

# High-mobility group box 1 as a surrogate prognostic marker in dogs with systemic inflammatory response syndrome

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## Abstract

**Objective** – To evaluate various surrogate markers associated with the inflammatory and counter-inflammatory responses with respect to mortality in dogs with systemic inflammatory response syndrome (SIRS).

**Design** – Prospective observational study.

**Setting** – Veterinary Teaching Hospital.

**Animals** – Twenty-eight dogs with naturally occurring diseases and SIRS from January 2007 to May 2009.

**Interventions** – Upon admission to the veterinary hospital, history and baseline data from the physical examination, including parameters previously defined for meeting SIRS criteria, were documented. Heparinized blood samples were collected and plasma cytokines interleukin-6 (IL-6), IL-10, and high-mobility group box 1 (HMGB1) were measured by sandwich ELISA.

**Measurements and Main Results** – In nonsurvivors, median plasma HMGB1 concentrations (0.718 µg/L, interquartile range [IQR]; 0.300–1.626 µg/L) and the ratio of HMGB1 to IL-10 (2.236, IQR; 0.972–5.367) were significantly increased as compared with those found in survivors (0.300 µg/L, IQR; 0.300–0.312 µg/L for HMGB1; 1.017, IQR; 0.862–1.126 for the ratio of HMGB1 to IL-10,  $P = 0.007$  and  $0.024$ , respectively). Plasma IL-6, IL-10, and the ratio of IL-6 to IL-10 were not significantly different between groups. Among the parameters studied, HMGB1 and the ratio of HMGB1 to IL-10 performed the best in discriminating outcome in dogs with SIRS according to receiver operator characteristic curve analysis.

**Conclusions** – Increases in plasma HMGB1 concentration and the ratio of HMGB1 to IL-10 may predict poorer outcomes in dogs with SIRS. The approach described may lead to reliable prognostic biomarkers and new therapeutic concepts in the study of SIRS in dogs.

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## Introduction

Systemic inflammatory response syndrome (SIRS) is a massive and global immune response to challenges of infectious or noninfectious origin.<sup>1</sup> The pathophysiology

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of SIRS is complex and numerous factors within the host's immune system interact to determine outcome.<sup>2</sup> Evidence suggests that endogenous cytokines have a major role in SIRS pathogenesis and that overstimulation of cytokine production contributes to poor outcomes.<sup>3–7</sup> However, further evidence suggests that SIRS outcome is not simply the consequence of excessive proinflammatory cytokine production, but rather an imbalance of pro- and anti-inflammatory mediators, suggesting a failure of inherent, protective mechanisms. Further knowledge of the factors influencing outcome in SIRS may lead to new diagnostic techniques and therapies for this life-threatening condition.<sup>8</sup> Thus, identification of biomarkers that could quantify disease severity and predict patient prognosis would be beneficial in both research studies and clinical applications.

Animal models and a human cohort study support the use of several markers of SIRS, including interleukin-1 (IL-1), tumor necrosis factor- $\alpha$ , IL-6, IL-10, C-reactive protein and lipopolysaccharide-binding protein to predict mortality in patients with SIRS.<sup>9–11</sup> In addition, ratios of proinflammatory to anti-inflammatory cytokines have been examined as prognostic indicators.<sup>12</sup> Recent studies have suggested that high-mobility group box 1 (HMGB1), a DNA-binding intranuclear protein, is a late activator of the inflammatory cascade that is released from necrotic cells into the extracellular milieu.<sup>13–15</sup> It acts as a damage-associated molecular pattern or *alarmin*, stimulating the release of numerous proinflammatory cytokines by human monocytes.<sup>15</sup> HMGB1 levels correlate with hospital mortality and also with the organ failure scores in human septic patients.<sup>13,16,17</sup>

In clinical veterinary medicine, a few studies have attempted to identify prognostic markers of SIRS in dogs.<sup>18–21</sup> Otto et al<sup>21</sup> reported that an increase in plasma tumor necrosis factor- $\alpha$  activity was predictive of mortality in naturally occurring parvoviral enteritis. In another report, plasma IL-6 levels correlated with disease severity and mortality in canine SIRS and sepsis.<sup>3</sup> Although measurement of HMGB1 concentration has shown promise in humans and laboratory animals, to our knowledge, HMGB1 has not been investigated as a biomarker in naturally occurring SIRS in dogs.

