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HMGB1 as a predictor of organ dysfunction and outcome in patients with severe sepsis

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Abstract *Objective:* To study the
predictive value of high mobility
group box-1 protein (HMGB1) and
hospital mortality in adult patients
with severe sepsis. *Study design:*
Prospective observational cohort
study in 24 ICUs in Finland.
Patients: Two hundred and forty-
seven adult patients with severe sep-
sis. *Measurements and main results:*
Blood samples for HMGB1 analy-
ses were drawn from 247 patients
at baseline and from 210 patients
72 h later. The mean APACHE II
and SAPS II scores were 24 (SD 9)
and 44 (SD 17), respectively. The
hospital mortality was 26%. The
serum HMGB1 concentrations were
measured first by semi-quantitative
Western immunoblotting (WB)
analysis. The median HMGB1
concentration on day 0 was 108%
(IQR 98.5–119) and after 72 h 107%
(IQR 98.8–120), which differed
from healthy controls (97.5%, IQR
91.3–106.5; $p = 0.028$ and 0.019,
respectively). The samples were re-
analysed by ELISA (in a subgroup of
170 patients) to confirm the results
by WB. The median concentration in
healthy controls was 0.65 ng/ml (IQR
0.51–1.0). This was lower than in
patients with severe sepsis (3.6 ng/ml,
IQR 1.9–6.5, $p < 0.001$). HMGB1
concentrations (WB and ELISA) did
not differ between hospital survivors
and non-survivors. In ROC analyses
for HMGB1 levels (WB) on day 0 and
72 h with respect to hospital mortality,
the areas under the curve were 0.51
and 0.56 (95% CI 0.40–0.61 and
0.47–0.65). *Conclusions:* Serum
HMGB1 concentrations were elevated
in patients with severe sepsis, but
did not differ between survivors and
non-survivors and did not predict
hospital mortality.

Keywords HMGB1 · Severe sep-
sis · Septic shock · Organ failure ·
Mortality · Outcome

Introduction

The prognosis of severe sepsis can be improved, as shown
by recent studies, but severe sepsis still continues to
have a mortality as high as 30% [1, 2]. Numerous studies
have emphasized the important role of proinflammatory
cytokines in sepsis. Despite their evident role in the
pathogenesis of severe sepsis and septic shock, attempts
to improve the prognosis of sepsis by inhibiting 'early'
cytokine-mediated inflammatory process have failed [3, 4].
Recent studies have suggested that high mobility group
box-1 protein (HMGB1), a DNA-binding intranuclear

protein, is a late activator of the inflammatory cascade when released into the extracellular space [5]. It has even been speculated that HMGB1 might be a target for anti-inflammatory treatment in severe sepsis and septic shock [6].

In an animal model of sepsis, the HMGB1 response is delayed, occurring at 16 h and remaining elevated at least 36 h after lipopolysaccharide (LPS) injection [5]. In vitro, macrophages and monocytes can be stimulated to secrete TNF with either LPS or HMGB1 [5, 7]. However, HMGB1 induces TNF release later than LPS (first peak 2–3 h after stimulation). The TNF-response to HMGB1 is also biphasic, with a second peak occurring 8–10 h after exposure [7]. The in vivo effects of HMGB1 are in line with those in vitro. In the lungs the intratracheal instillation causes cytokine release and pulmonary oedema [8], and in the gastrointestinal tract the intestinal barrier integrity is lost after exposure of HMGB1 [9]. In addition, a recent study in an animal model of acute lung injury suggests that HMGB1 may be an important mediator in ventilator-induced lung injury. Moreover, by blocking HMGB1, the lung injury can be attenuated [10]. There are only a few human studies on the role of HMGB1 in severe sepsis. In a rather heterogeneous population of sepsis patients, HMGB1 concentrations seem to be elevated for at least 1 week after admission, but there was no association between HMGB1 concentrations and severity of illness [11]. Originally, HMGB1 was measured in patients with severe sepsis by Western immunoblotting (WB) [5, 11]. Recently, an enzyme-linked immunosorbent assay (ELISA) has been introduced [12, 13].

