Plasma Levels of Hepcidin and Reticulocyte Haemoglobin during Septic Shock

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Keywords

Septic shock · Hepcidin · Reticulocyte haemoglobin · Heparin-binding protein

Abstract

Septic shock, a serious consequence of disseminated infection that has a high mortality, is due to a dysregulated, severe immune response triggered by the infection. Acute phase reactants play key roles in sepsis, for example, hepcidin regulating iron metabolism. Reticulocyte haemoglobin (Ret-He) depends on available iron in blood, indirectly regulated by hepcidin. This study aimed at exploring rapid changes in hepcidin and Ret-He in patients with septic shock receiving adequate antibiotic treatment. Fifteen patients, included within an hour of admission to the intensive care unit, were evaluated by microbiological tests and cultures, Sequential Organ Failure Assessment score, and plasma levels of hepcidin, Ret-He, heparin-binding protein (HBP), leucocytes, C-reactive protein, procalcitonin (PCT), and lactate. Samples were taken every morning for 7 consecutive days. Maximal levels of hepcidin (median 61 nmol/L; reference 1–12 nmol/L) were seen at the time of inclusion, then declining steadily similar to PCT and lactate levels. Ret-He values decreased transiently in response to increased hepcidin,

normalization occurred at 96 h upon decrease of hepcidin levels. Maximal levels of HBP were noted 24 h after inclusion. In conclusion, hepcidin promptly declined within the first 24 h in patients with septic shock receiving adequate antibiotic treatment in contrast to Ret-He and HBP.

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Introduction

Sepsis and septic shock are conditions caused by a dysregulated host response to an infection [1, 2]. The incidence of sepsis is increasing worldwide and hospital mortality rates are reported to be between 24 and >40% despite more efficient intensive care [1, 3, 4]. Early detection of sepsis and adequate treatment is crucial for survival, since every hour's delay of antibiotic administration has been reported to increase the mortality of septic shock by 7.6% after onset of hypotension [5]. The severity of sepsis is difficult to discriminate from other severe conditions of both infectious and non-infectious origin [6]. Clinical scoring systems for severely ill patients try to correlate organ function with severity of disease, as well as predict outcome for the patients [7]. The previously used term

“Severe Inflammatory Response Syndrome,” SIRS, has been abandoned by the scientific community since it includes many non-infectious inflammatory conditions. Sequential Organ Failure Assessment, SOFA, is an internationally used scoring system based on measurements of functions of the respiratory, cardiovascular, liver, coagulation, renal, and neurological systems [8]. Upon introduction by the Working Group on Sepsis-Related Problems of the European Society of Intensive Care Medicine, the SOFA score system is widely accepted [1]. A simplified version including only 2 fulfilled parameters, q-SOFA, has proven insufficient in excluding conditions other than sepsis [9]. Other objective parameters include measurements of different biomarkers as well as microbial identity in blood and urine, which cause the infection [10]. Identification of biomarkers with high specificity and sensitivity, which are helpful to distinguish sepsis from other inflammatory conditions and/or predict outcome for the patients, is highly warranted [11, 12]. Such biomarkers would also be important tools to evaluate the progression of disease and choice of therapy and response to therapy, including evaluation of the effect of administered antibiotics and an increasing clinical treatment challenge due to accelerating antibiotic resistance worldwide [12, 13].

Rapid changes in levels of circulating cytokines, acute phase reactants, and peripheral leucocytes accompany many different acute inflammatory conditions, including sepsis [14]. Similarly, parameters reflecting organ damage, such as increased creatinine due to kidney failure, are not sepsis specific [15]. These well-established inflammatory signatures have been the subject of numerous studies and are monitored in the daily routine care of septic patients [12]. Anaemia is a key marker of long-standing infection [16], and a decline of haemoglobin can be noted even early in severe infections [17, 18]. Iron is a growth-limiting factor for microbes/bacteria and lowered iron levels may protect against infection [19, 20], a notion supported by studies showing elevated morbidity and mortality upon iron supplementation in severely ill, infected patients [18]. Blood transfusion may be needed and used in septic patients in spite of the effects on microbial growth [21].

