Plasma HMGB-1 and Nucleosome Concentrations in Horses with Colic and Healthy Horses

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Background: Acute gastrointestinal disease occurs commonly in horses. Novel biomarkers might improve the understanding of SIRS and aid diagnosis and determination of prognosis.

Hypotheses: Increased plasma concentrations of the biomarkers HMGB-1 and nucleosomes are associated with severity of gastrointestinal lesions in horses; concentrations of these biomarkers will be greater in horses with lesions more likely to cause SIRS; and will provide additional information compared with standard biomarkers fibrinogen and SAA.

Animals: Thirty horses with gastrointestinal disease, 22 healthy horses.

Methods: Prospective study. Plasma samples taken on admission were used for measurement of HMGB-1, nucleosomes, fibrinogen, and SAA. Values were compared between healthy horses and those with gastrointestinal disease, and between horses with gastrointestinal disease grouped by lesion type (inflammatory, strangulating, and nonstrangulating). Correlations between biomarkers were assessed.

Results: Plasma concentrations of all biomarkers were significantly higher in horses with gastrointestinal disease compared to healthy horses ($P \leq .001$). HMGB-1 and nucleosomes were significantly higher in inflammatory and strangulating groups compared to healthy horses (3.5-fold and 5.4-fold increases, respectively, for HMGB-1 ($P < .05$) and 4.8-fold and 5.6-fold increases for nucleosomes ($P < .05$)), but concentrations in the group with nonstrangulating disease did not differ from healthy horses. There was significant correlation between HMGB-1 and nucleosomes (Spearman's $r = 0.623$; $P < .001$), and fibrinogen and SAA (Spearman's $r = 0.801$; $P < .001$) but not between other biomarkers.

Conclusions and Clinical Importance: High mobility group box-1 and nucleosomes might have use as biomarkers for horses with gastrointestinal disease. Further studies are required to determine kinetics and prognostic value of serial measurements of these biomarkers in horses.

Key words: HMGB-1; Horse; Nucleosomes; Systemic inflammatory response syndrome.

$\mathbf{T}$he systemic inflammatory response syndrome (SIRS) is generally defined in equine clinical practice as the presence of at least 2 of leukocytosis, leukopenia or >10% immature band neutrophils, hyperthermia, tachycardia or tachypnea.[1] It is a common complication of gastrointestinal disease (colic) and severe bacterial infections in horses, involving unbalanced activation of inflammatory pathways resulting in proinflammatory mediator dominance, which can lead to cardiovascular compromise, multiple organ failure, laminitis and death.[2–4] The investigation of biomarkers as useful aids in determining severity of colic lesions as

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Abbreviations:

HMGB-1	high mobility group box-1
DNA	deoxyribonucleic acid
SIRS	systemic inflammatory response syndrome
SAA	serum amyloid A

well as prognosis might lead to better assessment and management of these cases. These indices might also be useful for assessing the efficacy of novel therapies. Some biomarkers might also have potential as therapeutic targets in treating SIRS.

Fibrinogen is a nonspecific indicator of inflammation that is often increased in the presence of SIRS.[5] Fibrinogen also takes 24–72 h to reach peak concentrations in the plasma after the onset of inflammation, which makes it a relatively insensitive acute phase protein.[6] Although fibrinogen concentration is increased in severe inflammatory conditions, used alone it is unable to distinguish between strangulating and nonstrangulating lesions in horses with colic across a broad range of timeframes from presentation.[7] Changes in fibrinogen concentrations also do not necessarily agree with disease detection or progression.[6] Using more sensitive biomarkers, or a panel of different biomarkers, might enable greater discrimination between different conditions.

Serum amyloid A is a major acute phase protein that has several benefits over fibrinogen as a biomarker, including a more rapid and more pronounced increase in response to inflammatory disease, and greater sensitivity when used to indicate the presence of systemic inflammation in equine gastrointestinal disease.[6–9]

Because of these reasons, SAA has become a popular biomarker for inflammation in the horse. Like fibrinogen it is a nonspecific indicator of inflammation, however, the magnitude of increase might be suggestive of the cause of inflammation.[10]

High mobility group box-1 (HMGB-1) has recently been identified as a late-phase cytokine of sepsis in other species; although its role in SIRS in horses has not been investigated. Nucleosomes are disk-shaped complexes made up of histones surrounded by DNA, which are released mainly during apoptotic cell death,[11,12] the concentration of which increase in the serum of human patients with sepsis. Nucleosome concentrations differentiate between various degrees of disease severity, and survival versus nonsurvival.[13,14] To the authors' knowledge, nucleosome concentrations in equine SIRS have not been investigated previously.

The aims of this study were firstly to determine whether HMGB-1 and nucleosomes are present at higher concentrations in the plasma of horses with gastrointestinal disease compared to healthy horses; secondly to determine whether HMGB-1 and nucleosomes are present in higher quantities in horses with gastrointestinal lesions more likely to cause SIRS (strangulating lesions and inflammatory lesions) compared to those unlikely to cause SIRS (nonstrangulating lesions) and thirdly to compare plasma HMGB-1 and nucleosome concentrations with the existing biomarker, fibrinogen. A further aim was to determine whether biomarker concentrations were a useful predictor of survival.

