Royal
Veterinary
College
University of London

1   **Prospective cohort study evaluating risk factors for the development of pasture-associated laminitis**

2   **in the UK**

N.J Menzies-Gow, P.A. Harris[*] and J. Elliott

Royal Veterinary College, London UK.

[*]Equine Studies Group, WALTHAM Centre for Pet Nutrition, UK.

Corresponding Author: nmenziesgow@rvc.ac.uk

Word Count: 3861

3

4   **Summary**

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

6   **Reasons for performing the study:** Previous studies have predominantly investigated risk factors only

7   after disease occurrence.

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

9   **Study design**: Prospective cohort study.

10   **Methods:** Non-laminitic ponies $\geq$7 years old were recruited. Body condition score (BCS), height,

11   weight, crest height and thickness were measured and an overnight dexamethasone suppression test

12   performed. Plasma/serum adiponectin, leptin, triglyceride, basal insulin, insulin post dexamethasone,

13   insulin-like growth factor (IGF)-1, IGF binding protein (IGFBP)-1, IGFBP-3, C-reactive protein, von

14   Willebrand's factor (vWF), soluble (s) E-selectin and p-selectin concentrations were assayed. Follow-

15   up was obtained from owners annually for 3 years to ascertain occurrence of veterinary-diagnosed

16   pasture-associated laminitis. Data were analysed by multivariate logistic regression. ROC curves

17   analysis was performed for significant risk factors and cut off values determined.

18   **Results:** 446 animals were recruited; the median (interquartile range) age was 15 (10, 20) years; 50.4%

19   were mares and 49.6% geldings; the most common breeds were Welsh (36%), Shetland (17%) and cob

20   (9%); 72.2% were overweight/obese (BCS 7-9/9), 27.3% ideal weight (BCS 4-6/9) and 0.5%

21   underweight (BCS 1-3/9). After 1, 2 and 3 years, 18 (4%), 30 (7%) and 44/446 (10%) animals had had

22   laminitis. Plasma/serum [adiponectin],basal [insulin] and [insulin] post dexamethasone were

23   significantly (p$\leq$0.05) associated with laminitis occurrence cumulatively after 1, 2 and 3 years. The

24   accuracy to separate animals who did or did not develop laminitis determined using the area under

25   the ROC curves was good (basal [insulin] after 1 year), fair (all others) or poor ([insulin] post

26   dexamethasone).

27   **Main limitations:** Animals were evaluated at a single time point and biomarkers were assayed using

28   single assays.

29   **Conclusions:** Risk factors for future laminitis prior to disease occurrence include low plasma

30   adiponectin and high serum basal insulin or insulin post dexamethasone concentrations.

31

32   **Introduction**

33   A metabolic phenotype with similarities to human metabolic syndrome (HMS), including insulin

34   dysregulation (ID) [1], dyslipidaemia [2-4] and altered circulating adipokine concentrations [5-7] with

35   or without obesity appears to be associated with an increased risk of laminitis [8; 9]. Thus the same

36   pathologic mechanisms that underlie the cardiovascular diseases associated with HMS, including

37   changes in insulin signalling, inflammatory cytokines and endothelial dysfunction, could contribute to

38   laminitis risk. Multiple variables have been evaluated previously as laminitis risk factors [2-4]; [10], but

39   these studies have evaluated animals after disease occurrence and differences detected could reflect

40   the disease rather than a predisposition. Identifying risk factors prior to disease occurrence would

41   allow targeting of preventive management strategies. Potential risk factors requiring investigation

42   include obesity, ID, inflammatory cytokines and markers of endothelial dysfunction.

43   Intravenous infusion methods for ID identification are not practical in the field [11]. The

44   dexamethasone suppression test is a potential dynamic test for ID as exogenous cortisol analogues

45   antagonise insulin's actions resulting in increased endogenous insulin secretion. Previously laminitic

46   ponies (PLP) had a greater increase in insulin concentration post dexamethasone compared to

47   controls [12] in spring and summer [13]. Concentrations of the anti-inflammatory marker adiponectin

48   were significantly lower and plasma triglyceride concentrations were significantly higher in PLP in late

49   spring and winter [4].

4

50   Leptin is an adipose-derived hormone [14]; hyperleptinaemia in humans is associated with HMS, ID,

51   vascular inflammation [15] and endothelial dysfunction [16]. Insulin-like growth factor (IGF)-1 has

52   insulin-like metabolic actions and lower IGF-1 concentrations are associated with human obesity, ID

53   and HMS [17]. IGF binding proteins (IGFBPs) represent an important link between the insulin and IGF

54   systems and play important roles in human obesity and HMS [18]. Abnormal IGFBP expression is a

55   sensitive marker of ID, used to identify individual humans with ID at high cardiovascular risk and as an

56   early marker of HMS [18]. None of these factors/parameters has been assessed as predictors of

57   laminitis development prospectively.

58   The current gold standard non-invasive test of assessing endothelial function using endothelial-

59   dependent vasomotion in humans is flow mediated dilatation, however it is not suitable for equine

60   use due to a lack of accuracy or precision [19]. Additionally, various circulating molecules are used as

61   biomarkers of endothelial dysfunction including von Willebrand's factor (vWF) [20], soluble (s)E-

62   selectin [21] and P-selectin [22]. Thus it would be logical to evaluate these in animals prior to laminitis

63   occurrence.

64   The study aim was to investigate prospectively certain risk factors for the development of pasture-

65   associated laminitis in a cohort of animals with no known history of laminitis, including morphometric

66   measures of obesity and circulating concentrations of biomarkers of insulin dysregulation, adipokines

67   and endothelial dysfunction.

68

69   **Materials and Methods**

70   The study was approved by Royal Veterinary College Ethics and Welfare Committee and was

71   performed under the UK Veterinary Surgeons Act 1966. So that the dexamethasone suppression test

72   was performed for the benefit of the individual animal as a test for pituitary pars intermedia

73 dysfunction (PPID) and due to the fact that the youngest age of a case of PPID in the scientific literature

74 is 7 years [23; 24], only animals $\geq$7 years old could be included in the study.

