DISSERTATION

CIRCULATING MICRO RNA IN INSULIN RESISTANT HORSES

Submitted by

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ABSTRACT

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Insulin resistance is a prevalent pathophysiological disorder among domestic horses and is associated with many other conditions such as abnormal adiposity, chronic low-grade systemic inflammation, equine metabolic syndrome, and pituitary pars intermedia dysfunction. However, the processes leading to equine insulin resistance and associated conditions remain a subject of study. Studies in human and laboratory animals have shown that a class of small non-coding RNAs, known as microRNAs (miRNAs), are involved in the regulation of many biological processes and are associated with many disease.

MiRNAs have emerged as potential biomarkers in studies for the diagnosis and prognosis of many diseases, including type 2 diabetes and metabolic syndrome. Profiling circulating miRNAs allows researchers to understand further the mechanisms involved in many diseases, including type 2 diabetes mellitus and metabolic syndrome in humans, and it has excellent potential for equine medicine.

In our pilot study, we hypothesized that insulin resistant horses would have a different circulating miRNA profile than those that are healthy. 6 mares were selected from an initial population to represent the most insulin-sensitive (n = 3) and insulin resistant (n = 3) states. Serum samples were collected for miRNA profiling of these animals, investigating the presence and relative amount of 340 equine miRNAs. Results showed different miRNA profiles between groups, with a total of 14 miRNAs differently expressed between insulin resistant and insulin sensitive animals. Furthermore, results of this preliminary study suggested circulating miRNA profiles as potential

new tools for evaluating the mechanisms of insulin resistance in horses and the development of novel diagnosis and treatment methods for this condition in equines.

The current study aimed to follow up the pilot study by increasing the number of animals undergoing miRNA profiling and including both horses and ponies for testing. It was hypothesized that insulin resistant animals (horses and ponies) would have a different circulating miRNA profile than those that are healthy. Additionally, circulating miRNA profiles of horses and ponies were also compared.

Two initial populations, one of horses and one of ponies, were screened for their insulin sensitivity state using basal proxies for insulin and glucose. Selected animals, 12 non-pregnant Thoroughbred/ Thoroughbred-cross and 12 non-pregnant Welsh/Dartmoor mares were evaluated for insulin sensitivity with the frequent sampling intravenous glucose tolerance test (FSIGTT), and serum samples collected for miRNA profiling. The quantification of miRNAs was done through qRT-PCR analysis performed to investigate the presence and relative amount of 340 equine miRNAs. Confirmation by quantitative real-time polymerase chain reaction revealed that miRNA was present in the serum of all animals. After diagnosis based on the FSIGTT results, mares were divided into groups representing their insulin sensitivity state: insulin sensitive (IS, n = 13, ten horses and three ponies) or insulin resistant (IR, n = 11, two horses and nine ponies) animals, and their miRNA profile compared.

Results demonstrated that from the 340 miRNAs analyzed, 13 miRNAs were differentially expressed between insulin resistant and insulin sensitive horses, 15 differently expressed between insulin resistant and insulin sensitive ponies, 17 differently expressed between horses and ponies, and 14 differently expressed between insulin resistant and insulin sensitive animals, horses and ponies combined ($p<0.05$), with 4 of these miRNAs already noted when comparing horses versus ponies. In the horse groups, three miRNAs were expressed in the insulin resistant

group only. Finally, eight circulating miRNAs are proposed as potential regulators of equine insulin resistance.

The results of this study, in addition to our preliminary investigation, suggest potential new tools that could be used to understand further the mechanisms involved in equine insulin resistance and associated conditions and for the development of new, practical and efficient diagnosis and prognosis methods for this condition in horses.

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INTRODUCTION

Insulin resistance is a prevalent pathophysiological disorder among domestic horses and is associated with many other conditions such as abnormal adiposity, chronic low-grade systemic inflammation, equine metabolic syndrome, and pituitary pars intermedia dysfunction. Insulin resistance has become a rising topic of interest in research due to its association with endocrinopathic laminitis, where insulin resistant horses have an increased predisposition for developing laminitis. Coffman and Colles, 1983, first documented this association between insulin resistance and the development of laminitis. Furthermore, Field and Jeffcott, 1989, reported that ponies that were obese and ponies that had previously suffered laminitis were more intolerant to oral glucose loading and showed a greater response in plasma insulin levels after glucose loading, than regular ponies or Standardbred horses. These findings supported the relevance of insulin effectiveness and the regulation of carbohydrate and lipid metabolism in the pathogenesis of laminitis. In addition, Garcia and Beech, 1986, demonstrated that horses with clinically diagnosed pituitary adenoma have resting hyperglycemia and a delayed return of glucose values to baseline after intravenous glucose injection, indicating an apparently reduced tissue sensitivity to insulin in these animals. Since then, many researchers have directed their attention to the pathophysiology of equine insulin resistance. Although much has been learned in the past decades, the understanding regarding processes leading to equine insulin resistance and associated conditions remains limited.

It is known that genetic factors such as breed, environmental factors such as chronic overnutrition, lack of exercise, and the combination of these factors are critical players for the development of equine insulin resistance. The diagnosis of insulin resistance is most accurately assessed by performing dynamic tests such as the frequently sampled intravenous glucose tolerance (FSIGT) test with minimal model analysis. However, this test is highly time-consuming, expensive, and

impractical for field situations, which brings opportunities for new strategies for diagnosing insulin resistant animals. Since their discovery by Lee and colleagues in 1993, microRNAs (miRNAs) have emerged as exciting regulators of biological processes and levels of specific miRNAs circulating in the bloodstream can serve as potential biomarkers for the diagnosis and prognosis of many diseases, including type 2 diabetes in humans and mice. This class of small noncoding RNAs function as post-transcriptional regulators of gene expression and with important roles in diverse biological processes. Profiling of miRNA has become a powerful and efficient tool in disease research, including type 2 diabetes mellitus and metabolic syndrome in humans, and has great potential for equine medicine.

The current study hypothesized that insulin resistant horses and ponies would exhibit differential circulating miRNA profiles when compared to healthy animals. The characterization of circulating miRNA profiles associated with equine insulin resistance can help with the identification of candidate genes essential for equine insulin resistance and its associated conditions, as well as gathering information that could contribute for the development of future diagnosis and treatment of equine insulin resistance.

CHAPTER 1: LITERATURE REVIEW

1.1. Overview of insulin secretion and action

Insulin is a polypeptide hormone secreted by pancreatic beta-cells and regulates energy homeostasis by coordinating storage, mobilization, and utilization of fat and glucose in various organs, mainly muscle, adipose tissue, and the liver [1,2]. It also promotes cell division and growth through mitogenic effects [3].

Many factors stimulate insulin secretion. In humans, insulin secretion in response to glucose in healthy individuals is biphasic. In the first phase, insulin is released rapidly within 1 minute, peaking at 3-5 minutes, and lasting about 10 minutes after administration of an intravenous bolus of glucose in humans. The second phase is slower and not apparent until 10 minutes after glucose administration, lasts proportionally to the administrated glucose concentration, and continues for as long as the hyperglycemia (excess of glucose in the bloodstream) persists. The amount of insulin secreted in the bloodstream during the first phase is the insulin already synthesized and stored in secretory granules in pancreatic cells, while the portion of insulin released in second phase counts with newly synthesized insulin in addition to the already stored [4–6]. Horses, like humans, have also been shown to have a biphasic insulin secretion under both oral and intravenous glucose loads, with peaks of insulin measured at 180 minutes after starting the oral glucose load and 10 minutes after intravenous glucose load [7].

Insulin secretion is dependent on several factors. For example, insulin secretion following an intravenous glucose load is stimulated by the total dose of glucose and glucose rate of administration. Following an oral glucose load, insulin secretion also depends on the total dose of glucose and its rate of administration. However, there are additional factors that determine the appropriate response of insulin secretion after the oral load [4,5]. These additional factors include:

(I) diet composition, (II) gastro-intestinal hormones, (III) neural input associated with glucose ingestion. More specifically, (I) diet composition, influences gastric emptying and gastrointestinal motility, affecting glucose absorption. For example, increased dietary fiber leads to increase in the viscosity of liquids in the digestive tract, slowing the diffusion of glucose in the intestinal lumen. It also causes glucose molecules to interact with molecules in dietary fiber, preventing transport of glucose from the intestine, and can inhibit the activity of amylase enzymes, which convert starch to glucose [8]; (II) gastro-intestinal hormones, which response depends on relative proportions and forms of various nutrients, can facilitate downstream hormonal responses such as insulin release, and send signals to the brain to control feed intake. For example, glucose-dependent insulinotropic polypeptide (GIP), which enhances glucose-stimulated insulin secretion (incretin effect), delays gastric emptying and signals satiety. In horses, plasma GIP concentrations increase after oral glucose tolerance test, which stimulates insulin secretion more than intravenously glucose infusion [7,9], whereas intravenous glucose infusion has no effect on plasma GIP concentrations [7]; and (III) neural input associated with glucose ingestion. The parasympathetic nervous system, which is activated during gastric stimulation, can increase insulin secretion. In contrast, the sympathetic nervous system, which in contrast to the parasympathetic nervous system, prepares the body for intense physical activity, is activated during stress and can cause suppression in insulin secretion [5,10,11].

Non-glucose stimuli for insulin secretion also include other nutrient substrates like (I) proteins (amino acids): Arginine and leucine are the two most potent amino acids in insulin secretion stimulation, and it has been demonstrated that supplementation of dietary leucine can augment serum insulin response to an oral glucose load in horses [12]; and (II) fats (fatty acids and glycerol): Glycerol can be converted to glucose via gluconeogenic pathways stimulating insulin secretion. In addition, non-esterified fatty acids can reduce peripheral insulin sensitivity and insulin synthesis, increase hepatic glucose output, and decrease glucose-stimulated insulin secretion,

which can lead to decreased insulin effectiveness overtime. These nutrients can be either absorbed from the gastrointestinal tract or endogenously produced and released from specialized cells in body tissues [5,12,13]. In the horse, it has been demonstrated that the entero-insular axis, which includes neuronal and endocrine compounds, can intensify the glucose-induced pancreatic insulin secretion upon the stimulus of food absorption load [7].

One of the main physiological actions of insulin is the regulation of plasma glucose by stimulating glucose uptake by insulin-dependent cells. The major site of glucose absorption in horses maintained on conventional pasture forage diets is in the proximal and mid sections of the small intestine. Glucose is transported across the equine intestinal membrane through the sodium/glucose cotransporter isoform 1 (SGLT1). Glucose is then transported to the circulation from the intestine cells by GLUT-2 in a concentration gradient manner. Another significant source of glucose is provided by short-chain fatty acids absorbed from the horse's caecum and colon. These regions contain a diverse community of anaerobic bacteria, protozoa and fungi, which metabolize dietary carbohydrates into monomeric sugars, then into pyruvate and finally into short-chain fatty acids, such as mainly acetate, propionate and butyrate. Absorbed propionate is utilized in the liver as the precursor of gluconeogenesis, being converted into glucose for energy utilization in the horse metabolism [14,15]. Once released into the circulation, these glucose molecules will stimulate insulin action and be uptake by insulin-dependent cells.

Glucose uptake by these cells is facilitated by diffusion via transmembrane glucose transporters (GLUT). Different isoforms of GLUT can be glucose concentration gradient dependent, such as GLUT-2, or insulin dependent, such as GLUT-1 and GLUT-4 [2]. Skeletal muscle is the major collaborator to insulin-induced glucose uptake in the body. When there is not enough physiological response to the insulin action, the ability of the skeletal muscle to buffer increases in plasma glucose decreases, leading to short and long term metabolic abnormalities [16].

Insulin also regulates lipid mobilization by inhibiting hormone-sensitive lipase (HSL) in adipose tissues and stimulating lipoprotein lipase (LPL) in peripheral tissues. Thus, decreased response

to the hormone action can lead to a high level of plasma triglyceride concentration, hyperlipidemia. Hyperlipemia in horses is associated with lipemia (visual plasma turbidity) and infiltration of organs (mainly liver and kidneys) by lipids [17]. The excessive amount of fatty acids in tissues can lead to lipotoxicity, inflammation, mitochondrial dysfunctions, impaired insulin response and decreased glucose uptake [16]. Furthermore, excess adiposity is associated with increased lipid influx into the liver and increased *de novo* hepatic lipogenesis, promoting hepatic triglyceride accumulation and leading to non-alcoholic fatty liver disease (NAFLD) [18].

In the pancreas insulin is initially stored in vesicles within the beta-cells of the pancreatic islets of Langerhans. GLUT-2 transporters within the membrane of pancreatic cells are responsible for the uptake of circulating glucose through facilitated diffusion. Once into the pancreatic cells, the glucose is metabolized for ATP generation through glycolysis. The increase of intracellular ATP concentration results in closure of ATP-sensitive K^+ channels and subsequent depolarization of the cell membrane and the opening of Ca^{++} channels. In humans, the increase of intracellular Ca^{++} levels triggers the formation of a synaptosomal-associated receptor proteins (SNAREs) complex and in consequence, the fusion of the insulin vesicles with the plasma membrane and the release of the insulin hormone into the extracellular environment through exocytosis [2].

As insulin is released into the blood stream, it starts promoting glucose uptake by target tissues, particularly skeletal muscle, liver and adipose tissues. Insulin regulates the activity of GLUT-4 by inducing its translocation from the intracellular compartment to the plasma membrane, resulting in increased glucose uptake in response to high blood glucose concentrations via signaling cascade pathways [2]. Insulin interacts with its targets via cell membrane insulin receptors, which are a subfamily of receptor tyrosine kinases. When insulin binds to the alpha-subunit of the insulin receptor, it triggers autophosphorylation of the beta-subunits and the translocation of insulin receptor substrate (IRS)-1 to the plasma membrane occurs.

At the plasma membrane, IRS-1 undergoes tyrosine phosphorylation and stimulates phosphatidyl (PI)-3-kinase (PI3-K) activation. This process causes a cascade of events, including PI3K-dependent kinases activation, serine/threonine kinase (Akt) activation, and GLUT-4 translocation to the cell membrane, resulting in glucose uptake by the cells [19–22]. The insulin signaling pathway and its main physiological actions in the horse can be found in Figure 1. Furthermore, interferences in these signaling cascade pathways, from the insulin receptor to the downstream signal proteins, can have detrimental impacts on insulin action resulting in insulin resistance [22].

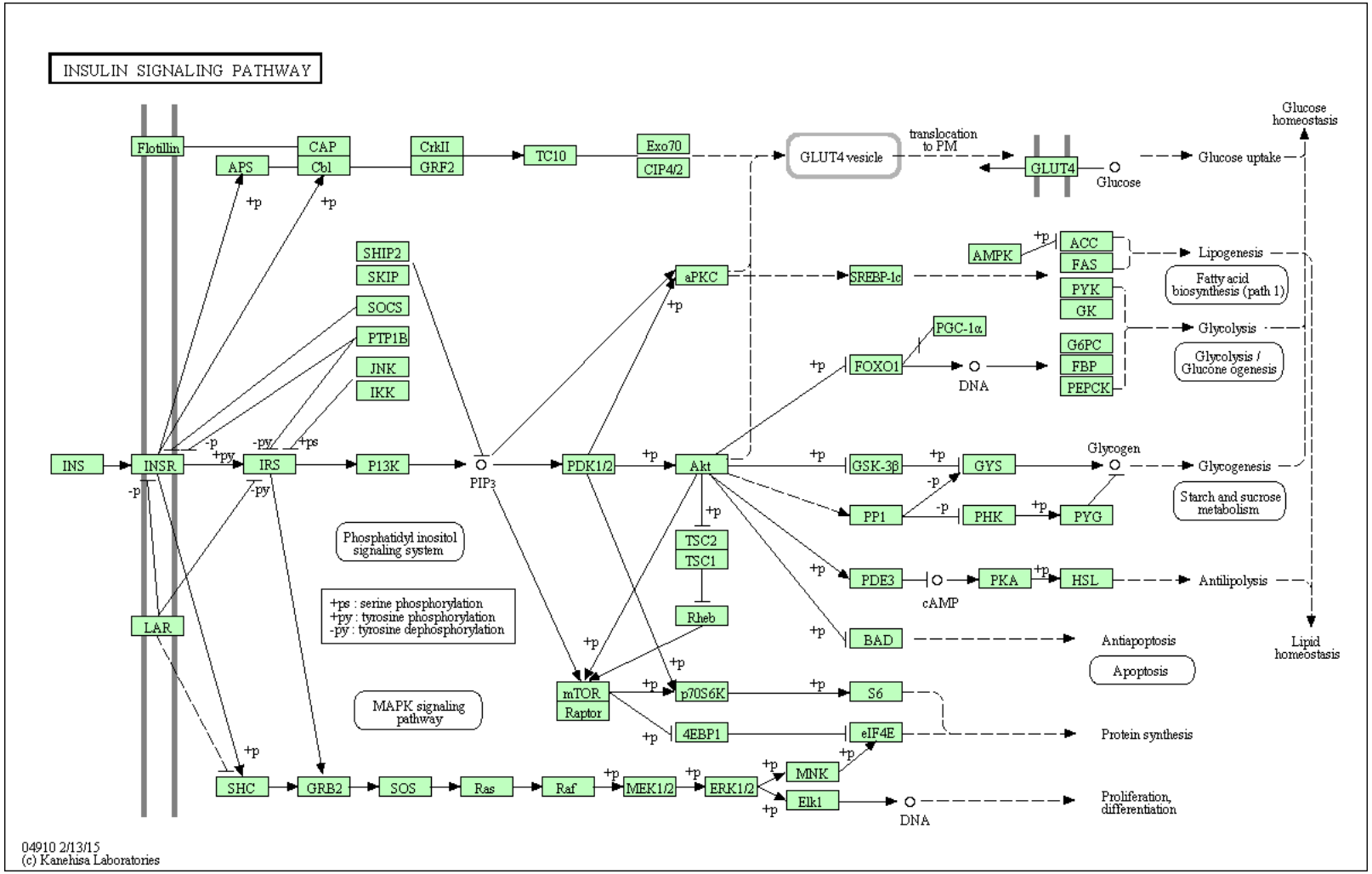

Figure 1 Equus caballus (horse) insulin signaling pathway. Insulin hormone (INS) binding to its receptor (INSR) leads to phosphorylation of insulin receptor substrates (IRS). This allows IRS to interact with regulatory subunit of phosphoinositide 3-kinase (PI3K). PI3K activates Akt, which leads to (1) deactivation of glycogen synthase kinase 3 (GSK-3), and activation of glycogen synthase (GYS), resulting on glycogen synthesis; (2) translocation of GLUT4 vesicles, which can also occur through CAP/Cbl/TC10 pathway, from intracellular pool to the plasma membrane for glucose uptake by the cell; (3) mTOR-mediated activation of protein synthesis. Both IRS and INSR can interact to GRB2, which can lead to activation of mitogen-activated protein kinase (MAPK) and mitogenic responses in the form of gene transcription, leading to cell proliferation and/or differentiation. ***Original source:*** *KEGG: Kyoto Encyclopedia of Genes and Genomes. DIANA-miRPath v3 pathway analysis (Vlachos et al., 2015).*

1.2. General pathophysiology of insulin resistance

Insulin resistance is a complex metabolic abnormality that mainly affects the ability of peripheral tissues (liver, skeletal muscle and adipose tissue) to respond to insulin action. It is defined as a clinical state in which a normal or elevated insulin level produces an attenuated biologic response [23] and it can lead to impairment of plasma glucose utilization, development of hyperglycemia, and compensatory hyperinsulinemia. The compensatory hyperinsulinemia occurs as pancreatic beta-cells increase insulin secretion, in response to peripheral insulin resistance in target tissues, to maintain normal blood glucose levels [3]. These processes force hyper-secretion of insulin, generating cellular stress and progressive beta-cell failure [21,22,24]. Additionally, although compensatory hyperinsulinemia happens to compensate for decreased insulin action in certain resistant pathways, other unaffected pathways can be overstimulated by the action of insulin [25]. In humans, elevated plasma insulin concentrations can enhance very-low-density lipoprotein (VLDL) synthesis, leading to hypertriglyceridemia and formation of fatty plaques in the arteries. Additionally, insulin is known to be atherogenic, also tending to promote the formation of fatty plaques in the arteries. Therefore, compensatory hyperinsulinemia state can also lead to hypertension and atherosclerotic cardiovascular disease [26]. It has been demonstrated that summer pastures can induce hypertension and insulin resistance in ponies predisposed to laminitis [27]. Horses with endocrinopathic laminitis exhibit significant vascular dysfunction in laminar vessels and in facial skin arteries, and it has been suggested that the vascular dysfunction that occurs in horses due to hyperinsulinemia is similar to in humans, based on an in vitro model [28,29].

There are different forms of insulin resistance due to the different molecular mechanisms that may be involved in this disorder. Insulin resistance can occur due to decreased sensitivity to the hormone, decreased responsiveness to the hormone, and combinations of these two factors, which can be accessed via dose-response curves. Additionally, a decrease in insulin action at the cellular level may occur in three different levels: (I) prior to the interaction of insulin with its cellular

receptor. This level includes factors that reduce free insulin concentration, including increased insulin degradation or insulin binding to structures others than its cellular receptor, such as anti-insulin antibodies as it happens in the case of type 1 diabetes; (II) at the level of the insulin receptor, which reflects alterations in the insulin receptor affinity or cellular concentration resulting in decreased biologic response to insulin; or (III) post insulin and its receptor interaction. This level may include any change in the post-receptor events, which can affect a large number of intracellular steps in insulin action.

The distinction of these levels is important because levels (I) and (II) are usually associated with decreased sensitivity (when there is not enough insulin hormone interacting with its receptor), whereas level (III) is more likely to produce decreased responsiveness (when the insulin hormone interacts with its receptor but the downstream pathways are compromised) [30]. There are many factors that can affect this metabolic condition. In humans, chronic overnutrition stimulates insulin secretion, triglyceride synthesis, obesity and finally, down-regulation of insulin receptors and signaling cascade events during insulin action [22,31]. Lack of physical activity can induce insulin resistance in skeletal muscle [32] while exercising can improve insulin sensitivity [33]. Another relevant factor is pregnancy, where adaptive responses diverting glucose and lipids to the developing fetus result in decreased insulin sensitivity [34]. In addition, stress and sleep deprivation can be factors able to decrease insulin sensitivity as well [3].

1.3. Insulin resistance in equines

Just like in humans, insulin plays many roles in the horse metabolism, including stimulation of glucose uptake by cells, lipogenesis, diminishing lipolysis, DNA synthesis and cell replication. Insulin resistance in horses is also defined as the failure of tissues to respond appropriately to insulin. However, in contrast to humans, insulin resistant horses usually show hyperinsulinemia with normoglycemia or mild hyperglycemia [25,30,35,36]. In addition, insulin resistance in horses

has been associated with many factors such as chronic overnutrition, lack of physical activity, pregnancy, and stress [25,37].