Materials and Methods

Banked plasma samples collected on admission from 30 horses presenting for gastrointestinal disease were used. Whole blood was collected into lithium heparin vacutainer tubes and centrifuged within 1 h of collection. All plasma samples were stored at −80°C before analysis. Time between collection and HMGB-1 and nucleosome analysis was 2–3 years. Banked plasma samples collected from 22 normal horses who reside at the University for research purposes, also collected into lithium heparin vacutainer tubes, which had been stored at −80°C for 1–3 years were used as healthy controls. Collection of blood samples from all horses was approved by the University of Melbourne's Animal Ethics Committee.

Cases were divided into groups using a system defined previously.[15,16] Briefly, cases were allocated to either a group comprising inflammatory lesions, a group comprising strangulating lesions, or a group comprising nonstrangulating lesions. Cases were allocated to the inflammatory group if their main diagnosis was gastrointestinal tract rupture, duodenitis–proximal jejunitis, colitis, typhlocolitis or peritonitis. Cases were allocated to the strangulating group if their main diagnosis was a small intestinal strangulating obstruction (volvulus, incarceration or intussusception), or large colon volvulus. Cases were allocated to the nonstrangulating group if their main diagnosis was large colon impaction or other obstruction (eg, enterolith), large colon displacement, nonstrangulating small intestinal obstruction or infiltrative bowel disease.[15,16] Diagnosis was reached by clinical and clinicopathological evaluation, exploratory celiotomy, necropsy or all methods.

High mobility group box-1 was measured using a commercially available ELISA kit[a] according to the manufacturer's instructions.

Briefly, a standard curve was prepared using porcine HMGB-1, over the concentration range 0–80 ng/mL. Plasma samples and standards were pipetted into their respective wells, which were pre-coated with purified anti-HMGB-1 antibody. Analysis of all samples and standards was performed in duplicate. Wells were incubated at 37°C for 20 h. After washing, the assays were developed using an enzyme conjugate and TMB substrate. Optical density was read at 450 nm using a microplate reader,[b] and sample values determined from the standard curve using microplate reader software.[c] In order to validate the assay for equine samples, firstly the intra-assay coefficient of variation (CV) was calculated. This was done by multiple measurements (3 × in duplicate) of samples containing high, medium or low concentrations of HMGB-1, and expressing the CV as the standard deviation as a percentage of the mean. Recovery was determined by spiking plasma from a normal horse with a standard solution (320 ng/mL) at a dilution of 1:10. This was then compared to an aliquot of unspiked plasma. Dilutional parallelism was determined by making serial dilutions of a positive plasma control and comparing to the shape of the standard curve.

Nucleosome concentration was measured using a commercially available Cell Death Detection ELISA[d] according to the manufacturer's instructions. Briefly, plasma samples were added to Streptavidin-coated wells in triplicate, along with a positive control (DNA-histone complex), negative control (incubation buffer) and blank (reagents only). Following this, 80 μL of immunoreagent was added. The immunoreagent comprised anti-histone biotin antibody (biotin-labeled monoclonal mouse antibody; clone H11-4), which binds to the Streptavidin-coated wells, and binds the histone component of nucleosomes in the sample; and also an anti-DNA antibody (peroxidase-conjugated monoclonal mouse antibody; clone MCA-33), which binds to the DNA component of nucleosomes (double and single-stranded DNA), and to the color-reaction substrate. This was then incubated at room temperature for 2 h on a microplate shaker at 300 rpm. After incubation, the wells were evacuated and washed three times with incubation buffer. Color reaction substrate was then added, followed by stop solution once the color reaction had developed sufficiently (approximately 5 min). Optical density was read at 405–490 nm on the same microplate reader as above, with blank values then subtracted from the delta value. As no purified nucleosome standard was available for this assay (only positive and negative controls), the precise nucleosome concentrations could not be determined. Therefore, the nucleosome levels were expressed as absorbance units and relative changes between groups were compared. The coefficient of variation in the assay was determined as described above for the HMGB-1 assay. Recovery of spiked samples and dilutional parallelism could not be determined for this assay because of the lack of an available standard, and therefore this was a semiquantitative assay.

Fibrinogen was measured by heat precipitation using the Miller method, which has been well described elsewhere.[17] Serum amyloid A was measured using a commercially available ELISA kit[e] as previously described.[18]

Statistical analyses were performed using commercially available software.[f] The Shapiro–Wilk test was used to test for normality, and data were found to not be normally distributed. Results were expressed as medians (range). For HMGB-1, nucleosomes, fibrinogen and SAA, a Mann–Whitney test was used to compare colic vs. normal horses, and a Kruskal–Wallis test with Dunn's multiple comparison was used for comparisons between groups. A Mann–Whitney test was also used to compare biomarker concentrations in survivors vs. nonsurvivors. Spearman's rank correlation coefficient was used to determine whether there was a correlation between any of the biomarkers measured. A P value of <.05 was considered significant.

