate if any of the biomarkers could predict clinical outcome evaluated by SOFA-score and conversely if SOFA score could predict any of the biomarker outcomes.

Paper 2

The same overall inclusion and exclusion protocol was conducted as in paper 1, but herein we added patients with community acquired critical illness other than those defined by the sepsis-3 criteria. We included patients within 24 hours of admission to the hospital in need of intensive care with an expected ICU stay of at least 3 days in need of assisted ventilation and/or vasopressor support. Clinical evaluation was performed with SAPS 3 score at ICU admission and SOFA score daily. Biomarkers and SOFA score were evaluated for seven consecutive days. Demographics explained in paper 1 were performed as well as assessing hospital mortality, 28 day, and 180-day mortality.

Extensive statistical analyses were performed on this cohort. Mann Whitney U-test and Bonferroni correction method were performed to compare admission data of biomarkers, temperature, and clinical scores between the sepsis and non-sepsis groups. Clustered box plots for respective biomarker illustrated the dynamics over time. A receiver operating characteristics (ROC) curve for hepcidin, HBP, CRP, PCT, lactate, and WBC was performed to evaluate the diagnostic power of each biomarker in sepsis patients. In addition, logistic regression was performed to analyse ROC curves combining two biomarkers, in this case hepcidin and HBP together with PCT and CRP, respectively. Sensitivities, specificities, positive and negative predictive values were calculated from cross tabulations. Wilcoxon´s signed rank test illustrated the biomarkers dynamics in relation to inclusion values.

Mann Whitney U test was also performed to analyse potential associations between admission values of biomarkers and clinical evaluation tools and survival at 180-days. A binary logistic regression model with an interaction effect was used to explore if admission values of hepcidin and HBP could predict 28- and 180-days mortality in respective patient group. A Spearman´s signed rank correlation was utilized to analyse correlation between biomarkers and SOFA- score and SAPS 3 at admittance.

Paper 3

The same patient cohort as in paper 2 was used to explore if there were any associations between hepcidin and HBP levels in serum and renal failure among critically ill patients with sepsis or non-septic conditions. Renal failure was defined according to KDIGO guidelines. Admission levels of hepcidin and HBP were investigated as to their correlation with development of AKI stage 2-3, peak creatinine, and the need for RRT in septic and non-septic patients. Patients with a known chronic kidney disease (CKD) were excluded.

Mann Whitney U-test was used to compare medians. Person´s Chi-Squared test was performed to investigate differences in 28day mortality between the two groups (sepsis and non-sepsis). Regression models were created to examine the effects of either hepcidin or HBP on the development of AKI stage 2-3, the need for renal

replacement therapy, and peak serum creatinine concentrations, respectively. If significant results were achieved in the regression models, the model was expanded with an interaction variable to evaluate and adjust for the possible interaction between sepsis and hepcidin or HBP. Furthermore, an adjusted (by age and sex) logistic regression model was created to examine the association between serum concentrations of hepcidin and HBP, respectively, and AKI stage 2-3. A linear regression model was created to estimate if hepcidin and HBP correlated with peak creatinine. Finally, a bivariate regression model was utilized using admission values of hepcidin and HBP, respectively, to analyse potential correlations between these biomarkers with the requirement of dialysis.

Paper 4

In collaboration between the Department of Medical Biochemistry and Microbiology, Biomedical Center, Uppsala University and the Sci-Life Laboratory Uppsala we performed an exploratory observational study using the Proseek® Multiplex^{96x96} Proteomics Tool (Olink, Uppsala, Sweden) analysing 272 biomarkers (Supplementary material) with sera from 30 of the patients who were representative of the whole patient cohort from paper 2. The 272 biomarkers were from the three panels, inflammation, immune response, and organ damage. The 272 biomarkers were analysed on days 1, 3, and 7 and compared to previous results on hepcidin, HBP, conventionally used biomarkers, CRP, PCT, white blood count, lactate, and clinical evaluation tools, SAPS3 and SOFA-score. All blood samples, data, and clinical scores were in place on day 1, 3 and 7 on the patients included in this part of the study.

Proseek® Multiplex^{96x96} Proteomic data were analysed using univariate linear regression comparing the sepsis and non-sepsis groups, adjusted for gender. P-values were calculated and adjusted for multiple tests using the Benjamini-Hochberg procedure. Adjusted p-values (q-values) were computed, and statistical significance defined as $q<0,05$.

Thesis results

Paper 1

In total 15 patients with septic shock were enrolled, all with a history of an infection, organ dysfunction, and need of vasopressor support. In 11 out of 15 patients a relevant microbial finding was detected, and all patients received adequate empiric antibiotics, according to later reports on microbial resistance, already at admission before any antibiogram was available. Serum hepcidin was analysed by LC MS/MS methodology.

Median hepcidin levels peaked at admission followed by a fast decline already at 24 hours continuing during the next 72 hours in line with PCT and lactate. Five patients developed secondary complications such as pleural effusion and ventilator associated pneumonia (VAP) and in those patients a secondary elevation of hepcidin occurred.

Median Ret-He was within the lower normal range at admission followed by a decrease during the next 72 hours followed by an elevation after 96 hours reaching normal values at 120 hours.

Median levels of CRP, HBP, and WBC peaked 24 hours after admittance to the ICU, followed by a decrease during the following days. SOFA score also peaked at 24 hours post inclusion, and a general clinical improvement of the entire cohort was registered at 144 hours when SOFA score was registered at its lowest level. No patient died within the seven-day study period in the ICU, but one patient died within 28 days follow up.

A significant, positive correlation was found between hepcidin levels and PCT, CRP, and leukocytes, respectively, whereas a negative correlation was found with Ret-He. A linear mixed model was utilized demonstrating that PCT, CRP, and Ret-He significantly predicted SOFA-score, whereas SOFA-score only significantly predicted PCT and CRP. There was no significant correlation between hepcidin and SOFA-score either way.

Paper 2

A total of 164 patient were enrolled, 100 with community acquired sepsis (97/100 fulfilling the sepsis-3, septic shock criteria) and 64 critically ill patients without having an infection at admission. The study showed that both hepcidin and HBP levels were elevated at inclusion (time zero) and were positively associated with the diagnosis of sepsis in contrast to non-septic conditions. Hepcidin presented superior sensitivity and specificity compared to HBP, but inferior to CRP and PCT (Fig 6.). An AUC>0,8 was reached with hepcidin or HBP, respectively, combined with CRP or PCT, respectively. Hepcidin levels peaked at admission, and presented a statistically significant sharp decrease at 24, 48, and 72 hours compared to inclusion values. HBP increased after admission and peaked after 24 hours followed by a statistically significant decrease at 72 hours compared to inclusion levels. In figure 7. the decline as percentage of initial values in septic patients of hepcidin, CRP, PCT, lactate, WBC, and HBP are plotted into a diagram illustrating the daily changes, variation over time, related to the median of respective inclusion value set at 100%. High initial values of hepcidin in septic patients correlated with 180-day survival. Furthermore, a significant association was noted between low initial hepcidin levels and 180-day mortality. A negative correlation was noted between hepcidin and SAPS 3 score.