Hepcidin, a small peptide hormone controlling plasma levels of iron, structurally related to chemokines, is produced in the liver. The synthesis of hepcidin is regulated by iron levels in plasma and by the inflammatory cytokine interleukin-6. The hormone reduces serum levels of iron by blocking the iron transporter ferroportin in enterocytes and thereby blocking the uptake of iron. Sec-

ond, hepcidin blocks ferroportin-dependent export of iron from macrophages participating in erythrocyte turnover. Hepcidin also inhibits the release of stored iron from hepatocytes [22]. Since hepcidin synthesis is upregulated by interleukin-6, it acts as an acute phase reactant [23]. Injection of LPS in humans results in increased hepcidin excretion in urine, maximal concentrations observed at 6 h post-injection, paralleled by a significant decrease in serum iron [24]. Increased hepcidin levels in plasma contribute to the observed reduction of erythropoiesis in community-acquired pneumonia [25]. Elevated serum hepcidin levels have also been reported in children with severe infection, as well as in adults suffering from sepsis [26–28]. It remains, however, unclear whether monitoring hepcidin levels is useful for discriminating between sepsis and other severe conditions that require intensive care treatment. Furthermore, it is unclear whether such monitoring can be useful to evaluate disease progression including therapeutic response and non-infectious complications in sepsis patients. Hepcidin was initially studied in the context of antimicrobial peptides, since hepcidin exerts such effects on many different pathogens including those most commonly associated with septic shock [29].

Reticulocytes are immature erythrocytes present in the circulation. Iron is an essential component of haemoglobin and circulating iron is required within the first 2 days of erythrocyte maturation, otherwise the erythrocytes undergo apoptosis [30]. Picogram levels of haemoglobin in reticulocytes, referred to as reticulocyte haemoglobin (Ret-He) equivalent, below 28–29 pg/L, indicate iron deficiency and impaired maturation of reticulocytes [31, 32]. Ret-He measurements serve as an indicator of iron deficiency secondary to an increase in hepcidin, since hepcidin inhibits the efflux of iron and decreases available iron. Hepcidin indirectly regulates the content of haemoglobin in reticulocytes [25], and low Ret-He values are reported to correlate with increased need of blood transfusion in critically ill patients [21]. The aim of the present study was to investigate the dynamics of hepcidin and Ret-He to serve as biomarkers for septic shock in patients admitted to the Intensive Care Unit at the tertiary hospital of Helsingborg, Sweden.

Materials and Methods

Patient Selection

Fifteen patients, ≥ 18 years of age, with septic shock were included in this single-centre, prospective, observational pilot study. Patients had to be admitted to the intensive care unit (ICU) within

Table 1. Demographic data of patients. All 15 patients suffered from septic shock

Patient	No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Age, years, median 60 (IQR 46–76)		46	29	78	18	77	20	48	51	70	61	70	80	47	79	67
Gender		M	F	F	M	F	M	F	M	F	M	M	M	M	M	M
Focus of infection																
Pyelonephritis	5							1	1				1	1	1	
Pneumonia	4	1		1						1						1
Tonsillitis	2				1		1									
Endometritis	1		1													
Epiglottitis	1										1					
Cholangitis	1											1				
Wound	1					1										
No. of comorbidities ^a		1	0	1	0	2	0	1	5	5	2	2	2	4	2	1
Antibiotics, days		7	>7	3	>7	>7	6	>7	6	7	>7	>7	>7	>7	>7	>7
Positive cultures																
Blood		0	1	1	1	0	0	1	1	0	0	1	0	0	1	1
Other ^b		0	1	1	1	1	0	1	1	1	1	0	0	1	1	0
Sensitive antibiograms		0	1	1	1	1	0	1	1	0	1	1	0	1	1	1
ICU parameters																
Days in ICU		3	7	7	>7	>7	3	4	5	>7	>7	>7	4	7	>7	>7
Intubation days		0	0	0	0	>7	0	0	0	>7	5	7	0	0	0	7
Catecholamine days		3	4	2	3	6	3	4	5	2	4	7	4	6	5	>7
Dialysis days		0	0	0	4	3	0	0	4	0	0	5	0	>7	0	>7
Peak SOFA median 10 (IQR 6–12)		11	10	5	20	16	10	8	14	12	11	19	10	12	15	18
28 days mortality		0	0	0	0	1	0	0	0	0	0	0	0	0	0	0

SOFA, Sequential Organ Failure Assessment; ICU, intensive care unit. ^a Comorbidities including hypertension, diabetes mellitus type II, rheumatoid arthritis, chronic obstructive pulmonary disease, ischemic heart disease, and hypothyroidism. ^b Microbial agent from sputum, throat, cervix, or urine (including urine antigen).

24 h of arrival to the hospital. All patients were diagnosed with septic shock, fulfilling the 2016 updated sepsis and septic shock guidelines [2]. Exclusion criteria included blood transfusion or surgery within 7 days preceding the current hospitalization. Patients were included into the study between May 2014 and December 2014. All subjects received broad-spectrum antibiotics according to local treatment guidelines for septic shock after adequate blood cultures had been obtained.

Definitions

According to the updated sepsis guidelines of 2016, patients have to have a suspected infection and a Sequential Organ Failure Assessment (SOFA) score ≥ 2 to fulfil the criteria of sepsis [1]. SOFA is based on measurement of respiratory, cardiovascular, hepatic, coagulation, renal, and neurological system functions [33]. The definition of septic shock is when a patient has persistent hypotension despite adequate fluid resuscitation and needs vasopressors to maintain a mean arterial pressure) of ≥ 65 mm Hg with a simultaneous serum lactate level > 2 mmol/L [2].

