WHOLE BLOOD TRANSCRIPTOME ANALYSIS OF INSULIN SENSITIVE AND INSULIN DYSREGULATED MARES

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WHOLE BLOOD TRANSCRIPTOME ANALYSIS OF INSULIN SENSITIVE AND INSULIN DYSREGULATED MARES

An Undergraduate Thesis
Submitted to the Undergraduate Thesis Committee of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements of College Honors in Roger Hadfield Ogden Honors College

by
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ABSTRACT

Equine Metabolic Syndrome (EMS) is a metabolic condition in horses that can lead to catastrophic inflammation of the hoof known as laminitis, among other debilitating symptoms. Although there is no single test for this condition, the most common method of diagnosis is the determination of insulin dysregulation. Horses with EMS typically have high resting insulin concentrations and an exaggerated insulin response to either oral or intravenous glucose. The objective of this experiment was to map the whole blood transcriptome of insulin sensitive and insulin dysregulated horses to determine differentially expressed genes in diseased and non-diseased states. Blood samples were collected from four insulin sensitive and four insulin dysregulated horses residing at the LSU Agricultural Center horse unit. RNA-sequence analysis of whole blood mRNA revealed 896 genes with changed expression in insulin dysregulated horses, 561 upregulated and 335 downregulated. Commonly upregulated pathways include inflammatory and immune responses and steroid biosynthesis. This study is the first to describe transcriptomic differences in the whole blood of insulin sensitive and insulin dysregulated horses.
CHAPTER 1. INTRODUCTION

Equine metabolic syndrome (EMS) is a term used to describe a group of factors that predispose horses to potential diseases, namely laminitis, a severe inflammatory condition of the connective tissue in the hoof. There is no single laboratory test that can definitively diagnosis EMS; however, the biggest factor associated with EMS is insulin dysregulation. Insulin, a hormone secreted by the pancreas, is responsible for moving glucose across cell membranes for either immediate use or storage. As a result, blood glucose decreases in response to insulin secretion. When normal insulin concentrations are unable to move glucose, as in the case of EMS, the pancreas compensates by secreting even more insulin resulting in high circulating levels of insulin, known as hyperinsulinemia. As a result, blood glucose is normal in horses with EMS; however, blood insulin concentrations are typically two-fold higher than normal (Arana Valencia et al., 2014). Chronically elevated concentrations of insulin have been shown to contribute greatly to laminitis (de Laat et al., 2010). Severe cases of laminitis usually result in euthanasia of the horse due to inability to walk or stand comfortably.

One well known risk factor for developing EMS is obesity, particularly fat deposition in certain regions of the body. Obese horses typically have high levels of circulating leptin, a hormone produced by fat cells, and high triglycerides (Frank et al., 2006). These metabolic imbalances can potentially lead to consequences other than laminitis, such as decreased appetite, weight loss, elevated liver enzymes and liver disease, to name a few. While many horses have outward symptoms of EMS, some appear healthy despite underlying metabolic imbalances.

The prevalence of EMS among horse breeds is still largely unknown, although ponies appear at greatest risk of developing hyperinsulinemia and laminitis. Muno et al. (2009) surveyed 300 horses in the United States and found that 22% of them had elevated concentrations of insulin. Breed, sex, and level of exercise did not appear to be risk factors for EMS. Similarly, a study conducted by Pleasant et al. (2013) also found no breed predispositions to EMS. The main factor appears to be hyperinsulinemia and increased body fat. Interestingly, at the LSU horse unit, many of the horses with elevated insulin do not
have the obvious signs of EMS. The most insulin insensitive horse residing there is a 25 year old Arabian with a very normal body weight and body condition. Understanding the unique differences in insulin dysregulated horses is critical to developing clinical therapies to treat EMS.