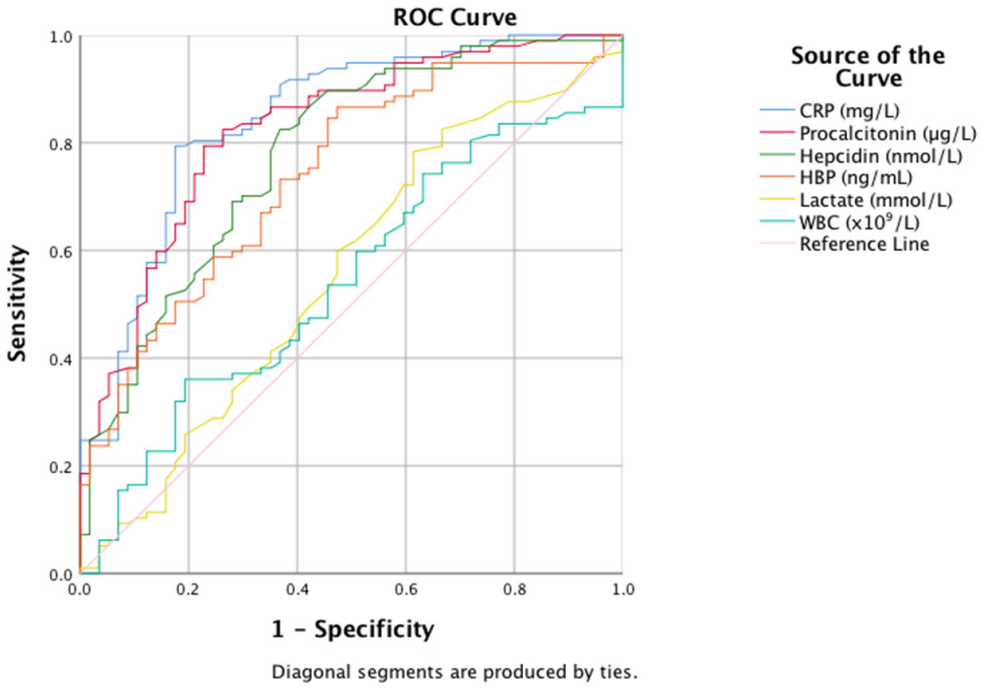

Figure 6. Receiver-operating characteristics curves of C-reactive protein (CRP), procalcitonin, hepcidin, heparin-binding protein (HBP), lactate, and white blood count (WBC) prediciting sepsis/septic shock diagnosis.

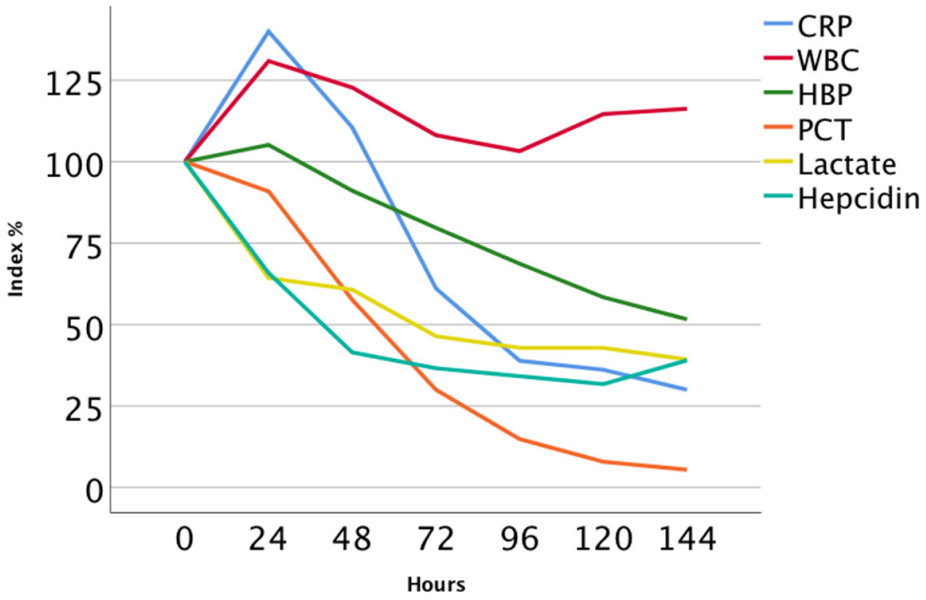

Figure 7. Relative changes in biomarker levels in septic patients. Dynamics related to initial values set at 100% for each biomarker.

Paper 3

A total of 140 patients out of the total 164 with community acquired critical illness admitted to the ICU within 24 hours were included in this sub study. Twenty-four patients were excluded due to a previous diagnose of kidney disease. Of the 140, 85 were diagnosed with severe sepsis/septic shock and the remaining 55 patients had other non-septic, critical disease in need of ICU care. 52 % of the sepsis patients and 33 % of the non-sepsis patients were diagnosed with AKI stage 2-3 at inclusion. The need for renal replacement therapy (RRT) was 20% and 15%, respectively, in the two groups. In 27/85 sepsis patients and 12/55 non-sepsis patients baseline creatinine was not available at inclusion, hence the MDRD equation was utilized, assuming a GFR of 75 mL/min/1,73m^2. Hepcidin levels at inclusion were significantly higher in the sepsis group compared to non-septic patients as noted already by the study reported in paper 2, but no significant correlation between hepcidin and the development of stage 2-3 AKI was detected in either the septic or the non-septic group. Neither were any significant correlations between hepcidin and peak creatinine levels, nor with the need for RRT detected. HBP significantly correlated with the development of AKI stage 2-3, peak creatinine levels, and the need for RRT in septic patients.