In humans, the main concerns related to insulin resistance are related to cardiovascular diseases and type 2 diabetes. However, the main concerns associated to the insulin resistance in horses is due to its implication as a major predisposing condition for the pathogenesis of laminitis [38,39]. Risk factors for laminitis in horses include the of accumulation of rapidly fermentable nonstructural carbohydrates in the pasture, and animals with an equine metabolic syndrome (EMS) phenotype. It appears that most of the cases of laminitis are associated with high NSC pasture grazing, obesity, and insulin resistance [40,41].

1.4. Insulin resistance and pasture-associated laminitis

Laminitis is a painful and devastating condition of horses and ponies that has major economic and welfare implications [42,43]. It is a common and debilitating clinical presentation in equine practice [44]. A national study in 2000 estimated that 50% of horse operations with 3 or more horses have one or more lame horses annually. The incidence of laminitis, although less common, is estimated to affect approximately 13% of operations and 2% of horses annually. Furthermore, grazing lush pasture was the single most common perceived cause (45.6%) of laminitis, indicating that the majority of laminitis cases could be prevented by improving grazing management [45]. Additionally, insulin resistance has been shown to have a major impact as a predisposing condition for laminitis [25].

Although not well characterized yet, there may be many mechanisms by which insulin resistance can lead to laminitis. In humans, it has been demonstrated that insulin resistance can alter vasodilatory responses by inducing downregulation of the PI3K pathway and subsequent reduction in nitric oxide production leading to decreased blood flow and increased blood pressure. Additionally, compensatory hyperinsulinemia can lead to excessive stimulation of the MAPK pathway, involved on cell growth and gene expression (Figure 1), and production of endothelin 1

(ET-1), a potent vasoconstrictor, and reactive oxygen species [28,46]. During the state of insulin resistance, the lamellar tissue, which is non-insulin-dependent, may experience altered blood flow and increased glucose flux, affecting the basal cell polarity resulting in lamellar failure. Additionally, insulin resistance may play an important role in the development of laminitis through changes in the modulation of the MAPK pathway and vascular responsiveness during hyperinsulinemia [47].

Additionally, in 2000, a US survey indicated that grazing lush pasture was the single most commonly perceived cause of laminitis (45.6%), followed by feed problems and complications of injury, obesity, or pregnancy (26.9%), grain overload, colic or diarrhea, and retained placenta (12.1%), and unknown causes (15.4%) [48]. This data reinforces the importance of the term "pasture-associated laminitis" since most laminitis cases occur in horses and ponies kept at pasture. The condition occurs at times of rapid grass growth and the accumulation of specific carbohydrates in pasture forage, such as fructans, starches and sugars [43]. Furthermore, more operations and horses seem to be affected by laminitis in spring compared with winter [48], which coincide with seasonal changes in light intensity and duration, and temperature that can affect the non-structural carbohydrates (NSC) content of forages [49].

High NSC content (which includes simple sugars, starch, and fructans) in pasture grasses is another factor to play an important role in the relationship between insulin and laminitis. Excessive NSC consumption decreases insulin sensitivity while leading to altered bacterial flora in the large intestine of horses and increased production of compounds that have the potential to cause peripheral vasoconstriction, playing an important role in the pathogenesis of laminitis [50].

The majority of horses that are presented with laminitis result from developed from secondary factors. The majority of laminitis cases are consistent with the diagnosis of endocrinopathic laminitis. The term endocrinopathic laminitis refers to laminitis developing from putative hormonal influences, rather than occurring in association with pro-inflammatory and intestinal conditions, and are commonly seen in association to Equine Cushing's Disease (Pituitary Pars Intermedia

Dysfunction, PPID) or Equine Metabolic Syndrome (EMS) [41,51]. It has been suggested that endocrinopathic laminitis occurs mostly in response to vascular dysfunctions due to the incidence of metabolic disorders and associated endocrine disturbances, such as hyperinsulinemia. Therefore, laminitis may be considered a clinical sign of disease rather than a disease itself [28,52].

1.5. The relevance of equine metabolic syndrome

The term equine metabolic syndrome (EMS) has been adopted to describe equines with a higher risk for developing laminitis as a result of underlying insulin resistance [41,53,54]. Furthermore, EMS is a clinical syndrome that shares some of the same features of metabolic syndrome (MetS) in human medicine and incorporates three principal components: increased generalized or regional adiposity, insulin resistance with hyperinsulinemia, and laminitis predisposition [38,54]. Other components include dyslipidemia, altered blood adipokine concentrations, systemic inflammation, and seasonal arterial hypertension [1,25,27,55–58]

Insulin resistance and obesity are both well associated with EMS and it seems that there is an interaction between these two conditions. Obesity occurs when there is overaccumulation of body fat and is also described as a chronic inflammatory state [59]. It may induce insulin resistance via accumulation of adipose tissue in non-adipose insulin dependent organs and by increasing pro-inflammatory adipose tissue factors that can have negative impacts on the insulin signaling cascade pathway and GLUT4 translocation [60]. However, the whole process involved in this interaction in equines is not well understood yet.

There are two main factors predisposing equines to this metabolic condition. One factor is genetic, such as breed predisposition, where pony breeds, Morgan horses, Paso Finos, Arabians, Saddlebreds, Quarter horses, and Tennessee Walking horses are more predisposed to develop EMS. The other factor is environmental, such as overfeeding and lack of exercise, which

increases the chance for obesity and decrease insulin sensitivity leading to insulin resistance [25,38].

Although there is little epidemiological data on the prevalence of EMS, many studies have investigated the occurrence of the individual components of EMS, indicating a significant number of at-risk individuals [44]. For example, despite the limited information available on the prevalence of obesity in horses in the United States, it has been indicated that obesity has been affecting a much higher proportion of horses than previously reported [61]. In addition, hyperinsulinemia has been shown to be associated with increasing age, supplementary feeding, increased leptin and triglyceride concentrations [44], and that an apparent seasonal variation exists in the plasma concentrations of both insulin and glucose in normal horses [62].

Regarding the frequency of all forms of equine laminitis, a prevalence of up to 34% has been estimated, as indicated by a systematic review, in which more than half of the data included referral hospitals and research herds. The review also indicated that the frequency of naturally-occurring laminitis is variable, and dependent on the population studied [63,64]. Additionally, up to 90% of laminitis cases occur in association with PPID or EMS [51], although there is no occurrence report solely attributed to EMS [52,65]. However several factors can be associated with an increase in risk for laminitis, such as weight gain, seasonal variation, new access to grass, owner-reported history of laminitis, and existing endocrinopathic (pituitary pars intermedia dysfunction and equine metabolic syndrome) disease [65]. Therefore, identification of at risk individuals is important for the early diagnosis and effective management of EMS and characterized conditions, such as insulin resistance [66].

Diets high in starch and sugar are especially detrimental to metabolic animals due to excessive NSC content. Therefore, diets high in fat and fiber are recommended for horses and ponies at risk for metabolic disease [67]. Moreover, the main adopted treatment for EMS is weight reduction

in combination with dietary modification and exercise in horses without painful or unstable laminitis [52].

1.6. Equine Cushing's disease/Pituitary pars intermedia dysfunction

The equine pituitary gland lies in the sella turcica at the base of the brain, connected to the hypothalamus by the pituitary stalk. The equine pituitary gland is divided into different lobes, and the pars intermedia is one of them, consisting of melanotrope cells and producing pro-opiomelanocortin (POMC) [68,69]. POMC yields adrenocorticotropin (ACTH), melanocyte-stimulating hormones (MSHs), b-endorphin, corticotropin-like intermediate lobe peptide (CLIP), lipotropins, and several other small peptides through post-translational processing. Additionally, the activity of the equine pars intermedia is inhibited by dopamine and stimulated by thyrotropin-releasing hormone (TRH) [70,71].

Pituitary pars intermedia dysfunction (PPID), or Cushing's disease, describes a collection of clinical signs attributed to a chronic elevation of circulating glucocorticoids (hypercortisolism or hyperadrenocorticism), being a common endocrinopathy neurodegenerative disease with loss of dopaminergic inhibitory input to the melanotropes of the pars intermedia that mainly affects aged horses and ponies [72,73].

Similar to EMS, genetic factors such as breed may play a role in the development of PPID, with at-risk breeds including ponies and Morgan horses [74]. In addition, the activity of the equine pars intermedia has a seasonal rhythm, an adaptation that is believed to help animals to prepare for the metabolic and nutritional conditions of winter [75,76]. The clinical signs of PPID in horses include the development of an abnormal hair coat, such as hirsutism, muscle wasting or sarcopenia, polyuria and polydipsia, excessive sweating, immunosuppression, lethargic behavior, reproductive infertility, neurologic impairment, and similar to EMS, endocrinopathic laminitis [74,77–83]. Importantly, PPID also causes abnormal adipose tissue distribution and insulin

resistance, which can cause chronic inflammation and oxidative stress via mitochondrial impairment, increasing the progression of PPID [74,84]. Although hyperinsulinemia is not specific to PPID, excessive production of glucocorticoids has been shown to cause abnormal fat distribution, elevation of plasma lipid levels, and glucose intolerance, leading to hyperinsulinemia [53,85]. Hyperglycemia may occur in advanced cases when compensatory hyperinsulinemia fails and pancreatic insufficiency develops. Additionally, glucocorticoids can directly influence insulin actions due to the antagonist action of excess circulating cortisol to insulin in animals with PPID [86,87] and inhibit insulin secretion from pancreatic beta-cells [88].

1.7. Insulin sensitivity and exercise

Exercising is an effective way to improve insulin sensitivity and other risk factors for metabolic syndrome [89,90]. Studies have shown that exercising can improve insulin sensitivity by mechanisms involved in skeletal muscle GLUT4 content, weight loss, and improvement of signal transduction in the glucose metabolism pathway [89,91–93]. Additionally, because exercise and insulin utilize different signaling pathways leading to GLUT4 translocation for glucose uptake, a single bout of exercise increases skeletal muscle glucose uptake via insulin-independent mechanisms and can improve insulin sensitivity for glucose uptake for 24 to 48 hours [93,94]. In contrast, frequent exercise results in a persistent improvement of insulin action in skeletal muscle. These improvements have been associated with increased metabolism in skeletal muscle and subsequent changes in the expression and activity of enzymes involved in glucose uptake and insulin signal transduction in skeletal muscle. Other associations include increased lipid utilization and turnover as frequent exercising results in an increased oxidative capacity of skeletal muscle through up-regulation of lipid oxidation and the expression of proteins involved in mitochondrial biogenesis [94], increased muscle capillarization, and improved glycemic control [95].

Acute exercise seems to have an immediate and intensity dependent effect on improving insulin sensitivity in pre-diabetic adults [96], and studies have supported that short-term exercise training can also increase insulin sensitivity in horses [97,98]. It has been demonstrated in horses that short-term (7 days) low-intensity (30min at a trot to a heart rate between 120 and 140 beats/min) exercise can affect glucose metabolism for 24 hours after exercise in horses, improving insulin sensitivity for a short period without changes in body weight and in the diet [97].

Exercise training in horses has been studied as a tool to protect animals against diet-induced insulin resistance as it is the case for animals fed high concentrates and high soluble carbohydrates diets. Turnout alone may not be sufficient to improve insulin sensitivity in horses under diet-inducing insulin resistance. In addition, either continuous light or moderate exercise, performed at least 5 days a week, can reduce the risk of insulin resistance in horses fed high amounts of concentrate [99]. In addition to walking and trotting, submaximal aqua-training exercise has also been shown to increase insulin-stimulated glucose uptake in skeletal muscle and decrease its plasma concentration, resulting on reduced insulin resistance in horses. Therefore, submaximal aqua-training exercise becomes a low impact training alternative for equine insulin resistance management [100]. Overall, exercise training has beneficial outcomes in insulin sensitiveness in horses even at low intensity and low impact exercises.

1.8. Diagnosing insulin resistance

There are several methods available to diagnose insulin resistance and they can either assess nonspecific indications of insulin resistance, less specific methods, which indirectly access insulin sensitivity by using relative faster and simpler tests, or be quantitative, which directly access insulin sensitivity in the individual by using more complex tests.

Less specific methods indicate the possibility of insulin resistance, but with no conclusive diagnosis. Usually, these methods are used as prognostic screening tests and in combination

with other information, such as the animal's history. The biggest downside of these methods is that they usually do not discriminate the source of failure of glucose homeostasis: inadequate secretion of insulin by pancreatic beta-cells versus a decrease in insulin sensitivity of tissues; or the stage of progression of the insulin resistance [101]. Nonspecific methods include: (1) basal hyperglycemia test, which measures fasting plasma glucose concentrations but does not discriminate the source of failure on glucose homeostasis; (2) basal hyperinsulinemia test, which measures fasting plasma insulin concentrations, but may give false-negative responses during the decompensation stage while the insulin resistance progresses [102]; (3) glucose tolerance test, which assesses plasma glucose response to either oral or intravenous admiration of glucose. Oral glucose tolerance test may become challenging given intrinsic factors to the animal, such as rate of consumption, gastric emptying, and intestinal absorption of the glucose dose. On the other hand, intravenous glucose tolerance test avoids the complications described for oral glucose tolerance, but still cannot discriminate the source of failure on glucose homeostasis; (4) insulin tolerance test, which uses the area under the glycemic curve, after glucose tolerance test with and without concurrent administration of insulin to access insulin sensitivity of the individual. However, the administration of exogenous insulin may induce responses of endogenous counter-regulatory hormones [101].

The quantitative methods are more specific and conclusive for insulin resistance, but much more complex and technically demanding, being time-consuming, expensive, and impractical for field situations. Quantitative methods include (1) insulin suppression test, which uses somatostatin and octreotide to suppress endogenous insulin concentrations while measures the ability of a fixed-rate infusion of exogenous insulin to dispose of a glucose load. Infusion rates of glucose and insulin are controlled during the whole test period. However, there may be significant variations on the effects of the insulin suppressant drugs, steady-state of insulin and glucose plasma concentrations; (2) euglycemic-hyperinsulinemic clamp technique, which works by controlling plasma glucose and insulin concentrations to achieve a desired hyperinsulinemic

plateau. It requires special equipment, trained personnel, and is labor intensive. It can differentiate insulin sensitivity from insulin secretion. However, there are significant variations between studies given lack of standardization of the technique and reference ranges for horses [101]; (3) minimal model of glucose-insulin dynamics analysis, which is a nonlinear regulatory model that is fitted to data obtained by use of a frequently sampling intravenous glucose tolerance test (FSIVGTT). This model can generate calculated variables that are used for physiologic interpretations being able to differentiate between compensated and decompensated stages of insulin resistance. It is currently used as the “gold standard” [102].

The minimal model quantitatively analyzes the glucose and insulin responses due to intravenous glucose tolerance testing and evaluates the interaction between glucose and insulin in glucose disposal [102,103]. This model has been primarily used in human studies of diabetes and successfully adapted to examine glucose-insulin dynamics in horses [104]. There are four parameters analyzed in this test: Glucose effectiveness (Sg), which describes the glucose-mediated glucose disposal rate; insulin sensitivity (Si), the efficiency of insulin to accelerate cellular glucose uptake; acute insulin response to glucose (AIRg), which quantifies endogenous insulin secretion in response to the glucose dose; and the disposition index (DI), the product of SI and AIRg, which is a measure of the ability of the combined effect of insulin secretion (AIRg) and insulin efficiency (SI) to prevent hyperglycemia [102,103]. Once those 4 parameters are calculated, they can be compared to parameters of insulin resistance for horses using reference quintiles. The reference quintiles allow for comparisons of individual data with data collected from a large population and facilitate the diagnosis and characterization of clinical cases [102]. However, accurately diagnosing insulin resistance can still be challenging given the non-specificity, lack of accuracy, and the overall time, skill and financial demand when performing the currently available tests. Furthermore, studies in human and laboratory animals have shown that a class of small noncoding RNAs is involved in the regulation of many biological processes, and has also been demonstrated to be associated with many diseases. These RNAs, known as

miRNAs (miRNAs), have emerged as potential biomarkers in studies for the diagnosis and prognosis of many diseases, including type 2 diabetes and metabolic syndrome.

1.9. An introduction to microRNAs

Studies have demonstrated that the DNA molecules previously known as "junk DNA" are actively transcribed and can code for a class of non-protein-coding RNA molecules, microRNAs (miRNAs) [105,106]. This class of RNAs is known to be very small, around 21–25-nucleotides, and to negatively regulate gene expression at the post-transcriptional level [107]. Furthermore, evidence has highlighted the importance of miRNAs in a variety of biological processes and disease development, and that by studying them researchers may be able to develop new and improved methods to address the prevention, diagnosis, and treatment of major diseases.

MiRNAs are part of regulatory RNAs that use simple complementary base-pairing interactions to regulate other nucleic acid species. MiRNAs generally down-regulate cytoplasmic mRNAs by repressing translation and destabilizing target mRNAs (mRNA decay). Synthesized miRNAs can be found in the cellular microenvironment and the extracellular environment. Those found in the extracellular environment are called “circulating miRNAs” or “extracellular miRNAs” (ECmiRNAs). Cellular miRNAs (tissue miRNAs) are found in the cellular microenvironment and can act inside the cell by repressing translation and destabilizing local mRNAs. ECmiRNAs are relatively stable and can be found in several biological fluids, such as serum, plasma, saliva, tears, urine, breast milk, seminal and follicular fluids. In addition, while some circulating miRNAs could potentially be in residual amounts with no (or very little) biological relevance, others may function as an important intercellular communication system in the body [108].

1.10. MicroRNAs synthesis

Genes that transcribe for miRNAs are located in either intergenic regions or in intronic regions. However, the majority of them are found in intergenic regions. Intergenic miRNAs, found in noncoding regions between genes, are transcribed as independent transcription units. In contrast, intronic miRNAs, derived from the intron regions of gene transcripts (embedded into a gene), are processed from the introns of their hosting transcription units [109–113]. Either RNA polymerase II (Pol II) or polymerase III (Pol III) can be responsible for their transcription process, but it seems that Pol II is the main polymerase in these processes [114–116]. The product of this transcription is known as primary miRNAs (pri-miRNAs), which can be processed into mature miRNAs [116]. The formation of mature miRNAs occurs by the action of two RNase III-type proteins, Drosha and Dicer [116,117] (Figure 2).

Transcribed pri-miRNAs are processed by the nuclear RNase III Drosha, which cleaves pri-miRNAs at the stem of the hairpin structure to form precursor miRNAs (pre-miRNAs). The pre-miRNAs are exported from the nucleus to the cytoplasm via the RanGTP-dependent nuclear transport receptor exportin-5 (EXP-5) to become mature miRNAs. Once they are in the cytoplasm, these pre-miRNAs are further processed into mature miRNAs by Dicer, which cleaves the miRNA strand. Finally, the miRNA duplex is incorporated into an Argonaute (Ago) family protein complex to create the miRNA-induced silencing complex (miRISC), which mediates gene silencing [116,118–121].

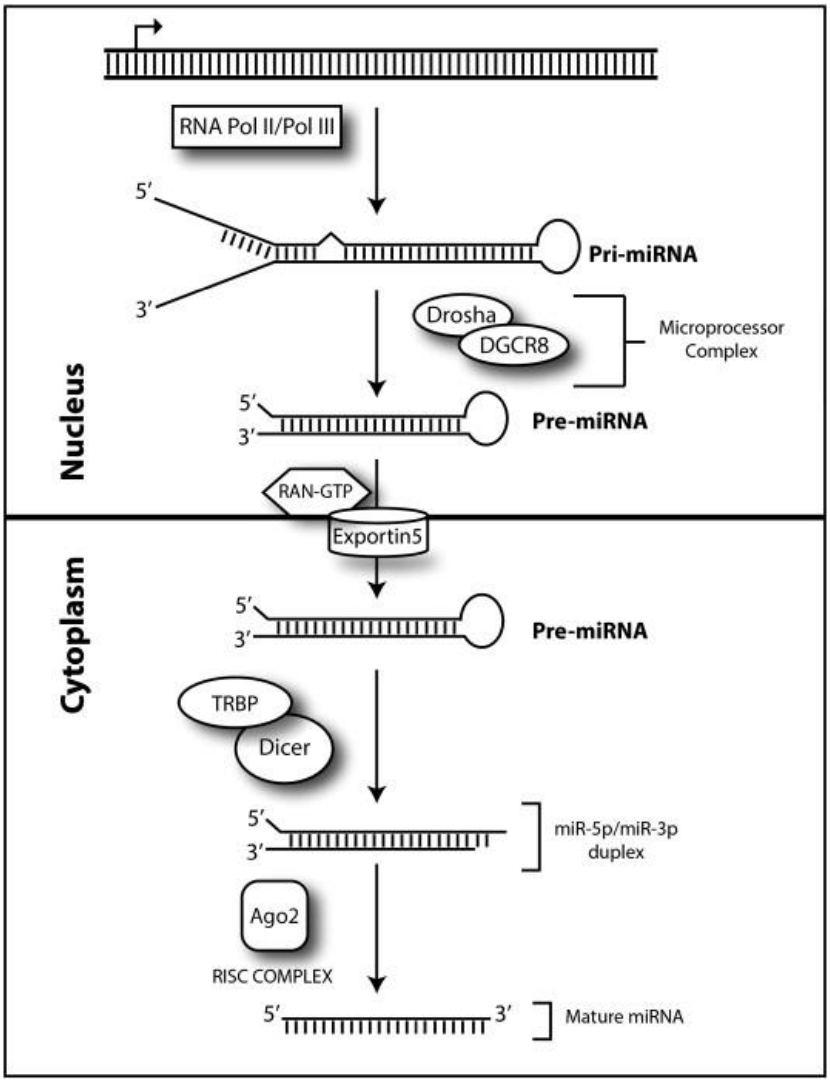

Figure 2 microRNA synthesis and mechanism of gene regulation. MicroRNAs are transcribed by RNA polymerase II (not shown) and form RNA precursors called pri-miRNAs. Pri-miRNAs are processed in the nucleus by Drosha and DGCR8, resulting in the pre-miRNAs. Pre-miRNAs are then exported into the cytoplasm by the exportin 5. Once in the cytoplasm, the pre-miRNAs undergo an additional processing step by Dicer. Once Dicer cleaves the pre-miRNA stem-loop, two complementary short RNA molecules are formed. One is integrated into the RISC complex, and the remaining strand is degraded. After this phase, miRNAs can exert their regulatory effects on messenger RNAs (mRNAs) via mRNA cleavage or translational repression, depending on the level of complementarity between the miRNA and its target mRNA. Original source: [122]

1.11. MicroRNAs mechanisms of action

The miRISC acts post-transcriptionally by recognizing and interacting with its target messenger RNAs (mRNAs) causing gene repression. There are two mechanisms by which miRISC can down-regulate gene expression: translational repression and mRNA cleavage. Although the

mechanisms of action of these processes are still being investigated, there is strong evidence suggesting that the number, type, and position of mismatches in the miRNA-mRNA duplex are crucial during the activation of degradation or translational repression mechanisms [116,123]. The degree of which the miRNA complements its target mRNA seems to be a major determinant in the regulation of these mechanisms: a high degree of complementarity results on mRNA cleavage mechanism process, while mismatches omit degradation and facilitate the translational repression mechanism [116] (Figure 2).