75

76 **Animals**

77 Sample size calculations assuming 80% power and 95% confidence indicated that 200-700 animals

78 were required to detect a 5- to 10-fold increase in laminitis risk in ID compared to non-ID animals

79 (assuming 2% laminitis risk over 2 years in non-ID animals and exposed:unexposed ratio of 1:10). Thus

80 the prospective cohort study aimed to recruit approximately 400 client-owned ponies $\geq$7 years old

81 with no previous known history of laminitis to the study. Animals were not cases nor were they

82 recruited via veterinary practices or referral hospitals. Instead they were healthy animals recruited to

83 the study directly from owners in response to posts on social media sites, requests placed in equine

84 lay magazines and letters sent to all equine charities and riding/livery establishments within a 50 mile

85 radius of the Royal Veterinary College by the study organiser. All suitable animals were included in the

86 study and there was no randomisation. A standard clinical examination of each animal was performed

87 by a single experience equine veterinarian and ponies were excluded if they displayed clinical signs of

88 acute, chronic or previous laminitis (including lameness affecting 2 or more feet, increased digital

89 pulses, characteristic stance of leaning back on the heels, divergent hoof growth rings) or pituitary

90 pars intermedia (PPID; including hypertrichosis) at the time of recruitment to the study. In addition,

91 ponies were excluded from the study if they were subsequently identified as having an abnormal

92 cortisol response to the dexamethasone suggestive of PPID. The study was fully explained to the

93 owners, a factsheet provided and informed consent obtained. Historical information was obtained by

94 direct questioning of the owner. All animals were evaluated and samples collected in summer

95 (August).

96

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

7

119 measured by the pathology laboratory at Bell Equine Veterinary Clinic, Kent, UK and plasma vWF

120 antigen concentrations were measured by a commercial laboratory[m]. All of these methods have been

121 previously validated for use in the horse apart from IGF-1, IGFBP-1, IGFBP-3, sE-selectin and P-selectin.

122 Thus these assays were validated by determining intra- and inter-assay variability (to assess precision

123 and repeatability; using pooled samples from 5 animals measured three times), dilutional parallelism

124 (using samples from 5 animals diluted 1 in 2, 1 in 4 and 1 in 8 within the working range of the assay)

125 and spiked recovery (to assess specificity; using the assay standard diluted in equine plasma).

126

127 **Follow up**

128 Owners of animals recruited to the study were contacted after 12, 24 and 36 months in order to

129 ascertain whether individual animals had suffered from pasture-associated laminitis diagnosed by a

130 veterinarian in the preceding 12 months.

131

132 **Data Analysis**

133 Data were analysed using the SPSS software program[n]. Univariable logistic regression was first used

134 to assess risk factors including morphometric data and blood analytes associated with the outcome

135 (namely laminitic after 1 year or not; laminitic after 2 years or not; laminitic after 3 years or not)

136 individually. Correlations between those risk factors with $p<0.1$ were tested by calculating Pearson's

137 correlation coefficient. Interactions between risk factors were tested by calculating A*B for significant

138 risk factors and entering risk factors A and B and A*B into a multivariable logistic regression model. If

139 the p value for the interaction term A*B was >0.05, there was not significant interaction between risk

140 factors A and B. Risk factors with $p<0.1$ were then entered into a multivariable logistic regression and

141 any risk factors with $p>0.05$ sequentially removed until all the risk factors had $p\leq0.05$ in the final

142 model. ROC curves were constructed for those risk factors remaining in the final model. The accuracy

143 of the test to separate animals into those which did or did not subsequently develop laminitis was

144 determined by calculating the area under the curve (AUC) whereby an area of 0.90-1 is excellent, 0.80-

145 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

146 ROC curve were then used to determine the cut off value which maximised specificity and sensitivity

147 and the corresponding positive (PPV) and negative predictive values (NPV) using these cut-off values

148 were calculated.

149

150 **Results**

151 Four hundred and forty six ponies aged (median [interquartile range]) 15 (10, 20) years were recruited

152 to the study. Of these 50.4% were mares and 49.6% were geldings. A range of pony breeds were

153 represented including Welsh (36.4%), Shetland (17%), cob (9.4%), New Forest (9%), crossbreed (7.6%)

154 and other (20.6%). The majority (72.2%) of animals were overweight or obese (BCS 7-9), with 27.3%

155 being ideal weight (BCS 4-6) and 0.5% being underweight (BCS 1-3).

156

157 After 1, 2 and 3 years cumulatively 18 (4%), 30 (7%) and 44 (10%) had developed pasture-associated

158 laminitis, 416 (93%), 374 (84%) and 348 (78%) remained non laminitic, and 12 (3%), 42 (9%) and 54

159 (12%) had been euthanased for reasons other than laminitis, respectively.

160

161 Values for crest height, crest thickness, body condition score, plasma/serum concentrations of

162 biomarkers and serum insulin response to an overnight dexamethasone suppression test in all animals

163 and those that subsequently developed laminitis and those that remained non laminitic after 1, 2 and

164 3 years are shown in Table 1.

165

166 The validation of the ELISAs used to measured plasma IGF-1, IGFBP-1, IGFBP-3, sE-selectin and P-

167 selectin concentrations revealed that they performed satisfactorily (Online supplementary

168 information, table 1).

169

170 Most of the pairwise correlations were weak (Pearson's correlation coefficient <0.2) apart from basal

171 insulin and insulin post dexamethasone (Pearson's correlation coefficient 0.68). The only significant

172 interaction between risk factors was between basal insulin and post dexamethasone insulin

173 concentration after 2 (p=0.03) and 3 years (p=0.02).