The aim of this study was to evaluate the value of HMGB1 in the prediction of organ failure and hospital mortality in an adult patient population with severe sepsis and septic shock. In addition, we evaluated the relation of HMGB1 to severity of illness and development and type of organ dysfunction.

Patients and methods

Patient selection

This study was a part of the Finnsepsis study, a prospective observational cohort study of incidence and outcome of severe sepsis in Finland [14]. All adult consecutive ICU admission episodes (4500) in 24 intensive care units were screened for severe sepsis over a 4-month period (from 1 November 2004 to 28 February 2005). Patients were eligible if they fulfilled the American College of Chest Physicians/Society of Critical Care Medicine (ACCP/SCCM) criteria for severe sepsis or septic shock [15]. Study entry (day 0) was the time when these criteria were first met. All patients or their next of kin gave written consent for participation in the study. APACHE II (Acute Physiology, Age, Chronic Health Evaluation) and SAPS II (Simplified Acute Physiology Score) scores [16, 17], organ dysfunction as evaluated with the SOFA (Sequential Organ Failure Assessment) score and maximum SOFA scores [18, 19], and ICU and hospital mortalities were recorded.

Blood samples

Arterial blood samples for HMGB1 analyses were drawn after informed consent within 24 h of the study entry (day 0) and 72 h thereafter. The reason for exclusion was failure to obtain consent. Blood for serum samples was collected in glass vacuum tubes without gel separation (BD Vacutainer 369032) and the samples were prepared within 60 min of sampling. More specifically, after 30–45 min of serum separation at room temperature the samples were centrifuged at 3,100 rpm ($\sim 1,500\,g$) for 15 min. The supernatant was pipetted carefully to polypropylene tubes, avoiding contamination from the buffy coat. The samples were stored at –80°C for later analysis. Serum samples from healthy volunteers ($n = 10$) were analysed as controls. A pooled sample was made from healthy volunteers.

The blood samples of healthy controls and day 0 samples of a subgroup of patients ($n = 170$) were also analysed by ELISA (HMGB1 ELISA subgroup). This subgroup was selected from patients with sufficient original blood samples for a re-analysis. Random Number Generator (SPSS 15.0) was used to create a subgroup in proportion of survivors (74%) and non-survivors (26%). All results refer to the original HMGB1 analyses with Western immunoblotting method unless mentioned otherwise (HMGB1 ELISA subgroup).

Details of the analyses by these methods are presented in the ESM.

Statistical analyses

Data are presented as medians and interquartile range (25th–75th percentile; IQR), absolute values and percentages, or means $\pm$ SD. The nonparametric data of survivors and non-survivors were compared with the Mann–Whitney U test and categorical variables with the chi-square test. To determine the prognostic accuracy of HMGB1 at both time points, the receiver operating characteristic (ROC) curves were constructed and the areas under the curve (AUC) with 95% confidence intervals (CI) were calculated. The Spearman correlation was used to test the relations between the estimated sepsis onset time and HMGB1 concentrations. The level of $p < 0.05$ was considered statistically significant in all tests. The analyses were performed using the SPSS 15.0 software (SPSS, Chicago, IL, USA).

