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# 9 Highlights

10	٠	Adipose tissue global gene expression differs between normal and previously
11		laminitic ponies.
12	•	The differences are greater in summer compared to winter.
13	•	The differences are not reproduced by feeding a diet mimicking spring grass in
14		winter.
15	•	Similar changes are not detected in peripheral blood mononuclear cells.
16		

#### 17 Abstract

The aims of the study were to determine whether adipose tissue global gene expression (i) 18 19 differs between never laminitic (NL) and previously laminitic (PL) ponies; (ii) is influenced by 20 season and/or a diet designed to simulate spring grass and (iii) differences seen also occur 21 systemically in peripheral blood mononuclear cells (PBMCs). Subcutaneous adipose tissue 22 and PBMCs were obtained from six NL and six PL ponies on three occasions; summer, winter (season study) and in winter after consuming a diet simulating spring grass for seven days 23 24 (diet study). Adipose tissue global gene expression was determined using a 44K equine specific microarray, validated using multiplex quantitative real time PCR (qRT-PCR) and 25 analysed using GeneSpring software and Ingenuity Pathway Analysis. PBMC gene expression 26 27 was quantified using qRT-PCR. The total number of genes whose expression differed (≥2-28 fold change, p≤0.01) between PL and NL ponies was greater in summer (192 genes) compared to winter (58 genes); 40/192 genes influenced by disease in the summer were 29 30 also seasonally regulated and were predominantly associated with inflammation. The genes 31 modified by dietary intervention and PBMC gene expression did not follow the same pattern 32 as the season study. Thus, adipose tissue global gene expression differed between NL and PL ponies most in summer compared to winter, and these differentially expressed genes 33 predominantly related to inflammation. 34

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36 Key words: gene; expression; adipose; laminitis; inflammation

#### 38 **1. Introduction**

Laminitis is a painful condition of the equine foot that is currently divided into three forms, 39 40 namely endocrinopathic, sepsis-associated and supporting limb laminitis [1]. Endocrinopathic laminitis is the commonest form of the disease in the UK occurring in 41 approximately 1.5-4% of the population [2, 3]. Endocrinopathic laminitis encompasses 42 43 laminitis associated with equine metabolic syndrome (EMS), pituitary pars intermedia dysfunction (PPID) and corticosteroid therapy and it is associated with insulin dysregulation 44 (ID) and the detrimental effects this has on the laminae [4]. Equine metabolic syndrome is 45 currently defined as a cluster of clinical abnormalities associated with an increased risk of 46 laminitis. The central feature of EMS is ID [5], which may manifest as hyperinsulinaemia, 47 excessive insulin response to oral carbohydrate or tissue insulin resistance. Additional 48 49 features include hypertriglyceridaemia [6-8], altered circulating concentrations of adipose tissue derived hormones (adipokines) [9-11] and obesity. Some adipokines antagonise the 50 51 actions of insulin further exacerbating ID and obesity is also associated with altered production of inflammatory mediators; thus, adipose tissue may impact glycaemic control, 52 inflammation and cardiovascular function [12]. EMS-associated laminitis appears to occur 53 following ingestion of carbohydrate from the pasture, particularly in spring and summer, 54 possibly due to exacerbation of the hyperinsulinaemia due to an excessive insulin response 55 [7, 13]. 56

57 Much of the understanding of EMS is extrapolated from human metabolic syndrome (HMS) 58 in which obesity, ID, hypertension, plasma dyslipidaemia and altered circulating adipokine 59 concentrations are associated with an increased risk of cardiovascular disease [14]. Thus, 60 similar pathologic mechanisms that underlie the cardiovascular disease associated with

61 HMS, including changes in insulin signalling, adipose-tissue derived inflammatory cytokine production and endothelial dysfunction [14], could contribute to EMS-associated laminitis. 62 DNA microarrays and real-time PCR have been used widely to investigate the genetic and 63 64 molecular basis of obesity, ID and metabolic syndrome in human medicine. For example, ID has been shown to be associated with altered adipose expression of insulin signalling genes 65 [15] and dietary carbohydrate modification induced alterations in adipose gene expression 66 in people with HMS [16]. However, DNA microarrays have yet to be applied to equine 67 68 laminitis.

Rather than utilising DNA microarrays, previous studies investigating the role of adipose 69 tissue in laminitis predisposition and equine metabolic syndrome have focussed on the 70 expression of a small number of genes associated with inflammatory mediators and have 71 produced conflicting results [17, 18]. Whilst visceral adipose tissue appears to play the 72 greatest role in HMS, instead the nuchal ligament adipose tissue depot appears to have a 73 74 unique biological behaviour in the horse and is more likely to adopt an inflammatory 75 phenotype than other depots [18]. The pattern of adipose gene expression in normal and 76 previously laminitic animals when they are consuming summer pasture and a hay-based diet in winter has not been previously explored. Thus, the aims of the study were to determine 77 whether: 1) nuchal ligament adipose tissue global gene expression differs between normal 78 79 and previously laminitic ponies and whether this is influenced by season; 2) a dietary 80 intervention designed to simulate spring grass mimics any observed seasonal changes in gene expression; and 3) the changes seen in adipose tissue gene expression also occur 81 systemically in peripheral blood mononuclear cells. 82

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#### 84 2. Material and Methods

The study was approved by the Royal Veterinary College Ethics and Welfare Committee and carried out under a Home Office Licence (PED1AA054).

