*Microbiol Immunol* 2013; 57: 150–158
doi: 10.1111/1348-0421.12016

ORIGINAL ARTICLE

# Diagnostic value of serum concentrations of high-mobility group-box protein 1 and soluble hemoglobin scavenger receptor in brucellosis

Ayşe Oğuz Ayarcı[1], Emel Yılmaz[1], Deniz Sığırlı[2], Ferah Budak[3], Güher Göral[3] and Haluk Barbaros Oral[3]

[1]Department of Infectious Diseases and Clinical Microbiology, Uludag University Faculty of Medicine, Bursa, Turkey, [2]Department of Biostatistics, Uludag University Faculty of Medicine, Bursa, Turkey and [3]Department of Immunology, Uludag University Faculty of Medicine, Bursa, Turkey

## ABSTRACT

**Both cluster of differentiation (CD)4$^{+}$ and CD8$^{+}$ T lymphocytes play key roles in immunity to *Brucella*, in part because they secrete interferon (IFN)-γ and activate bactericidal functions in macrophages. Therefore, use of markers of macrophage activation may have diagnostic and prognostic significance. High-mobility group-box 1 protein (HMGB1), a late-onset pro-inflammatory cytokine, is secreted by activated macrophages. Soluble hemoglobin scavenger receptor (sCD163) is a specific marker of anti-inflammatory macrophages. The aim of this study was to investigate the diagnostic value of HMGB1 and sCD163 concentrations in brucellosis and its various clinical forms. Serum HMGB1 and sCD163 concentrations in 49 brucellosis patients were compared with those in 52 healthy control subjects. Both serum HMGB1 and sCD163 concentrations were significantly higher in brucellosis patients than in healthy controls ($P < 0.001$). There were no statistically significant differences in serum concentrations of HMGB1 and sCD163 between cases of acute, subacute and chronic brucellosis. Additionally, serum HMGB1 concentrations were positively correlated with sCD163 concentrations, whereas neither HMGB1 nor sCD163 concentrations were correlated with C-reactive protein concentrations, white cell counts or erythrocyte sedimentation rates. Therefore, serum concentrations of HMGB1 and sCD163 may be diagnostic markers for brucellosis, but neither can be used to differentiate the three different forms of this disease (acute, subacute and chronic).**

**Key words** Brucella, high-mobility group-box 1 protein, immunity, soluble hemoglobin scavenger receptor.

Brucellosis, a systemic infectious disease caused by small, gram-negative coccobacilli of the genus Brucella, is a widespread zoonosis. This disease remains an important public health problem, mainly in the Mediterranean region, the Middle East, and central and southern America. Transmission to humans occurs through direct contact with infected animals, consumption of infected milk and other unpasteurized dairy products, or occasionally via inhalation. The clinical manifestations of brucellosis include arthritic symptoms in 20–85% of patients (1). The diagnosis is not difficult if the clinical presentation of the disease and associated laboratory findings are typical. However, varied and sometimes misleading manifestations of localized, subacute or chronic disease can lead to misdiagnoses (2). In addition, patients may appear to have recovered clinically while

**Correspondence**
Haluk Barbaros Oral, Department of Immunology, Uludag University Faculty of Medicine, Gorukle Campus 16059, Nilufer, Bursa, Turkey. Tel: +90 224 2954114; fax: +90 224 2954149; email: oralb@uludag.edu.tr

Received 30 October 2012; revised 28 November 2012; accepted 1 December 2012.

**List of Abbreviations:** 2-ME, 2-mercaptoethanol; AUC, area under the curve; CD, cluster of differentiation; CI, confidence interval; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; HMGB1, high-mobility group-box 1 protein; IFN, interferon; IL, interleukin; ROC, receiver operating characteristics; sCD163, soluble hemoglobin scavenger receptor; STA, standard tube agglutination; Th, T helper; TLR, Toll-like receptor; TNF-α, tumor necrosis factor-alpha; WBC, white blood cell.

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their laboratory findings remain abnormal. In such cases, it is difficult to decide whether to terminate or continue antimicrobial therapy.

