Relationships among inflammatory cytokines, obesity, and insulin sensitivity in the horse
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*J ANIM SCI* 2007, 85:1144-1155.
doi: 10.2527/jas.2006-673 originally published online January 30, 2007

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http://www.journalofanimalscience.org/content/85/5/1144
ABSTRACT: Recent studies associate obesity and insulin resistance in horses with development of abnormal reproductive function and debilitating laminitis. The factors contributing to insulin resistance in obese horses are unknown. However, human studies provide evidence that elevated inflammatory cytokines such as tumor necrosis factor α (TNFα), IL1, and IL6 play direct roles in development of obesity-associated insulin resistance. Thus, inflammation may be a key link between obesity and insulin resistance in horses. The aim of the current investigation was to examine possible relationships between obesity, inflammatory cytokines, and insulin sensitivity (IS) in the horse. Age was recorded and BCS and percent body fat (% FAT) were determined as measures of obesity in 60 mares. In addition, blood mRNA expression of IL1, IL6, and TNFα and circulating concentrations of TNFα protein (TNFp) were determined in each mare. Finally, fasted concentrations of insulin were determined, and IS was determined using the hyperinsulinemic, euglycemic clamp. Significant correlations between several variables provided evidence for the design of 4 population regression models to estimate relationships between measures of obesity, inflammatory factors, and IS in the sample population. The results of these analyses revealed that IS decreased as BCS and % FAT increased (P < 0.001) in the sample population. Additionally, increased IL1 (P < 0.05) and TNFp (P < 0.01) were associated with decreased IS. However, increased TNFα (P < 0.001) was associated with decreased IS only in mares 20 yr of age and older. Increased BCS and % FAT were associated with increased expression of TNFα (P = 0.053) and IL1 (P < 0.05), and increased TNFp (P < 0.05). Surprisingly, increased BCS and % FAT were associated with decreased IL6 expression (P = 0.05) in mares <20 yr of age. Finally, evaluation of the influence of obesity and inflammatory cytokines on IS within the same model suggested that BCS and % FAT (P < 0.001) with TNFα [mRNA (P = 0.07) and protein (P < 0.05)] are inversely associated with IS independently of one another. Combined, these results provide the first evidence associating obesity with increased inflammatory factors in the horse. Furthermore, the results suggest that an interrelationship exists among obesity, inflammatory cytokines, and IS in the horse and emphasize the need for further studies to elucidate the nature of these relationships.

Key words: interleukin-1, interleukin-6, inflammation, insulin resistance, tumor necrosis factor α

INTRODUCTION

Insulin regulates energy homeostasis by coordinating storage, mobilization, and utilization of FFA and glucose in adipose tissue, liver, and muscle (Ruan and Lodish, 2003). Reduced insulin sensitivity (IS), or insulin resistance, is a suppressed ability of insulin to induce glucose uptake into cells (Xu et al., 2003). Insulin resistance in horses is well documented, and recent studies associate obesity and insulin resistance in the
horse with development of abnormal reproductive function (Vick et al., 2006) and debilitating laminitis (Coffman and Colles, 1983; Field and Jeffcott, 1989; Pass et al., 1998). Insulin resistance has a number of etiologies and occurs as a result of injury, sepsis, and in conditions of obesity (Fernandez-Real and Ricart, 2003; Xu et al., 2003). Whereas injury and sepsis have long been defined as conditions characterized by elevated inflammation, obesity is now described as a mild but chronic inflammatory state (Das, 2001; Ramos et al., 2003). The specific factors contributing to development of insulin resistance in horses are unknown. However, human studies provide evidence that elevations in circulating concentrations of inflammatory cytokines such as IL1 (He et al., 2006), IL6 (Vozarova et al., 2001), and tumor necrosis factor α (TNFα) play direct roles in development of obesity-associated insulin resistance (Dandona et al., 2004; Krogh-Madsen et al., 2006). Thus, inflammatory cytokines may be a key link between obesity and insulin resistance in the horse. The following study was designed to address possible relationships between measurements of obesity, inflammatory cytokines, and IS in the horse. Our results provide new evidence that interleukin expression of the inflammatory cytokines TNFα, IL1 and IL6, circulating concentrations of TNFα protein (TNFp), age, and IS in the horse.

MATERIALS AND METHODS

Horses

Animal experimentation was conducted in accordance with accepted standards of humane animal care, and all procedures were approved by the Institutional Animal Care and Use Committee of the University of Kentucky. Mares (Equus caballus) of mixed light horse breed were maintained on pasture consisting primarily of bluegrass and orchard grass throughout the duration of all experiments with ad libitum access to water and a trace-mineralized salt block (Champions Choice, Cargill Inc., Minneapolis, MN).

Treatments

Sixty mares were included in this study, which was conducted in autumn. Age and measurements of BW, BCS, and percent body fat (% FAT) were recorded for each mare (Table 1). Duplicate blood samples were collected during the same week for measurement of TNFp and mRNA expression of TNFα, IL1, and IL6. Finally, IS was determined for each mare using the hyperinsulinemic, euglycemic clamp (HEC) procedure (see description below). An individual mare was used only once for determination of each of the respective variables.

Collection of Blood Samples

For each mare, blood was collected on a Monday and Friday of the same week. Blood samples were collected twice on each of the 2 d at approximately 0830 and 1600 to examine possible diurnal fluctuations in cytokine gene expression (Petrovsky et al., 1998). At each sampling time, 2.5 mL of blood were collected into each of 2 PAXgene tubes (Qiagen, Valencia, CA) according to the manufacturer’s instructions for subsequent RNA isolation. The PAXgene tubes were first stored at room temperature overnight and then at −20°C until RNA isolation. An additional 6 mL of blood were collected into a glass tube at each sampling time and allowed to clot at 4°C overnight. The following day, serum was harvested and stored at −20°C until assayed for circulating concentrations of TNFp.