174

175 For the data obtained after 1 year, the risk factors taken into the multivariate regression analysis

176 included plasma/serum adiponectin, basal insulin, insulin post dexamethasone and p-selectin

177 concentrations. Apart from p-selectin, these all proved to be significant risk factors for the

178 development of laminitis and so remained in the multivariate analysis (Table 2).

179

180 For the data obtained after 2 years, the risk factors taken into the multivariate regression analysis

181 included plasma/serum CRP, adiponectin, IGF-1, basal insulin and insulin post dexamethasone

182 concentrations. Those that proved to be significant risk factors for the development of laminitis and

183 so remained in the multivariate analysis were plasma adiponectin, plasma IGF-1, serum basal insulin

184 and serum insulin post dexamethasone concentrations (table 2).

185

186 For the data obtained after 3 years, the risk factors taken into the multivariate regression analysis

187 included plasma/serum adiponectin, basal insulin, insulin post dexamethasone and IGFBP-3

188 concentrations. Apart from IGFBP-3, these all proved to be significant risk factors for the development

189 of laminitis and so remained in the multivariate analysis (Table 2)

190

191 None of the morphometric data proved to be significant risk factors for the development of laminitis

192 in 1, 2 or 3 years.

193

194 The ROC AUC and the cut off values which maximised the specificity and the sensitivity, as well as the

195 corresponding PPV and NPV are shown in Table 3. There was no improvement in these values when

196 variables were combined.

197

198 **Discussion**

199 Consistent risk factors for the future development of laminitis in animals prior to disease occurrence

200 in the present study included low plasma adiponectin and high basal and post dexamethasone serum

201 insulin concentrations.

202

203 The hormone adiponectin has anti-diabetic, anti-atherogenic and anti-inflammatory properties in

204 humans and rodents [30; 31] and circulating concentrations are decreased in obese individuals and in

205 patients with HMS [32], type 2 diabetes [30] and cardiovascular disease [33]. Despite being specifically

206 secreted by adipocytes, a strong negative correlation exists between adiponectin concentrations and

207 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.

233

234   The link between laminitis and insulin dysfunction has been investigated and hyperinsulinaemia

235   and/or insulin dysregulation has been reported in previously laminitic animals in a number of studies

236   [2; 3; 42]. Hyperinsulinaemia and insulin dysregulation may predispose to laminitis by triggering

237   disturbances in vascular function through downregulation of the phosphatidylinositol 3-kinase (PI-3K)

238   pathway and hence reduction in production of the vasodilator nitric oxide (NO) in the face of

239   continued vasoconstrictor production [43]. Alternatively, as previously discussed, insulin may be

240   excessively stimulating lamellar IGF-1 receptors, leading to inappropriate epithelial cell proliferation

241   with lamellar weakening and consequent laminitis [44]. Previously, serum basal insulin concentrations

242   >32μiu/ml had good sensitivity (100%) and specificity (80%) for predicting clinical laminitis in the

243   following 3 months in previously laminitic animals [6]. Similarly, in the present study increased serum

244   insulin concentrations in non-laminitic animals was significantly associated with the subsequent

245   development of laminitis. ROC curve analysis revealed that the accuracy of serum basal insulin

246   concentration to separate animals into those which do or do not go on to develop laminitis

247   cumulatively in 1, 2 or 3 years was good (after 2 years) to fair (after 1 and 3 years) and a cut of value

248   of 21.8μiu/ml gave acceptable sensitivity (78%) and specificity (67%) values. This value is virtually

249   identical to that proposed as a value above which is consistent with insulin dysregulation previously

250   [9]. However it must be acknowledged that animals in the present study were not fasted prior to blood

251   sampling.

252

253   Single measurements of serum insulin concentration are affected by a number of factors including

254   diet, exercise, stress and time of day [11; 45]. Thus dynamic tests of endocrine function are often

255   advocated to detect ID. The dexamethasone suppression test (DST) is a potential dynamic test for ID

256   as exogenous cortisol analogues antagonise insulin resulting in increased endogenous insulin

257 secretion. Previously laminitic ponies had a greater increase in serum insulin post dexamethasone

258 compared to control ponies [12] which was seen only in spring and summer [13] and a cut off value of

259 75μiu/ml was suggested to distinguish groups of previously laminitic animals from controls. Similarly

260 in the present study, an exaggerated insulin response to dexamethasone was associated with the

261 subsequent development of laminitis in non laminitic ponies. ROC curve analysis revealed that the

262 accuracy of serum insulin post dexamethasone to separate animals into those which do or do not go

263 on to develop laminitis cumulatively in 1, 2 or 3 years was fair to poor and a cut of value of 105.6μiu/ml

264 gave fair sensitivity (69%) and specificity (68%) values for the development of laminitis in the next 12

265 months. It should be acknowledged that the study was designed prior to the recent increase in

266 popularity of oral sugar or glucose tests as a dynamic test of ID. To the authors' knowledge the insulin

267 response to dexamethasone has not been directly compared to the OGT or OST, thus it is not possible

268 to extrapolate these results to those tests.

269

270 Insulin-like growth factor (IGF)-1 is primarily produced in the liver from growth hormone metabolism

271 prior to secretion into the circulation. It has short-term insulin-like metabolic actions and long-term

272 growth factor-like effects on cell proliferation and differentiation. Lower IGF-1 concentrations are

273 associated in other species with obesity, insulin resistance, HMS [17], type 2 diabetes [46] and

274 increased risk of cardiovascular disease [47]; however the precise mechanism(s) behind these

275 apparent inverse relationships remains elusive. The median plasma IGF-1 concentrations in those

276 animals that remained non-laminitic in the present study were similar to those previously reported in

277 adult horses [48; 49]; whilst the median plasma IGF-1 concentrations in those ponies that

278 subsequently developed laminitis after 2 years was significantly lower. Plasma IGF-1 concentrations

279 have not been measured in previously laminitic ponies; however season and body condition score

280 have been shown to have an effect in other populations of horses [50; 51]. Plasma IGF-1

281 concentrations were significantly higher in the summer compared to the winter [50] and in overweight

14

282   compared to underweight mares [51]. In the present study, samples were collected from all of the

283   ponies at the same time of the year (August) and body condition score was not a risk factor for

284   laminitis development. Whilst in people, IGF-1 concentrations have been reported to decrease with

285   age [52], there is no evidence for aging being a factor in changes of IGF-1 in adult horses [48] or in the

286   present study population. Thus it would appear that for unknown reasons low IGF-1 concentrations

287   were only associated with an increased risk of developing laminitis after 2 years and not after 1 or 3

288   years suggesting that IGF-1 concentration is not directly associated with an increased risk.

