TITLE: Seasonal and dietary influences on adipose tissue and systemic gene expression in control and previously laminitic ponies

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Seasonal and dietary influences on adipose tissue and systemic gene expression in control and previously laminitic ponies.

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Highlights

- Adipose tissue global gene expression differs between normal and previously laminitic ponies.
- The differences are greater in summer compared to winter.
- The differences are not reproduced by feeding a diet mimicking spring grass in winter.
- Similar changes are not detected in peripheral blood mononuclear cells.

Abstract

The aims of the study were to determine whether adipose tissue global gene expression (i) differs between never laminitic (NL) and previously laminitic (PL) ponies; (ii) is influenced by season and/or a diet designed to simulate spring grass and (iii) differences seen also occur systemically in peripheral blood mononuclear cells (PBMCs). Subcutaneous adipose tissue 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 (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 analysed using GeneSpring software and Ingenuity Pathway Analysis. PBMC gene expression was quantified using qRT-PCR. The total number of genes whose expression differed (≥2-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 also seasonally regulated and were predominantly associated with inflammation. The genes modified by dietary intervention and PBMC gene expression did not follow the same pattern 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 predominantly related to inflammation.

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

1. Introduction

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

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

HMS, including changes in insulin signalling, adipose-tissue derived inflammatory cytokine production and endothelial dysfunction [14], could contribute to EMS-associated laminitis. DNA microarrays and real-time PCR have been used widely to investigate the genetic and 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 [15] and dietary carbohydrate modification induced alterations in adipose gene expression in people with HMS [16]. However, DNA microarrays have yet to be applied to equine laminitis.

Rather than utilising DNA microarrays, previous studies investigating the role of adipose tissue in laminitis predisposition and equine metabolic syndrome have focussed on the expression of a small number of genes associated with inflammatory mediators and have produced conflicting results [17, 18]. Whilst visceral adipose tissue appears to play the greatest role in HMS, instead the nuchal ligament adipose tissue depot appears to have a unique biological behaviour in the horse and is more likely to adopt an inflammatory phenotype than other depots [18]. The pattern of adipose gene expression in normal and 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 whether: 1) nuchal ligament adipose tissue global gene expression differs between normal and previously laminitic ponies and whether this is influenced by season; 2) a dietary 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 systemically in peripheral blood mononuclear cells.

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).

2.1. Animals

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 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 5-8; winter BCS median 5, range 3-7) and 6 were non-laminitic (NL) ponies (defined as never 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 healthy and had no clinical signs of laminitis during the studies. BCS was estimated by a single experienced observer using a 9-point scale [19]. None of the animals had clinical signs consistent with pituitary pars intermedia dysfunction (apart from laminitis; PL ponies only) and basal ACTH concentrations were within the seasonally adjusted reference range in all animals.

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®, Sakura, The Netherlands). Samples were sectioned (5 µm) using a microtome (Leica RM2125, Leica Microsystems [UK] LTD, Milton Keynes, UK), mounted onto SuperFrost®plus slides (VWR International, Leicestershire, UK) and then stained with haematoxylin and eosin using a standard laboratory protocol. Sections were analysed with a Leica DM4000B upright microscope (Leica Microsystems [UK] LTD), magnification X 10 and images taken on a Leica DC500 camera (Leica Microsystems [UK] LTD). The type of tissue obtained by punch biopsy was validated as adipose tissue by the appearance of adipocytes (Supplementary 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^{o}C until further analysis.

2.3. Study design

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

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 of year (season study). The second part of the study (the dietary intervention study) 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 days) designed to mimic spring pasture (ReadiGrass™ [Dried ryegrass pastures; non-structural carbohydrate content 216.4 g/kg dry matter; Friendship Estates Ltd, Doncaster, UK; at 1 % dry matter body weight (kg)] and haylage (NSC content 93.7 g/kg dry matter; at 1 % dry matter body weight [kg]).

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].

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.

