Inflammation-induced hepcidin-25 is associated with the development of anemia in septic patients: an observational study

Lucas T van Eijk, Joyce JC Kroot, Mirjam Tromp, Johannes G van der Hoeven, Dorine W Swinkels, Peter Pickkers

Abstract

Introduction: Anemia is a frequently encountered problem during inflammation. Hepcidin is an interleukin-6 (IL-6)-induced key modulator of inflammation-associated anemia. Human sepsis is a prototypical inflammatory syndrome, often complicated by the development of anemia. However, the association between inflammation, hepcidin release and anemia has not been demonstrated in this group of patients. Therefore, we explored the association between hepcidin and sepsis-associated anemia.

Methods: 92 consecutive patients were enrolled after presentation on the emergency ward of a university hospital with sepsis, indicated by the presence of a proven or suspected infection and ≥2 extended systemic inflammatory response syndrome (SIRS) criteria. Blood was drawn at day 1, 2 and 3 after admission for the measurement of IL-6 and hepcidin-25. IL-6 levels were correlated with hepcidin concentrations. Hemoglobin levels and data of blood transfusions during 14 days after hospitalisation were retrieved and the rate of hemoglobin decrease was correlated to hepcidin levels.

Results: 53 men and 39 women with a mean age of 53.3 ± 1.8 yrs were included. Hepcidin levels were highest at admission (median[IQR]: 17.9[10.1 to 28.4]nmol/l and decreased to normal levels in most patients within 3 days (9.5[3.4 to 17.9]nmol/l). Hepcidin levels increased with the number of extended SIRS criteria (P = 0.0005). Highest IL-6 levels were measured at admission (125.0[46.3 to 330.0]pg/ml) and log-transformed IL-6 levels significantly correlated with hepcidin levels at admission (r = 0.28, P = 0.015), day 2 (r = 0.51, P < 0.0001) and day 3 (r = 0.46, P < 0.0001). Twelve patients received one or more blood transfusions during the first 2 weeks of admission, not related to active bleeding. These patients had borderline significantly higher hepcidin level at admission compared to non-transfused patients (26.9[17.2 to 53.9] vs 17.9[9.9 to 28.8]nmol/l, P = 0.052). IL-6 concentrations did not differ between both groups. Correlation analyses showed significant associations between hepcidin levels on day 2 and 3 and the rate of decrease in hemoglobin (Spearman's r ranging from -0.32, P = 0.03 to -0.37, P = 0.016, respectively).

Conclusions: These data suggest that hepcidin-25 may be an important modulator of anemia in septic patients with systemic inflammation.

Introduction

Inflammation-associated anemia represents an important and highly prevalent clinical problem. In 2000, Krause et al. described a peptide that was later called ‘hepcidin’ based on its hepatic expression and antimicrobial activity [1,2].

This β-defensin-like peptide was found to be a principle regulator of systemic iron homeostasis. In concordance with this dual function, its expression is modulated by systemic iron requirements and inflammatory stimuli, as it is induced by cytokines such as IL-6 [3]. Its role in the development of anemia was first suggested in 2001 [4]. Since then it has been demonstrated that hepcidin is a central modulator of inflammation-associated anemia, not only by controlling the expression of ferroportin on intestinal cells.
and macrophages [5], but also via a direct inhibitory effect
of hepcidin on erythropoiesis [6]. In humans, increased
concentrations of hepcidin were detected in patients with
chronic infections and severe inflammatory diseases [7].
The association of increased concentrations of hepcidin
with anemia has been determined in patients suffering
from chronic inflammation [8], chronic kidney disease [9],
and cancer [10]. In addition, acute systemic inflammation
evoked by experimental endotoxemia in humans resulted
in an increase in hepcidin release, associated with a
decrease in serum iron [11]. Nevertheless, the association
between the innate immune response, hepcidin release
and consequent decrease in hemoglobin (Hb) has not
been established in patients with an acute systemic
inflammation.

Human sepsis is a prototypical acute inflammatory
syndrome frequently complicated by the development
of anemia. As the incidence of sepsis is high [12], determi-
nation of this putative pathway of the development of
anemia is of clinical importance. Therefore, we explored
the correlation between IL-6 and hepcidin, and the sub-
sequent rate of Hb decrease and number of blood trans-
fusions received in septic patients.