During translation repression, there are currently three main models suggesting the mechanisms of how miRNA represses translation of target mRNAs [116]: (i) presence of factors (miRISCs, GW182, or downstream factors) competing with eIF4E for binding to the mRNA 5′ cap structure resulting in failure of the translation initiation process [124,125]; (ii) miRISCs prevent mRNAs from circularizing, resulting in translation inhibition [126,127]; (iii) miRISCs inhibit 60S ribosomal subunit assembly with the 40S preinitiation complex, resulting in translation repression [128,129]. The cleavage or degradation mechanism process is believed to be catalyzed by many mechanisms, such as Ago proteins, deadenylation, decapping, and exonucleolytic digestion of mRNA [116,127,130,131].

1.12. Extracellular/Circulating microRNAs

There are several different ways by which ECmiRNAs can be secreted from cells to the extracellular environment and act as mediators of cell-to-cell communication. Currently, there are five known mechanism of possible miRNA secretion: (i) exosomes, (ii) microvesicles, (iii) apoptotic bodies, (iv) high-density lipoprotein (HDL), and (v) Ago protein complex [108,121].

Exosomes are formed by internalization of the cell membrane to produce endosomes and can function as intercellular transporters of one cell contents, such as lipids, proteins, DNAs, mRNAs,

and miRNAs, to another [108,121,132–135]. It appears that cells have a complex process with active mechanisms for exosome formation, content, and release [108,136]. Additionally, exosome content, such as mRNA, from one cell can be functional in another cell, suggesting practical relevance as a cell to cell interaction mechanism [134].

Similar to exosomes, microvesicles are membrane-bound vesicles that can carry a variety of molecules, including miRNAs. However, microvesicles differ from exosomes on their release mechanism, biogenesis and biophysical properties [108,137–139]. In the context of ECmiRNAs, it has been demonstrated that miRNAs transported by exosomes or microvesicles can influence biological processess by changing expression of genes involved in cell-to-cell interactons [108].

During apoptosis, cells release apoptotic bodies, or apoptotic vesicles. These extracellular vesicles are larger than exosomes or microvesicles and represent residues of apoptotic cells [140,141]. Additionally, apoptotic bodies can also carry molecules such as miRNAs, mRNAs and fragments of DNA [142]. However, it has not been determined whether the loading of miRNAs into apoptotic bodies are specific and selective or whether it is a random process in response to cellular collapse [108].

The secretion of miRNAs can also be done through lipoproteins, such as HLD, that have a great affinity to bind water-insoluble materials enabling them to carry nucleic acids and to be used as gene delivery agents, including miRNAs [143,144]. Differently from extracellular vesicles, HDL molecules are believed to carry mostly small non-coding RNAs, instead of both miRNAs and mRNAs. Furthermore, HDL coupled miRNAs have also been considered of biological relevance in the transport of ECmiRNAs [108,143]. Finally, ECmiRNAs can be released through binding with Argonaut protein families, particularly Ago2, which are important components of the RISC complex, delivering miRNAs from donor cells to recipient cells [108].

1.13. MicroRNAs and the diagnosis of metabolic diseases

A single miRNA has the potential to regulate multiple genes. Therefore, it also has the potential to impact many different biological processes [145]. It has been indicated that miRNAs can play important roles in disease development, progression, prognosis, diagnosis and evaluation of treatment response [146–151]. Moreover, miRNAs have been investigated as important regulators of biological processes and potential biomarkers for disease, and miRNA expression profiling has been shown to be significantly informative in studying diseases. For example, miRNA profiles can be used to reflect the developmental lineage and differentiation state of tumors [152], and differences in miRNA profiles can allow identification of healthy and non-healthy individuals [153].

In the case of insulin resistance, several miRNAs have been identified as having a physiological role in tissues associated with the development and progression of diabetes [145], such as the regulation of insulin biosynthesis, secretion and beta-cell survival in pancreatic beta-cells, insulin sensitivity regulation in the skeletal muscle and adipose tissue, and glucose and lipid metabolism in the liver [154].

Researchers have aimed to identify the profile of circulating miRNAs in type 2 diabetes and its response to changes in insulin sensitivity in humans. It has been proposed an inter-organ crosstalk controlled by miRNAs, which are able to target the expression of key components required for glucose homeostasis. These miRNAs can be tissue-specific or differentially expressed in different organs, such as the pancreas, insulin targeting tissues like adipose tissue and skeletal muscle, and even the brain. Additionally, circulating miRNAs may serve as long-distance communicators between tissues [154].

Cross-sectional studies have identified differences in circulating miRNA profile of 10 miRNAs in type 2 diabetic patients compared to individuals with normal glucose tolerance. Their findings showed increased miR-140-5p, miR-142-3p, and miR-222 and decreased miR-423-5p, miR-

125b, miR-192, miR-195, miR-130b, miR-532-5p, and miR-126 in patients with type 2 diabetes, associating the components of this profile to insulin action. Although it remained unclear whether changes in the levels of the 10 circulating miRNAs reflected deregulation of the miRNAs in the cellular level, from a clinical point of view, the results allowed enough evidence for future analyses using circulating miRNAs to examine the pathophysiology of type 2 diabetes [155]. Additionally, it has been found that circulating levels of miR-486, miR-146b, and miR-15b are increased in obese children and patients with type 2 diabetes and that they contribute to the pathological processes of obesity and type 2 diabetes. MiR-486 is implicated in accelerating preadipocyte proliferation and myotube glucose intolerance, while miR-146b and miR-15b are engaged in the suppression of high concentration glucose-induced pancreatic insulin secretion [156].

Although miRNAs studies seem to be focused on diseases in humans, the role of miRNAs in domestic animals, such as horses, has also been the subject of study [157]. For example, the regulation by miRNAs has been investigated in the adaptive response to extreme endurance exercise, suggesting miR-21-5p, miR-181b-5p and miR-505-5p as being putative regulators of the response to endurance exercise [158]. In addition, circulating miRNAs have also been used to differentiate specific breed types. For example, miR-122, miR-200, and miR-483 have been shown to be overexpressed, and miR-328 underexpressed in ponies compared to Warmbloods [159]. However, many disorders have not, or only scarcely, been investigated for miRNAs role in horses health and disease, making it an emerging and exciting field of the equine medicine [157]. Therefore, miRNAs have potential as important tools in disease-related studies and understanding their role and regulatory mechanisms in metabolism are crucial for developing novel and effective strategies towards disease prevention and management.

1.14. Hypothesis

Insulin is a crucial hormone secreted by pancreatic beta-cells that regulates energy homeostasis and cellular development. In equines, insulin resistance seems to play a major role in the development of endocrinopathic laminitis, a very painful and devastating condition in equines. Although much progress has been made, there is much still to learn regarding the pathophysiology of equine insulin resistance. In this sense, the use of miRNA profiling has become a powerful and efficient tool in disease research in humans and laboratory animals, which brings a great opportunity to further evaluate the mechanisms that lead to insulin resistance in horses. Therefore, we hypothesized that, by exploring the differences in circulating miRNA profiles between insulin resistant and healthy animals, it would be possible to identify potential key regulatory players of this condition in horses. We expect the results of our studies to help in the evaluation of miRNA-disease interactions in horses while revealing critical information to contribute to the development of novel diagnostic techniques and strategies to prevent and manage equine insulin resistance.

CHAPTER 2: POSSIBLE ROLE OF MICRORNA IN EQUINE INSULIN RESISTANCE: A PILOT STUDY[1]

2. Summary of Pilot Study

MicroRNAs (miRNAs) are a class of small endogenous single-stranded noncoding RNA molecules that have important roles in several biological processes. Research in human and laboratory animals has shown that miRNAs can regulate genes associated with type 2 diabetes mellitus and metabolic syndrome, and that the levels of specific miRNAs circulating in the bloodstream can serve as potential biomarkers for the diagnosis and prognosis of these diseases. We hypothesized that insulin-resistant (IR) horses would have a different circulating miRNA profile than those that are healthy. Fifteen nonpregnant mares housed at the Virginia Tech Middelburg Agricultural Research and Extension Center were evaluated for insulin sensitivity, with the frequent sampling intravenous glucose tolerance test. Selected mares, representing the most insulin-sensitive (IS, n = 3) and IR (n = 3) states, and paired for age, weight, and body condition, underwent miRNA profiling. Serum samples were collected, miRNA extracted, and microarray analysis performed to investigate the presence and relative amount of 340 equine miRNAs. Confirmation by quantitative real-time polymerase chain reaction revealed that miRNA was present in the serum of all animals. Results demonstrated different miRNA profiles between groups: Six miRNAs were expressed only in IS mares, five miRNAs were found to have lower quantity in IR mares relative to the IS ones, and three miRNAs were higher quantity in IR mares relative to the IS ones. The novel results of this preliminary study suggest potential new tools that could be developed for the diagnosis and treatment of metabolic syndrome in horses.

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2.1. Introduction

Insulin resistance (IR) is a prevalent pathophysiological disorder among domestic horses, and a key player in a large constellation of comorbid conditions, which includes obesity and regional adiposity, chronic low-grade systemic inflammation, equine metabolic syndrome, pituitary pars intermedia dysfunction, and endocrinopathic laminitis [38]. Exact mechanisms for how, why, and when IR occurs in horses are not completely elucidated, and remain active areas of research [160,161]. Myriad factors, similar to those in human sufferers of IR, may play a part, including reduced density of cell-surface insulin receptors, malfunction of these receptors, defects in the internal cell signaling pathways, and breakdown of the internal glucose-shuttling machinery within the cell [162].

Complicating matters is the dependence of IR on both environmental and intrinsic factors. Contemporary management practices, including unrestricted access to pasture, relatively sedentary lifestyles, and high-glycemic concentrated diets, have been associated with increased insulin resistance and compensatory increase in insulin secretion. Genotypes that favored fat and glycogen storage were beneficial for populations facing cycles of feast and famine, but present-day exposure to an overfed state has led to increased incidence of obesity and IR [163]. Because regulation of glucose homeostasis is a core requirement for any animal, insulin dysregulation may manifest across the body, affecting systems that regulate immunology, circulation, reproduction, and growth [164,165].

Insulin measurement, demonstrating hyperinsulinemia either fasting, fed or sugar challenged, is sufficient to diagnose insulin resistance. However, diagnosis of IR is performed most accurately by performing dynamic tests such as the euglycemic hyperinsulinemic clamp or the frequently sampled intravenous glucose tolerance (FSIGT) test with minimal model analysis [165]. However, these tests are time-consuming, expensive, and impractical for field situations. Single-sample and

proxy assessments of glucose and insulin have been developed [102], but these tests lack specificity, rendering them of limited diagnostic or prognostic applicability to individual animals.

Since their discovery in 1993, microRNAs (miRNAs) have emerged as exciting key regulators of biological processes and promising biomarkers for disease. These small (~22 nucleotides in length) noncoding RNA molecules bind to specific mRNA targets and promote their degradation or translational inhibition, effectively “turning off” genes in a post-transcriptional manner. One miRNA may regulate hundreds of mRNAs, and thus single miRNAs may have a substantial influence on gene expression networks. For this reason, differential miRNA expression patterns between healthy and diseased animals are especially rich in biological and physiological information, as variation in expression of hundreds of mRNAs may be revealed in the expression patterns of one or a few regulatory miRNA [166].

Profiling of miRNA has quickly become a powerful and attractive tool in disease research that offers two distinct benefits [167]. First, measuring miRNA expression facilitates better understanding of the molecular mechanisms involved in the pathology of interest, as it can allow system-level studies of gene regulation, especially when paired with mRNA data. Secondly, miRNAs have been shown to be incredibly robust in circulation and are measurable to a much higher sensitivity than cell-free circulating proteins. For this reason, miRNA profiling is of great interest to scientists wishing to develop biomarkers for diverse biomedical applications, including cancer, autoimmune, cardiovascular, and metabolic diseases [166,168].

In human metabolic research, miRNAs have already been identified, which can separate groups of patients into clinically relevant classes, such as prediabetic or type 2 diabetic versus healthy cohorts [169,170]. A large suite of miRNAs has been directly implicated in aberrant beta-cell development and functioning, adipogenesis, skeletal muscle insulin signaling pathways, and glucose and lipid metabolism [169,171,172]. In some cases, these miRNAs are detectable several

years before signs of type 2 diabetes mellitus (T2D) or metabolic syndrome manifest clinically [168,173].

In this study, it was hypothesized that IR horses would exhibit differential circulating miRNA profiles when compared with healthy animals. The characterization of circulating miRNA profiles associated with equine IR, followed by the identification of potential gene target components of pathways triggered by, or contributing to, the pathology of insulin resistance could help on the development of simple, noninvasive novel biomarkers that would assist clinicians in the diagnosis and treatment of equine insulin resistance. Therefore, this study aimed to measure insulin sensitivity in a group of animals and to compare miRNAs between insulin-resistant (IR) and insulin sensitive horses. Identification of candidate genes important for equine insulin resistance and its associated conditions was also pursued.

2.2. Material and Methods

Animals and Sampling

Initially, 15 nonpregnant light horse type Warmblood cross mares maintained on ad libitum pasture at the Virginia Tech Middleburg Agricultural Research and Extension Center (Middleburg, VA) were metabolically characterized using the FSIGT [104], a “gold standard” dynamic method for measuring equine insulin sensitivity. Briefly, on the evening before the test, horses were housed overnight in stalls allowed ad libitum access to water and hay with known composition and low sugar content to maintain an unperturbed state before sampling. On the morning of the test, baseline blood samples were taken at 30 and 0 minutes, and a dose of 50% dextrose (300 mg/kg body weight [BWT]; Vedco, Inc, St Joseph, MO) was administered intravenously over 30 seconds via indwelling jugular catheters. At 20-minute postdextrose, insulin (20 mIU/kg BWT; Humulin R, Lilly, Lake Forest, IL) was administered intravenously, and blood sampling continued at 2–30minute intervals until 180 minutes postdextrose infusion. Horses were provided ad libitum

access to water throughout the FSIGT, and all necessary precautions were taken to ensure the test was performed in a quiet environment to minimize untoward effects on insulin concentrations. The FSIGT was performed in May 2014, when the animals are usually more likely to develop metabolic syndrome symptoms because of rich pasture conditions. Pasture nutrient content at the time of the FSIGT is shown in Table 1.

Sample Handling and Insulin Sensitivity Analysis

Plasma tubes were spun at 2,000g for 10 minutes at 4C to separate plasma. Serum tubes were allowed to clot for 1 hour at room temperature before centrifuging at 1,500g for 10 minutes at 4C to separate serum. Plasma and serum samples were aliquoted into 2.5 mL cryovial tubes and stored at 80C for later use in downstream analyses. All FSIGT tests were performed in the span of 1 week to reduce variation because of the time of collection. Plasma was analyzed for glucose by enzymatic assay using the YSI 2700 SELECT Biochemistry Analyzer (Yellow Springs, OH) with YSI 2365 glucose membranes, YSI 2747 glucose/L-Lactate standard, and YSI 2357 buffer concentrate kit. Insulin was analyzed using Siemens Coat-A-Count Insulin Radioimmunoassay kit (Siemens, Los Angeles, CA), previously validated for horses [174]. Insulin levels above the kit standards were diluted with charcoal-stripped equine plasma [175]. Insulin sensitivity index, acute insulin response to glucose (within 20 minutes after glucose administration), glucose effectiveness, and disposition index were determined by the minimal model of glucose and insulin dynamics using specialized software (MINMOD Millennium, version 6.02) [102]. Serum collected during the 30-minute baseline measurement of the FSIGT was used for further analyses.

Isolation of RNA and cDNA Synthesis

Total RNA was isolated from equine serum samples according to manufacturer's protocols using TRI Reagent BDRNA/DNA/Protein Isolation Reagent for Blood Derivatives (Molecular Research Center, Cincinnati, OH). Samples were lysed and separated by chloroform into RNA and protein layers. RNA was precipitated with isopropanol and washed with 75% ethanol. All samples were then treated with DNAfree DNase Treatment and Removal Reagent (Invitrogen/Life Sciences, Grand Island, NY) to remove any DNA contamination. RNA purity values were assessed using the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). Typically, 260/280 values of above 1.8 are considered acceptable for RNA quantification by polymerase chain reaction (PCR). For this RNA isolation, using chloroform causes the nanodrop reading to have lower 260/280 values and therefore values of 1.7 were accepted for PCR. Samples were then stored at 80C until further quantitative realtime PCR (qRT-PCR) analysis. cDNA was generated using miScript (Qiagen Valencia, CA) per the manufacturer's protocol. 4µL 5X miScript RT Buffer, 1µL reverse transcriptase mix and 1µg of RNA and water were used in a 20µL solution and incubated for 60 min at 37°C and then 5 min at 95°C. cDNA reaction was then immediately added to qRT-PCR master mix for PCR analysis [176].

Selection of miRNA and Primer Design

Equine miRNA forward primer sequences were obtained by an in-silico detection model [177] and validated in the horse via small RNA-seq. Of all the 340 equine miRNAs, 250 were conserved human miRNAs and 75 were equine-specific miRNAs with human homologues, but different nucleotide sequences. The remaining 15 miRNAs were equine-specific, but had no apparent human homologue [176].

MiRNAs were considered present if the cross-point cycle (Cp) value was <40.0 and the melt curves appeared normal. Samples were run in duplicate and normalized to internal controls, that is, miRNAs present in all samples with a standard deviation (SD) between samples of less than one.

Real-time Quantitative PCR

Quantitative real-time polymerase chain reaction was performed using the LightCycler 480 PCR system (Roche). The following three controls were used: One master mix RTrxn control as a negative control and two master mix RTrxn controls with one random primer each. Quantitative real-time polymerase chain reaction was performed on 384 well plates. Each well contained 6 µL of total reaction including 2X QuantiTect SYBR Green PCR Master Mix, 10X miScript Universal reverse primer (Qiagen), and miRNA specific forward primer and 0.1 µL of cDNA. Analysis was performed using the LightCycler 480 PCR system. Cycle conditions began with reaction initiation at 95C for 15 minutes, followed by 45 cycles of 94C for 15 seconds to denature, 55C for 30 seconds to anneal, and 70C for 30 seconds for extension. All plates run in duplicate, and miRNAs were considered present at a cycle number of <37 as well as confirmed appropriate by amplification curves and singular melt peaks.

Pathway Analysis

DIANA TOOLS miRPath, version 3 [178], using miRNA differentially and uniquely expressed. Predicted targets were recorded using DIANA-microT-CDS algorithm, and pathways were considered significant with $p \leq 0.05$ [179].

Normalization of miRNA Expression Data and Statistical Analysis

Normalization occurred by taking the geometric mean of all miRNA genes expressed in all samples that had an SD ≤1. Two tailed t tests were performed to compare miRNA profiles between groups. Significant results considered when $p<0.05$ and a trend when $p<1$.

2.3. Results

From the fifteen mares used in the study, the three most insulin sensitive and the three most insulin resistant were chosen for the miRNA profiling. The three most insulin resistant horses had elevated values for beta pancreatic secretion relative to reference range indicating a high insulin secretion to maintain euglycemia. These horses also had insulin sensitivity values within the lowest quintile reported for horse populations. In contrast, the three most insulin sensitive horses fell within the normal beta-pancreatic response and highest quintile of the population for insulin sensitivity [102]. Minimal model results used to select the horses with the highest (n=3) and lowest (n=3) insulin sensitivity for miRNA profiling are demonstrated on tables 2a and 2b.

Table 1 Results of the evaluated minimal model parameters for each animal in the study.

Insulin Resistant					
AIRg (mu.l^-1.min)	SI ((mu/l)^-1.min^-1)	Sg (min^-1)	DI	Age	BCS
582.25	0.00	0.01	1.57	15	8
835.77	0.36	0.01	304.47	12	7.5
647.87	0.68	0.01	441.21	18	8
Insulin Sensitive					
AIRg (mu.l^-1.min)	SI ((mu/l)^-1.min^-1)	Sg (min^-1)	DI	Age	BCS
54.87	13.40	0.02	735.05	15	6.5
96.20	4.22	0.01	827.46	13	7
107.16	5.95	0.01	637.19	13	6

Values shown as mean ± standard error values.

Table 2 Results of the evaluated minimal model parameters.

Parameters	Insulin Resistant	Insulin Sensitive
AIRg (mu.l^-1.min)	**688.63 ± 75.96**	**86.08 ± 15.92**
SI ((mu/l)^-1.min^-1)	**0.35 ± 0.19**	**7.86 ± 2.81**
Sg (min^-1)	**0.01 ± 0.0**	**0.01 ± 0.0**
DI	**249.08 ± 129.89**	**733.23 ± 54.93**
Age	**15 ± 1.73**	**13.67 ± 0.66**
BCS	**7.8 ± 0.16**	**6.5 ± 0.28**

Values shown as mean ± standard error values.

Weight and ages were not significantly different ($p>0.5$) between groups. However, body condition scores (BCS) were lower in insulin sensitive mares ($p<0.05$).

Results demonstrated different miRNA profiles between groups. Six miRNAs were expressed only in insulin sensitive mares ($p<0.001$): mir-208a, mir-299, mir-302a, mir-381, mir-769b, mir-371-3p. Five were found to have lower quantity in insulin resistant mares relative to the insulin sensitive ones: mir-147b ($p=0.05$), mir-370 ($p=0.09$), mir-376c ($p=0.02$), mir-139-5p ($p= 0.14$), mir-1839 ($p<0.001$). Three miRNAs were higher quantity in insulin resistant mares relative to the insulin sensitive ones ($P<0.10$): mir-129a-5p ($p=0.06$), mir-770 ($p=0.02$), mir-140-3p ($p=0.06$). Some of these miRNAs have already been related to metabolic syndrome, obesity, and/or related diseases, in other species as showed by previous studies.

2.4. Discussion

MiRNAs found in IS mares' group only

Mir-208a has been implicated in cardiac metabolism, obesity, insulin sensitivity and plasma lipids levels. Inhibition of mir-208a in mice confers resistance to high fat diet-induced obesity and improves systemic insulin sensitivity and glucose tolerance. MED13 and mir-208a have been identified as central components of the heart in regulation of systemic metabolism. MED13 is negatively regulated by a heart-specific miRNA, mir-208a. MED13 is a regulatory subunit of the mediator complex and regulates systemic energy homeostasis [180].

