

Prediction of incipient pasture-associated laminitis from hyperinsulinaemia, hyperleptinaemia and generalised and localised obesity in a cohort of ponies

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Keywords: horse; laminitis; obesity; cresty neck; insulin; leptin

Summary

Reasons for performing study: The ability to predict ponies at increased risk of laminitic episodes, when exposed to nutrient dense pasture, would facilitate management to avoid disease.

Objectives: To identify variables and clinically useful cut-off values with reproducible diagnostic accuracy for the prediction of ponies that subsequently developed laminitis when exposed to nutrient dense pasture.

Methods: A cohort of predominantly Welsh and Dartmoor ponies from a closed herd was evaluated in March 2006 (n = 74) and March 2007 (n = 57). Ponies were categorised as never laminitic or previously laminitic according to reported laminitic history and as clinically laminitic (CL) if laminitis was observed within 3 months following evaluation. Body condition score (BCS), cresty neck score (CNS), girth and neck circumferences (NC), withers height, blood pressure and hoof surface temperature, and plasma insulin, glucose, triglyceride, leptin, cortisol, ACTH, uric acid and TNF- α concentrations were measured. Analysis of sensitivity, specificity and receiver operating characteristic curves was used to evaluate the diagnostic accuracy for a variable to predict CL ponies.

Results: Variables with diagnostic accuracy for the prediction of CL ponies included insulin, leptin, BCS, CNS, and NC:height ratio. Specific cut-off values of insulin (>32 μ U/l), leptin (>7.3 ng/ml), BCS (\geq 7), CNS (\geq 4) and NC:height ratio (>0.71) had reproducible diagnostic accuracy for the prediction of laminitis. Combining tests did not result in higher diagnostic accuracy than individual tests of insulin or leptin during either evaluation.

Conclusions: Tests of insulin and leptin concentrations and measures of generalised (BCS) and localised (CNS or NC:height ratio) obesity were beneficial in the prediction of laminitic episodes.

Potential relevance: These results highlight the importance of monitoring and reducing insulin concentration, and generalised and regional obesity in ponies to reduce risk of laminitis.

Abbreviations

BCS	Body condition score
CL	Clinically laminitic
CNS	Cresty neck score
LR	Likelihood ratio
MIRG	Modified insulin-to-glucose ratio
NL	Never laminitic
PL	Previously laminitic
PLMS	Prelaminitic metabolic syndrome
RISQI	Reciprocal of the square root of insulin
ROC	Receiver operating characteristic
gROC	Global ROC
lROC	Local ROC

Introduction

Pasture-associated laminitis has major economic and welfare implications, accounting for an estimated 54% of cases of equine laminitis for which the initial cause is identifiable (Anon 2000). Physiological or genetic factors may predispose some ponies more than others to recurrent or pasture-associated laminitis (Treiber *et al.* 2006). Identification of susceptible ponies could facilitate the prediction of laminitic episodes and allow for implementation of countermeasures (Harris *et al.* 2006).

Increased risk for pasture-associated laminitis in apparently healthy ponies has been characterised by a set of risk factors known as the prelaminitic metabolic syndrome (PLMS) (Treiber *et al.* 2006). These factors include insulin resistance (reciprocal of the square root of insulin [RISQI] <0.32 [μ U/l]^{-0.5}), increased insulin secretory response (modified insulin-to-glucose ratio [MIRG] >5.6 μ U_{insulin}²/[10.1 mg_{glucose}]), hypertriglyceridaemia (>570 mg/l), and obesity (body condition score >6 on a scale of 1–9, with localised fat deposits on neck and tailhead). Ponies identified by the PLMS by surpassing 3 or more criteria were 10 times more likely to develop laminitis than those not identified by PLMS, according to subsequent occurrence of laminitis in the study group.

Metabolic syndrome in man relates factors of obesity, insulin resistance, hypertension, hypertriglyceridaemia and hyperglycaemia

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[Paper received for publication 28.05.08; Accepted 27.06.08]

to risk for *type 2* diabetes and cardiovascular disease (Anon 1999, 2001). Recent research has demonstrated a number of similar factors, including insulin resistance, hyperinsulinaemia, obesity, hypertension and increased uric acid concentrations in ponies with a history of laminitis (Treiber *et al.* 2006, 2007; Bailey *et al.* 2007, 2008). Additionally, laminitis is a common sequela to pituitary *pars intermedia* dysfunction, defined by elevated ACTH concentration, and observed in 70% of horses with laminitis in a primary-care ambulatory setting (Donaldson *et al.* 2004).

Obesity has been associated with insulin resistance, hyperinsulinaemia, altered lipid metabolism and increased inflammatory cytokine expression (Hoffman *et al.* 2003; Frank *et al.* 2006; Vick *et al.* 2007). Obesity may therefore be related to laminitis either directly or indirectly through its associated metabolic factors. In man, regional fat accumulation centrally (visceral adipose) is more closely linked to disease risk than generalised obesity (Murphy and Bloom 2006). However, it has been proposed that, in horses and ponies, adipose tissue distributed specifically on the crest of the neck ('cresty neck') could indicate or contribute to hyperinsulinaemia, insulin resistance or risk for laminitis (Johnson 2002; Frank *et al.* 2006; Carter *et al.* 2008).

Considering these relationships with laminitic history, it was hypothesised that measurement of the above mentioned variables would provide useful tests for the prediction of laminitic episodes. The objective of the study was to identify variables and clinically useful cut-off values with reproducible diagnostic accuracy for the prediction of ponies that subsequently developed laminitis when exposed to nutrient dense pasture within 3 months of evaluation. Additionally, it was tested whether clustering variables into a syndrome, such as the PLMS, provided stronger diagnostic accuracy than assessing individual variables alone.

