Veterinary Immunology and Immunopathology 127 (2009) 286–294

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

Effect of body condition, body weight and adiposity on inflammatory cytokine responses in old horses

Amanda A. Adams [a], Madhu. P. Katepalli [a], Katharina Kohler [a], Stephanie E. Reedy [a], J.P. Stilz [a], Mandi M. Vick [a], Barry P. Fitzgerald [a], Laurie M. Lawrence [b], David W. Horohov [a,*]

[a] Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY 40546-0099, USA
[b] Department of Animal Science, University of Kentucky, Lexington, KY 40546-0215, USA

ARTICLE INFO

Article history:
Received 1 July 2008
Received in revised form 16 September 2008
Accepted 20 October 2008

Keywords:
Inflammation
Adiposity
Dietary restriction
Aging
Horses

ABSTRACT

Advanced age is associated with a low-grade, systemic inflammatory response characterized by increased inflammatory cytokine production both *in vitro* and *in vivo*, termed inflamm-aging. It is also known that increased white adipose tissue, associated with obesity, leads to increased production of inflammatory cytokines. To date, it is unknown whether increased adiposity contributes to the age-related increased inflammatory status. Here we show that peripheral blood mononuclear cells (PBMC) from old horses compared to young horses have increased inflammatory cytokine production; moreover, fat old horses compared to thin old horses have even greater frequencies of lymphocytes and monocytes producing inflammatory cytokines. Therefore, we proposed that decreasing adiposity in old horses would reduce age-associated increases of inflammatory cytokines both *in vitro* and *in vivo*, and increasing adiposity in old horses would increase these measurements. To test this hypothesis further, eight old obese horses (20–28 year) were assigned to two consecutive treatments, dietary restriction (DR) during weeks 1–12 and increased dietary intake (DI) during weeks 13–30. Body weight, body condition score (BCS) and percent body fat were measured weekly. PBMC were stimulated *in vitro* and interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) production was measured by intracellular staining. Levels of nascent IFNγ and TNFα mRNA expression were examined by RT-PCR. Serum concentrations of TNFα protein were also measured weekly. Reducing body weight and fat in old horses significantly reduced the percent of IFNγ and TNFα positive lymphocytes and monocytes, and serum levels of TNFα protein. Further, when weight and fat increased in these old horses there was a significant increase in inflammatory cytokine production. Regression analysis also revealed significant relationships. These findings demonstrate that age-related obesity potentially plays a role in the dysregulation of inflammatory cytokine production by the immune system with age or inflamm-aging in the horse.

1. Introduction

Advanced age is associated with a low-grade, chronic inflammatory state termed "inflamm-aging" (Franceschi et al., 2000). Older individuals have 2–4-fold increases in circulating levels of interleukin (IL)-6 (Harris et al., 1999; Pedersen et al., 2003; Roubenoff et al., 1998), tumor necrosis factor alpha (TNF)-α (Bruunsgaard and Pedersen,

* Corresponding author at: 108 Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY 40546-0099, USA. Tel.: +1 859 257 4757; fax: +1 859 257 8542.
E-mail address: DWHoro2@uky.edu (D.W. Horohov).

0165-2427/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetimm.2008.10.323

2003) and C-reactive protein (CRP) (Krabbe et al., 2004; Pedersen et al., 2003) associated with increased morbidity and mortality. Inflamm-aging has also been characterized by increases in inflammatory cytokine production by peripheral blood mononuclear cells (PBMC) *in vitro* (Engwerda et al., 1996; Fagiolo et al., 1993; Sandmand et al., 2003). Though these data indicate that dysregulation of inflammatory cytokines occurs with aging, the mechanism responsible for this process remains unclear. While a number of factors probably contribute to the increased inflammatory response in the elderly, recent attention has focused on the role of fat tissue (Pedersen et al., 2003).

Obesity is now considered to be a chronic mild inflammatory state (Dandona et al., 2004; Rajala and Scherer, 2003). The incidence of obesity in the elderly is rising and will likely continue to increase (Arterburn et al., 2004). Fat mass not only increases with age but is also redistributed, resulting in greater intraabdominal fat or white adipose tissue (WAT) (Villareal et al., 2005). WAT is composed of adipocytes, fibroblasts, endothelial cells and macrophages (Berg and Scherer, 2005; Weisberg et al., 2003). These resident adipocytes and macrophages have been shown to secrete a variety of pro-inflammatory cytokines such as TNFα, IL-6 and IL-1 in humans and rodents (Hotamisligil et al., 1993; Kershaw and Flier, 2004; Tilg and Moschen, 2006; Weisberg et al., 2003). Moreover, a recent review documented the close relationship between lymphatics and adipose tissue (Harvey, 2008). While the exact source of circulating inflammatory cytokines remains unclear; there is no doubt that obese individuals have increased amounts and reductions in body fat can decrease these levels (Bastard et al., 2000; Bruun et al., 2003; Clement et al., 2004; Dandona et al., 1998; Fontana and Klein, 2007; Xydakis et al., 2004). Nevertheless, few studies have measured changes in inflammatory cytokines with loss of body weight and fat in old obese subjects (de Mello et al., 2008; Nicklas et al., 2004).

Though the relationships between adipose tissue and the immune system in aging remain largely unknown; it has been suggested that elevated body fat may play a role in contributing to increased inflammation with age (Pedersen et al., 2003). Thus, we have investigated this relationship using the old obese horse as a model. In support of this model, horses are a relatively long-lived species with a mean lifespan of 25 years (equivalent to 71 year of age in people) and a maximum life span approaching 40 years (Mauderly and Hahn, 1982; McFarlane et al., 2001). We have recently shown that adipocytes from the horse are also capable of producing inflammatory cytokine TNF-α (Vick et al., 2008). In addition, obese adult horses have increased circulating levels of inflammatory cytokines (Vick et al., 2007). Here we show that PBMC from old horses compared to young adults have increased inflammatory cytokine production; moreover, fat old horses compared to thin old horses have even greater frequencies of lymphocytes and monocytes producing inflammatory cytokines. Therefore, we proposed that decreasing adiposity in old horses would reduce age-associated increases of inflammatory cytokines both *in vitro* and *in vivo*, and increasing adiposity in old horses would increase these measurements.