Measurement of BW, BCS, and % FAT

Body weights were determined using a portable agricultural scale (model 700, Tru Test Inc., Mineral Wells, TX). Body condition score was evaluated using the average of scores given by 3 individuals on a scale of 1 to 9 (1 = emaciated and 9 = extremely obese; Henneke et al., 1983). Fat thickness on the croup at approximately 11 cm cranial to the tailhead and 10 cm lateral to the midline was measured by ultrasound, and this measurement was used to calculate % FAT using the following equation: % FAT = [(5.4 × ultrasonic fat depth in cm) + 2.47] (Kane et al., 1987). The variables BCS and % FAT were each used as measures of obesity in subsequent statistical analyses.

HEC Procedure

The HEC is an established procedure for determining IS of peripheral tissues (DeFronzo et al., 1979). The HEC procedure was performed using a method adapted for use in the horse as previously described (Powell et al., 2002). Before the HEC procedure, mares were housed in individual 4 × 4 m boxed stalls and fasted

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**Table 1. Mean and range for each item measured**

<table>
<thead>
<tr>
<th>Item</th>
<th>Mean ± SEM</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, kg</td>
<td>526 ± 8</td>
<td>408</td>
<td>688</td>
</tr>
<tr>
<td>BCS</td>
<td>6 ± 0</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>% FAT</td>
<td>13.5 ± 0.8</td>
<td>2.5</td>
<td>27.6</td>
</tr>
<tr>
<td>AGE, y</td>
<td>16 ± 11</td>
<td>3</td>
<td>29</td>
</tr>
<tr>
<td>IL1</td>
<td>1.59 ± 0.10</td>
<td>0.37</td>
<td>4.05</td>
</tr>
<tr>
<td>IL6</td>
<td>1.74 ± 0.36</td>
<td>0.11</td>
<td>13.81</td>
</tr>
<tr>
<td>TNFp</td>
<td>1.39 ± 0.12</td>
<td>0.16</td>
<td>5.04</td>
</tr>
<tr>
<td>TNFp, pg/mL</td>
<td>830.7 ± 171.3</td>
<td>156.0</td>
<td>5,000.0</td>
</tr>
<tr>
<td>Insulin, μIU/mL</td>
<td>26.5 ± 4.1</td>
<td>3.0</td>
<td>129.3</td>
</tr>
<tr>
<td>IS</td>
<td>117.2 ± 9.8</td>
<td>28.3</td>
<td>281.4</td>
</tr>
</tbody>
</table>

1. n = 60.
2. % FAT = percent body fat; TNFα = tumor necrosis factor α; TNFp = tumor necrosis factor α protein; and IS = insulin sensitivity.
3. The average of 3 scores from separate observers; values ranged from 1 to 9.
4. Units = fold-change in expression of each cytokine compared with the calibrator’s value of 1.
5. Given as estimated area under the curve of the plot of glucose infusion rates over time in arbitrary units.
for 12 to 14 h. Mares were fasted overnight and 2 indwelling catheters (16 ga., 13 cm, AbboCath, Abbott Lab., Abbott Park, IL) were inserted into the jugular vein on each side of the neck for blood collection and administration of insulin and glucose. Blood samples (three, 6 mL each) were collected at 10-min intervals before beginning the HEC and were averaged to determine the basal concentrations of fasted insulin and glucose. Concentrations of blood glucose were measured using a handheld glucose meter (One Touch, Johnson and Johnson, New Brunswick, NJ) previously validated in physiological saline; I0516, Sigma, St. Louis, MO). Simultaneously, an infusion of insulin was initiated at a rate of 1.2 mU/kg of BW of bovine insulin in physiological saline; I0516, Sigma, St. Louis, MO). Simultaneously, an infusion of insulin was initiated at a rate of 1.2 mU·kg of BW·min⁻¹ for 120 min. Previous use of this insulin infusion rate has resulted in sustained hyperinsulinemia between 60 and 85% above physiological concentrations for individual mares throughout the clamp (Powell et al., 2002). Two minutes after insulin infusion, a glucose solution (50%, wt/vol; Butler Co., Lexington, KY) was administered at an initial rate of 30 mL/h.

To monitor blood glucose concentrations, blood samples (5 mL) were collected every 10 min for the first 40 min of the clamp and then every 5 min for the remainder of the 120-min procedure. Based on glucose values obtained at each sampling time, the rate of glucose infusion was adjusted to maintain the euglycemic conditions determined from the baseline samples. Euglycemia was maintained within 0.5 ± 0.02 mmol/dL (n = 60) throughout the duration of the HEC (Figure 1). Steady-state glucose infusion was achieved in the final 30 min of the 120-min clamp. Glucose infusion rates were then converted from milliliters per hour to milligrams-kilograms of BW⁻¹·minute⁻¹.

**Insulin RIA**

Fasted circulating concentrations of insulin were measured in each of the 3 serum samples that were collected from each mare before beginning the HEC. Insulin concentrations from all samples were determined within a single assay using a commercially available RIA kit (Coat-a-count, Diagnostic Products Corp., Los Angeles, CA) and procedures validated for equine serum as described previously (Freestone et al., 1991). The limit of detection for insulin assays was 1.20 μIU/mL, and the intraassay CV was 11%. The average of the 3 samples was used as the basal insulin concentration.