While hormone analysis has revealed many abnormalities regarding EMS, less is known about genetic differences between healthy horses and insulin-dysregulated horses. The advent of ribonucleic acid (RNA) sequencing has allowed for the quantification and mapping of the entire genetic blueprint (transcriptome) of the cells in a sample. The availability of the transcriptome allows researchers to explore the functions of genes and determine if they are upregulated or downregulated during certain states. Insights into disease processes can lead to identifying markers for disease predisposition, progress, and prognosis. In this experiment, we sought to use this technology to compare the transcriptome of the blood between insulin sensitive and insulin dysregulated horses. Whole blood is easy to obtain and provides a system wide comparison as opposed to a tissue specific comparison.
CHAPTER 2. REVIEW OF LITERATURE

2.1 Insulin and Glucose Metabolism

Insulin, a hormone secreted from pancreatic beta cells, facilitates the transport of glucose across cell membranes (Hadley and Levine, 2006). This usually occurs after a meal when blood sugar levels are high. Insulin binds to cell surface receptors on different tissues of the body activating glucose transporters (GLUT) which transport glucose across the plasma membrane by means of facilitated diffusion. When normal concentrations of insulin are insufficient to transport glucose, a horse is considered insulin resistant. Insulin resistance can be caused by a malfunction in several different endocrine pathways such as glucose transporters, insulin receptors, signaling pathways, or protein malfunction (Bertin and de Laat, 2017). Insulin resistance is the prevailing diagnostic tool to determine EMS. Insulin resistance is identified when there is a lowered ability of sufficient insulin concentrations to lower glucose levels in the bloodstream (Frank et al, 2010).

Glucose, ingested through feedstuffs, must be transported using glucose transport proteins from intestinal lumen and circulation to target cells. Glucose enters enterocytes thorough sodium-dependent glucose transporter proteins. These proteins are mainly located in the small intestine and kidney. There are thirteen currently known members of the sugar transport family. GLUT1-GLUT4 are the glucose transporters. GLUT1 and GLUT3 are located in the plasma membrane, are dependent on insulin, and are responsible for basal glucose transport. GLUT4 is located within organelles. GLUT4 can quickly be transported in response to insulin and increase tissue uptake of glucose 10-to-40-fold within a few minutes (Wattle and Pollitt, 2004).

Blood glucose levels are monitored by glucose sensors, namely, pancreatic beta cells. When blood glucose levels rise, these cells are activated, and insulin secretion is induced. Insulin is the primary molecule responsible for control of glucose levels. The main function of insulin is to enhance glucose
uptake after a meal into fat and muscle cells, as well as inhibiting production and release of glucose from the liver (Wattle and Pollitt, 2004).

2.2 Equine Metabolic Syndrome

Equine metabolic syndrome (EMS) refers to a clinical syndrome in horses and ponies typically characterized by obesity and insulin resistance, akin to type 2 diabetes mellitus in humans. Affected horses are usually “easy keepers” because of their low-calorie requirement. The enhanced metabolic efficiency can be considered a risk factor for the syndrome. This syndrome was not introduced until 2002, making it a relatively new topic of study in veterinary medicine (Frank et al, 2010). Cresty neck scores assess the amount of adipose tissue deposited on the neck region of the animal. Cresty neck scores 3 or great on a range of 0-5 are also common indicators of horses and ponies that are predisposed to the syndrome (Fitzgerald et al, 2019). The spectrum at which this disorder can affect is much larger than we know at this time (Frank et al, 2010).

Horses with EMS are considered compensated insulin resistant. Normal insulin concentrations are insufficient to transport glucose, so the pancreas compensates by increasing production and secretion of insulin. As a result, the insulin response to a meal is much greater than that of a horse without EMS. When glucose is administered, either orally or intravenously, insulin will be hypersecreted and remain increased for a prolonged period of time compared to insulin sensitive horses (Morgan et al, 2015).

The prevalence of EMS is difficult to identify, since there are no specific parameters in diagnosing a horse. However, obesity is shown to be present in 19-40% of domesticated horses and hyperinsulemia is observed in 22-29% of equine populations. Endocrinopathic laminitis has been shown to account for 89% of laminitis cases. This shows us that in the general domesticated equine population, there is a relatively high number of at-risk horses (Frank et al, 2010).