87 **2.1. Animals** 

88 Twelve mixed native breed ponies were used in the study. Six were previously laminitic (PL) ponies (defined as having 2 or more episode of laminitis in the last 5 years as diagnosed by 89 90 an experienced equine veterinary surgeon based on clinical signs and radiography; 5 mares, 1 gelding; aged between 12-23 years; weighing 314 ± 29 kg; summer BCS median 6.5, range 91 5-8; winter BCS median 5, range 3-7) and 6 were non-laminitic (NL) ponies (defined as never 92 93 previously having laminitis; all mares; aged between 15-20 years; weighing 292 ± 25 kg; summer BCS median 5.5, range 3-7; winter BCS median 5, range 4-7). All ponies were 94 healthy and had no clinical signs of laminitis during the studies. BCS was estimated by a 95 single experienced observer using a 9-point scale [19]. None of the animals had clinical signs 96 consistent with pituitary pars intermedia dysfunction (apart from laminitis; PL ponies only) 97 and basal ACTH concentrations were within the seasonally adjusted reference range in all 98 animals. 99

#### 100 **2.2.** Collection of adipose tissue and isolation of peripheral blood mononuclear cells

A single subcutaneous adipose tissue biopsy specimen was obtained from the mid cervical
region using a 6 mm biopsy punch (Kruuse, Langeskov, Denmark) under sedation
(detomidine; 10 μg/kg; Domosedan [10 mg/ml IV] solution of injection, Elanco Animal
Health, Hampshire, UK). Incisions were closed in a single layer with suture material (2-0
Ethilon; Ethicon Endo-Surgery [Europe] GmbH, Norderstedt, Germany).

The adipose tissue biopsy specimen was washed in sterile Dulbecco's PBS (without calcium magnesium and phenol red, pH 7.2) and then cut into two pieces (approximate ratio 3:1). The smaller piece was placed into 5 ml of 4 % paraformaldehyde and stored at room temperature. Three days later the tissue was removed and placed into 5 ml of 70 % ethanol and stored at 4°C until further processing. The larger piece was snap frozen in liquid nitrogen and stored at -80°C until isolation of RNA.

Formalin-fixed samples were placed into a Vacuum Infiltration Processor (Tissue-Tek® VIP®, 112 113 Sakura, The Netherlands). Samples were sectioned (5 µm) using a microtome (Leica RM2125, Leica Microsystems [UK] LTD, Milton Keynes, UK), mounted onto SuperFrost®plus 114 slides (VWR International, Leicestershire, UK) and then stained with haematoxylin and eosin 115 using a standard laboratory protocol. Sections were analysed with a Leica DM4000B upright 116 microscope (Leica Microsystems [UK] LTD), magnification X 10 and images taken on a Leica 117 DC500 camera (Leica Microsystems [UK] LTD). The type of tissue obtained by punch biopsy 118 119 was validated as adipose tissue by the appearance of adipocytes (Supplementary 120 information figure 1).

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood collected into heparin (17 IU/ml; Becton, Dickinson and Company, New Jersey, USA) as previously described. The cell pellet was snap frozen in liquid nitrogen and stored at -80°C until further analysis.

125 2.3. Study design

126 There were two parts to the study. The first part of the study involved the collection of 127 adipose tissue biopsy specimens and whole blood on two occasions, namely in winter whilst

128 being fed ad libitum haylage (January) and then summer whilst grazing pasture (June), to compare adipose and PBMC gene expression between NL and PL ponies at these two times 129 of year (season study). The second part of the study (the dietary intervention study) 130 131 involved the collection of adipose tissue biopsy specimens only in the following winter (January). Biopsies were collected before and immediately after consuming a diet (for 7 132 days) designed to mimic spring pasture (ReadiGrass™ [Dried ryegrass pastures; non-133 structural carbohydrate content 216.4 g/kg dry matter; Friendship Estates Ltd, Doncaster, 134 UK; at 1 % dry matter body weight (kg)] and haylage (NSC content 93.7 g/kg dry matter; at 1 135 136 % dry matter body weight [kg]).

137 2.4. Oral glucose test (OGT)

An oral glucose test (1g/kg body weight in a small amount of chaff; blood collected after 0, 90, 120, 150 and 180 min) was performed the day after collection of each adipose tissue sample. Serum insulin concentrations were determined using a commercially available RIA kit (Coat-A-Count Insulin RIA; Siemens) following the manufacturer's instructions which had previously been validated in our laboratory for equine samples [20].

143 **2.5. RNA isolation and cDNA synthesis** 

A pestle and mortar was used to break up the frozen adipose tissue before homogenisation. Ribonucleic acid (RNA) was isolated from the adipose tissue samples using the RNeasy Lipid Tissue Mini Kit (Qiagen Ltd, West Sussex, UK) by use of an RNeasy mini spin column following the manufacturer's protocol and stored at -80°C until analysis. Contaminating DNA was removed by DNase treatment followed by RNA clean up according to the manufacturer's protocol (Qiagen Ltd, West Sussex, UK).

Total RNA was isolated from PBMC using a RNeasy kit (Qiagen) following the manufacturer's protocol. Ribonucleic acid samples (500 ng) were treated with DNase I, following the manufacturer's protocol (Life Technologies Ltd, Paisley, UK). First-strand cDNA synthesis was carried out using M-MLV Reverse Transcriptase, following the manufacturer's protocol (Affymetrix UK Ltd, High Wywcombe, UK). Samples were stored at -20°C until multiplex qRT-PCR analysis.

156 RNA concentration was determined using the Tecan infinite pro 200 plate reader using i-157 control software (Tecan Group Ltd, Mannedorf, Switzerland). RNA quality was assessed on 158 an Agilent 2100 Bioanalyser (Agilent Technologies UK Ltd, Cheshire, UK). RIN values ranged 159 from 7.2-8.8 for the adipose tissue and 7.3-8.5 for the PBMCs.

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2.6.

