Prospective cohort study evaluating risk factors for the development of pasture-associated laminitis in the UK

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Keywords (6): horse, laminitis, insulin, adiponectin, IGF-1, dexamethasone

Word Count: 3861

Ethical considerations: This study follows international, national, and/or institutional guidelines for humane animal treatment and complies with relevant legislation in the country in which the study was conducted. It was approved by the ethics review committee at the institution at which the studies were conducted and was performed under the UK Veterinary Surgeons Act. Informed owner consent was obtained for all animals included in the study.

Competing Interests: Professor Harris is employed by one of the study funders.

Source of Funding: PetPlan Charitable Trust, British Veterinary Association Animal Welfare Foundation Norman Hayward Fund, Laminitis Trust and WALTHAM Centre for Pet Nutrition

Acknowledgements: Some of this data was presented at the European College of Equine Internal Medicine Annual Congress 2015. The authors would like to thank Miss Katya Potter for her help in collecting data.

Authorship: The study was designed by Dr Menzies-Gow and Professors Elliott and Harris. Dr Menzies-Gow executed the study. Data analysis and interpretation, and preparation of the manuscript were undertaken by Dr Menzies-Gow and Professors Elliott and Harris.

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Summary

Background: Certain individuals appear predisposed to recurrent pasture-associated laminitis.

Reasons for performing the study: Previous studies have predominantly investigated risk factors only after disease occurrence.

Objectives: To investigate pasture-associated laminitis risk factors prior to disease occurrence.

Study design: Prospective cohort study.

Methods: Non-laminitic ponies ≥7 years old were recruited. Body condition score (BCS), height, weight, crest height and thickness were measured and an overnight dexamethasone suppression test performed. Plasma/serum adiponectin, leptin, triglyceride, basal insulin, insulin post dexamethasone, insulin-like growth factor (IGF)-1, IGF binding protein (IGFBP)-1, IGFBP-3, C-reactive protein, von Willebrand's factor (vWF), soluble (s) E-selectin and p-selectin concentrations were assayed. Follow-up was obtained from owners annually for 3 years to ascertain occurrence of veterinary-diagnosed pasture-associated laminitis. Data were analysed by multivariate logistic regression. ROC curves analysis was performed for significant risk factors and cut off values determined.

Results: 446 animals were recruited; the median (interquartile range) age was 15 (10, 20) years; 50.4% were mares and 49.6% geldings; the most common breeds were Welsh (36%), Shetland (17%) and cob (9%); 72.2% were overweight/obese (BCS 7-9/9), 27.3% ideal weight (BCS 4-6/9) and 0.5% underweight (BCS 1-3/9). After 1, 2 and 3 years, 18 (4%), 30 (7%) and 44/446 (10%) animals had had laminitis. Plasma/serum [adiponectin],basal [insulin] and [insulin] post dexamethasone were significantly ($p \leq 0.05$) associated with laminitis occurrence cumulatively after 1, 2 and 3 years. The accuracy to separate animals who did or did not develop laminitis determined using the area under the ROC curves was good (basal [insulin] after 1 year), fair (all others) or poor ([insulin] post dexamethasone).

Main limitations: Animals were evaluated at a single time point and biomarkers were assayed using single assays.

Conclusions: Risk factors for future laminitis prior to disease occurrence include low plasma adiponectin and high serum basal insulin or insulin post dexamethasone concentrations.

Introduction

A metabolic phenotype with similarities to human metabolic syndrome (HMS), including insulin dysregulation (ID) [1], dyslipidaemia [2-4] and altered circulating adipokine concentrations [5-7] with or without obesity appears to be associated with an increased risk of laminitis [8; 9]. Thus the same pathologic mechanisms that underlie the cardiovascular diseases associated with HMS, including changes in insulin signalling, inflammatory cytokines and endothelial dysfunction, could contribute to laminitis risk. Multiple variables have been evaluated previously as laminitis risk factors [2-4]; [10], but these studies have evaluated animals after disease occurrence and differences detected could reflect the disease rather than a predisposition. Identifying risk factors prior to disease occurrence would allow targeting of preventive management strategies. Potential risk factors requiring investigation include obesity, ID, inflammatory cytokines and markers of endothelial dysfunction.