The purpose of the present study was to examine HMGB1 as well as IL-6 as prognostic surrogate markers for mortality in dogs with SIRS. In addition, because outcome in dogs with SIRS may be associated with imbalances of pro- and anti-inflammatory cytokines, we also evaluated the ratios of HMGB1 to IL-10 and IL-6 to IL-10.

## Materials and Methods

This was a prospective, observational study of dogs with SIRS. Eligible subjects were derived from the entire canine population hospitalized by one service of the Department of Internal Medicine from January 2007 to May 2009. Diagnostic criteria for SIRS were based on the study by de Laforcade et al<sup>22</sup> and a diagnosis of SIRS required 2 or more of the following: hypo- or hyperthermia ( $<37.8^{\circ}\text{C}$  or  $>39.4^{\circ}\text{C}$ ), tachycardia (heart rate  $>140/\text{min}$ ), tachypnea (respiratory rate  $>20/\text{min}$ ), leukocytosis ( $>16 \times 10^9$  cells/L [ $>16 \times 10^3$  cells/ $\mu\text{L}$ ]) or leukopenia ( $<6 \times 10^9$  cells/L [ $<6 \times 10^3$  cells/ $\mu\text{L}$ ]) or  $>3\%$  band forms in the WBC count. The present study was inclusive of infectious and noninfectious causes of SIRS. Before enrollment in this study, informed consent was obtained from each owner. All aspects of this study

were approved by the Committee on Bioethics at our institution.

Details regarding patient history, onset of clinical signs, and baseline data from the physical examination were recorded. Blood was immediately collected by venipuncture into heparin-containing tubes and the separated plasma was stored at  $-80^{\circ}\text{C}$  until analysis. Mortality was defined as death or euthanasia due to poor prognosis. Survival was defined as discharge from the hospital.

## Laboratory assays

Plasma IL-6 and IL-10 were measured by sandwich ELISA based on commercially available matched canine antibody pairs.<sup>a</sup> Because the molecular structure of canine HMGB1 is identical to that of the human molecule,<sup>23</sup> a sandwich ELISA for human HMGB1<sup>b</sup> was used for the quantitative determination of canine HMGB1 concentrations in plasma. Absorbances were measured at A450–A540 with a microplate spectrophotometer<sup>c</sup> and sample concentrations were calculated<sup>d</sup> from standard curves of the commercially available proteins. The lower limits of assay detection were  $0.063 \mu\text{g}/\text{L}$  ( $0.063 \text{ ng}/\text{mL}$ ) for IL-6,  $0.031 \mu\text{g}/\text{L}$  ( $0.031 \text{ ng}/\text{mL}$ ) for IL-10, and  $0.300 \mu\text{g}/\text{L}$  ( $0.300 \text{ ng}/\text{mL}$ ) for HMGB1. Values below the detection limit of the assay were assigned the default values of  $0.063$  (IL-6),  $0.031$  (IL-10), and  $0.300$  (HMGB1)  $\mu\text{g}/\text{L}$  ( $\text{ng}/\text{mL}$ ) for statistical analysis. Sample assays were performed in duplicate. For negative controls, cytokine concentrations were measured in 12 clinically healthy beagles (1–2 years of age).

## Statistical analysis

Data are presented as the median and interquartile range (IQR) or as the mean (SD). A Shapiro-Wilk test was used to verify normality of the data.<sup>e</sup> Cytokine concentrations in survivors and nonsurvivors were compared by Mann-Whitney *U*-test. A two-tailed *P*-value  $<0.05$  was considered statistically significant. Receiver operator characteristic curves and the area under the curve were also calculated for the prognostic markers evaluated. Ninety-five percent confidence intervals (CIs) were reported for the area under the curve.