Data and Sample Collection

Blood samples for biomarker analyses were obtained at the time of inclusion and then every morning during 7 consecutive days. Cultures were obtained prior to administration of antibiotics, at inclusion, and repeated appropriately according to the progression of the patients' illness, from blood as well as from wounds,

urine, sputum, and nasopharynx. Antigen tests for group A streptococci as well as urine tests for *Legionella* and pneumococcal antigens were performed when appropriate. In case of suspected viral infection, PCR analyses were performed.

Clinical evaluation was performed by daily SOFA score evaluation. Data on patient demographics, cause of infection, comorbidities, the number of days on broad-spectrum antibiotics, positive cultures, length of stay at the ICU, severity scores, and 28 days mortality were collected (Tables 1, 2).

The study was approved by the Ethics Committee, University of Lund, Sweden, Dnr. 2014/195. Oral and written consent was collected either from the patient or next of kin, where the latter approved consent if the patient was medically unstable and thus not able to confirm upon inclusion; thus, delayed consent from the patient was accepted by the Ethics Committee (Dnr. 2014/195).

Laboratory Methods

Hepcidin was analysed using mass spectrometry [34] with a 6500 QTrap[®] (Sciex, Washington, DC, USA) at the Clinical Chemistry Laboratory at Lund University Hospital, Sweden. Analyses of Ret-He, leucocytes, C-reactive protein (CRP), procalcitonin (PCT), and lactate and all other routine blood chemistry analyses were performed at the Clinical Chemistry Laboratory, Helsingborg Hospital, Sweden. Heparin-binding protein (HBP) analyses were performed with an in-house sandwich ELISA at the Biomedical Centre in Lund, Sweden, as described earlier [35].

Table 2. Microbial findings from positive locations at time of inclusion

Subject	Culture and/or pneumococcal antigen in urine	Organ with positive culture (including pneumococcal antigen in urine)	Bacteria identified
1	Negative		
2	Positive	Blood + cervix	Group A <i>Streptococcus</i>
3	Positive	Blood + urine antigen	<i>Streptococcus pneumoniae</i>
4	Positive	Blood	<i>Fusobacterium necrophorum</i>
5	Positive	Wound, sputum, and nasopharynx	<i>Staphylococcus aureus</i>
6	Negative		
7	Positive	Blood + urine	<i>Klebsiella pneumoniae</i>
8	Positive	Blood + nephrostomy	<i>Escherichia coli</i>
9	Positive	Urine antigen	<i>S. pneumoniae</i>
10	Positive	Trachea	<i>S. pneumoniae</i> , <i>Haemophilus influenzae</i> , <i>Citrobacter freundii</i>
11	Positive	Blood	<i>Citrobacter</i> species, <i>Klebsiella oxytoca</i> , <i>Proteus mirabilis</i> , <i>Enterococcus faecalis</i>
12	Negative		
13	Positive	Urine	<i>S. aureus</i>
14	Positive	Blood + urine antigen	<i>E. coli</i> , <i>S. pneumoniae</i>
15	Positive	Blood + urine antigen	<i>S. pneumoniae</i>

Statistics

Results were evaluated by IBM Statistical Package for the Social Sciences (SPSS) Statistics® for Windows and presented as median values (IQR). Clinical status of the patients was compared with values of hepcidin, Ret-He, leucocytes, CRP, PCT, lactate, HBP, and haemoglobin. Values registered at time zero represented baseline inclusion values from blood samples drawn at the ICU. Decline as percentage of initial values of hepcidin, CRP, PCT, lactate, leucocytes, and HBP were plotted into a diagram with daily changes related to the median of respective inclusion value set at 100%, to illustrate the variation over time, and response to therapy. The data could not be regarded as normally distributed due to the limited study population, with no normal distribution of symptoms in cases of sepsis. Pearson correlation analyses were performed to evaluate correlations between hepcidin, Ret-He, leucocytes, CRP, PCT, lactate, HBP, and haemoglobin.

Due to repeated measurements, linear mixed models were used depending on the outcome variable. The mixed model analyses were performed using the lme4 package in R. A p value ≤ 0.05 was considered statistically significant [36, 37]. The biomarkers hepcidin, HBP, PCT, CRP, and lactate were log2-transformed through the whole analysis.

Results

Patients

All patients included fulfilled the criteria for septic shock, with organ failure and need of vasopressors. Results were based on a total number of 15 patients, all completing the study and all discharged from the ICU with

improved condition (demographics shown in Table 1). Ten patients were male (67%) and 5 female (33%); the median age of the patients was 61 years, with no gender difference. The main locations of infections were respiratory and urinary tract.