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

2.6. 44K equine specific gene expression one-colour array

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

2.7. 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

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 PBMCs (Supplementary information Table 2). Additionally, 3 housekeeping genes (Hypoxanthine Phosphoribosyltransferase 1 [HPRT1]; Ribosomal Protein L32 [RPL32], Glyceraldehyde-3-Phosphate Dehydrogenase [GAPDH]) were included as controls. GenomeLab GeXP Analysis System (qRT-PCR; Beckman Coulter, California, USA) was utilised using a single RNA sample from either adipose tissue or PBMC. Equine mRNA sequences from Nucleotide (**http://www.ncbi.nlm.nih.gov/nuccore**), were imported into the GeXP eXpress Profiler software (Beckman Coulter) and multiplex qRT-PCR primers designed using the following parameters, maximum PCR product = 300 base pairs (bp), minimum PCR product = 100 bp, minimum separation size 7 bp, PCR products were generated between 137-237 bp, and *Kan(r)* at 325 bp. Each primer had a Forward universal primer: AGGTGACACTATAGAATA and a Reverse universal primer: GTACGACTCACTATAGGGA attached to a gene specific primer sequence (ThermoFisher Scientific, Hertfordshire, UK). Reverse transcription reactions were carried out using the GeXP start kit (Beckman Coulter) according to the manufacturer's protocol. Reactions were carried out using the GeXP PCR kit (Beckman Coulter), according to the manufacturer's protocol. PCR amplification conditions were 95^{o}C for 10mins, followed by 94°C for 30 s, 55°C for 1 min and 70°C for 1 min, repeated for 35 cycles. PCR reaction products were resolved by capillary electrophoresis and detected by the laser on the CEQ 8000 genetic analysis system (Beckman Coulter, serial 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 Profiler software. The raw data were then imported into the analysis module of the GeXP

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

197 **2.8. Statistical analysis**

198 Results are expressed as mean ± SEM. Unless otherwise stated, statistical analysis was
199 performed in GraphPad™ prism version 6 (GraphPad™ Software Inc. California, USA, for
200 windows). Normality of the distribution of the data was assessed using the Kolmogorov–
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),
203 respectively. The area under curve (AUC) for serum insulin responses to oral glucose
204 ($AUC_{insulin}$) was calculated for each individual pony and results similarly compared between
205 groups (PL vs. NL ponies) and seasons (summer vs. winter). Adipose tissue gene expression
206 data files were analysed using GeneSpring GX V12.5 software (Agilent Technologies).
207 Samples were quantile normalised. Gene lists of ≥2 fold change were created. After
208 consultation with a statistician (Dr Yu-Mei Chang, The Royal veterinary College), it was
209 decided that one way and two way ANOVAs were not suitable as the samples were paired
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
212 disease state effects between season and moderated *t*-tests to compare between groups
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
215 winter PL vs. NL ponies; and the following comparisons for the dietary intervention study: PL
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™ prism version 6 (GraphPad™ Software Inc. California, USA, for windows) and significance accepted at $p \leq 0.05$.

3. Results

3.1 Comparison of pony morphometric parameters and OGT $AUC_{insulin}$ between previously 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 $AUC_{insulin}$ was significantly ($p \leq 0.05$) 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 (≥2-fold change, p≤0.01) by dietary intervention did not follow the same pattern as that seen in the season study (Supplementary information Table 1). Before dietary intervention, gene expression in the adipose tissue of PL and NL ponies was similar with only 58 genes differentially expressed between groups (Figure 1B). After dietary intervention, the number of genes differentially expressed between PL and NL 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 that only 3 genes (2 of which were un-annotated) were modified between both disease

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, with only 10 genes similarly regulated in both studies. Of these 10 genes, *matrix metalloproteinase 9* (*MMP9*), *EOMES*, *Macrophage Scavenger Receptor 1* (*MSR1*), *Dendrocyte Expressed Seven Transmembrane Protein* (*DCSTAMP*), *PDK4*, *Ubiquitin Specific 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 ponies), but did not have the same regulation for the season study and the dietary intervention study.

3.6 Validation of adipose tissue gene expression using multiplex real time quantitative 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, *N-acylsphingosine 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.

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 patterns were apparent.

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.