#### 44K equine specific gene expression one-colour array

DNase treated RNA isolated from adipose tissue was submitted to the Centre for Genomic 161 Research, The University of Liverpool (Liverpool, UK). RNA was labelled with cyanine 3-CTP 162 (Agilent Technologies) using a low input quick amp labelling kit (Agilent Technologies) 163 following the manufacturers protocol. Then cRNA was purified using an RNeasy mini spin 164 column kit (Qiagen) and hybridised to a 44 K equine specific array (Agilent) for 17 hours 165 (10g, 65°C in Tecan HS Pro hybridisation station, Tecan Group Ltd). The chip was scanned 166 using an Agilent DNA microarray scanner Model G250C (Agilent Technologies). All samples 167 passed all quality controls. 168

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- 2.7. 1

#### Multiplex quantitative real time PCR (qRT-PCR)

Twelve genes of interest (Supplementary information Table 1) including genes with a fold change of >4 or 2-4 up and down or no fold change (control) were chosen from the

172 microarray to be validated by multiplex quantitative real time PCR using adipose tissue. Six genes of interest relating to inflammation were chosen to be similarly quantified using 173 PBMCs (Supplementary information Table 2). Additionally, 3 housekeeping genes 174 (Hypoxanthine Phosphoribosyltransferase 1 [HPRT1]; Ribosomal Protein L32 [RPL32], 175 Glyceraldehyde-3-Phosphate Dehydrogenase [GAPDH]) were included as controls. 176 GenomeLab GeXP Analysis System (gRT-PCR; Beckman Coulter, California, USA) was utilised 177 using a single RNA sample from either adipose tissue or PBMC. Equine mRNA sequences 178 from Nucleotide (http://www.ncbi.nlm.nih.gov/nuccore), were imported into the GeXP 179 eXpress Profiler software (Beckman Coulter) and multiplex qRT-PCR primers designed using 180 the following parameters, maximum PCR product = 300 base pairs (bp), minimum PCR 181 product = 100 bp, minimum separation size 7 bp, PCR products were generated between 182 137-237 bp, and *Kan(r)* at 325 bp. Each primer had a Forward universal primer: 183 184 AGGTGACACTATAGAATA and a Reverse universal primer: GTACGACTCACTATAGGGA 185 attached to a gene specific primer sequence (ThermoFisher Scientific, Hertfordshire, UK). Reverse transcription reactions were carried out using the GeXP start kit (Beckman Coulter) 186 according to the manufacturer's protocol. Reactions were carried out using the GeXP PCR kit 187 (Beckman Coulter), according to the manufacturer's protocol. PCR amplification conditions 188 were 95°C for 10mins, followed by 94°C for 30 s, 55°C for 1 min and 70°C for 1 min, 189 repeated for 35 cycles. PCR reaction products were resolved by capillary electrophoresis and 190 detected by the laser on the CEQ 8000 genetic analysis system (Beckman Coulter, serial 191 192 number 306 6606). After amplified fragments were separated, the peaks were gated, and quantified using CEQ 8000 Genetic Analysis software (Version 10.0) of the GeXP eXpress 193 194 Profiler software. The raw data were then imported into the analysis module of the GeXP

eXpress Profiler software and genes of interest were normalised against the geomean of thethree housekeeping genes, as previously described [21].

#### 197 **2.8.** Statistical analysis

198 Results are expressed as mean ± SEM. Unless otherwise stated, statistical analysis was performed in GraphPad<sup>™</sup> prism version 6 (GraphPad<sup>™</sup> Software Inc. California, USA, for 199 windows). Normality of the distribution of the data was assessed using the Kolmogorov-200 201 Smirnov test. An unpaired t test and a paired t test were used to compare pony 202 morphometrics between groups (PL vs. NL ponies) and seasons (summer vs. winter), respectively. The area under curve (AUC) for serum insulin responses to oral glucose 203 (AUC<sub>insulin</sub>) was calculated for each individual pony and results similarly compared between 204 groups (PL vs. NL ponies) and seasons (summer vs. winter). Adipose tissue gene expression 205 data files were analysed using GeneSpring GX V12.5 software (Agilent Technologies). 206 207 Samples were quantile normalised. Gene lists of ≥2 fold change were created. After consultation with a statistician (Dr Yu-Mei Chang, The Royal veterinary College), it was 208 decided that one way and two way ANOVAs were not suitable as the samples were paired 209 210 by season but not groups and this could not be appropriately accounted for in GeneSpring. 211 Consequently, data were analysed in GeneSpring using paired *t*-tests to compare within disease state effects between season and moderated *t*-tests to compare between groups 212 213 and significance set at p<0.01. The following comparisons were made for the season study: 214 PL ponies summer vs. winter, NL ponies summer vs. winter, summer PL vs. NL ponies and winter PL vs. NL ponies; and the following comparisons for the dietary intervention study: PL 215 216 ponies before vs. after dietary intervention, NL ponies before vs. after dietary intervention, 217 PL vs. NL ponies before dietary intervention and PL vs. NL ponies after dietary intervention.

The gene lists that included the gene symbols, Entrez Gene, fold change and *p* value were created. These lists were then loaded into Ingenuity Pathway Analysis (IPA) V119043121 software (Ingenuity, California, USA) for pathway analysis of top canonical pathways, networks, molecular and cellular functions and genes.

qRT-PCR results are expressed as mean ± SEM. A linear mixed model was used to assess data generated in the multiplex qRT-PCR assays (SPSS, PASW Statistics, Version 20, IBM, Illinois, USA; for Windows). Season (January or June) or dietary intervention (before or after feeding diet to mimic spring grass) and group (PL or NL ponies) were treated as fixed effects and pony was considered as a random effect in the mixed effects model. Statistical analysis of gene expression in PBMC was performed in GraphPad<sup>™</sup> prism version 6 (GraphPad<sup>™</sup> Software Inc. California, USA, for windows) and significance accepted at p≤0.05.

229

#### 230 3. **Results**

#### **3.1** Comparison of pony morphometric parameters and OGT AUC<sub>insulin</sub> between previously

232 laminitic (PL) and non-laminitic (NL) ponies

There were no significant differences between the PL and NL ponies with respect to age and height, weight and BCS (season and diet studies). The BCS, but not body weight, significantly (p<0.05) increased in the summer compared to the winter in PL ponies, but not in NL ponies. In both summer and winter the AUC<sub>insulin</sub> was significantly ( $p\leq0.05$ ) greater in PL ponies compared to NL ponies.

# 3.2. Global changes in adipose tissue gene expression between PL and NL ponies in summer and winter (season study)

The total number of genes whose expression differed (≥2-fold change, p≤0.01) between PL
and NL ponies was greater in the summer (192 genes), compared to the winter (58 genes;
Figure 1A). Within groups, 250 genes were differentially expressed between summer and
winter in the PL ponies group, and 340 within the NL ponies group.