Mir-302a is a negative regulator of adipocyte differentiation; associated with hepatic cholesterol, fatty acid and glucose metabolism. Mir-302a is involved in adipocyte differentiation via interaction with 3'-untranslated region of peroxisome proliferator-activated receptor gamma (PPARγ) mRNA in mice, suggesting that mir-302a may negatively regulate PPARγ expression through this direct binding. Expression of mir-302a is decreased during adipocyte differentiation (it might potently inhibit adipogenesis), and it might govern the lipid droplet formation and adipocyte-specific gene expression [181]. Mir-302a has also been suggested to have important role in the control of hepatic metabolism upon high lipid stimulus. Maternal high fat diet in mice prior to conception, pregnancy and lactation induces reduction of hepatic mir-302a level in offspring. Decrease in mir-302a expression in hepatocytes coincides with a marked nine-fold increase in the expression of the mir-302a target gene ELOVL6, which is involved in the formation of long-chain fatty acids. Upregulation of ELOVL6 gene expression may predispose the liver to a hepatic insulin resistance, one of the major characteristics of metabolic syndrome [181]. Mir-302a is also involved in cholesterol transport and efflux. The regulation of mir-302a has showed to be inversely correlated with cellular cholesterol levels. It specifically targets the 3'UTR of mouse and human ABCA1, which has the ability to stimulate the efflux of cholesterol from cells in the periphery playing an important role in the anti-atherosclerotic mechanism [182].

Another miRNA, mir-381, has been shown to be up-regulated in obese ovine and their offspring [183], and to have a trend for a stronger upregulation during the brown adipocyte differentiation in mice [182]. Another miRNA found only in insulin sensitive mares Mir-769b, is from the same family of mir-769-3p which has been related to patatin-like phospholipase domain containing 3 (PNPLA3), a gene consistently involved in the genetic susceptibility of nonalcoholic fatty liver disease in humans [184]. MiR-371b-5p, same family of mir-371-3p, was showed to be up-regulated in type 2 diabetic nephropathy (DN) patients compared to healthy and type 2 diabetic patients [185].

MiRNAs found in lower quantity in IR vs. IS group

Mir-370 is involved in the expression of other genes that affect lipid metabolism. It acts via mir-122 and may have a causative role in the accumulation of hepatic triglycerides by modulating initially the expression of several genes, such as sterol regulatory element binding protein 1c (SREBP-1c), diacylglycerol acyltransferase-2 (DGAT2), and carnitine palmitoyl transferase 1α (Cpt1α, which controls fatty acid oxidation) [186]. In humans, plasma levels of miR-122 and miR-370 are increased in patients with hyperlipidemia and positively correlated with blood levels of total cholesterol, triglyceride, low density lipoprotein cholesterol (LDL-C), and associated with coronary artery disease presence [187]. Mir-370 has been shown to be upregulated in myocardial microvascular endothelial cells from type 2 diabetic Goto-Kakizaki rats compared with those in Wistar rats [188].

Mir-376c is up-expressed in pancreatic islets in rats of type 2 diabetes. In addition, miRNAs from the same family of mir-376c have been shown to have implication in pancreatic beta-cells regulation and pancreatic tissue growth (mir-376) [189], and may be used as predictive biomarker in obesity (mir-376a) [190].

Mir-139–5p has been shown to block adipogenesis, in rats, via directly targeting the 3' untranslated regions of Notch1 and IRS1 mRNAs, a key member of Notch signaling and IRS1/PI3K/Akt insulin signaling, respectively [191].

MiRNAs found in higher quantity in IR vs. IS group

It has been suggested that miR-129-3p, same family as mir-129a-5p, may play a role in glucose and lipid metabolism [192]. MiR-770-5p, same family as mir-770, is increased in human patients with type 2 diabetes [193]. Mir-140-3p has been considered to be at some extension a specific and relevant miRNA for type 2 diabetes. Individuals with type 2 diabetes mellitus have been shown to have mir-140-3p upregulated, in comparison to individuals with gestational diabetes mellitus, and down regulated in comparison to individuals with type 1 diabetes mellitus [194]. Expression of mir-140, same family of mir-140-3p, is significantly higher in individuals with normal glucose tolerance versus individuals with type 2 diabetes, and it is believed to play a role in the link between adipose tissue dysfunction and the development of obesity associated disorders including type 2 diabetes [195]. In addition, mir-140-5p has potential to be a biomarker for risk estimation and classification of morbidly obese human patients [196].

Several of the miRNAs differently expressed between groups were also related to fat metabolism. That could be due to the differences in the BCS between both groups as well.

2.5 Conclusion

The results of this preliminary study could support the proposal that insulin resistant horses exhibit differential miRNA profiles from healthy animals. These differences in miRNA profiles may be used as potential biomarkers for helping to diagnose insulin resistance in equines. However, due to the small number of horses in the current study, more studies are required to validate our preliminary results.

CHAPTER 3: CIRCULATING MICRORNAS IN EQUINE INSULIN RESISTANCE

3. Introduction

Insulin is a hormone secreted by pancreatic beta-cells that regulates energy homeostasis in various organs, particularly muscle, adipose tissue, and the liver [1,2]., while also promoting cell division and growth through mitogenic effects [3]. Insulin secretion is controlled by many factors [4,5] and interferences in its action can lead to detrimental conditions [22].

In the horse, insulin resistance has been associated with obesity and regional adiposity, chronic low-grade systemic inflammation, equine metabolic syndrome, pituitary pars intermedia dysfunction, and endocrinopathic laminitis [38]. However, the mechanisms surrounding this condition are not completely clarified, demanding more research in the area.

Evidence has shown that microRNAs (miRNAs), a class of small non-coding protein RNA molecules, play important roles in a variety of biological processes and diseases [105]. It has been suggested that circulating miRNAs serve as long-distance communicators between tissues [154], and they have great potential as efficient tools in studying disease-related conditions such as type 2 diabetes and metabolic syndrome in humans and laboratory animals [155,156].

In a preliminary study, we demonstrated that insulin resistant horses differentially express circulating microRNAs compared to healthy animals. It was proposed that by studying the differences in circulating miRNA profiles between insulin resistant and healthy animals, it would be possible to gather more information regarding the mechanisms surrounding this condition in horses, which can lead to the development of potential biomarkers for diagnosing insulin resistance in equines. However, due to the small number of horses in the study, more studies

were required to validate the preliminary results [197]. Therefore, this study aimed to investigate the association of circulating miRNA profiles to this disease in equines.

We proposed that changes in miRNA profiles in equine serum lead to altered gene expression, which has a great impact in metabolic dysfunctions, such as insulin resistance. Therefore, insulin resistant animals, horses and ponies, would show different circulating miRNA profiles from healthy animals. The aims of the present study were to (1) measure insulin sensitivity in a group of animals, (2) compare circulating miRNAs between serums of insulin resistant and sensitive equines, horses and ponies, (3) characterize circulating miRNA profiles associated with equine insulin resistance and finally (4) identify candidate genes important for equine insulin resistance and its associated conditions.

3.1. Material and Methods

Ethics statement

This study was approved by the Virginia Tech Institutional Animal Care and Use Committee. IACUC#: 15-046, Virginia Polytechnic Institute and State University.

Animal selection

Animals in each herd were maintained on pasture with ad libitum access to forage, water, and mineral prior to the start of the study. Prior knowledge of two diverse but metabolically well-characterized populations (Thoroughbred/ Thoroughbred-cross horses and Welsh/Dartmoor ponies) were used to pre-select animals. These equines were screened by using proxy estimations of insulin sensitivity from single measurements of fasting glucose and insulin [102]. Based on the data from blood samples and individual animal history, animals were finally selected (24 animals; 12 from each herd) and divided into two main groups: 12 (6 horses and 6

ponies) suspected to be insulin resistant (IR) and 12 (6 horses and 6 ponies) controls suspected to be insulin sensitive (IS) matched for age, weight, breed and body condition. After the selection, the frequently-sampled intravenous glucose tolerance (FSIGT) test with minimal model analysis was performed to more accurately diagnose insulin resistant in those animals [104], and circulating miRNA profiling done (Figure 3).

Sampling, and sample processing

Animal sampling occurred in winter months to reduce variability in blood parameters or body condition due to pasture conditions. Additionally, only non-pregnant mares were used. Diets were designed to meet NRC recommendations for idle horses, and forage samples collected and analyzed prior to animal sampling to confirm nutrient values.

Prior to sampling, animals were maintained overnight in stalls to maintain an unperturbed state prior to sampling. Horses were allowed ad libitum access to water and hay with known composition and low sugar content. Screenings and FSIGT tests occurred between 8 and11AM.

All FSIGT tests were performed in the span of one week to reduce variation due to the time of collection. Briefly, after collection of baseline blood samples (-30 and 0 min), a 50% w/v dextrose solution (0.3 g/kg BW administered over 2 min) was infused and blood samples obtained at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, and 19 min after the end of the glucose infusion. At 20 min after glucose administration, insulin (10 IU/kg BW) was infused via catheter and subsequent blood samples collected at 22, 23, 24, 25, 27, 30, 25, 40, 50, 60, 70, 80, 90, 100, 120, 150 and 180 min post-glucose infusion. After each blood sample collection, an equivalent volume of sterile 0.9% saline containing 5 U/mL of heparin was used to flush the catheter. Sampling occurred via jugular venipuncture into 10 mL Vacutainer (BD, Dickinson, MA) tubes coated with EDTA (to recover plasma for insulin analysis), potassium oxalate (to recover plasma for glucose analysis) or without

anticoagulant (to recover serum for miRNA profiling, taken at baseline before FSIGTT). Plasma tubes were spun at 2000g for 10 minutes at 4°C to separate plasma. Serum tubes were allowed to clot for one hour at room temperature before centrifuging at 1500g for 10 minutes at 4°C to separate serum. Plasma and serum samples were aliquoted into 2.5 mL microcentrifuge tubes and stored at -80°C for later downstream analyses.

Plasma was analyzed for glucose by enzymatic assay using the YSI 2700 SELECT Biochemistry Analyzer (Yellow Springs, OH) with YSI 2365 glucose membranes, YSI 2747 glucose/L-Lactate standard, and YSI 2357 buffer concentrate kit. Insulin was analyzed using Siemens Coat-A-Count Insulin Radioimmunoassay kit (Siemens, Los Angeles, CA), previously validated for horses [174]. Insulin levels above the kit standards were diluted with charcoal-stripped equine plasma [175].

Insulin sensitivity index (SI), acute insulin response to glucose (AIRg), glucose effectiveness (Sg) and disposition index (DI) were determined by the minimal model of glucose and insulin dynamics using specialized software (MinMod Millenium 6.02) [102]. Areas under the insulin and glucose curve were calculated using Riemann sums (trapezoidal rule) [198,199]. Serum collected during the -30 min baseline measurement of the FSIGT was used for miRNA profiling analyses.

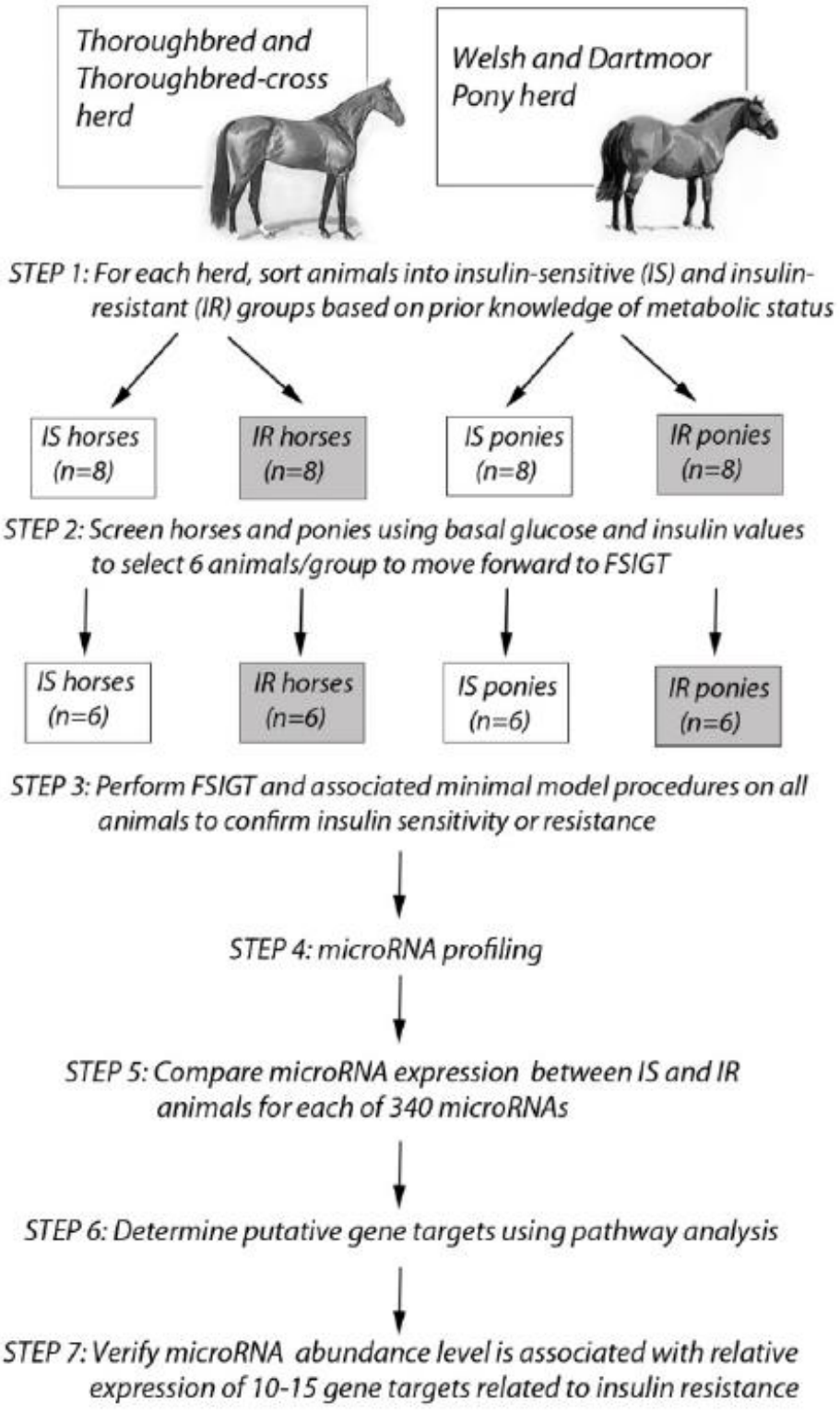

Figure 3 Schematic illustration of research protocol used in this study.

MicroRNA isolation and profiling

Total RNA was isolated from equine serum samples according to manufacturer's protocols using TRI Reagent BD-RNA/DNA/Protein Isolation Reagent for Blood Derivatives (Molecular Research Center, Cincinnati, OH). Samples were lysed and separated by chloroform into RNA and protein layers. RNA was precipitated with isopropanol and washed with 75% ethanol. All samples were then treated with DNA-free DNase Treatment and Removal Reagent (Invitrogen/Life Sciences, Grand Island, NY) to remove any DNA contamination. RNA purity values were assessed using the Nanodrop Spectrophotometer ND-1000 (Thermo Scientific, Wilmington, DE). Typically, 260/280 values above 1.8 are considered acceptable for RNA quantification by PCR. For this RNA isolation using chloroform causes the Nanodrop reading to have lower 260/280 values and therefore values of 1.7 were accepted for PCR. Samples were then stored at -80°C until further qRT-PCR analysis. cDNA was generated using miScript (Qiagen Valencia, CA) per the manufacturer's protocol. 4µL 5X miScript RT Buffer, 1µL reverse transcriptase mix and 1µg of RNA and water were used in a 20µL solution and incubated for 60 min at 37°C and then 5 min at 95°C. cDNA reaction was then immediately added to the qRT-PCR master mix for PCR analysis [176].

Equine miRNA forward primer sequences were obtained by an *in silico* detection model [177] and validated in the horse via small RNA-seq. Of all the 340 equine miRNAs used in this study, 250 were conserved human miRNAs and 75 were equine-specific miRNAs with human homologues but different nucleotide sequences. The remaining 15 miRNAs were equine-specific but had no apparent human homologue [176]. Qiagen qRT-PCR protocol was performed on 384 well plates with three previously-validated endogenous controls: RNU43, snoRNA, and U1 snRNA, a negative control (no primer), and 340 previously-validated equine miRNAs. Each well contained 6 µL of total reaction including 2X QuantiTect SYBR Green PCR Master Mix, 10X miScript Universal reverse primer (Qiagen), and miRNA specific forward primer and 0.1 µL of cDNA. qRT-

PCR was performed using the LightCycler480 PCR system (Roche). Cycle conditions run at 95°C for 15 minutes, followed by 45 cycles of 94°C for 15 seconds for denaturation, annealing at 55°C for 30 seconds, and extension at 70°C for 30 seconds, ending in a melt curve analysis to confirm single cDNA amplification. miRNAs were considered present at a cycle number of less than 37 as well as confirmed appropriate by amplification curves and singular melt peaks. Samples were run with biological and technical replicates and normalized to internal controls, i.e., miRNAs present in all samples with a standard deviation between samples of less than one.

Normalization ofmiRNA Expression Data and Statistical Analysis

Normalization was done by delta CT = Ct(target) - Ct(mean of the sample), which has been showed to have high efficiency identifying select housekeeping genes for large-scale miRNA expression studies utilizing the geometric mean of all miRNA genes expressed in samples that had an SD ≤1 [200].

A Shapiro-Wilk normality test was performed to determine normal distribution. Variables were transformed and analyzed on the log scale when necessary. Two-tailed t-tests were performed to compare miRNA profiles between groups. Significant results were considered when $p<0.05$.

Pathway Analysis

A posterior pathway analysis was performed with DIANA TOOLS miRPath v.3 [178] using miRNA differentially and uniquely expressed. Predicted targets were recorded using DIANA-microT-CDS algorithm and pathways were considered significant with $p \leq 0.05$ [179]. Additional data, such as miRNA-disease associations and miRNA-gene interactions, were collected from other database sources, including DIANNA TOOLs TarBase v.8 [201], and DisGeNET RDF v4.0 [202,203].

3.2. Results

Pastures and hays analysis

Results of feed analysis for horses can be found in Table 3. Horses pastures had similar nutritional content.

Table 3 Chemical analysis (on a dry matter basis) of the cool season grass pastures and hay for horses.

Analysis Parameters	Pasture 1	Pasture 2	Hay
DM (%)	98.2	96.3	96.5
DE (Mcal/Kg)	2.06	2.04	1.85
CP (%)	9.5	12.1	8.6
Estimated Lysine (%)	0.33	0.42	0.3
ADF (%)	38.5	38.2	45.5
NDF (%)	64.9	64.9	72
WSC (%)	6.5	7.9	4.4
ESC (%)	5.5	7.1	3.7
Starch (%)	0.5	0.1	0.1
NFC (%)	16	13.3	10.3
Estimated fructan (%)	1	0.8	0.7
NSC (%)	7	8	4.5

Dry Matter (DM) (%), Digestible Energy (DE) (Mcal/Kg), Crude Protein (%), Estimated Lysine (%), Acid Detergent Fiber (ADF) (%), Neutral Detergent Fiber (aNDF) (%), WSC (Water Sol. Carbs.) (%), ESC (Simple Sugars) (%), Starch (%), Non- Fiber Carb. (NFC), Non-structural carbohydrates (NSC) (%).

Results of feed analysis for ponies can be found in Table 4. Ponies pastures had similar nutritional content. However, the nutritional content of the diet fed for ponies was different from the diet fed for horses, mainly in regarding the NSC content (simple sugars, starch, and fructans).

Table 4 Chemical analysis (on a dry matter basis) of the cool season grass pastures and hay for ponies.

Analysis Parameters	Pasture1	Pasture2	Pasture3	Pasture4	Hay
DM (%)	95.8	95.7	95.6	95.5	95.8
DE (Mcal/Kg)	2.36	2.46	2.28	2.37	2.17
CP (%)	19.7	23.7	20.6	16.7	11.5
Estimated Lysine (%)	0.68	0.92	0.8	0.65	0.45
ADF (%)	30.5	26.2	32.4	29.4	32.5
NDF (%)	50.8	45.1	52.2	50.2	58.6
WSC (%)	13.2	18.6	12.8	19.8	12.6
ESC (%)	10.5	14	9.6	12.4	10.3
Starch (%)	0.2	0.1	0.1	0.1	0.8
NFC (%)	19.9	20.4	16.4	22.3	19.9
Estimated fructan (%)	2.7	4.6	3.2	7.4	2.3
NSC (%)	13.4	18.7	12.9	19.9	13.4

Dry Matter (DM) (%), Digestible Energy (DE) (Mcal/Kg), Crude Protein (%), Estimated Lysine (%), Acid Detergent Fiber (ADF) (%), Neutral Detergent Fiber (aNDF) (%), WSC (Water Sol. Carbs.) (%), ESC (Simple Sugars) (%), Starch (%), Non- Fiber Carb. (NFC), Non-structural carbohydrates (NSC) (%)

Groups description

Animals in the current study were qualified as insulin resistant (IR) or insulin sensitive (IS) according to several parameters. Because of the high dose of intravenous glucose and low exogenous insulin dose given during the *FSIGT* test, minimal model parameters were not the first parameter used to diagnose insulin resistant animals. Therefore, groups were selected based on the combination of their minimal model parameters, basal glucose and basal insulin values, and estimated areas under the curve (AUC) for both glucose and insulin. The cut-off values for being

considered insulin sensitive was basal insulin <20 to 30uIU/mL for ponies and horses, respectively [204–206].

Because the minimal model parameters were not efficient at diagnosing insulin resistant animals given the doses of exogenous insulin and glucose used in the test, it was not possible to discriminate the source of failure on glucose homeostasis. However, estimating total areas under curves for both glucose and insulin allows evaluation of the physiological response of glucose-insulin interactions. Normal individuals are expected to have a lower estimated area under the curve values for both glucose and insulin, while higher values are indicative of disturbances in glucose homeostasis. Therefore, the determining factors considered for selecting the groups were estimated area under the curves from the FSIGT test for glucose and for insulin (Tables 7 to 10).

Finally, animals' history of previous insulin resistance metabolic state or laminitis cases were also considered when diagnosing insulin resistant animals.

Considering these factors all together, there were 2 horses and 9 ponies diagnosed as insulin resistant (total of 11 insulin resistant animals) and 10 horses and 3 ponies diagnosed as insulin sensitive (total of 13 insulin sensitive animals). The final data of each group can be found on the tables below.

The selection of groups based on minimal model parameters was based on reference quintile values [102]. However, because insulin was too low and glucose too high compared to each other, only AIRg values, which quantify endogenous insulin secretion in response to the glucose dose, were considered. Groups considerably differed in AIRg quintiles for the minimal model parameters for horses (Table 5) and for ponies (Table 6).