289

290   There are six IGF binding proteins (IGFBPs) which link with IGF-1 and -2 and prevent them from being

291   degraded; they also facilitate IGF transport through body compartments. The interaction between

292   IGFs and their specific receptors is partly regulated by structural modifications inherent to the IGFBPs.

293   Whilst IGFBP expression has been suggested to be useful as a sensitive marker of ID, to identify

294   individuals with ID at high cardiovascular risk and as an early marker of HMS [18], concentrations of

295   IGFBP-1 or IGFBP-3 were not useful in the detection of animals at increased risk of development of

296   future laminitis.

297

298   Leptin is an adipose tissue derived hormone [14] and hyperleptinaemia is associated with HMS, insulin

299   resistance, vascular inflammation [15] and endothelial dysfunction [16]. In horses and ponies,

300   hyperleptinaemia is associated with hyperinsulinaemia [53], obesity [54] and previous laminitis in

301   some [6], but not all studies [7]. In addition, hyperleptinaemia could be used to predict clinical laminitis

302   in the following 3 months in previously laminitic animals [6]. However, whilst there was a weak

303   positive correlation between plasma leptin concentration and BCS ($p=0.03$, $r=0.14$), no such

304   correlation with serum insulin concentration was found and there was no association between plasma

305   leptin concentrations and subsequent development of laminitis was apparent in the present study.

306

307 Increased plasma triglyceride concentrations are associated with hyperinsulinaemia [53], obesity [54]

308 and previous laminitis [2-4]. However in agreement with the present study, they were not beneficial

309 in the prediction of clinical laminitis in the following 3 months in previously laminitic animals [6].

310

311 C-reactive protein (CRP) is an acute-phase protein and increased concentrations are associated with

312 insulin resistance [55], HMS [56; 57] and cardiovascular disease [58]. In horses, an increase in CRP

313 concentration has been reported in induced inflammation and laminitis, pneumonia, enteritis,

314 arthritis and after castration [59]. Other studies, however, reported that serum CRP concentration was

315 not affected by inflammatory disease [60; 61]. CRP concentrations have not been evaluated in

316 association with naturally occurring laminitis, but CRP concentrations were not significantly different

317 between control animals and hyperinsulinaemic obese horses [62]. The concentrations found in the

318 present study were similar to those reported in healthy horses in one study [28], but lower than those

319 reported in another [62] and they were not found to be a significant risk factor for the subsequent

320 development of laminitis.

321

322 Equine plasma vWF antigen concentrations have only been previously measured in association with

323 exercise [20] or as part of the investigation of clotting disorders [63; 64]. In humans, vWF

324 concentrations are increased in obesity [65], HMS [66] and insulin resistant patients [67]. However

325 plasma vWF antigen concentrations could not be used to predict the development of HMS in patients

326 with hypertension [68]. P-selectin and sE-selectin are markers of endothelial dysfunction in other

327 species [21; 22] and the role of endothelial dysfunction is important in HMS and ID and the

328 development of associated cardiovascular diseases [69]. Thus, it is logical to postulate that endothelial

329 dysfunction may play a role in the pathogenesis of laminitis associated with ID. However, none of the

330   biomarkers of endothelial dysfunction measured were associated with the subsequent development

331   of laminitis in the present study.

332

333   Whilst an association between previous laminitis and generalised and/or regional adiposity has been

334   reported [2; 6; 70] and generalised and/or regional adiposity could be used to predict clinical laminitis

335   in the following 3 months in previously laminitic animals [6], surprisingly no such association with

336   future laminitis was found in the present study. It should be acknowledged that the majority (72%) of

337   ponies in the study were overweight or obese, which is similar to previously reported figures of obesity

338   within the UK pony population in some studies [71], but much greater than those in other studies

339   evaluating both horses and ponies (30-45%) [72-75]. This high prevalence of obesity may have resulted

340   in it not being a discernible risk factor within the population studied; alternatively obesity alone may

341   not a significant risk factor for the future development of laminitis.

342

343   The main limitation of the present study was that animals were only examined at a single point in

344   time. There is no current evidence for the longevity or stability of these biomarkers in horses and it is

345   possible that both these and the morphometric data values changed considerably during the 3 years

346   over which the animals were subsequently followed which could in turn have had a significant impact

347   on the laminitis risk.

348

349   In conclusion, risk factors for the development of laminitis in previously non-laminitic animals in the

350   present study included low plasma adiponectin as well as high basal insulin and serum insulin post

351   dexamethasone concentrations. The accuracy of these to separate animals who did or did not develop

352   laminitis after 1, 2 or 3 years was good (serum basal [insulin] after 1 year), fair (all others) or poor

353 (serum [insulin] post dexamethasone) and cut off values with acceptable sensitivities and specificities

354 were generated. Combinations of these biomarkers did not improve their predictive value. However

355 it should be acknowledged that these cut-off values were generated using samples obtained at a single

356 time of the year (summer) and measured using single assays (radioimmunoassay) and radiography

357 was not performed so that it is possible that animals with pre-existing subclinical laminitis were

358 included. Surprisingly, the development of laminitis was not associated with regional or generalised

359 obesity, hyperleptinaemia or hypertriglyceridaemia. In addition there was no association with

360 circulating CRP, IGF-1, IGFBP-1, IGFBP-3, sE-selectin, p-selectin or vWF antigen concentrations. Further

361 prospective cohort studies that examine animals more frequently such that their morphometric and

362 metabolic variables are determined within a shorter time frame in relation to the onset of laminitis or

363 that include a much larger number of animals are warranted to assess these risk factors further.