*Brucella* spp are facultative intracellular gram-negative bacteria that can survive and multiply within host phagocytic cells and therefore lead to chronic infection in animals and humans (3). The immune strategies by which brucella evade intracellular killing mechanisms within phagocytic cells are not completely understood. Control of infection and eventual elimination of the bacteria is mediated by Th1 type immune responses, which primarily involve activation of macrophages (4). In response to various inflammatory stimuli, activated macrophages secrete a number of cytokines, such as TNF-$\alpha$, IL-1 and IL-12, and other inflammatory mediators, such as HMGB1 and sCD163 (4–7).

High-mobility group-box 1, previously known as a DNA-binding protein, is a highly conserved protein that is involved in maintenance of nucleosome structure and therefore regulates gene transcription. It also plays roles in DNA recombination, repair and replication (8). It consists of three domains: positively charged A and B DNA-binding boxes and a negatively charged C terminus. (9) The B box shows cytokine properties of HMGB1 (10), whereas the A box competes for the binding site with HMGB1 and downregulates an inflammatory cascade (11). Produced by macrophages, HMGB1 itself mediates stimulation of human monocytes to produce proinflammatory cytokines (12). HMGB1 has emerged progressively as a central player in the signalization and coordination of both septic and aseptic forms of inflammation (13, 14).

The membranous form of CD163 functions as a scavenger receptor that is responsible for endocytosis of haptoglobin–hemoglobin complexes (15). It is predominantly expressed by activated macrophages and monocytes (16, 17). The membrane-bound CD163 receptor can be cleaved by proteolytic enzymes and shed into the extracellular space (18–20). A soluble form of CD163 (sCD163) present in plasma and serum is considered to be a marker of macrophage activity (20).

In the present study, we investigated serum concentrations of HMGB-1 and sCD163 in Brucellosis patients. We also evaluated whether HMGB-1 and sCD163 could be used for discriminating between acute, subacute and chronic forms of brucellosis.

## MATERIALS AND METHODS

### Patients and clinical assessment

Serum samples were obtained from 49 patients with brucellosis (32 male and 17 female). The patients were classified as having acute, subacute and chronic brucellosis according to the duration of their disease: $< 8$ weeks, 8–52 weeks and $> 52$ weeks, respectively. There were 23 patients with acute, 15 with subacute and 11 with chronic brucellosis. The healthy control group consisted of 52 volunteers (40 male and 12 female).

Brucellosis was diagnosed on the basis of clinical, serological and bacteriological evidence. The diagnostic criteria required isolation of *Brucella* spp. from blood cultures (BACTEC 9050, Beckton-Dickinson Diagnostic Instrument System, Sparks, MD, USA) and/or a positive Rose-Bengal test, a single titer of $\geq$ 1/160 by a *Brucella* STA test or Coombs test, these findings being confirmed by a 2-ME test titer of $\geq$ 1/160, in association with compatible clinical findings. The healthy controls had no brucellosis history and no clinical or serological findings suggestive of brucellosis or any other disease. The study was approved by the Ethical Committee of Uludag University, Bursa, Turkey and all subjects gave written informed consent. All serum samples were kept at −80°C until use.

### Routine nonspecific–specific laboratory tests

Serum CRP concentrations were measured by the nephelometric method (Siemens, Erlangen, Germany), concentrations $< 0.05$ mg/dL being accepted as negative. WBCs were counted on a Sysmex SE 9000 (TOA; TOA, Kobe, Japan). The ESR was determined by the Westergreen method.

### Measurement of high-mobility group-box 1 protein and soluble hemoglobin scavenger receptor concentrations

Serum HMGB1 was measured in duplicate with a commercially available ELISA kit (Uscn Life Science, Wuhan, China) according to the manufacturer's instructions. The minimum detectable concentration of human HMG1 is 4.3 pg/mL (measuring range = 12.5–800 pg/mL). This range can be broadened by dilution of samples with high concentrations. Recovery of HMGB1 in this ELISA is 95–103%. Concentrations of serum sCD163 were measured in duplicate with a specific ELISA kit (R & D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The concentration of CD163 in each sample was determined by interpolation from a standard curve. The minimum detectable concentration of human sCD163 is 0.177 ng/mL (measuring range = 0.058–0.613 ng/mL). This range can be broadened by dilution of samples with high concentrations. Recovery of sCD163 in this ELISA is 101–110%.