Comparisons of these genes lists showed that 40/192 genes that were influenced by disease state in the summer were also seasonally regulated (between summer and winter). Of these 40 genes, 16 (3 of which are un-annotated) were seasonally regulated in PL ponies and 24 (7 of which are un-annotated) in NL ponies (Table 1). The 30 of these 40 genes that were annotated, were functionally classified to be associated with inflammation, transcription and cell growth and movement.

# 3.3 Global gene expression changes between PL and NL ponies following consumption of a diet designed to mimic spring grass

The number of genes modified ( $\geq$ 2-fold change, p $\leq$ 0.01) by dietary intervention did not 252 follow the same pattern as that seen in the season study (Supplementary information Table 253 1). Before dietary intervention, gene expression in the adipose tissue of PL and NL ponies 254 was similar with only 58 genes differentially expressed between groups (Figure 1B). After 255 dietary intervention, the number of genes differentially expressed between PL and NL 256 257 ponies was less (33 genes). In PL ponies, 79 genes were modified by dietary intervention, whilst only 39 genes were modified in NL ponies. Comparisons of these genes lists showed 258 that only 3 genes (2 of which were un-annotated) were modified between both disease 259

state and dietary intervention. The one annotated gene was *ankyrin repeat and SOCS box containing 14,* which plays a role in regulating a suppressor of cytokine signalling.

There was only one gene (*gonadotropin alpha 1 subunit*) that differed between PL and NL ponies in both winter seasons (i.e. the winter of the season study and the winter of the dietary intervention).

3.4 Top ten up-regulated and down-regulated genes in adipose tissue from NL and PL
 ponies in summer and winter (season study)

Analysis of the top 10 up-regulated genes for the season study revealed that 6/10 genes were associated with inflammation and extracellular matrix proteins when comparing PL vs. NL ponies in summer (Table 2). Furthermore, when comparing PL ponies in summer vs. winter, 9/10 top up-regulated genes were also associated with inflammation and extracellular matrix (ECM) proteins. These gene changes were not apparent when comparing PL vs. NL ponies in winter, nor when comparing NL ponies in summer vs. winter, where the top up-regulated genes do not appear to be grouped by role.

When comparing NL ponies in summer vs. winter, 7/10 top down-regulated genes were associated with cellular signalling and vasculature (Table 2). There does not appear to be any specific grouping of role for the top down-regulated genes when comparing PL ponies in summer vs. winter, PL vs. NL ponies in summer and PL vs. NL ponies in winter.

# 3.5 Top ten up-regulated and down-regulated genes in adipose tissue from NL and PL ponies before and after consumption of a diet designed to mimic spring grass (diet study).

Analysis of the top 10 up-regulated genes for the dietary intervention study revealed that 5 out of the top 7 were associated with cellular signalling and 6 out of the top 7 were associated with inflammation and cellular signalling alone when comparing before vs. after dietary intervention in PL and NL ponies respectively (Table 3). Three out of the top four and two out of the top seven up-regulated genes were associated with inflammation when comparing PL vs. NL ponies before and after dietary intervention respectively.

The top down-regulated genes do not appear to be grouped by role for NL before vs. after dietary intervention, before dietary intervention in PL vs. NL ponies or after dietary intervention in PL vs. NL ponies (Table 3). However, 4 out of the top 9 down-regulated molecules for PL ponies before vs. after dietary intervention were associated with inflammation.

The dietary intervention study produced incomplete gene overlap with the season study, 292 with only 10 genes similarly regulated in both studies. Of these 10 genes, matrix 293 metalloproteinase 9 (MMP9), EOMES, Macrophage Scavenger Receptor 1 (MSR1), 294 Dendrocyte Expressed Seven Transmembrane Protein (DCSTAMP), PDK4, Ubiquitin Specific 295 296 Peptidase 37 (USP37), Lymphocyte Antigen 9 (LY9) and Finkel-Biskis-Reilly Murine Sarcoma Virus (FBR-MuSV) Ubiquitously Expressed (FAU) appear to be modified by disease status (PL 297 ponies), but did not have the same regulation for the season study and the dietary 298 intervention study. 299

# 300 3.6 Validation of adipose tissue gene expression using multiplex real time quantitative 301 PCR

Adipose gene expression was validated using qRT-PCR for 9 genes in the season study and 7 genes in the dietary intervention study. Expression of an additional two genes, *Nacylsphingosine amidohydrolase 2B* (*ASAH2B*) and *serum amyloid A1* (*SAA1*), was below the sensitivity of the multiplex qRT-PCR assay and so not quantified.

In the season study, gene expression was significantly different between groups or within groups and between seasons for all comparisons for 6 of the 9 genes (Figure 2; CXCL10, EOMES, CD40L, PDK4, AGPAT6, FLRT2) as measured by both the microarray array and qRT-PCR. The patterns of gene expression were consistent between assays for the remaining 3 genes but significance was not reached for all comparisons using both assays (CALCB, LIPH, RGS1).

In the dietary intervention study, genes statistically significantly different in expression between groups or within groups and before and after dietary intervention were reproducible between microarray array and qRT-PCR analysis for 6 of the 7 genes measured (Figure 3; PDK4, CCL5, EOMES, RGS1, AGPAT6, FLRT2). The pattern of expression of the remaining gene, CXCL10, was consistent between the two assays but only statistically different in expression within NL before and after dietary intervention (Figure 3C) as measured by qRT-PCR but not microarray.

#### 319 3.7 Gene expression in PBMC

The six genes analysed in both the adipose tissue and the PBMC (RGS1, CXCL10, AGPAT6, EOMES, CCL5, CD40LG) did not show the same patterns in expression either between

groups or within groups between seasons (Figure 4). In addition, no alternative patternswere apparent.

