

## Glucocorticoid Receptor Density and Binding Affinity in Healthy Horses and Horses with Systemic Inflammatory Response Syndrome

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**Background:** Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis occurs in horses with systemic inflammatory response syndrome (SIRS). Peripheral resistance to glucocorticoids has not been investigated in horses.

**Objective:** To determine if glucocorticoid receptor (GR) function in horses can be measured using flow cytometry, and to use this information to evaluate HPA axis dynamics.

**Animals:** Eleven healthy adult horses in parts 1 and 2. Ten horses with SIRS and 10 age and sex matched controls in part 3.

**Methods:** Flow cytometry was used to evaluate GR density and binding affinity (BA) in 3 healthy horses in part 1. In part 2, exogenous ACTH was administered to eight healthy horses. Their cortisol response and GR properties were measured. In part 3, CBC, serum biochemistry, cortisol and ACTH, and GR properties were compared between controls without SIRS (n = 10) and horses with SIRS (n = 10), and between survivors and nonsurvivors (n = 4 and n = 6 respectively).

**Results:** Flow cytometry can be used to measure GR properties in equine PBMCs. No correlation was observed between plasma cortisol concentration and GR density or BA in healthy horses ( $r = -0.145$ ,  $P = .428$  and  $r = 0.046$ ,  $P = .802$  respectively). Nonsurvivors with SIRS had significantly decreased GR BA ( $P = .008$ ). Horses with triglyceride concentration > 28.5 mg/dL had increased odds of nonsurvival (OR=117; 95% CI, 1.94–7,060). GR BA <35.79% was associated with non-survival (OR = 30.33; 95% CI, 0.96–960.5).

**Conclusions and Clinical Importance:** Tissue resistance to glucocorticoids contributes to HPA axis dysfunction in adult horses with SIRS. These horses might benefit from treatment with exogenous glucocorticoids.

**Key words:** Cortisol; Critical illness-related corticosteroid insufficiency; Equine; Glucocorticoid receptor; Systemic inflammatory response syndrome.

Critical illness-related corticosteroid insufficiency (CIRCI), caused by disrupted regulation of the hypothalamic-pituitary-adrenal (HPA) axis, has been identified in both foals and adult horses with systemic inflammation from a variety of pathologic conditions including colitis, pleuropneumonia, neonatal sepsis, and colic.<sup>1–5</sup> Hypothalamic-pituitary-adrenal (HPA) axis dysregulation has been demonstrated in horses using serum cortisol concentrations, serum adrenocorticotropic hormone (ACTH) concentrations, and by performing ACTH stimulation tests to measure the ability of the adrenal glands to respond to increased demand for circulating cortisol.<sup>1–5</sup> In horses with HPA axis dysregulation a low basal cortisol concentration, inadequate cortisol response to ACTH-stimulation, increased or decreased endogenous ACTH concentration, and increased ACTH:cortisol ratio all have been associated with increases in morbidity and mortality.<sup>1–5</sup> Dysregulation of the HPA axis has been localized to either the

### Abbreviations:

HPA axis	hypothalamic-pituitary-adrenal axis
SIRS	systemic inflammatory response syndrome
GR	glucocorticoid receptor
BA	binding affinity
ACTH	adrenocorticotropic
CIRCI	critical illness related corticosteroid insufficiency
PBMC	peripheral blood mononuclear cell
PE	phycoerythrin
FITC	fluorescein isothiocyanate
Ig	immunoglobulin
TG	triglyceride

adrenal glands or the central nervous system. However, there are no studies in horses examining the role of peripheral resistance at the level of the glucocorticoid receptor (GR) in the development of CIRCI.

Several studies in humans and animals have investigated the contribution of tissue resistance to endogenous cortisol in the development of CIRCI using flow cytometry and biochemical binding assays of the GR.<sup>6–11</sup> These have shown that with severe acute inflammation decreases in the number and binding affinity of GR occur, suggesting that tissue resistance to cortisol plays a pivotal role in the development of CIRCI. Although still controversial, several studies in human medicine have suggested that treating septic patients who have CIRCI with a low dose of hydrocortisone can lead to improved shock reversal and survival.<sup>12–17</sup> This “physiologic” dose of glucocorticoid supplementation was derived from studies examining the maximal cortisol secretory rate in response to exogenous HPA axis stimulation with cosyntropin or by multiplying the daily endogenous cortisol production rate in healthy, unstressed individuals by various illness factors to

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account for the increases in cortisol production associated with stress and illness.<sup>18,19</sup> This lower cortisol dose was developed to avoid the negative effects of corticosteroids on immune function encountered in earlier studies of treating septic shock with high doses of glucocorticoids, which reported increased episodes of “superinfection” including new episodes of sepsis or septic shock.<sup>20</sup> At this time, it is not known whether this same approach would be beneficial in patients with peripheral cortisol resistance.

There were 3 objectives of this study. The first was to determine if commercially available human and mouse fluorochromes could be used to determine the GR density and binding affinity in equine peripheral blood mononuclear cells (PBMCs). The second was to determine if there was a correlation between increased plasma cortisol concentration and GR density or binding affinity in healthy adult horses. The third objective was to evaluate the HPA axis in adult horses presenting with systemic inflammatory response syndrome (SIRS), and to determine where any alterations in HPA axis function, GR density or binding affinity occur in these patients compared to healthy adults. We hypothesized that the same methodology that has been applied to other species would be appropriate for evaluating GR properties in the horse. Additionally, as the plasma cortisol concentration increased, we hypothesized that there would be a corresponding change in GR density and binding affinity. Lastly, we predicted that horses presenting with SIRS/CIRCI would have increased plasma concentrations of ACTH and decreased concentrations of cortisol or an alteration in their ACTH:cortisol ratio, and would have decreased GR density and binding affinity. Determining the sites of HPA axis dysfunction in horses with SIRS may help guide treatment in these patients, particularly with regard to the use of a “physiologic” dose of glucocorticoids.