Equine metabolic syndrome could be the result of genetic predisposal combined with environmental influence. From observations of type 2 diabetes mellitus research in humans and mice, it
can be accepted that multiple primary genetic risk alleles are associated with the alterations in insulin and glucose metabolism. These genes can also be assumed to have an epigenetic factor and be altered by environmental interactions (McCue et al, 2015).

2.3 Laminitis

Laminitis can be a devastating and debilitating condition of the hoof. The laminae are interdigitating connective tissues responsible for suspending the bone in the hoof (coffin bone) inside the hoof wall. Inflammation of the laminae, known as laminitis, can quickly lead to separation of the coffin bone from the hoof wall and eventual penetration through the sole of the hoof. Laminitis can develop on any of the horse’s hooves; however, the front hooves are more commonly affected. Laminitis can be developed from husbandry errors such as an excessive grain intake, high sugar pastures, excessive weight bearing due to injury, and too much work on hard surfaces. Laminitis can also be induced by endocrine disorders such as EMS and pituitary pars intermedia dysfunction. Although not completely understood, hyperinsulinemia has been directly implicated in EMS-associated laminitis (Asplin et al, 2007; de Laat et al, 2010).

Laminitis can cause very painful clinical symptoms, usually leading to lameness, weight shifting, camping out of front legs, and increased respiratory and heart rates. Studies suggest EMS-associated laminitis begins as a season problem, with flare ups associated with pasture changes increasing water-soluble carbohydrate content. Pastures have an increase in these types of forages in the summer and fall seasons. These non-structural carbohydrates include sugars, fructans, and starches (Raymond, 2009).

A link between insulin resistance and laminitis is still unknown. There have been studies attempting to replicate the local tissue insulin resistance in laminitis development, but none have been successful in uncovering a direct link. Extreme hyperinsulinemia will lead to the separation of the laminae from the hoof wall (McGowan, 2008).
2.5 Use of Transcriptomic Analysis to Study Diseases

Recently, RNA sequencing technology has increased diagnostic availability and certainty. Through RNA sequencing we can identify, annotate, and classify RNA sequence variants. Whole blood transcriptome analysis has been used in a similar study, in which the objective was to identify differentially expressed genes in bovine with respiratory disease and bovine without respiratory disease (Sun et al, 2020). This study concluded that whole blood transcriptome analysis was a useful diagnostic tool for an otherwise difficult to diagnose disease in a clinical setting. One useful finding of this study was that the results indicated that specific pathways and genes expressed in whole blood are associated with specific pathogens. Identifying these pathogens can lead veterinarians to a more targeted therapeutic approach to treating the disease. It was also concluded that the genes being upregulated in non-diseases animals could be protective mechanisms, reducing the likelihood of an animal to contract the disease. Once upregulated and downregulated genes are identified, animals can be diagnosed and treated more effectively (Sun et al, 2020).

A recent study done in feedlot beef cattle (Jiminex et al, 2021) compared whole blood transcriptomes of cows with Bovine Respiratory Disease (BRD) to those without. Previous studies of this disease only evaluated specific tissue and fluid cells from infection sites (lymph fluid and lung tissue). In this study conducted by the University of Alberta, RNA was extracted from whole blood, for ease of sampling and a bigger picture of the differences in gene expression between healthy and diseases cattle. Upon sequencing, it was discovered that the gene expression profiles were varied between the two groups. Results showed that the major pathways of the diseased cattle were associated with immune response. It was explained that BRD is very difficult to diagnose in a clinical setting, much like EMS. Researchers concluded that the results from whole blood transcriptome provided a general overview of the differential gene expression and health status of the individual animal, and by comparing these results from gene analysis in tissue and infection sites, commonalities can be further studied. RNA sequencing of
whole blood can point us in the direction of biomarkers for eventual early detection of disease and possible targeted therapeutic therapies.
CHAPTER 3. MATERIALS AND METHODS

3.1 Animals

All mares used in this study resided at the Louisiana State University Agricultural Experiment Station and have been residents for a minimum of five years. They were housed together and maintained outdoors on pasture consisting mainly of Coastal Bermudagrass and Bahiagrass. No concentrates were fed. All procedures were approved by the Institutional Animal Care and Use Committee of the Louisiana State University Agricultural Center.