364

365 **Manufacturers' Details**

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

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

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

369 [d] Adiponectin RIA kit, Merck Millipore, Missouri

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

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

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

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

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

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

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

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

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

379   [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 ± 1.1	4.9 ± 0.8	4.6 ± 1.1	0.36	5.0 ± 1.4	4.6 ± 1.1	0.54	4.9 ± 1.3	4.6 ± 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	68.4	11.6	<0.001	68.4	11.0	<0.001	42.8	10.8	<0.001
	(6.6, 30.6)	(20.2, 246.0)	(6.6, 29.77)		(21.9, 127.2)	(6.6, 27.9)		(21.2, 96.2)	(6.6, 24.6)	
Insulin post dexamethasone (µIU/ml)	46.9	194.0	46.4	0.02	269.9	43.9	0.002	192.2	42.5	<0.001
	(20.6, 146.6)	(39.6, 418.5)	(20.6, 142.1)		(60.3, 390.1)	(19.8, 124.5)		(55.3, 390.1)	(18.4, 121.2)	
CRP (µg/ml)	10.66	6.16	10.82	0.22	6.16	10.89	0.10	8.78	10.80	0.41
	(0.41, 25.97)	(2.85, 15.21)	(0.23, 26.26)		(1.70, 20.57)	(0.23, 26.26)		(1.69, 34.13)	(0.23, 25.28)	
IGF-1 (ng/ml)	225.8 ± 68.9	207.5 ± 71.7	226.6 ± 68.7	0.25	202.3 ± 72.9	227.5 ± 68.4	0.033	230.0 ± 74.9	225.4 ± 68.3	0.67
IGFBP-1 (ng/ml)	9.70	10.42	9.59	0.33	10.42	9.59	0.48	10.27	9.59	0.27
	(4.18, 25.23)	(4.08, 23.03)	(4.29, 25.41)		(3.95, 35.84)	(4.29, 25.14)		(3.44, 35.89)	(4.29, 24.88)	
IGFBP-3 (ng/ml)	25.48	43.62	25.26	0.30	29.80	25.42	0.44	25.16	25.99	0.12
	(10.91, 95.37)	(13.14, 53.80)	(10.80, 95.57)		(9.69, 58.16)	(11.04, 95.52)		(10.64, 53.80)	(10.91, 98.35)	
sE selectin (ng/ml)	3.34	3.85	3.21	0.70	4.76	3.08	0.28	3.59	3.21	0.60
	(1.23, 7.83)	(1.83, 9.82)	(1.23, 7.75)		(1.15, 10.59)	(1.24, 7.55)		(1.31, 9.80)	(1.23, 7.44)	
P selectin (ng/ml)	3.0	2.62	3.04	0.11	3.22	3.0	0.62	2.92	3.0	0.68
	(1.12, 10.4)	(1.88, 4.41)	(1.11, 11.17)		(1.82, 11.42)	(1.11, 10.37)		(1.48, 7.59)	(1.10, 10.59)	
vWF Ag (% of normal)	85.0	63.0	85.0	0.54	84.0	85.0	0.54	79.5	85.0	0.92
	(55.0, 117.0)	(44.5, 131.8)	(55.0, 116.0)		(56.0, 123.0)	(55.0, 116.3)		(55.0, 116.0)	(55.0, 117.5)	

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%

References

[1]   Treiber, K.H., Boston, R.C., Kronfeld, D.S., Staniar, W.B. and Harris, P.A. (2005) Insulin resistance and compensation in Thoroughbred weanlings adapted to high-glycemic meals. *J Anim Sci* **83**, 2357-2364.

[2]   Treiber, K.H., Kronfeld, D.S., Hess, T.M., Byrd, B.M., R.K., S. and Staniar, W.B. (2006) Evaluation of genetic and metabolic predispositions and nutritional risk factors for pasture-associated laminitis in ponies. *J Am Vet Med Assoc* **228**, 1538-1545.

[3]   Bailey, S.R., Habershon-Butcher, J., Ransom, K., Elliott, J. and Menzies-Gow, N.J. (2008) Hypertension and insulin resistance in a mixed-breed population of ponies predisposed to laminitis: Further characterizing pre-laminitic metabolic syndrome. *Am J Vet Res* **69**, 122-129.

[4]   Wray, H., Elliott, J., Bailey, S.R., Harris, P.A. and Menzies-Gow, N.J. (2013) Plasma concentrations of inflammatory markers in previously laminitic ponies. *Equine Vet J* **45**, 546-551.

[5]   Frank, N., Elliott, S.B., Brandt, L.E. and Keisler, D.H. (2006) Physical characteristics, blood hormone concentrations, and plasma lipid concentrations in obese horses with insulin resistance. *J Am Vet Med Assoc* **228**, 1383-1390.

[6]   Carter, R.A., Treiber, K.H., Geor, R.J., Douglass, L. and Harris, P.A. (2009) Prediction of incipient pasture-associated laminitis from hyperinsulinaemia, hyperleptinaemia and generalised and localised obesity in a cohort of ponies. *Equine Vet J* **41**, 171-178.

[7]   Schultz, N., Geor, R.J., Manfredi, J. and McCue, M.E. (2014) Factors associated with leptin and adiponectin concentrations in a large across breed cohort of horses and ponies. *Journal of veterinary internal medicine* **28**, 1114.

[8]   Johnson, P.J. (2002) The equine metabolic syndrome peripheral Cushing's syndrome. *Vet Clin North Am Equine Pract* **18**, 271-293.

[9]   Frank, N., Geor, R.J., Bailey, S.R., Durham, A.E. and Johnson, P.J. (2010) Equine metabolic syndrome. *J Vet Intern Med* **24**, 467-475.