**TNFα ELISA**

Concentrations of TNFp were determined using a commercially available equine-specific ELISA (Endogen, Rockford, IL). The assay was validated for serum samples with a minimum required dilution of 1:3 (vol/vol) in reagent diluent. Linearity of sample dilutions, defined as dilution corrected concentrations of TNFα that varied no more than 80 to 120% between doubling dilutions, was achieved in samples diluted between 1:3 and 1:30 in reagent diluent. Dilutional parallelism experiments resulted in an average percent recovery of 99.8% in dilutions ranging from 1:3 and 1:30. Intra- and interassay CV averaged 7.5 and 12%, respectively.

Ninety-six well plates were coated and blocked according to the manufacturer’s instructions. The equine TNFp standard that was provided was diluted 1:5 in reagent diluent containing 20% fetal equine serum to produce a high standard of 2,000 pg/mL, and 1:2 (vol/vol) serial dilutions were prepared to the lowest value of 15.6 pg/mL. Standard (100 μL) was added to individual wells in quadruplicate on each plate. Samples were diluted 1:5 in the reagent diluent, and 100 μL of each sample was added to individual wells in quadruplicate. The plates were then incubated for 1 h at room temperature. All remaining steps were conducted according to the manufacturer’s instructions. Absorbance was measured at A450. All samples were analyzed within a single assay, and the intraassay CV was 10.9%. The lower and upper dilution-corrected limits of detection were 156 and 5,000 pg/mL, respectively.

**RNA Isolation**

Total RNA was isolated from 2.5-mL samples of whole blood that were collected and stored in PAXgene blood RNA tubes in accordance with the protocol of the PAXgene blood RNA kit (PreAnalytics/Qiagen). A DNase treatment was performed on the column for quality assurance before RNA was eluted from the filter and stored at −80°C until analyzed.

**Relative Quantification of Cytokine Gene Expression by Real-Time Reverse Transcription-PCR**

Total RNA (500 ng) was diluted in 39 μL of nuclease-free water (Qiagen) and combined with 41 μL of reverse
transcription master mix [3 μL (20 U/μL) of avian myeloblastosis virus reverse transcription, 4 μL of oligo dT primer (0.5 μg/μL), 2 μL of RNAsin (40 U/μL), 8 μL of dNTP (10 mM), 8 μL of avian myeloblastosis virus buffer, and 16 μL of MgCl₂ (25 mM; all reagents from Promega, Madison, WI)] for each reverse transcription reaction. Reactions were incubated at 42°C for 15 min, 95°C for 5 min, and 3°C for 5 min in a thermocycler. Samples of cDNA were then stored at −20°C until further analyzed.

Cytokine gene expression was measured in cDNA samples using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Equine-specific TNFα, IL6, IL1, and beta-glucuronidase (β-GUS) primer-probe sets were designed for this purpose (Assays-by-Design, Applied Biosystems). The β-GUS was used as a housekeeping gene (Breathnach et al., 2006), and its expression did not vary more than 1-fold from the mean under the conditions of this experiment. Real-time reverse transcription-PCR primers and probe were designed to be intron-spanning by comparison of equine mRNA sequence with homologous splice sites on human sequences. For each primer/probe combination, a real-time assay utilizing genomic DNA and reverse transcription-negative RNA samples was employed to ensure that the primers and probes did not amplify DNA. The selected primers and probes failed to amplify genomic DNA or reverse transcription-negative RNA samples.

The PCR reactions were incubated at 95°C for 1 min, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. Each reaction contained 20 μL of master mix [12.5 μL of FAST TaqMan Universal PCR Master Mix (Applied Biosystems), 1.25 μL of 20X assay mix for the gene of interest (primer-probe set; Applied Biosystems), 6.25 μL of nuclelease-free water (Qiagen), and 5 μL of the reverse-transcription reaction product. All reactions were performed in duplicate wells. Changes in cytokine gene expression were calculated by relative quantification using the ∆∆CT method (Livak and Schmittgen, 2001), where ∆∆CT = (Avg. gene of interest CT - Avg. β-GUS CT)mare - (Avg. gene of interest CT - Avg. β-GUS CT)calibrator, and CT is defined as the amplification cycle (C) at which the gene reaches a threshold (T) level of fluorescence. Fold-changes in gene expression were calculated as 2−ΔΔCT. The results are expressed as the mean fold-change in gene expression compared with a calibrator of each sample. The calibrator was the animal with the median CT value for each individual gene.

Statistical Analysis

Conversions and Correlations. Glucose infusion rates were plotted over time for each individual mare, beginning with a glucose infusion rate of 0 at time 0. These sets of data (glucose infusion rate over time) were then used to calculate an area under the curve value for each mare. The purpose of using area under the curve was to convert a set of several data points into a single data value for the IS variable per mare to include in the correlation and regression analysis because all of the other variables in this study are represented by a single data value per mare. Area under the curve was calculated using the trapezoid rule in the R online statistical program (R Development Core Team, 2006). In addition, morning and afternoon cytokine protein and mRNA expression were not statistically different and were therefore averaged for simplicity in analyzing and reporting the results. Spearman correlations were calculated for all possible bivariate combinations using Sigma Stat 3.0 (Jandel Scientific, San Rafael, CA).

Regression Analysis. Four regression models were designed to further examine the relationships revealed by the correlation data by allowing simultaneous analysis of the influence of several variables on a particular outcome. The models were developed to analyze the data set using PROC REG, with stepwise model selection criteria (SAS Inst. Inc., Cary, NC). For all models, variables with P-values of <0.50 entered the model and variables with P-values <0.10 remained in the model and are reported in the results. The models were designed to investigate the hypothetical relationships among obesity, inflammatory cytokines, and IS in the horse, as depicted in Figure 2.