Four insulin-resistant mares, aged 9, 14, 15, and 20 years, were selected based on previous assessments of insulin sensitivity. Four age matched, insulin-sensitive horses were selected for comparison. To confirm insulin dysregulation, all eight mares were subjected to an intravenous insulin challenge and an intravenous glucose challenge prior to blood sampling for RNA extraction.

3.2 Insulin Response to Glucose

On the evening prior to glucose challenge, mares were housed overnight in stalls with unlimited access to water, but not hay (~ 12 hr fast). The next morning, 14-gauge indwelling catheters were inserted into the jugular vein and kept patent using a 0.05% sodium citrate solution. After all catheters were inserted, mares were left undisturbed for an hour to reduce any stress associated artifacts. Blood samples were drawn via the catheter -10 and 0 minutes prior to glucose infusion (100 mg/kg BW as a 50% solution; Durvet Inc., Blue Springs, MO) and continued 5, 10, 15, 20, 25, 30, 35, and 45 minutes after glucose infusion. Blood samples were placed into 6 mL-plastic tubes containing K$_3$EDTA (Vacuette, Greiner Bio-One, Monroe, NC). Plasma was harvested after centrifugation and stored at -20°C. Insulin concentrations were determined by immunoradiometric assay (Immuno-Biological Laboratories, Inc., Minneapolis, MN) and means compared by analysis of variance (ANOVA) accounting for repeated sampling using GraphPad Prism (GraphPad Software, La Jolla, CA).
3.3 Blood Collection and RNA Extraction

Blood samples from all eight mares were collected into Tempus™ Blood RNA tubes (ThermoFisher Scientific, Waltham, MA) and RNA extracted using MagMAX™ for Stabilized Blood Tubes RNA Isolation Kit (ThermoFisher) according to manufacturer recommendations. RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

3.4 Library Preparation

Total RNA was used as input material for the RNA sample preparations. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in First Strand Synthesis Reaction Buffer(5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3’ ends of DNA fragments, Adaptor with hairpin loop structure were ligated to prepare for hybridization. To select cDNA fragments of preferentially 370–420 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. Libraries were then sequenced on the Illumina HiSeq × platform with 150-bp pair-end reads.

3.5 RNA Sequencing Data Analysis

The RNA-seq analysis was performed using Partek flow (Partek). Multiplexed sequencing reads that passed quality control filters were trimmed to remove low-quality reads and adaptors. The quality of the reads after filtering was assessed, followed by alignment to the equine genome EquCab3.0 by STAR (2.5.3a) with default parameters. Approximately 10 million reads per individual sample were generated. Individual mapped reads were quantified to calculate fragments per kilo-base of exon per million mapped
fragments (FPKM) values. Genes with FPKM > 0.1 in at least one sample were kept for downstream analysis, consisting of Pearson correlation, principle component analysis (PCA) and cluster analysis using R (R Foundation for Statistical Computing, version 3.6.0.) Differential gene expression analysis was performed by the Partek Flow GSA algorithm with default parameters. The genes were deemed differentially expressed if they provided a false discovery rate of < 0.05 and fold change > 2. Ingenuity pathway analysis (Qiagen) and KEGG were used to reveal gene ontology and pathways associated with the differentially expressed genes (DEGs) identified.
CHAPTER 4. RESULTS

4.1 Insulin Response to Glucose

Insulin was stimulated (P < .0001) in both groups of mares after glucose infusion. There was an effect of insulin sensitivity (P < 0.01) on insulin concentrations as well as a treatment by time interaction (P < 0.0001; Figure 1).

Figure 1. Plasma insulin concentrations in insulin resistant and sensitive mares after intravenous glucose infusion (100 mg/kg body weight).