## Materials and Methods

### *Animals*

Three groups of horses were evaluated. Four healthy adult horses from a herd maintained at the Marion duPont Scott Equine Medical Center and 4 healthy client-owned horses were included in the first two parts of the study. Horses were determined to be healthy before inclusion by lack of abnormalities identified on physical examination. For the second part of the study, 10 adult horses presenting to the Marion DuPont Scott Equine Medical Center that met the criteria for SIRS, and 10 adult horses presenting for orthopedic or ophthalmic evaluation were included. The study design was approved by the Virginia Tech University Institutional Animal Care and Use Committee, and informed owner consent was obtained before enrollment of any client owned horses.

### *Study Design*

In part 1, 30 mL of peripheral venous blood was collected by jugular venipuncture into glass tubes containing potassium-EDTA at 8 a.m. on 3 separate days for flow cytometry analysis. Peripheral blood mononuclear cells were isolated from these samples and collected by density gradient centrifugation using Ficoll-Hypaque<sup>a</sup>. Cells were separated into 9 aliquots containing  $1 \times 10^6$  cells per

sample. Preliminary trials were performed with various volumes of primary and secondary antibody (results not shown), and the protocol described below was developed. The first aliquot served as a control for autofluorescence. Two aliquots were used to determine CD44 expression on PBMCs. Ten microliter Phycoerythrin (PE)-Mouse IgG<sup>b</sup> was added to the first tube as an isotype control and 10  $\mu$ L of PE-anti-mouse CD44<sup>c</sup> was added to the second tube. These were incubated in the dark at 4°C for 10 minutes. After incubation, cells were washed with 2 mL phosphate buffered saline (PBS) and centrifuged ( $400 \times g$  for 10 minutes at 4°C). The resulting supernatant was discarded, and the tubes were gently vortexed to resuspend the cells. The cells were washed two more times. One milliliter of PBS was added to each sample after the third wash.

Three aliquots were used to determine the density of GR in the cytosol of PBMCs. PBMCs were surface stained with PE-CD44 as described above. Next, PBMCs were permeabilized by adding 0.25 mL of CytoFix/CytoPerm solution<sup>d</sup> per tube and incubating the tubes in the dark at 4°C for 15 minutes. Two milliliters Cyto-Fix/CytoPerm wash buffer<sup>d</sup> then was added followed by centrifugation ( $400 \times g$  for 10 minutes at 4°C). The resulting supernatant was discarded, and cells were resuspended in 1 mL PBS. Ten microliters purified mouse antiglucocorticoid receptor<sup>e</sup> then was added to the first sample, and an equal volume of purified mouse IgG<sup>f</sup> was added to the other two aliquots as an isotype control for the primary antibody and a control for the secondary antibody, which was added next. These samples were allowed to incubate in the dark at 4°C for 10 minutes. Next, 10  $\mu$ L of a fluorescein isothiocyanate (FITC) goat anti-mouse secondary antibody<sup>g</sup> was added to the sample containing the anti-mouse GR antibody and to one of the aliquots stained with mouse isotype control. These samples were allowed to incubate in the dark at 4°C for 10 min, washed 3 times as described above, and then resuspended in 1 mL of PBS.

Two aliquots were used to assess GR binding affinity. Peripheral blood mononuclear cells were surface stained with PE-CD44 and permeabilized as described above. One sample was incubated with 5  $\mu$ g of dexamethasone<sup>h</sup> for 10 minutes at 37°C, and then  $1.5 \times 10^{-7}$  M of fluorescein-labeled dexamethasone<sup>i</sup> was added to both tubes. These tubes were incubated in the dark at room temperature for 1 hour, gently mixing the sample every 10 minutes. The cells then were washed with PBS as described above. Finally, one aliquot was used to determine cell viability. Cells were surface stained for CD44 as described above and then 2  $\mu$ L of propidium iodide solution<sup>j</sup> was added to the sample. This mixture was allowed to incubate in the dark at room temperature for 5 minutes before analysis using flow cytometry.

Cell samples were run on a FACSCalibur flow cytometer<sup>k</sup>, and analyzed by CELLQuest Software<sup>l</sup>. Twenty-thousand gated events were acquired per sample. A dot plot of forward scatter and side scatter enabled visualization of voltage adjustments in order to move PBMCs into a gated region. CD44<sup>+</sup> and GR+ cells were identified and gated on PE (FL2) and FITC (FL1) plots respectively. The relative density of GR (DeltaGR%) was expressed as the percentage of PBMCs that were positive for both the PE and FITC antibodies minus the percentage of isotype control PBMCs that were positive for both. CD44<sup>+</sup> and FITC-dexamethasone+ cells also were identified and gated on these plots. The relative binding affinity of GR (Delta BA%) was determined in the same manner as DeltaGR%. Cell viability was determined by evaluating cells that were positive for both the PE and PI stain (FL3).