#### 324 **3.8** Ingenuity Pathway Analysis of pathways altered by disease state or season

Four out of the 5 top canonical pathways that differed between PL and NL ponies in the summer were associated with inflammation (Table 4). Between seasons, pathways associated with epithelial junctions and vasculature were modified in NL ponies, whilst pathways associated with inflammation and cellular processes were modified in PL ponies.

The most frequently represented networks identified in adipose tissue were associated with inflammation, the cell cycle and lipid metabolism. When comparing PL vs. NL ponies in summer, 3/5 top networks were associated with inflammation or immune responses. (Table 5).

Analysis of the top molecular and cellular functions showed lipid metabolism, molecular transport and small molecule biochemistry to both differ between PL ponies in summer vs. winter and NL ponies in summer vs. winter. Cellular functions such as development, growth and proliferation and movement were different in the summer between PL and NL ponies (Supplementary information Table 3).

Analysis of the predicted top upstream regulators showed that 5 molecules were predicted activators (LPS, HIF1A, TNF, CSF1, TGFB) and 1 molecule (Alpha Catenin) a predicted inhibitor of downstream genes differentially expressed in PL ponies in summer vs. winter (Table 6). Three different molecules (MYC, CSF2, TBX2) were predicted activators of downstream genes in NL in summer vs. winter. Lipopolysaccharide (LPS), a predicted activator in summer vs. winter in PL ponies, was also identified in the comparison PL vs. NL

ponies in summer, but had an activation score below the set threshold (1.9). The most marked predictions were when comparing PL vs. NL ponies in summer, where 9 molecules were predicted activators and 3 molecules were predicted inhibitors of downstream genes (Table 6). These included a number of molecules associated with initiating inflammatory responses or induced by LPS such as IL-1, TLR4 and IL-18.

349

#### 350 **4 Discussion**

The present study sought to determine whether adipose tissue global gene expression 351 differed between normal (NL) and previously laminitic (PL) ponies and whether these 352 353 differences were influenced by season. The total number of genes whose expression differed significantly between the two groups was greater in the summer (192 genes), 354 compared to the winter (58 genes). Of the 192 genes that were influenced by disease state 355 in the summer, 40 were also seasonally regulated and these were functionally classified to 356 be associated predominantly with inflammation, but also regulation of transcription and cell 357 growth and movement. Pathway analysis of these significant genes revealed that four out of 358 the five top pathways were associated with inflammation, the top 5 networks were 359 associated with inflammation, the cell cycle and lipid metabolism and the 5 top upstream 360 regulators were associated with initiating inflammatory responses. Thus, in the season in 361 which the risk of laminitis is greatest, it would appear that the differences in adipose tissue 362 global gene expression are mainly associated with inflammation. 363

Local expression of inflammatory molecules [22] and upregulation of inflammation pathways [23] by adipose tissue depots is reported to play a central role in the onset of local

366 and systemic insulin resistance in HMS; however, studies investigating the role of adipose tissue and inflammatory mediators in equine metabolic syndrome and laminitis 367 368 predisposition have produced conflicting results. Initial studies found that obesity in horses 369 was correlated with systemic inflammation [24, 25], but these findings were confounded by failure to control for age. In ponies with historical laminitis, circulating concentrations of the 370 pro-inflammatory mediator tumour necrosis factor- $\alpha$  were not correlated with obesity [26]; 371 372 there were no differences in systemic markers of inflammation in equids fed to promote obesity compared to equids fed to maintain weight [27]; and markers of systemic 373 inflammation were not related to obesity per se [28]. However, other studies have shown 374 that markers of systemic inflammation are increased in EMS ponies compared to obese 375 insulin sensitive ponies [29]. In studies evaluating adipose tissue expression of a small 376 number of specific genes, although adipose tissue location was associated with differences 377 378 in expression of inflammation-related genes [17], there was no difference in gene 379 expression between insulin sensitive and insulin resistant horses [18]. This is the first study to explore adipose tissue global gene expression and provides evidence to support a role for 380 inflammation in the predisposition to laminitis. 381

Any alterations in gene expression associated with season may be due to changes in diet that inevitably occur with respect to the pasture growth and/or carbohydrate content, or may be due to non-dietary environmental changes. In order to explore this further, the animals were fed a dietary intervention designed to simulate spring grass in winter to determine whether this would mimic the observed seasonal changes in adipose tissue gene expression. The genes modified by dietary intervention did not follow the same pattern as

that seen in the season study with only 10 genes similarly regulated in both studiessuggesting that the seasonal changes were not the result of changes in diet alone.

390 In order to determine whether the seasonal changes seen in adipose tissue global gene expression are reflected systemically, expression of specific genes associated with 391 392 inflammation was explored in peripheral blood mononuclear cells. In agreement with 393 previous studies comparing adipose tissue and PBMC gene expression in obese humans,[30, 31] the six genes that were analysed in both the adipose tissue and the PBMC (RGS1, 394 CXCL10, AGPAT6, EOMES, CCL5, CD40LG) did not show the same patterns in expression 395 between groups or seasons. Additionally, whilst it has been demonstrated that PBMCs 396 might contribute to the low-grade chronic inflammation that characterises obesity and HMS 397 [32, 33], this does not appear to hold true for the genes examined in the present study. 398

The limitations of the study were that a small number of animals were used in each group 399 400 and there will have been variation in the weather conditions from one winter to the next winter, which are poorly controllable. In addition, in order to corroborate the study findings 401 402 further and determine their functional significance, further analysis of the relevant genes at the protein level should have been performed using techniques such as western blotting. 403 However, due to ethical constraints, only very small adipose tissue samples were obtained, 404 meaning that there was insufficient sample available to allow this to be undertaken. Finally, 405 406 due to the age of the animals included in the study, it is possible that age-related changes 407 affected the transcriptome of the adipose tissue. Indeed, it is of particular relevance that 408 evidence of systemic inflammation appears to increase with age in horses [24, 25]. 409 However, there was no significant difference in the ages of the two groups of animal (NL vs

PL ponies) such that both groups will have been equally affected and age should not havebeen a confounding factor.