Paper 4

In total 30 patients from the larger cohort were included in this study, 17 with septic shock and 13 with non-septic critical illness. The selected patients were representative for the whole cohort according to all parameters recorded so far. The following twenty-two out of 272 Proseek® Multiplex^{96x96} inflammatory mediators turned out significantly elevated in septic patients on day 1; ADGRG1 (Adhesion G protein coupled receptor 1), CALCA (Calcitonin Related Polypeptide Alpha), CCL (Cysteine-Cysteine Motif Chemokine) 3, 4, 19, 20, and 23, CLEC6A (C type Lectin Domain Family 6 Member A, also called Dectin 2), CXCL (Cysteine X Cysteine Motif Chemokine) 10 and 11, IL (Interleukin) 6, 8, 17A, and 18R1, LAP-TGF-β1 (Latency-Associated Peptide-Transforming Growth Factor-beta 1), LILRB4 (Leukocyte Ig like Receptor Subfamily B Member 4), MCP-3 (Monocyte Chemotactic Protein 3), MILR1 (Mast Cell Immunoglobulin like Receptor 1), NOS3 (Nitric Oxide Synthase 3), OPG (Osteoprotegerin), OSM (Oncostatin M) and TNFSF14 (Tumor Necrosis Factor Ligand Superfamily Member 14). By day three NOS3 and LILRB4 were still significantly elevated, as well as HCLS1 (Hematopoietic Cell Specific Lyn Substrate 1), SIT1 (Signaling Threshold Regulating Transmembrane Adapter 1), and PIK3AP1 (Phosphoinositide 3 Kinase Adapter Protein 1). Similarly, the NPX levels of CALCA, IL18R, and TNFSF14 did not decrease between days 1 and 3, although significant differences were not found compared with non-septic patient values, since these had increased by this time. HCLS1 and SIT1 remained significantly elevated in septic patients from day 3 to 7. CXCL11 dynamics differed from other chemokines, as the levels were elevated in septic patients on day 1, decreased at day 3 to the same levels as seen in the non-septic patients, followed by a significant increase on day 7. Between days 3 and 7, NOS3 increased in non-septic patients, and simultaneously decreased in septic patients, converging at comparable levels by day 7. LILRB4, PIK3AP1, and HCLS1 attained comparable levels in both groups by day 7.

Thesis discussion

Paper 1

Hepcidin acts as an acute phase reactant and correlates with conventionally used biomarkers such as PCT and CRP in septic shock patients. Hepcidin reached peak values at inclusion and declined rapidly in response to adequate antibiotic treatment suggesting that hepcidin could be valuable for evaluation of treatment success. Complications are of great concern in the ICU setting and are difficult to detect. We observed a trend of a secondary hepcidin elevation in patients with complications, thus implicating that repetitive hepcidin measurements could reveal complications earlier than the other investigated biomarkers in this cohort, which, if at all, increased about 24 hours later than hepcidin. At admission Re-He was in the lower range and continued to decrease in line with previous reports in patients with pneumonia and sepsis (228, 229). In this limited number of patients Ret-He values significantly predicted the clinical outcome measured by SOFA score, in contrast to hepcidin.

Limitations

Per definition this was a pilot study with only 15 subjects and the results must be assessed with high precaution. A larger sample size and validation cohort would have added credibility. Obvious limitations due to the sample size is that the complication subgroup is very small and therefore of limited value, although triggering an interest for further study. Additional parameters describing the iron metabolism are missing, *e.g.,* iron, transferrin, and ferritin that would have added value to the report.

Paper 2

The significantly larger patient cohort in paper 2 made it possible to evaluate the results statistically. Hepcidin levels discriminated community acquired septic shock from other critical illness at admission in patients in need of care in the ICU. All admitted critically ill patients were subject to identical sampling and to evaluation at the end of the study. As for the prediction of septic shock, hepcidin showed higher sensitivity, specificity, positive and negative predictive values compared to HBP, although inferior to CRP and PCT. Combination tests with CRP or PCT, respectively, improved sensitivity and specificity both for hepcidin and HBP,

respectively. In line with previous experimental reports that hepcidin induces hypoferremia and acts as a defence mechanism against bacterial infection, we here report a potential protective effect of hepcidin in a clinical setting, where elevated hepcidin at admission correlated positively with survival at 180 days follow up. High hepcidin levels were significantly associated with lower SAPS3 score (123, 230, 231).

Hepcidin possesses both a direct antimicrobial effect but also an indirect antimicrobial effect by reducing iron availability for siderophile microorganisms when an infection occurs. (130, 140, 232). A prolonged overexpression of hepcidin can be harmful for its host due to increased risk for adverse effects such as cognitive and cardiovascular dysfunction, but also an increased risk of secondary, nosocomial infection (233, 234). In our study we observed the highest levels of hepcidin at inclusion followed by a significant daily decline. 98/100 of the included septic patients received adequate empiric antibiotic treatment at inclusion, suggesting that the daily decline observed in hepcidin, can implicate adequate therapeutic response to administered empiric treatment. In addition, repeated measurements of hepcidin could be of value in view of therapeutic targets to upregulate hepcidin in order to minimize harmful iron availability, as well as to downregulate hepcidin if a harmful prolonged iron deficiency persists to minimize later complications. A prolonged iron deficiency is reported to be associated with poorer outcome in ICU-patients, *e.g.* increased one-year mortality, higher risk of cognitive and cardiovascular impairment, as well as higher risk of developing nosocomial infections (234-237).

Limitations

There are several limitations in paper 2, *e.g.* the cohort is observational, single-centre and relatively small. Every eligible patient admitted to the ICU <24 hours was not included due to reasons such as high workload, moving of patients due to lack of available beds, and other prioritizations by the staff, often unknown to the research team, in the ICU. Patients were included and treated by the regular staff at the ICU, thus no extra personnel resources were available. On the other hand, the research team was not involved by any means in care or treatment of the patients, thus the risk for bias was limited. Power calculation was not executed upfront to estimate the statistical strength. A training and validation set of patients would have given the results more credibility. There are over time some missing values of variables that can have influenced the statistical analysis. There is a discrepancy of comorbidities with an overrepresentation of cardiovascular disease and CKD in the sepsis group. Previous reports have shown that hepcidin values are elevated in CKD patients due to inflammation and decreased renal clearance that may have influenced the hepcidin levels already at inclusion (238), although this was not the case when later analysed in paper three.

Paper 3

High serum iron levels are associated with increased short- and long-term mortality in ICU patients with AKI (239). Furthermore, clinical observations report an association between high urinary levels of hepcidin and reduced risk of AKI in patients undergoing cardiac surgery, suggesting a protective effect of elevated hepcidin (240, 241). Contrary to these reports, we could not support the hypothesis that increased serum hepcidin protects patients from developing AKI, need for RRT therapy, or survival neither in the septic nor in the non-septic group. This is in line with a report by Leaf *et al.* where 807 critically ill patients with AKI were studied and lower concentrations of hepcidin was significantly associated with mortality (242). In line with previous reports, we could confirm that heparin binding protein correlated with AKI, the need for RRT, as well as with peak creatinine in septic patients (225-227).

Limitations

Similar limitations as listed under paper 2 are also relevant for paper 3, *i.e.,* the cohort is observational and single centre. As per statistical analysis the cohort was small, hence there is a risk of a type 2 error. Furthermore, it would have given more credibility if we had measured collected urine output from the start of ICU admission to have a more accurate result as we cannot solely rely on the creatinine measurements.