Results

There were 30 horses with gastrointestinal disease, comprising 16 females, 3 entire males and 11 geldings. Breeds represented were Thoroughbred (15), Warmblood (6), Standardbred (2), Quarter Horse (1), Arabian (2), Pony breeds (3) and 1 horse for which breed was unknown. These horses were aged between 2 and 29 years, with a mean age of 11 years. There were 22 healthy horses, comprising 10 females and 12 geldings. Breeds represented in this group were Standardbred (11), Pony breeds (6) and Andalusians and their crosses (5). The normal horses were aged between 4 and 20 years with a mean age of 8.8 years. Of the 30 horses presenting with gastrointestinal disease, there were 11 in the inflammatory group, 12 in the strangulating group and 7 in the nonstrangulating group. Distribution of horses between groups and types of lesions diagnosed are presented in Table 1.

HMGB-1

The overall coefficient of variation for the HMGB-1 ELISA was $14.6 \pm 4.1\%$, although the precision of the assay varied, with CVs from 6.8 to 27.8% when the plasma concentrations were at the highest end of the scale. Dilution of high plasma concentrations of HMGB-1 in dilution buffer produced a curve that deviated slightly from parallel (slopes 0.0155 and 0.0116 for standard curve and plasma dilution, respectively), suggesting that there could have been some interfering substances in the plasma, and this was reflected in the poor recovery of the high added concentrations of HMGB-1 in the spiked plasma (22.1%). Therefore, this assay might have underestimated the actual concentration of HMGB-1 in the plasma, however, the main aim of the study was to focus on comparison between groups of horses.

Results for HMGB-1 are shown in Fig 1. When comparing all horses with colic to the healthy group, plasma concentrations of HMGB-1 were significantly higher in horses with colic (median 12.2 ng/mL, range 3.3–46.6 ng/mL) compared to healthy horses (median 3.5 ng/mL, range 0.1–11.9 ng/mL), $P < .001$. When comparing plasma levels of HMGB-1 between the different categories of gastrointestinal disease, a significant

difference was found when comparing the inflammatory (3.5-fold increase, median 12.2 ng/mL, range 7.9–35.2 ng/mL) or strangulating groups (5.4-fold increase, median 18.8 ng/mL, range 3.3–46.6 ng/mL) to the healthy group ($P < .05$), but not when comparing the nonstrangulating group (median 8.0 ng/mL, range 4.4–21.5 ng/mL) to the healthy group ($P > .05$). The strangulating group had the highest maximum concentration of HMGB-1 at 46.6 ng/mL (range 3.3–46.6 ng/mL). A significant difference was not found when comparing between groups of horses with gastrointestinal disease ($P > .05$).
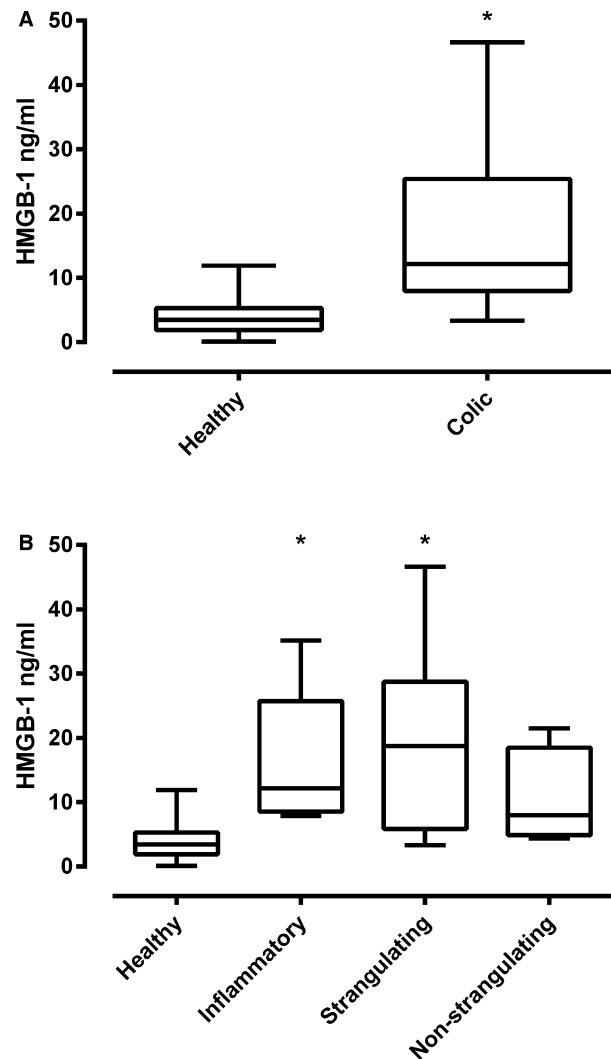

Fig 1. Plasma concentrations of HMGB-1 in all horses with gastrointestinal disease (colic, n = 30) compared to healthy horses (n = 22), A; and between healthy (n = 22), inflammatory (n = 11), strangulating (n = 12), and nonstrangulating (n = 7) groups; B. Data are expressed as the median and interquartile range (boxes), with the whiskers representing the range. Asterisks show significant differences from the healthy group. HMGB-1 concentrations were significantly higher in horses with gastrointestinal disease compared to healthy horses ($P < .001$; A). HMGB-1 concentrations were significantly higher in the inflammatory and strangulating groups compared to the healthy horses ($P < .05$; B).

Table 1. Group categorizations and types of lesions diagnosed in each group.