Clinical scoring, SOFA scores, for the patients at inclusion varied between 4 and 17 (median 10, IQR 6–12). Maximal median SOFA score was seen 24 h after inclusion (Fig. 3). A general clinical improvement at 144 h (day 7) correlated with a prompt decrease in SOFA score for the whole study group. Ten of the 15 patients were treated at the ICU throughout the whole study period, 2 patients were discharged from the ICU to regular medical wards after 48 h, 2 more after 72 h, and 1 patient after 96 h; the maintaining patients in the ICU were thus in more severe conditions during the last days of their study period, reflected by higher SOFA scores. Blood samples were collected from patients in the medical wards in line with the study protocol, whereas SOFA score measurements were only performed in the ICU. Patients treated during all 7 days at the ICU were thus more critically ill reflected by increased SOFA scores when less ill patients were discharged from the ICU. Median SOFA scores in 13 patients were compared to median values of hepcidin (Fig. 3), and SOFA scores were missing in 2 patients; these were excluded in the figure.

Fig. 1. a–h Boxplots representing median values of hepcidin (**a**), Ret-He (**b**), procalcitonin (PCT) (**c**), CRP (**d**), HBP (**e**), lactate (**f**), leucocytes (**g**), and haemoglobin (**h**) over the 7-day study period. Ret-He, reticulocyte haemoglobin; PCT, procalcitonin; CRP, C-reactive protein; HBP, heparin-binding protein.

Fig. 2. Relative changes in biomarker levels in septic patients. Dynamics related to initial values set at 100% for each biomarker.

Microbiology of Patient Samples

Microbial cultures were obtained from various sites, positive in 11 out of 15 patients, yielding slightly more Gram-positive than Gram-negative bacteria (Table 2). *Streptococcus pneumoniae* were found in 4 of 15 patients, *Staphylococcus aureus* and *Escherichia coli* were isolated from 2 patients each, whereas the other 3 patients exhibited *Streptococcus pyogenes*, *Fusobacterium necrophorum*, or polymicrobial growth (Table 2). The antibiograms of all isolated bacteria demonstrated susceptibility to the antibiotics administered empirically already in the emergency room before the patients were included into the study. Blood cultures were obtained from all patients included, with complete antibiograms from 8 of the patients. One patient suffered from pyelonephritis of a kidney transplant, with urine culture growing *S. aureus* at inclusion, and a few days later, during antibiotic treatment, blood cultures grew the same staphylococcal strain. Another patient suffering from severe pneumonia had polymicrobial cultures from the lower respiratory tract early after inclusion into the study. One patient, presenting with a severe sacral wound infection, had wound cultures revealing the same bacteria as found in cultures from sputum as well as nasopharynx. One patient, suffering from

Fig. 3. Variations in median of hepcidin (blue) and SOFA score (green). SOFA score was missing in 2 patients; thus, the graph represents 13 patients. SOFA, Sequential Organ Failure Assessment.

pneumonia, exhibited a positive pneumococcal antigen in urine, though all cultures were negative.

The first dose of antibiotics was administered within <1 h after admission to the hospital in 13/15 patients. Antibiotics were administered 3 and 11 h after entering the emergency department, respectively, in 2 patients.

Biomarkers

At the time of inclusion, all values of hepcidin, PCT, CRP, HBP, lactate, and leucocytes were elevated compared to reference values (Fig. 1a–g). Ret-He values at admission were within the lower normal range (Fig. 1b). Haemoglobin values were below the lower reference level for men (median 120 g/L, reference interval for women 117–153 g/L and for men 134–170 g/L) at admission and anaemia continued throughout the study in all patients (Fig. 1h). The maximal levels noted for hepcidin, PCT, and lactate were noted at admission, contrary to levels of Ret-He, CRP, HBP, and leucocytes, where the maximal values were reached later during the stay in the ICU.

Hepcidin

Normal reference values for hepcidin are set to 1–12 nmol/L in adults according to the local Reference Laboratory, Lund University Hospital, Region Skåne, Sweden [34, 38, 39]. Median hepcidin concentrations had already reached the maximal levels at the time of inclusion (median 61 nmol/L, mean 68 nmol/L), with a fast and steady decline during the next 96 h (Fig. 1a). In 3 patients, hepcidin levels reached maximum at 24 h and in 1 patient at 48 h after inclusion into the study. Five patients developed secondary complications exhibiting a second in-

Table 3. Pearson correlation analyses including the biomarkers, hepcidin, Ret-He, haemoglobin, CRP, PCT, HBP, lactate, and leucocytes