412

#### 413 **5** Conclusions

In conclusion, it would appear that the differences in adipose tissue global gene expression between normal and previously laminitic ponies in summer, the season in which laminitis risk is increased, predominantly relate to genes, pathways, networks and pathway regulators associated with inflammation. This suggests that laminitis predisposition may be associated with an inflammatory phenotype to the subcutaneous adipose tissue. However, there was no evidence of a contribution from PBMCs.

420

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421

#### 422 Figure Legends

Figure 1: Venn diagram demonstrating the overlap of genes with  $\geq$ 2-fold change in adipose tissue expression generated using an equine 44K microarray in non-laminitic (NL; n=6) and previously laminitic (PL; n=6) ponies A) in summer compared to winter (season study) and B) before and after being fed a diet designed to mimic spring grass (diet study). A Represents up and  $\downarrow$  represents down regulated and number in brackets represents the total number of differentially expressed genes for that comparison.

significantly (p<0.05) different results. The

429	
430	Figure 2: Validation of 9 genes with A) >4-fold, B) 2-4-fold or C) no change in adipose tissue
431	expression generated using an equine 44K microarray in non-laminitic (NL; n=6) and
432	previously laminitic (PL; n=6) ponies in summer compared to winter (season study). Each
433	column represents mean $\pm$ SEM. * denotes significantly (p<0.05) different results. The

patterns of gene expression were consistent between the microarray array and gRT-PCR 434 assays for all of the nine genes assessed, but significance was not reached for all 435 436 comparisons for three of these genes (CALCB, LIPH, RGS1).

437

Figure 3: Validation of 7 genes with A) >4-fold, B) 2-4-fold or C) no change in adipose tissue 438 expression generated using an equine 44K microarray in non-laminitic (NL; n=6) and 439 previously laminitic (PL; n=6) ponies before and after being fed a diet designed to mimic 440 441 spring grass (diet study). Each column represents mean ± SEM. \* denotes significantly 442 (p<0.05) different results. The patterns of gene expression were consistent between the microarray array and qRT-PCR assays for all of the seven genes assessed, but significance 443 444 was not reached for all comparisons for one of these genes (CXCL10)

445

Figure 4: Adipose tissue inflammatory gene expression determined using the 44K microarray 446 compared to peripheral blood mononuclear cell inflammatory gene expression determined 447 using multiplex real time quantitative PCR in non-laminitic (NL: n=6) and previously laminitic 448 (PL; n=6) ponies. Each column represents mean  $\pm$  SEM. \* denotes significantly (p<0.05) 449 different results. The patterns of gene expression were not consistent between adipose 450 451 tissue and peripheral blood mononuclear cells either between groups or seasons.

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# Tables

**Table 1:** Forty genes identified using a 44K equine microarray as being differentially expressed ( $\geq$ 2-fold change, p $\leq$ 0.01) in adipose tissue between seasons (summer vs. winter) and/or disease state (previously laminitic [PL] vs. non-laminitic [NL] ponies; n=6/group). A) 16 genes differentially expressed in summer PL vs. NL as well as PL summer vs. winter, (3 genes are un-annotated and not listed) and B) 24 genes differentially expressed in both summer PL vs. NL and NL summer vs. winter, 7 of which are un-annotated and not listed. \*denotes gene symbol not yet available.

A)

Major classification	Gene name	Gene symbol
Inflammation	Immunoglobulin-like transcript 11 A	ILT11A
	Connective tissue growth factor-like	CTGF
	T-cell immunoglobulin and mucin domain-containing protein 4-	TIMD4
	like	
Cell Growth	Fibroblast growth factor 12-like	FGF12
and movement	Rhophilin-2-like	RHPN2
	Tyrosine-protein kinase FRK-like	FRK
	Excitatory amino acid transporter 3-like	SLC1A1
	GATS Protein-Like 2	CASTOR2
Extracellular matrix	Matrix Metallopeptidase 9	ММР9
Liver function	Solute Carrier Organic Anion Transporter Family, Member 1A2	SLCO1A2
Proteoglycan	Biglycan	BGN
Pseudogene	Short transient receptor potential channel 2-like	TRPC2
Unknown	FANCD2 opposite strand	FANCD2OS
function		

B)

Major	Gene name	Gene symbol
classification		
Lipid mediators	Acyl-CoA Synthetase Long-Chain Family Member 3	ACSL3
Cellular	N-Acylsphingosine Amidohydrolase (Non-Lysosomal	ASAH2
	Ceramidase) 2	RGS1
	Regulator Of G-Protein Signalling 1	CDKN3
	Cyclin-dependent kinase inhibitor 3-like, transcript variant 2	LOC100630765
	Coiled-coil domain-containing transmembrane protein C7orf53	*
	homolog	
Central nervous	Glutamate Receptor, Metabotropic 8	GRM8
system	5-Hydroxytryptamine (Serotonin) Receptor 3A, Ionotropic	HTR3A
Transcription	Glutamate-Rich WD Repeat Containing 1	GRWD1
	Homeobox protein CDX-4-like	CDX4
	Sex Comb On Midleg-Like 4 (Drosophila)	SCML4
	Storkhead Box 1	STOX1
	Ankyrin Repeat And Death Domain Containing 1A	ANKDD1A
Inflammation	SH2 domain-containing protein 1A-like	SH2D1A
	Similar to granzyme A	GZMA
	Interleukin-1 family member 9-like	IL1f9
Smell	Olfactory receptor 2K2-like	OR2K2
Bone	Osteocrin-like	OSTN

**Table 2:** The top five up ( $\uparrow$ ) and five down-regulated ( $\downarrow$ ) genes in adipose tissue grouped by role in non-laminitic (NL; n=6) and previously laminitic (PL; n=6) ponies in summer and winter (season study). Gene lists were generated using IPA ( $\geq$ 2-fold change, p $\leq$ 0.01). If the gene was also differentially expressed, but not in the top 5 genes for other group comparisons in the table, the value for the fold change is also included in brackets. Genes underlined also appear in Table 3.