Results

Blood samples for the HMGB1 analysis were obtained from 247 out of 470 (52.6%) patients of the whole Finnsepsis study population. Two-hundred and forty seven samples were obtained at baseline (day 0) and 210 samples were taken 72 h later. Fourteen patients died before the second sample was obtained. The HMGB1 concentration from one patient could not be measured for technical reasons. For 22 patients, samples were not available. For the ELISA analysis, 228/247 (92%) blood samples were available. For practical reasons, a HMGB1 ELISA subgroup of 170 patients was randomly selected in proportion to survivors (126/170, 74%) and non-survivors (44/170, 26%). Basic characteristics, disease severity, organ failure and mortality in the HMGB1 study group were similar to other Finnsepsis patients except that there were more patients with community-acquired infections in the HMGB1 group (Table 1). The HMGB1 ELISA subgroup did not differ from the original HMGB1 patient group in age ($p = 0.15$), sex ($p = 0.61$), APACHE II ($p = 0.88$) and SAPS II ($p = 0.12$) scores, SOFA scores (day 1 and SOFAmax, $p = 0.26$ and $p = 0.71$, respectively), or hospital mortality ($p = 0.99$). The mean age of the healthy controls was 34 ± 6 years (M/F 5/5).

In septic patients, the median HMGB1 concentration on day 0 was 108% (IQR 98–119) and after 72 h 107% (IQR 99–120). HMGB1 concentrations were higher than the median HMGB1 concentration 97.5% (IQR 91.3–106.5) in healthy controls at both time points ($p = 0.028$ and $p = 0.019$, respectively). There were no differences in HMGB1 values between males and females (day 0 $p = 0.89$; 72 h $p = 0.06$). Age also did not have any influence on HMGB1 levels ($p = 0.89$ and $p = 0.98$).

HMGB1 concentrations in survivors, non-survivors and healthy controls on day 0 are presented in Fig. 1a. The median HMGB1 concentrations did not differ between survivors (108%, IQR 99–118) and non-survivors (106%, IQR 98–122, $p = 0.93$) on day 0. Accordingly, no difference between survivors and non-survivors was detected at 72 h [107%, (IQR 99–121) vs. 104% (IQR 98–116), $p = 0.24$]. In ROC analyses with death as the outcome, the areas under the curve (AUC) for HMGB1 concentrations on day 0 and at 72 h were 0.51 and 0.56 (95% CI 0.40–0.61 and 0.47–0.65), respectively.

The HMGB1 concentrations on day 0 were not higher in patients with severe organ dysfunction (maximum SOFA score 3–4) than in milder dysfunction in different organ systems (Table 2). Patients with the most severe cardiovascular failure (SOFA 4; $n = 98$) had lower HMGB1 concentrations at 72 h than on day 0 [103% (IQR 96–114) vs. 106% (IQR 97–117), $p = 0.001$]. However, in the ROC analysis, this was not predictive for hospital mortality (AUC 0.45, 95% CI 0.32–0.62, $p = 0.65$). Patients with severe haematological failure (SOFA 3–4) also had lower HMGB1 concentrations at 72 h than on day 0 [103 % (IQR 96–115) vs. 109% (IQR 97–118), $p = 0.032$]. Organ dysfunction in other systems did not have any effect on HMGB1 concentrations between day 0 and 72 h. HMGB1 levels on day 0 and at 72 h and corresponding SOFA

**Table 1** Comparison of HMGB1 study cohort with the rest of the Finnsepsis study patients

	HMGB1 patients	Other Finnsepsis patients	p
Number of patients	247	223	
Age	59.3 (15.6)	59.9 (14.8)	0.68
Male	171 (69.2%)	145 (65%)	0.35
APACHE II	23.8 (9.1)	24.6 (9.0)	0.36
SAPS II	43.5 (16.9)	46.0 (16.8)	0.14
SOFA on day 1	8.3 (3.7)	8.5 (3.6)	0.68
SOFAmax	10.9 (4.3)	10.4 (4.4)	0.33
Infection type			
Community acquired	161 (65.2%)	116 (52.0%)	0.015
Hospital acquired	80 (32.4%)	100 (44.8%)	0.015
Source of infection			
Pulmonary	100 (40.5%)	98 (43.9%)	0.46
Intra-abdominal	82 (33.2%)	68 (30.5%)	0.53
Skin or soft tissue	24 (9.7%)	24 (10.8%)	0.71
Urinary tract	11 (4.5%)	11 (4.9%)	0.81
Other	34 (13.8%)	29 (13.0%)	0.81
Blood culture positive	70 (28.3%)	58 (26.0%)	0.57
Postoperative admission	65 (26.3%)	71 (31.8%)	0.19
ICU mortality	33 (13.4%)	40 (17.9%)	0.17
Hospital mortality	65 (26.3%)	68 (30.5%)	0.32