	Correlations							
	hepcidin, nmol/L	Ret-He, pg	haemoglobin, g/L	CRP, mg/L	PCT, µg/L	lactate, mmol/L	HBP, ng/mL	leucocytes, ×10 ⁹ /L
Hepcidin, nmol/L								
Pearson correlation	1	−0.229*	0.175	0.580**	0.406**	0.066	0.006	−0.380**
Sig. (2-tailed)		0.029	0.077	0.000	0.000	0.555	0.955	0.000
N	103	91	103	103	100	82	97	103
Ret-He, pg								
Pearson correlation	−0.229*	1	0.174	−0.192	−0.182	0.403**	0.160	0.166
Sig. (2-tailed)	0.029		0.095	0.065	0.087	0.000	0.139	0.113
N	91	280	93	93	90	74	87	93
Haemoglobin, g/L								
Pearson correlation	0.175	0.174	1	0.007	−0.101	0.294**	0.058	−0.418**
Sig. (2-tailed)	0.077	0.095		0.942	0.311	0.007	0.571	0.000
N	103	93	105	105	102	83	99	105
CRP, mg/L								
Pearson correlation	0.580**	−0.192	0.007	1	0.461**	0.200	0.152	0.035
Sig. (2-tailed)	0.000	0.065	0.942		0.000	0.070	0.133	0.721
N	103	93	105	105	102	83	99	105
PCT, µg/L								
Pearson correlation	0.406**	−0.182	−0.101	0.461**	1	0.198	0.104	0.206*
Sig. (2-tailed)	0.000	0.087	0.311	0.000		0.076	0.316	0.038
N	100	90	102	102	102	81	96	102
Lactate, mmol/L								
Pearson correlation	0.066	0.403*	0.294**	0.200	0.198	1	0.399**	0.011
Sig. (2-tailed)	0.555	0.000	0.007	0.070	0.076		0.000	0.920
N	82	74	83	83	81	83	78	83
HBP, ng/mL								
Pearson correlation	0.006	0.160	0.058	0.152	0.104	0.399**	1	0.228*
Sig. (2-tailed)	0.955	0.139	0.517	0.133	0.316	0.000		0.023
N	97	87	99	99	96	78	99	99
Leucocytes, ×10 ⁹ /L								
Pearson correlation	−0.380**	0.166	−0.418**	0.035	0.206*	0.011	0.228*	1
Sig. (2-tailed)	0.000	0.113	0.000	0.721	0.038	0.920	0.023	
N	103	93	105	105	102	83	99	105

Ret-He, reticulocyte haemoglobin; CRP, C-reactive protein; PCT, procalcitonin; HBP, heparin-binding protein. * Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed).

crease of hepcidin concentrations during the complication period (Fig. 4b). Patients without complications showed a steady decline of hepcidin concentrations approaching reference levels of hepcidin at the end of the study (Fig. 4a).

Determination of Ret-He Levels

Initial median value of Ret-He (reference interval 28–35 pg) was 28 pg, in the lower range of the reference interval. Ret-He <28–29 pg indicates iron deficiency and impaired erythropoiesis [31, 32]. In this study, Ret-He concentrations continuously decreased during the first 72 h after admission. A response indicating beneficial effect of treat-

ment of the patients with decreasing acute phase reactants including hepcidin was noted with increased Ret-He values with a 96-h delay (Fig. 1b). Total reticulocyte numbers and Ret-He showed covariation in all patients (data not shown). For a few samples, reticulocytes in the patient's blood were below $10 \times 10^9/L$, reported not detectable by the Sysmex[®] XN-10 method used (9/105 samples).

Comparison over Time and Response to Therapy

Daily median values of the biomarkers hepcidin, PCT, CRP, HBP, lactate, and leucocytes were calculated and displayed as percentage of median arrival values set to 100% (Fig. 2). A significant daily decline was shown

Fig. 4. a Hepcidin levels (nmolG/mL) in 5 patients with complications during the 7-day period in the ICU. **b** Hepcidin levels in 10 patients without complications during the 7-day period in the ICU. ICU, intensive care unit.

for hepcidin, PCT, and lactate during the first 48 h. Concentrations of PCT (reference level $<0.05 \mu\text{g/L}$) were highest at inclusion (median $95 \mu\text{g/L}$), parallel with hepcidin, and with a steady daily decline observed throughout the entire study period (Fig. 1c). On day 7 (144 h), the median PCT concentration was $1.8 \mu\text{g/L}$. A minor increase of median CRP (reference interval $<3\text{--}10 \text{ mg/L}$) was observed during the first 24 h ($245\text{--}247 \text{ mg/L}$) (Fig. 1d), followed by a decline registered at 48 h after inclusion (Fig. 1d). The concentrations of HBP (reference interval $<15\text{--}30 \text{ ng/mL}$) were elevated in 13/15 patients at inclusion (median 173 ng/mL), followed by a minor increase during the first 24 h (median 177.9 ng/mL), with a daily decline thereafter with some fluctuations in a few patients (Fig. 1e). Lactate concentrations (reference interval $0.5\text{--}2.2 \text{ mmol/L}$) were highest at inclusion (median 3.6), followed by a steady decline during the next 72 h (Fig. 1f). Leucocyte counts (reference interval $3.5\text{--}8.8 \times 10^9/\text{L}$) reached maximal levels after 24 h and remained increased throughout the whole study period (Fig. 1g). A decline of biomarker levels, including hepcidin, showed covariation with the clinical improvement of the patients according to SOFA score (Fig. 3).