4 Discussion

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

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
367 tissue and inflammatory mediators in equine metabolic syndrome and laminitis
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
370 failure to control for age. In ponies with historical laminitis, circulating concentrations of the
371 pro-inflammatory mediator tumour necrosis factor-α were not correlated with obesity [26];
372 there were no differences in systemic markers of inflammation in equids fed to promote
373 obesity compared to equids fed to maintain weight [27]; and markers of systemic
374 inflammation were not related to obesity *per se* [28]. However, other studies have shown
375 that markers of systemic inflammation are increased in EMS ponies compared to obese
376 insulin sensitive ponies [29]. In studies evaluating adipose tissue expression of a small
377 number of specific genes, although adipose tissue location was associated with differences
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
380 to explore adipose tissue global gene expression and provides evidence to support a role for
381 inflammation in the predisposition to laminitis.

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

388 that seen in the season study with only 10 genes similarly regulated in both studies
389 suggesting 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
391 expression are reflected systemically, expression of specific genes associated with
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,
394 31] the six genes that were analysed in both the adipose tissue and the PBMC (RGS1,
395 CXCL10, AGPAT6, EOMES, CCL5, CD40LG) did not show the same patterns in expression
396 between groups or seasons. Additionally, whilst it has been demonstrated that PBMCs
397 might contribute to the low-grade chronic inflammation that characterises obesity and HMS
398 [32, 33], this does not appear to hold true for the genes examined in the present study.

399 The limitations of the study were that a small number of animals were used in each group
400 and there will have been variation in the weather conditions from one winter to the next
401 winter, which are poorly controllable. In addition, in order to corroborate the study findings
402 further and determine their functional significance, further analysis of the relevant genes at
403 the protein level should have been performed using techniques such as western blotting.
404 However, due to ethical constraints, only very small adipose tissue samples were obtained,
405 meaning that there was insufficient sample available to allow this to be undertaken. Finally,
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 have been a confounding factor.

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.

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Figure Legends

Figure 1: Venn diagram demonstrating the overlap of genes with ≥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). ↑Represents up and ↓ represents down regulated and number in brackets represents the total number of differentially expressed genes for that comparison.

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 ± SEM. * denotes significantly ($p<0.05$) different results. The
434 patterns of gene expression were consistent between the microarray array and qRT-PCR
435 assays for all of the nine genes assessed, but significance was not reached for all
436 comparisons for three of these genes (CALCB, LIPH, RGS1).

437

438 **Figure 3:** Validation of 7 genes with A) >4-fold, B) 2-4-fold or C) no change in adipose tissue
439 expression generated using an equine 44K microarray in non-laminitic (NL; n=6) and
440 previously laminitic (PL; n=6) ponies before and after being fed a diet designed to mimic
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
443 microarray array and qRT-PCR assays for all of the seven genes assessed, but significance
444 was not reached for all comparisons for one of these genes (CXCL10)

445

446 **Figure 4:** Adipose tissue inflammatory gene expression determined using the 44K microarray
447 compared to peripheral blood mononuclear cell inflammatory gene expression determined
448 using multiplex real time quantitative PCR in non-laminitic (NL: n=6) and previously laminitic
449 (PL; n=6) ponies. Each column represents mean ± SEM. * denotes significantly ($p<0.05$)
450 different results. The patterns of gene expression were not consistent between adipose
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 (≥2-fold change, p≤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* *Connective tissue growth factor-like* *T-cell immunoglobulin and mucin domain-containing protein 4-like*	*ILT11A* *CTGF* *TIMD4*
Cell Growth and movement	*Fibroblast growth factor 12-like* *Rhophilin-2-like* *Tyrosine-protein kinase FRK-like* *Excitatory amino acid transporter 3-like* *GATS Protein-Like 2*	*FGF12* *RHPN2* *FRK* *SLC1A1* *CASTOR2*
Extracellular matrix	*Matrix Metallopeptidase 9*	*MMP9*
Liver function	*Solute Carrier Organic Anion Transporter Family, Member 1A2*	*SLCO1A2*
Proteoglycan	*Biglycan*	*BGN*
Pseudogene	*Short transient receptor potential channel 2-like*	*TRPC2*
Unknown function	*FANCD2 opposite strand*	*FANCD2OS*

B)