Paper 4

In this pilot study, we investigated cytokines, chemokines, and other soluble inflammatory mediators, all together 272 and compared them with conventional biomarkers, HBP, and hepcidin in septic shock and non-septic critically ill patients. 25/272 of the inflammatory mediators turned out to be significantly elevated in septic patients compared to non-septic patients. Our findings regarding inflammatory mediators concurred with the results of previous studies showing that IL-6, IL-8, and IL-18 were significantly elevated in sepsis patients (243-246). Unexpectedly, a relatively low number (25/272) of the Proseek® Multiplex^{96x96} inflammatory mediators were significantly increased in the septic patients. This finding shows that despite the severity of septic shock, the early phase of the innate immune response is not characterized by a broad upregulation of inflammatory mediators. Of the significant elevated Proseek® Multiplex^{96x96} inflammatory mediators on day 1, 17 of these rapidly had declined and normalized at day 3 most likely due to adequate antibiotic treatment. PIK3AP1, LILRB4, SIT1, NOS3, HCLS1 and IL18-R1 remained elevated on day 3, that speculatively can reflect a two-phase immune response, possibly involving ongoing endothelial damage and neutrophil activation and NETosis. Hepcidin co-varied with *e.g.,* IL-6 and oncostatin M (OSM), where the latter two are reported to stimulate hepcidin,

underlining that hepcidin is part of the early inflammatory response (143, 247). Monitoring Proseek® Multiplex^{96x96} inflammatory mediators in combination with conventional biomarkers and possibly other variables, *e.g*., age, sex, comorbidities *etc.* might be the path forward in future targeted personalized medicine.

Limitations

This study was on an exploratory level with few included patients and our conclusions must be taken with precaution. Measurements with Proseek® Multiplex^{96x96} yielded expression levels and not absolute concentrations of each biomarker, which made it difficult to compare correlations between biomarkers and inflammatory mediators.

Concluding discussion

In summary, while the search for an optimal biomarker for sepsis and septic shock remains challenging, this thesis has provided valuable insights into the complexities surrounding biomarker identification in critical care settings. The ideal biomarker is a surrogate marker of a biological state or process and should be an indicator of a normal, objective, biological process. Other important attributes of optimal biomarkers are that they should be reliable, easily measured, biologically available, non-invasive, produce rapid results, measurable with little or no variability, demonstrate high sensitivity and specificity, vary rapidly in response to treatment, possess predictive and prognostic value of outcome and they should be inexpensive (248, 249).

As expected, none of the biomarkers investigated in this thesis have all these attributes for discriminating sepsis/septic shock from other critical illnesses in the ICU. Obvious reasons are the diverse appearance of critical illness in the ICU, both sepsis and other critical illnesses, the differing host immune status and response to the disease, and the influence of sex, gender, age, genetics, comorbidities, medications, virulence factors *etc*.

In community acquired conditions the prehospital duration of illness is usually not known and may differ by days, thus having impact on the first sampling results.

An important issue when performing studies on biomarkers in an ICU setting like this is the difference in time to the first sampling even after admittance to the ICU, heterogenicity of patients, and interindividual response to the dysregulated immune response to infection. Due to several factors such as *e.g.* age, patient delay to seek health care, immune status, and virulence factors our patients are at different stages of their infection.

In view of this no inflammatory mediator will ever be the fittest, generalizable for all individuals. Combination tests with two biomarkers in paper 2 gave a better ROC curve, suggesting that combination strategy of biomarkers can be beneficial.

Personalized medicine with help from artificial intelligence are upcoming diagnostic tools, where many different variables might be of value/interest, *i.e.* conventional biomarkers, inflammatory mediators, but also variables such as clinical evaluation tools, age, sex, ethnicity, comorbidities, kidney function, immune suppression, epidemiological status, microbial aetiology, surgery, liver failure, osteosynthesis material, diet, medication *etc*. Important to have in mind for future methods for diagnosis with biomarkers in combination with artificial intelligence to predict disease and outcome would still be to achieve the fulfilment of the following criteria of "the perfect" biomarker; reliability, easily measured, biologically available, non-invasive, produce rapid results, measurable with little or no variability, demonstrate high sensitivity and specificity, vary rapidly in response to treatment, possess predictive and prognostic value of outcome and importantly they should also be inexpensive.

Measurement of hepcidin with LC-MS/MS methodology is presently still somewhat restricted to rather few and specialised laboratories making the use of hepcidin in clinical practise limited. The advantage of using the LC-MS/MS methodology is the measurement of the bioactive form of hepcidin, whereas most immunoassay methods (ELISA) measure all hepcidin isoforms (137). Oppen *et al*. highlighted the need of a standardized method with validations and external quality assessment programs before implementation of hepcidin measurements into clinical practise (186). Given that future medicine heads towards more personalized treatment and that sophisticated analyses will be more widely available it is possible that hepcidin measurements in sepsis diagnosis will become more common (191).

As with other biomarkers triggered by IL-6 *e.g.,* CRP, hepcidin has the same disadvantage of not being specific to infections and non-selective for sepsis/septic shock. Hepcidin levels are also elevated in many different cancer forms, *e.g.,* multiple myeloma, lymphoma, small cell lung cancer, kidney cancer, brain cancer and also in inflammatory conditions such as inflammatory bowel disease, is elevated in CKD patients but also under healthy conditions such as pregnancy (250-253).

In this thesis we have investigated a community acquired well-defined group of critically ill patients in the ICU. We have explored the included biomarkers in a routine clinical setting, eager to find out and compare the respective biomarker´s predictive and prognostic values of outcome and dynamics to a given, standardized therapy. Sepsis treatment has not really changed that much over time, still primarily focused on antibiotic therapy, fluid resuscitation, supportive care (vasopressors, ventilation and renal replacement therapy) securing oxygenation, when necessary in the ICU, and focuses of finding the source of infection (107-109). We do not really have any optimal optional treatment, except for maybe corticosteroids and intravenous immunoglobulin (IVIG), the latter in treatment of invasive group A streptococcal disease, when it comes to regulating the dysregulated immune response of sepsis and septic shock (107, 254).

As sepsis management continues to evolve, our understanding of the immunological landscape remains crucial. The rationale on focusing on hepcidin is due to hepcidin´s key regulation of iron metabolism, leading to iron deficiency in inflammation. Hepcidin reduces harmful catalytic free iron and reduces vital iron availability for microorganisms to thrive, thus making hepcidin evolutionary protective to its host (233). We have further investigated HBP and an upstream of inflammatory mediators, among them cytokines and chemokines that in a complex immune modulatory state leads to the dysregulated immune response that sepsis and septic shock represents. Promising experimental results and observational trials shed light on *i.e.,* the protective features of hepcidin. As of now there are ongoing clinical trials with administration of exogenous hepcidin, but also experimental models on hepcidin antagonists (177, 179-181, 183-185).