Intravenous infusion methods for ID identification are not practical in the field [11]. The dexamethasone suppression test is a potential dynamic test for ID as exogenous cortisol analogues antagonise insulin's actions resulting in increased endogenous insulin secretion. Previously laminitic ponies (PLP) had a greater increase in insulin concentration post dexamethasone compared to controls [12] in spring and summer [13]. Concentrations of the anti-inflammatory marker adiponectin were significantly lower and plasma triglyceride concentrations were significantly higher in PLP in late spring and winter [4].

Leptin is an adipose-derived hormone [14]; hyperleptinaemia in humans is associated with HMS, ID, vascular inflammation [15] and endothelial dysfunction [16]. Insulin-like growth factor (IGF)-1 has insulin-like metabolic actions and lower IGF-1 concentrations are associated with human obesity, ID and HMS [17]. IGF binding proteins (IGFBPs) represent an important link between the insulin and IGF systems and play important roles in human obesity and HMS [18]. Abnormal IGFBP expression is a sensitive marker of ID, used to identify individual humans with ID at high cardiovascular risk and as an early marker of HMS [18]. None of these factors/parameters has been assessed as predictors of laminitis development prospectively.

The current gold standard non-invasive test of assessing endothelial function using endothelial-dependent vasomotion in humans is flow mediated dilatation, however it is not suitable for equine use due to a lack of accuracy or precision [19]. Additionally, various circulating molecules are used as biomarkers of endothelial dysfunction including von Willebrand's factor (vWF) [20], soluble (s)E-selectin [21] and P-selectin [22]. Thus it would be logical to evaluate these in animals prior to laminitis occurrence.

The study aim was to investigate prospectively certain risk factors for the development of pasture-associated laminitis in a cohort of animals with no known history of laminitis, including morphometric measures of obesity and circulating concentrations of biomarkers of insulin dysregulation, adipokines and endothelial dysfunction.

Materials and Methods

The study was approved by Royal Veterinary College Ethics and Welfare Committee and was performed under the UK Veterinary Surgeons Act 1966. So that the dexamethasone suppression test was performed for the benefit of the individual animal as a test for pituitary pars intermedia

dysfunction (PPID) and due to the fact that the youngest age of a case of PPID in the scientific literature is 7 years [23; 24], only animals ≥7 years old could be included in the study.

Animals

Sample size calculations assuming 80% power and 95% confidence indicated that 200-700 animals were required to detect a 5- to 10-fold increase in laminitis risk in ID compared to non-ID animals (assuming 2% laminitis risk over 2 years in non-ID animals and exposed:unexposed ratio of 1:10). Thus the prospective cohort study aimed to recruit approximately 400 client-owned ponies ≥7 years old with no previous known history of laminitis to the study. Animals were not cases nor were they recruited via veterinary practices or referral hospitals. Instead they were healthy animals recruited to the study directly from owners in response to posts on social media sites, requests placed in equine lay magazines and letters sent to all equine charities and riding/livery establishments within a 50 mile radius of the Royal Veterinary College by the study organiser. All suitable animals were included in the study and there was no randomisation. A standard clinical examination of each animal was performed by a single experience equine veterinarian and ponies were excluded if they displayed clinical signs of acute, chronic or previous laminitis (including lameness affecting 2 or more feet, increased digital pulses, characteristic stance of leaning back on the heels, divergent hoof growth rings) or pituitary pars intermedia (PPID; including hypertrichosis) at the time of recruitment to the study. In addition, ponies were excluded from the study if they were subsequently identified as having an abnormal cortisol response to the dexamethasone suggestive of PPID. The study was fully explained to the owners, a factsheet provided and informed consent obtained. Historical information was obtained by direct questioning of the owner. All animals were evaluated and samples collected in summer (August).

Weight, condition score and crest measurements

A measuring stick was used to measure the height of the ponies at the withers and the weight of the ponies was estimated using a weigh tape[a] around the girth. Body condition score (BCS) was assessed by a single experienced equine veterinarian giving a grade on a scale of 1 to 9 [25] over 6 areas of the body and then calculating the mean. Callipers were used to measure the height and thickness of the crest of the neck above the nuchal ligament at the point half way between the poll and the withers.

Overnight dexamethasone suppression test

Blood samples were collected by jugular venepuncture before and 19 hours after the intramuscular administration of dexamethasone[b] (44 μg/kg).

Blood sample collection

Jugular venous blood samples were collected into EDTA and heparinised vacutainer tubes[c] for plasma separation and into plain vacutainer tubes for serum preparation. Plasma tubes were kept in ice until centrifugation at 3,000 x g for 10 minutes. Plasma was harvested, aliquoted and frozen at -80°C until analysed. Serum tubes were incubated in a 37°C water bath for 30 minutes, centrifuged, aliquoted and frozen at -80°C.