Linear regression modeling was used to develop 4 general models. Each of models 1 through 3 was designed to examine the possible relationship represented by the corresponding numbered directional lines in Figure 2. All models included age (AGE) as a covariate. Although obesity and IS have been correlated in many recent studies, model 1 was designed to examine the influence of obesity on IS within the population sampled for this study and after adjusting for age (Jeffcott et al., 1986; Powell et al., 2002; Cartmill et al., 2003). Model 1 used the following equation, in which IS represents the response variable, OB represents a measure of obesity, OB × AGE represents the interaction between obesity and age, and ε represents the residual error:

\[ IS = \beta_0 + \beta_1 OB + \beta_2 AGE + (\beta_3 OB \times AGE) + \epsilon. \]
Model 1 was estimated for each of 2 measures of obesity, where BCS and % FAT were each used as the OB variable.

Model 2 was designed to examine the influence of inflammatory cytokines on IS in the sample population. Model 2 is given by the following equation, in which IS represents the response variable; IL1, IL6, and TNFα represent mRNA expression of each inflammatory cytokine in blood; TNFp represents circulating protein concentrations of TNFα; and “INT” represents the set of betas for all possible 2-way interactions (all other terms are as defined in model 1):

\[
IS = \beta_0 + \beta_1 IL1 + \beta_2 IL6 + \beta_3 TNF_\alpha + \epsilon. \tag{model 2}
\]

Model 3 examined the influence of obesity on individual inflammatory factors within the sample population. The general model is given as follows, where INF represents individual inflammatory factors (all other terms are as defined in models 1 and 2):

\[
INF = \beta_0 + \beta_1 OB + \beta_2 AGE + (\beta_3 OB \times AGE) + \epsilon. \tag{model 3}
\]

Model 3 was estimated in a total of 8 combinations, where the 4 individual inflammatory factors (IL1, IL6, TNFα, and TNFp) were each used as the INF variable and the 2 individual measures of obesity (% FAT and BCS) were each used as the OB variable.

Model 4 combined models 1 and 3 to examine whether inflammatory factors or obesity influence IS independently of one another. Model 4 was estimated twice; the 2 individual measures of obesity (% FAT and BCS) were each used as the OB variable. Model 4 is represented by the following equation (all terms are as defined in models 1, 2, and 3):

\[
IS = \beta_0 + \beta_1 IL1 + \beta_2 IL6 + \beta_3 TNF_\alpha + \beta_4 TNFp + \beta_5 OB + \beta_6 AGE + \epsilon. \tag{model 4}
\]

Additionally, insulin was also substituted for the IS variable in all relevant models. Parameter estimates ± SE, variable P-values, model fit (F-test) P-values, and Mallows C(p) are reported in the results. Mallows C(p) is an estimation of model fit and has an ideal value equal to the number of independent variables (p) plus 1. Mallows C(p) greater than (p + 1) indicates that too few independent variables are included in the model, whereas values less than (p + 1) indicate that too many independent variables are included in the model (Neter et al., 1985). In models in which significant interactions between variables occurred, the data set was subdivided into 2 groups based on the interaction variables and the models were reanalyzed for each of the subgroups to discern the nature of the interaction. Interactions with AGE were analyzed by dividing the data into 2 groups of younger mares, those under 20 yr of age (n = 32), and older mares, those 20 yr of age and older (n = 28) based on evidence of immune-related changes in horses older than 20 yr of age (Horohov et al., 2002).

**RESULTS AND DISCUSSION**

**Variable Selection and Sample Population Statistics**

Mean ± SEM and range (minimum and maximum) values for all variables measured in the sample population are presented in Table 1. Multiple variables were measured for each of the broad categories of obesity and inflammatory cytokines. Currently, most equine studies involving obesity or body fat composition use the BCS system developed by Henneke et al. (1983). The study by Henneke et al. (1983) produced a standard which by rapid visual evaluation of body fat can be conducted, though the method can be subject to individual bias. Estimation of % FAT using the method developed by Westervelt et al. (1976) and Kane et al. (1987) is a more quantitative system of measurement; however, due to inherent differences in these 2 systems, BCS and % FAT were both measured as putative determinants of degree of obesity.

Inflammatory cytokine expression was measured using real-time reverse transcription-PCR. Real-time reverse transcription-PCR is both a highly sensitive and highly reproducible method of quantifying mRNA expression (Pagliarulo et al., 2004; Funato and Takeda, 2006). Blood mRNA expression of 3 inflammatory cytokines relevant to obesity and insulin resistance (IL1, IL6, and TNFα) were included in this investigation (Vozarova et al., 2001; Krogh-Madsen et al., 2004; He et al., 2006). Additionally, circulating protein concentrations of TNFα, the cytokine perhaps most directly related to IS in recent studies (Hotamisligil, 1999b; Kushibiki et al., 2001; Krogh-Madsen et al., 2006), were also included as a variable. Measurement of TNFp provided information about systemic levels of this cytokine and allowed comparison of protein to mRNA expression. An ELISA for measurement of equine-specific IL1 and IL6 protein has not yet been developed. Finally, in addition to determining IS using the HEC procedure, fasted concentrations of circulating insulin were included as an additional variable for the purpose of comparing with IS.

**Spearman Correlation Analysis**

Spearman correlation coefficients for all bivariate combinations are presented in Table 2. Body condition score and % FAT were highly correlated with one another, and both variables exhibited a positive correlation with the inflammatory factors IL1, TNFα, and TNFp, as well as fasted circulating concentrations of insulin. Insulin sensitivity was negatively correlated with BCS, % FAT, AGE, TNFα, TNFp, and insulin.
Interestingly, there was no significant correlation between mRNA expression and circulating protein concentrations of TNFα. The apparent differences between cytokine message produced in blood and the amount of protein in circulation may have been due to posttranscriptional and posttranslation regulation of TNFα or due to the contribution of protein in circulation from other cell populations in the body, such as adipose tissue (Hotamisligil et al., 1993). Results from the Spearman correlation analysis provide preliminary evidence for a relationship between obesity, inflammatory cytokines, and IS in the horse. The correlation coefficients resulting from analysis of the sample population support previous studies associating obesity with decreased IS, or insulin resistance in the horse (Powell et al., 2002; Hoffman et al., 2003; Vick et al., 2006). Additionally, the correlation results indicate that inflammatory factors increase as obesity increases, and concurrently, IS decreases with increasing levels of the inflammatory factors measured in this study.