## Results

During the study period, 142 client-owned dogs were considered for inclusion in the study. Twenty-eight dogs of 13 different breeds met inclusion criteria and were enrolled. Twelve of 28 (43%) dogs were male, 2 of 12 were castrated, and 16 of 28 (57%) were female, 3 of 16 were spayed. The dogs had a median age of 5 years (range, 2 mo to 14 y). Dogs included in study had the

following diagnoses: trauma (eg, bite wounds or motor vehicle accident;  $n = 4$ ), pyometra ( $n = 3$ ), canine parvoviral enteritis ( $n = 3$ ), heart failure ( $n = 2$ ), peritonitis from gastrointestinal tract perforation ( $n = 2$ ), and 1 of each of the following, canine distemper virus infection, bacterial glomerulonephritis, acute hepatitis, pulmonary thromboembolism, gastroenteritis sequel to foreign body ingestion, fever of unknown origin, bacterial mastitis, acute pancreatitis, Cushing's syndrome, severe hemorrhage, immune-mediated hemolytic anemia, rectal prolapse, ethylene glycol toxicosis, and bacterial pyoderma. The mean (SD) for body temperature, pulse rate, respiration rate, and WBC counts were 39.3°C (1.1°C), 143/min (22/min), 43/min (11/min), and  $19.1 \times 10^9$  cells/L ( $13.9 \times 10^9$  cells/L), respectively. The overall in-hospital mortality rate was 64.3% (18/28) for the 28 dogs included in this study. Of the nonsurvivors, only 1 case was euthanized, a dog with acute hepatitis and liver failure. When survivors and nonsurvivors were compared, none of the physical parameters showed significant differences between the 2 groups.

There was no detectable IL-6 or HMGB1 in plasma of healthy control dogs. Median plasma HMGB1 concentrations in nonsurvivors (0.718 µg/L, IQR; 0.300–1.626 µg/L) were significantly higher than those found in survivors (0.300 µg/L, IQR; 0.300–0.312 µg/L,  $P = 0.007$ ). In nonsurvivors, the mean plasma IL-6 concentration (0.126 µg/L, IQR; 0.063–1.348 µg/L) was twice the concentration found in the survivors (0.063 µg/L, IQR; 0.063–0.289 µg/L); however, this difference was not significantly different between the groups ( $P = 0.252$ ). The mean plasma IL-10 concentration was not statistically different between groups ( $P = 0.415$ ). The ratio of HMGB1 to IL-10 was significantly increased in the nonsurvivors as compared with survivors ( $P = 0.024$ ), whereas the ratio of IL-6 to IL-10 did not differ significantly between groups ( $P = 0.270$ ) (Table 1 and Figure 1).

In receiver operator characteristic curve analysis with death as the outcome, the following area under the curve values were calculated: IL-6 – 0.622 (95% CI, 0.416–0.829); IL-10 – 0.594 (95% CI, 0.361–0.828); IL-6:IL-10 – 0.628 (95% CI, 0.417–0.838); HMGB1 – 0.794 (95% CI, 0.631–0.958); and HMGB1/IL-10 – 0.761 (95% CI, 0.581–0.941). HMGB1 and HMGB1/IL-10 were found to be the best discriminator between survivors and nonsurvivors in this comparative receiver operator characteristic curve analysis (Figure 2).

## Discussion

To the authors' knowledge this is the 1st report to relate initial plasma HMGB1 concentrations to prognosis in dogs with SIRS. Dogs with SIRS and increased plasma concentration of HMGB1 were associated with worse outcome regardless of the etiology of SIRS. Similar to the findings in our study, Wang *et al*<sup>13</sup> found that human patients who died with sepsis-associated SIRS had significantly higher HMGB1 concentrations than survivors.

The prognostic value of measuring HMGB1 may be due, in part, to its kinetics. After LPS administration in a murine model of endotoxemia, serum HMGB1 concentrations peaked at 16–32 hours, which is later when compared with other cytokines, including IL-6. Wang *et al*<sup>13</sup> described HMGB1 as a late mediator of endotoxin lethality. In a human cohort study concerning the kinetics of HMGB1, plasma HMGB1 concentrations remained high in a majority of the septic patients for up to 1 week after hospitalization.<sup>17</sup> The ability to measure a surrogate marker late in the course of disease may offer distinct advantages in veterinary medicine because few veterinary clinical patients are presented to veterinarians within a short time from the onset of clinical signs. The results of the present study demonstrate that a HMGB1 response can be readily detected