Group (number)	Diagnoses (number)
Inflammatory (11)	Colitis (6), peritonitis (5)
Strangulating (12)	Small intestinal incarceration (6), small intestinal volvulus (2), jejunojejunal intussusception (1), large colon volvulus (3)
Nonstrangulating (7)	Large colon displacement (2), lymphocytic–plasmacytic enteritis (1), nonstrangulating ileocecal intussusception (1), large colon obstruction (3)

Nucleosomes

The coefficient of variation for the nucleosome ELISA was $14.4 \pm 3.1\%$. Results of the nucleosome assays are shown in Fig 2. A significant difference was found when comparing plasma nucleosome-associated absorbance between healthy horses and horses with gastrointestinal disease, with a 5.0-fold increase in nucleosome-associated absorbance in the group with gastrointestinal disease ($P < .001$). Similar to HMGB-1, there was no significant difference between the groups of horses with gastrointestinal disease, however, there was a significant difference between the inflammatory group compared to the healthy group (4.8-fold increase, $P < .05$) and strangulating group compared to the healthy group (5.6-fold increase, $P < .05$). There was no
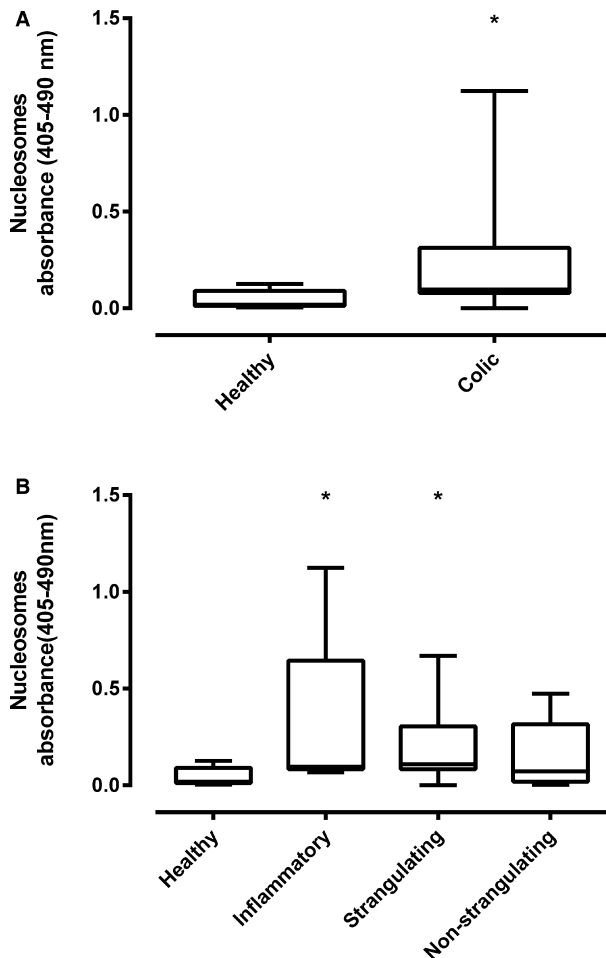
significant difference between horses in the nonstrangulating group and healthy horses (3.7-fold increase, $P > .05$). The inflammatory group had the highest nucleosome measurement with an absorbance of 1.237 units (range 0.068–1.237).

Fibrinogen

Results for fibrinogen are shown in Fig 3. Fibrinogen values were available from 6 healthy horses (all Stan-

Fig 2. Plasma concentrations of nucleosomes in all horses with gastrointestinal disease (colic, n = 30) compared to healthy horses (n = 22), A; and between healthy (n = 22), inflammatory (n = 11), strangulating (n = 12), and nonstrangulating (n = 7) groups; B. Data are expressed as the median and interquartile range (boxes), with the whiskers representing the range. Asterisks show significant differences from the healthy group. Nucleosome absorbance was significantly higher in horses with gastrointestinal disease compared to healthy horses ($P < .001$; A). As for HMGB-1, nucleosome concentrations were significantly higher in the inflammatory and strangulating groups compared to the healthy horses ($P < .05$; B).
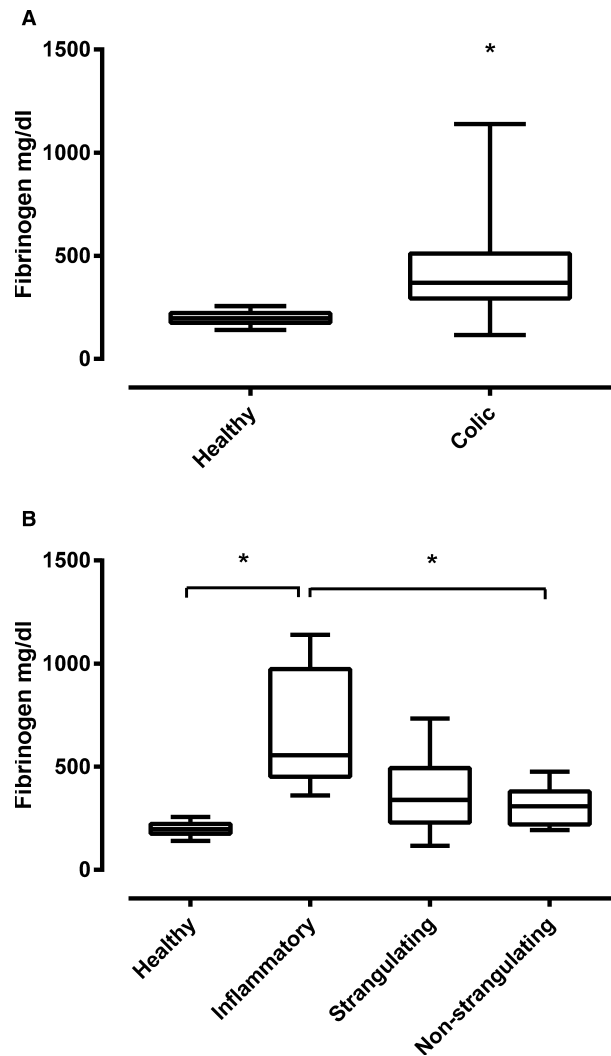
Fig 3. Plasma concentrations of fibrinogen in all horses with gastrointestinal disease (colic, n = 28) compared to healthy horses (n = 6), A; and between healthy, inflammatory (n = 10), strangulating (n = 11), and nonstrangulating (n = 7) groups; B. Data are expressed as the median and interquartile range (boxes), with the whiskers representing the range. Asterisks show significant differences from the healthy group, or between groups where indicated. Fibrinogen concentrations were significantly higher in horses with gastrointestinal disease compared to healthy horses ($P = .001$; A). Unlike the other two biomarkers, fibrinogen was only significantly higher in the inflammatory group compared to healthy horses ($P < .05$, B). Fibrinogen was also significantly higher in the inflammatory group compared to the nonstrangulating group ($P < .05$, B).