Mediator Analysis

Plasma adiponectin[d] [26] and leptin[e] [26] and serum insulin[f] [27] concentrations were measured using radioimmunoassays. Plasma total IGF-1,[g] IGFBP-1,[h] IGFBP-3,[i] C-reactive protein[j] [28], P-selectin[k] and sE-selectin[l] concentrations were measured using ELISAs. Plasma triglyceride concentrations were

measured by the pathology laboratory at Bell Equine Veterinary Clinic, Kent, UK and plasma vWF antigen concentrations were measured by a commercial laboratory[m]. All of these methods have been previously validated for use in the horse apart from IGF-1, IGFBP-1, IGFBP-3, sE-selectin and P-selectin. Thus these assays were validated by determining intra- and inter-assay variability (to assess precision and repeatability; using pooled samples from 5 animals measured three times), dilutional parallelism (using samples from 5 animals diluted 1 in 2, 1 in 4 and 1 in 8 within the working range of the assay) and spiked recovery (to assess specificity; using the assay standard diluted in equine plasma).

Follow up

Owners of animals recruited to the study were contacted after 12, 24 and 36 months in order to ascertain whether individual animals had suffered from pasture-associated laminitis diagnosed by a veterinarian in the preceding 12 months.

Data Analysis

Data were analysed using the SPSS software program[n]. Univariable logistic regression was first used to assess risk factors including morphometric data and blood analytes associated with the outcome (namely laminitic after 1 year or not; laminitic after 2 years or not; laminitic after 3 years or not) individually. Correlations between those risk factors with $p<0.1$ were tested by calculating Pearson's correlation coefficient. Interactions between risk factors were tested by calculating A*B for significant risk factors and entering risk factors A and B and A*B into a multivariable logistic regression model. If the p value for the interaction term A*B was >0.05, there was not significant interaction between risk factors A and B. Risk factors with $p<0.1$ were then entered into a multivariable logistic regression and any risk factors with $p>0.05$ sequentially removed until all the risk factors had $p\leq0.05$ in the final

model. ROC curves were constructed for those risk factors remaining in the final model. The accuracy of the test to separate animals into those which did or did not subsequently develop laminitis was determined by calculating the area under the curve (AUC) whereby an area of 0.90-1 is excellent, 0.80-0.90 good, 0.70-0.80 fair, 0.60-0.70 poor and 0.50-.60 fail [29]. In addition, the co-ordinates of the ROC curve were then used to determine the cut off value which maximised specificity and sensitivity and the corresponding positive (PPV) and negative predictive values (NPV) using these cut-off values were calculated.

Results

Four hundred and forty six ponies aged (median [interquartile range]) 15 (10, 20) years were recruited to the study. Of these 50.4% were mares and 49.6% were geldings. A range of pony breeds were represented including Welsh (36.4%), Shetland (17%), cob (9.4%), New Forest (9%), crossbreed (7.6%) and other (20.6%). The majority (72.2%) of animals were overweight or obese (BCS 7-9), with 27.3% being ideal weight (BCS 4-6) and 0.5% being underweight (BCS 1-3).

After 1, 2 and 3 years cumulatively 18 (4%), 30 (7%) and 44 (10%) had developed pasture-associated laminitis, 416 (93%), 374 (84%) and 348 (78%) remained non laminitic, and 12 (3%), 42 (9%) and 54 (12%) had been euthanased for reasons other than laminitis, respectively.

Values for crest height, crest thickness, body condition score, plasma/serum concentrations of biomarkers and serum insulin response to an overnight dexamethasone suppression test in all animals and those that subsequently developed laminitis and those that remained non laminitic after 1, 2 and 3 years are shown in Table 1.

The validation of the ELISAs used to measured plasma IGF-1, IGFBP-1, IGFBP-3, sE-selectin and P-selectin concentrations revealed that they performed satisfactorily (Online supplementary information, table 1).

Most of the pairwise correlations were weak (Pearson's correlation coefficient <0.2) apart from basal insulin and insulin post dexamethasone (Pearson's correlation coefficient 0.68). The only significant interaction between risk factors was between basal insulin and post dexamethasone insulin concentration after 2 ($p=0.03$) and 3 years ($p=0.02$).

For the data obtained after 1 year, the risk factors taken into the multivariate regression analysis included plasma/serum adiponectin, basal insulin, insulin post dexamethasone and p-selectin concentrations. Apart from p-selectin, these all proved to be significant risk factors for the development of laminitis and so remained in the multivariate analysis (Table 2).