Although the correlation results provided a first look at the relationships between pairs of variables, the results do not account for potential confounding variables. Therefore, results from the Spearman correlation analysis were used to hypothesize 4 population regression models. For instance, the lack of correlation between the BCS and % FAT, these variables were not included simultaneously as predictor variables in any model to avoid collinearity. Similarly, insulin was not included simultaneously as a predictor with models involving IS.

**Model 1: Influence of Obesity on IS**

Equation parameter estimates ± SE, $P$-values for individual variables and overall $F$-tests, and Mallows C(p) values for model 1 are listed in Table 3. Mallows C(p) values were equal to the ideal value of $(p + 1)$ for both model 1 regression fits. The results revealed that IS decreases with increasing BCS and % FAT. Age classification was a significant factor in influencing IS in both fits of model 1, and there was an AGE × BCS ($P = 0.059$) and an AGE × % FAT ($P = 0.090$) interaction term in the model. Insulin was also substituted for the IS variable in model 1; however, the overall model $F$-test $P$-value was not significant and no variables remained in the model.

To elucidate the nature of the interaction between AGE and OB in this model, the data were divided into 2 groups of mares under 20 yr of age and mares 20 yr of age and older and a BCS main effect model was fit in both groups. Body condition score was inversely ($P < 0.01$) related to IS in both age groups. However, based on the parameter estimates for BCS in both models ($−21.60 ± 11.26$ in mares 20 yr of age and older vs. $−42.04 ± 6.71$ in mares younger than 20 yr of age), the effect of BCS on the rate of change in IS is twice as large in younger mares vs. older mares. Results were similar when % FAT was fit as the OB variable in both groups (data not shown). These results indicate that a significant relationship between measures of obesity and IS exists in the sample population; however, the degree to which BCS influences IS depends on the age of the mare.

**Model 2: Influences of Inflammatory Cytokines on Insulin Sensitivity**

Equation parameter estimates ± SE, $P$-values, and Mallows C(p) values for model 2 are listed in Table 4. The Mallows C(p) for the stepwise selection of model 2 was close to the ideal value of $(p + 1)$. Whereas TNFα expression and age were not initially correlated with IS and did not remain in the model, there was a significant AGE × TNFα interaction. Therefore, the model was

### Table 2. Spearman correlation coefficients for individual pairs of items measured

<table>
<thead>
<tr>
<th>Item²</th>
<th>BCS</th>
<th>% FAT</th>
<th>AGE</th>
<th>IL1</th>
<th>IL6</th>
<th>TNFα</th>
<th>TNFp</th>
<th>Insulin</th>
<th>IS⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, kg</td>
<td>NS³</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.26*</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>BCS⁴</td>
<td>0.90***</td>
<td>NS</td>
<td>0.40***</td>
<td>NS</td>
<td>0.27*</td>
<td>0.29*</td>
<td>0.53***</td>
<td>−0.57***</td>
<td>−0.64***</td>
</tr>
<tr>
<td>% FAT</td>
<td>NS</td>
<td>0.42**</td>
<td>NS</td>
<td>0.38**</td>
<td>0.27*</td>
<td>0.47***</td>
<td>NS</td>
<td>−0.36***</td>
<td>NS</td>
</tr>
<tr>
<td>AGE, y</td>
<td>NS</td>
<td>−0.56***</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>−0.64***</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>IL1⁵</td>
<td>NS</td>
<td>0.32*</td>
<td>NS</td>
<td>0.27*</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>IL6⁵</td>
<td>NS</td>
<td>NS</td>
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<td>NS</td>
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<tr>
<td>TNFα⁵</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.34*</td>
<td>−0.32*</td>
<td>NS</td>
<td>−0.28*</td>
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<tr>
<td>TNFp, pg/mL</td>
<td>NS</td>
<td>NS</td>
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<td>Insulin, µIU/mL</td>
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</tr>
</tbody>
</table>

¹$n = 60$. Units of measure for all items in row 1 are listed in column 1.
²% FAT = percent body fat; TNFα = tumor necrosis factor α; TNFp = tumor necrosis factor α protein; and IS = insulin sensitivity.
³NS = not significant.
⁴The average of 3 scores from separate observers; values ranged from 1 to 9.
⁵Units = fold-change in expression of each cytokine compared with the calibrator’s value of 1.
⁶Given as estimated area under the curve of the plot of glucose infusion rates over time in arbitrary units.

$^*$ $P < 0.10$, $^*$ $P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. 

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refitted to include AGE and TNF$_{\alpha}$ as covariates along with the corresponding interaction term, and the results are listed as model 2R in Table 4. In model 2R, IL1 and TNFp exhibited an inverse relationship with IS. Interestingly, there was a positive relationship between IL6 and IS in model 2R. Insulin was also substituted for the IS variable in model 1; however, the overall model $F$-test $P$-value was not significant and no variables remained in the model. To elucidate the nature of the interaction between AGE and TNF$_{\alpha}$ in model 2R, the data were divided into 2 groups of mares under 20 yr of age and mares 20 yr of age and older, and the model was fit in both groups with IL1, IL6, TNF$_{\alpha}$, and TNFp included as independent variables. The results of this analysis revealed that TNF$_{\alpha}$ was inversely related to IS ($P < 0.001$) in mares 20 yr of age and older but was not significant in mares younger than 20 yr of age (data not shown).