For part 2, horses were placed in a stall the evening before performing an ACTH stimulation test. The horses were weighed, and a physical examination was performed. An IV jugular catheter<sup>m</sup> was placed aseptically in the right or left jugular vein of each horse 12 hours before intervention. The catheter was irrigated every 6 hours with heparinized saline. Horses were housed in individual stalls, and orchard grass hay and water were offered

free choice. An ACTH stimulation test was performed at 8 a.m. the next morning, using 0.5 µg/kg of cosyntropin<sup>n</sup> administered IV. Ten milliliters of venous blood samples were collected into a sterile glass tubes containing potassium-EDTA at baseline, and 4, 8, and 24 hours post-ACTH injection for measurement of plasma cortisol concentration. Samples were centrifuged within 20 min of collection at 5°C, 400 g for 15 minutes, and then plasma was aliquoted and stored at -30°C until shipment for hormone concentration analysis. Thirty milliliters of venous blood was obtained and placed in tubes containing potassium-EDTA for GR density and binding affinity determination at each time point, and stored at 4°C for a maximum of 1 hour before PBMC isolation.

In part 3, horses were included in the SIRS group if they met  $\geq 2$  of the following criteria on presentation: (1) leukocytosis or leukopenia (white blood cell count  $>12,000$  cells/ $\mu\text{L}$  or  $<5,000$  cells/ $\mu\text{L}$ ) or the presence of  $\geq 10\%$  band neutrophils, (2) hyperthermia (rectal temperature  $>101.5^\circ\text{F}$ ), (3) tachycardia (heart rate  $>60$  beats per minute), or (4) tachypnea (respiratory rate  $>30$  breaths per minute).<sup>21-23</sup> Horses presenting to the hospital for orthopedic or ophthalmic evaluation were used as age- and sex-matched controls. Data collected on presentation included history, primary complaint and baseline physical examination findings. Blood variables assessed included CBC, serum biochemistry, plasma fibrinogen concentration, plasma hormone concentrations (ACTH and cortisol), and GR density and binding affinity.

Blood samples were collected by direct jugular venipuncture into tubes containing potassium-EDTA for measurement of plasma endogenous ACTH and cortisol concentrations, CBC, plasma fibrinogen concentration and GR density and binding affinity. Blood was collected into plastic tube containing sodium heparin for serum biochemistry analysis. Complete blood count serum biochemistry, and plasma fibrinogen concentrations were determined immediately. The CBC was performed using an automatic blood cell counter<sup>o</sup> and manual differential cell count. Plasma fibrinogen concentration was determined using the heat precipitation method. Serum biochemistry analysis was performed using an automated chemistry analyzer<sup>p</sup>. A total of 10 mL of venous blood was obtained for hormone assays, and centrifuged within 20 minutes of collection at 5°C, 400 g for 15 minutes. Plasma then was aliquoted and stored at -30°C until shipment for hormone concentration analysis. A total of 20 mL of venous blood was obtained for GR density and binding affinity determination, which was stored at 4°C for a maximum of 1 hour before PBMC isolation.

### Hormone Assays

Plasma ACTH and cortisol concentrations were determined using an automated chemiluminescent enzyme immunoassay<sup>q</sup> previously validated for horses.<sup>24,25</sup>

### Statistical Analysis

Statistical analysis was performed using commercial statistical software (GraphPad Prism 6<sup>r</sup> and SAS<sup>s</sup>). Data sets were tested for normality by the Shapiro-Wilk statistic. A student *t*-test or Mann-Whitney test was used to compare CBC, serum biochemistry variables, hormone concentrations, and GR density and binding affinity between healthy controls in part 1 of this study, and between healthy controls and SIRS cases and survivors and non-survivors in part 3. Repeated measures analysis of variance (ANOVA) with multiple comparisons conducted using Tukey's test was used to compare the PBMC count and cell viability. The Friedman test was used to evaluate changes in cortisol concentrations, Delta GR% and Delta BA% over the 3 days in part 1, and over the 24-hour study period in part 2 of the study, with Dunn's multiple

comparison test used when significant differences were found. Spearman's rank correlation test was used to evaluate for an effect of cortisol on Delta GR% and Delta BA% in part 2, and to evaluate the relationship between plasma hormone concentrations, Delta GR% and Delta BA%, and serum biochemistry and CBC variables in part 3 of the study.

Receiver-operator curve (ROC) analysis was performed on plasma ACTH and cortisol concentrations, ACTH:cortisol ratio, GR variables, and serum triglyceride (TG) concentrations. The cut-points for these results determined by the sensitivity and specificity combination with the highest likelihood ratio, then were used to assess these results in this population using Fisher's exact test and to determine an OR for nonsurvival. Hypothesis tests were 2-tailed, and statistical significance was set at  $P < .05$  for all analyses. Data are expressed as mean  $\pm$  standard deviation, or median and range as appropriate.

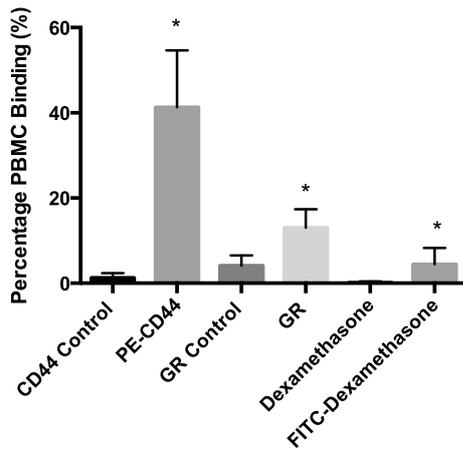
## Results

### Part 1

Mean age of the horses included in part 1 of the study was  $13.3 \pm 7.5$  years. All 3 horses were female, and consisted of a Quarter Horse, a Paint Horse, and a Thoroughbred. The median PBMC count recovered from these horses on all 3 days was  $1.7 \times 10^6$  cells/mL. There was no significant variation among horses or by day of collection on PBMC recovery. The mean PBMC viability was  $95.16 \pm 1.65\%$ . There was no significant effect of horse or day of collection on PBMC viability.