[10]   Hinckley, K.A. and Henderson, I.W. (1996) The epidemiology of equine laminitis in the UK. In: *35th Congress of the British Equine Veterinary Association*, Warwick. p 62.

[11]   Firshman, A.M. and Valberg, S.J. (2007) Factors affecting clinical assessment of insulin sensitivity in horses. *Equine Vet J* **39**, 567-575.

[12]   Bailey, S.R., Menzies-Gow, N.J., Harris, P.A., Habershon-Butcher, J.L., Crawford, C., Berhane, Y., Boston, R.C. and Elliott, J. (2007) Effect of dietary fructans and dexamethasone administration on the insulin response of ponies predisposed to laminitis. *J Am Vet Med Assoc* **231**, 1365-1373.

[13]   Borer, K.E. (2011) *Laminitis and insulin resistance in ponies*, University of London (Royal Veterinary College).

[14]   Mattu, H.S. and Randeva, H.S. (2012) The role of Adipokines in Cardiovascular Disease. *J Endocrinol*.

[15]   Wannamethee, S.G., Tchernova, J., Whincup, P., Lowe, G.D., Kelly, A., Rumley, A., Wallace, A.M. and Sattar, N. (2007) Plasma leptin: associations with metabolic, inflammatory and haemostatic risk factors for cardiovascular disease. *Atherosclerosis* **191**, 418-426.

[16]   Beltowski, J. (2006) Role of leptin in blood pressure regulation and arterial hypertension. *J Hypertens* **24**, 789-801.

[17]   Lam, C.S., Chen, M.H., Lacey, S.M., Yang, Q., Sullivan, L.M., Xanthakis, V., Safa, R., Smith, H.M., Peng, X., Sawyer, D.B. and Vasan, R.S. (2010) Circulating insulin-like growth factor-1 and its binding protein-3: metabolic and genetic correlates in the community. *Arterioscler Thromb Vasc Biol* **30**, 1479-1484.

[18]   Ruan, W. and Lai, M. (2010) Insulin-like growth factor binding protein: a possible marker for the metabolic syndrome? *Acta Diabetol* **47**, 5-14.

[19]   Finding, E.J.T., Menzies-Gow, N.J., Harris, P.A. and Elliott, J. (2014) Assessment of non-invasive methods for measuring endothelial function in the horse. *J Vet Intern Med* **28**, 1123.

[20]   Smith, J.M., Meyers, K.M., Barbee, D.D., Schott, H. and Bayly, W.M. (1997) Plasma von Willebrand factor in thoroughbreds in response to high-intensity treadmill exercise. *Am J Vet Res* **58**, 71-76.

[21]   Torres, M.R. and Sanjuliani, A.F. (2012) Effects of weight loss from a high-calcium energy-reduced diet on biomarkers of inflammatory stress, fibrinolysis, and endothelial function in obese subjects. *Nutrition*.

[22]   Dallmeier, D., Larson, M., Vasan, R., Keaney, J., Jr., Fontes, J., Meigs, J., Fox, C. and Benjamin, E. (2012) Metabolic Syndrome and Inflammatory Biomarkers: a community-based cross-sectional Study at the Framingham Heart Study. *Diabetol Metab Syndr* **4**, 28.

[23]   Orth, D.N., Holscher, M.A., Wilson, M.G., Nicholson, W.E., Plue, R.E. and Mount, C.D. (1982) Equine Cushing's disease: plasma immunoreactive proopiolipomelanocortin peptide and cortisol levels basally and in response to diagnostic tests. *Endocrinology* **110**, 1430-1441.

[24]   Heinrichs, M., Baumgartner, W. and Capen, C.C. (1990) Immunocytochemical demonstration of proopiomelanocortin-derived peptides in pituitary adenomas of the pars intermedia in horses. *Vet Pathol* **27**, 419-425.

[25]   Henneke, D.R., Potter, G.D., Kreider, J.L. and Yeates, B.F. (1983) Relationship between condition score, physical measurements and body fat percentage in mares. *Equine Vet J* **15**, 371-372.

[26]   Kearns, C.F., McKeever, K.H., Roegner, V., Brady, S.M. and Malinowski, K. (2006) Adiponectin and leptin are related to fat mass in horses. *Vet J* **172**, 460-465.

[27]   Reimers, T.J., Cowan, R.G., McCann, J.P. and Ross, M.W. (1982) Validation of a rapid solid-phase radioimmunoassay for canine, bovine, and equine insulin. *Am J Vet Res* **43**, 1274-1278.

[28]   Cywinska, A., Szarska, E., Gorecka, R., Witkowski, L., Hecold, M., Bereznowski, A., Schollenberger, A. and Winnicka, A. (2012) Acute phase protein concentrations after limited distance and long distance endurance rides in horses. *Res Vet Sci* **93**, 1402-1406.

[29]   Greiner, M., Pfeiffer, D. and Smith, R.D. (2000) Principles and practical application of the receiver-operating characteristic analysis for diagnostic tests. *Prev Vet Med* **45**, 23-41.

[30]   Hotta, K., Funahashi, T., Arita, Y., Takahashi, M., Matsuda, M., Okamoto, Y., Iwahashi, H., Kuriyama, H., Ouchi, N., Maeda, K., Nishida, M., Kihara, S., Sakai, N., Nakajima, T., Hasegawa, K., Muraguchi, M., Ohmoto, Y., Nakamura, T., Yamashita, S., Hanafusa, T. and Matsuzawa, Y. (2000) Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. *Arterioscler Thromb Vasc Biol* **20**, 1595-1599.

[31]   Trujillo, M.E. and Scherer, P.E. (2006) Adipose tissue-derived factors: impact on health and disease. *Endocr Rev* **27**, 762-778.

[32]   Bremer, A.A., Devaraj, S., Afify, A. and Jialal, I. (2011) Adipose tissue dysregulation in patients with metabolic syndrome. *J Clin Endocrinol Metab* **96**, E1782-1788.