For the data obtained after 2 years, the risk factors taken into the multivariate regression analysis included plasma/serum CRP, adiponectin, IGF-1, basal insulin and insulin post dexamethasone concentrations. Those that proved to be significant risk factors for the development of laminitis and so remained in the multivariate analysis were plasma adiponectin, plasma IGF-1, serum basal insulin and serum insulin post dexamethasone concentrations (table 2).

For the data obtained after 3 years, the risk factors taken into the multivariate regression analysis included plasma/serum adiponectin, basal insulin, insulin post dexamethasone and IGFBP-3 concentrations. Apart from IGFBP-3, these all proved to be significant risk factors for the development of laminitis and so remained in the multivariate analysis (Table 2)

None of the morphometric data proved to be significant risk factors for the development of laminitis in 1, 2 or 3 years.

The ROC AUC and the cut off values which maximised the specificity and the sensitivity, as well as the corresponding PPV and NPV are shown in Table 3. There was no improvement in these values when variables were combined.

Discussion

Consistent risk factors for the future development of laminitis in animals prior to disease occurrence in the present study included low plasma adiponectin and high basal and post dexamethasone serum insulin concentrations.

The hormone adiponectin has anti-diabetic, anti-atherogenic and anti-inflammatory properties in humans and rodents [30; 31] and circulating concentrations are decreased in obese individuals and in patients with HMS [32], type 2 diabetes [30] and cardiovascular disease [33]. Despite being specifically secreted by adipocytes, a strong negative correlation exists between adiponectin concentrations and the BMI of human patients [34; 35] with a similar observation made in horses [26]. Previously laminitic

208 ponies have been shown to have significantly lower plasma adiponectin concentrations compared to
209 non-laminitic ponies irrespective of season [4]. Lower plasma adiponectin concentrations may
210 promote a decreased anti-inflammatory capacity in previously affected horses. We report for the first
211 time that lower adiponectin concentrations occur in animals prior to clinical signs of laminitis
212 indicating that hypoadiponectinaemia may be a risk factor for laminitis rather than solely a
213 consequence of the disease. ROC curve analysis revealed that the accuracy of adiponectin
214 concentrations to separate animals into those which do or do not go on to develop laminitis in 1, 2 or
215 3 years was fair and a cut of value of 2.50μg/ml gave acceptable sensitivity (78%) and specificity (79%)
216 values.
217
218 The link between hypoadiponectinaemia and laminitis risk in the present study was not due to obesity
219 as obesity was not a risk factor of laminitis and there was no correlation between plasma adiponectin
220 concentrations and morphometric measures of obesity. In other species, adiponectin promotes
221 vasorelaxation through increased vascular expression of endothelial nitric oxide (NO) synthase and
222 prostacylin (PGI_2) synthase [36] and via opening of smooth muscle cell K^+ channels [37]. Thus, low
223 adiponectin concentrations may increase the risk of future laminitis through decreased vasorelaxation
224 of the equine digital vasculature. Alternatively, there is evidence in humans that there is cross-talk
225 between adiponectin and both the insulin (InsR) and IGF-1 (IGF-1R) receptors [38]. Adiponectin in
226 association with insulin is able to induce activation of InsR and IGF-1R and activate the downstream
227 intracellular signalling pathways [38]. Supraphysiologic hyperinsulinaemia causes laminitis in healthy
228 equids [39]; at high concentrations insulin can bind to and activate InsR, IGF-1R and InsR/IGF-1R [40];
229 and IGF-1R and InsR have been detected in lamellar epithelial and endothelial cells respectively [41].
230 Thus, hypoadiponectinaemia and hyperinsulinaemia could potentially combine to alter lamellar
231 epithelial and endothelial InsR and IGF-1R expression resulting in epithelial proliferation and
232 endothelial dysfunction and consequent laminitis. These hypotheses require further investigation.