To the authors’ knowledge, there are no studies in any species relating blood mRNA expression of IL6 to IS. Whereas there is little information on blood mRNA expression of IL6 in relation to insulin sensitivity, several studies positively correlate serum concentrations of IL6 protein with obesity and insulin resistance in humans (Vozarova et al., 2001; Bluher et al., 2005). Additionally, there is evidence that a considerable portion of circulating IL6 protein is produced by adipose tissue and not blood in humans (Mohamed-Ali et al.,

---

**Table 3.** Stepwise regression analysis of model 1: influence of measures of obesity on insulin sensitivity$^1$

<table>
<thead>
<tr>
<th>Model</th>
<th>Dependent variable$^2$</th>
<th>Parameter parameters$^2$</th>
<th>Parameter estimate ± SE</th>
<th>Variable $P$-value</th>
<th>Model $P$-value$^3$</th>
<th>Mallows C (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IS$^4$</td>
<td>Intercept</td>
<td>512.41 ± 74.53</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>4.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BCS$^5$</td>
<td>−55.70 ± 12.43</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AGE, yr</td>
<td>−11.68 ± 3.99</td>
<td>0.005</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BCS × AGE$^6$</td>
<td>1.32 ± 0.69</td>
<td>0.059</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>IS$^4$</td>
<td>Intercept</td>
<td>354.10 ± 40.68</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>4.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>% FAT</td>
<td>−12.77 ± 2.92</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AGE, yr</td>
<td>−7.66 ± 2.10</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>% FAT × AGE$^6$</td>
<td>0.28 ± 0.16</td>
<td>0.090</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^1$n = 60. Variables included in the analysis are given in the model statements. Only variables remaining in the model ($P < 0.10$) are shown here.

$^2$IS = insulin sensitivity, and % FAT = percent body fat.

$^3$P-value corresponding to the overall model $F$-test.

$^4$Given as estimated area under the curve of the plot of glucose infusion rates over time in arbitrary units.

$^5$The average of 3 scores from separate observers; values range between 1 and 9.

$^6$Represents the interaction between the variables.

---

**Table 4.** Stepwise regression analysis of model 2: influence of inflammatory factors on IS$^1$

<table>
<thead>
<tr>
<th>Model</th>
<th>Dependent variable$^2$</th>
<th>Parameter parameters$^2$</th>
<th>Parameter estimate ± SE</th>
<th>Variable $P$-value</th>
<th>Model $P$-value$^3$</th>
<th>Mallows C (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>IS$^4$</td>
<td>Intercept</td>
<td>182.76 ± 22.94</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>4.79</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL1$^5$</td>
<td>−24.23 ± 11.94</td>
<td>0.048</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL6$^5$</td>
<td>9.36 ± 3.33</td>
<td>0.007</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TNFp, pg/mL</td>
<td>−0.02 ± 0.01</td>
<td>0.008</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AGE × TNF$\alpha$$^6$</td>
<td>−1.17 ± 0.45</td>
<td>0.012</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2R</td>
<td>IS$^4$</td>
<td>Intercept</td>
<td>129.81 ± 44.29</td>
<td>0.005</td>
<td>&lt;0.001</td>
<td>4.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL1$^5$</td>
<td>−31.49 ± 12.93</td>
<td>0.019</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL6$^5$</td>
<td>13.41 ± 4.43</td>
<td>0.004</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TNF$\alpha$$^5$</td>
<td>−35.47 ± 24.18</td>
<td>0.149</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TNFp, pg/mL</td>
<td>−0.02 ± 0.01</td>
<td>0.004</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AGE, yr</td>
<td>3.02 ± 2.29</td>
<td>0.193</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AGE × TNF$\alpha$$^6$</td>
<td>−2.87 ± 1.23</td>
<td>0.024</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^1$n = 60. Variables included in the analysis are given in the model statements. Only variables remaining in the model ($P < 0.10$) are shown here.

$^2$IS = insulin sensitivity, TNF$\alpha$ = tumor necrosis factor $\alpha$ expression, and TNFp = tumor necrosis factor $\alpha$ protein.

$^3$P-value corresponding to the overall model $F$-test.

$^4$Estimated area under the curve of the plot of glucose infusion rates over time in arbitrary units.

$^5$Units = fold-change in expression of each cytokine compared with the calibrator’s value of 1.

$^6$Represents the interaction between the variables.
is no significant relationship between the 2 variables in mares over 20 yr of age (results not shown). Using % FAT as the OB variable, results were similar to using BCS as the OB variable with the exception that increasing % FAT was associated with increasing TNFα expression, but not with TNFp in the sample population.

The finding that IL6 expression decreases with degree of obesity in younger mares is supported by a recent study suggesting that expression of IL6 in blood negatively correlate with degree of obesity in humans (O’Rourke et al., 2006). Here again, expression of IL6 in blood may not have provided a complete picture of IL6 activity in the sample population. However, there is some in vitro evidence that IL6 may exert an insulin sensitizing effect when present without high concentrations of insulin (Al-Khalili et al., 2006). This finding may provide some speculation in regards to what was observed in vivo in our sample population.