Data are presented as absolute values (percentages) or mean (standard deviation).
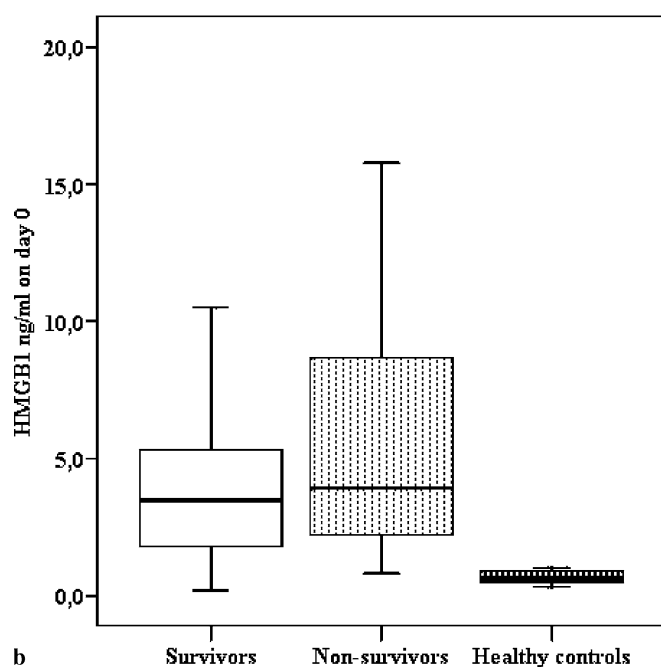*APACHE*, Acute Physiology, Age and Chronic Health Evaluation; *SAPS*, Simplified Acute Physiology Score; *SOFA*, Sequential Organ Failure Assessment score on the following day after study entry; *SOFAmax*, maximum SOFA score
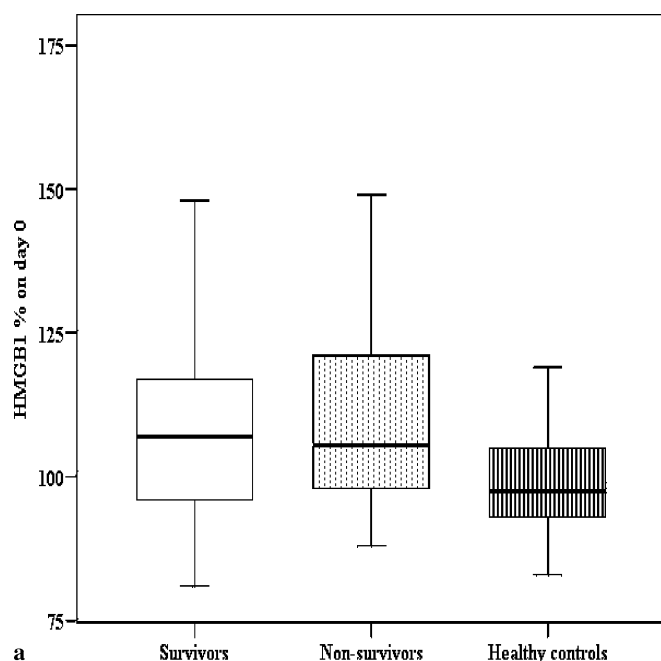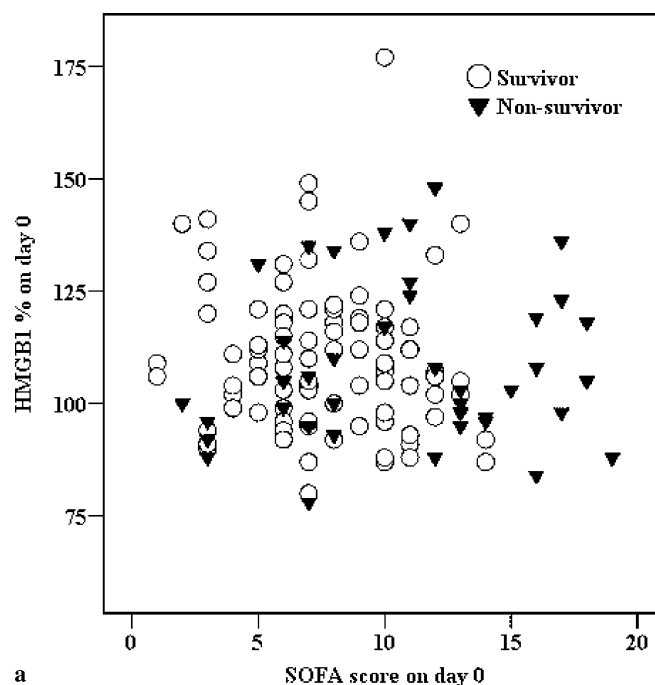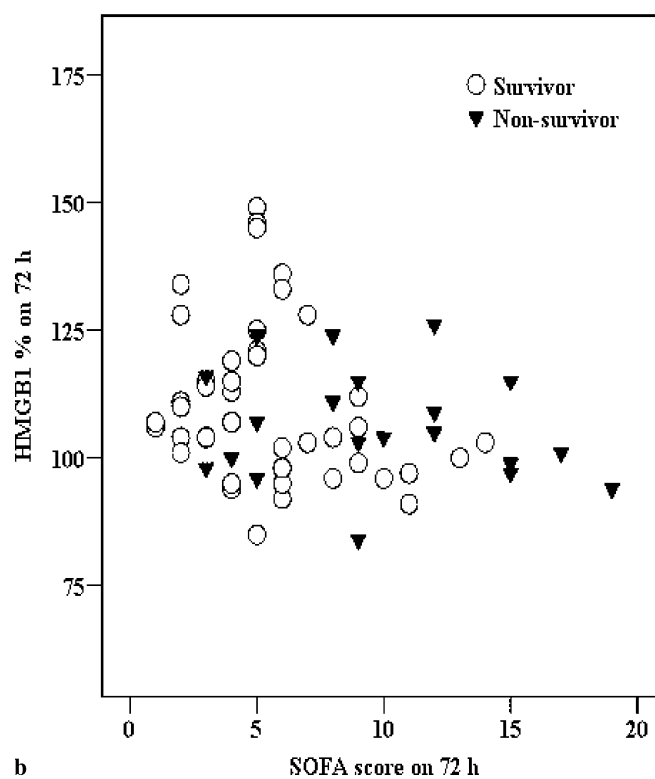
Fig. 1 HMGB1 levels on day 0 in survivors, non-survivors and healthy controls as measured by **a** Western immunoblotting (WB; %) or **b** ELISA (ng/ml). There was no difference in HMGB1 levels between survivors and non-survivors ($p = 0.93$ for WB, $p = 0.17$ for ELISA), but both survivors and non-survivors had higher HMGB1 levels than healthy controls (respectively $p = 0.032$ and $p = 0.048$ for WB; $p < 0.001$ and $p < 0.001$ for ELISA)