Materials and methods

Animals and study design

All procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee. Following a cohort study design, a single herd of ponies was evaluated in March 2006 (*Evaluation 1*) and March 2007 (*Evaluation 2*). Eligibility criteria for inclusion in the study included being fully or partially descended from pony bloodlines, residing at the selected farm in northern Virginia, age ≥ 3 years, female and apparently healthy at the time of initial evaluation. Ponies age <3-years-old were excluded based on a lack of laminitic episodes in this age range according to farm records, and males were excluded as mares were almost exclusively available for evaluation. For *Evaluation 1*, 74 ponies fitting the selected criteria were recruited and sampled on 3 consecutive days in March 2006 between 0800 and 1200 h. Ponies were used in *Evaluation 2* if they had participated in *Evaluation 1* and were apparently healthy at the time of re-evaluation. For *Evaluation 2*, 57 ponies were evaluated on 2 consecutive days in March 2007.

Diagnosis of laminitis

Ponies that were reported in farm records to have exhibited one or more episode of idiopathic lameness, characteristic of laminitis, were categorised as previously laminitic (PL). Identification of previous laminitis episodes was performed by farm personnel knowledgeable in the identification of lameness due to laminitis as

opposed to other causes of lameness. Unaffected ponies were categorised into a never laminitic (NL) control group. Following initial evaluations, episodes of acute pasture-associated laminitis were documented in spring and early summer (April to June, 2006 and 2007). One of the investigators (R.G.) performed complete physical and lameness examinations to confirm suspected episodes of laminitis. These ponies were subsequently allocated to a clinically laminitic (CL) group for each evaluation.

Sample collection and analysis

During *Evaluation 1*, linear measurements of withers height, girth and waist circumferences, and neck circumference (NC) at half of neck length were measured with a tape measure. Waist circumference was measured as the abdominal circumference two-thirds the distance from the point of the shoulder (intermediate tubercle of the humerus) to the point of the hip (*tuber coxae*). Three experienced evaluators rated body condition score (BCS) on a scale of 1–9 and cresty neck score (CNS) of neck adiposity on a scale of 0–5 (Henneke *et al.* 1983; Carter *et al.* 2008). The median score for each pony was used for analysis. Systolic, diastolic and mean blood pressures were measured with an oscillometric device (Colin 8800)¹ and pressure cuff applied to the base of the tail. Five consecutive measurements were taken from each pony, with the median value used for analysis. Hoof surface temperature was measured with a portable infrared laser thermometer (IR Man)² at the dorsal midpoint of the hoof wall for each hoof. Ponies were removed from direct sunlight onto an asphalt barn aisle for ≥ 5 min before hoof temperature measurement. Ambient temperature was recorded each day.

Basal blood samples were collected by jugular venipuncture between 0800 and 1200 h, before concentrate or cereal grains were fed. Samples were collected into evacuated tubes containing sodium heparin or potassium EDTA as anticoagulant. Plasma was separated by centrifugation within 30 min of sample collection, and stored at -20°C until analysis. Plasma glucose, triglyceride and uric acid concentrations were assayed enzymatically by commercial kits and an automated analyser (CX5 Chemistry Analyzer)³. Plasma insulin (Coat-A-Count Insulin)⁴, leptin (Multi-species Leptin RIA)⁵, cortisol (Coat-A-Count Cortisol)⁴ and ACTH (DiaSorin ACTH)⁶ concentrations were measured by commercial radioimmunoassay previously validated for use in equine plasma (Freestone *et al.* 1991; McManus and Fitzgerald 2000; McFarlane *et al.* 2006). Plasma TNF α was measured by a commercially available equine-specific ELISA (Equine TNF α Screening Set)⁷ previously validated in equine samples (Vick *et al.* 2007). Assays were performed in duplicate.

During *Evaluation 2*, BCS, CNS, withers height, girth circumference, NC and blood pressure, and plasma insulin, glucose, triglyceride and leptin concentrations were measured as described for *Evaluation 1*. Plasma ACTH concentrations were analysed at a commercial laboratory⁸ by an automated chemiluminescent enzyme immunoassay system (Immulite)⁹ previously validated in equine plasma (Perkins *et al.* 2002).

Statistical analyses

Values for RISQI and MIRG were calculated as described previously (Treiber *et al.* 2005). Results of the Shapiro-Wilk test revealed that not all variables were normally distributed within laminitis group: therefore, data are reported as median (95%

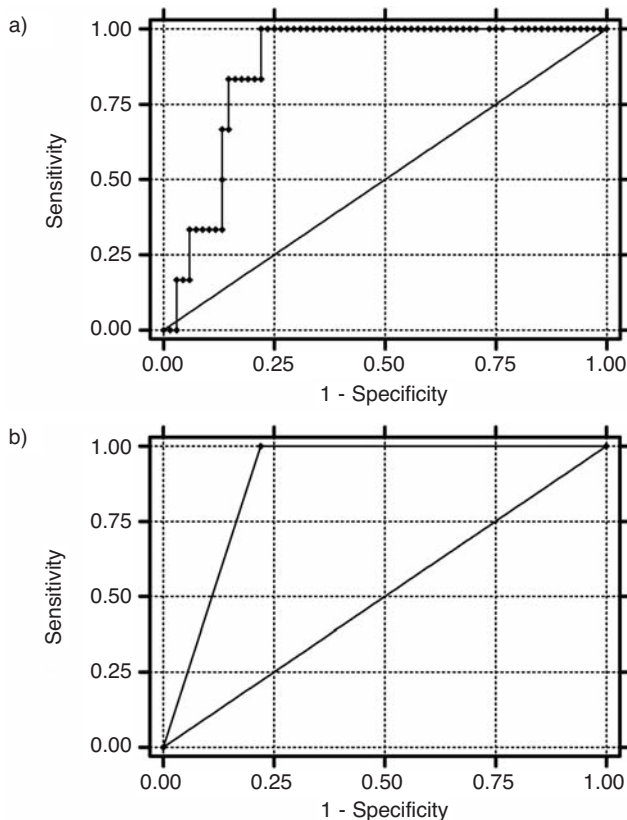


Fig 1: Example receiver operating characteristic (ROC) plots for the prediction of CL ponies in Evaluation 1 from insulin concentration among all insulin values in a global ROC plot (a) and at a specified cut-off value of $>32 \mu\text{U/ml}$ in a local ROC plot (b).

confidence intervals for the median) and laminitis groups (NL, PL, and CL) were compared by Kruskal-Wallis tests with Dunn's multiple comparison post test applied between groups. Differences in medians between evaluations were assessed by Wilcoxon's matched pairs test. Values of $P < 0.05$ were considered significant.