dardbred females, mean age 6 years, range 4–14 years) and 28 horses with gastrointestinal disease (fibrinogen values were not available from one warmblood mare with colitis in the inflammatory group, and a pony gelding with small intestinal strangulation from the strangulating group).

Fibrinogen concentration was significantly higher in horses with gastrointestinal disease (median 370 mg/dL, range 116–1140 mg/dL) compared to healthy horses (median 200 mg/dL, range 141–256 mg/dL) $P = .001$. Fibrinogen concentration was also significantly higher when comparing the inflammatory group (median 560 mg/dL, range 360–1140 mg/dL) to the healthy horses ($P < .05$), However, comparison between the strangulating group (median 340 mg/dL, range 116–734 mg/dL) and healthy horses, and nonstrangulating group (median 310 mg/dL, range 193–476 mg/dL) and healthy horses were not significantly different ($P > .05$). When comparing the inflammatory group to the nonstrangulating group, fibrinogen was significantly higher in the inflammatory group ($P < .05$), however, comparisons between all other colic groups did not reveal a significant difference ($P > .05$).

Serum Amyloid A

Intra-assay coefficient of variation for the SAA ELISA used in this study was <10%.[15] Serum amyloid A values were measured for 22 healthy horses and 29 horses with gastrointestinal disease. Results are shown in Fig 4. Plasma SAA concentrations were significantly higher in horses with gastrointestinal disease (median 400 μg/mL, range 1.2–1480 μg/mL) compared to healthy horses (median 0.8 μg/mL, range 0–3.6 μg/mL) ($P < .001$). There were no significant differences between groups of horses with gastrointestinal disease ($P > .05$). Median (range) for each group was 709 (276–1480) μg/mL for the inflammatory group, 282 (1.2–1349) μg/mL for the strangulating group, and 265 (5.9–587) μg/mL for the nonstrangulating group. Each of these groups was significantly different from the healthy group ($P < .05$).

Biomarker Correlations and Survival

There was significant correlation between HMGB-1 and nucleosomes (n = 30, Spearman's $r = 0.623$, $P < .001$), and fibrinogen and SAA (n = 28, Spearman's $r = 0.801$, $P < .001$) (Fig 5). When considering horses for which data of all measured biomarkers was available (n = 28), there was no significant difference in concentration of any biomarker between survivors (n = 19) and nonsurvivors (n = 9) (Table 2).