Ultimately, this thesis emphasizes the importance of a nuanced understanding of biomarkers in critical illness and the need for ongoing research to enhance diagnostic and therapeutic strategies in sepsis and septic shock.

Future research/Clinical implication

There is still a knowledge gap of biomarker´s potential role in discriminating critically ill patients from sepsis patients, where in the best of worlds, larger multi-centre studies should be performed with optimal statistical pre-calculations and statistical power, including a healthy validation group, without any risk of selection bias, where all eligible patients should be included. Multi-centre studies should include microbial multi-resistant environments where the risk potentially might be higher of empiric antibiotic treatment failure already at inclusion. From a clinician´s perspective, preferably, investigations should not only focus on biomarker´s role as a predictive or prognostic instrument, but also focus on the dynamic behaviour over time, potentially giving us a biomarker that responds quickly, giving us a hint of being on the right track when it comes to *i.e.* antibiotic treatment.

We are probably facing a future of more personalized medicine, with more advanced at times expensive target directed therapies. There are several challenges though in our part of the world where we are facing an elderly population with shrinking labour force, that may strain future financial resources. Maybe with help from artificial intelligence we can explore and develop new ways to predict and prognosticate critical illness where preferably rather few inexpensive biomarkers can be included in the algorithms.

Fever is part of our evolutionary protection against microorganisms, where increased temperature in the host makes it more difficult for bacteria to thrive, resulting in a better immune response (255). Hepcidin seem to have somewhat a similar evolutionary protective attribute, reducing harmful iron promptly, including reducing available iron as a nutrient for microorganisms to thrive and proliferate, when inflammation including sepsis occurs (127). This pinpoints that hepcidin should be further exploited as a potentially important biomarker and should be investigated as being a therapeutic target in a wide range of inflammatory disorders including sepsis.

Research on hepcidin in an emergency room setting collecting samples from all available subjects would give further value, understanding hepcidin´s behaviour in all patients' groups with an extra interest in inflammatory disorders.

Future research to develop better ELISA methods and more available test kits (bedside) for hepcidin, detecting the bioactive form hepcidin-25 is warranted, including reduction of analysis costs. It is also important to further clear out if LC-MS/MS and ELISA tests are equivalent in giving reliable results.

Populärvetenskaplig sammanfattning på svenska

Sepsis orsakar cirka 20 procent av samtliga dödsfall globalt. Förutom att drabbas av ett svårt akut kliniskt tillstånd lider många överlevande av negativa efterverkningar av sepsis. För klinikern är diagnostik och behandling utmanande och för samhället är kostnaden hög ur både hälsoekonomiskt och resursperspektiv.

Sepsis är volatilt, och utgör hos vissa patienter ett katastrofalt sjukdomstillstånd vilket uppstår när kroppens immunförsvar överreagerar vid en infektion. Symptombilden varierar beroende på faktorer såsom ålder, komorbiditet, patientens immunstatus och mikrobiologisk etiologi, vilket medför att identifieringen av sepsis i tidigt skede kan vara svår. Nuvarande klinisk identifiering av sepsispatienter bygger på kliniska screeningsverktyg som mäter vitalparametrar som exempelvis blodtryck, andningsfrekvens, och syremättnad, men rapporter föreligger om normala vitalparametrar initialt hos en tredjedel av de drabbade patienterna. Här kan biomarkörer utgöra ett viktigt screeningsupplement för identifiering av sepsis, prognostisering men även som ett utvärderingsinstrument för bekräftelse på att insatt empirisk behandling leder till förbättring med b la. normalisering av olika biomarkörer i blodet. Behandlande läkare får likaså möjlighet att ändra ickefungerande behandling liksom att avsluta antibiotikabehandlingen så snabbt som möjligt för att undvika onödig belastning, dels på patienten men även i ett större perspektiv på miljön för att minska risken för antibiotikaresistens.

Syftet med avhandlingen var att undersöka diagnostiska biomarkörer hos patienter som vårdades på intensivvårdsavdelningen, IVA, i Helsingborg. Fokus lades på att undersöka biomarkörer som diskriminerade sepsispatienter från andra kritiskt intensivvårdskrävande patienter i tidigt skede, undersöka biomarkörernas dynamik över första vårdveckan, samt och om markörerna kunde vara prognostiserande för tillfrisknande likväl som för mortalitet.

Helsingborg var det första sjukhuset i Norden att rutinmässigt analysera anemi-biomarkören hepcidin. Hepcidin är ett protein som är huvudregulator för kroppens järnmetabolism. Experimentella försök har visat att hepcidin prompt påverkas och snabbt ökar vid frisättning av interleukin-6 vilket leder sker vid inflammatoriska tillstånd. Det innebär att hepcidin är ett så kallat akutfasprotein vid inflammatoriska tillstånd, såsom sepsis. Ur ett evolutionärt perspektiv är hepcidins snabba reglering av järnmetabolismen logisk, eftersom tillgängligt järn i blodet vid inflammation snabbt minskar. Järn är en viktig tillväxtfaktor inte endast för människan utan även t.ex. för bakteriers, svampars och parasiters tillväxt. Oxidativt järn i blodet frisätts vid inflammation vilket bland annat skadar kroppens endotel och DNA.

<u>Arbete 1</u>

Det första arbetet utgjorde ett pilotprojekt där vi undersökte och jämförde konventionella biomarkörer samt det kliniska evalueringsverktyget SOFA score

med hepcidin och en annan järn biomarkör, Reticulocyt Hemoglobin (Ret-He), under en period av sju dygn, hos intensivvårdskrävande patienter med septisk chock vilka kommit till sjukhuset senast 24 timmar innan ankomsten till IVA. Hepcidin hade nått sitt högsta värde redan vid ankomsten till IVA, för att därefter sjunka korrelerat till klinisk förbättring med adekvat behandling. Hepcidin korrelerade signifikant med de vanligast använda infektionsbiomarkörerna C-reaktivt protein (CRP) och procalcitonin (PCT). Ret-He sjönk de första 72 timmarna, därefter steg Ret He nivåerna och var normaliserade efter 144 timmar. Normaliserade Ret-He värden korrelerade med förbättrat klinisk status, i detta fall mätt med SOFA score.