Materials and methods
Subjects and sampling
This is an explorative observational study in which data
of the subjects were retrieved from a prospectively
aggregated database of patients with sepsis. Following
Dutch law, the local Institutional Review Board of Arnhem-Nijmegen indicated that no formal approval was
required for this study. Patients were informed, but no
written consent was necessary. Ninety-two consecutive
septic patients were enrolled after presentation on the
emergency ward and subsequent hospital admission.
Sepsis was defined by the presence of two or more
extended criteria for systemic inflammation (body tem-
perature >38.3 or < 36°C, acutely altered mental status,
shivering, heart rate >90 bpm, systolic blood pressure
<90 mmHg or mean arterial pressure < 65 mmHg,
respiratory rate >20 breaths/min, hyperglycemia in
absence of diabetes) and a proven or suspected source
of infection [13]. The total number of extended systemic
inflammatory response syndrome (SIRS) scores were cal-
culated. Patients were given usual care according to the
guidelines of the Surviving Sepsis Campaign [14]. Blood
was drawn at day one, two and three of admission for
the measurement of IL-6 and hepcidin-25. Hb measure-
ments were not taken as part of a protocol, but Hb
levels that were determined as part of standard hospital
care during the first 14 days after hospital admission
were retrieved and used for further analysis. Hemoblo-
brin levels were checked regularly, but not every day in
every patient. Also the accompanying indices of mean
corpuscular volume (MCV), mean cell Hb (MCH), and
red cell distribution width (RDW) were analyzed. Blood
transfusions during hospital stay were recorded. We
hypothesized that the effect of sustained elevated levels
of hepcidin could be first seen in the Hb level after a per-
iod of 7 to 14 days. This was based on the assumption
that erythrocytes circulate for approximately 120 days. If
erythropoiesis would be abrogated by hypoferremia due
to an increased hepcidin level, it would therefore take
approximately 12 days to reduce the Hb levels by 10%.
A decrease of 10% was considered a clinically relevant
and reliably detectable difference. However, due to a possible
direct inhibitory effect of hepcidin on erythropoiesis, and
a reduced erythrocyte half-life during inflammation, a
detectable reduction of Hb from day seven onwards was
anticipated.

Laboratory measurements
IL-6 levels were measured on an Immulite 2500 (Siemens,
Breda, The Netherlands), based on a solid-phase, enzyme-
labelled, chemiluminescent sequential immunometric
method. Serum hepcidin-25 measurements were
performed by a combination of weak cation exchange
chromatography and time-of-flight mass spectrometry
(ToF-MS), using a Microflex LT matrix-enhanced laser
desorption/ionisation TOF-MS platform (Bruker
Daltonics, Bremen, Germany). An internal standard (syn-
thetic hepcidin-24; Peptide International Inc., Louisville,
KT, USA) was used for quantification [15,16].

Calculations and statistical analysis
Log-transformed IL-6 concentrations were correlated
with hepcidin-25 concentrations using Pearson’s correla-
tion coefficient. Heparidin-25 was correlated with the
rate of decrease of Hb between day 1 and 14, using
Spearman’s correlation coefficient. The rate of decrease
of Hb was calculated per patient by linear regression
using all available Hb measurements. If Hb levels were
not measured at days 7 to 14, or if patients received a
blood transfusion during their stay, they were excluded
from this analysis. Heparidin levels at admission (prior to
any transfusion) of patients who received a blood trans-
fusion during the first 14 days of hospitalization were
compared with patients who did not.

To test whether the presence of comorbidity affected
the rate of Hb decrease, we divided different forms of
comorbidity into eight categories (chronic kidney dis-
ease, hematologic, malignancy, pulmonary, rheumatic/
autoimmune, cardiologic, urologic, and other) and
performed a step-wise multi-variate analysis in which hep-
cidin levels and the eight categories of comorbidity were
added to the model. If a comorbidity was found to sig-
nificantly attribute to the prediction of Hb decrease, it
was left in the model, but otherwise discarded. Data are
expressed as mean ± standard error of the mean or median (25th to 75th percentile) depending on their distribution. Correlations were expressed as Spearman's correlation coefficient, except for the correlations between hepcidin and log-transformed IL-6 concentrations that were expressed as Pearson's r. Paired observations over time were tested with Wilcoxon matched-pairs test and unpaired observations with a Mann-Whitney test.