4.2 RNA Sequencing

Of all 896 differentially expressed gene transcripts found from the 8 mares, 561 were upregulated and 335 were downregulated (Figure 2). Of the 896, 10 gene transcripts were found to be the most significantly different. These gene transcripts were determined most significant based on the adjusted p-value of ≤ 0.05. RNA sequencing of the 4 insulin sensitive horses and 4 insulin dysregulated horses revealed five gene transcripts that were absent from one or the other group. Two differential gene
transcripts were absent from the insulin sensitive horses, and three differential gene transcripts are absent from the insulin resistant horses.

**Figure 2.** Volcano plot of global RNA sequencing data (n = 19,402 total genes). The x-axis shows the fold change of genes in different samples. The y-axis shows the statistical significance of gene expression level changes. The smaller the corrected p-value, the bigger -\log_{10}(\text{corrected p value}), the more significant the difference. Each point represents a gene, wherein blue indicates no difference, red indicates upregulation, and green dots indicate downregulated differentially expressed genes. Select gene annotations are provided.
Hierarchical clustering analysis confirmed that insulin resistant (R1-4) and insulin sensitive (S1-4) animals are two distinct populations, based on their whole blood relative RNA abundance (Figure 3).

**Figure 3.** Hierarchical clustering heatmap of differentially expressed (P ≤ 0.05) genes (n = 896), as determined by fragments per kilobase of exon per million mapped fragments (FPKM) cluster analysis, clustered by log10(FPKM+1) values. Red denotes genes with relatively high expression levels, whereas green denotes genes with comparatively low expression levels. N.B.
**Figure 4.** KEGG Enrichment GO plot, which shows the relationship between the top 20 most significantly enriched GO terms (padj.), by grouping similar terms together. The ratio is the number of corresponding gene transcripts in each group in relation to the total number of gene transcripts identified in the plot. The color of the dot is correlated to the significance, as measured by the adjusted p-value. The diameter of the dot represents the total gene transcripts found in the specific ontology grouping.
Figure 5. Using Panther gene library, pie charts were assembled grouping gene ontologies of the 896 differentially expressed gene transcripts. Graphs A and B depict the molecular functions, with A being the down-regulated gene transcripts, and B being the up-regulated gene transcripts. Graphs C and D depict the biological processes of the differentially expressed gene transcripts. Graph C representing the down-regulated gene transcripts, and graph D representing the up-regulated gene transcripts.
Figure 6. Logarithmic (log$_2$) fold change [log(FC)] of relative gene transcript (RNA) abundance in whole blood from insulin resistant vs. sensitive (control) horses. Genes exhibiting an adjusted $P \geq 0.05$ are excluded.
CHAPTER 5. DISCUSSION

RNA sequencing uncovered 19,402 gene transcripts from the 8 blood samples that were sequenced. Of the 896 differentially expressed gene transcripts, 561 were upregulated in insulin resistant horses and 335 were downregulated. The transcripts were clustered together based on frequency, showing a clear distinction in expression between the insulin sensitive mares and insulin resistant mares. Within the 896, the most significantly enriched gene ontology groups were discovered using the KEGG gene library. The top 20 gene ontology groups were all related to disease and signaling pathways. The top three groups with the highest gene ratio and most significant adjusted p-values were Influenza A, Hepatitis C, and Kaposi Sarcoma-associated herpes virus infection. These data show a correlation between immune response and the most significant differentially expressed gene transcripts in insulin resistant mares. In other words, the most significantly upregulated gene transcripts in insulin resistant mares were associated with immune function.

When looking at the individual transcripts, using Panther gene library, the up-regulated and down-regulated transcripts were sorted into ontology classifications as well. For both groups, the most common ontologies for molecular functions were binding and catalytic activity. For biological processes, the gene ontologies were more diverse with no clear dominant ontology. The three most represented ontologies for biological process of differentially expressed gene transcripts were biological regulation, cellular process, and metabolic process.