Table 5 Acute insulin response to glucose (AIRg) values, and respective quintiles, for horses.

Parameter	AIRg (mu.l^-1.min)			
Group	IR		IS	
Mean	1,022[a] (1,012; 1,031)	5[th]	197[b] (173; 224)	3[rd]

Values are shown as mean (95% confidance interval). Different letters (a or b) indicate significance between groups (p<0.05).

Table 6 Acute insulin response to glucose (AIRg) values, and respective quintiles, for ponies.

Parameter	AIRg (mu.l^-1.min)			
Groups	IR		IS	
Mean	839[a] (736; 956)	5[th]	210[b] (151; 292)	3[rd]

Values are shown as mean (95% confidance interval). Different letters (a or b) indicate significance between groups (p<0.05).

Insulin resistant and insulin sensitive horses differed for basal insulin and total area under the insulin curve (Table 7) but did not differ for basal glucose and total area under the glucose curve (Table 8).

Table 7 Values of basal insulin and area under the insulin curve (AUCi) for insulin resistant (IR) versus insulin sensitive (IS) horses (H).

Parameter	Basal Insulin (uIU/mL)	
Group	IR	IS
Mean	29.2[a] (21.4; 40.0)	12.9[b] (11.6; 14.4)
Parameter	**AUCi (µIU/mL × min)**	
Group	IR	IS
Mean	26,190[a] (40,174; 17,074)	5,685[b] (4,987; 6,480)

Values are shown as mean (95% confidance interval). Different letters (a or b) indicate significance between groups (p<0.05).

Table 8 Values of basal glucose and area under the glucose curve (AUCg) for insulin resistant (IR) versus insulin sensitive (IS) horses (H).

Parameter	Basal Glucose (mg/dL)		AUCg (mg/dL × min)	
Group	IR	IS	IR	IS
Mean	88.4±4.5	90.06±1.5	27313.7±12	28849.27±12

Values are shown as mean ± standard error. Different letters (a or b) indicate significance between groups (p<0.05).

Insulin resistant and insulin sensitive ponies did not differ for basal glucose and area under the glucose curve (Table 10) but differed for basal insulin and total area under the insulin curve (Table 9).

Table 9 Values of basal insulin and area under the insulin curve (AUCi) for insulin resistant (IR) versus insulin sensitive (IS) ponies (P).

Parameter	Basal Insulin (uIU/mL)	
Group	IR	IS
Mean	35.5[a] (29.3; 43.1)	15.0[b] (11.6; 19.3)
Parameter	**AUC Insulin (µIU/mL × min)**	
Group	IR	IS
Mean	24,510[a] (21,270; 28,244)	8,192[b] (5,855; 11,463)

Values are shown as mean (95% confidance interval). Different letters (a or b) indicate significance between groups (p<0.05).

Table 10 Values of basal glucose and area under the glucose curve (AUCg) for insulin resistant (IR) versus insulin sensitive (IS) ponies (P).

Parameter	Basal Glucose (mg/dL)		AUCg (mg/dL × min)	
Group	IR	IS	IR	IS
Mean	88.67±2.53	94.24±4.1	27476.44±964.3	32730.4± 3212.7

Values are shown as mean ± standard error. Different letters (a or b) indicate significance between groups (p<0.05).

Both groups for horses (Table 11) and for ponies (Table 12) did not differ for body weight and body condition score.

Table 11 Values of body weight (BW) and body condition score (BCS) for insulin resistant (IR) versus insulin sensitive (IS) horses (H).

Parameter	BW (kg)		BCS	
Group	IR	IS	IR	IS
Mean	655.5±2.5	635±29	7.5±0.5	6.2±0.3

Values are shown as mean ± standard error.

Table 12 Values of body weight (BW) and body condition score (BCS) for insulin resistant (IR) versus insulin sensitive (IS) ponies (P).

Parameter	BW (kg)		BCS	
Group	IR	IS	IR	IS
Mean	321.8±21.5	384±47	6.8±0.25	6.8±0.16

Values are shown as mean ± standard error.

MicroRNA profiles

Of the 340 miRNAs analyzed, several were found to be in different relative quantity between groups ($p<0.05$). There were 13 miRNAs differently expressed between insulin resistant and insulin sensitive horses (Table 6a), 15 differently expressed between insulin resistant and insulin sensitive ponies (Table 6b), 17 differently expressed between horses and ponies (Table 6c), and 14 differently expressed between insulin resistant and insulin sensitive animals, horses and ponies combined (Table 6d), with 4 of these miRNAs already noted when comparing horses versus ponies. In the horse groups, 3 miRNAs were expressed in one group only, IR, and 1 miRNA was repeated in our previous study, mir-140-3p in horses [197]. Additionally, DIANA TOOLS pathway analysis indicated that many of these significant miRNAs have already been

associated with type 2 diabetes, metabolic syndrome, hyperinsulinism, hyperglycemia, obesity, or insulin resistance in either humans or mouse (Tables 17, 18, Appendix).

MicroRNAs significantly different between insulin resistant and insulin sensitive horses:

From the 13 miRNAs quantitatively different between groups, 8 were higher in the insulin resistant group: eca-mir-200b, eca-mir-421, eca-mir-216b, eca-mir-103, eca-mir-217, eca-mir-7, eca-mir-507, and eca-mir-140-3p; 3 were expressed in insulin resistant group only: eca-mir-539, eca-mir-544b, and eca-mir-1597; and 2 were higher in insulin sensitive group: eca-mir-433 and eca-mir-30e (Table 13).

Table 13 Normalized Ct values of significantly different miRNAs between groups: insulin sensitive (IS) and insulin resistant (IR) horses.

miRNA	IS	IR
ecamir200b	2.83±0.50	6.42±0.77
ecamir421	1.35±0.35	4.62±0.77
ecamir216b	1.63±0.45	4.80±2.32
ecamir103	1.08±0.40	4.12±0.15
ecamir217	1.16±0.39	3.95±1.84
ecamir7	1.93±0.23	4.54±0.912
ecamir507	0.29±0.21	1.75±0.41
ecamir1403p	-1.47±0.15	-0.34±0.68
ecamir539	0±0	0.98±0.98
ecamir544b	0±0	0.84±0.84
ecamir1597	0±0	0.63±0.63
ecamir433	-0.81±0.09	-1.49±0.21
ecamir30e	4.27±0.23	2.86±0.07

Values are shown as mean ± standard error.

MiRNAs significantly different between insulin resistant and insulin sensitive ponies:

From the 15 miRNAs quantitatively different between groups, 4 were higher in the insulin sensitive group: eca-mir-187, eca-mir-323-3p, eca-mir-23b, eca-mir-195; and 11 higher in the insulin resistant: eca-mir-1291a, eca-mir-15b, eca-mir-133a, eca-mir-10a, eca-mir-324-5p, eca-let-7a, eca-let-7d, eca-mir-331, eca-mir-423-5p, eca-mir-200c, eca-mir-204b (Table 14).

Table 14 Normalized Ct values of significantly different miRNAs between groups: insulin sensitive (IS) and insulin resistant (IR) ponies.

miRNA	IR	IS
ecamir1291a	6.39±0.58	1.11±1.69
ecamir15b	4.21±0.37	0.5±1.44
ecamir133a	-8.93±1.21	-16.6±1.21
ecamir10a	4.84±0.28	1.1±1.93
ecamir3245p	4.09±0.86	-3.8±3.8
ecalet7a	3.86±0.55	-2.72±3.61
ecalet7d	4.41±0.65	-3.91±4.76
ecamir187	-1.82±0.35	0.14±0.5
ecamir331	2.78±0.6	-4.46±4.46
ecamir4235p	3.18±0.26	0.25±1.99
ecamir200c	4.59±0.56	-2.76±5.39
ecamir3233p	-8.61±0.16	-7.47±0.75
ecamir204b	2.23±0.94	-4.46±4.46
ecamir23b	1±0.3	2.92±1.24
ecamir195	2.715±0.323	4.133±0.466

Values are shown as mean ± standard error.

MicroRNAs significantly different between ponies and horses, and insulin resistant and insulin sensitive animals:

Furthermore, we compared circulating miRNA profiles between ponies and horses, combining animals regardless of whether they were insulin resistant or insulin sensitive. There were 17 miRNAs quantitatively different between groups, from which 15 were higher in horses: eca-mir-150, eca-mir-192, eca-mir-708, ec-amir-223, eca-mir-21, eca-mir-23a, eca-mir-1302c5, eca-mir-

217, eca-mir-215, eca-mir-92a, eca-mir-32, eca-mir-421, eca-mir-330, eca-mir-30d, eca-mir-197; and 2 higher in ponies: eca-mir-222, eca-mir-154 (Table 15).

Table 15 Normalized Ct values of significantly different miRNAs between groups: combined horses versus combined ponies.

miRNA	P	H
ecamir150	1.9±0.49	4.26±0.29
ecamir192	0.65±0.48	3.18±0.47
ecamir708	2.66±0.34	4.25±0.26
ecamir223	0.07±0.62	2.44±0.34
ecamir222	4.71±0.34	2.77±0.57
ecamir21	-0.67±0.34	0.97±0.47
ecamir23a	-0.13±0.33	1.2±0.38
ecamir154	4.59±0.34	3.24±0.41
ecamir1302c5	1.79±0.73	3.76±0.45
ecamir217	-2.06±1.53	1.63±0.51
ecamir215	2.24±0.38	3.56±0.44
ecamir92a	-0.25±0.54	1.22±0.37
ecamir32	-2.01±1.42	1.36±0.56
ecamir421	-1.68±1.59	1.9±0.48
ecamir330	-2.23±1.5	1.15±0.53
ecamir30d	2.11±0.64	3.55±0.24
ecamir197	0.19±1.43	3.25±0.29

Values are shown as mean ± standard error

Then, we compared insulin resistant with insulin sensitive animals, combining groups independently of being horses or ponies, there were 14 miRNAs differently expressed between

groups. However, 4 of these miRNAs overlapped the ones found significant between horses and ponies: eca-mir-192, eca-mir-223, eca-mir-23a, eca-mir-21. From the 10 remaining miRNAs quantitatively different between groups, 6 were higher in the insulin resistant group: eca-let-7d, eca-mir-331, eca-mir-1291a, eca-let-7a, eca-mir-15b, eca-mir-200b; and 4 higher in the insulin sensitive group: eca-mir-193a-5p, eca-mir-23b, eca-mir-150, eca-mir-122 (Table 16).

Table 16 Normalized Ct values of significantly different miRNAs between groups: combined insulin resistant animals versus combined insulin sensitive animals.

miRNAs	IS	IR
ecalet7d	0.86±1.14	4.20±0.67
ecamir331	-0.59±0.99	2.27±0.59
ecamir1291a	3.07±0.52	5.67±0.69
ecalet7a	1.15±0.88	3.72±0.47
ecamir15b	2.37±0.55	4.268±0.38
ecamir200b	2.78±0.49	4.24±0.58
ecamir193a5p	4.99±0.37	3.76±0.50
ecamir23b	2.52±0.33	1.26±0.37
ecamir150	3.75±0.42	2.29±0.49
ecamir122	3.20±0.45	0.84±0.96

Values are shown as mean ± standard error

3.3. Discussion

In the current study, a total of 13 miRNAs were found differentially expressed between insulin resistant and insulin sensitive horses. Our preliminary study [197] found 6 circulating miRNAs that were expressed only in insulin sensitive mares, 5 miRNAs that were found in lower quantity in insulin resistant mares, and 3 miRNAs that were found in higher quantity in insulin resistant mares, totaling 14 miRNAs differently expressed between groups in horses. In addition, the miRNA profiles were different between the preliminary and current studies. The different results between studies are most likely due to seasonal variations during sample collections between studies. Although both studies used the same population of horses, the samples were not collected in the same seasonal period and included different animals from the herd. In the preliminary study, samples were collected in the spring and had higher levels of NSC (over 15.4% of DM), whereas the current samples were collected in the winter, with NSC no higher than 8% of DM. Seasonal changes can induce variations in the nutritional content of the pasture, especially in the way that carbohydrates from grass are stored [207,208]. Changes in diet have been shown to affect the levels of miRNAs [209,210]. Pastures with high NSC content can predispose horses to many diseases including insulin resistance [27,211,212].

The pilot investigation had a smaller sample size, with only 6 horses (3 insulin resistant and 3 insulin sensitive). However, the current study had a larger sample size of 12 horses and had an uneven distribution of insulin resistant versus sensitive equines (2 insulin resistant and 10 insulin sensitive horses). In contrast to our pilot study, there was no variation in body condition between insulin resistant and insulin sensitive horses. The body condition score had a smaller range in insulin resistant horses (between 7 and 8, n=2) whereas the range was larger in insulin sensitive horses (between 5.5 and 8, n=10). However, the non-significance in this parameter may be due to the small number of animals in the insulin resistant group (n=2).

In contrast to the previous study, the current group of animals included a population of ponies for circulating miRNA profiling. Insulin sensitive ponies and insulin sensitive horses did not differ for AIRg, the area under the insulin curves, or basal insulin. The same applied for insulin resistant ponies versus insulin resistant horses, demonstrating homogeneity between insulin resistant groups (horses and ponies) and insulin sensitive groups (horses and ponies) for these parameters. Furthermore, 15 miRNAs were differentially expressed between insulin resistant and insulin sensitive ponies. There is no current data available to compare our results with, but studies using humans and mice have shown many of the found miRNAs to be relevant in insulin resistance and associated conditions (Tables 17 and 18, Appendix).

Finally, 10 miRNAs were differentially expressed between insulin resistant and insulin sensitive animals (horses and ponies combined) indicating potential similarity in the mechanisms of insulin resistance in Thoroughbred/ Thoroughbred-cross horses and Welsh/Dartmoor ponies.

Despite the differences in the results of the studies, both investigations were able to show differences in miRNA profiles between insulin resistant and insulin sensitive animals. These differences are potentially relevant to equine insulin resistance based on research conducted in humans and mice (Tables 17 and 18, Appendix).

Possible breed associated differences in circulating microRNA profiles

In addition to comparing circulating miRNA profiles between insulin resistant and insulin sensitive animals, this study also compared circulating miRNA profiles between animal breeds: horses versus ponies. Our results found 17 differently expressed miRNAs between these groups, supporting circulating miRNAs as potential tools to differentiate specific breed types, which has already been suggested [159].

Only miR-122 and miR-154 were found in higher quantity in ponies compared to horses. The remaining 15 miRNAs were in higher quantity in horses compared to ponies. It is recognized that

there are breed-related differences in glucose and insulin dynamics in equines, and that ponies and certain horse breeds, such as Morgans, Paso Finos, and quarter horses, are more commonly affected by insulin resistance and endocrinopathic laminitis [213,214]. Therefore, the difference in circulating miRNA profiles between these breed groups may also be associated with their predisposition to insulin resistance and associated conditions.

In this study, ponies were more prone to be insulin resistant whereas horses were more prone to be insulin sensitive. Therefore, the metabolic differences between groups (ponies versus horses) may have interfered in the results. For future studies, it would be recommended to pair more metabolically homogeneous groups and to explore the metabolic differences between breeds in association to their circulating miRNA profiles.

Insulin sensitivity testing

Influences of the non-structural carbohydrate content in the pastures

The combination of genetic predisposition, in addition to nutritional factors, such as high fractions of NSC, had a noticeable impact on the metabolic state of the animals in this study. Most of the horses (10 out of 12) were diagnosed as insulin sensitive, and most ponies (9 out of 12) were diagnosed as insulin resistant.

It is recommended for horses and ponies with insulin resistance to be offered feed with less than 10% to 12% NSC (which includes simple sugars, fructans, and starches) of dry matter [40,215]. The horses in this study were offered feed with the NSC lower (4.5 to 8%) than this recommendation, whereas the ponies had higher NSC content (12.9 to 19.9%). The content of NSC in the feed can have a significant impact on postprandial insulin response and digestive fermentation, resulting in chronic metabolic disorders associated with high glycemic and insulinemic responses and laminitis [208,216–218]. For future studies, it would be recommended

to equally control for feed content factors for both breeds by moving groups to dry lots at least one month before test sampling and offer them the same diet.

The diagnosis of insulin resistant animals: Minimal model parameters

The reason for not using the SI for the minimal model parameter analysis was due to the low dose of endogenous insulin and a high dose of intravenous dextrose during the FSGIT tests, which generated false positives when diagnosing insulin resistant animals based on minimal model parameters. Therefore, minimal model parameters were not definitive factors for diagnosing insulin resistant animals. The dose of exogenous insulin appears to not have been enough to cause significant physiological response given the dose of intravenous dextrose. However, all horses were given the same doses, which allowed for comparison in areas under the insulin and glucose curves.

Although the minimal model parameters were not definitive to select groups, insulin resistant horses and ponies had all AIRg values in the 5th reference quintile, in contrast to the insulin sensitive animals, which had AIRg values around the 3rd reference quintile. Higher values of AIRg indicate higher release of endogenous insulin to acquire normal (or close to) physiological response. This increase in insulin secretion to achieve physiological homeostasis is indicative of insulin resistance [102,103].

The diagnosis of insulin resistant animals: Estimated areas under the curve

The main factors for diagnosing animals in this study were the estimated area under the curve for the FSGIT tests for insulin and basal insulin concentration. Insulin resistant animals have higher areas under the insulin curve as a result of not being able to mediate glucose uptake as efficiently as an insulin sensitive animal.

In our pilot study, the area under the curve for insulin for insulin resistant horses had a mean of 24494 (95% confidence interval: 22457; 26717) µIU/mL × min, while in the current study, the mean for insulin resistant horses was 26190 (95% confidence interval: 17074; 40174) µIU/mL × min and 24510 (95% confidence interval: 21270; 28244) µIU/mL × min for insulin resistant ponies. The area under the insulin curve for insulin sensitive horses in our pilot study had a mean of 6758 (95% confidence interval: 4653; 9816) µIU/mL × min, while in the current study, the mean was 5685 (95% confidence interval: 4987; 6480) µIU/mL × min for insulin sensitive horses and 8192 (95% confidence interval: 5855; 11463) µIU/mL × min for insulin sensitive ponies. These results confirm that higher concentrations of insulin are required to maintain normal levels circulating glucose, as can be noticed in the values for basal glucose and estimated area under the curve for glucose (Tables 8 and 10). Based on their area under the curve for both glucose and insulin in this study, insulin resistant horses and ponies indicated a hyperinsulinemic-normoglycemic physiological state.

MicroRNAs review: Horses

Our results showed that horses with insulin resistance had higher levels of circulating miR-200b. It has been suggested that decreased expression of miR-200 family miRNAs (miR-200s), including miR-200a, miR-200b, and miR-200c, contributes to hepatic insulin resistance [219]. Hepatic insulin resistance refers to impaired suppression of glucose production (gluconeogenesis) by insulin in hepatocytes. Additionally, hepatic insulin resistance is suggested to be an underlying cause of metabolic syndrome in humans and linked to hyperglycemia, dyslipidemia and increased inflammatory factors [220]. In addition, decreased levels of miR-200s have been observed during elevated serum concentration of interleukin-6 (IL-6). The administration of IL-6 has been shown to induce insulin resistance through down-regulation of miR-200s resulting in impaired activation of the PI3K/AKT pathway and synthesis of glycogen

[219]. Adipose tissue is one of the main sources of inflammatory mediators including IL-6, and elevated IL-6 levels have been reported in obese humans [221]. Therefore, it is possible that the higher level of circulating miR-200b in insulin resistant horses is also associated with their increased body condition score, overweight. However, because of the small number of animals in this group, it is not possible to confirm this hypothesis. Additionally, the expression levels of circulating miR-200b-3p has been shown to be higher in horses with acute laminitis and decreasing after routine laminitis treatment [222]. Both insulin resistance and incidence of laminitis are associated with equine metabolic syndrome and therefore, miR-200b may have great potential as a biomarker for this metabolic condition

MiR-421-3p has been observed to be upregulated in the liver of mice fed a high-fat diet and causally linked to the development of hepatic insulin resistance. Additionally, i*n silico* target analysis identified miR-421-3p as having putative binding sites for the 3'UTRs of insulin receptor (INSR*) and* insulin receptor substrate 1 (*IRS-1*) genes [223]. Although there was no significant difference in body weight and BCS between insulin resistant and insulin sensitive horses, it is possible that impairment in the fat metabolism of insulin resistant animals caused differences in circulating levels of miR-421 between groups. Future studies including the lipid profile of insulin resistant in contrast to insulin sensitive animals can be helpful in addressing this possibility.

It has been shown that mir-103 targets caveolin-1, a critical regulator of the insulin receptor, and expression of miR-103 is upregulated in the liver and fat tissues of obese mice. Increased activity of miR-103 can induce impaired glucose homeostasis, and silencing miR-103 is concomitant with stabilization of the insulin receptor, enhanced insulin signaling, decreased adipocyte size and enhanced insulin-stimulated glucose uptake [224]. If similar to mice, the higher concentration of circulating miR-103 in insulin resistant horses found in this study may be due to the expected increased concentration of miR-103 in the liver and fat tissues.

Increased concentration of serum miR-217 has been associated with the development of proteinuria in type 2 diabetes human patients and may be involved in the development of diabetic kidney disease by promoting chronic inflammation, renal fibrosis, and angiogenesis in humans [225]. Additionally, it has been proposed that blocking miR-217 might improve insulin resistance under high glucose diet in mice [226]. Furthermore, phosphatase and tensin homologue (PTEN) is a tumor suppressor that serves as a negative regulator of PI3K/PTEN/Akt signaling pathway and is downregulated by miR-217. The PI3K/PTEN/Akt signaling cascades have important effects on glucose uptake via translocation of GLUT-4, and overexpression of PTEN inhibits GLUT-4 translocation and glucose transport [227–229]. In our current study, mir-217 was found in higher quantity in insulin resistant animals, as expected for humans with type 2 diabetes. The increase in circulating miR-217 may be given to an attempt of the organism to increase glucose uptake by cells and alleviate the hyperglycemia stress associated with insulin resistance. However, the actual role of miR-214 in equine metabolism during the state of insulin resistance remains unclear.

In mouse muscle cells, overexpression of miR-7 was shown to downregulate expression of IRS-1 and to inhibit insulin-stimulated Akt phosphorylation and glucose uptake, inducing insulin resistance [230]. Likewise, our study found that miR-7 was in higher quantity in insulin resistant compared to insulin sensitive horses.

In contrast to our previous study [197], miR-140-3p was found to be in higher quantity in insulin resistant horses in this study. Downregulation of miR-140-3p has been associated with calorie restriction [231], while its upregulation has been associated with diabetes, specifically type 2 diabetes [196]. The difference in the miR-140-3p levels in this and our previous study may be due to a combination of seasonal variations since our first study performed the FSIGT test in May versus January for our current study, and animal composition, since the body condition score between insulin sensitive horses in both studies differs from each other. However, more studies are necessary to investigate the source of variation and the relationship between circulating miR-

140-3p levels and equine insulin resistance. Additionally, mir-140, the same family of mir-140-3p, is believed to play a role in the link between adipose tissue dysfunction and the development of obesity-associated disorders such as type 2 diabetes [195].