For each variable, true positives (TP), true negatives (TN), false positives (FP) and false negatives (FN) were determined based on the ability of a test to distinguish CL ponies from all other ponies at each measured value or at a specified cut-off value. Sensitivity and specificity were calculated as $\text{TP}/(\text{TP} + \text{FN})$ and $\text{TN}/(\text{FP} + \text{TN})$, respectively (Gibson 1990). To determine the diagnostic accuracy of a test, the true positive fraction (sensitivity) was plotted against the true negative fraction (1 - specificity) in receiver operating characteristic (ROC) plots and the area under the curve was calculated using the trapezoidal rule (Zweig and Campbell 1993). Areas under the ROC curves were considered significant if there was rejection of the null hypothesis that the ROC area equals 0.50 by a one-tailed test ($\alpha = 0.05$).

Continuous variables were used in global ROC (gROC) plots to determine the ability of the test to distinguish between CL and all other ponies over all decision thresholds (Fig 1a). Continuous variables with $\text{gROC} > 0.50$ ($P < 0.05$) or part of the original PLMS criteria were then transformed into binary variables (positive or negative) by categorisation based on a cut-off value and local ROC (lROC) plots were generated (Fig 1b). An optimum cut-off value was chosen based on a maximum area under the lROC curve value, at which point there was a balanced minimum of false-positive and false-negative results. Likelihood ratios were calculated as the ratio of the true-positive rate to the false-positive rate (LR^+) and the ratio of the false-negative rate to the true-negative rate (LR^-) (Biggerstaff 2000). Areas under the ROC

TABLE 1: Median values of variables for never laminitic (NL), previously laminitic (PL), and clinically laminitic (CL) groups of ponies measured in March 2006 during Evaluation 1. Area under the global receiver operating characteristic curve (gROC) was used as a measure of diagnostic accuracy for the prediction of laminitis. Values in () are 95% confidence intervals

	NL (n = 32)	PL (n = 36)	CL (n = 6)	gROC
Blood variables				
Triglyceride, mg/l	390 ^a (330–470)	530 ^b (480–580)	470 ^{a,b} (310–1220)	0.58 (0.28–0.88)
Insulin, $\mu\text{U/l}$	8.8 ^a (7.1–14.6)	20.5 ^b (10.1–36.9)	59.5 ^b (35.3–333)	0.88* (0.80–0.96)
Glucose, mg/l	910 (890–950)	960 (920–1010)	930 (890–1040)	0.53 (0.34–0.73)
RISQI, $[\mu\text{U/l}]^{0.5}$	0.34 ^a (0.26–0.38)	0.22 ^b (0.16–0.32)	0.13 ^b (0.05–0.17)	0.88 [†] * (0.80–0.96)
MIRG, $\mu\text{U}_{\text{insulin}}^2/[\text{l.l.mg}_{\text{glucose}}]$	4.9 (4.0–6.6)	5.1 (3.8–7.6)	11.4 (-315–12.8)	0.67 (0.28–1.0)
Leptin, ng/ml	4.9 ^a (3.4–6.3)	5.8 ^a (4.0–6.8)	11.3 ^b (5.6–16.0)	0.87* (0.70–1.0)
Cortisol, $\mu\text{g/l}$	56.0 (49.0–65.0)	56.0 (52.0–66.0)	44.0 (36.0–120.0)	0.66 [†] (0.38–0.95)
ACTH, pg/ml	75.0 ^a (57.2–87.1)	86.4 ^b (80.7–97.6)	51.8 ^a (20.5–100.4)	0.78 [†] * (0.54–1.0)
Uric acid, mg/l	5.0 (4.0–5.0)	5.0 (5.0–5.0)	4.5 (3.0–5.0)	0.63 [†] (0.41–0.85)
TNF- α , pg/ml	373 ^a (204–725)	1839 ^b (1007–2527)	1341 ^{ab} (493–2406)	0.59 (0.41–0.76)
Adiposity measurements				
Body condition score	6.5 ^a (6.0–7.0)	7.0 ^{ab} (6.5–7.5)	7.5 ^b (7.0–8.0)	0.75* (0.63–0.87)
Girth:height ratio	1.30 (1.26–1.34)	1.33 (1.29–1.35)	1.37 (1.31–1.47)	0.75* (0.56–0.94)
Waist:height ratio	1.45 (1.40–1.50)	1.48 (1.46–1.53)	1.45 (1.39–1.68)	0.49 (0.25–0.73)
Cresty neck score	2.3 ^a (2.0–3.0)	3.0 ^b (3.0–4.0)	4.3 ^c (3.5–5.0)	0.89* (0.80–0.99)
NC:height ratio	0.64 ^a (0.61–0.66)	0.66 ^a (0.64–0.71)	0.73 ^b (0.71–0.80)	0.90* (0.81–0.98)
Blood pressure				
Systolic, mmHg [‡]	119 (112–130)	121 (112–126)	111 (99–127)	0.71 [†] * (0.50–0.92)
Diastolic, mmHg [‡]	60 (53–65)	59 (55–62)	53 (45–63)	0.72 [†] * (0.50–0.95)
Mean, mmHg [‡]	84 (78–87)	81 (76–87)	74 (67–85)	0.72 [†] * (0.52–0.91)
Hoof surface temperature, °C				
	14.6 ^a (10.7–18.7)	21.4 ^b (19.5–24.2)	21.8 ^{ab} (14.4–24.2)	0.65 (0.47–0.83)