2. Materials and methods

2.1. Experimental animals

Sixteen old (mean age 24.4; range 20–28) and seven young (mean age 4.3; range 4–5) female horses (*Equus caballus*) of mixed-breed were used in this study. All horses were maintained at the Department of Veterinary Science's Maine Chance Farm, Lexington, KY. Each horse had a thorough veterinary medical exam to exclude horses with evidence of illness, trauma, lameness, weight loss or abnormal coat shedding (as a sign of Cushing's). All blood samples were drawn via aseptic venipuncture after feeding was completed. Husbandry procedures were in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research, U.S. Department of Agriculture. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

2.2. Measurement of body weight, BCS and adiposity (% body fat)

Body weight was measured each week before feedings (0830 h) using a portable agriculture scale (model 700, Tru Test Inc., Mineral Wells, TX). Body condition score (BCS) was determined at random time points using the Henneke scoring system which uses a scale of 1–9 (1 = emaciated and 9 = extremely obese; Henneke et al., 1983). Individual horse scores were determined by using the average score given by 3 trained persons. Percent body fat was also determined weekly before feedings by using ultrasound measurement of croup fat thickness at approximately 11 cm caudal from the tail head and 10 cm off the midline. This measurement was used to calculate percent body fat using the equation: % body fat = [5.4*(ultrasound fat depth in cm) + 2.47] (Kane et al., 1987). Obese horses are defined by a BCS $\geq$ 7 and % body fat $\geq$ 15 (Vick et al., 2007, 2006).

2.3. Dietary treatments

All horses were housed and fed daily at 0830 h in 16-m^2 individual stalls. All horses had free access to water at all times. Two consecutive treatments, dietary restriction (DR, weeks 1–12), assigned to eight of the ten old horses and increased dietary intake (DI, weeks 13–30), assigned to seven (one horse died due to unrelated cause) of the ten old obese horses. The two remaining fat old horses were not subjected to dietary treatments and served as controls. The mares were initially fed a maintenance diet (NRC, 1989), consisting of mixed grass hay (DE, Mcal/kg 0.37, 13.3% crude protein and 19.4% non-fiber carbohydrate), for 4 weeks and thereafter the diet was restricted every 2 weeks to 80%, 70% and 60% of the maintenance diet until the targeted BCS ($\leq$7) and % body fat ($\leq$15) was obtained. Afterwards, dietary intake was increased every 2 weeks from 60% to 70% to 80% and finally to 100% of the maintenance diet until targeted percent body fat ($\geq$15) was achieved. All diets were supplemented daily for each horse with a balancing ration of 1.0 kg of oats (DE, Mcal/kg 2.85 and 11.8% crude protein) and 0.22 kg of Gro N'Win (Buckeye Nutrition, Edmond, OK) supplement (32.0% crude protein, 6.0% starch and 5.0% crude fat).

2.4. IFNγ and TNFα intracellular staining

Heparinized blood samples were collected once a week. PBMC were purified by Ficoll-Paque Plus™ (Amersham Biosciences, Piscataway, NJ) gradient centrifugation and cultured in 24-well plates with c-RPMI [RPMI-1640 (Gibco, Grand Island, NY) with 2.5% fetal equine serum (FES; Sigma–Aldrich, St. Louis, MO), 100 U/ml penicillin/streptomycin (Sigma), and 55 μM 2-mercaptoethanol (Gibco)] media alone or c-RPMI media with phorbol 12-myristate 13-acetate (PMA; 25 ng/ml; Sigma) and ionomycin (1 μM; Sigma), or lipopolysaccharide (LPS, 1 μg/ml; Sigma) at 37 °C 5% CO_2 for 6 h. In addition, brefeldin A (10 μg/ml; Sigma) was added to all wells during the 6 h incubation period. Following the incubation, cells were fixed in 2% paraformaldehyde (Sigma) and stored overnight at 4 °C. The next day intracellular staining for IFNγ was performed using a method we have previously described (Breathnach et al., 2006). Intracellular staining for TNFα was performed using a similar procedure with an anti-equine TNFα monoclonal antibody (HL801, kindly provided by Dr. Rob MacKay, University of Florida). All IFNγ and TNFα stained samples were resuspended in FACS flow for flow cytometric acquisition and analysis. Lymphocyte and monocyte gates were determined using forward and side-scatter. Markers were set using the isotype control antibody-labeled cells from each horse for each treatment and used to determine the percent of IFNγ-producing or TNFα-producing lymphocytes (L) and monocytes (M) in the corresponding media or stimulated cultures.

2.5. RT-PCR (real-time) quantitation of cytokine gene expression in vivo

Approximately 3.0 ml of whole blood was collected from the jugular vein into PAXgene™ blood RNA tubes (Qiagen/PreAnalytiX, Valencia, CA). The tubes were incubated at room temperature for 24 h and then stored at −20 °C until assayed. Once thawed, total RNA was extracted using the PAXgene™ blood RNA extraction kit (Qiagen) using the manufacturer's protocol. Reverse transcription reactions were performed, as previously described (Breathnach et al., 2006), using 1.0 μg of each RNA sample and reverse transcription master mix (Promega, Madison, WI). The reactions were incubated at 42 °C for 15 min and 95 °C for 5 min. The cDNA was stored at −20 °C until analyzed by RT-PCR.

Cytokine gene expression was measured by RT-PCR using equine specific intron-spanning primer/probe sets for IFNγ and TNFα (Breathnach et al., 2006). Each reaction contained 5 μl cDNA and 20 μl of master mix containing the following: 6.25 μl nuclease-free water (Qiagen); 1.25 μl 20X assay mix for primer/probe set of interest (Applied Biosystems); and 12.5 μl TaqMan™ (Applied Biosystems, Foster City, CA). All reactions were incubated in duplicate wells for 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s in an Applied Biosystems 7500 sequence detection system. The $\Delta\Delta C_T$ method was used to calculate relative changes in cytokine gene expression (Livak and Schmittgen, 2001). Beta-glucouronidase (β-GUS) was used as the housekeeping gene. The calibrator used was calculated from the mean ΔC_T for all baseline samples of each horse for each individual gene. Results are expressed as relative quantity (RQ) calculated as $2^{-\Delta\Delta CT}$.

2.6. TNFα ELISA

Weekly serum was separated from peripheral blood and stored at −20 °C until assayed. Serum TNFα protein was measured using a commercially available equine TNFα ELISA kit (Endogen, Rockford, IL). This assay was validated for equine serum samples diluted at a minimum of 1:3 in reagent diluent (Vick et al., 2007). All steps of the ELISA were performed by the manufacturer's instructions except for the following: the recombinant TNFα standard provided was prepared in 20% FES and reagent diluent to make a top standard of 2000 pg/ml and a low standard of 15.6 pg/ml; all samples were diluted 1:5 in reagent diluent and added to 96-well plates in quadruplicate; and single absorbance was measured at A_{450}.