To evaluate the density and binding affinity of GR by equine PBMCs, we first identified mononuclear cells from each sample. Cells first were analyzed without any added antibodies, and dot diagrams were used to visualize cells separated by side scatter and forward scatter. Cells with characteristic forward and side scatter were identified as lymphocytes and monocytes, and these cells were gated for further sampling. CD44 is a glycoprotein expressed on all leukocytes, endothelial cells, hepatocytes, and mesenchymal cells, and thus was used in this study to identify both lymphocytes and monocytes. Binding of an isotype control for the CD44 antibody was used to control for the CD44 antibody and to account for any background effect of the flow cytometer and analysis software. This then was compared to samples incubated with the CD44 antibody. The median percentage of PBMCs that bound the isotype control was 0.70% (range, 0.51-3.92%) and the median percentage of PBMCs that bound the CD44 antibody was 44.20% (range, 23.10-64.68%). These values were significantly different ( $P < .0001$ ), and there was no significant effect of horse or day on these measurements.

After identification of the CD44<sup>+</sup> population of cells, the density of GR in this cell population was determined. Dot diagrams were used to visualize expression of two receptors simultaneously. After correcting for the FITC-goat anti-mouse immunoglobulin (Ig) control, the mean percentage of PBMCs that bound both the GR and CD44 antibodies was  $8.87 \pm 4.61\%$ . There was a significant difference between the FITC-control cells and the cells expressing GR ( $P < .0001$ ), and there was no significant effect of horse or time on these measurements. Using this same population of CD44<sup>+</sup>



**Fig 1.** Percentage peripheral blood mononuclear cell binding for CD44 control, PE-CD44, GR control, FITC-GR, dexamethasone control and FITC-dexamethasone. Asterisk (\*) indicates a significant difference between the control and fluorescent antibody.

cells, the binding affinity of the GR in the PBMCs was determined. Again, dot diagrams were used to visualize expression of the two receptors simultaneously. There was a significant difference between the control group, where the receptors were blocked by dexamethasone, and those incubated with FITC-dexamethasone ( $P < .0001$ ). Again, there was no significant effect of horse or time on these measurements. After correcting for the control samples, the median percentage of PBMCs that bound both the FITC-dexamethasone and CD44 antibodies was 2.15% (range, 0.97–11.29%). Figure 1 summarizes these results.

### Part 2

This group consisted of 4 mares and 4 geldings, with a mean age of  $12.0 \pm 6.7$  years. There were 3 Paint Horses, 2 Quarter Horses, 2 Thoroughbreds, and 1 Warmblood cross. All were considered healthy based on physical examination. The mean PBMC count recovered from these horses at all 4 time points was  $2.4 \times 10^6 \pm 1.0 \times 10^6$  cells/mL, and the mean PBMC viability was  $92.46 \pm 3.33\%$ . There was no significant variation among horses or time points for cell count or viability.

The median plasma cortisol concentration at baseline was 4.9  $\mu\text{g/dL}$  (range, 3.2–6.1  $\mu\text{g/dL}$ ). The median cortisol concentration 4 hours after the administration of ACTH was 5.6  $\mu\text{g/dL}$  (range, 4.8–7.4  $\mu\text{g/dL}$ ). The cortisol concentrations at baseline and 4 hours were significantly higher than the 8 hour post-ACTH administration cortisol concentration of 1.4  $\mu\text{g/dL}$  (range, 1.1–2.7  $\mu\text{g/dL}$ ;  $P = .0221$  and  $P = .0006$  respectively). The median Delta GR% over the course of the 24-hour period was 16.28% (range, 1.21–53.63%), and there was no significant difference in these results at any time period. The median Delta BA% over the course of the 24-h period was 45.00% (range, 7.01–90.98%), and there were no significant differences in this value among horses or over the 24 hour of analysis.

There was no correlation between cortisol concentration and Delta GR% ( $r = -0.145$ ,  $P = .428$ ) or Delta BA% ( $r = 0.046$ ,  $P = .802$ ). However, there was a significant correlation between Delta GR% and Delta BA% ( $r = -0.483$ ,  $P = .005$ ).

### Part 3

Twenty horses were included in this part of the study, 10 controls and 10 SIRS cases. Of the SIRS cases, 4 horses survived to discharge and the other 6 were because of perceived poor prognosis by the attending clinician. The mean age was  $12.7 \pm 4.1$  years, and there was no significant difference in age between SIRS cases and controls or survivors and nonsurvivors. The mean PBMC count was  $1.6 \times 10^6 \pm 0.6 \times 10^6$  cells/mL and the mean PBMC viability was  $95.28 \pm 2.58\%$ . There was no significant difference between SIRS cases and controls for either PBMC count or viability.