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

Table 2: The top five up (↑) and five down-regulated (↓) 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 (≥2-fold change, p≤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 Classification	Gene symbol	NL Summer vs. Winter; fold change	PL Summer vs. Winter; fold change	Summer PL vs. NL; fold change	Winter PL vs. NL; fold change
Inflammation	*SAA1*	18.19 (↑)			
	MSR1		6.88 (↑)		
	DCSTAMP		6.71 (↑)		
	CXCL10			4.74 (↑)	
	LY9			3.99 (↑)	
	PIGR				2.88 (↑)
Transcription regulators	*SCML4*	3.28 (↓)		4.32 (↑)	
	PAX6		11.51 (↓)		
	EOMES			3.28 (↑)	
	TFAP2B			5.83 (↓)	
	SRY				2.6 (↑)
Extracellular matrix proteins	*COL6A5*		8.10 (↑)		
	ITGA11		5.88 (↑)		
	MMP9		5.04 (↑)	3.78 (↑)	
	COL4A3				3.87 (↓)
Central nervous system	*GRM8*	3.96 (↓)		(3.12)	2.67 (↓)
	NCAM2				
Calcium channel	*CACNA1G*				2.43 (↑)
Transferase	*MBOAT4*				2.41 (↑)
Keratin	*KRT25*	147.51 (↑)			
	KRT73	12.39 (↑)			
Cell signalling	*ASAH2B*	12.45 (↑)		(-3.97)	
	ABCB11	(-2.86)			2.65 (↓)
	ARPP21	4.87 (↓)			
	RGS1	3.56 (↓)		(2.79)	

	GJB1 *PLS1* *FAU* *MX2*		(2.94) 8.40 (↓)	7.17 (↓) 6.37 (↓)	3.26 (↓)
Vasculature	*F12* *HBB* *CALCB*	11.64 (↑) 12.76 (↓)		56.71 (↓)	
Hormone	*SLC16A10* *HTR3A*	(3.38)	11.05 (↓)	6.52 (↓)	
Lipid mediators	*PNLIPRP3*		9.17 (↓)		
Mitochondrial	*PDK4*		(3.00)		3.36 (↓)
Acyl-CoA	*ACOX2*		16.29 (↓)		

Table 3: The top five up (↑) and five down-regulated (↓) 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 (≥2-fold change, p≤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 symbol	NL after vs. before diet; fold change	PL after vs. before diet; fold change	After diet PL vs. NL; fold change	Before diet PL vs. NL; fold change
Inflammation	*TNFRSF19*		3.48 (↑)		2.18 (↓)
	MSR1		3.58 (↓)		
	MASP1	2.86 (↑)			
	IL1RN			2.99 (↑)	
	ITGAL			2.63 (↑)	
	MME			2.11 (↓)	
	IRG1				5.05 (↑)
	ADAMDEC1				3.80 (↑)
Tumour suppressor	*PTCH2*		3.17 (↑)		
Cell signalling	*P2RX1*		3.08 (↑)		
	ATP1B2		2.27 (↑)		
	ASAH2B		2.61 (↑)		
	FAU		4.49 (↓)		
	RGS1		3.03 (↓)		
	ATP2B4	2.24 (↑)			
	ST6GALNAC1	2.05 (↑)			
	CCNE1	2.29 (↑)			
	WDR4			2.316 (↑)	
Mitochondria	*PDK4*		4.53 (↓)		
	HOGA1	2.13 (↑)			
Kidney	*CLCN5*			2.234 (↑)	
Extra cellular matrix	*HPSE*	4.75 (↓)	(2.55)		2.679 (↓)
	MMP9				6.831 (↑)
Transcription regulators	*PEG10*	2.83 (↓)			
	EOMES				3.196 (↑)
	ZNF541				3.096 (↑)

	EGR2				2.403 (↓)
Prostaglandin synthesis	*HPGD*	2.98 (↓)	4.678 (↓)		
Vasculature	*FGFR2* *PAFAH2*	2.44 (↓) 2.70 (↓)			
Central nervous system	*DDC*			2.09 (↓)	
Steroid hormone synthesis	*STAR*			2.514 (↓)	
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 (≥2-fold change, p≤0.01)