The link between laminitis and insulin dysfunction has been investigated and hyperinsulinaemia and/or insulin dysregulation has been reported in previously laminitic animals in a number of studies [2; 3; 42]. Hyperinsulinaemia and insulin dysregulation may predispose to laminitis by triggering disturbances in vascular function through downregulation of the phosphatidylinositol 3-kinase (PI-3K) pathway and hence reduction in production of the vasodilator nitric oxide (NO) in the face of continued vasoconstrictor production [43]. Alternatively, as previously discussed, insulin may be excessively stimulating lamellar IGF-1 receptors, leading to inappropriate epithelial cell proliferation with lamellar weakening and consequent laminitis [44]. Previously, serum basal insulin concentrations >32μiu/ml had good sensitivity (100%) and specificity (80%) for predicting clinical laminitis in the following 3 months in previously laminitic animals [6]. Similarly, in the present study increased serum insulin concentrations in non-laminitic animals was significantly associated with the subsequent development of laminitis. ROC curve analysis revealed that the accuracy of serum basal insulin concentration to separate animals into those which do or do not go on to develop laminitis cumulatively in 1, 2 or 3 years was good (after 2 years) to fair (after 1 and 3 years) and a cut of value of 21.8μiu/ml gave acceptable sensitivity (78%) and specificity (67%) values. This value is virtually identical to that proposed as a value above which is consistent with insulin dysregulation previously [9]. However it must be acknowledged that animals in the present study were not fasted prior to blood sampling.

Single measurements of serum insulin concentration are affected by a number of factors including diet, exercise, stress and time of day [11; 45]. Thus dynamic tests of endocrine function are often advocated to detect ID. The dexamethasone suppression test (DST) is a potential dynamic test for ID as exogenous cortisol analogues antagonise insulin resulting in increased endogenous insulin

secretion. Previously laminitic ponies had a greater increase in serum insulin post dexamethasone compared to control ponies [12] which was seen only in spring and summer [13] and a cut off value of 75μiu/ml was suggested to distinguish groups of previously laminitic animals from controls. Similarly in the present study, an exaggerated insulin response to dexamethasone was associated with the subsequent development of laminitis in non laminitic ponies. ROC curve analysis revealed that the accuracy of serum insulin post dexamethasone to separate animals into those which do or do not go on to develop laminitis cumulatively in 1, 2 or 3 years was fair to poor and a cut of value of 105.6μiu/ml gave fair sensitivity (69%) and specificity (68%) values for the development of laminitis in the next 12 months. It should be acknowledged that the study was designed prior to the recent increase in popularity of oral sugar or glucose tests as a dynamic test of ID. To the authors' knowledge the insulin response to dexamethasone has not been directly compared to the OGT or OST, thus it is not possible to extrapolate these results to those tests.

Insulin-like growth factor (IGF)-1 is primarily produced in the liver from growth hormone metabolism prior to secretion into the circulation. It has short-term insulin-like metabolic actions and long-term growth factor-like effects on cell proliferation and differentiation. Lower IGF-1 concentrations are associated in other species with obesity, insulin resistance, HMS [17], type 2 diabetes [46] and increased risk of cardiovascular disease [47]; however the precise mechanism(s) behind these apparent inverse relationships remains elusive. The median plasma IGF-1 concentrations in those animals that remained non-laminitic in the present study were similar to those previously reported in adult horses [48; 49]; whilst the median plasma IGF-1 concentrations in those ponies that subsequently developed laminitis after 2 years was significantly lower. Plasma IGF-1 concentrations have not been measured in previously laminitic ponies; however season and body condition score have been shown to have an effect in other populations of horses [50; 51]. Plasma IGF-1 concentrations were significantly higher in the summer compared to the winter [50] and in overweight

compared to underweight mares [51]. In the present study, samples were collected from all of the ponies at the same time of the year (August) and body condition score was not a risk factor for laminitis development. Whilst in people, IGF-1 concentrations have been reported to decrease with age [52], there is no evidence for aging being a factor in changes of IGF-1 in adult horses [48] or in the present study population. Thus it would appear that for unknown reasons low IGF-1 concentrations were only associated with an increased risk of developing laminitis after 2 years and not after 1 or 3 years suggesting that IGF-1 concentration is not directly associated with an increased risk.

There are six IGF binding proteins (IGFBPs) which link with IGF-1 and -2 and prevent them from being degraded; they also facilitate IGF transport through body compartments. The interaction between IGFs and their specific receptors is partly regulated by structural modifications inherent to the IGFBPs. Whilst IGFBP expression has been suggested to be useful as a sensitive marker of ID, to identify individuals with ID at high cardiovascular risk and as an early marker of HMS [18], concentrations of IGFBP-1 or IGFBP-3 were not useful in the detection of animals at increased risk of development of future laminitis.