Model 3: Influence of Obesity on Inflammatory Cytokines

Equation parameter estimates ± SE, P-values, and Mallows C(p) values for model 3 are listed in Table 5. Mallows C(p) was higher than the ideal value of (p + 1) when model 3 was fitted with IL1 as the response variable, but were equal to the ideal value of (p + 1) in the remaining 6 fits of model 3. Model 3 revealed slight variation in the relationship between the 2 measures of obesity (BCS and % FAT) with the 4 individual inflammatory factors. When BCS was entered as the OB variable, results indicate that IL1 and TNFp both increase with increasing BCS in the sample population. However, TNFα expression was not related (P = 0.188) to BCS. Age was a significant factor in the relationship between TNFp and BCS, and TNFp increased with AGE in the model. There was also an AGE × BCS interaction when IL6 was entered as the INF variable. Additional analysis revealed that IL6 decreased with increased BCS in mares under 20 yr of age (P = 0.10), but there

<table>
<thead>
<tr>
<th>Model</th>
<th>Dependent variable2</th>
<th>Regression parameters2</th>
<th>Parameter estimate ± SE</th>
<th>Variable P-value</th>
<th>Model P-value3</th>
<th>Mallows C (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>IL14</td>
<td>Intercept</td>
<td>0.58 ± 0.45</td>
<td>0.199</td>
<td>0.024</td>
<td>3.06</td>
</tr>
<tr>
<td>3</td>
<td>IL14</td>
<td>BCS5</td>
<td>0.17 ± 0.07</td>
<td>0.024</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>IL14</td>
<td>% FAT</td>
<td>0.03 ± 0.02</td>
<td>0.047</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>IL64</td>
<td>Intercept</td>
<td>12.68 ± 3.22</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>4.00</td>
</tr>
<tr>
<td>3</td>
<td>IL64</td>
<td>BCS5</td>
<td>−1.33 ± 0.54</td>
<td>0.016</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>IL64</td>
<td>AGE, yr</td>
<td>−0.52 ± 0.17</td>
<td>0.004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>IL64</td>
<td>BCS × AGE6</td>
<td>0.06 ± 0.03</td>
<td>0.065</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>IL64</td>
<td>Intercept</td>
<td>9.22 ± 1.78</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>4.00</td>
</tr>
<tr>
<td>3</td>
<td>IL64</td>
<td>% FAT</td>
<td>−0.34 ± 0.13</td>
<td>0.011</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>IL64</td>
<td>AGE, yr</td>
<td>−0.37 ± 0.09</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>IL64</td>
<td>% FAT × AGE6</td>
<td>0.01 ± 0.01</td>
<td>0.053</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>TNFα4</td>
<td>Intercept</td>
<td>0.81 ± 0.32</td>
<td>0.015</td>
<td>0.0526</td>
<td>2.00</td>
</tr>
<tr>
<td>3</td>
<td>TNFα4</td>
<td>% FAT</td>
<td>0.04 ± 0.02</td>
<td>0.053</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>TNFp, pg/mL</td>
<td>Intercept</td>
<td>−1,348.18 ± 879.72</td>
<td>0.131</td>
<td>0.0419</td>
<td>3.00</td>
</tr>
<tr>
<td>3</td>
<td>TNFp, pg/mL</td>
<td>BCS5</td>
<td>260.11 ± 125.30</td>
<td>0.043</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>TNFp, pg/mL</td>
<td>AGE, yr</td>
<td>39.20 ± 21.67</td>
<td>0.076</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1n = 60. Variables included in the analysis are given in the model statements. Only variables remaining in the model (P < 0.10) are shown here.
2TNFp = tumor necrosis factor α protein in serum, and % FAT = percent body fat.
3P-value corresponding to the overall model F-test.
4Units = fold-change in expression of each cytokine compared with the calibrator’s value of 1.
5The average of 3 scores from separate observers using the Henneke scoring chart; values range between 1 and 9.
6Represents the interaction between the variables.
Age and % FAT also remained in the model with a significant AGE × % FAT interaction.

The AGE × OB variable interaction was then analyzed by dividing the data into 2 groups of mares under 20 yr of age and mares 20 yr of age and over, and the model was fit in both groups. Both OB variables (BCS and % FAT) were significant in each of the 2 age groups. However, based on the parameter estimates for BCS (−14.43 ± 7.58 in mares 20 yr of age vs. −14.99 ± 6.91 in mares younger than 20 yr of age), the effect of BCS on the rate of change in IS is more than twice as large in younger mares vs. older mares. Results were similar when % FAT was fit in both groups (data not shown).

Several conclusions can be drawn from the results of model 4. Whereas OB seems to influence IL1 and IL6 in model 3, and concurrently IL1 and IL6 seem to influence the outcome of IS in model 2, the effect of OB seems to override the effects of IL1 and IL6 when all variables are entered together in model 4. It is likely that IL1 and IL6 do not significantly contribute to degree of IS when degree of OB is taken into account in the sample population. Blood TNFα mRNA expression and TNFp each influenced IS, depending on which variable was entered as the OB variable (% FAT or BCS). Thus it appears they each influence IS, even after adjusting for degree of obesity. The results from model 4 provide evidence that TNFα is the most influential of the inflammatory factors measured in this study. This is supported by numerous studies in other species describing not only the relationship of TNFα to obesity, but also its direct ability to induce insulin resistance (Hotamisligil et al., 1993; Hotamisligil, 1999a; Kushibiki et al., 2001).