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## Statistical analysis

The Shapiro–Wilk test was used to test the normality of variables, the Kruskal–Wallis test for comparing more than two groups for continuous variables and the Mann–Whitney *U*-test for comparing two groups for which the Kruskal–Wallis test had indicated significant differences. An independent sample Student's *t*-test was used for comparing two groups, for continuous variables meeting normality assumption. Continuous variables are presented as median (minimum value − maximum value) or mean ± standard deviation. Categorical variables are expressed by counts and percentages. Comparisons between the groups were performed with the Pearson $X^2$ test for categorical variables. ROC curve analysis was performed for assessing the ability of sCD163 and HMGB1 to differentiate between cases and controls and to determine optimal cut-off values for sCD163 and HMGB1. Significance concentration was taken as $\alpha = 0.05$. Statistical analyses were performed with IBM SPSS Statistics version 20.0.

## RESULTS

The mean age of patients was 46.02 ± 15.80 years and of controls 33.80 ± 8.48 ($P < 0.001$). The two groups were homogenous in terms of sex ($P = 0.285$). All of the healthy controls had negative Rose-Bengal and STA tests and ESRs in the normal range (data not shown).

The symptoms, clinical findings and routine laboratory results of patients are shown in Tables 1 and 2. The commonest symptoms were fever, back pain and arthralgia (Table 1), whereas the commonest clinical findings were fever and osteoarticular involvement (Table 1). Brucella species were isolated from blood cultures in 10 patients (20.4%). All the *Brucella* species were identified as *Brucella melitensis*. The median titration concentrations of *Brucella* STA and 2-ME were 1:320 and 1:80, respectively (Table 2).

**Table 1.** Symptoms of brucellosis patients ($n = 49$)

| Symptom | *n* | % |
|---|---|---|
| Fever | 33 | 67.34 |
| Back pain | 19 | 38.77 |
| Arthralgia | 19 | 38.77 |
| Malaise | 18 | 36.73 |
| Sweating | 14 | 25.57 |
| Headache | 8 | 16.32 |
| Myalgia | 8 | 16.32 |
| Anorexia | 3 | 6.12 |
| Vomiting | 2 | 4.08 |
| Nausea | 2 | 4.08 |

**Table 2.** Clinical findings and routine laboratory results in brucellosis patients ($n = 49$)

| Clinical findings | *n* | % |
|---|---|---|
| Fever (38°C or higher) | 18 | 36.73 |
| Osteoarticular involvement | 14 | 25.57 |
| Hepatomegaly | 3 | 6.12 |
| Splenomegaly | 2 | 4.08 |
| Epididymitis | 1 | 2.04 |
| Endocarditis | 1 | 2.04 |
| **Laboratory results** | **Median** | **Minimum–maximum** |
| WBC count (× 1000/μL) | 5.87 | 2.64–13.2 |
| CRP (mg/dL) | 0.41 | 0.31–10.3 |
| STA | 1:320 | 1:10–2560 |
| 2-ME | 1:80 | 1:20–1280 |
| ESR (mm/hr) | 22 | 2–116 |

Both serum HMGB1 and sCD163 concentrations were significantly higher in brucellosis patients than in healthy controls. The median serum HMGB1 concentration was 170.65 (13.19–188.23) ng/mL in brucellosis patients, whereas it was 77.09 (0.00–182.84) ng/mL in the controls ($P < 0.001$; Fig. 1). The median serum sCD163 concentration was 1.27 (0.37–2.13) mg/L in brucellosis patients, whereas it was 0.57 (0.21–1.52) mg/L in the healthy controls ($P < 0.001$; Fig. 2). There were

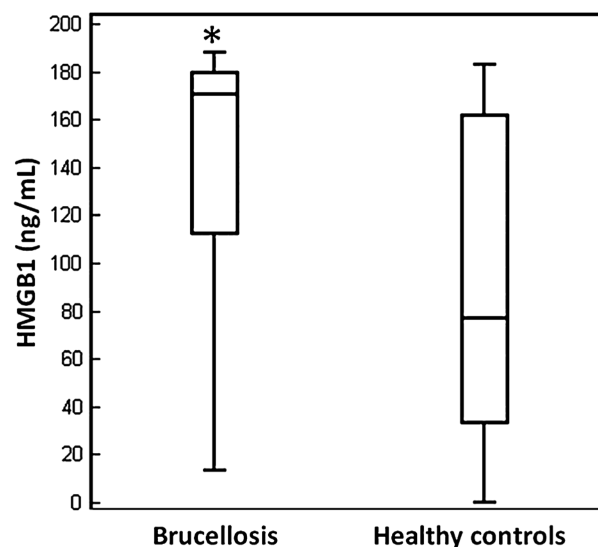


**Fig. 1. Box plot for high-mobility group-box 1 protein (HMGB-1) concentrations in brucellosis cases and controls.** The boxes represent the values within the 25th to 75th percentile. The central horizontal lines represent the medians. The vertical lines extend from the minimum to the maximum values. *$P < 0.001$, brucellosis cases versus healthy controls.