Discussion

All four biomarkers investigated in this study were significantly increased in horses with gastrointestinal disease compared to healthy horses. However, no significant difference was found when comparing HMGB-1 or nucleosomes between the different gastrointestinal
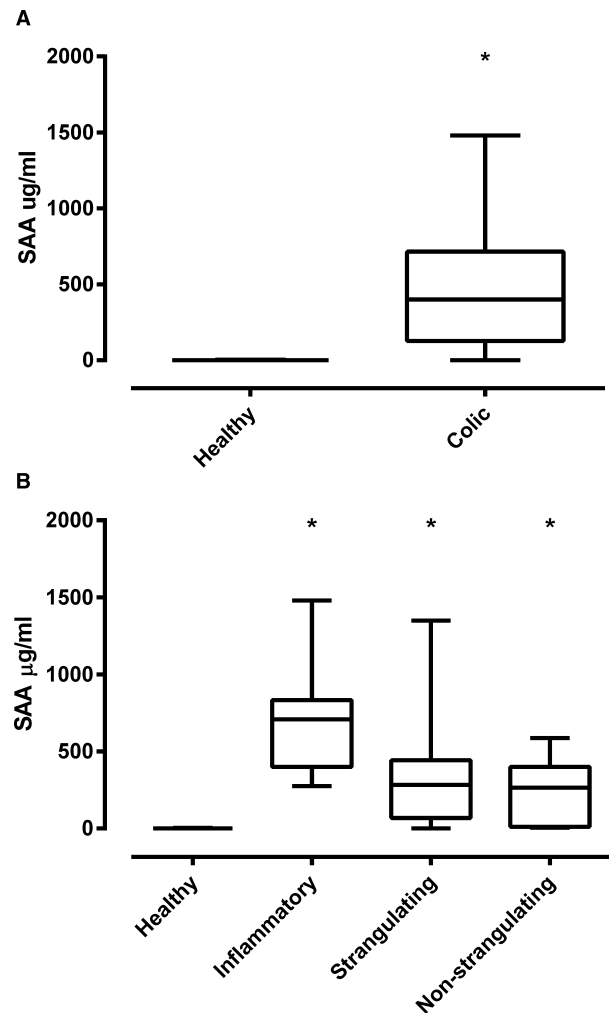
Fig 4. Plasma concentrations of Serum amyloid A (SAA) in all horses with gastrointestinal disease (colic, n = 30) compared to healthy horses (n = 22), A; and between healthy, inflammatory (n = 11), strangulating (n = 12) and nonstrangulating (n = 7) groups; B. Data are expressed as the median and interquartile range (boxes), with the whiskers representing the range. Asterisks show significant differences from the healthy group. Plasma concentrations of SAA were significantly higher in horses with gastrointestinal disease compared to healthy horses ($P < .001$; A). Serum amyloid A was significantly higher in all groups compared to healthy horses ($P < .05$, B), but not significantly different between groups of horses with gastrointestinal disease ($P > .05$; B).

lesion groups, therefore these biomarkers did not appear able to distinguish between different types of gastrointestinal lesions on samples taken at admission. Fibrinogen was able to distinguish between the inflammatory and nonstrangulating groups, but not between other groups which also limits its usefulness for this purpose. Serum amyloid A distinguished between healthy horses and those with gastrointestinal disease, but not between different lesion types. HMGB-1 and nucleosomes might provide information on different aspects of the disease process, although serial samples over a period of time are likely required in order for
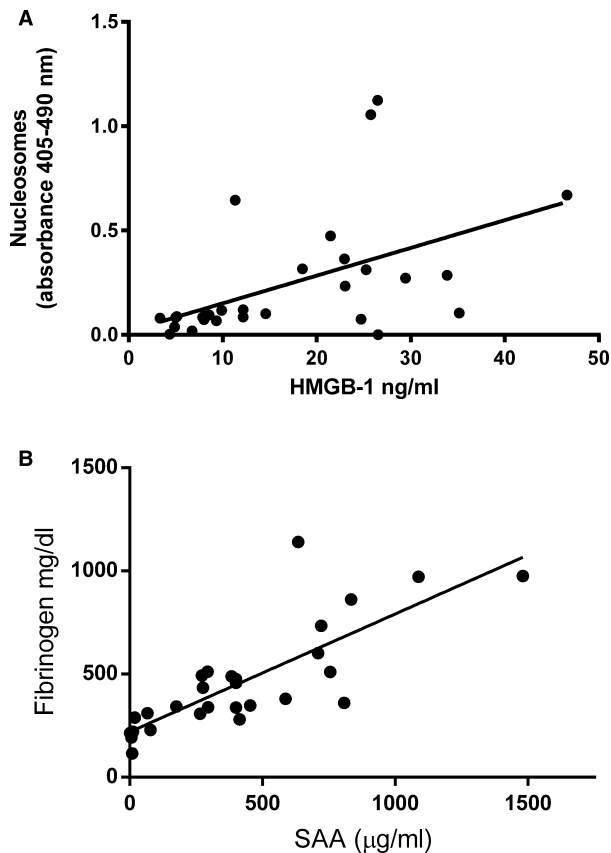
Fig 5. Correlation between HMGB-1 and nucleosomes (n = 30), and fibrinogen and SAA (n = 28). There was significant correlation between HMGB-1 and nucleosome concentrations (Spearman's $r = 0.623$, $P < .001$; A). There was also significant correlation between fibrinogen and SAA (Spearman's $r = 0.801$, $P < .001$; B).

these markers to provide sufficient discrimination between different types of gastrointestinal disease.

Fibrinogen was significantly higher only in the inflammatory group compared to the healthy group. The significant increase in fibrinogen in horses with inflammatory disorders but not strangulating or nonstrangulating lesions on admission agrees with a previous study in which the same system for grouping cases was used.[19] The results of this study, as well as our results show that fibrinogen is not useful in discriminating between different types of colic lesions. Fibrinogen is easily measured and is widely used to determine the presence of inflammatory disorders in horses, however,

it is a nonspecific marker of inflammation and the lag between onset of inflammation and peak fibrinogen levels limits its usefulness in the acute stages of equine colic.[6] It is likely that horses categorized in the inflammatory group had a longer duration from onset of disease to presentation, thereby accounting for the higher fibrinogen concentrations in this group. As samples were taken only on admission, follow-up fibrinogen concentrations are not available, and would likely be confounded by other differences in the groups other than primary disease process, such as abdominal surgery for the strangulating group. Despite a delayed appearance in the circulation of HMGB-1 and nucleosomes, based on the results of human and rodent studies, we had anticipated that these biomarkers would be more specific for the types of lesions encountered in equine colic. They did, however, have the advantage of discriminating between healthy horses and both inflammatory and strangulating lesions, whereas fibrinogen could only distinguish inflammatory lesions from healthy horses.