Fig. 2 HMGB1 levels on day 0 and at 72 h and corresponding SOFA scores at both time points in survivors and non-survivors

scores in both survivors and non-survivors are shown in Fig. 2.

HMGB1 concentrations were associated with the severity of the total score of organ dysfunction for differ-ent organ systems only in patients with highest SOFAmax quartile (SOFAmax score 14–24). Patients with more severe organ failure had lower HMGB1 concentrations on 72 h (103%, IQR 96–115) than patients with milder

Table 2 HMGB1 levels (%) according to the grade of organ dysfunction. Concentrations are presented as median (interquartile range). The *p* value refers to HMGB1 concentrations on day 0 between organ dysfunction groups (SOFAmax 0–2 and SOFAmax 3–4)

Organ dysfunction	*n*	SOFAmax 0–2	*n*	SOFAmax 3–4	*p*
Cardiovascular	56	112.5 (98.3–123.0)	170	106 (98–117.3)	0.06
Respiratory	70	110.5 (97.8–121.3)	156	106.5 (98–117.8)	0.20
Haematological	166	108 (98–119)	60	108.5 (97–118)	0.73
Renal	177	107 (98–118)	49	112 (97.5–122)	0.39
Hepatic	214	108.5 (98–119)	12	102 (92.5–117.3)	0.37

organ failure (SOFAmax score 1–7; 112%, IQR 101–129, $p = 0.005$). HMGB1 levels on day 0 did not differ between patients with positive and negative blood cultures at either time point ($p = 0.83$ and $p = 0.19$).

In those patients who survived beyond 72 h, the change in HMGB1 concentration (ΔHMGB1; HMGB1 72 h – HMGB1 day 0) was positive in 53.8% ($n = 113$) and negative in 46.2% ($n = 97$). There was no difference in mortality between patients with increasing HMGB1 (24/113, 21.2%) and those with decreasing HMGB1 (21/101, 20.8%; $p = 0.94$), but increasing ΔSOFA (SOFAmax–SOFA score on day 1) had a clear association with mortality ($p < 0.001$, Mann–Whitney). There was a correlation between SOFA scores and HMGB1 concentrations at the first time point the blood samples were taken ($p < 0.001$), but not at 72 h ($p = 0.87$). The duration of severe sepsis before the hospital admission and the study entry could be estimated in 149 patients with community-acquired infection. No association was found between the HMGB1 concentrations on day 0 and the different times of onset of community-acquired severe sepsis ($r = 0.11$ by Spearman, $p = 0.18$) or with the survival ($p = 0.64$) (Fig. 3).

The healthy controls had a very low median concentration of HMGB1 (0.65 ng/ml, IQR 0.51–1.0) as analysed by ELISA. In contrast, the ELISA subgroup had a median HMGB1 concentration of 3.6 ng/ml (IQR 1.9–6.5, $p < 0.001$). The median HMGB1 concentrations also did not differ between survivors (3.6 ng/ml, IQR 1.8–5.8) and non-survivors (3.9 ng/ml, IQR 2.1–8.7; $p = 0.17$) in the ELISA subgroup (Fig. 1b). In ROC analyses with death as the outcome, the AUC for HMGB1 concentrations in the HMGB1 ELISA subgroup was 0.57 (95% CI 0.47–0.67).

Discussion

Finnsepsis was a prospective observational study that covered almost the entire (90.6%) adult population of Finland [14]. The incidence of sepsis was somewhat lower than in previous studies, and the outcome of severe sepsis (ICU mortality of 15.5% and hospital mortality of 28.3%) was comparable with recent international studies. The main finding of this study was that even though HMGB1 increases moderately in patients with severe sepsis and septic shock compared with healthy volunteers, it does not have a predictive value for organ failure and outcome.