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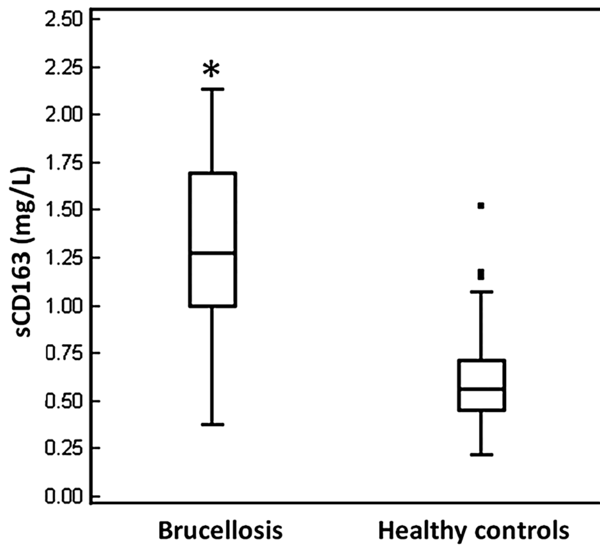


**Fig. 2. Box plot for soluble hemoglobin scavenger receptor (sCD163) concentrations in brucellosis cases and controls. The boxes represent the values within the 25th to 75th percentile.** The central horizontal lines represent the medians. The vertical lines extend from the minimum to the maximum values, excluding "outside" and "far out" values which are displayed as separate points. An outside value is defined as a value that is smaller than the 25th percentile minus 1.5 times the interquartile range, or larger than the 75th percentile plus 1.5 times the interquartile range; these are shown by black square markers. *$P < 0.001$, Brucellosis cases versus healthy controls.

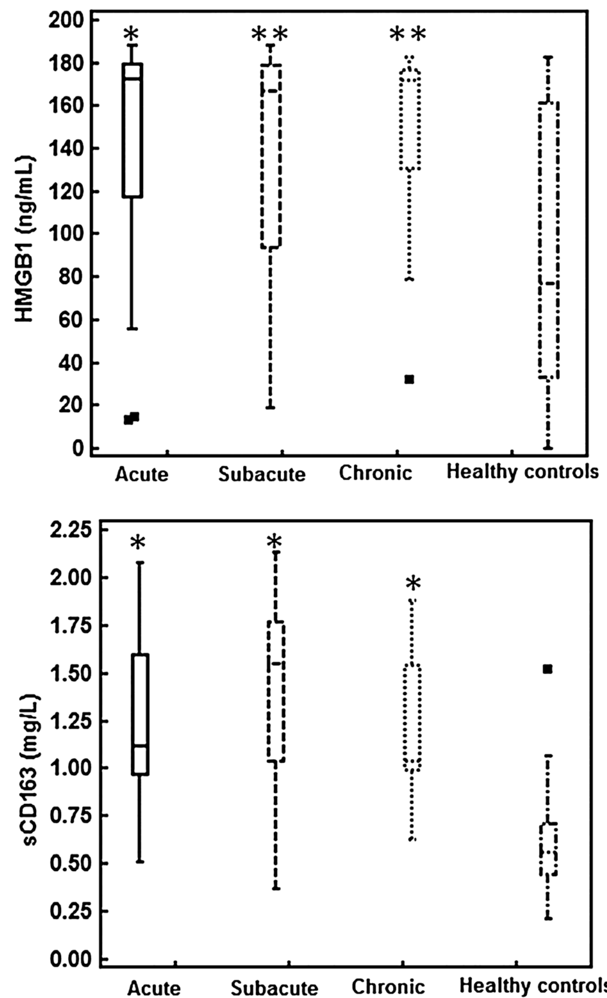


**Fig. 3. Box plots for high-mobility group-box 1 protein (HMGB-1) and soluble hemoglobin scavenger receptor (sCD163) concentrations in cases of acute ($n = 23$), subacute ($n = 15$) and chronic ($n = 11$) brucellosis.** The central boxes represent the values within the 25th to 75th percentile. The central horizontal lines represent the medians. The vertical lines extend from the minimum to the maximum values, excluding "outside" and "far out" values which are displayed as separate points. An outside value is defined as a value that is smaller than the 25th percentile minus 1.5 times the interquartile range, or larger than the 75th percentile plus 1.5 times the interquartile range; these are shown by black square markers. *$P < 0.001$, **$P < 0.010$, Brucellosis cases versus healthy controls.