Leptin is an adipose tissue derived hormone [14] and hyperleptinaemia is associated with HMS, insulin resistance, vascular inflammation [15] and endothelial dysfunction [16]. In horses and ponies, hyperleptinaemia is associated with hyperinsulinaemia [53], obesity [54] and previous laminitis in some [6], but not all studies [7]. In addition, hyperleptinaemia could be used to predict clinical laminitis in the following 3 months in previously laminitic animals [6]. However, whilst there was a weak positive correlation between plasma leptin concentration and BCS ($p=0.03$, $r=0.14$), no such correlation with serum insulin concentration was found and there was no association between plasma leptin concentrations and subsequent development of laminitis was apparent in the present study.

Increased plasma triglyceride concentrations are associated with hyperinsulinaemia [53], obesity [54] and previous laminitis [2-4]. However in agreement with the present study, they were not beneficial in the prediction of clinical laminitis in the following 3 months in previously laminitic animals [6].

C-reactive protein (CRP) is an acute-phase protein and increased concentrations are associated with insulin resistance [55], HMS [56; 57] and cardiovascular disease [58]. In horses, an increase in CRP concentration has been reported in induced inflammation and laminitis, pneumonia, enteritis, arthritis and after castration [59]. Other studies, however, reported that serum CRP concentration was not affected by inflammatory disease [60; 61]. CRP concentrations have not been evaluated in association with naturally occurring laminitis, but CRP concentrations were not significantly different between control animals and hyperinsulinaemic obese horses [62]. The concentrations found in the present study were similar to those reported in healthy horses in one study [28], but lower than those reported in another [62] and they were not found to be a significant risk factor for the subsequent development of laminitis.

Equine plasma vWF antigen concentrations have only been previously measured in association with exercise [20] or as part of the investigation of clotting disorders [63; 64]. In humans, vWF concentrations are increased in obesity [65], HMS [66] and insulin resistant patients [67]. However plasma vWF antigen concentrations could not be used to predict the development of HMS in patients with hypertension [68]. P-selectin and sE-selectin are markers of endothelial dysfunction in other species [21; 22] and the role of endothelial dysfunction is important in HMS and ID and the development of associated cardiovascular diseases [69]. Thus, it is logical to postulate that endothelial dysfunction may play a role in the pathogenesis of laminitis associated with ID. However, none of the

biomarkers of endothelial dysfunction measured were associated with the subsequent development of laminitis in the present study.

Whilst an association between previous laminitis and generalised and/or regional adiposity has been reported [2; 6; 70] and generalised and/or regional adiposity could be used to predict clinical laminitis in the following 3 months in previously laminitic animals [6], surprisingly no such association with future laminitis was found in the present study. It should be acknowledged that the majority (72%) of ponies in the study were overweight or obese, which is similar to previously reported figures of obesity within the UK pony population in some studies [71], but much greater than those in other studies evaluating both horses and ponies (30-45%) [72-75]. This high prevalence of obesity may have resulted in it not being a discernible risk factor within the population studied; alternatively obesity alone may not a significant risk factor for the future development of laminitis.

The main limitation of the present study was that animals were only examined at a single point in time. There is no current evidence for the longevity or stability of these biomarkers in horses and it is possible that both these and the morphometric data values changed considerably during the 3 years over which the animals were subsequently followed which could in turn have had a significant impact on the laminitis risk.

In conclusion, risk factors for the development of laminitis in previously non-laminitic animals in the present study included low plasma adiponectin as well as high basal insulin and serum insulin post dexamethasone concentrations. The accuracy of these to separate animals who did or did not develop laminitis after 1, 2 or 3 years was good (serum basal [insulin] after 1 year), fair (all others) or poor

(serum [insulin] post dexamethasone) and cut off values with acceptable sensitivities and specificities were generated. Combinations of these biomarkers did not improve their predictive value. However it should be acknowledged that these cut-off values were generated using samples obtained at a single time of the year (summer) and measured using single assays (radioimmunoassay) and radiography was not performed so that it is possible that animals with pre-existing subclinical laminitis were included. Surprisingly, the development of laminitis was not associated with regional or generalised obesity, hyperleptinaemia or hypertriglyceridaemia. In addition there was no association with circulating CRP, IGF-1, IGFBP-1, IGFBP-3, sE-selectin, p-selectin or vWF antigen concentrations. Further prospective cohort studies that examine animals more frequently such that their morphometric and metabolic variables are determined within a shorter time frame in relation to the onset of laminitis or that include a much larger number of animals are warranted to assess these risk factors further.