2.7. Statistics

Statistical analysis was performed using Sigma Stat (Systat Inc., Richmond, CA). One-way repeated measures ANOVAs (Holm–Sidak method) were used when data was normally distributed to determine differences in each of the measurements over time (1–12 weeks) and (13–30 weeks). The TNFα pg/ml protein data were $\log_{10}$-transformed for statistical analysis. Before and after, paired *t*-tests were used to determine differences between baseline and DR, DR and DI, and DI and baseline data. Linear regression analysis was used to assess the degree of association between the changes (Δ) in percent body fat and inflammatory cytokines. Delta was calculated for each horse as the difference between each time period and week 1 for DR, and for DI it was calculated as the difference between each time period and week 12. Univariate linear regressions were performed on pooled data (Δ values for each horse) during weeks 1–12 and then during weeks 12–30. Significant differences were determined at the level of ($p < 0.05$).

3. Results

3.1. Effect of body condition status on inflammatory cytokine production by PBMC

Old horses, like aged humans, have evidence of inflamm-aging (Franceschi et al., 2000) as indicated by significantly ($p < 0.05$) increased frequencies of IFNγ and TNFα positive lymphocytes (L) and monocytes (M) after stimulation with PMA and ionomycin (Fig. 1a). To determine the effect of adiposity on intracellular inflammatory cytokine production, the old horses were divided into fat and thin groups based on body weight, % body fat and BCS (Table 1). Fat horses had a significant increase in the percent TNFα positive lymphocytes (L) and monocytes (M) following stimulation when compared to thin horses (Fig 1b).

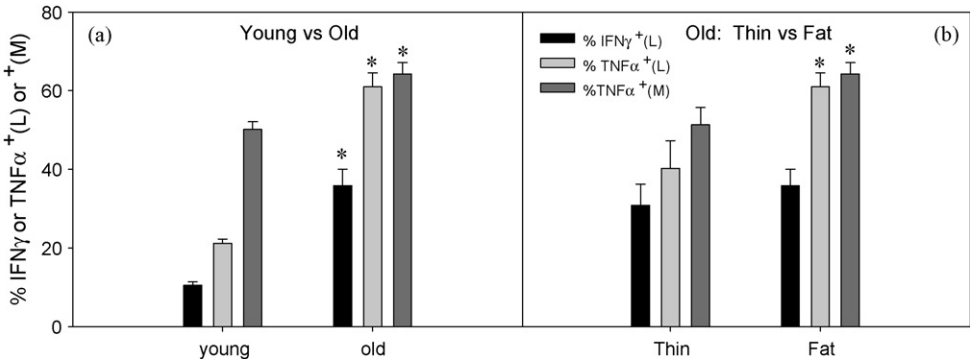

Fig. 1. (a) Old horses ($n = 7$) had significantly ($p < 0.05$) increased frequencies (mean + S.E.) of IFNγ and TNFα positive lymphocytes (L) and monocytes (M) following stimulation with PMA and ionomycin when compared to young ($n = 7$). (b) Fat old horses ($n = 7$) had similar frequencies (mean + S.E.) of IFNγ positive (L) ($p > 0.05$) and significantly increased frequencies of TNFα positive (L) and (M) (*$p < 0.05$) after stimulation with PMA and ionomycin when compared to thin ($n = 6$) old horses.

3.2. Effect of DR and DI on body weight, % body fat and BCS

Dietary restriction significantly altered body weight, percent body fat and BCS during a 12-week period as illustrated in Fig. 2a–c. Overall, DR resulted in a 10.28% loss of initial mean body weight, 24.06% loss of initial mean body fat, and a loss of 2.073 mean BCS (Table 2). Increasing the diet during weeks 12–30 significantly ($p < 0.05$) returned mean body weight and mean % body fat to baseline levels, though mean BCS remained significantly lower compared to baseline mean BCS (Fig. 2, Table 2).

3.3. Inflammatory cytokine (IFNγ and TNFα) production by stimulated PBMC is affected by changing body weight, % body fat and BCS

Because both aging and increased adiposity are associated with increased inflammatory cytokine production, we examined the effect of altering percent body fat in old horses. Decreasing body fat over time significantly reduced the percentage of IFNγ (Fig. 3a) and TNFα (Fig. 3b) positive, PMA and ionomycin stimulated lymphocytes. There was no effect of LPS stimulation on IFNγ and TNFα production from lymphocytes over time (data not shown). The frequencies of TNFα positive, PMA and ionomycin stimulated monocytes were significantly decreased ($p < 0.05$) during weeks 1–12 (Fig. 3c). However, the percent of LPS stimulated TNFα positive monocytes was significantly ($p < 0.05$) increased during weeks 6 and 7. Increasing body fat over time significantly ($p < 0.05$) increased the percent of IFNγ and TNFα positive, PMA and ionomycin stimulated lymphocytes and monocytes (Fig. 3a–c). There was no change in IFNγ and TNFα production from lymphocytes stimulated with LPS during the DI period (data not shown). There was no change in the percent of TNFα positive monocytes after LPS stimulation. In comparing before DR and after DI values, there was a significant increase in IFNγ and TNFα positive, PMA and ionomycin stimulated lymphocytes and monocytes but no difference after LPS stimulated PBMC (Table 3). After DI the percent of IFNγ positive lymphocytes and TNFα positive monocytes returned to baseline levels; while the percent TNFα positive lymphocytes increased but still remained different to baseline values. Overall, during both DR and DI periods there was no significant difference in the frequencies of IFNγ and TNFα positive, non-stimulated (cRPMI media alone) lymphocytes and monocytes. Within the two control mares there was no change (baseline vs DR vs DI) in mean ± S.E. in IFNγ (30.0 ± 6.2 vs 30.1 ± 3.9 vs 29.2 ± 5.2) and TNFα (54.9 ± 3.9 vs 53.3 ± 3.0 vs 48.5 ± 1.4) positive lymphocytes stimulated with PMA. Further there was no change in the mean ± S.E. in TNFα positive

Table 1
Mean body weight, % body fat and body condition score (BCS) of fat and thin old horses.