Expression of miR-30e has been found to be higher in abdominal subcutaneous adipose tissue in normal glucose tolerance compared to newly diagnosed type 2 diabetes humans, being considered to play a role in the link between adipose tissue dysfunction and the development of obesity associated disorders including type 2 diabetes [232]. Additionally, downregulation of miR-30e has been reported during high-fat diet-induced obesity in mice [233], and in human patients with type 2 diabetes [234] [196]. Similarly, our insulin resistant horses had lower levels of miR-30e. No relevant data for insulin resistance and associated conditions was found for miR-216b, miR-507, miR-539, miR-544b, miR-1597, and miR433 in our review.

MicroRNAs review: Ponies

MiR-15b has been shown as a potential predictive biomarker in obesity, being higher in obese individuals when compared to controls, diabetic, and obese diabetic individuals [192]. Furthermore, miR-15b has been reported to target the INSR, and overexpression of miR-15b impairs the insulin signaling cascade, as well as insulin stimulated glycogen storage in hepatocytes, is linked to the pathogenesis of hepatic insulin resistance in saturated fatty acid induced obesity [235]. Similar to these studies, miR-15b was higher in the insulin resistant group, even though there was no significant difference in body weight and BCS between pony's groups.

MiR-133a is expressed in both brown adipose tissue white adipose tissue and regulates adipocyte browning in vivo [236]. MiR-133a has been reported as being down-regulated in the liver of db/db mice (genetically mutated mouse in which leptin receptors do not function properly)

compared with control [237]. However, in our study, insulin resistant ponies were found to have higher levels of circulating miR-133a.

MiR-10a has been reported as being dysregulated in human type 2 diabetic muscles [238–240]. Additionally, it has been reported that miR-10a overexpression improves kidney damage of diabetes, while diabetes induces the decrease of miR-10a level in the kidney [241]. Our results showed that levels of circulating miR-10a were higher in insulin resistant animals. Further studies are necessary to investigate the physiological impacts of high levels of circulating miR-10a in equines and its association with insulin resistance.

MiR-331 has been shown to be involved in glucose metabolism [242], and reported as being dysregulated in human type 2 diabetic muscles [238–240].

A study found inverse associations of miR-423-5p with obesity measures and markers of inflammation [243], and it has been suggested as a potential biomarker for risk estimation and classification of morbidly obese patients [192,244]. Lastly, miR-423-5p has also been reported to be differentially expressed in subjects with prediabetes and type 2 diabetes patients compared to control subjects. The same study also found that miR-423-5p had sex-specific associations with prediabetes or diabetes [245]. In our study, higher levels of circulating miR-423-5p were associated with insulin resistance in ponies in accordance with the previous study in humans [241]. Mir-187 has been reported to be expressed in higher levels in pancreatic islets of human diabetic versus non-diabetic donors [246,247]. In the current study, mir-187 was found in lower circulating levels in insulin resistant versus insulin sensitive horses, indicating that circulating levels of miRNAs may be different from their tissue levels.

It has been suggested that let-7 family miRNAs, including let-7a and let-7d, represents a potential therapeutic target for the treatment of type 2 diabetes. It has been demonstrated that l*et-7* regulates multiple aspects of glucose metabolism. Global and pancreas-specific overexpression

of *let-7* in mice results in impaired glucose tolerance and reduced glucose-induced pancreatic insulin secretion. In addition, global knockdown of the let-7 family can prevent and treat impaired glucose tolerance in mice with diet-induced obesity [248,249]. Furthermore, it has been shown that let-7a and let-7d are direct translational repressors of the IL-13 gene and their expression increases in skeletal muscle from type 2 diabetes patients [250]. Let-7 is also involved in the regulation of FAM174A gene [201]. This gene has been found to be near significant genetic markers for elevated insulin values and increased the frequency of laminitis in Arabian horses. Therefore, FAM174A has been proposed to have an important role in the risk for equine metabolic syndrome [251].

Insulin resistant ponies had higher levels of circulating mir-200c, miR-200s family, compared to insulin sensitive ponies. Similar to horses, this difference may be related to hepatic insulin resistance and excess adipose tissue [219], and laminitis [222] in ponies. Additionally, miR-324-5p levels have been found to be increased in the serum of type 1 diabetic children [252], which were also elevated in insulin resistant ponies compared to sensitive group, indicating a possible link between this miRNA and type 1 and type 2 diabetes.

Although there were higher levels of miR-195 were found in the serum of insulin sensitive animals, studies have shown that miR-195 is upregulated in the liver of hyperglycemic mice [253]. Levels of miR-195 have been shown to be upregulated in the liver, but not in the skeletal muscle, of diet-induced obese mice. Additionally, miR-195 can suppress INSR buy targeting INSR 3′UTR directly. It can also be induced by saturated fatty acid palmitate, and is causally involved in the development of insulin resistance induced by saturated fatty acid [254]. Because our study focused on circulating miRNAs, it is not possible to know whether levels of miR-195 have increased in the liver of insulin resistant ponies or not. More studies are necessary to evaluate the association between the levels of miR-195 in the circulation and in the liver of horses. No

relevant data for insulin resistance and associated conditions were found for miR-1291a, miR-323-3p, miR-204b, miR-23b, and miR-195 in our review.

MicroRNAs review: Insulin resistant versus insulin sensitive animals combined

Finally, from the 10 miRNAs differently expressed between insulin resistant horses and ponies combined versus insulin sensitive horses and ponies combined, 7 miRNAs were shown to be differently expressed in either the insulin resistant versus insulin sensitive horses analysis: miR-200b; or the insulin resistant versus insulin sensitive ponies analysis: let-7a, let-7d, miR-23b, miR-1291a, miR-15b, and miR-331. From the remaining 3 miRNAs, miR-150 has been suggested to participate in the regulation of insulin signaling, with two potential targets being proposed for miR-150: CBL, which specifically targets activated protein tyrosine kinases and regulates their signaling, and GLUT-4. MiR-150 has been shown to be upregulated in adipose and liver tissues of type 2 diabetic mice [255]. In contrast, it has been reported that miR-150 modulates adipose tissue function, and that miR-150 deficiency significantly impairs the insulin signaling pathway in mice under obese stress [256]. Furthermore, our study indicated that insulin sensitive animals had higher levels of circulating miR-150. However, more studies are necessary to address the physiological impact of miR-150 in equine health.

Inhibition in a diet-induced obesity mouse model of miR-122 has been reported to decrease plasma cholesterol levels and to improve liver steatosis, fat in the liver [257]. However, more studies are required to evaluate the physiological impact of decreased circulating levels of miR-122 in insulin resistant equines. Additionally, miR-193-5p has been reported to regulate insulin-like growth factor 2 (IGF-2) and to be an active angiogenic factor in diabetic cardiomyopathy, a condition that makes it hard for the heart to deliver blood to the body, in rats [258]. Furthermore, expression of miR-193 in the liver has been reported to have a linear relationship with plasma

triacylglycerol levels [253]. Insulin resistant animals had lower levels of circulating miR-193a-5p. Even though it may be related to changes in blood pressure and lipid profile in our animals, the actual impact of this miRNA in horses remains to be clarified.

Relevant microRNAs for equine insulin resistance

A significant quantity of miRNAs described here has shown importance to insulin resistance and related conditions in other species (mainly humans and mice) based on the information found during our miRNA literature reviews. However, our current data do not allow us to conclude which ones are directly acting on insulin resistance and related conditions in equines.

Our investigation of miRNAs associated with equine insulin resistance also included database analysis: (1) DIANNA TOOL mirPath v.3 for miRNA pathway analysis and miRNA-disease associations [178]; (2) TarBase v.8 for miRNA-gene interactions [201]; and (3) DisGeNET RDF v4.0 for genes and variants associated with human disease [202,203]. This data altogether allows the selection of the most probable key regulators associated with insulin resistance in horses.

The DIANNA TOOL mirPath v.3 analysis indicated that at least 12 of all the 33 circulating miRNAs found in different relative quantities in this study have been reported to be linked to at least 3 conditions associated to insulin resistance in either humans or mice: miR-200b, miR-193a-5p, let7, miR-10a, miR-133a, miR-15b, miR-200b, miR-23b, miR-30e, miR-324-5p, miR-421, and miR-423-5p (Tables 17 and 18, Appendix).

In addition, 15 of these 33 miRNAs have been reported to be linked to genes associated with insulin resistance in humans [201]. It includes 8 of the top 10 scored gene associations for this disease [202,203]: Peroxisome proliferator-activated receptor gamma (PPARG): miR-324; Tumor necrosis factor (TNF): miR-1291; Adrenoceptor beta 2 (ADRB2): let-7 and miR-23b; Insulin receptor (INSR): miR-30e, miR-10a, miR-23b, miR-195, let-7, and miR-122; Glucokinase (GCK):

miR-331; Protein kinase AMP-activated catalytic subunit alpha 1 (PRKAA1): miR-217, miR-421, miR-30e, miR-103a, miR-216b, let-7, miR-10a, miR-23b, miR-133a, and miR-122; Insulin receptor substrate 1 (IRS1): miR-103a and miR-195; and Insulin (INS): miR-200b. Additionally, 7 of these miRNAs are associated with at least 2 of these genes: let-7, miR-103a, miR-10a, miR-122, miR-195, miR-23b, and miR-30e.

Finally, 8 miRNAs were both linked to (1) at least 3 conditions associated with insulin resistance in either humans or mince and to (2) at least one of the genes associated with insulin resistance in humans: let-7, mir-10a, mir-133a, mir-200b, mir-324, mir-421, mir-23b, and mir-30e. Therefore, these 8 miRNAs are most likely to be key regulators associated with insulin resistance in horses. Additionally, these 8 miRNAs are likely to have great potential as biomarkers for insulin resistance and associated conditions, such as hyperinsulinism, hyperglycemia and obesity, in equines. Unfortunately, there is not much data on equine insulin resistance and miRNA profiles currently available for equines as compared to other species, such as humans and mice, in which to compare results.

Because we used circulating miRNA profiles, we are not able to define where these significantly different miRNAs were being released from, which cells they were targeting, and how they were being regulated. Therefore, to confirm our hypothesis, future studies focusing on specific tissue cells and metabolic pathways are required for better understanding the regulatory mechanisms involving these miRNAs in equine health and disease.

CHAPTER 4: DISCUSSION

Both studies demonstrated significant differences in circulating microRNA (miRNA) patterns between insulin resistant and insulin sensitive animals. However, the profiles of insulin resistant animals differed between studies. The inconsistency between the studies may be due to environmental variations and metabolic status of the animals. The FSIGT test for the first study was done in May, 2014, while it was done in January, 2016, for the second study.

Even though the similarity of the circulating miRNA profiles associated with a disease is expected, there is no current information regarding changes in circulating miRNA profiles in equines through the seasons of the year, which can occur and may affect the miRNA profiling of animals. The insulin sensitive horses for the first study were not obese animals, whereas the insulin sensitive animals for the second study included obese animals in the insulin sensitive group of horses. This fact may be enough to generate significant changes between data. For future studies, it would be recommended to compare miRNA profiles of obese noninsulin resistant animals to obese insulin resistant animals, and nonobese noninsulin resistant animals to nonobese insulin resistant animals.

Although there were differences in the miRNA profile between studies, both identified differential circulating miRNA profiles between healthy and sick animals. Several miRNAs identified to be differentially expressed between groups have already been reported to be associated with diseases related to insulin resistance. Therefore, these studies support the vast potential of miRNAs as tools for predicting and managing insulin resistance and related conditions in horses.

The current study suggests eight circulating miRNAs as possible vital regulators of insulin resistance in horses: let-7, mir-10a, mir-133a, mir-200b, mir-324, mir-421, mir-23b, mir-30e. All of these miRNAs have been previously reported in association with many diseases in humans and mice, including insulin resistance, obesity, and type 2 diabetes. They are also associated with

genes linked to insulin resistance in humans. Therefore, it is possible that the mechanisms by which these miRNAs regulate gene expression and cause diseases in horses are similar to the ones in humans and mice. More studies are required to identify the specific roles of these circulating miRNAs in equine health.

CHAPTER 5: CONCLUSION

The results of these studies support a potential novel strategy to study insulin resistance in horses. There is substantial evidence supporting the association of circulating miRNA profiles with the development of insulin-resistance in horses. Although the use of miRNA as biomarkers for this metabolic condition in equines is still a work in progress, several miRNAs have been identified as possible critical regulators in the incidence of the disease. Many questions are still waiting to be answered regarding how these miRNAs impact equine insulin resistance. The current study suggests eight circulating miRNAs as possible key regulators of insulin resistance in horses, which are expected to be used to investigate the prognosis of equine insulin resistance further. Future studies are required to confirm the similarity of our results and to identify possible roles miRNAs play in equine health and disease. By identifying the role of miRNAs in equine health and disease, it will be possible to use new strategies to understand the mechanisms involved in equine insulin resistance and associated conditions and develop practical and efficient diagnosis and prognosis methods for this condition in horses.

REFERENCES

1. Vick, M.M., Adams, A.A., Murphy, B.A., Sessions, D.R., Horohov, D.W., Cook, R.F., Shelton, B.J. and Fitzgerald, B.P. (2007) Relationships among inflammatory cytokines, obesity, and insulin sensitivity in the horse. *J. Anim. Sci.* **85**, 1144–1155.

2. Nirmalan, N. and Nirmalan, M. (2017) Hormonal control of metabolism: regulation of plasma glucose. *Anaesth. Intensive Care Med.* . http://linkinghub.elsevier.com/retrieve/pii/S1472029917301728.

3. Wilcox, G. (2005) Insulin and insulin resistance. *Clin. Biochem. Rev.* **26**, 19–39. http://www.ncbi.nlm.nih.gov/pubmed/16278749%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC1204764.

4. DeFronzo, R. a, Tobin, J.D. and Andres, R. (1979) Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am. J. Physiol.* **237**, E214–E223.

5. Pfeifer, M. a, Halter, J.B. and Porte, D. (1981) Insulin secretion in diabetes mellitus. *Am. J. Med.* **70**, 579–588.

6. Robertson, R.P. and Guest, R.J. (1978) Reversal by methysergide of inhibition of insulin secretion by prostaglandin E in the dog. *J. Clin. Invest.* **62**, 1014–1019.

7. Dühlmeier, R., Deegen, E., Fuhrmann, H., Widdel, A. and Sallmann, H.P. (2001) Glucose-dependent insulinotropic polypeptide (GIP) and the enteroinsular axis in equines (Equus caballus). *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* **129**, 563–575.

8. Lingling Liu, William L.Kerr, Fanbin Kong, Derek R.Dee, M.L. (2018) Influence of nano-fibrillated cellulose (NFC) on starch digestion and glucose absorption. *Carbohydr. Polym.* **196**, 146–153.

9. Hampton, S.M., Morgan, L.M., Tredger, J.A., Cramb, R. and Marks, V. (1986) Insulin and C-peptide levels after oral and intravenous glucose. Contribution of enteroinsular axis to insulin secretion. *Diabetes* **35**, 612–616.

10. Kajinuma H. Kaneto A, Kuzuya T, et al. (1968) Effects of methacholine on insulin secretion in man. *J Clin Invest* **28**, 1384–1388.

11. Porte D Jr, Woods SC, S.P. (1980) Neural control of the pancreatic islet and its relation to stress hyperglycemia. *Marliss EB, ed. Diabetes Mellit. a Pathophysiol. approach to Clin. Pract.* .

12. Floyd, J.C., Fajans, S.S., Conn, J.W., Knopf, R.F. and Rull, J. (1966) Stimulation of insulin secretion by amino acids. *J. Clin. Invest.* **45**, 1487–1502.

13. Amery, C. and Nattras, M. (2000) Fatty acids and insulin secretion. *Diabetes, Obes. Metab.* **2**, 213–231. http://www.embase.com/search/results?subaction=viewrecord&from=export&id=L460183

83%5Cnhttp://dx.doi.org/10.1080/17482970601076354%5Cnhttp://sfx.hul.harvard.edu/sfx_local?sid=EMBASE&issn=17482976&id=doi:10.1080%2F17482970601076354&atitle=Dietary+fatty+acid.

14. Dyer, J., Merediz, E. Fernandez-Castaño Salmon, K.S.H., Proudman, C.J., Edwards, G.B. and Shirazi-Beechey, S.P. (2010) Molecular characterisation of carbohydrate digestion and absorption in equine small intestine. *Equine Vet. J.* **34**, 349–358. http://doi.wiley.com/10.2746/042516402776249209.

15. Shirazi-Beechey, S.P. (2008) Molecular insights into dietary induced colic in the horse. In: *Equine Veterinary Journal*. pp 414–421.

16. Capel, F., Acquaviva, C., Pitois, E., Laillet, B., Rigaudière, J.P., Jouve, C., Pouyet, C., Gladine, C., Comte, B., Vianey Saban, C. and Morio, B. (2015) DHA at nutritional doses restores insulin sensitivity in skeletal muscle by preventing lipotoxicity and inflammation. *J. Nutr. Biochem.* **26**, 949–959.

17. Johnson, P.J. (2014) Dyslipidemias. In: *Robinson's Current Therapy in Equine Medicine: Seventh Edition*. pp 578–581.

18. Smith, B.W. and Adams, L.A. (2011) Non-alcoholic fatty liver disease. *Crit. Rev. Clin. Lab. Sci.* **48**, 97–113.

19. Nandi, A., Kitamura, Y., Kahn, C.R. and Accili, D. (2004) Mouse models of insulin resistance. *Physiol. Rev.* **84**, 623–647. http://www.ncbi.nlm.nih.gov/pubmed/15044684.

20. Burgering, B.M. and Coffer, P.J. (1995) Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* **376**, 599–602. http://www.ncbi.nlm.nih.gov/pubmed/7637810.

21. Defronzo, R.A. (2009) Banting Lecture. From the triumvirate to the ominous octet: a new paradigm for the treatment of type 2 diabetes mellitus. *Diabetes* **58**, 773–95. http://www.ncbi.nlm.nih.gov/pubmed/19336687%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2661582.

22. Sah, S.P., Singh, B., Choudhary, S. and Kumar, A. (2016) Animal models of insulin resistance: A review. *Pharmacol. Reports* **68**, 1165–1177.

23. Cefalu, W.T. (2001) Insulin resistance: cellular and clinical concepts. *Exp. Biol. Med. (Maywood).* **226**, 13–26.

24. Duque-Guimarães, D.E. and Ozanne, S.E. (2013) Nutritional programming of insulin resistance: Causes and consequences. *Trends Endocrinol. Metab.* **24**, 525–535.

25. Treiber, K.H., Kronfeld, D.S. and Geor, R.J. (2006) Insulin Resistance in Equids: Possible Role in Laminitis. *J. Nutr* **136**, 2094–2098.

26. DeFronzo, R.A. (1997) Insulin resistance: A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidaemia and atherosclerosis. *Neth. J. Med.* **50**, 191–197.

27. Bailey, S.R., Habershon-Butcher, J.L., Ransom, K.J., Elliott, J. and Menzies-Gow, N.J. (2008) Hypertension and insulin resistance in a mixed-breed population of ponies predisposed to laminitis. *Am. J. Vet. Res.* **69**, 122–129.

28. Morgan, R.A., Keen, J.A., Walker, B.R. and Hadoke, P.W.F. (2016) Vascular dysfunction in horses with endocrinopathic laminitis. *PLoS One* **11**.

29. Venugopal, C.S., Eades, S., Holmes, E.P. and Beadle, R.E. (2011) Insulin resistance in equine digital vessel rings: An in vitro model to study vascular dysfunction in equine laminitis. *Equine Vet. J.* **43**, 744–749.

30. Ronald Kahn, C. (1978) Insulin resistance, insulin insensitivity, and insulin unresponsiveness: A necessary distinction. *Metabolism.* **27**, 1893–1902.

31. Bray, G.A., Lovejoy, J.C., Smith, S.R., Delany, J.P., Lefevre, M., Hwang, D., Donna, H., York, D. a, Ryan, D.H., York, D. a, Donna, H. and York, D. a (2002) The influence of different fats and fatty acids on obesity, insulin resistance and inflammation. *J. Nutr.* **132**, 2488–2491.

32. Hirose, M., Kaneki, M., Sugita, H., Yasuhara, S. and Martyn, J.A. (2000) Immobilization depresses insulin signaling in skeletal muscle. *Am. J. Physiol. Endocrinol. Metab.* **279**, E1235-41. http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=11093909&retmode=ref&cmd=prlinks%5Cnpapers2://publication/uuid/EF5ABD10-24A0-43C7-AFFC-04265F0314A0.

33. Dohm, G.L. (2002) Invited Review: Regulation of skeletal muscle GLUT-4 expression by exercise: Fig. 1. *J. Appl. Physiol.* **93**, 782–787. http://jap.physiology.org/lookup/doi/10.1152/japplphysiol.01266.2001.

34. Vorona, R.D., Winn, M.P., Babineau, T.W., Eng, B.P., Feldman, H.R. and Ware, J.C. (2005) Overweight and obese patients in a primary care population report less sleep than patients with a normal body mass index. *Arch. Intern. Med.* **165**, 25–30.

35. Treiber, K.H., Kronfeld, D.S., Hess, T.M., Byrd, B.M. and Splan, R.K. (2005) Pre-laminitic metabolic syndrome in genetically predisposed ponies involves compensated insulin resistance. *J. Anim. Physiol. Anim. Nutr. (Berl).* **89**, 430–431.

36. Wylie, C.E. and Collins, S.N. (2010) Equine insulin resistance: The quest for sensitivity. *Vet. J.* **186**, 275–276.

37. Coffman, J.R. and Colles, C.M. (1983) Insulin Tolerance in Laminitic Ponies. *Can. J. Comp. Med.* **47**, 347–351.

38. Frank, N., Geor, R.J.J., Bailey, S.R.R., Durham, A.E.E., Johnson, P.J.J. and American College of Veterinary Internal Medicine (2010) Equine metabolic syndrome. *J. Vet. Intern. Med.* **24**, 467–75. http://doi.wiley.com/10.1111/j.1939-1676.2010.0503.x%0Ahttp://doi.wiley.com/10.1111/j.1939-1676.2010.0503.x%0Ahttp://content.ebscohost.com/ContentServer.asp?T=P&P=AN&K=50275416&S=R&D=a9h&EbscoContent=dGJyMMvl7ESeqLI4v%2BbwOLCmr0%2BeprZSrqa4SrKWxWXS&Conten.

39. Field, J.R. and Jeffcott, L.B. (1989) Equine laminits - Another hypothesis for pathogenesis. *Med. Hypotheses* **30**, 203–210.