^{a,b}Values in the same row with different letters indicate differences ($P < 0.05$) between laminitis groups within each variable. *Area under the gROC curve significantly greater ($P < 0.05$) than 0.50; gROC did not differ ($P > 0.05$) between variables greater than 0.50. [†]Inverse values were used for ROC calculations due to negative association with risk for laminitis [‡]NL, n = 30; PL, n = 35; CL, n = 5.

curves and likelihood ratios are reported with 95% confidence intervals (Altman *et al.* 2000; Biggerstaff 2000) and were compared using 95% confidence intervals for differences between variables. Intercooled Stata Version 9.2¹⁰ was used for statistical computations.

Results

Evaluation 1

Evaluated ponies were descended predominantly from Welsh (59%) or Dartmoor (17%) bloodlines or were crossbred (21%) with horse bloodlines. According to farm manager-reported histories of laminitis, 42 of the 74 ponies presented for evaluation were categorised as PL (25 pregnant, 17 nonpregnant; age 7–34 years) and 32 were categorised as NL (18 pregnant, 14 nonpregnant; age 3–29 years). Six PL ponies developed pasture-associated laminitis during April or May 2006 (i.e. 2–3 months after evaluation) and were subsequently recategorised into a CL group (2 pregnant, 4 nonpregnant; age 7–22 years).

Median (95% confidence interval) values for measured test variables are reported in Table 1. Fore- and hindfoot temperatures were similar ($P>0.05$), therefore the median temperature of all 4 hooves for each pony was used for analysis. Hoof temperatures were different ($P<0.05$) between days of evaluation, with higher hoof temperatures corresponding to a

greater ambient temperature. Potential confounding factors of age and pregnancy status were evaluated in all variables. Girth:height ratio and triglyceride and uric acid concentrations were higher ($P<0.05$) in pregnant than nonpregnant ponies. Hoof wall temperature and TNF α concentrations were positively associated ($P<0.05$) with age.

Diagnostic accuracy of continuous variables to distinguish CL ponies from all other ponies was assessed by area under the gROC curve (Table 1). Tests of insulin, RISQI, leptin, ACTH, BCS, girth:height, CNS, NC:height, BCS and blood pressure measurements had gROC areas >0.50 ($P<0.05$), indicating an ability to distinguish between ponies that did and did not develop clinical laminitis.

Predictions by individual tests were compared to combinations of tests, including: 1) the original PLMS as previously defined (Treiber *et al.* 2006); 2) a modified PLMS with the same variables as the original, but cut-off values specific for the prediction of CL in the present study (3 of 4 criteria: BCS ≥ 7 and CNS ≥ 4 , TG >940 mg/l, RISQI <0.17 [$\mu\text{u/l}$]^{-0.5}, MIRG >11.3 $\mu\text{u}_{\text{insulin}}^2/[\text{10.l.mg}_{\text{glucose}}]$); and 3) a new cluster of variables chosen for their superior ability to predict CL (3 of 4 criteria: BCS ≥ 7 , CNS ≥ 4 , basal insulin >32 $\mu\text{u/l}$, leptin >7.3 ng/ml) (Table 2). The new cluster had a higher IROC than the original PLMS, whereas the modified PLMS criteria IROC did not differ from either the original PLMS or new cluster of variables. Combining tests did not result in higher IROC than the individual tests.

TABLE 2: Diagnostic statistics for the prediction of laminitis in ponies in Evaluation 1 (n = 74) using a defined cut-off value for each variable are sensitivity, specificity, area under the local receiver operating characteristic curve (IROC), and log likelihood ratios for the positive (LR+) and negative (LR-) tests. Values in () are 95% confidence intervals

	Cut-off	Sensitivity, %	Specificity, %	IROC	LR+	LR-
Blood variables						
Triglyceride, mg/l	>940	33 ^b (10–70)	99 ^a (92–100)	0.66 ^b (0.54–0.77)	22.7 ^{ab} (2.4–215.2)	0.7 (0.4–1.2)
Insulin, $\mu\text{u/l}$	>32	100 ^a (61–100)	78 ^b (67–86)	0.89 ^a (0.80–0.95)	4.5 ^{bcd} (2.9–7.1)	0.0 (0.0–1.5)
RISQI, [$\mu\text{u/l}$] ^{-0.5}	<0.17	100 ^a (61–100)	78 ^b (67–86)	0.89 ^a (0.80–0.95)	4.5 ^{bcd} (2.9–7.1)	0.0 (0.0–1.5)
MIRG, $\mu\text{u}_{\text{insulin}}^2/[\text{10.l.mg}_{\text{glucose}}]$	>11.3	67 ^{ab} (30–90)	97 ^a (90–99)	0.82 ^{ab} (0.72–0.90)	22.7 ^a (5.2–99.4)	0.3 (0.1–1.1)
Leptin, ng/ml	>7.3	83 ^{ab} (44–97)	78 ^b (67–86)	0.81 ^{ab} (0.70–0.89)	3.8 ^{bcd} (2.1–6.7)	0.2 (0.0–1.3)
ACTH, pg/ml	<58	83 ^{ab} (44–97)	79 ^b (68–87)	0.83 ^{ab} (0.71–0.90)	4.0 ^{bcd} (2.2–7.3)	0.2 (0.0–1.3)
Adiposity measurements						
Body condition score	≥ 7	100 ^a (61–100)	44 ^c (33–56)	0.72 ^b (0.60–0.81)	1.8 ^e (1.4–2.2)	0.0 (0.0–2.7)
Girth:height ratio	>1.30	100 ^a (61–100)	41 ^c (30–53)	0.71 ^b (0.59–0.80)	1.7 ^e (1.4–2.1)	0.0 (0.0–2.9)
Cresty neck score	≥ 4	83 ^{ab} (44–97)	78 ^b (67–86)	0.81 ^{ab} (0.70–0.89)	3.8 ^{bcd} (2.1–6.7)	0.2 (0.0–1.3)
NC:height ratio	>0.71	100 ^a (61–100)	79 ^b (68–87)	0.90 ^a (0.80–0.95)	4.9 ^{bc} (3.0–7.7)	0.0 (0.0–1.5)
Mean blood pressure, mmHg[#]						
	<76	83 ^{ab} (44–97)	69 ^b (57–79)	0.76 ^{ab} (0.64–0.85)	2.7 ^{cde} (1.6–4.5)	0.2 (0.0–1.5)
Combined criteria						
Original PLMS	Treiber <i>et al.</i> 2006 [†]	67 ^{ab} (30–90)	66 ^b (54–76)	0.66 ^b (0.54–0.77)	2.0 ^{de} (1.0–3.8)	0.5 (0.2–1.6)
Modified PLMS	New cut-offs [‡]	67 ^{ab} (30–90)	97 ^a (90–99)	0.81 ^{ab} (0.72–0.90)	22.7 ^a (5.2–99.4)	0.3 (0.1–1.1)
New cluster	New tests [§]	100 ^a (61–100)	79 ^b (68–87)	0.90 ^a (0.80–0.95)	4.9 ^{bc} (3.0–7.7)	0.0 (0.0–1.5)