Measurements	Fat ($n = 7$)	Thin ($n = 6$)
Body weight	527 ± 16.5^a	493 ± 11.0^a
% Body fat	20.8 ± 2.31^a	5.47 ± 0.994^b
BCS	7.25 ± 0.103^a	4.76 ± 0.484^b

[a,b]Means (±S.E.) in the same row with different superscript letters differ significantly ($p < 0.05$).

Table 2
Mean body weight, % body fat and BCS before (baseline) dietary restriction (week 1), after dietary restriction (DR) (week 12), and after increased caloric intake (DI) (week 30).

Measurements	Baseline	DR	DI
Body weight	522.3 ± 15.31^a	468.6 ± 14.12^b	512.7 ± 14.03^a
% Body fat	22.73 ± 2.295^a	17.26 ± 1.438^b	23.82 ± 2.162^a
BCS	7.094 ± 0.168^a	5.021 ± 0.157^b	6.129 ± 0.137^c

[a–c]Means (±S.E.) in the same row with different superscript letters differ significantly ($p < 0.05$).

Table 3
Intracellular staining of IFNγ and TNFα production by lymphocytes (L) or monocytes (M) following stimulation with PMA and ionomycin or LPS, before DR (baseline), after DR, and DI.

Measurements	Baseline	DR	DI
% IFNγ (L)-PMA	34.56 ± 3.816^a	29.78 ± 3.719^b	34.05 ± 4.385^a
% TNFα (L)-PMA	60.88 ± 3.113^a	44.84 ± 3.681^b	53.32 ± 3.648^c
% TNFα (M)-PMA	62.76 ± 2.886^a	43.35 ± 3.046^b	67.06 ± 2.053^a
% TNFα (M)-LPS	32.32 ± 3.467^a	27.15 ± 2.898^a	31.44 ± 3.092^a

[a–c]Means (±S.E.) in the same row with different superscript letters differ significantly ($p < 0.05$).

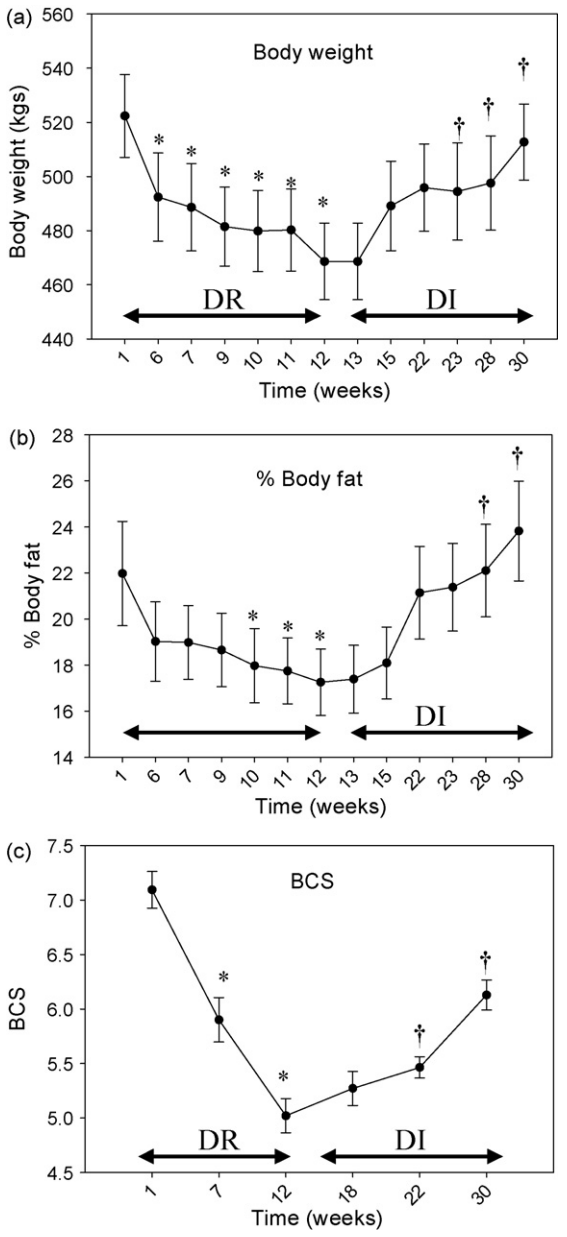

Fig. 2. The effect of dietary restriction, DR (weeks 1–12) and increased dietary intake, DI (weeks 12–30) on average (±S.E.) (a) body weight, (b) percent body fat and (c) body condition score (BCS). DR significantly decreased mean body weight, % body fat and BCS from week 1 to 12 in a group of old horses ($n = 8$). DI significantly increased all three measurements from week 12 to 30 in a group of old horses ($n = 7$). *Significantly different from baseline (week 1) or †statistically different from DR (week 12) ($p < 0.05$).