Results
Demographic data
Demographic data of the subjects are displayed in Table 1. Two patients died during hospitalization. Blood culture results are presented in Table 2. Twenty percent of the patients had a positive blood culture. This relatively low percentage is probably due to the fact that in most cases the general practitioner had already initiated antimicrobial therapy before admission to the hospital.

IL-6 and hepcidin
IL-6 was highest at admission (125.0 (46.3 to 330.0) pg/ml), and decreased on day two (37.2 (16.8 to 112.8) pg/ml) and day three (19.5 (7.4 to 55.7) pg/ml). A similar pattern was observed for hepcidin levels, being highest at admission (17.9 (10.1 to 28.4) nmol/l) and declining to 9.5 (3.4 to 17.9) nmol/l on day three, which is still increased compared with control values. Log-transformed IL-6 levels correlated significantly with hepcidin levels on admission, day two and day three, (Pearson’s r = 0.28, P = 0.015; r = 0.512, P< 0.0001; r = 0.458, P< 0.0001, respectively; Figure 1a). Also, the number of extended SIRS criteria present correlated with hepcidin levels (Figure 1b).

Hepcidin and hemoglobin
Hb was 12.0 (11.2 to 13.4) g/dl at admission and decreased to an average of 11.3 (10.3 to 12.8) g/dl at day 7 to 14 (P = 0.004) in patients who did not receive a blood transfusion. During hospitalization the Hb levels decreased at least 0.8 g/dl in 69 (86%) of 80 patients who did not receive a blood transfusion. There was no correlation between hepcidin levels and Hb levels at admission (r = 0.21, P = 0.07). Hepcidin levels on day one of admission did not correlate

Table 1 Demographic data of the subjects

<table>
<thead>
<tr>
<th></th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>92 (100)</td>
</tr>
<tr>
<td>Male/female</td>
<td>53/39 (58/42)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>53.3 ± 1.8</td>
</tr>
<tr>
<td>ICU admissions</td>
<td>3 (3)</td>
</tr>
<tr>
<td>Deaths</td>
<td>2 (2)</td>
</tr>
<tr>
<td>Median hospital length of stay (days)</td>
<td>6 (4-11)</td>
</tr>
<tr>
<td>Number of SIRS criteria present</td>
<td>2.5 ± 0.9</td>
</tr>
<tr>
<td>Number of patients transfused</td>
<td>12 (13)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Site of infection</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>28 (30)</td>
</tr>
<tr>
<td>Abdomen</td>
<td>12 (13)</td>
</tr>
<tr>
<td>Urinary tract</td>
<td>24 (26)</td>
</tr>
<tr>
<td>Skin/soft tissue</td>
<td>4 (4)</td>
</tr>
<tr>
<td>Bone/joint</td>
<td>3 (3)</td>
</tr>
<tr>
<td>Blood</td>
<td>2 (2)</td>
</tr>
<tr>
<td>Cerebral</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Other</td>
<td>3 (3)</td>
</tr>
<tr>
<td>Unknown</td>
<td>9 (10)</td>
</tr>
<tr>
<td>No infectious focus</td>
<td>6 (7)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Comorbidity</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>36 (39)</td>
</tr>
<tr>
<td>Chronic kidney disease</td>
<td>13 (14)</td>
</tr>
<tr>
<td>Hematologic disease</td>
<td>7 (8)</td>
</tr>
<tr>
<td>Malignancy</td>
<td>8 (9)</td>
</tr>
<tr>
<td>Lung disease</td>
<td>6 (7)</td>
</tr>
<tr>
<td>Rheumatic / autoimmune disease</td>
<td>2 (2)</td>
</tr>
<tr>
<td>Cardial disease</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Urological disease</td>
<td>5 (5)</td>
</tr>
<tr>
<td>Other</td>
<td>14 (15)</td>
</tr>
</tbody>
</table>

Data are expressed as absolute numbers and percentages of total, mean ± standard error of the mean or median (25th to 75th percentile). Multivariate analysis demonstrated that comorbidities were not independently associated with hemoglobin decrease. SIRS, systemic inflammatory response syndrome.