^{a–e}Comparisons between variables within each diagnostic statistic. Values in the same column with different superscripts differ ($P<0.05$) between variables, with “a” representing the variables with the greatest value for each statistic. Only IROC values greater ($P<0.05$) than 0.50 were considered in comparisons. [†]Surpass 3 of 4 criteria: BCS >6 , TG >560 mg/l, RISQI <0.32 , MIRG >5.6 ; [‡]Surpass 3 of 4 criteria: BCS ≥ 7 and CNS ≥ 4 , TG >940 mg/l, RISQI <0.17 , MIRG >11.3 ; [§]Surpass 3 of 4 criteria: BCS ≥ 7 , CNS ≥ 4 , insulin >32 $\mu\text{u/l}$, leptin >7.3 ng/ml. [#]n = 70.

Evaluation 2

Fifty-seven of the ponies that were evaluated in 2006 were re-evaluated in 2007. Reasons for withdrawal from the study between *Evaluations 1* and 2 included being sold, located in inaccessible pastures or deceased. Twenty-seven were NL (19 pregnant, 8 nonpregnant; age 4–21 years) and 30 were PL (11 pregnant, 19 nonpregnant; age 8–24 years). Eight PL ponies (3 pregnant, 5 nonpregnant; age 9–15 years) developed pasture-associated laminitis in April or May 2007. Two ponies were CL in both *Evaluations 1* and 2. In the 57 ponies assessed during both evaluations, triglyceride, leptin, and ACTH concentrations decreased ($P<0.001$), NC:height increased ($P<0.037$) and mean blood pressure decreased ($P<0.028$) from *Evaluation 1* to 2, with no differences between evaluations in all other variables ($P>0.05$).

Cut-off values established in *Evaluation 1* were used in *Evaluation 2* in order to examine the within herd accuracy of these cut-offs on the second evaluation's data. Median (95% confidence interval) values for variables are reported in Table 3. Variables with reproducible diagnostic accuracy (gROC greater ($P<0.05$) than 0.50 in *Evaluations 1* and 2) included insulin, RISQI, leptin, BCS, CNS, and NC:height (Table 3). Variables with reproducible diagnostic accuracy at specified cut-off values (IROC >0.50 [$P<0.05$] in *Evaluations 1* and 2) included insulin, RISQI, MIRG, leptin, BCS, CNS, and NC:height (Table 4). Combining tests into the new cluster of variables had greater IROC than the original or modified PLMS. However, combining tests did not result in higher IROC than the individual tests of insulin, leptin, RISQI or MIRG.

Discussion

In a closed herd of predominantly Welsh and Dartmoor ponies, variables with reproducible diagnostic accuracy for the prediction of laminitic episodes included basal insulin and leptin concentrations, RISQI and measures of generalised (BCS) and localised (CNS or NC:height ratio) obesity. Although the original PLMS criteria or combinations of new criteria predicted laminitis with reproducible diagnostic accuracy, their areas under the IROC curves were not higher than the individual tests from which they were produced.

Previously established PLMS criteria were determined on the basis of their ability correctly to differentiate PL from NL ponies historically, which was then applied for the prediction of CL cases (Treiber *et al.* 2006). The present study analysed variables for their ability to differentiate CL ponies from ponies that did not develop laminitis. These risk factors identify ponies during times of low pasture carbohydrate levels (nonstructural carbohydrates $<10\%$ of dry matter during March 2006 and 2007; K.H. Treiber, unpublished data) that will be more likely to develop laminitis in the upcoming months when exposed to high carbohydrate pasture (nonstructural carbohydrates $>15\%$ of dry matter; K.H. Treiber, unpublished data). This focuses on variables relevant to current risk of laminitis rather than previous conditions which may no longer apply. The small number of ponies that develop laminitis each year and the variability of measured variables create the necessity of using statistical analyses, such as ROC plots and likelihood ratios.