**Table 1:** Selected parameters in survivors and nonsurvivors among 28 dogs with SIRS

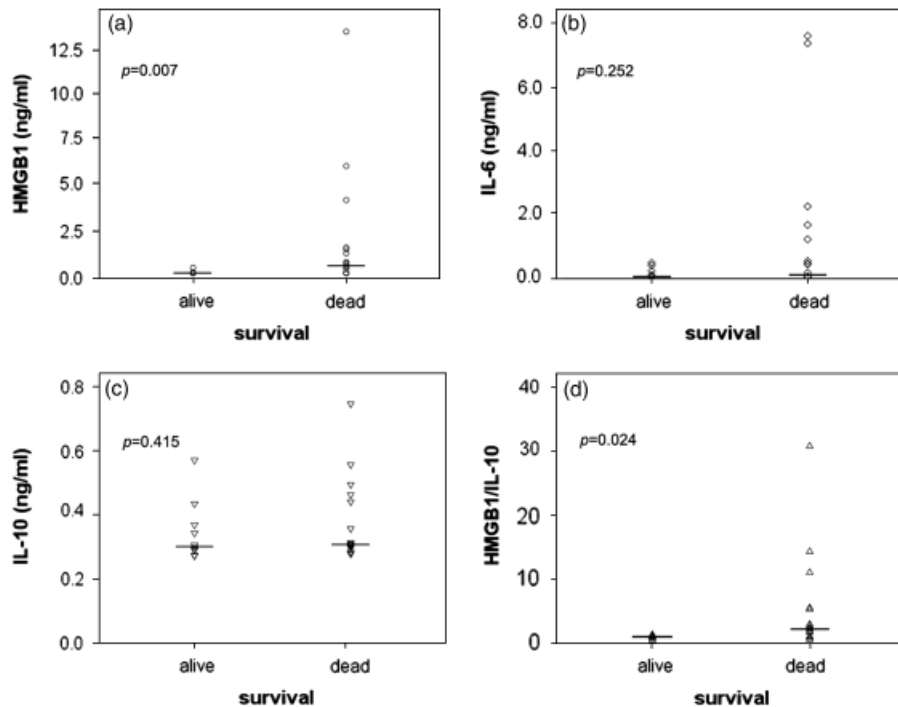
Variable	Survivors ( $n = 10$ )	Nonsurvivors ( $n = 18$ )	P-value
T (°C)	38.9 (38.1–40.2)	39.8 (38.0–40.2)	0.79*
PR (beats/min)	145 (124–160)	150 (120–180)	0.68*
RR (breaths/min)	48.0 (32.5–85.0)	63.0 (29.0–90.0)	0.71†
WBC count ( $10^9$ cells/L)	9.90 (6.65–15.0)	11.9 (1.60–33.1)	0.58*
IL-6 (µg/L)	0.063 (0.063–0.289)	0.126 (0.063–1.348)	0.25†
IL-10 (µg/L)	0.302 (0.286–0.385)	0.307 (0.298–0.446)	0.42†
IL-6/IL-10	0.224 (0.165–0.967)	0.239 (0.204–3.910)	0.27†
HMGB1 (µg/L)	0.300 (0.300–0.312)	0.718 (0.300–1.626)	0.007†
HMGB1/IL-10	1.017 (0.862–1.126)	2.236 (0.972–5.367)	0.024†

Data are presented as the median (interquartile range).

T, body temperature; PR, pulse rate; RR, respiration rate; HMGB1, high-mobility group box 1; SIRS, systemic inflammatory response syndrome.

\*Student's *t*-test.

†Mann-Whitney *U*-test.