Table 2 Blood culture results

<table>
<thead>
<tr>
<th>Organisms and culture sites</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organisms</td>
<td></td>
</tr>
<tr>
<td>Aeromonas species</td>
<td>1</td>
</tr>
<tr>
<td>Candida species</td>
<td>2</td>
</tr>
<tr>
<td>Citrobacter species</td>
<td>1</td>
</tr>
<tr>
<td>Corynebacterium jeikeium</td>
<td>1</td>
</tr>
<tr>
<td>Coxiella species</td>
<td>4</td>
</tr>
<tr>
<td>Enterobacter species</td>
<td>1</td>
</tr>
<tr>
<td>Enterococcus species</td>
<td>5</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>10</td>
</tr>
<tr>
<td>Haemophilus influenzae</td>
<td>1</td>
</tr>
<tr>
<td>Klebsiella species</td>
<td>2</td>
</tr>
<tr>
<td>Morganella species</td>
<td>1</td>
</tr>
<tr>
<td>Pseudomonas species</td>
<td>3</td>
</tr>
<tr>
<td>Salmonella species</td>
<td>1</td>
</tr>
<tr>
<td>Staphylococcus species</td>
<td>2</td>
</tr>
<tr>
<td>Streptococcus species</td>
<td>3</td>
</tr>
<tr>
<td>Viral infection (positive serological test)</td>
<td>6</td>
</tr>
<tr>
<td>No pathogen cultured</td>
<td>50</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sites</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>18</td>
</tr>
<tr>
<td>Urine</td>
<td>18</td>
</tr>
<tr>
<td>Other</td>
<td>4</td>
</tr>
<tr>
<td>Multiple organisms</td>
<td>2</td>
</tr>
<tr>
<td>Multiple sites</td>
<td>2</td>
</tr>
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</table>

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with the rate of decrease in Hb ($r = -0.13$, $P = 0.39$). Hepcidin on day two and day three significantly correlated with the rate of decrease of Hb ($r = -0.32$, $P = 0.03$ and $r = -0.37$, $P = 0.016$; Figure 1c).

Twelve patients received one or more blood transfusions during the first two weeks of admission, not related to active bleeding. These patients had borderline significant higher hepcidin level at admission (preceding any blood transfusion) compared with non-transfused patients (26.9 (17.2 to 53.9) vs 17.9 (9.9 to 28.8) nmol/l, $P = 0.052$; Figure 1d).

MCV slightly increased during hospital admission from 86.0 (84.0 to 90.0) to an average of 88.5 (85.7 to 92.6) fl at day 7 to 14 ($P = 0.011$). RDW increased from 13.9 (13.1 to 15.4) to an average of 15.9 (14.2 to 17.0)% ($P = 0.002$). MCH remained unchanged during 14 days of follow up (from 1.84 (1.75 to 1.90) fmol to an average of 1.82 (1.76 to 1.90) fmol at day 7 to 14 ($P = 0.39$)). None of the changes in red cell indices correlated with the hepcidin levels on days one to three.

**Discussion**

In the present study, three novel findings emerged. This is the first study to show that: hepcidin-25 is increased during human sepsis; in septic patients the degree
inflammation, indicated by IL-6 levels and number of SIRS criteria present, is associated with the elevated concentrations of hepcidin; and persistently increased levels of hepcidin-25 at day two and day three after admission are associated with a decrease in Hb during hospitalization. Naturally, in patients who received a transfusion, the effect of hepcidin on Hb could not be determined and these patients were excluded from this part of the analysis. In a separate analysis, we showed that transfused patients showed a trend towards higher hepcidin levels than those who were not. These findings combined suggest that the observed association between elevated hepcidin and a decrease in Hb is likely to be an underestimation.

This study does not necessarily indicate a causal relation between elevated hepcidin and Hb decrease in septic patients. However, the causal relation of the induction of hepcidin by IL-6 and the development of anemia by sustaining elevated hepcidin levels has been shown by others in separate experiments [3,5,6,17]. This study is the first to address the combined measurement of hepcidin, IL-6 and Hb levels in patients and shows that hepcidin probably plays a role in sepsis-associated anemia. One may argue that the correlation between inflammation, hepcidin and anemia is an epiphenomenon, because more severely affected patients release higher levels of inflammatory parameters and also receive more fluids during their volume resuscitation, resulting in the more pronounced decrease in Hb. This appears not to be the case, because hepcidin concentrations at admission did not predict the decrease in Hb, in contrast to more prolonged elevations in hepcidin during the first three days. Moreover, the decrease in Hb was determined over 14 days, a period in which the effects of volume resuscitation should have diminished. Nevertheless, it is important that in addition to the physiological role of hepcidin, several other factors that lead to anemia during infection have been described, such as iatrogenic blood loss, inhibition of erythropoietin production [18], blunted erythropoietic response [18,19], and a decreased lifespan of erythrocytes, mediated by increased adherence to the vascular wall and phagocytosis by macrophages [20]. In addition, in these patients the presence of different comorbidities and the severity of the disease could have influenced the development of anemia. However, we were not able to express these variables in size and number, and therefore could not include these parameters as a continuous variable into a multivariate regression analysis. This may explain the relatively low correlation coefficients we found between hepcidin and Hb decrease.