The diagnostic accuracy of a test for the prediction of CL was assessed by evaluation of areas under the ROC curves (Fig 1). When a continuous variable is used in an ROC plot, a comprehensive picture is formed of the ability of the test to make the distinction being examined over all decision thresholds (Zweig and Campbell 1993). This was referred to as global ROC (gROC) and provides information on a variable's relationship to laminitis independent of any specified cut-off value. When interpreting gROC, an area under the curve of 0.80, for example, would mean that a CL pony would have a test value greater than that of a pony that did not develop laminitis 80% of the time. When a continuous variable was transformed into a binary variable (positive or negative) by categorisation based on a cut-off value, local ROC (IROC) plots were generated. Applying these cut-offs to the second year of data provides some degree of verification of the adequacy of values determined with the first year of data. Likelihood ratios correspond to the slopes of the ROC plot (Biggerstaff 2000). LR+ describes how probability of laminitis shifts with a positive test result, whereas LR- describes how the probability of laminitis shifts with a negative test result. A test with higher LR+ and lower LR- values was preferred. Although likelihood ratios provide useful information on test performance at a specified cut-off value, it is necessary to interpret values with

TABLE 3: Median values of variables for never laminitic (NL), previously laminitic (PL), and clinically laminitic (CL) groups of ponies measured in March 2007 during *Evaluation 2*. Area under the global receiver operating characteristic curve (gROC) was used as a measure of diagnostic accuracy for the prediction of laminitis. Values in () are 95% confidence intervals

	NL (n = 27)	PL (n = 22)	CL (n = 8)	gROC
Blood variables				
Triglyceride, mg/l [†]	250 (21–38)	440 (33–52)	480 (220–590)	0.67 (0.49–0.85)
Insulin, $\mu\text{u/l}$	9.8 ^a (7.0–22.4)	25.4 ^{ab} (14.5–30.7)	62.6 ^b (32.8–73.4)	0.87* (0.78–0.96)
RISQI, [$\mu\text{u/l}$] ^{0.5}	0.32 ^a (0.21–0.38)	0.20 ^{ab} (0.18–0.26)	0.13 ^b (0.12–0.17)	0.87 [†] * (0.78–0.96)
MIRG, $\mu\text{u}_{\text{insulin}}^2/10.1\text{mg}_{\text{glucose}}$	4.6 ^a (3.6–8.0)	7.5 ^{ab} (5.3–10.2)	10.8 ^b (8.1–13.2)	0.87* (0.77–0.98)
Leptin, ng/ml [‡]	3.7 ^a (2.6–5.3)	4.6 ^{ab} (2.3–6.7)	8.4 ^b (4.0–11.7)	0.78* (0.59–0.96)
ACTH, pg/ml	19.7 (16.8–28.4)	28.5 (20.2–37.5)	23.7 (15.4–46.6)	0.54 (0.36–0.73)
Adiposity measurements				
Body condition score	7.0 ^a (6.5–7.5)	7.3 ^{ab} (7.0–8.0)	8.0 ^b (7.0–9.0)	0.80* (0.66–0.94)
Girth:height ratio	1.33 (1.28–1.36)	1.33 (1.27–1.37)	1.35 (1.28–1.42)	0.63 (0.44–0.82)
Cresty neck score	2.5 ^a (2.0–3.0)	3.5 ^b (3.0–4.0)	3.8 ^b (3.0–4.5)	0.80* (0.67–0.93)
NC:height ratio	0.65 ^a (0.64–0.69)	0.70 ^b (0.65–0.74)	0.72 ^b (0.67–0.81)	0.75* (0.59–0.92)
Mean blood pressure, mmHg[§]	74 (68–85)	77 (67–82)	82 (61–93)	0.67 (0.40–0.93)

^{a,b}Values in the same row with different letters indicate differences ($P<0.05$) between laminitis groups within each variable. *Area under the gROC curve significantly greater ($P<0.05$) than 0.50; gROC did not differ ($P>0.05$) between variables greater than 0.50. [†]Inverse values were used for ROC calculations due to negative associations with risk for laminitis. [‡]PL, n = 21; [§]NL, n = 25; PL, n = 21; [§]NL, n = 13; PL, n = 10; CL, n = 7.

respect to sensitivity and specificity. For example, a test with high LR+ would indicate a high probability of laminitis with a positive test result. However, in the presence of low sensitivity and a high specificity, it would not identify many of the ponies that would develop laminitis due to an inappropriately high cut-off value.

As components of the original PLMS, it was hypothesised that triglyceride concentration, RISQI and MIRG would be useful for the prediction of insipient laminitis. Proxy measurements of RISQI and MIRG were developed as easily obtainable surrogates of minimal model parameters of insulin sensitivity and acute insulin response to glucose, respectively (Treiber *et al.* 2005). As RISQI is inversely proportional to insulin concentrations, it had the same ability as basal insulin concentration for the prediction of CL ponies. Both MIRG and triglyceride concentration had low diagnostic accuracy for the prediction of laminitis, with gROC not significantly different than 0.50 for one or both evaluations. Although triglyceride concentration differentiated NL from PL ponies, it predicted CL ponies with low diagnostic accuracy. Additionally, MIRG has limited value when insulin concentrations are >50 mu/l because its parabolic nature causes values to decline after insulin concentrations exceed 50 mu/l, and therefore restricts its utility of identifying laminitis risk in hyperinsulinaemic ponies.

Although surface temperature of the hoof wall was greater in PL compared to NL ponies, this measurement was not an effective predictor of laminitis in CL ponies. The usefulness of this measurement is limited due to the effect of ambient temperature

and the necessity of a controlled environment. Difference in hoof temperature between laminitis groups may be a response to previous occurrences of laminitis, such as vascular remodelling or a continual inflammatory response from previous damage.

Results of the current study do not support blood pressure or uric acid concentrations as predictive measurements for the occurrence of laminitis. These variables were previously evaluated in ponies with a history of laminitis, and differences between NL and PL ponies were observed during summer but not winter (Bailey *et al.* 2008). Although seasonal differences in variables may be part of the phenotype of ponies with a history of laminitis (and suspected predisposition for laminitis), these variables were not predictive of laminitic episodes and did not differ between NL and PL groups in March in the present study.