no statistically significant differences in serum concentrations of HMGB1 and sCD163 among acute, subacute and chronic cases with Brucellosis, whereas serum concentrations of HMGB1 and sCD163 in all of these clinical forms were significantly increased compared to those of healthy controls (Fig. 3). Also, no significant difference in serum HMGB1 and sCD163 concentrations were found when acute plus subacute and subacute plus chronic cases were compared with chronic and acute cases, respectively (data not shown).

A statistically significant positive correlation was found between HMGB1 and sCD163 concentrations ($r = 0.398$, $P = 0.005$; Fig. 4). Neither HMGB1 nor sCD163 concentrations were correlated with CRP, WBC count or ESR (Table 3).

For HMGB1, the AUC for distinguishing between patients with brucellosis and healthy controls was 0.744 ($P < 0.001$). Using a cutoff concentration of > 165.99 for HMGB1 concentrations yielded sensitivity and specificity values of 61.2 (95% CI95 = 46.2–74.8) and 80.8 (95% CI = 67.5–90.4), respectively (Fig. 5a). For sCD163, the AUC for distinguishing between patients with brucellosis and healthy controls was 0.920 ($P < 0.001$). Using a cutoff concentration of > 0.82 for sCD163 concentrations yielded sensitivity and specificity values of 89.80 (95% CI = 77.8–96.6) and 90.4 (95% CI = 79.0–96.8), respectively (Fig. 5b). When the performances of serum HMGB1 and sCD163 concentrations were compared, sCD163 was found to perform significantly better than HMGB1 ($P = 0.001$).

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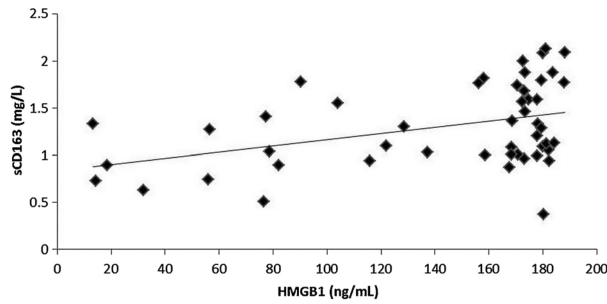


**Fig. 4. Line graph showing a positive correlation between serum high-mobility group-box 1 protein (HMGB1) concentrations and soluble hemoglobin scavenger receptor (sCD163) in patients with brucellosis ($r = 0.398$, $P < 0.005$).**

## DISCUSSION

Although host resistance to *Brucella* spp. is still incompletely understood, cell-mediated immunity seem to play a major role in immune responses against virulent *Brucella* infection (4). Both $CD4^+$ and $CD8^+$ T lymphocytes play critical roles in immunity to brucella (21), in part because they secrete IFN-$\gamma$ and activate the bactericidal functions of macrophages (22). Several studies have been demonstrated that Th1 cytokines confer resistance to (23, 24), whereas Th2 or T regulatory cytokines facilitate the development of brucellosis (25, 26). However, some cases with strong Th1 profiles reportedly have impaired monocyte and macrophage functions (27). This may be one of the mechanisms responsible for relapse or chronicity of brucella infections. In addition, macrophages present a variety of phenotypes when mediating different responses to various microenvironmental stimuli (28, 29). Macrophages are classically activated toward the M1 phenotype by microbial products or IFN-$\gamma$. M1 type macrophages express IL-$12^{high}$, IL-$23^{high}$ and IL-$10^{low}$ phenotypes and produce reactive nitrogen intermediates and proinflammatory cytokines such as IL-1β, TNF-α, IL-6, HMGB1 (12, 28, 30), whereas M2 "alternatively activated" type macrophages have IL-$12^{low}$,

**Table 3.** Correlations between high-mobility group-box 1 protein (HMGB1)/soluble hemoglobin scavenger receptor (sCD163) and the examined inflammatory markers in brucellosis cases ($n = 49$)

| HMGB1 versus | *r* | *P*-value | sCD163 versus | *r* | *P*-value |
|---|---|---|---|---|---|
| sCD163 | 0.398 | 0.005 | HMGB1 | 0.398 | 0.005 |
| ESR | — | 0.061 | ESR | — | 0.685 |
| CRP | — | 0.251 | CRP | — | 0.744 |
| WBC | — | 0.296 | WBC | — | 0.111 |

*r*, Pearson correlation coefficient.