When the CBCs of the SIRS cases and controls were compared, the WBC count in SIRS cases was found to be significantly higher ( $P = .0191$ ), as was the percentage of band neutrophils ( $P = .0031$ ) and eosinophils ( $P = .0007$ ), whereas SIRS cases had lower percentages of lymphocytes ( $P = .0045$ ) and monocytes ( $P = .0080$ ) compared to controls (Table 1). No significant differences were detected when the CBCs of survivors and nonsurvivors were compared. When serum biochemistry results of SIRS cases and controls were compared, the SIRS cases were found to have significantly higher activities or concentrations of AST ( $P = .0049$ ), BUN ( $P = .0002$ ), CK ( $P = .0173$ ), creatinine ( $P = .0489$ ), and LDH ( $P = .0231$ ), whereas their serum chloride concentration was found to be significantly lower ( $P = .0050$ ; Table 2). When the serum biochemistry of survivors and nonsurvivors were compared, the only significant difference detected was in their serum TG concentrations, with a median of 58 mg/dL (range, 29–612 mg/dL) in nonsurvivors compared to a median of 16 mg/dL (range, 10–28 mg/dL) in nonsurvivors ( $P = .0095$ ).

Plasma ACTH and cortisol concentrations, and ACTH:cortisol ratios were compared between SIRS cases and controls as well as survivors and nonsurvivors (Tables 3 and 4). The median baseline ACTH and cortisol concentrations of controls were significantly lower than those of the SIRS cases ( $P = .0002$  and  $P = .0232$ , respectively). The ACTH:cortisol ratios were not significantly different between SIRS cases and controls or survivors and nonsurvivors. Delta GR% and Delta BA% were compared between SIRS cases and controls as well as survivors and nonsurvivors (Tables 3 and 4). The Delta BA% was significantly decreased in nonsurvivors compared to survivors ( $P = .008$ ).

Receiver-operator curve analysis and the area under the curves (AUCs) were used to determine cut-off results for the plasma ACTH, plasma cortisol, ACTH:cortisol, and Delta BA% for predicting nonsurvival. This revealed that serum TG concentration  $> 28.5$  mg/dL and a Delta BA%  $> 35.79\%$  were associated with

**Table 1.** Complete Blood Cell Count values in healthy controls and horses presenting with SIRS, including: White Blood Cell Count (WBC), Neutrophils (Neuts), Band Neutrophils (Bands), Lymphocytes (Lymphs), Eosinophils (Eos), Monocytes (Monos), Toxic Morphology, Hematocrit (Hct), Platelet Count (Plt), Total Protein (TP), and Fibrinogen (Fib). Values with an asterisk (\*) indicate a significant difference between groups.

Group	WBC (cells/ $\mu$ L)	Neuts (%)	Bands (%)	Lymphs (%)	Eos (%)	Monos (%)	Toxic		Hct (%)	Plt ( $\times 10^3$ cells/ $\mu$ L)	TP (g/dL)	Fib (mg/dL)
							(#/total)					
Healthy Controls (n = 10)	7,300 $\pm$ 1,712	67.90 $\pm$ 10.84	0	29.00 $\pm$ 10.33	0	4.0 (1.0–18.0)	0/10	38.50 $\pm$ 6.654	206.1 $\pm$ 43.14	6.98 $\pm$ 0.649	250 (100–700)	
SIRS Cases (n = 10)	11,940 $\pm$ 5,437*	76.00 $\pm$ 16.76	4.50 (0–11.0)*	14.00 $\pm$ 10.32*	2.0 (0–3.0)*	1.0 (0–4.0)*	3/10	43.40 $\pm$ 13.91	185.7 $\pm$ 68.43	7.31 $\pm$ 1.76	200 (100–700)	
P-value	.019	.40	.003	.0045	.0007	.008	.21	.19	.82	.61	.50	

**Table 2.** Serum Biochemistry results in healthy controls and SIRS cases, including: Albumin, Alkaline Phosphatase (ALP), Amylase, Aspartate Aminotransferase (AST), Blood Urea Nitrogen (BUN), Calcium (Ca), Creatine Kinase (CK), Creatinine (Creat), Direct Bilirubin (D Bili), Gamma Glutamyl Transferase (GGT), Glucose, Sodium (Na), Potassium (K), Chloride (Cl), Lactate Dehydrogenase (LDH), Magnesium (Mg), Phosphorus (Phos), Total Bilirubin (T Bili), and Triglycerides (TG). Values with an asterisk (\*) indicate a significant difference between groups.

Group	Albumin (g/dL)	ALP (U/L)	Amylase (U/L)	AST (U/L)	BUN (mg/dL)	Ca (mg/dL)	CK (U/L)	Creat (mg/dL)	D Bili (U/L)	GGT (U/L)	Glucose (mg/dL)	Na (mEq/L)	K (mEq/L)	Cl (mEq/L)	LDH (U/L)	Mg (mg/dL)	Phos (mg/dL)	T Bili (U/L)	TG (mg/dL)
Healthy Controls (n = 10)	2.9 (2.6–3.4)	127.5 (79.0–298.0)	3.0 (0–6.0)	256 (163–336)	14 (11–21)	10.81 $\pm$ 0.735	205.5 (106–951)	1.2 (0.8–2.4)	0.1 (0–0.3)	21.5 (13–34)	100.5 (84–232)	134.7 $\pm$ 4.76	3.30 $\pm$ 0.43	100.6 $\pm$ 3.20	248 $\pm$ 113.2	1.97 $\pm$ 0.47	2.58 $\pm$ 0.57	1.35 (0.7–5.5)	21 (6–41)
SIRS Cases (n = 10)	2.95 (1.8–3.4)	170.5 (55.0–342.0)	6.5 (1.0–517.0)	391 (189–762)*	24.5 (15–67)*	10.49 $\pm$ 1.92	530 (150–3060)*	1.6 (0.8–2.8)*	0.1 (0–0.1)	21.5 (10–229)	159.5 (77–282)*	131.4 $\pm$ 4.27	3.15 $\pm$ 0.66	96.0 $\pm$ 3.23*	418.5 $\pm$ 185.1	1.62 $\pm$ 0.68	2.47 $\pm$ 1.05	2.7 (1.0–9.4)	35.5 (10–612)
P-value	.26	.44	.53	.005	.0002	.95	.017	.049	.58	.62	.022	.94	.89	.032	.089	.59	.44	.066	.059

**Table 3.** Median basal endogenous plasma ACTH concentration, total plasma cortisol concentration, basal ACTH:cortisol ratio, median Delta GR and mean Delta BA for SIRS cases and healthy controls. Values with an asterisk (\*) indicate a significant difference between groups.