Manufacturers' Details

[a] Weigh tape, Equi Life Ltd, Mead House, Dauntsey, Chippenham, Wilts. UK

[b] Colvasone, Norbrook Laboratories (GB) Ltd, Carlisle, UK

[c] Vacutainer, Becton-Dickinson Ltd, Oxford, UK

[d] Adiponectin RIA kit, Merck Millipore, Missouri

[e] Multi-species leptin RIA kit, Merck Millipore, Missouri

[f] Coat-a-count insulin assay, Diagnostic Products Corp, Los Angeles, California

[g] Human IGF-1ELISA, Mediagnost, Reutlingen, Germany

[h] Equine IGFBP-1 ELISA, BlueGene Biotech, Shanghai, China

[i] Equine IGFBP-3 ELISA, BlueGene Biotech, Shanghai, China

[j] Horse CRP ELISA, Kamita Biomedical Company, Seattle, WA

[k] Equine P-selectin ELISA, BlueGene Biotech, Shanghai, China

[l] Equine sE-selectin ELISA, BlueGene Biotech, Shanghai, China

[m]Animal Health Center Laboratory, Cornell University, Ithaca, NY

[n] SPSS Statistics, IBM Corporation, New York

Table 1

Median (interquartile range) or mean + SD values for morphometric and metabolic variables measured in 446 non-laminitic ponies that either remained non-laminitic or developed laminitis in the following 1, 2 or 3 years. Data were analysed using multivariate logistic regression and significance accepted at $p<0.05$

Variable	All ponies n=446	Laminitic after 1 year n = 18	Non laminitic after 1 year n = 428	P value	Laminitic after 2 years n=30	Non laminitic after 2 years n=416	P value	Laminitic after 3 years n=44	Non laminitic after 3 years n=402	P value
Crest height (cm)	5.1 (3.9, 6.2)	5.1 (3.9, 6.1)	4.9 (3.9, 6.8)	0.38	5.1 (3.9, 6.1)	5.2 (4.0, 7.6)	0.49	5.1 (3.9, 6.1)	4.9 (4.0, 6.9)	0.72
Crest thickness (cm)	4.6 $\pm$ 1.1	4.9 $\pm$ 0.8	4.6 $\pm$ 1.1	0.36	5.0 $\pm$ 1.4	4.6 $\pm$ 1.1	0.54	4.9 $\pm$ 1.3	4.6 $\pm$ 1.1	0.58
Body condition score	8 (6, 8)	8 (6, 8.25)	8 (6, 8)	0.74	8 (6, 8)	8 (6, 8)	0.73	7.5 (6, 8)	8 (6, 8)	0.38
Adiponectin (μg/ml)	3.72 (2.55, 5.06)	1.78 (1.39, 3.0)	3.77 (2.62, 5.06)	0.04	1.59 (1.29, 2.49)	3.87 (2.71, 5.07)	0.004	2.15 (1.42, 4.53)	3.86 (2.71, 5.06)	0.035
Leptin (ng/ml HE)	3.39 (1.98, 6.01)	2.80 (1.78, 7.90)	3.41 (1.98, 5.82)	0.32	3.93 (1.98, 6.34)	3.43 (1.98, 5.82)	0.51	3.60 (1.98, 4.86)	3.39 (1.98, 6.06)	0.74
Triglyceride (mmol/l)	0.28 (0.19, 0.44)	0.52 (0.33, 0.63)	0.27 (0.19, 0.43)	0.35	0.48 (0.21, 0.56)	0.27 (0.19, 0.43)	0.49	0.40 (0.21, 0.52)	0.28 (0.19, 0.43)	0.98