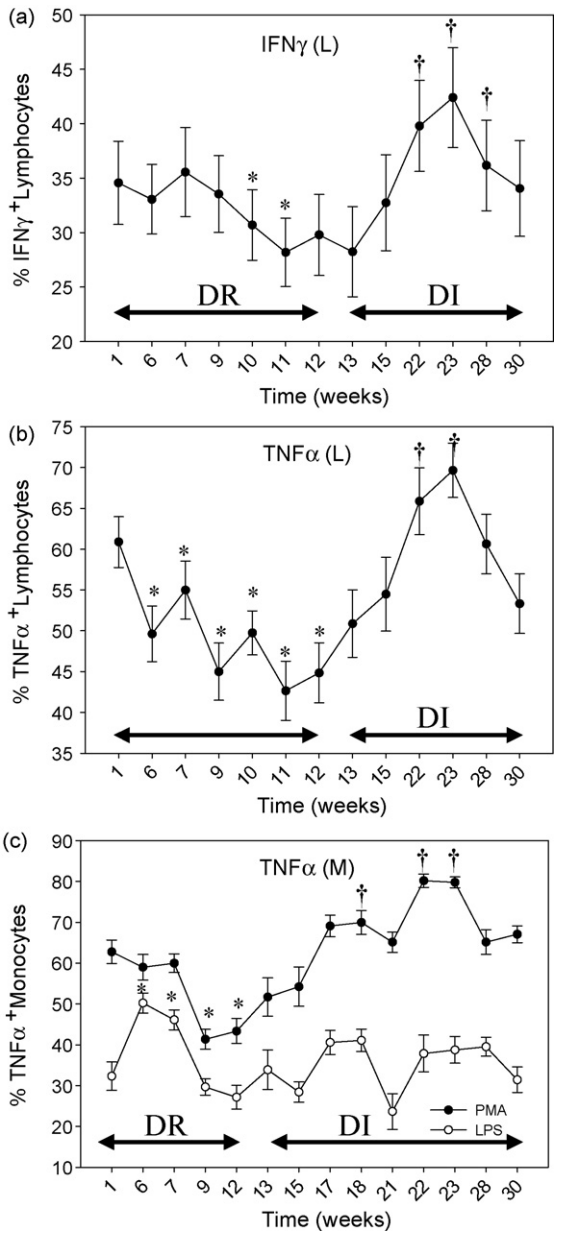

Fig. 3. (a) Mean ± S.E. percent of IFNγ positive lymphocytes (L), (b) TNFα positive lymphocytes (L) and (c) TNFα positive monocytes (M) following stimulation with PMA and ionomycin (solid circles) or LPS-stimulated monocytes (open circles) were altered by changing body condition and fat. DR significantly decreased the percent of IFNγ$^+$(L), TNFα$^+$(L) and TNFα$^+$(M) from eight old horses week 1–12 (*$p < 0.05$). Over time DI significantly increased the percentage IFNγ $^+$(L), TNFα$^+$(L) and TNFα$^+$(M) following stimulation with PMA and ionomycin from seven old horses ($^\dagger p < 0.05$).

monocytes stimulated with PMA (63.1 ± 7.4 vs 62.0 ± 5.4 vs 68.3 ± 1.5) or LPS (28.8 ± 5.3 vs 29.4 ± 2.9 vs 23.7 ± 3.8).

3.4. Serum TNFα protein (pg/ml) measured in serum decreased with DR and continued to decrease with DI

We have previously shown that TNFα protein is significantly increased in obese horses compared to lean, regardless of age (Vick et al., 2007). Here we determined whether changing body fat in old horses had an effect on TNFα protein measured in serum. Serum TNFα protein significantly decreased ($p < 0.05$) with decreasing adiposity during weeks 1–12 (Fig. 4). In addition, there was a significant ($p < 0.05$) difference in serum TNFα levels before and after DR (Table 4). When body fat was increased during DI, serum TNFα protein levels continued to

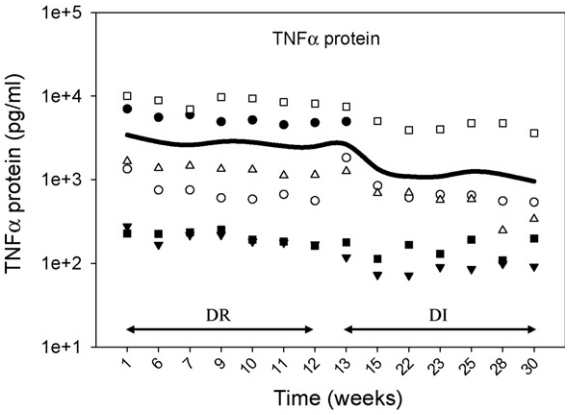

Fig. 4. Serum TNFα protein (pg/ml) decreased with DR (weeks 1–12) in a group of old horses ($n = 6$) and continued to decrease in a group of old horses ($n = 5$) during DI (weeks 12–30) ($p < 0.05$). Two horses had pg/ml values higher than the standard curve and were not included. Each symbol (□, ●, ○, △, ■, ▼) represents a different horse and the solid line (—) represents the mean.

decrease significantly ($p < 0.05$) during the initial weeks 12–15; however, RM ANOVA revealed no significant change during weeks 15–30. While the levels of serum TNFα remained constant during weeks 15–30 they did not return to baseline values (Table 4). Serum TNFα protein remained constant in one of the control mares (other control mare had pg/ml values higher than the standard curve and was not included) when comparing baseline, DR and DI values (3359 pg/ml vs 3163 pg/ml vs 3360 pg/ml), respectively.

3.5. Gene expression of inflammatory cytokines before DR (baseline), after DR, and DI are not altered

To determine whether altering body composition and fat modulates nascent expression of inflammatory cytokines in peripheral blood cells, we assessed mRNA levels in peripheral blood using RT-PCR. There was not a significant decrease in IFNγ and TNFα mRNA expression during weeks 1–12. Though there was a weak and non-significant increase in nascent TNFα and a significant ($p < 0.05$) increase in the mRNA expression of IFNγ during weeks 12–30 (Table 5).

Table 4
Serum TNFα protein (pg/ml) before (baseline) DR, after DR and DI.

Measurements	Baseline	DR	DI
TNFα protein	3420 ± 1671^{a}	2488 ± 1331^{b}	951.6 ± 663.8^{b}

[a,b]Means (±S.E.) in the same row with different superscript letters differ significantly ($p < 0.05$).

Table 5
Relative quantitation (RQ) of nascent gene expression of IFNγ and TNFα in peripheral blood before DR (baseline), after DR and DI.

Measurements	Baseline	DR	DI
IFNγ RQ	1.05 ± 0.370^{a}	0.792 ± 0.132^{a}	1.75 ± 0.242^{b}
TNFα RQ	1.08 ± 0.174^{a}	0.965 ± 0.098^{a}	1.032 ± 0.214^{a}

[a,b]Means (±S.E.) in the same row with different superscript letters differ significantly ($p < 0.05$).

3.6. Regression analysis revealed relationships between changes (Δ) in % body fat and inflammatory measures during DR and DI

To further investigate the relationship between inflammatory cytokines and adiposity, we used univariate linear regressions on pooled data (Δ) during weeks 1–12 (Table 6a) and then during weeks 12–30 (Table 6b). While regression analysis revealed a significant relationship between changes in body weight and percent body fat, there were differences in correlating each of these

Table 6a
Linear regression analysis of the change (Δ) in % body fat and body weight with inflammatory measures during DR[a].

Body condition measurements	Inflammatory cytokines					
	Δ % Body fat	% IFNγ (L)	% TNFα (L)	% TNFα (M) PMA	% TNFα (M) LPS	TNFα (pg/ml) (log 10)
Δ Body weight	$R = 0.613$ $p < 0.001$	$R = 0.540$ $p < 0.001$	$R = 0.412$ $p < 0.005$	$R = 0.510$ $p < 0.001$	$R = 0.665$ $p < 0.001$	$R = 0.394$ $p = 0.017$
Δ % Body fat	– –	$R = 0.444$ $p < 0.005$	$R = 0.115$ $p = 0.479$	$R = 0.472$ $p < 0.005$	$R = 0.050$ $p = 0.803$	$R = 0.463$ $p = 0.004$

[a] *R*-value and *p*-value are shown for pooled data.