### Table 6. Stepwise regression analysis of model 4: influence of obesity and inflammation on IS

<table>
<thead>
<tr>
<th>Model</th>
<th>Response variable</th>
<th>Regression parameters</th>
<th>Parameter estimate ± SE</th>
<th>Variable P-value</th>
<th>Model P-value</th>
<th>Mallows C (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>IS</td>
<td>Intercept</td>
<td>525.36 ± 72.21</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>5.69</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TNFp, pg/mL</td>
<td>−18.18 ± 7.29</td>
<td>0.016</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BCS</td>
<td>−53.81 ± 12.05</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AGE, yr</td>
<td>−11.55 ± 3.85</td>
<td>0.004</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AGE × BCS</td>
<td>1.33 ± 0.66</td>
<td>0.051</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>IS</td>
<td>Intercept</td>
<td>364.36 ± 40.81</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>5.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FAT %</td>
<td>−12.24 ± 2.92</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AGE, yr</td>
<td>−7.49 ± 2.08</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TNFp</td>
<td>−13.60 ± 7.48</td>
<td>0.076</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AGE × % FAT</td>
<td>0.28 ± 0.16</td>
<td>0.088</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1n = 60. Variables included in the analysis are given in the model statements. Only variables remaining in the model (P < 0.10) are shown here.
2IS = insulin sensitivity, TNFp = tumor necrosis factor α protein, TNFα = tumor necrosis factor α expression, and % FAT = percent body fat.
3P-value corresponding to the overall model F-test.
4Units are estimated area under the curve of the plot of glucose infusion rates over time in arbitrary units.
5The average of 3 scores from separate observers; values range between 1 and 9.
6Represents the interaction between the variables.

### Age-Related Associations

The results from Spearman correlations and population regression models 1 through 4 revealed associations between age and IS, obesity, and inflammatory cytokines. Spearman analysis (Table 2) indicated that insulin sensitivity decreased with age in the sample population. Further analysis using model 1 regression fits (Table 3) indicated an interaction between the influence of obesity and age on insulin sensitivity. Obesity played a much greater role in influencing IS in younger mares than older mares in the sample population. This indicates a propensity for reduced IS in older mares, regardless of degree of obesity. Relationships between age and TNFα (blood mRNA expression and serum protein) were revealed by models 2 and 3. In model 2R (Table 4), elevated TNFα was associated with decreased insulin sensitivity only in the older mares in the sample population, whereas model 3 (Table 5) indicated that TNFp increased with age in the sample population even after BCS is taken into account. The evidence provided by models 1 through 3 highlight a possible relationship between aging, inflammatory cytokines, and insulin sensitivity in the horse similar to that observed in humans (DeFronzo, 1979; Franceschi et al., 2000; Figaro et al., 2006).

### Links Among Obesity, Inflammation, and IS: Possible Role of Adipose Tissue

The association between obesity and a chronic inflammatory response in the horse is consistent with findings in other species (Miller et al., 1998; Ramos et al., 2003; Tilg and Moschen, 2006). Recent studies in other species revealed that increased inflammation in
relation to obesity may be due to direct secretion of a multitude of factors called adipokines from adipose tissue itself (Arner, 2005). Adipose tissue secretes the inflammatory cytokines TNFα, IL1, and IL6 along with factors such as serum amyloid A, resistin, leptin, and adiponectin that regulate the immune/inflammatory response and IS (Arner, 2005; Tilg and Moschen, 2006). Circulating concentrations of each of these factors increase with degree of obesity, and the majority (except IL1) are secreted by adipocytes themselves (Kern et al., 1995; Good et al., 2006; Tilg and Moschen, 2006). However, obesity is also associated with macrophage accumulation in adipose tissue (Weisberg et al., 2003), and several of the adipokines secreted by adipocytes, such as serum amyloid A, can act directly on macrophages to increase production of inflammatory cytokines such as TNFα, IL1, and IL6 as well as resistin (Tilg and Moschen, 2006; Yang et al., 2006).

Several of the inflammatory factors produced by adipose tissue can act directly or indirectly to reduce IS. For example, increased resistin expression induces increased TNF and IL6, and associated decreases in glucose transport and IS in adipocytes in vitro (Fu et al., 2006). Several in vitro and in vivo studies suggest that TNF, IL1, and IL6 can each directly impair insulin sensitivity by interfering with insulin-stimulated glucose uptake in peripheral tissues (Hotamisligil et al., 1996; Senn et al., 2003; He et al., 2006). Additionally, there is evidence that factors secreted by adipocytes can act in an endocrine manner to activate monocytes. For example, resistin can induce cytokine production in cultured macrophages, and its production is also regulated by the inflammatory cytokines TNFα, IL1, and IL6, creating a potential positive feedback loop (Lehrke et al., 2004; Silswal et al., 2005). Leptin can also exert an endocrine effect to increase cytokine production in blood monocytes (Sanchez-Margalet et al., 2003). Therefore, a continuous cycle of cross talk between adipocytes and monocytes (Suganami et al., 2005) may stimulate and perpetuate the proinflammatory status associated with obesity in humans and mice. Based on these studies, greater amounts of adipose tissue associated with obesity may be largely responsible for local and systemic increases in inflammation and decreases in IS in the horse. Although the underlying mechanisms are unknown, future studies investigating the role of adipose tissue in relation to obesity, inflammation, and IS may shed light on the nature of these interactions in the horse.

**Overall Conclusions**

Previous studies have associated obesity and insulin sensitivity in mares and highlighted obesity as a risk factor for laminitis and impaired reproductive function. The results of this investigation support previous associations as demonstrated by increased circulating concentrations of insulin and reduced IS in mares with high BCS and % FAT. These effects were exacerbated by aging and correlated with increased inflammatory response as demonstrated by significantly elevated TNFp in serum as well as elevated blood mRNA expression of TNFα in obese animals.

The results of this study provide the first evidence associating obesity with elevations in inflammatory cytokines in the horse. Additionally, this study demonstrates for the first time interrelationships between obesity, inflammatory cytokines, and IS in the horse, though considerable additional research is required to elucidate the nature of the mechanisms behind these interactions. Results from this study underscore the need for careful management, particularly in older animals. Finally, they highlight the importance of continued investigation of obesity, inflammation, and IS in relation to conditions such as laminitis in the horse to determine physiological guidelines for the susceptibility to and possible prevention of these conditions.

**LITERATURE CITED**