Major	Gene	NL	PL	Summer	Winter
Classification	symbol	Summer vs.	Summer vs.	PL vs.	PL vs.
		Winter; fold	Winter; fold	NL; fold	NL; fold
		change	change	change	change
Inflammation	SAA1	18.19 (个)			
	<u>MSR1</u>		6.88 (个)		
	DCSTAMP		6.71 (个)		
	CXCL10			4.74 (个)	
	LY9			3.99 (个)	
	PIGR				2.88 (个)
Transcription	SCML4	3.28 (↓)		4.32 (个)	
regulators	PAX6		11.51 (↓)		
	<u>EOMES</u>			3.28 (个)	
	TFAP2B			5.83 (↓)	
	SRY	Y			2.6 (个)
Extracellular	COL6A5		8.10 (个)		
matrix proteins	ITGA11		5.88 (个)		
	<u>MMP9</u>		5.04 (个)	3.78 (个)	
	COL4A3				3.87 (↓)
Central nervous	GRM8	3.96 (↓)		(3.12)	2 67 (.).
system	NCAM2				2.07 (\psi)
Calcium channel	CACNA1G				2.43 (个)
Transferase	MBOAT4				2.41 (个)
Keratin	KRT25	147.51 (个)			
	KRT73	12.39 (个)			
Cell signalling	<u>ASAH2B</u>	12.45 (个)		(-3.97)	
	ABCB11	(-2.86)			2.65 (↓)
	ARPP21	4.87 (↓)			
	<u>RGS1</u>	3.56 (↓)		(2.79)	

		ACCEPTED MA	NUSCRIPT		
	GJB1			7.17 (↓)	
	PLS1			6.37 (↓)	
	<u>FAU</u>		(2.94)		3.26 (↓)
	MX2		8.40 (↓)		
Vasculature	F12	11.64 (个)			
	HBB	12.76 (↓)		56.71	
	CALCB			(↓)	
Hormone	SLC16A10		11.05 (↓)		
	HTR3A	(3.38)		6.52 (↓)	
Lipid mediators	PNLIPRP3		9.17 (↓)		
Mitochondrial	<u>PDK4</u>		(3.00)	Q_	3.36 (↓)
Acyl-CoA	ACOX2		16.29 (↓)		

**Table 3**: The top five up ( $\uparrow$ ) and five down-regulated ( $\downarrow$ ) genes grouped by role in adipose tissue from non-laminitic (NL; n=6) and previously laminitic (PL; n=6) ponies before and after being fed a diet designed to mimic spring grass (diet study). Gene lists were generated using IPA ( $\geq$ 2-fold change, p $\leq$ 0.01). If the gene was also differentially expressed, but not in the top 5 genes for other group comparisons in the table, the fold change value is also included in brackets. Genes underlined also appear in table 2.

Major Classification	Gene	NL after vs.	PL after vs.	After diet	Before diet
	symbol	before diet;	before diet;	PL vs. NL;	PL vs. NL;
		fold change	fold change	fold change	fold change
Inflammation	TNFRSF19		3.48 (个)		2.18 (↓)
	<u>MSR1</u>		3.58 (↓)		
	MASP1	2.86 (个)			
	IL1RN			2.99 (个)	
	ITGAL			2.63 (个)	
	MME		Y	2.11 (↓)	
	IRG1	Y			5.05 (个)
	ADAMDEC1				3.80 (个)
Tumour suppressor	PTCH2		3.17 (个)		
Cell signalling	P2RX1		3.08 (个)		
	ATP1B2		2.27 (个)		
	<u>ASAH2B</u>		2.61 (个)		
	<u>FAU</u>		4.49 (↓)		
	<u>RGS1</u>		3.03 (↓)		
	ATP2B4	2.24 (个)			
	ST6GALNAC1	2.05 (个)			
	CCNE1	2.29 (个)			
	WDR4			2.316 (个)	
Mitochondria	<u>PDK4</u>		4.53 (↓)		
	HOGA1	2.13 (个)			
Kidney	CLCN5			2.234 (个)	
Extra cellular matrix	HPSE	4.75 (↓)	(2.55)		2.679 (↓)
	<u>MMP9</u>				6.831 (个)
Transcription	PEG10	2.83 (↓)			
regulators	<u>EOMES</u>				3.196 (个)
	ZNF541				3.096 (个)

	EGR2				2.403 (↓)
Prostaglandin	HPGD	2.98 (↓)	4.678 (↓)		
synthesis					
Vasculature	FGFR2	2.44 (↓)			
	PAFAH2	2.70 (↓)			
Central nervous	DDC			2.09 (↓)	
system					
Steroid hormone	STAR			2.514 (↓)	
synthesis				R	
Protein modification	USP37			2.452 (↓)	

**Table 4:** The top 5 canonical pathways activated or repressed in adipose tissue in non-laminitic (NL; n=6) and previously laminitic (PL; n=6)

ponies in summer and winter (season study) using IPA analysis. Up represents up-regulation and down represents down-regulation. Pathway

analysis was not completed between group (PL vs. NL) in the winter due to the small number of differentially expressed genes. Data was

generated in IPA using gene lists ( $\geq 2$ -fold change, p $\leq 0.01$ )