Comparison	Pathway	P value	Ratio	Genes
NL summer vs. winter	eNOS Signalling	p= 4.24E-03	4/155 (0.03)	CCNA2 (up), HSPA14 (up), KNG1 (down), VEGFA (down)
	Epithelial Adherens Junction Signalling	p= 6.22E-03	4/154 (0.03)	TUBA1C (up), TUBB4B (up), FGF1 (down), SORBS1 (down)
	Intrinsic Prothrombin Activation Pathway	p= 8.61E-03	2/37 (0.05)	F12 (up), KNG1 (down)
	PXR/RXR Activation	p= 4.62E-03	3/92 (0.03)	ABCB11 (down), ABCC2 (down), INSR (down)
	Remodelling of Epithelial Adherens Junctions	p= 4.81E-03	3/70 (0.04)	NME1 (up), TUBA1C (up), TUBB4B (up)
PL summer vs. winter	2-ketoglutarate Dehydrogenase Complex	p= 1.72E-02	1/9 (0.111)	DHTKD1 (down)
	Acetate Conversion to Acetyl-CoA	p= 2.14E-02	1/11 (0.091)	ACSL1 (down)
	LPS/IL-1 Mediated Inhibition of RXR Function	p= 2.67E-03	5/245 (0.02)	CHST11 (up), ACOX2 (down), ACSL1 (down), 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 Rheumatoid Arthritis	p=1.47E-04	4/100 (0.04)	CD40LG (up), TLR1 (up), TLR7 (up), TRB (up)
	CCR5 Signalling in Macrophages	p=6.57E-05	4/97 (0.04)	CCL5 (up), CCR5 (up), CD3G (up), TRB (up)
	Communication between Innate and Adaptive Immune Cells	p= 3.69E-07	6/112 (0.05)	CCL5 (up), CD40LG (up), CXCL10 (up), TLR1 (up), TLR7 (up), TRB (up)
	Hepatic Fibrosis / Hepatic Stellate Cell Activation	p= 7.05E-05	5/155 (0.032)	CCL5 (up), CCR5 (up), CD40LG (up), MMP9 (up), TGFB3 (up)
	Pathogenesis of Multiple Sclerosis	p= 2.35E-06	3/10 (0.3)	CCL5 (up), CCR5 (up), CXCL10 (up)

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 (≥2-fold change, p≤0.01).

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

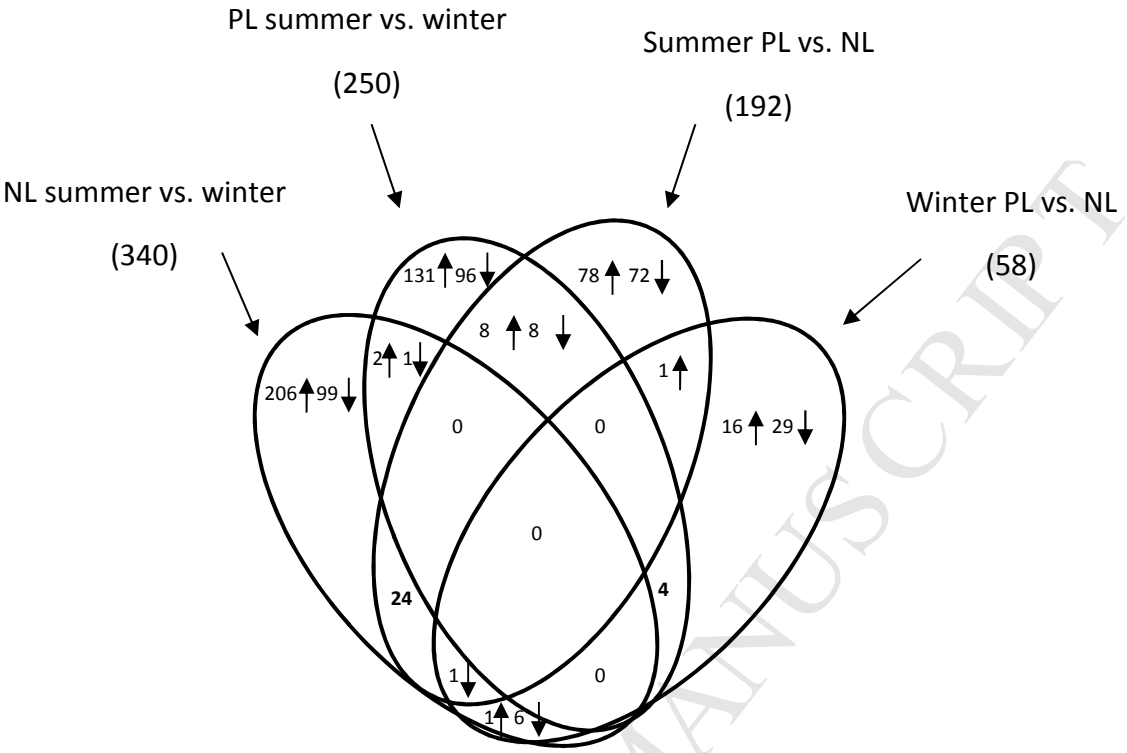
	Immune Response, Cell Death and Survival	
	Cellular Movement, Haematological System Development and Function, Immune Cell Trafficking	22
	Inflammatory Response, Cardiovascular System Development and Function, Tissue Morphology	17
	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry	15