HMGB1, originally named amphoterin [20], is considered an important late-phase cytokine in sepsis, as suggested by Tracey and colleagues [5]. It is secreted by immunostimulated macrophages and monocytes and, as described quite recently, intestinal epithelial cells [9]. HMGB1 is released from necrotic cells, but not from apoptotic cells [21]. So far, most of the data available showing that high HMGB1 is associated with the severity of sepsis are from animal studies. HMGB1 has been shown to be associated with shock [5] and acute lung injury [8, 22]. Ogawa et al. showed that ventilation with high tidal volumes increased HMGB1 concentrations in bronchoalveolar lavage fluid. Blocking HMGB1 with anti-HMGB antibody attenuated lung injury [10]. The kinetics of HMGB1 release may differ depending on the source of infection, and HMGB1 release also may occur at the site of infection (abdominal fluid in peritonitis, alveolar fluid in pneumonia) [23].
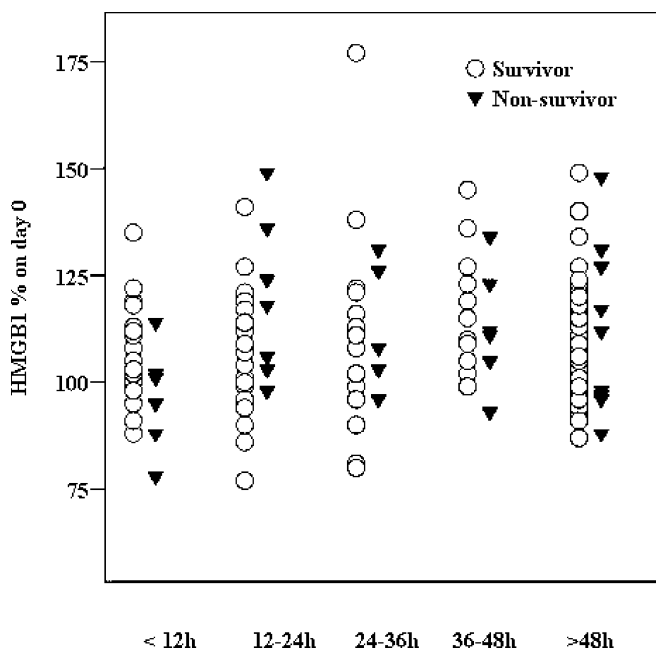

Fig. 3 Estimated time of onset of severe sepsis before hospital admission and HMGB1 levels on day 0. The onset of sepsis was estimated by local investigators at the study sites according to the time the first symptoms leading to community-acquired severe sepsis had begun

In human studies, high HMGB1 concentrations as measured by WB have been associated with increased mortality in severe sepsis only in one study with 25 patients [5]. The first study to investigate HMGB1 and its release and kinetics in severe sepsis patients was published recently [11] and it showed that HMGB1 stays elevated up to 1 week. There was no correlation of HMGB1 with disease severity or mortality. It has also been found that high HMGB1 levels were elevated in patients with severe acute pancreatitis. In that study, HMGB1 concentrations correlated with the degree of organ dysfunction, but not with mortality [24]. In surgical patients, high preoperative HMGB1 concentrations have been reported to be associated with sepsis and acute lung injury [25].

Our results agree with earlier human sepsis studies in which HMGB1 concentrations were elevated in severe sepsis patients but had no association with the outcome [11, 23]. However, our finding that in patients with septic shock HMGB1 concentrations were actually lower in more severe than in milder cardiovascular dysfunction differs from earlier data published by Sunden-Cullberg et al. [11]. In that study ($n = 33$) there was no difference in HMGB1 concentrations between patients with septic shock and patients with severe sepsis. In a recent study by Gibot et al., septic shock non-survivors had higher HMGB1 concentrations than survivors on day 3, but not at the time of hospital admission [26]. Although the HMGB1 ROC curve on day 1 did not predict mortality, the ROC curve on day 3 showed an AUC of 0.71 (95% CI 0.51–0.91). The SOFA scores and HMGB1 concentrations were also positively correlated in patients with septic shock. Unexpectedly, our patients with the most severe organ failure had lower HMGB1 concentrations than patients with milder organ dysfunction.

It has been postulated that apoptotic cell death may trigger sepsis-induced anergy and hence influence the function of the surviving immune cells [27–29]. It can be speculated that some of the patients in our study have had sepsis-induced apoptotic cell deaths instead of necrotic cell deaths. This could affect HMGB1 levels in the most severely ill patients with high SOFAmax scores.