Correlation between Biomarkers

Pearson correlation analyses were performed to investigate if there were any correlations between the analysed biomarkers (Table 3). Hepcidin demonstrated a statistically significant positive correlation with CRP, PCT, and

leucocytes and a statistically significant negative correlation with Ret-He. Ret-He, accept from correlating negatively to hepcidin, showed a significant positive correlation with lactate. A significant positive correlation was observed between HBP and leucocytes. Haemoglobin levels did not correlate significantly with either hepcidin or Ret-He.

Secondary Complications

Five of the 15 patients developed secondary complications during the 7-day study period, 3 of those developed significant fever reactions. Three patients had pleural effusion in need of drainage ($>1,000$, $1,500$ and $4,000 \text{ mL}$ fluid drained, respectively), and 2 of these developed fever at the time of complication. Another patient developed a radiologically confirmed nosocomial pneumonia with a fever reaction and 1 patient developed suspected aspiration pneumonia without any fever reaction. All complications occurred between study days 2 and 5.

Concentrations of hepcidin, PCT, CRP, and HBP were compared between patients with and without secondary complications (Fig. 4a, b, 5a, b). In patients with no secondary complications, hepcidin decreased steadily and was almost normalized by day 6 (144 h). Increased levels of hepcidin and CRP were noted at 72 h in patients suffering complications, whereas PCT continuously decreased also in those patients. A daily decrease of HBP was noted in patients without complications, but HBP was increased slightly in patients with complications on day 5 (120 h).

Fig. 5. a Biomarker levels of CRP (mg/L), HBP (ng/L), and PCT (µg/L) in 5 patients with complications during the 7-day period in the ICU. **b** Biomarker levels of CRP, HBP, and PCT in 10 patients without complications during the 7-day period in the ICU. CRP, C-reactive protein; HBP, heparin-binding protein; PCT, procalcitonin; ICU, intensive care unit.

Table 4. Mixed models analyses. Predictor of biomarkers compared to SOFA score as dependent variable (all biomarkers log2-transformed through the whole analysis).

Linear mixed models, SOFA as dependent variable		
predictor	effect size (CI)	p value
Hepcidin	−0.164 (−0.763; 0.436)	0.593
HBP	0.343 (−0.114; 0.799)	0.142
PCT	0.789 (0.463; 1.115)	<0.001
CRP	0.968 (0.461; 1.475)	<0.001
Lactate	0.356 (−0.469; 1.181)	0.398
Ret-He	−4.260 (−8.131; −0.388)	0.031

SOFA, Sequential Organ Failure Assessment; HBP, heparin-binding protein; PCT, procalcitonin; CRP, C-reactive protein; Ret-He, reticulocyte haemoglobin.

Table 5. Mixed models analyses. SOFA score as a predictor compared to the biomarkers hepcidin, HBP, PCT, CRP and lactate (all biomarkers log2-transformed through the whole analysis)

Linear mixed models, SOFA as predictor		
dependent variable	effect size (CI)	p value
Hepcidin	−0.030 (−0.113; 0.053)	0.477
HBP	0.078 (−0.029; 0.186)	0.153
PCT	0.293 (0.173; 0.414)	<0.001
CRP	0.066 (0.007; 0.125)	0.028
Lactate	0.018 (−0.024; 0.060)	0.405
Ret-He	−0.009 (−0.022; 0.004)	0.176

SOFA, Sequential Organ Failure Assessment; HBP, heparin-binding protein; PCT, procalcitonin; CRP, C-reactive protein; Ret-He, reticulocyte haemoglobin.

Prediction between Biomarkers and Clinical Outcome (SOFA Score)

Mixed models were utilized to evaluate if any of the biomarkers could predict clinical outcome as evaluated by SOFA score due to the repeated measurements of the biomarkers hepcidin, HBP, PCT, CRP, and lactate. Evaluation using a linear mixed model, PCT (p value <0.001), and CRP (p value <0.001) as well as Ret-He (p value 0.031) significantly predicted SOFA score in the patients of the study, whereas neither hepcidin (p value 0.593), HBP (p value 0.142), nor lactate (p value 0.398) predicted SOFA

score (Table 4). Conversely, when using a linear mixed model, SOFA score significantly predicted PCT (p value <0.001) and CRP (p value 0.028) but not values of hepcidin (p value 0.477), Ret-He (p value 0.176), HBP (p value 0.153), or lactate (p value 0.405) (Table 5).