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 (≥2-fold change, p≤0.01) and an activation score of > or < 2 was considered significant.

Comparison	Upstream molecule	Activation Score
NL summer vs. 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 α/β)	2.219
	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

A)



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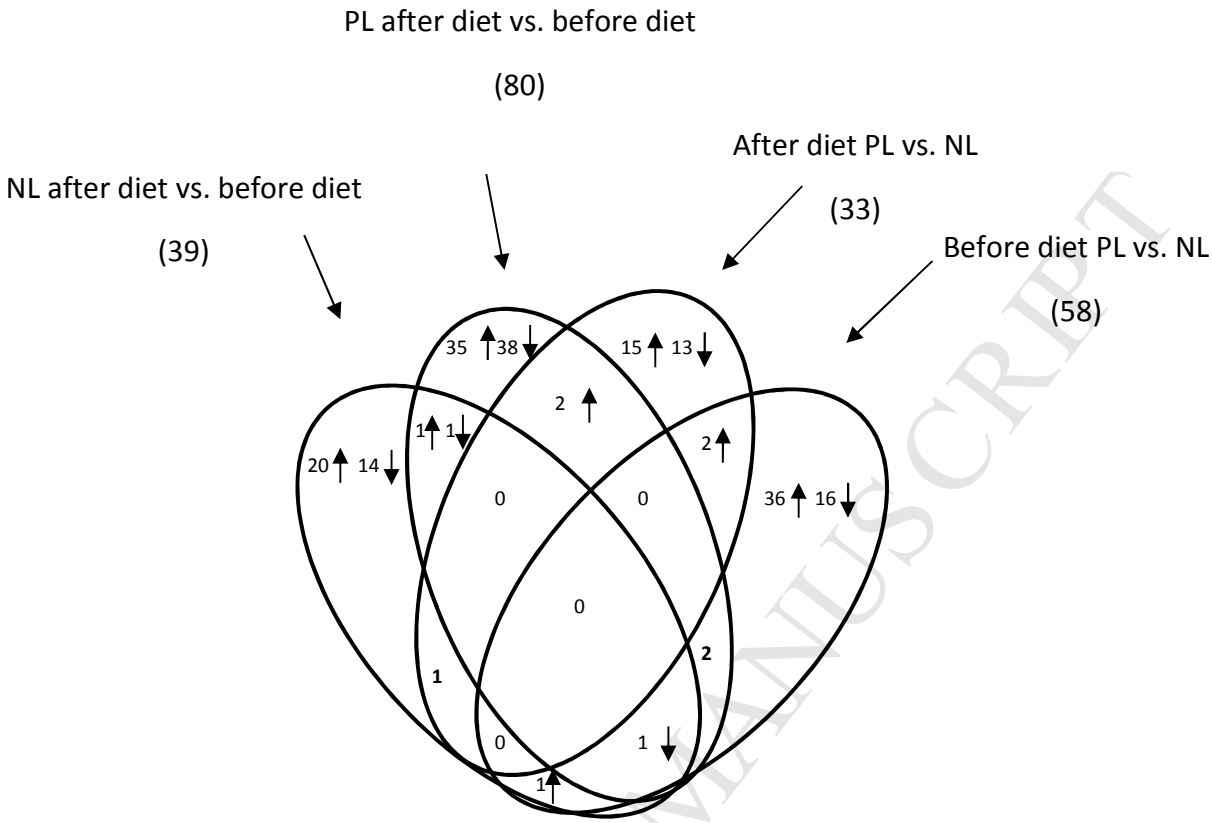
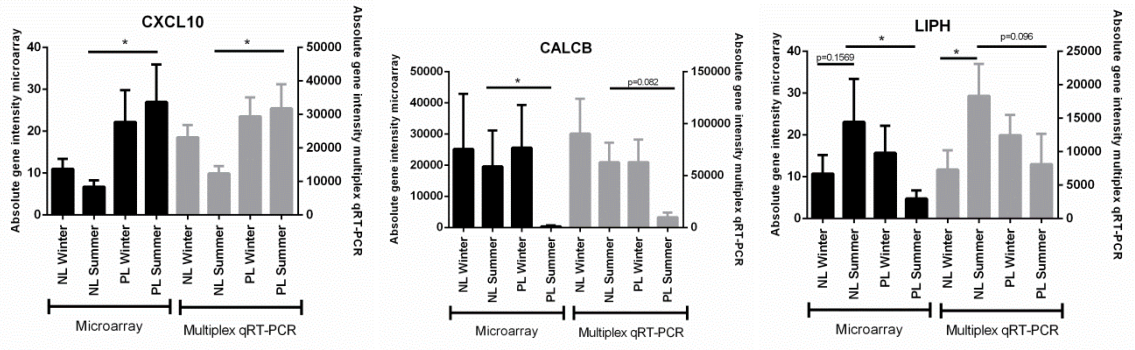