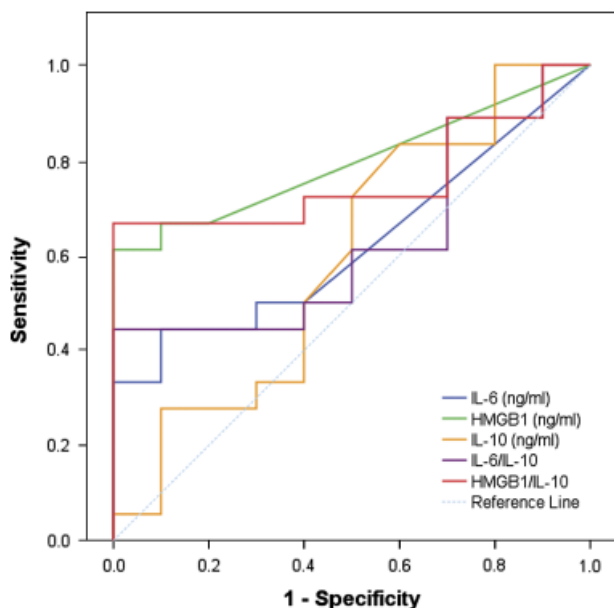


**Figure 1:** Plasma cytokine concentration in dogs with SIRS. Increased concentrations of plasma HMGB1 ( $P = 0.007$ ) (a) and the ratio of HMGB1 to IL-10 ( $P = 0.024$ ) (d) in the nonsurvivors compared with survivors among 28 dogs with SIRS (bars show means). Plasma IL-6 (b) and IL-10 (c) levels are not significantly different between groups. HMGB1, high mobility group box 1; SIRS, systemic inflammatory response syndrome.

in a heterogeneous population of dogs with SIRS. Thus, HMGB1 has the potential to be an important prognostic biomarker in dogs with SIRS. We have subsequently confirmed via Western blotting a clear band around

30 kDa in the serum from septic dog and also in tissues by using anti-HMGB1 polyclonal antibody (unpublished data). However, this method for HMGB1 detection is time consuming, and may have further limitations including possible cross-reaction with light chains of immunoglobulins as suggested by the other investigators.<sup>17</sup>

Interestingly, IL-6 concentrations were not related to outcome in this study. Our inability to identify a relationship between IL-6 concentration and outcome could be related to sampling time issues. In an experimentally induced canine model of endotoxemia, tumor necrosis factor- $\alpha$  and IL-1 $\beta$  concentrations peaked within 3 hours to initiate the immune response, then decreased to normal concentrations within a few hours.<sup>24</sup> In both animal models and human studies, IL-6 increased more gradually over the 1st hour and began to decrease after 6 hours.<sup>25–28</sup> IL-6 measured 6 hours after the initiation of experimental sepsis has been used to predict mortality.<sup>25</sup> However, the transient nature of many pro-inflammatory cytokines, including IL-6, would make them less reliable as late biomarkers and this may explain some of the results of our study. Alternately, the lack of significant differences in the IL-6 results could be related to the small sample size because there was a trend for higher values in the nonsurvivors as compared with survivors.



**Figure 2:** Receiver operating characteristic curves comparing the discriminating capabilities of prognostic markers between survivors and nonsurvivors.

In addition to our evaluation of individual cytokines, we examined ratios of pro- and anti-inflammatory cytokines for their potential use as biomarkers. The manifestation of SIRS may be more than the consequence of excessive proinflammatory cytokine production. SIRS may also result from a failure or imbalance of the protective mechanisms against systemic inflammation, and this was the reason we elected to examine the ratio of proinflammatory and anti-inflammatory cytokines as possible prognostic surrogate markers.<sup>12</sup> To our knowledge, this is the 1st study to specifically test the ratio of HMGB1 to IL-10 as a predictor of survival in dogs. Although plasma IL-10 concentrations alone were not predictive of survival, we did identify that the ratio of HMGB1 to IL-10 was significantly increased in the nonsurvivors as compared with the survivors ( $P = 0.024$ ). However, in this study, the ratio did not appear to have advantages over the use of HMGB1 alone. The ratio of IL-6 to IL-10 did not differ significantly between survivors and nonsurvivors. As with our IL-6 results, it is possible these results were related to the timing with regard to sample acquisition.

In conclusion, our data demonstrate a significant increase in plasma HMGB1 in nonsurvivors compared with survivors in dogs with SIRS and this finding supports the use of HMGB1 as a prognostic biomarker. Future studies determining the potential role of using HMGB1 in the diagnosis of various illnesses, including the differentiation of sepsis and nonsepsis-associated SIRS, are warranted.

## Footnotes

- <sup>a</sup> DuoSet ELISA Development Kit, R&D Systems, Minneapolis, MN.
- <sup>b</sup> HMGB1 Detection Set, Enzo Life Sciences AG, Lausen, Switzerland.
- <sup>c</sup> VersaMax, Molecular Devices Corp, Sunnyvale, CA.
- <sup>d</sup> Softmax Pro Software, version 5.3, Molecular Devices Corp, Boston, MA.
- <sup>e</sup> SPSS Software, version 15.0, SPSS, Chicago, IL.

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