Outcome and Mortality

Treatment response was satisfactory since all of the 15 patients consistently improved and were able to leave the ICU after initial care. Antibiotics administered empirically covered isolated pathogens, and no antibiotic resis-

tance was reported in the analysis performed at the Clinical Microbiology Laboratory in Lund. None of the 15 patients diseased during the stay in the ICU. Only 1 patient diseased on day 25 after admission, still during the same hospital stay, yielding an unexpectedly low 28-day mortality rate compared to other studies [3, 4].

Discussion/Conclusion

The primary outcome of the present observational pilot study on patients admitted to the ICU with septic shock was to investigate the short-term alterations in hepcidin, Ret-He, and HBP over time in response to clinical improvement. In the clinical routine, biomarker levels are included to evaluate the response and recovery from disease, and previous reports have shown hepcidin concentrations to be significantly increased in both children and adults with bacterial sepsis [26, 27, 40]. In this study, all patients with septic shock received adequate antibiotic treatment and hepcidin concentrations had reached maximal levels already at inclusion, with a steady decline, suggesting that hepcidin might be valuable for monitoring treatment success in patients with septic shock.

Hepcidin is an acute phase reactant responding to various cytokine alterations not only in sepsis but also in chronic disease associated with infections, cancer, and autoimmune diseases with secondary anaemia [41–43]. Hepcidin is the key regulator of iron metabolism, leading to functional iron deficiency in inflammation. The evolution of this protective mechanism in sepsis, which adds to many other anti-infective mechanisms, is due to that free iron, used by invading microbes, will be reduced in response to increased hepcidin concentrations [44]. Long-standing iron deficiency has negative effects for the host since iron is an essential component for the host innate immunity, for DNA and neurotransmitter synthesis, as well as for mitochondrial function [44]. Low levels of available iron can lead to critical illness complications itself manifested in dysfunction of the cardiopulmonary, cognitive, and neuromuscular systems and predispose for nosocomial infection [44]. In summary, balance of iron availability is crucial and therapeutic targets are under development for both hepcidin agonists and hepcidin inhibitors [44]. Complications often seen in patients treated in the ICU are oedema, pleural effusions, myocardial stress, and ventilator-associated pneumonia often due to intense fluid resuscitation [45, 46]. In this study, a secondary increase of hepcidin was noted in patients suffering complications in the ICU, mostly noted at 72 h after

inclusion. Daily measurements of hepcidin could reveal upcoming non-infectious complications at an early stage in critically ill patients and also support the decision-making in therapy, where a steady decline of hepcidin concentration supports the efficiency of the ongoing antibiotic treatment as well as other treatment strategies. In light of recent knowledge of iron metabolism, an increase in hepcidin will decrease iron availability, with an increased risk for critical illness complications [44]. Thus, regular monitoring of hepcidin would not only be valuable for evaluation of treatment success in patients with septic shock but also be of great value when looking at therapeutic targets in the up- or downregulation of hepcidin to minimize harmful iron availability.

The results of Ret-He concentrations in this study on patients with septic shock were in line with that Ret-He has been reported to decrease initially in patients with community-acquired pneumonia and in patients with sepsis [21, 25, 47]. Ret-He was normalized in response to decreasing hepcidin concentrations in the patients with septic shock. Normalizing Ret-He values correlated with improved clinical status, reflected by lower SOFA scores; thus, Ret-He could be valuable concerning the discontinuation of antibiotic treatment. Ret-He reached the normal reference interval in all patients at 144 h, correlating with hepcidin, PCT, and SOFA score, which were also normalized at 144 h. In contrast to hepcidin, Ret-He is a routine analysis in all clinical chemistry laboratories, at much lower cost than PCT analysis.

Plasma iron levels regulate Ret-He and thus the correlation seen between hepcidin and Ret-He in the present study would be expected as well as the noted correlation between HBP and leucocytes. Haemoglobin concentrations did not significantly correlate with hepcidin and Ret-He, probably due to the short study period.

Levels of CRP also increased in patients suffering complications but with some delay compared to hepcidin, whereas PCT did not increase at all in these non-septic complications. Previous reports state that PCT is selective for systemic inflammation in response to bacterial challenge but is usually not induced when having a local bacterial infection or colonization [48].

The results of the present small study suggest that combined measurements of hepcidin and PCT could be useful to distinguish non-septic from septic complications in the ICU, thus revealing upcoming non-infectious complications at an early stage in critically ill patients, and might support a decision to refrain from changing antibiotics.