[33]   Efstathiou, S.P., Tsioulos, D.I., Tsiakou, Y.E., Gratsias, Y.E., Pefanis, A.V. and Mountokalakis, T.D. (2005) Plasma adiponectin levels and five-year survival after ifrst-ever ischaemic stroke *Stroke* **36**, 1915-1919.

[34]   Arita, Y., Kihara, S., Ouchi, N., Takahashi, M., Maeda, K., Miyagawa, J., Hotta, K., Shimomura, I., Nakamura, T., Miyaoka, K., Kuriyama, H., Nishida, M., Yamashita, S., Okubo, K., Matsubara, K., Muraguchi, M., Ohmoto, Y., Funahashi, T. and Matsuzawa, Y. (1999) Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun* **257**, 79-83.

[35]   Yang, W.S., Lee, W.J., Funahashi, T., Tanaka, S., Matsuzawa, Y., Chao, C.L., Chen, C.L., Tai, T.Y. and Chuang, L.M. (2001) Weight reduction increases plasma levels of an adipose-derived anti-inflammatory protein, adiponectin. *J Clin Endocrinol Metab* **86**, 3815-3819.

[36]   Ouchi, N., Ohishi, M., Kihara, S., Funahashi, T., Nakamura, T., Nagaretani, H., Kumada, M., Ohashi, K., Okamoto, Y., Nishizawa, H., Kishida, K., Maeda, N., Nagasawa, A., Kobayashi, H., Hiraoka, H., Komai, N., Kaibe, M., Rakugi, H., Ogihara, T. and Matsuzawa, Y. (2003) Association of hypoadiponectinemia with impaired vasoreactivity. *Hypertension* **42**, 231-234.

[37]   Fesus, G., Dubrovska, G., Gorzelniak, K., Kluge, R., Huang, Y., Luft, F.C. and Gollasch, M. (2007) Adiponectin is a novel humoral vasodilator. *Cardiovasc Res* **75**, 719-727.

[38]   Ruan, H. and Dong, L.Q. (2016) Adiponectin signaling and function in insulin target tissues. *Journal of molecular cell biology*.

[39]   Asplin, K.E., Sillence, M.N., Pollitt, C.C. and McGowan, C.M. (2007) Induction of laminitis by prolonged hyperinsulinaemia in clinically normal ponies. *Vet J* **174**, 530-535.

[40]   Blakesley, V.A., Scrimgeour, A., Esposito, D. and Le Roith, D. (1996) Signaling via the insulin-like growth factor-I receptor: does it differ from insulin receptor signaling? *Cytokine & growth factor reviews* **7**, 153-159.

[41]   Kullmann, A., Weber, P.S., Bishop, J.B., Roux, T.M., Norby, B., Burns, T.A., McCutcheon, L.J., Belknap, J.K. and Geor, R.J. (2015) Equine insulin receptor and insulin-like growth factor-1 receptor expression in digital lamellar tissue and insulin target tissues. *Equine Vet J.*

[42]   Treiber, K.H., Kronfeld, D.S. and Geor, R.J. (2006) Insulin resistance in equids: possible role in laminitis. *J Nutr* **136**, 2094S-2098S.

[43]   Kim, J.A., Montagnani, M., Koh, K.K. and Quon, M.J. (2006) Reciprocal relationships between insulin resistance and endothelial dysfunction: molecular and pathophysiological mechanisms. *Circulation* **113**, 1888-1904.

[44]   de Laat, M.A., Pollitt, C.C., Kyaw-Tanner, M.T., McGowan, C.M. and Sillence, M.N. (2013) A potential role for lamellar insulin-like growth factor-1 receptor in the pathogenesis of hyperinsulinaemic laminitis. *Vet J* **197**, 302-306.

[45]   Pratt, S.E., Siciliano, P.D. and Walston, L. (2009) Variation of insulin sensitivity estimates in horses. *J Equine Vet Sci* **29**, 507-512.

[46]   Sandhu, M.S., Heald, A.H., Gibson, J.M., Cruickshank, J.K., Dunger, D.B. and Wareham, N.J. (2002) Circulating concentrations of insulin-like growth factor-I and development of glucose intolerance: a prospective observational study. *Lancet* **359**, 1740-1745.

[47]   Troncoso, R., Ibarra, C., Vicencio, J.M., Jaimovich, E. and Lavandero, S. (2014) New insights into IGF-1 signaling in the heart. *Trends in endocrinology and metabolism: TEM* **25**, 128-137.

[48]   Lygren, T., Hansen, S., Langberg, H., Fjeldborg, J., Jacobsen, S., Nielsen, M.O., Schjerling, P., Markussen, B., Thomsen, P.D. and Berg, L.C. (2014) Serum insulin-like growth factor 1 in the aging horse. *Veterinary clinical pathology / American Society for Veterinary Clinical Pathology* **43**, 557-560.

[49]   Popot, M.A., Bobin, S., Bonnaire, Y., Delahaut, P.H. and Closset, J. (2001) IGF -I plasma concentrations in non-treated horses and horses administered with methionyl equine somatotropin. *Res Vet Sci* **71**, 167-173.

[50]   Luszczyn'ski, J. and Pieszka, M. (2011) Effect of season on plasma concentration of insulin-like growth factor-I and 1.25-dihydroxycholecalciferol in horses. . *Acta Scientiarium Polonorum - Zootechnica* **10**, 45-54.

[51]   Salazar-Ortiz, J., Monget, P. and Guillaume, D. (2014) The influence of nutrition on the insulin-like growth factor system and the concentrations of growth hormone, glucose, insulin, gonadotropins and progesterone in ovarian follicular fluid and plasma from adult female horses (Equus caballus). *Reproductive biology and endocrinology : RB&E* **12**, 72.

[52]   Rosen, C.J. (2000) Growth hormone and aging. *Endocrine* **12**, 197-201.