Comparison	Pathway	P value	Ratio	Genes
NL summer vs.	eNOS Signalling	p= 4.24E-03	4/155	CCNA2 (up), HSPA14 (up), KNG1 (down), VEGFA
winter			(0.03)	(down)
	Epithelial Adherens Junction Signalling	p= 6.22E-03	4/154	TUBA1C (up), TUBB4B (up), FGF1 (down), SORBS1
			(0.03)	(down)
	Intrinsic Prothrombin Activation	p= 8.61E-03	2/37 (0.05)	F12 (up), KNG1 (down)
	Pathway	Y		
	PXR/RXR Activation	p= 4.62E-03	3/92 (0.03)	ABCB11 (down), ABCC2 (down), INSR (down)
	Remodelling of Epithelial Adherens	p= 4.81E-03	3/70 (0.04)	NME1 (up), TUBA1C (up), TUBB4B (up)
	Junctions			
PL summer vs.	2-ketoglutarate Dehydrogenase	p= 1.72E-02	1/9 (0.111)	DHTKD1 (down)
winter	Complex			
	Acetate Conversion to Acetyl-CoA	p= 2.14E-02	1/11	ACSL1 (down)
			(0.091)	
	LPS/IL-1 Mediated Inhibition of RXR	p= 2.67E-03	5/245	CHST11 (up), ACOX2 (down), ACSL1 (down),
	Function		(0.02)	PPARGC1A (down), SLCO1A2 (down)
	Melatonin Degradation III	p= 4.32E-03	1/6 (0.167)	MPO (up)
	TCA Cycle II (Eukaryotic)	p= 4.39E-03	2/41	DHTKD1 (down), OGDHL (down)

			(0.049)	
Summer PL vs. NL	Altered T Cell and B Cell Signalling in	p=1.47E-04	4/100	CD40LG (up), TLR1 (up), TLR7 (up), TRB (up)
	Rheumatoid Arthritis		(0.04)	A
	CCR5 Signalling in Macrophages	p=6.57E-05	4/97 (0.04)	CCL5 (up), CCR5 (up), CD3G (up), TRB (up)
	Communication between Innate and	p= 3.69E-07	6/112	CCL5 (up), CD40LG (up), CXCL10 (up), TLR1 (up),
	Adaptive Immune Cells		(0.05)	TLR7 (up), TRB (up)
	Hepatic Fibrosis / Hepatic Stellate Cell	p= 7.05E-05	5/155	CCL5 (up), CCR5 (up), CD40LG (up), MMP9 (up),
	Activation		(0.032)	TGFB3 (up)
	Pathogenesis of Multiple Sclerosis	p= 2.35E-	3/10 (0.3)	CCL5 (up), CCR5 (up), CXCL10 (up)
		06		

**Table 5**: The top 5 networks in adipose tissue in non-laminitic (NL; n=6) and previously laminitic (PL; n=6) ponies in summer and winter (season study) using IPA analysis. Up represents up-regulation and down represents down-regulation. Pathway analysis was not completed between group (PL vs. NL) in the winter due to the small number of differentially expressed genes. Data was generated in IPA using gene lists ( $\geq$ 2-fold change, p $\leq$ 0.01).

Comparison	Network	Consistency
		Score
NL summer vs.	Cell Cycle, Cardiac Damage, DNA Replication,	40
winter	Recombination, and Repair	
	Cellular Growth and Proliferation, Endocrine System	33
	Development and Function, Cellular Development	
	Cell Cycle, Developmental Disorder, Hereditary	28
	Disorder	
	Free Radical Scavenging, Small Molecule	25
	Biochemistry, Lipid Metabolism	
	Digestive System Development and Function, Lipid	23
	Metabolism, Molecular Transport	
PL summer vs.	Organ Morphology, Skeletal and Muscular System	33
winter	Development and Function, Cellular Movement	
	Lipid Metabolism, Small Molecule Biochemistry,	33
	Molecular Transport	
	Developmental Disorder, Hereditary Disorder,	29
	Metabolic Disease	
	Cellular Movement, Reproductive System	18
	Development and Function, Cellular Function and	
¥,	Maintenance	
	Endocrine System Development and Function, Tissue	16
	Morphology, Cellular Development	
Summer PL vs. NL	Cellular Development, Haematopoiesis, Cell-To-Cell	38
	Signalling and Interaction	
	Cell-To-Cell Signalling and Interaction, Cell-mediated	30

Immune Response, Cell Death and Survival	
Cellular Movement, Haematological System Development and Function, Immune Cell Trafficking	22
Inflammatory Response, Cardiovascular System Development and Function, Tissue Morphology	17
Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry	15

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**Table 6:** The top upstream regulators in adipose tissue in non-laminitic (NL; n=6) and previously laminitic (PL; n=6) ponies in summer and winter (season study). Pathway analysis was not completed between groups (PL vs. NL) in the winter due to the small number of differentially expressed genes. Positive and negative activation values represent predicted activation or inhibition of downstream molecules, respectively. Data was generated in IPA using gene lists ( $\geq$ 2-fold change, p $\leq$ 0.01) and an activation score of > or < 2 was considered significant.

		Activation
Comparison	Upstream molecule	Score
NL summer vs.	$\sim$	
winter	V-Myc Avian Myelocytomatosis Viral Oncogene Homolog (MYC)	2.777
	Colony Stimulating Factor 2 (Granulocyte-Macrophage) (CSF2)	2.388
	T-Box 2 (TBX2)	2
PL summer vs.		
winter	Lipopolysaccharide	2.986
	Hypoxia Inducible Factor 1, Alpha (HIF1A)	2.425
	Tumor necrosis factor (TNF)	2.348
	Colony Stimulating Factor 1 (Macrophage) (CSF1)	2.183
	Transforming Growth factor beta (Tgf β)	2.13
	Alpha catenin	-2.207
Summer PL vs. NL	Interleukin 1 (IL1)	2.558
	Angiotensinogen (Serpin Peptidase Inhibitor, Clade A, Member 8)	
	(AGT)	2.419
	Poly rI:rC-RNA	2.404
	Interferon alpha/beta (IFN $\alpha/\beta$ )	2.219
1	Interleukin 18 (IL18)	2.198
	Bleomycin	2.18
	Toll like receptor 4 (TLR4)	2.113
	5-O-mycolyl-beta-araf-(1->2)-5-O-mycolyl-alpha-araf-(1->1')-	
	glycerol	2
	Keratin 17 (KRT17)	2
	rosiglitazone	-2.385
	Nuclear Receptor Subfamily 1, Group H (Nr1h)	-2.219
	Insulin Induced Gene 1 (INSIG1)	-2

# Figure 1



B)

PL after diet vs. before diet



# Figure 2

А







С



# Figure 3

Α



В







Figure 4