40. Geor, R.J. (2010) Current concepts on the pathophysiology of pasture-associated laminitis. *Vet. Clin. North Am. - Equine Pract.* **26**, 265–276.

41. Johnson, P.J., Wiedmeyer, C.E., LaCarrubba, A., Seshu Ganjam, V.K. and Messer, N.T. (2010) Laminitis and the equine metabolic syndrome. *Vet. Clin. North Am. - Equine Pract.* **26**, 239–255.

42. Bailey, S.R., Marr, C.M. and Elliott, J. (2004) Current research and theories on the pathogenesis of acute laminitis in the horse. *Vet. J.* **167**, 129–142.

43. Geor, R.J. (2009) Pasture-Associated Laminitis. *Vet. Clin. North Am. - Equine Pract.* **25**, 39–50.

44. Morgan, R.A., Mcgowan, T.W. and Mcgowan, C.M. (2014) Prevalence and risk factors for hyperinsulinaemia in ponies in Queensland, Australia. *Aust. Vet. J.* **92**, 101–106.

45. Albert J. Kane, J.T.-D. and Willard C. Losinger, L.P.G. (2000) The Occurrence and Causes of Lameness and Laminitis in the U.S. Horse Population. *Proc. Annu. Conv. AAEP* **46**, 277–280.

46. Cusi, K., Maezono, K., Osman, A., Pendergrass, M., Patti, M.E., Pratipanawatr, T., DeFronzo, R.A., Kahn, C.R. and Mandarino, L.J. (2000) Insulin resistance differentially affects the PI 3-kinase- and MAP kinase-mediated signaling in human muscle. *J. Clin. Invest.* **105**, 311–320.

47. Burns, T.A. and Toribio, R.E. (2016) Metabolic Syndrome in Humans and Horses: The Relationship Between Obesity and Insulin Resistance. In: *Equine Laminitis*. pp 149–166. https://www.scopus.com/inward/record.uri?eid=2-s2.0-85018289397&doi=10.1002%2F9781119169239.ch18&partnerID=40&md5=b02c5bf3b2d010b412e7a1dd0e228925.

48. USDA (2000) Lameness and laminitis in U.S. horses. In: *National Animal Health Monitoring System*. p 1–34 #N318.0400.

49. Watts, K.A. (2004) Forage and pasture management for laminitic horses. *Clin. Tech. Equine Pract.* **3**, 88–95.

50. Bailey, S.R., Rycroft, A. and Elliott, J. (2002) Production of amines in equine cecal contents in an in vitro model of carbohydrate overload. *J. Anim. Sci.* **80**, 2656–2662.

51. Karikoski, N.P., Horn, I., McGowan, T.W. and McGowan, C.M. (2011) The prevalence of endocrinopathic laminitis among horses presented for laminitis at a first-opinion/referral equine hospital. *Domest. Anim. Endocrinol.* **41**, 111–117.

52. Morgan, R. a., Keen, J. a. and McGowan, C.M. (2015) Treatment of Equine Metabolic Syndrome: a clinical case series. *Equine Vet. J.* **0**, 1–5. http://doi.wiley.com/10.1111/evj.12445.

53. Johnson, P.J. (2002) The equine metabolic syndrome. Peripheral Cushing's syndrome. *Vet. Clin. North Am. - Equine Pract.* **18**, 271–293.

54. Geor, R. and Frank, N. (2009) Metabolic syndrome-From human organ disease to laminar failure in equids. *Vet. Immunol. Immunopathol.* **129**, 151–154.

55. Frank, N., Elliott, S.B., Brandt, L.E. and Keisler, D.H. (2006) Physical characteristics, blood hormone concentrations, and plasma lipid concentrations in obese horses with insulin resistance. *J. Am. Vet. Med. Assoc.* **228**, 1383–1390. http://avmajournals.avma.org/doi/abs/10.2460/javma.228.9.1383.

56. Cartmill, J.A., Thompson, D.L., Storer, W.A., Gentry, L.R. and Huff, N.K. (2003) Endocrine responses in mares and geldings with high body condition scores grouped by high vs. low resting leptin concentrations. *J. Anim. Sci.* **81**, 2311–2321.

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

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

59. Monteiro, R. and Azevedo, I. (2010) Chronic inflammation in obesity and the metabolic syndrome. *Mediators Inflamm.* **2010**.

60. Kahn, B. and Flier, J. (2000) Obesity and insulin resistance. *J. Clin. Invest.* **106**, 473–481. http://www.ncbi.nlm.nih.gov/pubmed/10953022%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC380258%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=380258&tool=pmcentrez&rendertype=abstract.

61. Thatcher, C.D., Pleasant, R.S., Geor, R.J. and Elvinger, F. (2012) Prevalence of Overconditioning in Mature Horses in Southwest Virginia during the Summer. *J. Vet. Intern. Med.* **26**, 1413–1418.

62. Muno, J.D. (2009) *Prevalence, Risk Factors and Seasonality of Plasma Insulin Concentrations in Normal Horses in Central Ohio. Ohio State Univ.* .

63. Wylie, C.E., Collins, S.N. and Verheyen, K.L.P. (2011) Frequency of equine laminitis: A sysematic review with quality appraisal of published evidence. *Vet. J.* **3**, 248–256.

64. Wylie, C.E., Collins, S.N., Verheyen, K.L.P. and Newton, J.R. (2012) Risk factors for equine laminitis: A systematic review with quality appraisal of published evidence. *Vet. J.* **193**, 58–66.

65. Wylie, C.E., Collins, S.N., Verheyen, K.L.P. and Newton, J.R. (2013) Risk factors for equine laminitis: A case-control study conducted in veterinary-registered horses and ponies in Great Britain between 2009 and 2011. *Vet. J.* **198**, 57–69.

66. Morgan, R.A., Keen, J.A. and McGowan, C.M. (2016) Treatment of equine metabolic syndrome: A clinical case series. *Equine Vet. J.* **48**, 422–426.

67. Quinn, R.W., Burk, A.O., Hartsock, T.G., Petersen, E.D., Whitley, N.C., Treiber, K.H. and Boston, R.C. (2008) Insulin Sensitivity in Thoroughbred Geldings: Effect of Weight Gain, Diet, and Exercise on Insulin Sensitivity in Thoroughbred Geldings. *J. Equine Vet. Sci.* **28**, 728–738.

68. Saiardi, A. and Borrelli, E. (1998) Absence of Dopaminergic Control on Melanotrophs Leads to Cushing's-Like Syndrome in Mice. *Mol. Endocrinol.* **12**, 1133–1139. http://press.endocrine.org/doi/10.1210/mend.12.8.0144.

69. Wilson, M.G., Nicholson, W.E., Holscher, M.A., Sherrell, B.J., Mount, C.D. and Orth, D.N. (1982) Proopiolipomelanocortin peptides in normal pituitary, pituitary tumor, and plasma of normal and cushing's horses. *Endocrinology* **110**, 941–954.

70. McFarlane, D., Beech, J. and Cribb, A. (2006) Alpha-melanocyte stimulating hormone release in response to thyrotropin releasing hormone in healthy horses, horses with pituitary pars intermedia dysfunction and equine pars intermedia explants. *Domest. Anim. Endocrinol.* **30**, 276–288.

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

72. McFarlane, D. (2007) Advantages and limitations of the equine disease, pituitary pars intermedia dysfunction as a model of spontaneous dopaminergic neurodegenerative disease. *Ageing Res. Rev.* **6**, 54–63.

73. Schott, H.C. (2002) Pituitary pars intermedia dysfunction: equine Cushing's disease. *Vet. Clin. North Am. - Equine Pract.* **18**, 237–270.

74. McFarlane, D., Johnson, P.J. and Schott, H.C. (2016) Pituitary Pars Intermedia Dysfunction. In: *Equine Laminitis*. pp 334–340.

75. McFarlane, D., Donaldson, M., McDonnell, S. and Cribb, A. (2004) Effects of season and sample handling on measurement of plasma alpha-melanocyte-stimulating hormone concentrations in horses and ponies. *Am. J. Vet. Res.* **65**, 1463–1468.

76. Logan, A. and Weatherhead, B. (1980) Photoperiodic dependence of seasonal changes in pituitary content of melanocyte-stimulating hormone. *Neuroendocrinology* **30**, 309–312. http://www.ncbi.nlm.nih.gov/pubmed/7383270.

77. Frank, N., Andrews, F.M., Sommardahl, C.S., Eiler, H., Rohrbach, B.W. and Donnell, R.L. (2006) Evaluation of the combined dexamethasone suppression/ thyrotropin-releasing hormone stimulation test for detection of pars intermedia pituitary adenomas in horses. *J. Vet. Intern. Med.* **20**, 987–93. http://www.ncbi.nlm.nih.gov/pubmed/16955827.

78. Narici, M. V. and Maffulli, N. (2010) Sarcopenia: Characteristics, mechanisms and functional significance. *Br. Med. Bull.* **95**, 139–159.

79. Kolk JH, Kalsbeek HC, van Garderen E, et al. van der (1993) Equine pituitary neoplasia : a clinical report of 21 cases (1990-1992). *Vet. Rec.* **133**, 594–597.

80. MacKay, R.J. (2008) Quantitative intradermal terbutaline sweat test in horses. *Equine Vet. J.* **40**, 518–520.

81. Glover, C.M., Miller, L.M., Dybdal, N.O., Lopez, A., Duckett, W.M. and McFarlane, D. (2009) Extrapituitary and Pituitary Pathological Findings in Horses with Pituitary Pars Intermedia Dysfunction: A Retrospective Study. *J. Equine Vet. Sci.* **29**, 146–153.

82. Donaldson, M.T., LaMonte, B.H., Morresey, P., Smith, G. and Beech, J. (2002) Treatment with pergolide or cyproheptadine of pituitary pars intermedia dysfunction (equine Cushing's disease). *J. Vet. Intern. Med.* **16**, 742–746.

83. Donaldson, M.T., Jorgensen, A.J.R. and Beech, J. (2004) Evaluation of suspected pituitary pars intermedia dysfunction in horses with laminitis. *J. Am. Vet. Med. Assoc.* **224**, 1123–1127. http://avmajournals.avma.org/doi/abs/10.2460/javma.2004.224.1123.

84. Garcia, M.C. and Beech, J. (1986) Equine intravenous glucose tolerance test: glucose and insulin responses of healthy horses fed grain or hay and of horses with pituitary adenoma. *Am. J. Vet. Res.* **47**, 570–572.

85. P.J. Johnson, N.T. Messer, V.K.G. (2004) Cushing's syndromes, insulin resistance and endocrinopathic laminitis. *Equine Vet. J.* 194–198.

86. C.E. Boujon, G.E. Bestetti, H.P. Meier, R. Straub, U. Junker, G.L.R. (1993) Equine pituitary adenoma: a functional and morphological study. *J Comp Pathol* **109**, 163–178.

87. Gehlen, H., May, A. and Bradaric, Z. (2014) Comparison of insulin and glucose metabolism in horses with pituitary pars intermedia dysfunction treated versus not treated with pergolide. *J. Equine Vet. Sci.* **34**, 508–513.

88. Andrews, R.C. and Walker, B.R. (1999) Glucocorticoids and insulin resistance: old hormones, new targets. *Clin. Sci.* **96**, 513–523. http://www.clinsci.org/cs/096/cs0960513.htm%5Cnhttp://www.clinsci.org/content/96/5/513.abstract.

89. Geor, R.J. (2010) Nutrition and exercise in the management of horses and ponies at high risk for laminitis. *J. Equine Vet. Sci.* **30**, 463–470.

90. Kim, E.S., Im, J.A., Kim, K.C., Park, J.H., Suh, S.H., Kang, E.S., Kim, S.H., Jekal, Y., Lee, C.W., Yoon, Y.J., Lee, H.C. and Jeon, J.Y. (2007) Improved insulin sensitivity and adiponectin level after exercise training in obese Korean youth. *Obesity* **15**, 3023–3030.

91. Ross, R., Dagnone, D., Jones, P.J.H., Smith, H., Paddags, A., Hudson, R. and Janssen, I. (2000) Reduction in obesity and related comorbid conditions after diet-induced weight loss or exercise-induced weight loss in men: A randomized, controlled trial. *Ann. Intern. Med.* **133**, 92–103.

92. Zierath, J.R. (2002) Invited review: Exercise training-induced changes in insulin signaling in skeletal muscle. *J. Appl. Physiol.* **93**, 773–81. http://jap.physiology.org/content/93/2/773.full-text.pdf+html.

93. Hawley, J.A. (2004) Exercise as a therapeutic intervention for the prevention and

treatment of insulin resistance. *Diabetes. Metab. Res. Rev.* **20**, 383–393.

94. Hawley, J.A. and Lessard, S.J. (2008) Exercise training-induced improvements in insulin action. In: *Acta Physiologica*. pp 127–135.

95. Borghouts, L.B. and Keizer, H.A. (2000) Exercise and Insulin Sensitivity: A Review. *Int. J. Sports Med.* **21**, 1–12. http://www.thieme-connect.de/DOI/DOI?10.1055/s-2000-8847.

96. Rynders, C.A., Weltman, J.Y., Jiang, B., Breton, M., Patrie, J., Barrett, E.J. and Weltman, A. (2014) Effects of exercise intensity on postprandial improvement in glucose disposal and insulin sensitivity in prediabetic adults. *J. Clin. Endocrinol. Metab.* **99**, 220–228.

97. Powell, D.M., Reedy, S.E., Sessions, D.R. and Fitzgerald, B.P. (2002) Effect of short-term exercise training on insulin sensitivity in obese and lean mares. *Equine Vet. J.* **34**, 81–84.

98. Stewart-Hunt, L., Pratt-Phillips, S., Mccutcheon, L.J. and Geor, R.J. (2010) Dietary energy source and physical conditioning affect insulin sensitivity and skeletal muscle glucose metabolism in horses. *Equine Vet. J.* **42**, 355–360.

99. Turner, S.P., Hess, T.M., Treiber, K., Mello, E.B., Souza, B.G. and Almeida, F.Q. (2011) Comparison of Insulin Sensitivity of Horses Adapted to Different Exercise Intensities. *J. Equine Vet. Sci.* **31**, 645–649.

100. Bonelli, F., Sgorbini, M., Meucci, V., Sighieri, C. and Baragli, P. (2017) How swimming affects plasma insulin and glucose concentration in Thoroughbreds: A pilot study. *Vet. J.* **226**, 1–3.

101. David S. Kronfeld, Kimberley H. Treiber, R.J.G. (2005) Comparison of nonspecific indications and quantitative methods for the assessment of insulin resistance in horses and ponies. *J. Am. Vet. Med. Assoc.* **226**, 712–719.

102. Treiber, K.H., Kronfeld, D.S., Hess, T.M., Boston, R.C. and Harris, P.A. (2005) Use of proxies and reference quintiles obtained from minimal model analysis for determination of insulin sensitivity and pancreatic beta-cell responsiveness in horses. *Am. J. Vet. Res.* **66**, 2114–2121.

103. Bergman, R.N., Ider, Y.Z., Bowden, C.R. and Cobelli, C. (1979) Quantitative estimation of insulin sensitivity. *Am. J. Physiol.* **236**, E667-77. http://ajpendo.physiology.org.ezproxy.uct.ac.za/content/236/6/E667.full-text.pdf+html%5Cnhttp://www.jci.org/articles/view/112886%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/443421.

104. Hoffman, R.M., Boston, R.C., Stefanovski, D., Kronfeld, D.S. and Harris, P.A. (2003) Obesity and diet affect glucose dynamics and insulin sensitivity in Thoroughbred geldings. *J. Anim. Sci.* **81**, 2333–2342.

105. Ardekani, A.M. and Naeini, M.M. (2010) The role of microRNAs in human diseases. *Avicenna J. Med. Biotechnol.* **2**, 161–179.

106. Lee, R.C., Feinbaum, R.L. and Ambros, V. (1993) The C. elegans heterochronic gene lin-

4 encodes small RNAs with antisense complementarity to lin-14. *Cell* **75**, 843–854.

107. He, L. and Hannon, G.J. (2004) MicroRNAs: Small RNAs with a big role in gene regulation. *Nat. Rev. Genet.* **5**, 522–531.

108. Sohel, M.H. (2016) Extracellular/Circulating MicroRNAs: Release Mechanisms, Functions and Challenges. *Achiev. Life Sci.* **10**, 175–186. http://linkinghub.elsevier.com/retrieve/pii/S2078152016300797.

109. Lau, N.C., Lim, L.P., Weinstein, E.G. and Bartel, D.P. (2001) An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. *Science (80-.).* **294**, 858–862.

110. Lagos-Quintana, M., Rauhut, R., Lendeckel, W. and Tuschl, T. (2001) Identification of novel genes coding for small expressed RNAs. *Science (80-.).* **294**, 853–858.

111. Ramalingam, P., Palanichamy, J.K., Singh, A., Das, P., Bhagat, M., Kassab, M.A., Sinha, S. and Chattopadhyay, P. (2014) Biogenesis of intronic miRNAs located in clusters by independent transcription and alternative splicing. *RNA* **20**, 76–87. http://rnajournal.cshlp.org/cgi/doi/10.1261/rna.041814.113.

112. Lin, S.L., Miller, J.D. and Ying, S.Y. (2006) Intronic microRNA (miRNA). *J. Biomed. Biotechnol.* **2006**.

113. Monteys, A.M., Spengler, R.M., Wan, J., Tecedor, L., Lennox, K.A., Xing, Y. and Davidson, B.L. (2010) Structure and activity of putative intronic miRNA promoters. *RNA* **16**, 495–505. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2822915&tool=pmcentrez&rendertype=abstract.

114. Lee, Y., Kim, M., Han, J., Yeom, K.-H., Lee, S., Baek, S.H. and Kim, V.N. (2004) MicroRNA genes are transcribed by RNA polymerase II. *EMBO J.* **23**, 4051–4060. http://emboj.embopress.org/cgi/doi/10.1038/sj.emboj.7600385.

115. Borchert, G.M., Lanier, W. and Davidson, B.L. (2006) RNA polymerase III transcribes human microRNAs. *Nat. Struct. Mol. Biol.* **13**, 1097–1101.

116. Wahid, F., Shehzad, A., Khan, T. and Kim, Y.Y. (2010) MicroRNAs: Synthesis, mechanism, function, and recent clinical trials. *Biochim. Biophys. Acta - Mol. Cell Res.* **1803**, 1231–1243.

117. Kim, V.N., Han, J. and Siomi, M.C. (2009) Biogenesis of small RNAs in animals. *Nat. Rev. Mol. Cell Biol.* **10**, 126–139.

118. Lund, E., Güttinger, S., Calado, A., Dahlberg, J.E. and Kutay, U. (2004) Nuclear Export of MicroRNA Precursors. *Science (80-.).* **303**, 95–98.

119. Maniataki, E. and Mourelatos, Z. (2005) A human, ATP-independent, RISC assembly machine fueled by pre-miRNA. *Genes Dev.* **19**, 2979–2990.

120. Ipsaro, J.J. and Joshua-Tor, L. (2015) From guide to target: Molecular insights into

eukaryotic RNA-interference machinery. *Nat. Struct. Mol. Biol.* **22**, 20–28.

121. Makarova, J.A., Shkurnikov, M.U., Wicklein, D., Lange, T., Samatov, T.R., Turchinovich, A.A. and Tonevitsky, A.G. (2016) Intracellular and extracellular microRNA: An update on localization and biological role. *Prog. Histochem. Cytochem.* **51**, 33–49.

122. Tétreault, N. and Guire, V. De (2013) MiRNAs: Their discovery, biogenesis and mechanism of action. *Clin. Biochem.* **46**, 842–845.

123. Aleman, L.M., Doench, J. and Sharp, P.A. (2007) Comparison of siRNA-induced off-target RNA and protein effects. *RNA* **13**, 385–395. http://www.rnajournal.org/cgi/doi/10.1261/rna.352507.

124. Mathonnet, G., Fabian, M.R., Svitkin, Y. V., Parsyan, A., Huck, L., Murata, T., Biffo, S., Merrick, W.C., Darzynkiewicz, E., Pillai, R.S., Filipowicz, W., Duchaine, T.F. and Sonenberg, N. (2007) MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F. *Science (80-.).* **317**, 1764–1767.

125. Eulalio, A., Huntzinger, E. and Izaurralde, E. (2008) GW182 interaction with Argonaute is essential for miRNA-mediated translational repression and mRNA decay. *Nat. Struct. Mol. Biol.* **15**, 346–353.

126. Wakiyama, M., Takimoto, K., Ohara, O. and Yokoyama, S. (2007) Let-7 microRNA-mediated mRNA deadenylation and translational repression in a mammalian cell-free system. *Genes Dev.* **21**, 1857–1862.

127. Wu, L., Fan, J. and Belasco, J.G. (2006) MicroRNAs direct rapid deadenylation of mRNA. *Proc. Natl. Acad. Sci.* **103**, 4034–4039. http://www.pnas.org/cgi/doi/10.1073/pnas.0510928103.

128. Chendrimada, T.P., Finn, K.J., Ji, X., Baillat, D., Gregory, R.I., Liebhaber, S.A., Pasquinelli, A.E. and Shiekhattar, R. (2007) MicroRNA silencing through RISC recruitment of eIF6. *Nature* **447**, 823–828.

129. Wang, Y., Juranek, S., Li, H., Sheng, G., Tuschl, T. and Patel, D.J. (2008) Structure of an argonaute silencing complex with a seed-containing guide DNA and target RNA duplex. *Nature* **456**, 921–926.

130. Behm-Ansmant, I., Rehwinkel, J., Doerks, T., Stark, A., Bork, P. and Izaurralde, E. (2006) mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. *Genes Dev.* **20**, 1885–1898.

131. Giraldez, A.J., Mishima, Y., Rihel, J., Grocock, R.J., Dongen, S. Van, Inoue, K., Enright, A.J. and Schier, A.F. (2006) Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. *Science (80-.).* **312**, 75–79.

132. Urbé, S., Sachse, M., Row, P.E., Preisinger, C., Barr, F.A., Strous, G., Klumperman, J. and Clague, M.J. (2003) The UIM domain of Hrs couples receptor sorting to vesicle formation. *J. Cell Sci.* **116**, 4169–4179. http://www.ncbi.nlm.nih.gov/pubmed/12953068.

133. Rechavi, O., Erlich, Y., Amram, H., Flomenblit, L., Karginov, F. V., Goldstein, I., Hannon,

G.J. and Kloog, Y. (2009) Cell contact-dependent acquisition of cellular and viral nonautonomously encoded small RNAs. *Genes Dev.* **23**, 1971–1979.

134. Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J.J. and Lötvall, J.O. (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* **9**, 654–659.

135. Simpson, R.J., Kalra, H. and Mathivanan, S. (2012) Exocarta as a resource for exosomal research. *J. Extracell. Vesicles* **1**.