Arbete 2

De positiva resultaten från vår pilotstudie ledde till en större studie där vi prospektivt inkluderande 164 patienter (100 med sepsis och 64 icke septiskt svårt sjuka) inlagda på intensivvårdsavdelningen i Helsingborg inom 24 timmar efter ankomst till sjukhus. Vi jämförde även här hepcidin med vanliga biomarkörer såsom C-reaktivt protein (CRP), procalcitonin (PCT), laktat, vita blodkroppar liksom med en relativt nyligen rapporterad sepsisbiomarkör, heparin bindande protein, HBP, samt kliniska evaluerings verktyg, under sju dygn i följd. Vi undersökte vidare om det förelåg någon korrelation mellan biomarkörer och död efter 28 respektive 180 dagar.

Hepcidinkoncentrationen visade sig vara signifikant högre i de septiska jämfört med de icke-septiska patienterna vid ankomsten och sjönk successivt och signifikant efter 24 och vidare vid 48 och 72 timmar då det nådde närmast normalvärden. 98 % av de septiska patienterna fick adekvat empirisk antibiotikabehandling det första dygnet vilket implicerade att hepcidins dynamik under dessa dygn reflekterade adekvat behandlingseffekt. Vid analys av sensitivitet och specificitet vid diagnostik av sepsis var CRP och PCT bättre än hepcidin. Kombinationstest av hepcidin med CRP alternativt PCT gav dock bättre sensitivitets och specificitetsutfall i sepsis gruppen. En statistisk signifikant negativ korrelation mellan hepcidin och det kliniska evaluerings-verktyget SAPS3 förelåg likaså. Höga värden av hepcidin vid ankomst var associerat med 180-dagars överlevnad i hela studiepopulationen liksom i sepsisgruppen.

Vår studie kunde således visa att hepcidin kan diskriminera septiska jämfört med icke septiska patienter vid ankomsten till IVA och att hepcidin prompt sjunker vid adekvat insatt behandling. Ett högre hepcidin värde vid ankomst tyder på skyddande effekt vid inflammation bl a. genom att minska fritt järn i blodet.

Arbete 3

Sepsis är den vanligaste orsaken till akut njursvikt bland kritisk sjuka patienter som vårdas på intensivvårdsavdelningar. Akut njursvikt indelas i tre allvarlighetsgrader, stadium 1-3. Klassificeringen grundas främst på patientens urinproduktion och kreatininvärdet. I arbete 3 använde vi oss av samma kohort som i arbete 2 där vi undersökte sambandet mellan respektive hepcidin och HBP vid ankomst till

intensivvårdsavdelningen och utvecklandet av akut njursvikt, dialys samt med högst uppmätta kreatininvärde. Resultaten av analyserna visade att det förelåg en signifikant korrelation mellan HBP och njursviktstadium 2-3 bland såväl sepsis- som icke-sepsis patienterna. I sepsis gruppen sågs även ett signifikant samband mellan HBP och behovet av dialys liksom med det högsta kreatininvärdet. Motsvarande signifikanta samband mellan hepcidin och njursviktsgrad 2-3, dialys eller högsta kreatininvärde förelåg inte i denna studie.

Arbete 4

I samarbete med SciLife laboratoriet vid Biomedicinskt Centrum, Uppsala universitet, genomförde vi en mindre studie med proteomteknik, Proseek®Multiplex^{96x96} Immunoassay från Olink, där vi analyserade 276 biomarkörer för inflammation, immunförsvar och organskada i 17 septiska och 13 icke-septiska vuxna IVA patienter från de patienter som inkluderats i den större studien, vilka var representativa för denna patientkohort. I blodprover tagna vid ankomst till IVA dag 1, 3 och 7 jämfördes ingående markörer i Proseek®Multiplex^{96x96} med konventionella biomarkörer såsom CRP och PCT men liksom med hepcidin och HBP. Av 272 proteiner i Proseek® Multiplex^{96x96} Immunoassay visade det sig att tjugofem av dessa var signifikant högre hos sepsis patienterna jämfört med den icke septiska gruppen. Ett flertal av dessa tjugofem biomarkörer är tidigare rapporterat signifikant förhöjda i sepsis patienter såsom interleukin (IL)-6 och IL-8. I vår studie fann vi att IL-17A och latency-associated peptide TGF-β1 var signifikant högre i vår sepsisgrupp jämfört med icke septiska patienter. Vissa cytokiner i Proseek®Multiplex^{96x96} sjönk dag 3 vilket kan implicera att korrekt behandling var given, då en samtidig klinisk förbättring sågs i sepsispatientgruppen. Oncostatin (OSM) och IL-6 samvarierade med hepcidin bland sepsis patienterna. OSM respektive IL-6 aktiverar hepcidin vilket understryker att hepcidin aktiveras tidigt vid sepsis och inflammation.

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Errata

1. In paper 1 we incorrectly have written plasma levels of hepcidin instead of serum hepcidin levels. It is serum hepcidin levels that have been measured. Strong correlations have been reported between serum and plasma hepcidin concentration, but there is also reported discrepancy with higher measured levels in plasma compared to serum (186, 196, 256, 257).

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Supplementary material:

Proseek®Multiplex^{96x96} Immunoassay Olink

Inflammation panel:

Adenosine Deaminase (ADA)	Fibroblast growth fact 5 (FGF-5)
Artemin (ARTN)	Fms-related tyrosine kinase 3 ligand (Flt3L)
Axin-1 (AXIN1)	Fractalkine (CX3CL1)
Beta-nerve growth factor (Beta-NGF)	Glial cell line-derived neurotrophic factor (GDNF)
Brain-derived neurotrophic factor (BDNF)	Hepatocyte growth factor (HGF)
Caspase 8 (CASP-8)	Interferon gamma (IFN-gamma)
C-C motif chemokine 19 (CCL19)	Interleukin-1 alpha (IL-1 alpha)
C-C motif chemokine 20 (CCL20)	Interleukin-10 (IL-10)
C-C motif chemokine 23 (CCL23)	Interleukin-10 receptor subunit alpha (IL-10RA)
C-C motif chemokine 25 (CCL25)	Interleukin-10 receptor subunit beta (IL-10RB)
C-C motif chemokine 28 (CCL28)	Interleukin-12 subunit beta (IL-12B)
C-C motif chemokine 3 (CCL3)	Interleukin-13 (IL-13)
C-C motif chemokine 4 (CCL4)	Interleukin-15 receptor subunit alpha (IL-15RA)
CD40L receptor (CD40)	Interleukin-17A (IL-17A)
CUB domain-containing protein 1 (CDCP1)	Interleukin-17C (IL-17C)
C-X-C motif chemokine 1 (CXCL1)	Interleukin-18 (IL-18)
C-X-C motif chemokine 10 (CXCL10)	Interleukin-18 receptor 1 (IL-18R1)
C-X-C motif chemokine 11 (CXCL11)	Interleukin-2 (IL-2)
C-X-C motif chemokine 5 (CXCL5)	Interleukin-2 receptor subunit beta (IL-2RB)
C-X-C motif chemokine 6 (CXCL6)	Interleukin-20 (IL-20)
C-X-C motif chemokine 9 (CXCL9)	Interleukin-20 receptor subunit alpha (IL-20RA)
Cystatin D (CST5)	Interleukin-22 receptor subunit alpha-1 (IL-22 RA1)