Group	ACTH Concentration (pg/mL)	Cortisol Concentration (µg/dL)	ACTH:Cortisol	Delta GR (%)	Delta BA (%)
Healthy Controls (n = 10)	28.75 (9.07–202.0)	4.46 (2.29–14.00)	4.85 (2.13–27.82)	33.06 (20.64–73.56)	31.06 ± 25.29
SIRS Cases (n = 10)	72.50* (39.90–689.0)	9.46* (0.81–41.20)	8.09 (4.49–97.14)	56.13 (40.67–68.43)	28.57 ± 25.36

**Table 4.** Median basal endogenous plasma ACTH concentration, total plasma cortisol concentration, basal ACTH:cortisol ratio, median Delta GR and mean Delta BA for survivors and nonsurvivors with SIRS. Values with an asterisk (\*) indicate a significant difference between groups.

Group	ACTH Concentration (pg/mL)	Cortisol Concentration (µg/dL)	ACTH:Cortisol	Delta GR (%)	Delta BA (%)
Survivors (n = 4)	54.54 (39.90–202.0)	9.33 (5.20–14.0)	6.43 (4.50–20.63)	55.35 ± 12.64	51.51 ± 24.53
Nonsurvivors (n = 6)	150.1 (60.00–689.0)	10.97 (0.81–41.20)	20.83 (4.69–97.14)	52.51 ± 19.98	13.28 ± 9.77*

an increased OR of non-survival (OR = 117; 95% CI, 1.94–7,060 and OR = 30.33; 95% CI, 0.96–960.5, respectively). Spearman correlation analysis identified no correlations between serum TG concentration and plasma ACTH or cortisol concentrations, ACTH:cortisol ratio or the Delta GR% and Delta BA%. A significant negative correlation was observed between plasma ACTH concentration and ACTH:cortisol and Delta BA% ( $r = -0.685$ ,  $P = .029$  and  $r = -0.697$ ,  $P = .025$  respectively). No correlation between Delta GR% and Delta BA% was observed ( $r = 0.176$ ,  $P = .627$ ).

### Discussion

Measurement of the density and binding affinity of the equine GR in PBMCs was readily performed using flow cytometry. Although no correlation was observed between endogenous cortisol concentration and either GR density or binding affinity, a decrease in the Delta BA% and an increase in serum TG concentrations were found to be significantly associated with increased OR of nonsurvival in these cases. These horses also had an increased, but not statistically significant, ACTH:cortisol ratio. These findings suggest that HPA axis dysfunction may occur at multiple levels in adult horses presenting with SIRS, and additional research is needed to further elucidate methods for rapidly detecting which horses are at risk of nonsurvival and those that may benefit from supplementation with glucocorticoids.

In this study, CD44 was chosen as a cell surface marker to identify mononuclear cells. This cell-surface glycoprotein is expressed on all leukocytes, endothelial cells, hepatocytes, and mesenchymal cells. In a pilot study, the PE-CD44 antibody was shown to be effective at identifying both equine lymphocytes and monocytes. There was no significant difference in the percentage of CD44<sup>+</sup> cells among horses on any given day of collection. Cell viability of PBMCs was determined using flow cytometry based on the uptake of propidium iodide by cells that were simultaneously labeled with

CD44. Propidium iodide is a DNA-binding dye that is known to pass only through the cell membrane of dead or dying cells.<sup>26</sup> The mean PBMC viability obtained for all three parts of this study was  $93.78 \pm 3.187\%$ . Although there is no standard value for the viability level below which a specimen yields data that is unacceptable for flow cytometry analysis, samples with decreased cell viability can have an effect on cell staining and can induce cell clumping.<sup>27</sup> Because all of the samples in this study had similar cell viability, we do not believe this contributed to any artifact in these results.

A mouse monoclonal GR antibody was used to detect and quantify the GR in equine PBMCs, using a technique previously validated in studies in rats, humans, and cattle.<sup>11,28–34</sup> In part 3, substantial variation in GR density among horses was detected, making it difficult to detect differences between SIRS cases and controls. In another study, the percentage of GR identified in human PBMC samples differed when monocytes or lymphocytes were examined individually.<sup>11</sup> In that study, when CD3<sup>+</sup> cells (T-lymphocytes) were examined in healthy individuals, a mean GR density of 72.7% was identified, whereas a mean of 43.6% was found in CD14<sup>+</sup> cells (monocytes).<sup>11</sup> Therefore, using separate cell surface markers to identify lymphocytes and monocytes might have helped decrease the variability in the GR density determined, and potentially may have highlighted differences in GR density between SIRS cases and controls as well as survivors and nonsurvivors.