Table 6b
Linear regression analysis of the change (Δ) in % body fat and body weight with inflammatory measures during DI[a].

Body condition measurements	Inflammatory cytokines					
	Δ % Body fat	% IFNγ (L)	% TNFα (L)	% TNFα (M) PMA	% TNFα (M) LPS	TNFα (pg/ml) (log10)
Δ Body weight	$R = 0.591$ $p < 0.001$	$R = 0.433$ $p < 0.01$	$R = 0.473$ $p < 0.01$	$R = 0.508$ $p < 0.001$	$R = 0.241$ $p = 0.09$	$R = 0.111$ $p = 0.468$
Δ % Body fat	– –	$R = 0.434$ $p < 0.01$	$R = .0.550$ $p < 0.001$	$R = 0.515$ $p < 0.001$	$R = 0.525$ $p < 0.001$	$R = 0.070$ $p = 0.969$

[a] *R*-value and *p*-value are shown for pooled data.

measurements with inflammatory cytokine changes. The changes (Δ) in decreasing body weight were significantly ($p < 0.05$) related to decreasing (Δ) for all inflammatory measurements (Table 6a). However, loss in percent body fat was related to only changes in the % IFNγ (L), % TNFα (M) PMA stimulated, and TNFα pg/ml (Table 6a). The changes (Δ) in increasing body weight were significantly ($p < 0.05$) associated with all inflammatory measurements except for changes in the % TNFα (M) LPS and TNFα pg/ml (Table 6b) Likewise, regression analysis showed relationships between changes in percent body fat and all inflammatory measurements except for TNFα pg/ml.

4. Discussion

In humans, both obesity (Dandona et al., 2004) and age-associated changes in the immune system (Franceschi et al., 2000) are associated with elevated levels of inflammatory cytokines. The effect of obesity on inflamm-aging remains largely unknown. Here, fat old horses were used as a model to investigate this relationship. Old horses, compared to young, have increased inflammatory cytokine production. Moreover, fat old horses have increased frequencies of lymphocytes and monocytes producing inflammatory cytokines. These results indicated that age-related obesity may contribute to inflamm-aging in the old horse. Thus, adiposity was manipulated over time while measuring inflammatory cytokines to further explore this association. Dietary-restriction resulted in significant weight loss, body fat loss and body condition loss during a twelve week period, followed by increased dietary intake which induced significant weight gain, body fat gain and body condition during an eighteen week period in the same group of old horses. Studies of obese elderly woman have also shown that short-term dietary manipulation results in weight loss (5–6% of initial body weight) and reduces body fat (by 10–12% of initial body fat mass); however these studies did not investigate the effect of increasing body weight and fat (Mazzali et al., 2006; Ryan and Nicklas, 2004).

Regression analysis revealed a significant relationship between percent body fat and IFNγ positive lymphocytes and TNFα positive monocytes. Reducing body weight and fat in old horses significantly reduced the percent of IFNγ and TNFα positive lymphocytes and TNFα positive monocytes. Further, when weight and fat increased in these old horses there was a significant increase in the percent of IFNγ and TNFα positive lymphocytes and monocytes. Although decreased body fat was associated with a significant reduction in the percentage of inflammatory cytokine positive PBMCs, these frequencies were still higher when compared to young adult horses. While adiposity contributes to inflamm-aging, it alone does not account for increased inflammatory cytokine production in the old horse.

While macrophages in obese adipose tissue are known to be the main source of TNFα (Suganami et al., 2005; Tilg and Moschen, 2006), it has been shown that circulating PBMCs in obese subjects have increased inflammatory cytokine mRNA expression, as well (Ghanim et al., 2004). Moreover, a recent study showed that weight and fat loss in older obese subjects decreased the expression of inflammatory cytokine mRNA in PBMC (de Mello et al., 2008). These results suggest some cross-talk occurs between adipose tissue and circulating lymphocytes and monocytes. Indeed, other adipocyte-derived cytokines such as leptin have been shown to increase the production of inflammatory cytokines TNFα and IL-6 from monocytes and macrophages (Gainsford et al., 1996) and the production of T helper cytokine IFNγ (Lord et al., 1998). It is known that IFNγ produced induces the production of TNFα from monocytes and macrophages (Farrar and Schreiber, 1993); which could then act in an autocrine or paracrine manner stimulating resident or peripheral macrophages and T cells to secrete inflammatory cytokines. Perhaps reducing adiposity dampens the inflammatory cycle by decreasing the activation and infiltration of resident macrophages (Bruun et al., 2006). Moreover, a recent review has provided evidence that there is a link between the function of lymphatic vessels and adipose tissue (Harvey, 2008). Thus, adipocyte and monocyte-derived inflammatory cytokines could contribute to alterations in immune function with age.

The effect of adiposity on the immune response to LPS was measured in stimulated PBMCs. During the period of weight and fat loss, the percentage of monocytes producing TNFα increased initially; however, during weight gain no change was observed. This is consistent with the decreased responsiveness to LPS in obese individuals (Lamas et al., 2002) and could account for their increased incidence of infectious illnesses and sepsis (Edelman, 1981; Gottschlich et al., 1993).

There was a significant reduction in serum levels of TNFα protein following weight loss. Also, regression analysis revealed a significant relationship between changes in percent fat and changes in TNFα protein. Circulating levels of TNFα protein have been associated with increased truncal fat mass in elderly subjects (Pedersen et al., 2003). Furthermore, others have shown a significant decrease in circulating TNFα protein with short-term weight and fat loss in older subjects (de Mello et al., 2008). Decreasing adiposity reduced IFNγ and TNFα mRNA expression, though not significantly. This is consistent with the adipose tissue being the source of the circulating TNFα. Surprisingly, when body weight and fat were increased there was a continuous decrease in TNFα during weeks 12–15; however, after week 15 the levels of circulating TNFα protein remained constant. At the same time there was no increase in TNFα mRNA expression in peripheral blood, though there was a significant increase in IFNγ mRNA. Perhaps short-term return of fat mass is not sufficient enough time to restore complete function of adipocytes or macrophages in fat tissue (Bedoui et al., 2005; Xydakis et al., 2004). If body fat would have continued to increase beyond week 30 perhaps the levels of circulating TNFα would have increased to pre-restriction levels.