Recent studies in patients with community-acquired pneumonia have found that patients with severe sepsis did not have higher HMGB1 levels than other patients with sepsis [30]. This is in agreement with our study, because we found no correlation between organ dysfunction and HMGB1 levels. Angus et al. found no significant increase or decrease in HMGB1 levels within 1 week, which confirms that our two samples taken within 72 h are representative [31]. However, we could not confirm higher HMGB1 levels in non-survivors than in survivors, as reported in other studies [30, 32].

WB for HMGB1 is time consuming and semi-quantitative, but at the time, this was the only available method for our research group to use. The method may have its limitations, because of possible cross-reaction with light chains of immunoglobulins as suggested by the other investigators [11]. Therefore, the baseline concentrations or samples from healthy volunteers give somewhat higher values than the method described by Parkkinen et al. [20]. Unfortunately, at the time, even after several attempts, we could not obtain a pure HMGB1 control. At the time of our investigation, commercial ELISA kits were not available. Since the time of the analyses, new ELISA methods have been published [13]. These methods are prone to the same confounding factors, immunoglobulins in general and HMGB1-binding anti-HMGB1 antibodies in particular [33]. We decided to use this newly available method in a subgroup of patients with severe sepsis and healthy volunteers to confirm our results by WB showing that HMGB1 levels are not elevated in healthy subjects. The results obtained by WB were indeed confirmed by ELISA. Therefore, the primary methodology (WB) used in the present investigation can be considered adequate.

Our study has other limitations. The samples were taken only at two time points, and patients could have been at different phases in the course of their sepsis. However, the time of onset of sepsis can only be estimated. The first sample was taken when patients met the criteria for severe sepsis or septic shock. In experimental endotoxin shock, HMGB1 levels increase after 8 h and remain elevated for at least 36 h [7]. In the present study, the onset time of sepsis could be estimated only in patients with community-acquired sepsis. The number of patients with a time of less than 12 h from onset of symptoms to ICU admission was very small. Therefore, it is conceivable that this 72 h time window was sufficient to detect a HMGB1 response if present.

In conclusion, although there is solid evidence demonstrating the importance of HMGB1 as a mediator of inflammation and as a marker of the severity of organ dysfunction, HMGB1 concentrations were only moderately elevated in severe sepsis patients, did not differ between survivors and non-survivors, and did not predict hospital mortality in patients with severe sepsis.

Appendix: Participants of the Finnsepsis Study

Hospital	Investigator	Hospital	Investigator
Satakunta Central Hospital	Dr. Vesa Lund	Kymenlaakso Central Hospital	Dr. Jussi Pentti, Dr. Seija Alila
Savonlinna Central Hospital	Dr. Markku Suvela		
Central Finland Central Hospital	Dr. Raili Laru-Sompa	Helsinki University Hospital	Dr. Ville Pettilä, Dr. Marjut Varpula, Dr. Marja Hynninen
South Savo Central Hospital	Dr. Heikki Laine		
North Karelia Central Hospital	Dr. Sari Karlsson		
Seinäjoki Central Hospital	Dr. Kari Saarinen	Helsinki University Hospital (Jorvi)	Dr. Tero Varpula
South Karelia Central Hospital	Dr. Seppo Hovilehto	Helsinki University Hospital (Peijas)	Dr. Rita Linko
Päijät-Häme Central Hospital	Dr. Pekka Loisa	Tampere University Hospital	Dr. Esko Ruokonen, Dr. Pertti Arvola
Kainuu Central Hospital	Dr. Tuula Korhonen		
Vaasa Central Hospital	Dr. Pentti Kairi	Kuopio University Hospital	Dr. Ilkka Parviainen
Kanta-Häme Central Hospital	Dr. Ari Alaspää	Oulu University Hospital	Dr. Tero Ala-Kokko, Dr. Jouko Laurila
Lappi Central Hospital	Dr. Outi Kiviniemi		
Central Pohjanmaa Central Hospital	Dr. Tadeusz Kaminski	Länsi-Pohja Central Hospital	Dr. Jorma Heikkinen

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