Fluorescent-labeled dexamethasone was used to determine the binding affinity of the GR in equine PBMCs. There was substantial variation in binding affinity, ranging from 5.47 to 56.64% in healthy horses and from 3.51 to 23.05% in nonsurvivors with SIRS. However, unlike GR density, a significant decrease in binding affinity was detected between survivors and nonsurvivors in this study. In another study examining periparturient Holstein cows, GR binding affinity was decreased in lymphocytes and monocytes of cows immediately post-

calving compared to the 28 days before calving, and this decrease persisted for up to 14 days.<sup>34</sup> Thus, the decrease in GR binding affinity detected with systemic inflammation in this study coincides with what has been detected in inflammatory states of other species.

When an ACTH stimulation test was performed on healthy horses to increase endogenously produced cortisol, we did not detect a correlation between plasma cortisol concentration and GR density or binding affinity. This finding was similar to what was reported in other studies in which no correlation between plasma cortisol concentrations and GR density or binding affinity were detected.<sup>10,35</sup> Additionally, in part 2 of the present study, GR density was not significantly different between groups, although horses with SIRS had significantly higher plasma cortisol concentrations. This observation suggests that the relationship between plasma cortisol concentration and GR behavior is complex, and most likely influenced by other local factors. In another study, human PBMCs incubated with IL-2 and IL-4 demonstrated decreased GR binding affinity that was reversed when the cells were incubated in medium without cytokines present.<sup>36</sup> Thus, there appears to be a local effect of inflammatory cytokines on GR binding affinity acting separately from the effects of circulating cortisol. Additionally, studies examining different GR isoforms have shown an increase in both GR $\alpha$  and GR $\beta$  in inflammatory states and a concomitant decrease in the GR $\alpha$ :GR $\beta$  ratio.<sup>37-40</sup> The GR $\beta$  isoform has been shown to have a dominant negative effect on GR $\alpha$ -induced transcriptional activity. Thus, in the presence of local inflammatory cytokines, an increase in GR $\beta$  also could explain the decreased binding affinity seen in this study.<sup>37</sup>

Horses with SIRS that did not survive had significantly decreased Delta BA% compared to survivors. Additionally, nonsurvivors had an increased, but not statistically significant, ACTH:cortisol ratio compared to survivors. In previous studies in foals, the ACTH:cortisol ratio has been used as a measure of decreased adrenal responsiveness to ACTH because cortisol concentrations should increase in proportion to ACTH concentrations. These findings suggest that HPA axis dysfunction occurs in horses with SIRS both at the level of the adrenal gland and the GR. The increased concentrations of ACTH and cortisol seen in horses with SIRS in this study are consistent with an appropriate response to critical illness. However, decreased cortisol release in relation to the increased ACTH concentrations in nonsurvivors suggested inadequate cortisol production and release by the adrenal glands. Extensive adrenal hemorrhage has been documented in up to 30% of critically ill humans who did not survive septic shock, and has been reported as a common finding on necropsy examination of horses suffering from endotoxic shock.<sup>12,41-43</sup>

In the clinical cases with SIRS, an increase in serum TG concentration was found to be associated with non-survival. In several human and animal models, increased concentrations of circulating endotoxin have been found to produce an increase in serum TG concen-

trations.<sup>44-47</sup> Mechanisms proposed to explain this finding include an increase in lipoprotein production by the liver in response to an increase in inflammatory cytokine concentrations, including TNF- $\alpha$  and IL-1, as well as a decrease in lipoprotein clearance caused by a decrease in lipoprotein lipase activity.<sup>44-47</sup> Hypertriglyceridemia has previously been documented in adult horses with SIRS, but has not been evaluated as a biomarker for determining prognosis or disease state in this species.<sup>48</sup> In a study of foals, a median TG concentration >100 mg/dL was observed in a population of septic foals, compared to medians of <100 mg/dL in sick nonseptic and healthy controls.<sup>49</sup> Additionally, the TG concentration in the nonsurvivor group was twice that of the concentration in the survivors.<sup>49</sup> Similarly, in a study of humans examining the utility of serum biomarkers in predicting in-hospital mortality, patients with higher serum TG concentrations (>300 mg/dL) had a higher mortality rate compared to those with lower serum TG concentrations (37% versus 7%).<sup>50</sup> In this study, we found that a TG concentration >28.5 mg/dL was highly associated with nonsurvival. Although the negative correlation between TG concentration and the Delta BA% was not significant, this may have been related to small size. Thus, increased TG concentrations are observed commonly in horses with SIRS, may have a role as a biomarker of HPA axis activation, and may be associated with poor prognosis for survival.

This study had several limitations. With regard to methodology, no validation study was performed. The protocol was modeled after similar studies performed with human, rat and bovine PBMCs, and part 1 showed no significant variability in our measurements in healthy horses. Additionally, although there are likely substantial differences in GR-binding affinity between dexamethasone used for our analysis in this study and endogenous cortisol, this same technique was used in a study of humans to successfully demonstrate cortisol resistance in PBMCs isolated from patients with systemic lupus erythematosus.<sup>11</sup> The relevance of this difference as it applies to this study remains unknown at this time, and may have an impact on the clinical relevance of these results. Lastly, cortisol concentration was measured at 4, 8, and 24 hours after ACTH administration rather than at earlier time points. In another study, administration of 0.5  $\mu$ g/kg of ACTH caused a significant increase in cortisol concentrations up to 4 hours after administration.<sup>5</sup> In a study of humans, storing blood samples at room temperature for prolonged periods caused granulocyte activation, which affected their buoyancy profile, and resulted in less efficient separation of PBMCs by density gradient procedures.<sup>51</sup> Additionally, another study indicated that delays in processing blood of 8 hours increased granulocyte contamination of PBMCs 2.3-fold compared to samples processed in <3 hours.<sup>52</sup> Because cell viability was crucial to the methodology used in this study, the time points for blood collection were distributed relatively widely to allow time for PBMC isolation between each sample collection to ensure appropriate cell viability. If additional studies are performed using this methodology,

having collection that coincide with peak cortisol concentrations at 30–90 minutes would be beneficial to determine if there is a more immediate effect on the GR.