The mechanism responsible for age-associated inflammation remains unknown. In this study the results show a strong relationship between changes in adiposity and inflammatory cytokine production by PBMC and circulating levels of inflammatory cytokines. These results

demonstrate that age-related obesity potentially plays a role in the dysregulation of inflammatory cytokine production by the immune system with age or inflamm-aging. We are not aware of other studies in which intracellular protein levels of inflammatory cytokines were measured in circulating PBMC of the obese aged, undergoing such manipulation of body weight and fat. Further understanding of what regulates chronic inflammation with age is critical and highly relevant to today's growing population of obese elderly and old horses.

Acknowledgments

This research was supported by the William Robert Mills endowment and the Jeffrey C. Hughes Foundation, University of Kentucky. The authors are very grateful to Mr. Lynn Ennis and the staff at the University of Kentucky's Maine Chance Farm for their technical assistance and care of the horses.

References

Arterburn, D.E., Crane, P.K., Sullivan, S.D., 2004. The coming epidemic of obesity in elderly Americans. J. Am. Geriatr. Soc. 52, 1907–1912.

Bastard, J.P., Jardel, C., Bruckert, E., Blondy, P., Capeau, J., Laville, M., Vidal, H., Hainque, B., 2000. Elevated levels of interleukin 6 are reduced in serum and subcutaneous adipose tissue of obese women after weight loss. J. Clin. Endocrinol. Metab. 85, 3338–3342.

Bedoui, S., Velkoska, E., Bozinovski, S., Jones, J.E., Anderson, G.P., Morris, M.J., 2005. Unaltered TNF-alpha production by macrophages and monocytes in diet-induced obesity in the rat. J. Inflamm. (Lond.) 2, 2.

Berg, A.H., Scherer, P.E., 2005. Adipose tissue, inflammation, and cardiovascular disease. Circ. Res. 96, 939–949.

Breathnach, C.C., Sturgill-Wright, T., Stiltner, J.L., Adams, A.A., Lunn, D.P., Horohov, D.W., 2006. Foals are interferon gamma-deficient at birth. Vet. Immunol. Immunopathol. 112, 199–209.

Bruun, J.M., Helge, J.W., Richelsen, B., Stallknecht, B., 2006. Diet and exercise reduce low-grade inflammation and macrophage infiltration in adipose tissue but not in skeletal muscle in severely obese subjects. Am. J. Physiol. Endocrinol. Metab. 290, E961–E967.

Bruun, J.M., Lihn, A.S., Verdich, C., Pedersen, S.B., Toubro, S., Astrup, A., Richelsen, B., 2003. Regulation of adiponectin by adipose tissue-derived cytokines: in vivo and in vitro investigations in humans. Am. J. Physiol. Endocrinol. Metab. 285, E527–E533.

Bruunsgaard, H., Pedersen, B.K., 2003. Age-related inflammatory cytokines and disease. Immunol. Allergy Clin. North Am. 23, 15–39.

Clement, K., Viguerie, N., Poitou, C., Carette, C., Pelloux, V., Curat, C.A., Sicard, A., Rome, S., Benis, A., Zucker, J.D., Vidal, H., Laville, M., Barsh, G.S., Basdevant, A., Stich, V., Cancello, R., Langin, D., 2004. Weight loss regulates inflammation-related genes in white adipose tissue of obese subjects. Faseb J. 18, 1657–1669.

Dandona, P., Aljada, A., Bandyopadhyay, A., 2004. Inflammation: the link between insulin resistance, obesity and diabetes. Trends Immunol. 25, 4–7.

Dandona, P., Weinstock, R., Thusu, K., Abdel-Rahman, E., Aljada, A., Wadden, T., 1998. Tumor necrosis factor-alpha in sera of obese patients: fall with weight loss. J. Clin. Endocrinol. Metab. 83, 2907–2910.

de Mello, V.D., Kolehmainen, M., Schwab, U., Mager, U., Laaksonen, D.E., Pulkkinen, L., Niskanen, L., Gylling, H., Atalay, M., Rauramaa, R., Uusitupa, M., 2008. Effect of weight loss on cytokine messenger RNA expression in peripheral blood mononuclear cells of obese subjects with the metabolic syndrome. Metabolism 57, 192–199.

Edelman, R., 1981. Obesity: does it modulate infectious disease and immunity? Prog. Clin. Biol. Res. 67, 327–337.

Engwerda, C.R., Fox, B.S., Handwerger, B.S., 1996. Cytokine production by T lymphocytes from young and aged mice. J. Immunol. 156, 3621–3630.

Fagiolo, U., Cossarizza, A., Scala, E., Fanales-Belasio, E., Ortolani, C., Cozzi, E., Monti, D., Franceschi, C., Paganelli, R., 1993. Increased cytokine production in mononuclear cells of healthy elderly people. Eur. J. Immunol. 23, 2375–2378.

Farrar, M.A., Schreiber, R.D., 1993. The molecular cell biology of interferon-gamma and its receptor. Annu. Rev. Immunol. 11, 571–611.

Fontana, L., Klein, S., 2007. Aging, adiposity, and calorie restriction. JAMA 297, 986–994.

Franceschi, C., Bonafe, M., Valensin, S., Olivieri, F., De Luca, M., Ottaviani, E., De Benedictis, G., 2000. Inflamm-aging. An evolutionary perspective on immunosenescence. Ann. NY Acad. Sci. 908, 244–254.

Gainsford, T., Willson, T.A., Metcalf, D., Handman, E., McFarlane, C., Ng, A., Nicola, N.A., Alexander, W.S., Hilton, D.J., 1996. Leptin can induce proliferation, differentiation, and functional activation of hemopoietic cells. Proc. Natl. Acad. Sci. U.S.A. 93, 14564–14568.

Ghanim, H., Aljada, A., Hofmeyer, D., Syed, T., Mohanty, P., Dandona, P., 2004. Circulating mononuclear cells in the obese are in a proinflammatory state. Circulation 110, 1564–1571.

Gottschlich, M.M., Mayes, T., Khoury, J.C., Warden, G.D., 1993. Significance of obesity on nutritional, immunologic, hormonal, and clinical outcome parameters in burns. J. Am. Diet Assoc. 93, 1261–1268.