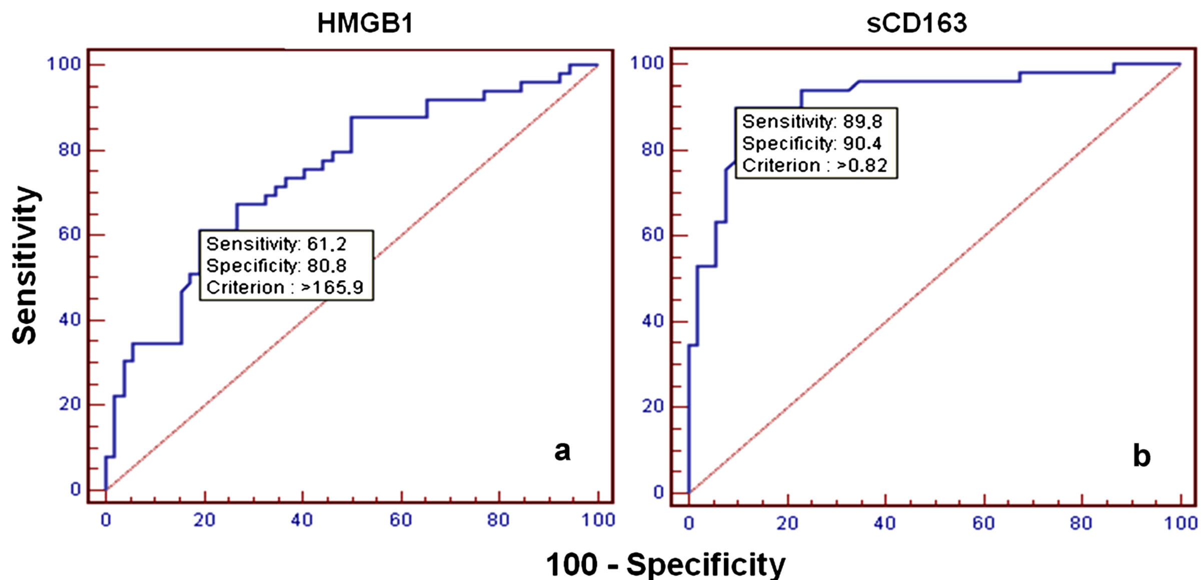


**Fig. 5. Receiver operating characteristics (ROC) curve for (a) serum HMGB1 and (b) sCD163 concentrations in patients with brucellosis and healthy controls.** Chosen cutoff values for univariate analysis are indicated.


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IL-23$^{low}$ and IL-10$^{high}$ phenotypes and express several receptors, such as hemoglobin scavenger receptor (CD163) (31). Therefore, in general terms, HMGB1 and CD163 are expressed predominantly on macrophages possessing proinflammatory and anti-inflammatory responses, respectively (12, 30, 32, 33).

Immune cells, especially monocytes/macrophages, dead and/or dying cells can secrete HMGB1 (34). Once released, HMGB1 generates a positive feedback loop and in turn triggers secretion of some proinflammatory cytokines by monocytes, thereby sustaining prolonged inflammation (35). As well as its DNA-binding features, HMGB1 can bind to various cellular receptors such as that for advanced glycation end products, TLR-2, TLR-4 and TLR-9, facilitating activation of the nuclear factor kappa B and mitogen-activated protein kinases (36–38). As a result, HMGB1 especially stimulates human monocytes and macrophages to produce proinflammatory cytokines, which include TNF-$\alpha$, IL-1$\beta$, IL-6, and macrophage inflammatory protein-1 (12).