[53]   Morgan, R.A., McGowan, T.W. and McGowan, C.M. (2014) Prevalence and risk factors for hyperinsulinaemia in ponies in Queensland, Australia. *Aust Vet J* **92**, 101-106.

[54]   Pleasant, R.S., Suagee, J.K., Thatcher, C.D., Elvinger, F. and Geor, R.J. (2013) Adiposity, plasma insulin, leptin, lipids, and oxidative stress in mature light breed horses. *J Vet Intern Med* **27**, 576-582.

[55]   Ingelsson, E., Hulthe, J. and Lind, L. (2008) Inflammatory markers in relation to insulin resistance and the metabolic syndrome. *Eur J Clin Invest* **38**, 502-509.

[56] Ryu, S.Y., Kim, K.S., Park, J., Kang, M.G. and Han, M.A. (2008) The association between circulating inflammatory markers and metabolic syndrome in Korean rural adults. *Journal of preventive medicine and public health = Yebang Uihakhoe chi* **41**, 413-418.

[57] Maleki, A., Rashidi, N., Aghaei Meybodi, H., Montazeri, M., Montazeri, M., Falsafi, F., Ghanavati, R., Foroughi, S. and Alyari, F. (2014) Metabolic syndrome and inflammatory biomarkers in adults: a population-based survey in Western region of iran. *International cardiovascular research journal* **8**, 156-160.

[58] Pischon, T., Hu, F.B., Rexrode, K.M., Girman, C.J., Manson, J.E. and Rimm, E.B. (2008) Inflammation, the metabolic syndrome, and risk of coronary heart disease in women and men. *Atherosclerosis* **197**, 392-399.

[59] Takiguchi, M., Fujinaga, T., Naiki, M., Mizuno, S. and Otomo, K. (1990) Isolation, characterization, and quantitative analysis of C-reactive protein from horses. *Am J Vet Res* **51**, 1215-1220.

[60] Lavoie-Lamoureux, A., Leclere, M., Lemos, K., Wagner, B. and Lavoie, J.P. (2012) Markers of systemic inflammation in horses with heaves. *J Vet Intern Med* **26**, 1419-1426.

[61] Pollock, P.J., Prendergast, M., Schumacher, J. and Bellenger, C.R. (2005) Effects of surgery on the acute phase response in clinically normal and diseased horses. *Vet Rec* **156**, 538-542.

[62] Holbrook, T.C., Tipton, T. and McFarlane, D. (2012) Neutrophil and cytokine dysregulation in hyperinsulinemic obese horses. *Veterinary Immunology and Immunopathology* **145**, 283-289.

[63] Brooks, M., Leith, G.S., Allen, A.K., Woods, P.R., Benson, R.E. and Dodds, W.J. (1991) Bleeding disorder (von Willebrand disease) in a quarter horse. *J Am Vet Med Assoc* **198**, 114-116.

[64] Macieira, S., Rivard, G.E., Champagne, J., Lavoie, J.P. and Bedard, C. (2007) Glanzmann thrombasthenia in an Oldenbourg filly. *Veterinary clinical pathology / American Society for Veterinary Clinical Pathology* **36**, 204-208.

[65] Giordano, P., Del Vecchio, G.C., Cecinati, V., Delvecchio, M., Altomare, M., De Palma, F., De Mattia, D., Cavallo, L. and Faienza, M.F. (2011) Metabolic, inflammatory, endothelial and haemostatic markers in a group of Italian obese children and adolescents. *European journal of pediatrics* **170**, 845-850.

[66] Huang, K., Zou, C.C., Yang, X.Z., Chen, X.Q. and Liang, L. (2010) Carotid intima-media thickness and serum endothelial marker levels in obese children with metabolic syndrome. *Archives of pediatrics & adolescent medicine* **164**, 846-851.

[67]   Ragab, A., Abousamra, N.K., Higazy, A. and Saleh, O. (2008) Relationship between insulin resistance and some coagulation and fibrinolytic parameters in patients with metabolic syndrome. *Laboratory hematology : official publication of the International Society for Laboratory Hematology* **14**, 1-6.

[68]   Lim, H.S., Lip, G.Y. and Blann, A.D. (2004) Plasma von Willebrand factor and the development of the metabolic syndrome in patients with hypertension. *J Clin Endocrinol Metab* **89**, 5377-5381.

[69]   El-Mesallamy, H.O., Hamdy, N.M., Salman, T.M. and Ibrahim, S.M. (2012) Adiponectin and sE-selectin concentrations in relation to inflammation in obese type 2 diabetic patients with coronary heart disease. *Angiology* **63**, 96-102.

[70]   Treiber, K., Carter, R., Gay, L., Williams, C. and Geor, R. (2009) Inflammatory and redox status of ponies with a history of pasture-associated laminitis. *Vet Immunol Immunopathol* **129**, 216-220.

[71]   Salonen, L.K., Hollands, T., Piercy, R.J. and Verheyen, K.L. (2009) Epidemiology of equine obesity in south-east England: Preliminary findings. In: *48th British Equine Veterinary Association Annual Congress*. p 247.

[72]   Giles, S.L., Nicol, C.J., Rands, S.A. and Harris, P.A. (2015) Assessing the seasonal prevalence and risk factors for nuchal crest adiposity in domestic horses and ponies using the Cresty Neck Score. *BMC veterinary research* **11**, 13.

[73]   Robin, C.A., Ireland, J.L., Wylie, C.E., Collins, S.N., Verheyen, K.L. and Newton, J.R. (2015) Prevalence of and risk factors for equine obesity in Great Britain based on owner-reported body condition scores. *Equine Vet J* **47**, 196-201.

[74]   Wyse, C.A., McNie, K.A., Tannahill, V.J., Murray, J.K. and Love, S. (2008) Prevalence of obesity in riding horses in Scotland. *Vet Rec* **162**, 590-591.

[75]   Stephenson, H.M., Green, M.J. and Freeman, S.L. (2011) Prevalence of obesity in a population of horses in the UK. *Vet Rec* **168**, 131.