Basal insulin (µIU/ml)	11.9 (6.6, 30.6)	68.4 (20.2, 246.0)	11.6 (6.6, 29.77)	<0.001	68.4 (21.9, 127.2)	11.0 (6.6, 27.9)	<0.001	42.8 (21.2, 96.2)	10.8 (6.6, 24.6)	<0.001
Insulin post dexamethasone (µIU/ml)	46.9 (20.6, 146.6)	194.0 (39.6, 418.5)	46.4 (20.6, 142.1)	0.02	269.9 (60.3, 390.1)	43.9 (19.8, 124.5)	0.002	192.2 (55.3, 390.1)	42.5 (18.4, 121.2)	<0.001
CRP (µg/ml)	10.66 (0.41, 25.97)	6.16 (2.85, 15.21)	10.82 (0.23, 26.26)	0.22	6.16 (1.70, 20.57)	10.89 (0.23, 26.26)	0.10	8.78 (1.69, 34.13)	10.80 (0.23, 25.28)	0.41
IGF-1 (ng/ml)	225.8 $\pm$ 68.9	207.5 $\pm$ 71.7	226.6 $\pm$ 68.7	0.25	202.3 $\pm$ 72.9	227.5 $\pm$ 68.4	0.033	230.0 $\pm$ 74.9	225.4 $\pm$ 68.3	0.67
IGFBP-1 (ng/ml)	9.70 (4.18, 25.23)	10.42 (4.08, 23.03)	9.59 (4.29, 25.41)	0.33	10.42 (3.95, 35.84)	9.59 (4.29, 25.14)	0.48	10.27 (3.44, 35.89)	9.59 (4.29, 24.88)	0.27
IGFBP-3 (ng/ml)	25.48 (10.91, 95.37)	43.62 (13.14, 53.80)	25.26 (10.80, 95.57)	0.30	29.80 (9.69, 58.16)	25.42 (11.04, 95.52)	0.44	25.16 (10.64, 53.80)	25.99 (10.91, 98.35)	0.12
sE selectin (ng/ml)	3.34 (1.23, 7.83)	3.85 (1.83, 9.82)	3.21 (1.23, 7.75)	0.70	4.76 (1.15, 10.59)	3.08 (1.24, 7.55)	0.28	3.59 (1.31, 9.80)	3.21 (1.23, 7.44)	0.60
P selectin (ng/ml)	3.0 (1.12, 10.4)	2.62 (1.88, 4.41)	3.04 (1.11, 11.17)	0.11	3.22 (1.82, 11.42)	3.0 (1.11, 10.37)	0.62	2.92 (1.48, 7.59)	3.0 (1.10, 10.59)	0.68
vWF Ag (% of normal)	85.0 (55.0, 117.0)	63.0 (44.5, 131.8)	85.0 (55.0, 116.0)	0.54	84.0 (56.0, 123.0)	85.0 (55.0, 116.3)	0.54	79.5 (55.0, 116.0)	85.0 (55.0, 117.5)	0.92

Table 2

Analysis of continuous variables put forward into the multivariable model for the future development of laminitis in 446 ponies in 1, 2 and 3 years.

	Odds Ratio			**95% Confidence interval**			**P value**		
	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3
Basal insulin	1.50	1.50	1.50	1.48 – 1.55	1.47-1.55	1.48-1.54	<0.001	<0.001	<0.001
Insulin post dexamethasone	1.22	1.21	1.21	1.10-1.31	1.10-1.32	1.11-1.31	0.001	<0.001	<0.001
Adiponectin	0.57	0.52	0.64	0.39-0.72	0.38-0.71	0.60-0.81	0.02	<0.001	0.005
IGF-1	-	0.90	-	-	0.84-0.93	-	-	0.04	-

Table 3

ROC curve analysis for all of the variables that remained within the multivariable logistic regression models. Cut off values were determined from the co-ordinates of the ROC curves which maximised the sensitivity and specificity for the ability of the test to divide animals that will and will not develop laminitis in the following 1, 2 and 3 years. The corresponding positive (PPV) and negative predictive values (NPV) using these cut-off values were calculated.

	After 1 year			**After 2 years**			**After 3 years**		
	Adiponectin	Basal insulin	Insulin post dexamethasone	Adiponectin	Basal insulin	Insulin post dexamethasone	Adiponectin	Basal insulin	Insulin post dexamethasone
Area under ROC curve	0.74	0.80	0.67	0.77	0.74	0.69	0.66	0.73	0.69
95% CI	0.6, 0.88	0.70, 0.90	0.52, 0.83	0.65, 0.88	0.64, 0.85	0.57, 0.81	0.55, 0.77	0.65, 0.81	0.60, 0.78
Significance	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Cut off value	2.50ng/ml	21.8μiu/ml	105.6μiu/ml	2.50ng/ml	21.8μiu/ml	59.4 μiu/ml	2.89ng/ml	21.2μiu/ml	54.7μiu/ml
Sensitivity	78%	78%	69%	80%	73%	70%	64%	73%	71%
Specificity	79%	67%	68%	80%	73%	58%	73%	73%	58%
PPV	13.2%	9.3%	8.3%	22.6%	15.3%	11.3%	20.6%	21.5%	15.8%
NPV	98.8%	98.6%	98.0%	98.2%	97.3%	96.8%	94.8%	95.9%	95.0%

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