Delta and Notch-like epidermal growth factor-related receptor (DNER)	Interleukin-24 (IL-24)
Eotaxin-1 (CCL11)	Interleukin-33 (IL-33)
Eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1)	Interleukin-4 (IL-4)
Fibroblast growth factor 19 (FGF-19)	Interleukin-5 (IL-5)
Fibroblast growth factor 21 (FGF-21)	Interleukin-6 (IL-6)
Fibroblast growth factor 23 (FGF-23)	Interleukin-7 (IL-7)
Interleukin-8 (IL-8)	Signaling lymphocytic activation molecule (SLAMF1)
Latency-associated peptide transforming growth factor beta 1 (LAP TGF-beta-1)	SIR2-like protein 2 (SIRT2)
Leukemia inhibitory factor (LIF)	STAM-binding protein (STAMBP)
Leukemia inhibitory factor receptor (LIF-R)	Stem cell factor (SCF)
Macrophage colony-stimulating factor 1 (CSF-1)	Sulfotransferase 1A1 (ST1A1)
Matrix metalloproteinase-1 (MMP-1)	T cell surface glycoprotein CD6 isoform (CD6)
Matrix metalloproteinase-10 (MMP-10)	T-cell surface glycoprotein CD5 (CD5)
Monocyte chemotactic protein 1 (MCP-1)	Thymic stromal lymphopoietin (TSLP)
Monocyte chemotactic protein 2 (MCP-2)	TNF-beta (TNFB)
Monocyte chemotactic protein 3 (MCP-3)	TNF-related activation-induced cytokine (TRANCE)
Monocyte chemotactic protein 4 (MCP-4)	TNF-related apoptosis-inducing ligand (TRAIL)
Natural killer cell receptor 2B4 (CD244)	Transforming growth factor alpha (TGF-alpha)
Neurotrophin-3 (NT-3)	Tumor necrosis factor (Ligand) superfamily, member 12 (TWEAK)
Neurturin (NRTN)	Tumor necrosis factor (TNF)
Oncostatin-M (OSM)	Tumor necrosis factor ligand superfamily member 14 (TNFSF14)
Osteoprotegerin (OPG)	Tumor necrosis factor receptor superfamily member 9 (TNFRSF9)
Programmed cell death 1 ligand 1 (PD-L1)	Urokinase-type plasminogen activator (uPA)
Protein S100-A12 (EN-RAGE)	Vascular endothelial growth factor A (VEGF-A)

Immune response panel:

Allergin-1 (ADA)	Histamine N-methyltransferase (HNMT)
Amphiregulin (AR) (AREG)	Importin subunit alpha-5 (KPNA1)
Aryl hydrocarbon receptor nuclear translocator (ARNT)	Inactive dipeptidyl peptidase 10 (DPP10)
Baculoviral IAP repeat-containing protein 2 (BIRC2)	Integral membrane protein 2A (ITM2A)
Beta-galactosidase (GLB1)	Integrin alpha-6 (ITGA6)
Butyrophilin subfamily 3 member A2 (BTN3A2)	Integrin alpha-11 (ITGA11)
CD83 antigen (CD83)	Integrin beta-6 (ITGB6)
Contactin-associated protein-like 2 (CNTNAP2)	Interferon lambda receptor 1 (IFNLR1)
Corneodesmosin (CDSN)	Interferon regulatory factor 9 (IRF9)
Corticosteroid 11-beta-dehydrogenase isozyme 1 (HSD11B1)	Interleukin-1 receptor-associated kinase 1 (IRAK1)
Coxsackievirus and adenovirus receptor (CXADR)	Interleukin-1 receptor-associated kinase 4 (IRAK4)
C-type lectin domain family 4 member A (CLEC4A)	Interleukin-5 (IL5)
C-type lectin domain family 4 member C (CLEC4C)	Interleukin-6 (IL6)
C-type lectin domain family 4 member D (CLEC4D)	Interleukin-10 (IL10)
C-type lectin domain family 4 member G (CLEC4G)	Interleukin-12 receptor subunit beta-1 (IL12RB1)
C-type lectin domain family 6 member A (CLEC6A)	Islet cell autoantigen 1 (ICA1)
C-type lectin domain family 7 member A (CLEC7A)	Keratin, type I cytoskeletal 19 (KRT19)
Cytoskeleton-associated protein 4 (CKAP4)	Leukocyte immunoglobulin-like receptor subfamily B member 4 (LILRB4)
Diacylglycerol kinase zeta (DGKZ)	Lymphocyte activation gene 3 protein (LAG3)
Discoidin, CUB and LCCL domain-containing protein 2 (DCBLD2)	Lymphocyte antigen 75 (LY75)
DNA fragmentation factor subunit alpha (DFFA)	Lysosome-associated membrane glycoprotein 3 (LAMP3)
Dual adapter for phosphotyrosine and 3-phosphotyrosine and 3-phosphoinositide (DAPP1)	Mannan-binding lectin serine protease 1 (MASP1)
Dynactin subunit 1 (DCTN1)	Merlin (NF2)
E3 ubiquitin-protein ligase TRIM21 (TRIM21)	Methylated-DNA--protein-cysteine methyltransferase (MGMT)
Egl nine homolog 1 (EGLN1)	Natural cytotoxicity triggering receptor 1 (NCR1)
Eotaxin (CCL11)	Natural killer cells antigen CD94 (KLRD1)
Eukaryotic translation initiation factor 4 gamma 1 (EIF4G1)	Neurabin-2 (PPP1R9B)
Eukaryotic translation initiation factor 5A-1 (EIF5A)	Neurotrophin-4 (NTF4)
Fc receptor-like protein 3 (FCRL3)	Nuclear factor of activated T-cells, cytoplasmic 3 (NFATC3)