CD163 is a monocyte/macrophage-specific scavenger receptor for haptoglobin–hemoglobin complexes. Its essential function is to bind hemoglobin–haptoglobin complexes, thereby removing hemoglobin from the circulation (39). In additon to this function, CD163-expressing macrophages play a role during the healing phase of acute inflammation, in chronic inflammation and in wound healing (40). It plays a role in the anti-inflammatory response, its expression being induced by IL-10 and glucocorticoid (20).

Several published studies have investigated HMGB1 and sCD163 concentrations in patients with some infections and inflammatory diseases (5, 41–50). Most previous studies have shown that HMGB1 is involved is septic inflammation; this was first described in an experimental mouse model of endotoxemia (51). First of all, serum HMGB1 concentrations are significantly increased in mice with experimentally induced endotoxemia or sepsis, as well as in sepsis-related conditions in humans (44, 47, 52). Secondly, inhibition of HMGB1 release and/or its activities by some potentially therapeutic agents, such as neutralizing antibodies, can rescue animals with severe sepsis (13). Moreover, significant concentrations of anti-HMGB1 antibodies develop progressively during the course of these diseases, particularly in patients who survive (53). On the other hand, concentrations of sCD163 are reportedly increased in various diseases that are characterized by abundant activated monocytes/macrophages, such as hemophagocytic syndrome, sepsis, coronary heart disease, sickle cell disease and type 2 diabetes mellitus (20, 48, 54–57). Only two studies have investigated both serum HMGB1 and sCD163 concentrations in patients with bacteremia and serious bacterial infections (48, 58). In a study comparing bacteremic and non-bacteremic patients, Gaini *et al.* found significantly higher concentrations of both HMGB1 and sCD163 in patients with bacteremia (48). In another study of Malawian pediatric patients with serious bacterial infections, HMGB1 concentrations, but not those of sCD163, were higher in patients with bacteremia than in controls. Interestingly, sCD163 concentrations were significantly higher in non-survivors than in survivors (58). In the present study, we have demonstrated, for the first time, that both serum HMGB1 and sCD163 concentrations are significantly higher in patients with brucellosis.

Gaini *et al.* have also shown that increases in HMGB1 concentrations, but not in those of sCD163, correlate with WBC counts and concentrations of other proinflammatory indicators and CRP (48). CRP is reportedly significantly positively correlated with serum HMGB1 concentrations according to some studies (45, 59–61); however, others found no correlations between HMGB1 and CRP (62–64). Neither HMGB-1 nor sCD163 concentrations correlated with WBC count and CRP in our study. According to our data, neither HMGB1 nor sCD163 directly reflect acute phase inflammatory responses. Our study also demonstrates that both pro- and anti-inflammatory mechanisms are activated during brucella infection, as evidenced by the increases we observed in both HMGB1 and sCD163 concentrations and the positive correlation between these two variables. It is very likely, however, that alternatively activated macrophages are also involved in the pathogenesis of brucellosis.

Interestingly, the concentrations of HMGB1 that we measured in our study and those reported in previous studies differ markedly. This is true not only of patients' HMGB1 concentrations, but also for the concentrations in control subjects (42, 44, 45). This is probably due to variability of the assay reagents used in the kits. The three different assay kits use different HMGB1-specific antibodies that may recognize distinct epitopes on HMGB1 protein. In addition, the antibodies are used either alone or in combination and at various concentrations. Further, the supplements to the reagents of the different test kits can also influence the test results. Finally, these variations between different ELISA kits and, therefore, the differences in measured HMGB1 epitopes, may be important in any investigations of the involvement of specific organ systems and their correlation with disease activity. This could be investigated in further studies. At present, we recommend that only ELISA kits from the same manufacturer should be used during any single study, including any follow-up investigations.

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Taken together, the significantly higher concentrations of HMGB-1 and sCD163 in brucellosis patients than in healthy controls infer that both markers might be useful for diagnosing brucellosis, especially when there are clinical findings suggestive of this disease. Further studies are needed to clarify the role of these cytokines in the pathogenesis and prognosis of *Brucella* infection in humans. Also, further prospective studies are needed to evaluate the effect of antibiotic treatment regimes on HMGB1 and sCD163 concentrations in order to determine whether such measurements can be used to monitor treatment efficacy.

## ACKNOWLEDGEMENTS

This work was supported by a grant from Uludag University, Bursa, Turkey (grant number UAP(T)-2010/04).

## DISCLOSURE

The authors declare no conflicts regarding financial interests.

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