Figure 2

A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p=0.096
p=0.082

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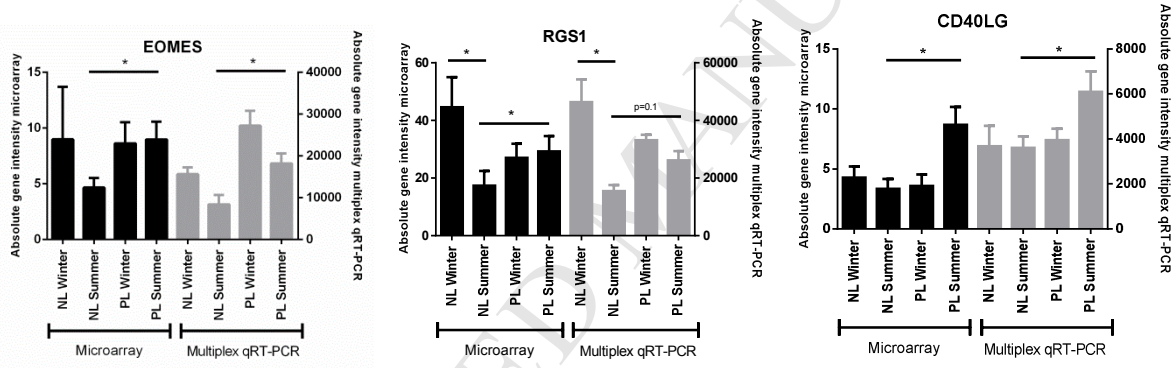
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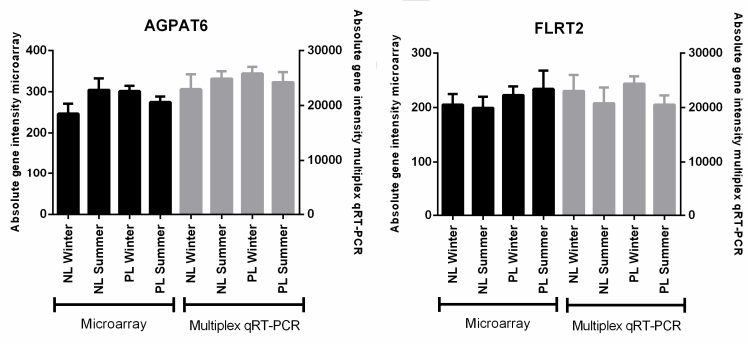
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PL Summer
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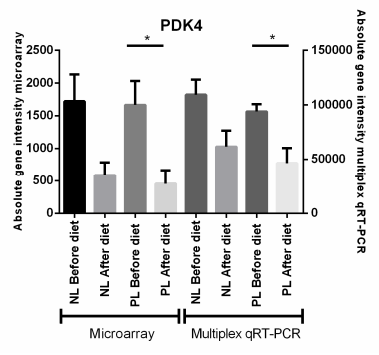
Multiplex qRT-PCR