Elevated lactate is known to reflect increased aerobic glycolysis activated by stress response through adrenergic

stimulation mirroring the organ dysfunction such as that of septic shock [49], and an elevated lactate concentration was seen in all patients in the present study. Lactate levels were normalized within the first 24 h upon treatment and did not increase in patients with complications. Elevated lactate, though, is also seen due to any kind of severe conditions, where organ dysfunction and hypoperfusion are observed, for example, cardiac arrest and hypoxia [50], though in the present study, the patients did not suffer any other condition leading to organ hypoperfusion than septic shock.

Levels of CRP were maximal at 36–50 h after inclusion [51] in all patients and thus decreasing at a later time point compared with hepcidin, PCT, and lactate. Previous reports show that HBP is significantly elevated in patients with severe sepsis/septic shock compared to patients with non-septic disease treated in the ICU, declining over time [52]. Median HBP levels in this study were elevated at inclusion, continued to increase for 24 h related to neutrophil counts [52], since neutrophils are the main source of HBP in the circulation [35].

SOFA score has proven valuable for grading organ failure, and thus, it would be desirable if sepsis biomarkers correlate to the score [53]. Median SOFA score in this study decreased in line with the sepsis biomarkers during the first 72 h. Since 5 patients were discharged from the ICU prior to the end of the 7-day study period, SOFA scores at time points beyond 72 h reflected fewer patients, with more severe conditions, reflected in an increased SOFA score at 120 h for the patients who were all still treated in the ICU.

Since we performed repeated measurements, it was possible to make analyses by mixed models showing that PCT, CRP, and Ret-He significantly predicted the clinical outcome as measured by SOFA score, and SOFA score also significantly predicted the values of the PCT and CRP. No significant prediction for hepcidin, HBP, and lactate compared to SOFA scores was found. The study population was small, and the patients SOFA scores varied considerably; thus, the clinical significance of the results may be disputed, though their support of earlier reports on that PCT and CRP are valuable in septic patients showed that our patient group did not differ from other patients with septic shock; thus, the reported results in the present study could be considered reliable.

Only 1 patient diseased within 28 days of the current hospital stay due to a non-related illness; thus, the overall mortality rate during the study was extremely low compared to previous studies [3, 4]. None of the patients needed blood transfusion as a consequence of sepsis.

Although small, the patient population of this study was comparatively representative as of septic patients compared

to previous reports [11, 18, 26]; thus, the values of hepcidin, Ret-He, and HBP were regarded valid for the diagnosis. The strength of this study was the daily monitoring of patients, samples taken at the same time of the day, every day for 7 consecutive days with very few missing or non-detectable samples (6/105 HBP, 2/105 hepcidin, 2/105 PCT, 12/105 Ret-He, 0/105 CRP, 21/105 lactate, and 0/105 leucocytes patient values were missing). HBP, a promising biomarker for predicting sepsis and sepsis-induced organ failure, was measured to shed light on how HBP might serve as a tool for evaluation of therapy and recovery from sepsis. The results in our study suggested that HBP as a biomarker for evaluating sepsis recovery at an early stage (the first 24 h) is not favourable compared to hepcidin or PCT.

The small number of patients in the study limited the statistical analyses to mixed models and Pearson correlation test. A control group without treatment is naturally unethical as of septic shock, and our aim was mainly to evaluate parameters in severely ill septic patients at a time when little was published in this field. Hepcidin is still not available as a bedside test nor is HBP. Another limitation of this study is that other important iron metabolism biomarkers such as free iron, ferritin, transferrin, and transferrin saturation were not analysed since the focus of the study was on sepsis biomarkers rather than anaemia.

Conclusion

Measuring hepcidin and Ret-He added valuable information to routinely used biomarkers for evaluating response and recovery over time in patients with septic shock receiving adequate antibiotic treatment. Hepcidin was superior to other biomarkers in detecting secondary complications in critically ill patients. The predictive values of the biomarkers to clinical response measured by SOFA score were statistically significant for CRP, PCT, and Ret-He. Larger studies are needed, including patients with other critical illness than septic shock, to confirm the utility of these parameters as well as of HBP that added valuable information in the early phase of septic shock.

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Statement of Ethics

This study was approved by the chairmen of the departments in Helsingborg as well as by the Regional Ethics Committee of Scania County, Lund, Sweden No. 2014/195. Subjects provided written informed consent. The study protocol was approved by the research institute's committee on human research.

Conflict of Interest Statement

Hansa Medical (Lund, Sweden) has filed patent applications on HBP as a diagnostic maker. H.H. is listed as inventor. All other authors have no conflicts of interest to declare.

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Author Contributions

Substantial contributions to the conception or design of the work: J.O. and C.R. The acquisition, analysis, or interpretation of data for the work: J.O., D.E., E.L., and C.R. Drafting the work or revising it critically for important intellectual content: J.O., D.E., E.L., C.R., and H.H.

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