Harris, T.B., Ferrucci, L., Tracy, R.P., Corti, M.C., Wacholder, S., Ettinger Jr., W.H., Heimovitz, H., Cohen, H.J., Wallace, R., 1999. Associations of elevated interleukin-6 and C-reactive protein levels with mortality in the elderly. Am. J. Med. 106, 506–512.

Harvey, N.L., 2008. The link between lymphatic function and adipose biology. Ann. NY Acad. Sci. 1131, 82–88.

Henneke, D.R., Potter, G.D., Kreider, J.L., Yeates, B.F., 1983. Relationship between condition score, physical measurements and body fat percentage in mares. Equine Vet. J. 15, 371–372.

Hotamisligil, G.S., Shargill, N.S., Spiegelman, B.M., 1993. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science 259, 87–91.

Kane, R., Fisher, M., Parrett, D., Lawrence, L., 1987. Estimating fitness in horses. In: Proceedings of the 10th Equine Nutrition Symposium. pp. 127–131.

Kershaw, E.E., Flier, J.S., 2004. Adipose tissue as an endocrine organ. J. Clin. Endocrinol. Metab. 89, 2548–2556.

Krabbe, K.S., Pedersen, M., Bruunsgaard, H., 2004. Inflammatory mediators in the elderly. Exp. Gerontol. 39, 687–699.

Lamas, O., Marti, A., Martinez, J.A., 2002. Obesity and immunocompetence. Eur. J. Clin. Nutr. 56 (Suppl. 3), S42–S45.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. Methods 25, 402–408.

Lord, G.M., Matarese, G., Howard, J.K., Baker, R.J., Bloom, S.R., Lechler, R.I., 1998. Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. Nature 394, 897–901.

Mauderly, J.L., Hahn, F.F., 1982. The effects of age on lung function and structure of adult animals. Adv. Vet. Sci. Comp. Med. 26, 35–77.

Mazzali, G., Di Francesco, V., Zoico, E., Fantin, F., Zamboni, G., Benati, C., Bambara, V., Negri, M., Bosello, O., Zamboni, M., 2006. Interrelations between fat distribution, muscle lipid content, adipocytokines, and insulin resistance: effect of moderate weight loss in older women. Am. J. Clin. Nutr. 84, 1193–1199.

McFarlane, D., Sellon, D.C., Gibbs, S.A., 2001. Age-related quantitative alterations in lymphocyte subsets and immunoglobulin isotypes in healthy horses. Am. J. Vet. Res. 62, 1413–1417.

Nicklas, B.J., Ambrosius, W., Messier, S.P., Miller, G.D., Penninx, B.W., Loeser, R.F., Palla, S., Bleecker, E., Pahor, M., 2004. Diet-induced weight loss, exercise, and chronic inflammation in older, obese adults: a randomized controlled clinical trial. Am. J. Clin. Nutr. 79, 544–551.

NRC, 1989. Nutrient Requirements of Horses. National Academy Press, Washington, DC, pp. 43–44, 90–110.

Pedersen, M., Bruunsgaard, H., Weis, N., Hendel, H.W., Andreassen, B.U., Eldrup, E., Dela, F., Pedersen, B.K., 2003. Circulating levels of TNF-alpha and IL-6-relation to truncal fat mass and muscle mass in healthy elderly individuals and in patients with type-2 diabetes. Mech. Ageing Dev. 124, 495–502.

Rajala, M.W., Scherer, P.E., 2003. Minireview: the adipocyte—at the crossroads of energy homeostasis, inflammation, and atherosclerosis. Endocrinology 144, 3765–3773.

Roubenoff, R., Harris, T.B., Abad, L.W., Wilson, P.W., Dallal, G.E., Dinarello, C.A., 1998. Monocyte cytokine production in an elderly population: effect of age and inflammation. J. Gerontol. A: Biol. Sci. Med. Sci. 53, M20–M26.

Ryan, A.S., Nicklas, B.J., 2004. Reductions in plasma cytokine levels with weight loss improve insulin sensitivity in overweight and obese postmenopausal women. Diab. Care 27, 1699–1705.

Sandmand, M., Bruunsgaard, H., Kemp, K., Andersen-Ranberg, K., Schroll, M., Jeune, B., 2003. High circulating levels of tumor necrosis factor-alpha in centenarians are not associated with increased production in T lymphocytes. Gerontology 49, 155–160.

Suganami, T., Nishida, J., Ogawa, Y., 2005. A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: role of free fatty acids and tumor necrosis factor alpha. Arterioscler. Thromb. Vasc. Biol. 25, 2062–2068.

Tilg, H., Moschen, A.R., 2006. Adipocytokines: mediators linking adipose tissue, inflammation and immunity. Nat. Rev. Immunol. 6, 772–783.

Vick, M.M., Adams, A.A., Murphy, B.A., Sessions, D.R., Horohov, D.W., Cook, R.F., Shelton, B.J., Fitzgerald, B.P., 2007. Relationships among inflammatory cytokines, obesity, and insulin sensitivity in the horse. J. Anim. Sci. 85, 1144–1155.

Vick, M.M., Murphy, B.A., Sessions, D.R., Reedy, S.E., Kennedy, E.L., Horohov, D.W., Cook, R.F., Fitzgerald, B.P., 2008. Effects of systemic inflammation on insulin sensitivity in horses and inflammatory cytokine expression in adipose tissue. Am. J. Vet. Res. 69, 130–139.

Vick, M.M., Sessions, D.R., Murphy, B.A., Kennedy, E.L., Reedy, S.E., Fitzgerald, B.P., 2006. Obesity is associated with altered metabolic and reproductive activity in the mare: effects of metformin on insulin sensitivity and reproductive cyclicity. Reprod. Fertil. Dev. 18, 609–617.

Villareal, D.T., Apovian, C.M., Kushner, R.F., Klein, S., 2005. Obesity in older adults: technical review and position statement of the American Society for Nutrition and NAASO, The Obesity Society. Am. J. Clin. Nutr. 82, 923–934.

Weisberg, S.P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R.L., Ferrante Jr., A.W., 2003. Obesity is associated with macrophage accumulation in adipose tissue. J. Clin. Invest. 112, 1796–1808.

Xydakis, A.M., Case, C.C., Jones, P.H., Hoogeveen, R.C., Liu, M.Y., Smith, E.O., Nelson, K.W., Ballantyne, C.M., 2004. Adiponectin, inflammation, and the expression of the metabolic syndrome in obese individuals: the impact of rapid weight loss through caloric restriction. J. Clin. Endocrinol. Metab. 89, 2697–2703.