The healthy horses used in the second part of this study had a short acclimatization period (15 hours) before performing the ACTH stimulation test. Although this time period may not have been long enough to eliminate all external effects of stress on their cortisol concentrations, all horses were maintained under the same housing and handling conditions in an effort to control for this effect. In part 3 of this study, horses presenting to the hospital for lameness or ophthalmic evaluations were used as controls. Inflammation associated with chronic lameness or ophthalmic disease could have caused some HPA axis activation, potentially affecting these results. We elected to use these cases as controls to account for the effect of shipping and the stress associated with being in a new environment on HPA axis analysis, because shipping horses even for short periods has been shown to produce changes in their cortisol concentrations.<sup>53</sup>

Although CIRCI has been more thoroughly documented in foals, we elected to examine the inflammatory response in adult horses because of concerns about subject availability. However, these adult horses presented with an array of disorders, introducing a substantial amount of variability in the SIRS population, with about half of these patients not meeting the criteria for sepsis or septic shock. Because the appropriate endocrine and HPA response to critical illness varies substantially among individuals and over the time course of disease, utilizing a small and heterogenous study group may have resulted in erroneous conclusions. Thus, it would have been ideal to examine a more uniform population (e.g. only horses with colitis). Additionally, although the SIRS cases were classified as survivors and nonsurvivors for analysis in this study, this classification also could have been affected by perceptions of a poor prognosis by the attending clinician. In such a small group of horses, this may have altered the results for the diagnostic utility of these variables. Therefore, this effect should be examined in a larger group of horses to determine if the associations found in this study are more broadly applicable. Lastly, it would have been beneficial to perform necropsy examinations on all of the nonsurvivors to determine if adrenal pathology was present. This would have helped to make a stronger argument for the presence or absence of adrenal dysfunction in these horses.

In summary, the commercially available PE-CD44 fluorochrome, mouse anti-glucocorticoid receptor with a secondary FITC-goat anti-mouse immunoglobulin, and FITC-dexamethasone can be used to evaluate the equine GR on PBMCs. In healthy horses, endogenous cortisol concentration was not correlated with either GR density or binding affinity. This finding supports previous work suggesting a complex interaction between cortisol and the cellular GR, also involving local factors, mainly inflammatory cytokines. Additional work in larger populations of horses is needed to determine

the effectiveness of easily measured variables such as the serum TG concentration, as potential proxies for evaluating the HPA axis. Additionally, it would be beneficial to examine GR activity in foals as well to determine if peripheral resistance to cortisol also occurs in neonates. Current research supports HPA axis dysfunction at the level of the adrenal gland, and has led to the suggestion of supplementing these patients with “physiologic” concentrations of glucocorticoids (e.g. hydrocortisone). However, it is unknown if decreased GR binding affinity also is present in these foals, and how it would affect their response to treatment with hydrocortisone.

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## Footnotes

- <sup>a</sup> Histopaque®-1077, Sigma-Aldrich, St. Louis, MO.
- <sup>b</sup> PE-Cy™5 Mouse IgG1 κ Isotype Control, BD Pharmingen™, San Jose, CA.
- <sup>c</sup> PE anti-mouse/human CD44, BioLegend, San Diego, CA.
- <sup>d</sup> BD CytoFix/CytoPerm™ Fixation/Permeabilization Solution Kit, BD Biosciences, San Jose, CA.
- <sup>e</sup> Purified Mouse Anti-Glucocorticoid Receptor, BD Transduction Laboratories™, San Jose, CA.
- <sup>f</sup> Purified Mouse IgG1 κ Isotype Control, BD Pharmingen™, San Jose, CA.
- <sup>g</sup> FITC Goat Anti-Mouse Ig, BD Pharmingen™, San Jose, CA.
- <sup>h</sup> Dexamethasone, Sigma-Aldrich, St. Louis, MO.
- <sup>i</sup> Dexamethasone Fluorescein, Life Technologies Corporation, Carlsbad, CA.
- <sup>j</sup> Propidium Iodide Staining Solution, BD Pharmingen™, San Jose, CA.
- <sup>k</sup> FACSCaliber, BD Biosciences, San Jose, CA.
- <sup>l</sup> CellQuest Software, Becton Dickinson and Company, San Jose, CA.
- <sup>m</sup> Abbocath, 14-gauge 5.5 inch PEP polymer, Medline Industries Inc, Mundelein, IL.
- <sup>n</sup> Cortrosyn®, Amphstar Pharmaceuticals, Rancho Cucamonga, CA.
- <sup>o</sup> AC-T diff™ Analyzer, Beckman Coulter®, Brea, CA.
- <sup>p</sup> Synermed© IR-500 Chemistry Analyzer, Synermed, Westfield, IN.
- <sup>q</sup> Immulite, Diagnostic Products Corporation, Los Angeles, CA.
- <sup>r</sup> GraphPad Prism Statistical Software, version 6, GraphPad Software Inc, San Diego, CA.
- <sup>s</sup> SAS, version, SAS Institute Inc, Cary, NC.

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*Off-label Antimicrobial Declaration:* Authors declare no off-label use of antimicrobials.

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