There are two known ways that hepcidin can result in inflammation-associated anemia. First, hepcidin can abrogate erythroid colony formation in situations where erythropoietin concentrations are reduced, as is the case during sepsis [6]. Furthermore, inflammation leads to sequestration of iron in cells resulting in a blocked transport of iron to the bone marrow. Considering the fact that the lifespan of erythrocytes of approximately 120 days might be shortened due to inflammation and the fact that hepcidin suppresses erythropoiesis itself, we hypothesized that if hepcidin is upregulated by inflammation and thereby suppresses serum iron levels, a measurable effect on Hb level was expected from seven days onwards after the diagnosis of sepsis. Furthermore, we anticipated a swift decrease in inflammation in treated septic patients. For these reasons we determined IL-6 and hepcidin levels for three days and the rate of Hb decrease within 7 to 14 days.

We were not able to demonstrate a relation of Hb decrease with a change in MCV, MCH, or RDW. This does not invalidate the hypothesis that increased hepcidin attributes to the development of anemia in these patients. It was previously shown that erythrocyte progenitor cells carry iron transporter ferroportin1B on their cell membrane [21]. During inflammation systemically elevated hepcidin down-regulates ferroportin on these cells, thereby preventing a loss of intracellular iron and the microcytic anemia that is seen in iron-deficiency anemia. Therefore, inflammation-associated anemia is not typically microcytic [22]. Moreover, the observed effect of hepcidin on the development on anemia may have been mediated by a direct inhibitory effect on erythropoiesis, rather than by blocking iron transport to the bone marrow by sequestration.

Interestingly, hepcidin at day one did not predict the rate of Hb decrease. Probably persistently elevated hepcidin levels are necessary to exert a relevant effect on Hb concentrations. The association we found may be an underestimation, because the patients in this study were already anemic at the time of presentation to the emergency ward and likewise it is possible that before presentation hepcidin levels were even more pronounced. Nevertheless, although statistically significant, the observed association between hepcidin and Hb levels is modest, indicating that other previously mentioned factors that influence Hb are likely to play a role.

Conclusions

Anemia during acute systemic inflammation evoked by sepsis is a frequently encountered clinical problem. Up to now, human data concerning the effect of hepcidin release on the development of anemia during sepsis were absent. Our study demonstrates that inflammation in septic patients is associated with increased hepcidin-25 concentrations. Moreover, the elevated hepcidin
concentrations observed in early sepsis negatively correlated with Hb levels during the hospital stay of these patients. These human in vivo correlations suggest that hepcidin release is a modulator of anemia in septic patients with systemic inflammation.

Key messages
- IL-6 concentrations and number of SIRS criteria present in septic patients are associated with increased hepcidin-25 concentrations.
- The increase in hepcidin concentrations observed in early sepsis correlates with the decrease in Hb levels during their hospital stay and patients with higher hepcidin concentrations tend to need more blood transfusions.
- The inflammation-hepcidin release-anemia pathway is present in patients with sepsis.

Abbreviations
Hb: hemoglobin; IL-6: interleukin 6; MCH: mean cell hemoglobin; MCV: mean corpuscular volume; RDW: red cell distribution width; SIRS: systemic inflammatory response syndrome; TOF-MS: time-of-flight mass spectrometry.

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Authors’ contributions
LTE participated in data collection, performed the statistical analysis and drafted the manuscript. JCK performed hepcidin measurements and participated in drafting the manuscript. MT collected demographic and SIRS data, built the database and collected the blood samples. JGH revised the manuscript and participated in the design of the study. DWS revised the manuscript and participated in the design of the study and was responsible for hepcidin measurements. PP conceived of the study, participated in its design and coordination and helped to draft the manuscript.

Competing interests
The authors declare that they have no competing interests.

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