Figure 3

A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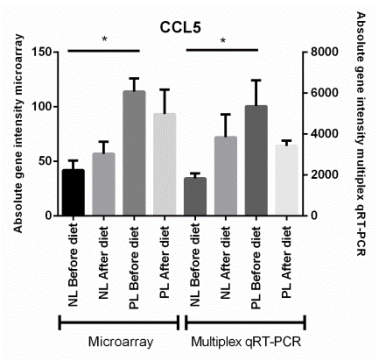
Multiplex qRT-PCR

B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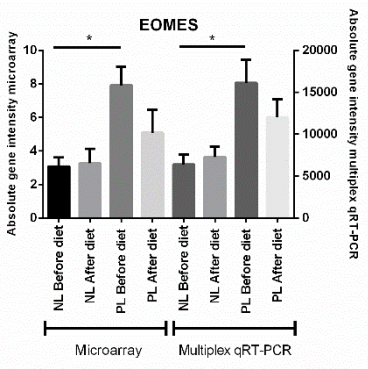
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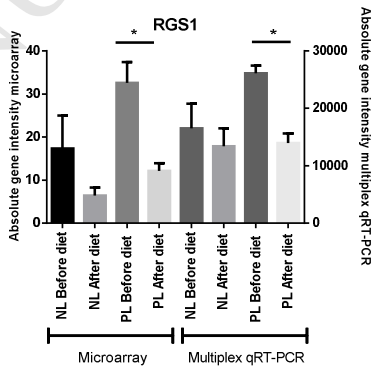
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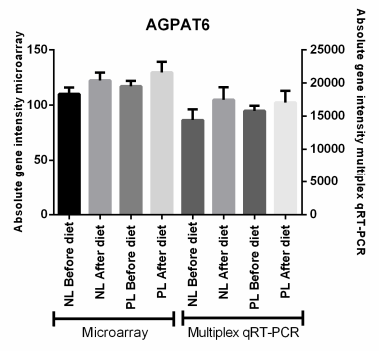
C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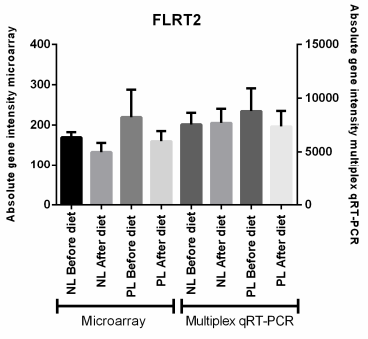
Microarray
Multiplex qRT-PCR


FLRT2
Absolute gene intensity microarray
Absolute gene intensity multiplex qRT-PCR
Microarray
Multiplex qRT-PCR

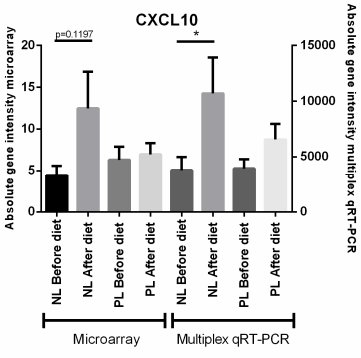

CXCL10
p=0.1197
Absolute gene intensity microarray
Absolute gene intensity multiplex qRT-PCR
Microarray
Multiplex qRT-PCR

Figure 4