The method of fibrinogen measurement is a limitation of the study. There are more accurate methods of measuring fibrinogen in equine plasma, however, at the time of sample collection for this study these were not readily available. The semiquantitative measurements obtained by using the Miller method for determining fibrinogen concentrations must be taken into consideration, however, as the same method was used across all groups the results were considered to be comparable.

Originally thought to be simply a transcription factor, HMGB-1 has recently been identified as a late-phase cytokine of sepsis in rodent experimental models and human clinical patients, appearing in the plasma 8–32 h after the onset of disease.[20,21] Increased concentrations of HMGB-1 have been found in septic human patients, and also in rodent models of sepsis.[20,22,23] This cytokine also shows potential as a therapeutic target for the treatment of SIRS, as it may contribute to the disease process over a prolonged period of time.[21] Therefore, it could also provide a wider therapeutic window than early phase cytokines such as tumor necrosis factor alpha.[21]

The presence of HMGB-1 has previously been established in synovial fluid of horses with joint disease,[24] using the same ELISA assay. The HMGB-1 protein is very well preserved between all mammalian species,[25,26] with the equine, porcine, and human HMGB-1 protein amino acid sequences sharing 99% homology.[24] This was confirmed during the present study by conducting a

Table 2. Median (range) of all biomarkers in survivors and nonsurvivors. None of the measured biomarkers were able to discriminate between survivors and nonsurvivors.

Biomarker	Survivors (n = 19)	Nonsurvivors (n = 9)	P-value
HMGB-1 (ng/mL)	12.2 (3.3–46.6)	14.6 (5.0–35.2)	.46
Nucleosomes (absorbance, 405–490 nm)	0.1 (0.0–1.1)	0.1 (0.0–1.1)	.66
Fibrinogen (mg/dL)	458.0 (116.0–1140.0)	339.0 (214.0–862.0)	.38
SAA (μg/mL)	400.0 (5.9–1480.0)	276.0 (1.2–834.0)	.31

BLAST analysis (NCBI protein database search). Therefore, the use of porcine recombinant HMGB-1 as the standard for this assay is justified. The validation data for the HMGB-1 assay suggested the presence of interfering substances in equine plasma. Therefore, our results might have underestimated the actual plasma concentrations, however, comparisons between groups are still valid. The poor performance of the HMGB-1 assay at high plasma concentrations is a limitation of the study and should be kept in mind when interpreting those results giving high plasma concentrations.

Nucleosomes are disk-shaped complexes made up of histones surrounded by DNA, which are released mainly during apoptotic cell death.[11,12] As nucleosomes result primarily from cell death, we expected their levels to be significantly higher in horses with strangulating lesions compared to other groups. The delay in their increase in the circulation for at least 24 h might have contributed to this, as horses were only sampled on presentation to the hospital. Therefore, horses with strangulating lesions likely presented very acutely in their disease, and thus had not had time to develop high plasma levels of nucleosomes despite having severe disease. It is also possible that nucleosome concentrations would increase more significantly in these horses after correction of the strangulating lesion and reperfusion had occurred. Inflammatory lesions such as colitis and peritonitis cause a massive inflammatory response in the horse, often causing SIRS.[27] Nucleosome concentrations have been shown to be increased in the serum of human patients with sepsis,[14] and furthermore, nucleosome levels have been able to differentiate between various degrees of disease severity, and survival versus nonsurvival.[13,14] Therefore, it is plausible that nucleosome concentrations would also be significantly increased in the cases investigated in the current study. However, there was no significant difference in nucleosome concentrations between survivors and nonsurvivors, although this might have been because of the low number of nonsurvivors.

Although designed for humans and rodents, the nucleosome assay used in this study also cross-reacts with other species.[12] Because of the heterogeneic nature of nucleosome size and composition, and difficulties in their purification, an absolute concentration or quantity of nucleosomes in a sample cannot be determined from a standard curve constructed using a particular source of these cell fragments.[12] Although recovery data could not be obtained for the nucleosome assay because of the lack of availability of a standard (for any species, including human and equine), this assay was considered semiquantitative, although again it was valid for the purposes of comparison between groups.