When looking at the gene transcripts with the largest fold change between our experimental group, and control group, and accounting for the adjusted p-value, the top 10 gene transcripts were evaluated. Solute Carrier Family 7, Member 4 (4SLC7A4) was the most significantly upregulated transcript in resistant mares. An exhaustive literature search, we failed to find information regarding the function of this gene other than its involvement in amino acid transport. The next most significant transcript, CCDC183, has been studied in hepatocellular carcinomas in humans. The upregulation of this
gene was shown to have a high correlation with hepatocellular carcinomas (Zhu et al, 2021). Next, the ASS1 transcript upregulation has been shown to be associated hepatocellular adenomas, and increased bleeding in hedgehog species (Sala et al 2020).

Hydroxysteroid (11-Beta) dehydrogenase (HSD11B1) is an enzyme responsible for converting cortisone to cortisol. Variations in the gene that encode for HSD11B have been linked to obesity and insulin resistance in humans. Overexpression of HSD11B1 in adipose tissue resulted in visceral obesity and metabolic syndrome in mice when fed a high-fat diet (Masuzaki et al, 2001). Previous studies involving humans observed that hydroxysteroid (11-Beta) dehydrogenase expression relates to a protective mechanism from general obesity in individuals with type-II diabetes (Chedid et al, 2019). This high expression in insulin resistant horses could also relate to a protective mechanism from obesity. Sulfiredoxin 1 (SRXN1) expression has also been studied to be a protective mechanism in diseased mammals. This gene transcript relates to protection of cells against stress-induced cellular damage (Zhu et al, 2021). Similarly, a study done of gene expression in type-II diabetes, marks Ubiquitin C (UBC) expression as a biomarker of development of the disease. The direct pathway in causation is still unclear (Feng et al, 2019).

There have been many different cellular functions proposed for ADAM Metallopeptidase Domain 12 (ADAM12), but it has been studied as a biomarker for breast and kidney cancer in humans. ADAM12 is strongly upregulated in cancerous tumors (Kveiborg et al, 2008; Gao et al, 2022).

RNA sequencing of diabetic vs. non-diabetic mice (type II) demonstrated an upregulation of PX Domain-Containing Protein Kinase-Like Protein (PXK) transcript in mice with type II diabetes (Zhu et al, 2018), similar to the findings in this study. Variations in this gene are likely due to autoimmune diseases, particularly lupus (Harley et al, 2008).
SYNCRIP, an RNA binding transcript, has been shown to play a significant role in mRNA sorting, but expression has not been related to specific disease pathways (Santangelo et al, 2016). However, sequencing revealed a down regulation of expression in correlation to horses with EMS.

The WNT11 gene transcript was completely absent from all four of the insulin sensitive mares sequenced. All four insulin resistant mares were found to have at least 1.1847 FPKM of WNT11. In a study done on human bone marrow, results showed that high glucose levels upregulate levels of WNT11 (Filteau et al, 2013). These results support the finding of WNT11 up-regulation as a biomarker for EMS, as EMS horses are unable to uptake glucose as efficiently as non-diseased horses. Horses with EMS exhibit a slow return to normal blood-glucose levels after a meal, as opposed to healthy horses, which are much more efficient in glucose use and storage as directed by insulin. In a recent study from 2020, WNT11 in mice correlated higher insulin sensitivity to the downregulation of the gene (Nie et al, 2021). It is possible that WNT11 is a significant biomarker for EMS. The up regulation of this gene corresponds to insulin resistance. Conversely, the downregulation, or absence, of this gene corresponds to insulin sensitivity.

Summary and Conclusion

RNA sequencing revealed a significant amount of differentially expressed gene transcripts between insulin sensitive and insulin resistant mares. Most of the upregulated gene transcripts in insulin resistant mares were related to immune function. Several genes were also differentially expressed in human and mice with type 2 diabetes mellitus, providing further evidence that EMS shares many similarities with type 2 diabetes. This is the first study to assess transcriptomic differences between horses with EMS and those without.
REFERENCES