Bailey *et al.* (2008) found that ACTH concentrations were similar between NL and PL ponies. In the present study, although ACTH concentration was higher in PL than NL during *Evaluation 1*, it was not a useful predictor of laminitis nor did it differ between laminitis groups during *Evaluation 2*. Additionally, the concentrations in *Evaluation 1* were higher than in *Evaluation 2*, perhaps due to annual variation or assay methodology.

Inclusion of CNS or NC:height in the PLMS obesity criterion complimented BCS, providing a standardised measurement of the localised fat deposits on the neck, which were referred to in the original PLMS. However, the current study cannot support a role of CNS independent of BCS for the prediction of laminitis since

TABLE 4: Diagnostic statistics for the prediction of laminitis in ponies in *Evaluation 2* (n = 57) using a defined cut-off value for each variable are sensitivity, specificity, area under the local receiver operating characteristic curve (IROC), and log likelihood ratios for the positive (LR+) and negative (LR-) tests. Values in () are 95% confidence intervals

	Cut-off	Sensitivity, %	Specificity, %	IROC	LR+	LR-
Blood variables						
Triglyceride, mg/l [†]	>940	0 ^d (0–32)	98 ^{ab} (89–100)	0.49 (0.36–0.63)	0.0 (0.0–84.0)	1.0 ^c (1.0–1.1)
Insulin, mu/l	>32	100 ^a (68–100)	80 ^{de} (66–89)	0.90 ^a (0.78–0.96)	4.9 ^{ab} (2.8–8.5)	0.0 (0.0–1.2)
RISQI, [mu/l] ^{-0.5}	<0.17	88 ^{ab} (53–98)	84 ^{cde} (71–91)	0.86 ^a (0.74–0.94)	5.4 ^{ab} (2.7–10.6)	0.1 ^a (0.0–0.9)
MIRG, mu _{insulin} ² /[10.l.mg _{glucose}]	>11.3	50 ^{bc} (22–78)	94 ^{abc} (83–98)	0.72 ^{ab} (0.58–0.83)	8.2 ^a (2.2–29.9)	0.5 ^{abc} (0.3–1.1)
Leptin, ng/ml [#]	>7.3	63 ^{abc} (31–86)	90 ^{bcd} (78–96)	0.76 ^{ab} (0.62–0.87)	6.1 ^{ab} (2.3–16.5)	0.4 ^{abc} (0.2–1.0)
ACTH, pg/ml	<58	100 ^a (68–100)	8 ^g (3–19)	0.54 (0.41–0.68)	1.1 ^d (1.0–1.2)	0.0 (0.0–13.1)
Adiposity measurements						
Body condition score	≥7	100 ^a (68–100)	29 ^f (18–42)	0.64 ^b (0.51–0.77)	1.4 ^c (1.2–1.7)	0.0 (0.0–3.3)
Girth:height ratio	>1.30	88 ^{ab} (53–98)	37 ^f (25–51)	0.62 (0.48–0.74)	1.4 ^{cd} (1.0–1.9)	0.3 ^{abc} (1.0–2.2)
Cresty neck score	≥4	50 ^{bc} (22–78)	80 ^{de} (66–89)	0.65 ^b (0.45–0.84)	2.5 ^{abc} (1.0–5.9)	0.6 ^{abc} (0.3–1.3)
NC:height ratio	>0.71	63 ^{abc} (31–86)	67 ^e (53–79)	0.65 ^b (0.51–0.77)	1.9 ^{bc} (1.0–3.7)	0.6 ^{abc} (0.2–1.4)
Mean blood pressure, mmHg[#]	<76	14 ^{cd} (3–51)	52 ^f (33–71)	0.33 (0.17–0.53)	0.3 ^e (0.1–1.1)	3.1 ^e (1.6–5.8)
Combined criteria						
Original PLMS	Treiber <i>et al.</i> 2006 [†]	100 ^a (68–100)	43 ^f (30–57)	0.71 ^b (0.58–0.83)	1.8 ^c (1.4–2.2)	0.0 (0.0–2.2)
Modified PLMS	New cut-offs [‡]	25 ^{cd} (7–59)	100 ^a (92–100)	0.63 ^b (0.49–0.76)	∞ (1.1–∞)	0.8 ^{bc} (0.5–1.1)
New Cluster	New tests [§]	86 ^{ab} (49–97)	87 ^{cd} (74–94)	0.87 ^a (0.75–0.95)	6.6 ^a (2.9–14.7)	0.2 ^{ab} (0.0–1.0)

^{a–f}Comparisons between variables within each diagnostic statistic. Values in the same column with different superscripts differ (P<0.05) between variables, with “a” representing the variables with the greatest value for each statistic. Only IROC values greater (P<0.05) than 0.50 or LR greater (P<0.05) than 0 were considered in comparisons. [†]Surpass 3 of 4 criteria: BCS >6, TG >560 mg/l, RISQI <0.32, MIRG >5.6; [‡]Surpass 3 of 4 criteria: BCS ≥7 and CNS ≥4, TG >940 mg/l, RISQI <0.17, MIRG >11.3; [§]Surpass 3 of 4 criteria: BCS ≥7, CNS ≥4, insulin >32 mu/l, leptin >7.3 ng/ml #n = 30.

all CL ponies displayed both generalised and localised obesity. In horses, generalised obesity has been linked to insulin resistance and inflammation (Hoffman *et al.* 2003; Vick *et al.* 2007), although researchers are only beginning to explore the possibility of a high-risk fat depot located along the crest of the neck (Frank *et al.* 2006; Bailey *et al.* 2008).