Overall, the concentrations of HMGB-1 and nucleosomes in the different groups showed similar patterns, being significantly higher in the inflammatory and strangulating groups compared to the healthy group. Furthermore, HMGB-1 and nucleosomes were well correlated, whereas neither fibrinogen nor SAA correlated with either of these two biomarkers. The differences between the biomarkers could be attributable to several reasons. Despite the differences between biomarkers measured, none could be considered clinically useful as an indicator for lesion type. Further investigation including using other biomarkers, and use of biomarkers cumulatively might provide greater delineation between lesion types.[28]

Both HMGB-1 and nucleosomes have been evaluated as prognostic indicators in human sepsis patients. HMGB-1 was found to be correlated with disease severity, number of organ dysfunctions and survival in human sepsis patients undergoing direct hemoperfusion with a polymyxin-B fiber column.[29] HMGB-1 has also been shown to be indicative of the development of sepsis in post-trauma human patients.[30] Nucleosome concentration has been shown to indicate the presence of sepsis, organ dysfunction and survival in human patients.[13] This biomarker has also been shown to be indicative of the need for renal support treatment in human sepsis patients.[31] Nucleosome concentrations have also been shown to differentiate between patients with variable severities of sepsis.[14]

The fact that HMGB-1 and nucleosomes were significantly increased in both these groups compared to healthy horses could be attributable to the way that they are released into the circulation. Nucleosomes are released during the later stages of cell death by apoptosis, which despite being largely a noninflammatory event, has been shown to be an important form of cell death and contributor to multiple organ dysfunction syndrome in human sepsis patients.[32,33] Apoptosis, as opposed to necrosis, has also been shown to be substantial, and perhaps the major contributor to cell death in intestinal ischemia and reperfusion.[34,35] Further, active nucleosome release has been shown to occur from stimulated neutrophils during inflammation.[36] HMGB-1 is released both passively during cell necrosis and apoptosis, as well as actively by inflammatory cells during inflammation.[21,25,37–39] Further, apoptotic cells might stimulate macrophages to secrete HMGB-1.[39] Because nucleosomes and HMGB-1 are in close relationship in the nuclei of cells, they can often be released together, and have been found to form complexes.[26,40–42] Therefore, it is not surprising that similar results were found for both biomarkers in this study, as well as correlation between the two. Fibrinogen, on the other hand, is formed in response to SIRS as part of the intimate relationship between inflammation and coagulation,[5,7] and therefore enters the circulation through a quite different mechanism. As fibrinogen and SAA are both acute phase proteins and so share a similar mechanism for release (produced by the liver in response to inflammatory cytokine production),[6] it is again not surprising that these biomarkers were correlated with each other but not with HMGB-1 or nucleosomes.

It is also possible that the timing of appearance of these substances into the circulation may affect the differences seen between groups of lesions. HMGB-1 can increase in the circulation as early as 8 h after the onset of disease but might not appear for up to 32 h[21], whereas fibrinogen and nucleosomes usually do not increase maximally for at least 24 h.[6,11] As many horses

with strangulating lesions present acutely because of the extremely painful nature of these lesions, they most likely have not had time for fibrinogen to increase appreciably at the time of presentation. However, if this were simply the case we would not expect enough time to have passed for a significant increase in nucleosomes in the strangulating group either.

Despite having a significant correlation, SAA was not superior to fibrinogen in its ability to distinguish between groups of horses with gastrointestinal disease. Serum amyloid A increases rapidly after the onset of inflammation,[6] however, still a significant difference between groups of horses with gastrointestinal disease was not detected. This is in contrast to another study where SAA was shown to be superior to fibrinogen in detecting inflammation in horses with surgical colic in the perioperative period.[8] However, direct comparison between that study and the current study is difficult because of differences in study design. In another study, SAA was not able to detect a difference between similar groups of horses with gastrointestinal disease in the early stages of disease, but was able to detect some differences between groups as it was followed over time.[7] However, again comparisons between that study and the current study are difficult as time from onset of clinical signs to blood sampling was not evaluated in the current study.

Limitations of this study include the consideration that samples were only taken at a single time point (on admission) with no precise indication of duration of clinical signs before presentation for many cases. It would likely be more useful to evaluate these biomarkers serially during hospitalization, with attention paid to duration of clinical signs before presentation, in order to better characterize their roles in equine SIRS. As mentioned already, the benefit of measuring SAA over several sequential time points to detect a difference between similar gastrointestinal disease categories has been demonstrated in another study.[7] Another way to evaluate this would be by using an experimental model of endotoxemia, which would allow for more controlled conditions. However, to see an effect on these later phase biomarkers experimentally, endotoxemia would have to be maintained for at least 48 h, which is possible although not commonly undertaken.[43] A further limitation of the study is the relatively low numbers of cases in each classification, however, for these biomarkers to be clinically useful in the assessment of individual cases, ideally they should show clear disease-related differences without large populations being required.

In conclusion, like fibrinogen and SAA, plasma concentrations of HMGB-1 and nucleosomes are increased in horses with gastrointestinal disease compared to healthy horses. Further studies, preferably including serial sampling over time and larger numbers of cases, are required to better characterize the role of these late-phase biomarkers in equine SIRS. As HMGB-1 itself acts as a late-phase cytokine and causes inflammation, investigation into its potential benefit as a therapeutic target is also warranted.

Footnotes

[a] HMGB-1 ELISA; IBL International, Hamburg, Germany
[b] Synergy H1 Hybrid Microplate Reader; BioTek, Winooski VT
[c] Gen5 Microplate Reader Software; BioTek, Winooski VT
[d] Cell Death Detection ELISA Plus; Roche Diagnostics, Mannheim, Germany
[e] Tridelta Development Ltd, Kildare, Ireland
[f] Graphpad Prism version 6 La Jolla, CA

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