Fc receptor-like protein 6 (FCRL6)	Parathyroid hormone/parathyroid hormone-related peptide receptor (PTH1R)
Fibroblast growth factor 2 (FGF2)	PC4 and SFRS1-interacting protein (PSIP1)
FXYD domain-containing ion transport regulator 5 (FXYD5)	Peroxiredoxin-1 (PRDX1)
Hematopoietic lineage cell-specific protein (HCLS1)	Peroxiredoxin-5, mitochondrial (PRDX5)
Phosphoinositide 3-kinase adapter protein 1 (PIK3AP1)	Stanniocalcin-1 (STC1)
Plexin-A4 (PLXNA4)	Stromal cell-derived factor 1 (CXCL12)
Polypeptide N-acetylgalactosaminyltransferase 3 (GALNT3)	T-cell-specific surface glycoprotein CD28 (CD28)
Probable ATP-dependent RNA helicase DDX58 (DDX58)	Thioredoxin-dependent peroxide reductase, mitochondrial (PRDX3)
Protein FAM3B (FAM3B)	TNF receptor-associated factor 2 (TRAF2)
Protein HEXIM1 (HEXIM1)	TRAF family member-associated NF-kappa-B activator (TANK)
Protein kinase C theta type (PRKCQ)	Transcription factor AP-1 (JUN)
Protein sprouty homolog 2 (SPRY2)	Transcription regulator protein BACH1 (BACH1)
Protein-arginine deiminase type-2 (PADI2)	Triggering receptor expressed on myeloid cells 1 (TREM1)
SH2 domain-containing protein 1A (SH2D1A)	Tripartite motif-containing protein 5 (TRIM5)
SH2B adapter protein 3 (SH2B3)	Tryptase alpha/beta-1 (TPSAB1)
Signaling threshold-regulating transmembrane adapter 1 (SIT1)	Tumor necrosis factor receptor superfamily member EDAR (EDAR)
SRSF protein kinase 2 (SRPK2)	Zinc finger and BTB domain-containing protein 16 (ZBTB16)

Organ damage panel:

Artemin (ARTN)	Interleukin-20 (IL-20)
Axin 1 (AXIN1)	Interleukin-24 (IL-24)
Brain-derived neurotrophic factor (BDNF)	Interleukin-33 (IL-33)
Beta-nerve growth factor (bNGF)	Latency-associated peptide transforming growth factor beta 1 (LAP TGF-beta-1)
Caspase 8 (CASP8)	Leukemia inhibitory factor (LIF)
C-C motif chemokine 2 (CCL2)	Matrix metalloproteinase-1 (MMP1)
C-C motif chemokine 3 (CCL3)	Matrix metalloproteinase-10 (MMP10)
C-C motif chemokine 4 (CCL4)	Neurturin (NRTN)
C-C motif chemokine 7 (CCL7)	Neurotrophin-3 (NT3)
C-C motif chemokine 8 (CCL8)	Oncostatin-M (OSM)
C-C motif chemokine 11 (CCL11)	Adenosine deaminase, (ADA)
C-C motif chemokine 13 (CCL13)	Cluster of differentiation 5, (CD5)
C-C motif chemokine 19 (CCL19)	Cluster of differentiation 6, (CD6)
C-C motif chemokine 20 (CCL20)	Cluster of differentiation 40, tumor necrosis factor receptor superfamily member 5, (CD40)
C-C motif chemokine 23 (CCL23)	Natural killer cell receptor 2B4, (CD244)
C-C motif chemokine 25 (CCL25)	CUB domain-containing protein 1, (CDCP1)
C-C motif chemokine 28 (CCL28)	Delta and Notch-like eepidermal growth factor-related receptor, soluble (sDNER)
Macrophage colony-stimulating factor 1 (CSF1)	Fms-realted tyrosine kinase 3 ligand, (Flt3L)
Cystadin D (CST5)	Hepatocyte growth factor (HGF)
C-X-C motif chemokine 1 (CXCL1)	Interleukin-2 receptor subunit beta (IL2RB)
C-X-C motif chemokine 5 (CXCL5)	Interleukin-10 receptor subunit alpha (IL10RA)
C-X-C motif chemokine 6 (CXCL6)	Interleukin-10 receptor subunit beta (IL10RB)
C-X-C motif chemokine 9 (CXCL9)	Interleukin-15 receptor subunit alpha (IL15RA)
C-X-C motif chemokine 10 (CXCL10)	Interleukin-18 receptor 1 (IL18R1)
C-X-C motif chemokine 11 (CXCL11)	Interleukin-20 receptor subunit alpha (IL20RA)
Eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1)	Interleukin-22 receptor subunit alpha-1 (IL22RA1)
Extracellular newly identified receptor for advanced glycation endproducts binding protein, protein S100-A12 (EN.RAGE)	Sirtuin 2 (SIRT2)
Fibroblast growth factor 5 (FGF5)	C-X3-C motif ligand 1, fractalkine (CX3CL1)
Fibroblast growth factor 19 (FGF19)	Leukemia inhibitory factor receptor (LIFR)
Fibroblast growth factor 21 (FGF21)	Osteoprotegerin (OPG)

Fibroblast growth factor 23 (FGF23)	Programmed cell death 1 ligand 1 (PDL1)
Glial cell line-derived neurotrophic factor (GDNF)	Stem cell factor (SCF)
Interferon gamma (IFNG)	Signaling lymphocytic activation molecule (SLAMF1)
Interleukin-1 alpha (IL1A)	Sulfotransferase 1A1 (ST1A1)
Interleukin-2 (IL2)	STAM-binding protein (STAMBP)
Interleukin-4 (IL-4)	Transforming growth factor alpha (TGFA)
Interleukin-5 (IL-5)	TNF-beta (TNFB)
Interleukin-6 (IL-6)	Tumor necrosis factor receptor superfamily member 9 (TNFRSF9)
Interleukin-7 (IL-7)	Tumor necrosis factor ligand superfamily member 14 (TNFSF14)
Interleukin-8 (IL-8)	TNF-related apoptosis-inducing ligand (TRAIL)
Interleukin-10 (IL-10)	TNF-related activation-induced cytokine (TRANCE)
Interleukin-12 beta (IL12B)	Tumor necrosis factor ligand superfamily member 12 (TWEAK)
Interleukin-13 (IL-13)	Thymic stromal lymphopoietin (TSLP)
Interleukin-17A (IL17A)	Tumor necrosis factor (TNF)
Interleukin-17C (IL-17C)	Urokinase-type plasminogen activator (uPA)
Interleukin-18 (IL-18)	Vascular endothelial growth factor A (VEGFA)

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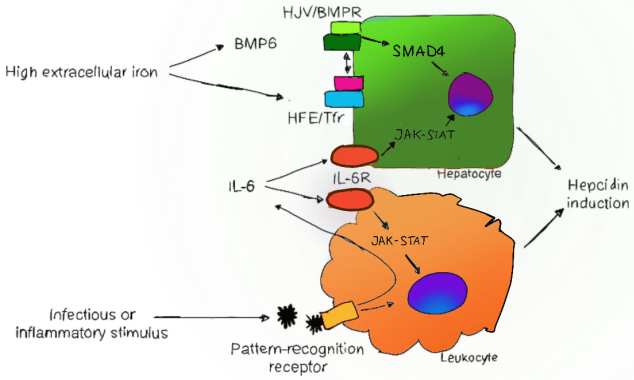

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