Laminitic episodes observed in the ponies in the present study were highly associated with the presence of obesity and obesity-related factors, including high BCS, high CNS, and elevated insulin and leptin concentrations. It is hypothesised that, in this population, a chronic state of overnutrition and/or reduced physical activity contributed to the development of obesity (generalised and localised) which then contributed to an insulin resistant state with concurrent hyperinsulinaemia (Hoffman *et al.* 2003; Vick *et al.* 2007). Therefore, it is suggested that the tests presented here are most applicable to cases where an obesity-associated change in metabolism has increased the risk of laminitis. It is uncertain whether these tests would predict laminitis when obesity is not present. Additionally, the data in the present study were drawn from a closed herd of inbred ponies and further studies are therefore needed to determine the generality of results to wider populations (Carter *et al.* 2006).

Insulin concentration had reproducibly high diagnostic accuracy as a continuous variable and with a cut-off value >32 mu/l in *Evaluations 1* and *2*. Low insulin sensitivity and/or high insulin concentrations have been associated with laminitic predisposition in previous studies (Coffman and Colles 1983; Jeffcott *et al.* 1986; Treiber *et al.* 2006, 2007; Bailey *et al.* 2007, 2008), and experimental induction of continuous hyperinsulinaemia (>1000 mu/l) while maintaining euglycaemia induced laminitis in healthy ponies (Asplin *et al.* 2007). Although hyperinsulinaemia effectively predicted laminitis in the current study, dependence on a single factor, rather than a combination of multiple risk factors, may limit sensitivity or generality. Insulin concentration can vary substantially with changes in dietary composition that occur seasonally and diurnally, as observed in grazing horses (McIntosh *et al.* 2007). Additionally, a recent study by Bailey *et al.* (2007) demonstrated that basal insulin concentrations were not consistently different between NL and PL mixed-breed ponies, especially when fed a low glycaemic diet. A more reliable identifier of the laminitis-predisposed phenotype was insulinaemic response to a challenge of i.v. glucose, oral inulin or i.m. dexamethasone. Dynamic tests, or measuring insulin in response to a stimulus, could facilitate prediction of a pony's response to stressors that may induce laminitis, including changes in pasture carbohydrate levels. However, even dynamic tests of insulin sensitivity display inter-day variability, with coefficients of variation of 24–33% reported for insulin-modified frequently sampled i.v. glucose tolerance tests (Pratt *et al.* 2005; Frank *et al.* 2008).

Leptin concentration had high diagnostic accuracy as a continuous variable, with a cut-off value >7.3 ng/ml in *Evaluations 1* and *2*. Leptin is primarily produced and secreted from adipose tissue at levels that are proportional to body condition (Buff *et al.* 2002); and its secretion is stimulated by insulin and inversely related to insulin sensitivity in obese horses (Cartmill *et al.* 2003, 2005; Frank *et al.* 2006). Additionally, obesity is related to a state of relative insulin resistance (Hoffman *et al.* 2003; Vick *et al.* 2007), with adipose tissue deposited specifically along the crest of the neck associated with insulin concentration or glucose tolerance (Frank *et al.* 2006; Carter *et al.* 2008). Although previous studies have demonstrated that basal

insulin concentration is a useful indicator of insulin sensitivity (Treiber *et al.* 2005; Frank *et al.* 2006), measurement of variables associated with insulin sensitivity that do not fluctuate as acutely as insulin concentration, such as leptin, BCS or CNS, could complement the prediction of laminitis.

Although a predisposition to develop laminitis may be genetically or physiologically determined, actual development of laminitis is dependent on interactions between the animal and its environment. Evaluations of the current study were performed in March, presumably before environmental stressors, such as changes in pasture composition, affect metabolism. Although sampling at this time of year provides the greatest opportunity to implement countermeasures to avoid laminitis, repeating evaluation under various environmental conditions would provide a broader perspective of the factors contributing to laminitis.

In summary, using a cohort of ponies from a single inbred herd, tests of insulin and leptin concentrations as well as measures of generalised (BCS) and localised (CNS or NC:height ratio) obesity were beneficial in the prediction of laminitic episodes resulting from exposure to nutrient dense pasture with relatively high levels of nonstructural carbohydrates within 3 months of evaluation. These results highlight the importance of monitoring and reducing insulin concentration and obesity in ponies in order to improve metabolic health and therefore reduce laminitis risk.

Acknowledgements

Research was supported by the Bernice Barbour Foundation, the estate of the late Paul Mellon, the Virginia Horse Industry Board, the WALTHAM Centre for Pet Nutrition, and the John Lee Pratt Graduate Fellowship Program in Animal Nutrition at Virginia Tech. We are grateful to Dr David Kronfeld for intellectual contribution and study initiation. Other contributors to this work include Burt Staniar, Tania Cubitt, Lindsey George, Louisa Gay, Tracy Smith, Kate Myers, Lindsey Williamson and Matt Utt. A special thanks to ponies and their owners and managers.

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- ²MIKRON Instrument Company, Oakland, New Jersey, USA.
- ³Beckman Coulter Inc, Fullerton, California, USA.
- ⁴Diagnostic Products Corporation, Los Angeles, California, USA.
- ⁵Linco Research Inc, St Charles, Missouri, USA.
- ⁶DiaSorin, Stillwater, Minnesota, USA.
- ⁷Endogen, Rockford, Illinois, USA.
- ⁸Animal Health Diagnostic Center, Ithaca, New York, USA.
- ⁹Siemens Medical Solutions Diagnostics, Los Angeles, California, USA.
- ¹⁰Stata-Corp, College Station, Texas, USA.

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Author contributions The initiation, conception, planning and writing of this study were by R.A.C., K.H.T., R.J.G. and P.A.H. Pathology by R.J.G. Execution by R.A.C., K.H.T. and R.J.G. Statistics by R.A.C. and L.D.