136. Yáñez-Mó, M., Siljander, P.R.M., Andreu, Z., Zavec, A.B., Borràs, F.E., Buzas, E.I., Buzas, K., Casal, E., Cappello, F., Carvalho, J., Colás, E., Cordeiro-Da Silva, A., Fais, S., Falcon-Perez, J.M., Ghobrial, I.M., Giebel, B., Gimona, M., Graner, M., Gursel, I., Gursel, M., Heegaard, N.H.H., Hendrix, A., Kierulf, P., Kokubun, K., Kosanovic, M., Kralj-Iglic, V., Krämer-Albers, E.M., Laitinen, S., Lässer, C., Lener, T., Ligeti, E., Line, A., Lipps, G., Llorente, A., Lötvall, J., Manček-Keber, M., Marcilla, A., Mittelbrunn, M., Nazarenko, I., Nolte-'t Hoen, E.N.M., Nyman, T.A., O'Driscoll, L., Olivan, M., Oliveira, C., Pállinger, É., Portillo, H.A. Del, Reventós, J., Rigau, M., Rohde, E., Sammar, M., Sánchez-Madrid, F., Santarém, N., Schallmoser, K., Ostenfeld, M.S., Stoorvogel, W., Stukelj, R., Grein, S.G. Van Der, Helena Vasconcelos, M., Wauben, M.H.M. and Wever, O. De (2015) Biological properties of extracellular vesicles and their physiological functions. *J. Extracell. Vesicles* **4**, 1–60.

137. Aharon, A., Katzenell, S., Tamari, T. and Brenner, B. (2009) Microparticles bearing tissue factor and tissue factor pathway inhibitor in gestational vascular complications. *J. Thromb. Haemost.* **7**, 1047–1050.

138. Chironi, G.N., Boulanger, C.M., Simon, A., Dignat-George, F., Freyssinet, J.M. and Tedgui, A. (2009) Endothelial microparticles in diseases. *Cell Tissue Res.* **335**, 143–151.

139. Lee, Y., Andaloussi, S. El and Wood, M.J.A. (2012) Exosomes and microvesicles: Extracellular vesicles for genetic information transfer and gene therapy. *Hum. Mol. Genet.* **21**.

140. Belting, M. and Wittrup, A. (2008) Nanotubes, exosomes, and nucleic acid-binding peptides provide novel mechanisms of intercellular communication in eukaryotic cells: Implications in health and disease. *J. Cell Biol.* **183**, 1187–1191.

141. Beyer, C. and Pisetsky, D.S. (2010) The role of microparticles in the pathogenesis of rheumatic diseases. *Nat. Rev. Rheumatol.* **6**, 21–29.

142. Zernecke, A., Bidzhekov, K., Noels, H., Shagdarsuren, E., Gan, L., Denecke, B., Hristov, M., Köppel, T., Jahantigh, M.N., Lutgens, E., Wang, S., Olson, E.N., Schober, A. and Weber, C. (2009) Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection. *Sci. Signal.* **2**.

143. Vickers, K.C., Palmisano, B.T., Shoucri, B.M., Shamburek, R.D. and Remaley, A.T. (2011) MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nat. Cell Biol.* **13**, 423–435.

144. Kim, S.I., Shin, D., Choi, T.H., Lee, J.C., Cheon, G.J., Kim, K.Y., Park, M. and Kim, M.

(2007) Systemic and specific delivery of small interfering RNAs to the liver mediated by apolipoprotein A-I. *Mol. Ther.* **15**, 1145–1152.

145. Kantharidis, P., Wang, B., Carew, R.M. and Lan, H.Y. (2011) Diabetes complications: The microRNA perspective. *Diabetes* **60**, 1832–1837.

146. Calin, G.A. and Croce, C.M. (2006) MicroRNA-cancer connection: The beginning of a new tale. *Cancer Res.* **66**, 7390–7394.

147. Barger, J.F. and Nana-Sinkam, S.P. (2015) MicroRNA as tools and therapeutics in lung cancer. *Respir. Med.* **109**, 803–812.

148. Esquela-Kerscher, A., Trang, P., Wiggins, J.F., Patrawala, L., Cheng, A., Ford, L., Weidhaas, J.B., Brown, D., Bader, A.G. and Slack, F.J. (2008) The let-7 microRNA reduces tumor growth in mouse models of lung cancer. *Cell Cycle* **7**, 759–764. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18344688.

149. Yu, S.L., Chen, H.Y., Chang, G.C., Chen, C.Y., Chen, H.W., Singh, S., Cheng, C.L., Yu, C.J., Lee, Y.C., Chen, H.S., Su, T.J., Chiang, C.C., Li, H.N., Hong, Q.S., Su, H.Y., Chen, C.C., Chen, W.J., Liu, C.C., Chan, W.K., Chen, W.J., Li, K.C., Chen, J.J.W. and Yang, P.C. (2008) MicroRNA Signature Predicts Survival and Relapse in Lung Cancer. *Cancer Cell* **13**, 48–57.

150. Yang, N., Coukos, G. and Zhang, L. (2008) MicroRNA epigenetic alterations in human cancer: one step forward in diagnosis and treatment. *Int. J. Cancer* **122**, 963–968.

151. Jiang, Q., Wang, Y., Hao, Y., Juan, L., Teng, M., Zhang, X., Li, M., Wang, G. and Liu, Y. (2009) miR2Disease: A manually curated database for microRNA deregulation in human disease. *Nucleic Acids Res.* **37**.

152. Lu, J., Getz, G., Miska, E.A., Alvarez-Saavedra, E., Lamb, J., Peck, D., Sweet-Cordero, A., Ebert, B.L., Mak, R.H., Ferrando, A.A., Downing, J.R., Jacks, T., Horvitz, H.R. and Golub, T.R. (2005) MicroRNA expression profiles classify human cancers. *Nature* **435**, 834–838.

153. Bhomia, M., Balakathiresan, N.S., Wang, K.K., Papa, L. and Maheshwari, R.K. (2016) A Panel of Serum MiRNA Biomarkers for the Diagnosis of Severe to Mild Traumatic Brain Injury in Humans. *Sci. Rep.* **6**.

154. Mao, Y., Mohan, R., Zhang, S. and Tang, X. (2013) MicroRNAs as pharmacological targets in diabetes. *Pharmacol. Res.* **75**, 37–47.

155. Ortega, F.J., Mercader, J.M., Moreno-Navarrete, J.M., Rovira, O., Guerra, E., Esteve, E., Xifra, G., Martínez, C., Ricart, W., Rieusset, J., Rome, S., Karczewska-Kupczewska, M., Straczkowski, M. and Fernández-Real, J.M. (2014) Profiling of circulating microRNAs reveals common microRNAs linked to type 2 diabetes that change with insulin sensitization. *Diabetes Care* **37**, 1375–1383.

156. Cui, X., You, L., Zhu, L., Wang, X., Zhou, Y., Li, Y., Wen, J., Xia, Y., Wang, X., Ji, C. and Guo, X. (2018) Change in circulating microRNA profile of obese children indicates future

risk of adult diabetes. *Metabolism.* **78**, 95–105.

157. Kolk, J.H. van der, Pacholewska, A. and Gerber, V. (2015) The role of microRNAs in equine medicine: a review. *Vet. Q.* **35**, 88–96.

158. Mach, N., Plancade, S., Pacholewska, A., Lecardonnel, J., Rivière, J., Moroldo, M., Vaiman, A., Morgenthaler, C., Beinat, M., Nevot, A., Robert, C. and Barrey, E. (2016) Integrated mRNA and miRNA expression profiling in blood reveals candidate biomarkers associated with endurance exercise in the horse. *Sci. Rep.* **6**.

159. Pacholewska, A., Mach, N., Mata, X., Vaiman, A., Schibler, L., Barrey, E. and Gerber, V. (2016) Novel equine tissue miRNAs and breed-related miRNA expressed in serum. *BMC Genomics* **17**.

160. Kaczmarek, K., Janicki, B. and Głowska, M. (2016) Insulin resistance in the horse: A review. *J. Appl. Anim. Res.* **44**, 424–430.

161. Gandarillas, M., Matus, J.T., Márquez-Hernández, R.I. and Vargas-Bello-Pérez, E. (2015) Development of insulin resistance in horses (Equus caballus): Etiologic and molecular aspects. *Cienc. e Investig. Agrar.* **42**.

162. Summers, S.A. (2006) Ceramides in insulin resistance and lipotoxicity. *Prog. Lipid Res.* **45**, 42–72.

163. Neel, J. V (1962) Diabetes Mellitus: A "Thrifty" Genotype Rendered Detrimental by "Progress"? *Am. J. Hum. Genet.* **14**, 363–362. http://sdps.ctrl.ucla.edu/nutrigen/pages/publicview/Epi 258 Fall 2010/Epi 258/Neel 2b.pdf.

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

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

166. Pritchard, C.C., Cheng, H.H. and Tewari, M. (2012) MicroRNA profiling: Approaches and considerations. *Nat. Rev. Genet.* **13**, 358–369.

167. Chugh, P. and Dittmer, D.P. (2012) Potential pitfalls in microRNA profiling. *Wiley Interdiscip Rev RNA* **3**, 601–616. http://www.ncbi.nlm.nih.gov/pubmed/22566380%5Cnhttp://onlinelibrary.wiley.com/doi/10.1002/wrna.1120/abstract.

168. Guay, C. and Regazzi, R. (2013) Circulating microRNAs as novel biomarkers for diabetes mellitus. *Nat Rev Endocrinol* **9**, 513–521. http://www.ncbi.nlm.nih.gov/pubmed/23629540.

169. Rottiers, V. and Näär, A.M. (2012) MicroRNAs in metabolism and metabolic disorders. *NRottiers, V., Näär, A. M. (2012). MicroRNAs Metab. Metab. Disord. Nat. Rev. Mol. Cell Biol. 13(4), 239–250. doi10.1038/nrm3313ature Rev. Mol. Cell Biol.* **13**, 239–250. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4021399&tool=pmcentrez&rendertype=abstract.

170. Raitoharju, E., Oksala, N. and Lehtimäki, T. (2013) MicroRNAs in the atherosclerotic plaque. *Clin. Chem.* **59**, 1708–1721.

171. Fernandez-Valverde, S.L., Taft, R.J. and Mattick, J.S. (2011) MicroRNAs in beta-cell biology, insulin resistance, diabetes and its complications. *Diabetes* **60**, 1825–1831.

172. Chen, H., Lan, H.Y., Roukos, D.H. and Cho, W.C. (2014) Application of microRNAs in diabetes mellitus. *J. Endocrinol.* **222**.

173. Karolina, D.S., Tavintharan, S., Armugam, A., Sepramaniam, S., Pek, S.L.T., Wong, M.T.K., Lim, S.C., Sum, C.F. and Jeyaseelan, K. (2012) Circulating miRNA profiles in patients with metabolic syndrome. *J. Clin. Endocrinol. Metab.* **97**, E2271-6. http://www.ncbi.nlm.nih.gov/pubmed/23032062.

174. Freestone, J.F., Shoemaker, K., Bessin, R. and Wolfsheimer, J.K. (1992) Insulin and glucose response following oral glucose administration in well-conditioned ponies. *Equine Vet. J. Suppl.* 13–17.

175. Borer KE, Berhane Y, Menzies-Gow NJ, et al. (2009) Measurement of concentrations of insulin in equine serum that exceed the working range of radioimmunoassay kits. *48th Br Equine Vet Assoc* .

176. Nulton, L. (2014) Cross-kingdom microRNA detection and influence of diet on endogenous equine micrornas. *Color. State Univ. Thesis Submiss.* .

177. Zhou, M., Wang, Q., Sun, J., Li, X., Xu, L., Yang, H., Shi, H., Ning, S., Chen, L., Li, Y., He, T. and Zheng, Y. (2009) In silico detection and characteristics of novel microRNA genes in the Equus caballus genome using an integrated ab initio and comparative genomic approach. *Genomics* **94**, 125–131.

178. Vlachos, I.S., Zagganas, K., Paraskevopoulou, M.D., Georgakilas, G., Karagkouni, D., Vergoulis, T., Dalamagas, T. and Hatzigeorgiou, A.G. (2015) DIANA-miRPath v3.0: Deciphering microRNA function with experimental support. *Nucleic Acids Res.* **43**, W460–W466.

179. Reczko, M., Maragkakis, M., Alexiou, P., Grosse, I. and Hatzigeorgiou, A.G. (2012) Functional microRNA targets in protein coding sequences. *Bioinformatics* **28**, 771–6. http://www.ncbi.nlm.nih.gov/pubmed/22285563.

180. Grueter, C.E., Rooij, E. Van, Johnson, B.A., Deleon, S.M., Sutherland, L.B., Qi, X., Gautron, L., Elmquist, J.K., Bassel-Duby, R. and Olson, E.N. (2012) A cardiac MicroRNA governs systemic energy homeostasis by regulation of MED13. *Cell* **149**, 671–683.

181. Jeong, B.C., Kang, I.H. and Koh, J.T. (2014) MicroRNA-302a inhibits adipogenesis by suppressing peroxisome proliferator-activated receptor γ expression. *FEBS Lett.* **588**, 3427–3434.

182. Meiler, S., Baumer, Y., Toulmin, E., Seng, K. and Boisvert, W.A. (2015) MicroRNA 302a is a novel modulator of cholesterol homeostasis and atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **35**, 323–331.

183. Hoekstra, M., Sluis, R.J. van der, Kuiper, J. and Berkel, T.J.C. Van (2012) Nonalcoholic fatty liver disease is associated with an altered hepatocyte microRNA profile in LDL receptor knockout mice. *J. Nutr. Biochem.* **23**, 622–8. http://www.sciencedirect.com/science/article/pii/S0955286311000945.

184. Yan, X., Huang, Y., Zhao, J.X., Rogers, C.J., Zhu, M.J., Ford, S.P., Nathanielsz, P.W. and Du, M. (2013) Maternal obesity downregulates microRNA let-7g expression, a possible mechanism for enhanced adipogenesis during ovine fetal skeletal muscle development. *Int. J. Obes.* **37**, 568–575.

185. Keller, P., Gburcik, V., Petrovic, N., Gallagher, I.J., Nedergaard, J., Cannon, B. and Timmons, J.A. (2011) Gene-chip studies of adipogenesis-regulated microRNAs in mouse primary adipocytes and human obesity. *BMC Endocr. Disord.* **11**.

186. Sookoian, S. and Pirola, C.J. (2012) PNPLA3, the triacylglycerol synthesis/hydrolysis/storage dilemma, and nonalcoholic fatty liver disease. *World J. Gastroenterol.* **18**, 6018–6026.

187. Delić, D., Eisele, C., Schmid, R., Baum, P., Wiech, F., Gerl, M., Zimdahl, H., Pullen, S.S. and Urquhart, R. (2016) Urinary exosomal miRNA signature in type II diabetic nephropathy patients. *PLoS One* **11**.

188. Iliopoulos, D., Drosatos, K., Hiyama, Y., Goldberg, I.J. and Zannis, V.I. (2010) MicroRNA-370 controls the expression of MicroRNA-122 and Cpt1α and affects lipid metabolism. *J. Lipid Res.* **51**, 1513–1523. http://www.jlr.org/lookup/doi/10.1194/jlr.M004812.

189. Esguerra, J.L.S., Bolmeson, C., Cilio, C.M. and Eliasson, L. (2011) Differential glucose-regulation of microRNAs in pancreatic islets of non-obese type 2 diabetes model Goto-Kakizaki rat. *PLoS One* **6**.

190. Gao, W., He, H.W., Wang, Z.M., Zhao, H., Lian, X.Q., Wang, Y.S., Zhu, J., Yan, J.J., Zhang, D.G., Yang, Z.J. and Wang, L.S. (2012) Plasma levels of lipometabolism-related miR-122 and miR-370 are increased in patients with hyperlipidemia and associated with coronary artery disease. *Lipids Health Dis.* **11**.

191. Wang, X.H., Qian, R.Z., Zhang, W., Chen, S.F., Jin, H.M. and Hu, R.M. (2009) MicroRNA-320 expression in myocardial microvascular endothelial cells and its relationship with insulin-like growth factor-1 in type 2 diabetic rats. *Clin. Exp. Pharmacol. Physiol.* **36**, 181–8. http://www.ncbi.nlm.nih.gov/pubmed/18986336.

192. Pescador, N., Pérez-Barba, M., Ibarra, J.M., Corbatón, A., Martínez-Larrad, M.T. and Serrano-Ríos, M. (2013) Serum Circulating microRNA Profiling for Identification of Potential Type 2 Diabetes and Obesity Biomarkers. *PLoS One* **8**.

193. Mi, L., Chen, Y., Zheng, X., Li, Y., Zhang, Q., Mo, D. and Yang, G. (2015) MicroRNA-139-5p suppresses 3T3-L1 preadipocyte differentiation through notch and irs1/pi3k/akt insulin signaling pathways. *J. Cell. Biochem.* **116**, 1195–1204.

194. Raitoharju, E., Seppälä, I., Oksala, N., Lyytikäinen, L.P., Raitakari, O., Viikari, J., Ala-Korpela, M., Soininen, P., Kangas, A.J., Waldenberger, M., Klopp, N., Illig, T., Leiviskä, J., Loo, B.M., Hutri-Kähönen, N., Kähönen, M., Laaksonen, R. and Lehtimäki, T. (2014)

Blood microRNA profile associates with the levels of serum lipids and metabolites associated with glucose metabolism and insulin resistance and pinpoints pathways underlying metabolic syndrome. The cardiovascular risk in Young Finns Study. *Mol. Cell. Endocrinol.* **391**, 41–49.

195. Wang, C., Wan, S., Yang, T., Niu, D., Zhang, A., Yang, C., Cai, J., Wu, J., Song, J., Zhang, C.Y., Zhang, C. and Wang, J. (2016) Increased serum microRNAs are closely associated with the presence of microvascular complications in type 2 diabetes mellitus. *Sci. Rep.* **6**.

196. Collares, C.V.A., Evangelista, A.F., Xavier, D.J., Rassi, D.M., Arns, T., Foss-Freitas, M.C., Foss, M.C., Puthier, D., Sakamoto-Hojo, E.T., Passos, G.A., Donadi, E.A., C.V., C., A.F., E., D.J., X., D.M., R., T., A., M.C., F.-F., M.C., F.-F., D., P., E.T., S.-H. and G.A., P. (2013) Identifying common and specific microRNAs expressed in peripheral blood mononuclear cell of type 1, type 2, and gestational diabetes mellitus patients. *BMC Res. Notes* **6**, 491. http://www.ncbi.nlm.nih.gov/pubmed/24279768%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4222092%5Cnhttp://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=emed11&NEWS=N&AN=24279768%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcg.

197. Costa Santos, H. da, Hess, T., Bruemmer, J. and Splan, R. (2018) Possible Role of MicroRNA in Equine Insulin Resistance: A Pilot Study. *J. Equine Vet. Sci.* **63**, 74–79.

198. Rasmussen, J.J., Schou, M., Selmer, C., Johansen, M.L., Gustafsson, F., Frystyk, J., Dela, F., Faber, J. and Kistorp, C. (2017) Insulin sensitivity in relation to fat distribution and plasma adipocytokines among abusers of anabolic androgenic steroids. *Clin. Endocrinol. (Oxf).* **87**, 249–256.

199. Tai, M.M. (1994) A mathematical model for the determination of total area under glucose tolerance and other metabolic curves. *Diabetes Care* **17**, 152–154.

200. Mestdagh, P., Vlierberghe, P. Van, Weer, A. De, Muth, D., Westermann, F., Speleman, F. and Vandesompele, J. (2009) A novel and universal method for microRNA RT-qPCR data normalization. *Genome Biol.* **10**.

201. Karagkouni, D., Paraskevopoulou, M.D., Chatzopoulos, S., Vlachos, I.S., Tastsoglou, S., Kanellos, I., Papadimitriou, D., Kavakiotis, I., Maniou, S., Skoufos, G., Vergoulis, T., Dalamagas, T. and Hatzigeorgiou, A.G. (2018) DIANA-TarBase v8: A decade-long collection of experimentally supported miRNA-gene interactions. *Nucleic Acids Res.* **46**, D239–D245.

202. Piñero, J., Bravo, Á., Queralt-Rosinach, N., Gutiérrez-Sacristán, A., Deu-Pons, J., Centeno, E., García-García, J., Sanz, F. and Furlong, L.I. (2017) DisGeNET: A comprehensive platform integrating information on human disease-associated genes and variants. *Nucleic Acids Res.* **45**, D833–D839.

203. Piñero, J., Queralt-Rosinach, N., Bravo, À., Deu-Pons, J., Bauer-Mehren, A., Baron, M., Sanz, F. and Furlong, L.I. (2015) DisGeNET: A discovery platform for the dynamical exploration of human diseases and their genes. *Database* **2015**.

204. R. Morgan, J. Keen, C.M. (2016) REVIEW: Equine Metabolic Syndrome. *Equine Laminitis* 329–333.

205. McGowan, C.M., Frost, R., Pfeiffer, D.U. and Neiger, R. (2004) Serum insulin concentrations in horses with equine Cushing's syndrome: response to a cortisol inhibitor and prognostic value. *Equine Vet. J.* **36**, 295–298.

206. Frank, N. (2008) Endocrinopathic laminitis, obesity-associated laminitis, and pasture-associated laminitis. *AAEP Proc.* 341–346.

207. Metson, A.J., Gibson, E.J., Hunt, J.L. and Saunders, W.M.H. (1979) Seasonal Variations in Chemical Composition of Pasture 3. Silicon Aluminum Iron Zinc Copper and Manganese. *New Zeal. J. Agric. Res.* **22**, 309–318.

208. Longland, A.C. and Byrd, B.M. (2006) Pasture Nonstructural Carbohydrates and Equine Laminitis. *J. Nutr.* **136**, 2099S–2102S.

209. Becker, N. and Lockwood, C.M. (2013) Pre-analytical variables in miRNA analysis. *Clin. Biochem.* **46**, 861–868.

210. Witwer, K.W. (2012) XenomiRs and miRNA homeostasis in health and disease. *RNA Biol.* **9**, 1147–1154. http://www.tandfonline.com/doi/abs/10.4161/rna.21619.

211. McFarlane, D., Paradis, M.R., Zimmel, D., Sykes, B., Brorsen, B.W., Sanchez, A. and Vainio, K. (2011) The effect of geographic location, breed, and pituitary dysfunction on seasonal adrenocorticotropin and α-melanocyte-stimulating hormone plasma concentrations in horses. *J. Vet. Intern. Med.* **25**, 872–881.

212. Donaldson, M.T., McDonnell, S.M., Schanbacher, B.J., Lamb, S. V., McFarlane, D. and Beech, J. (2005) Variation in plasma adrenocorticotropic hormone concentration and dexamethasone suppression test results with season, age, and sex in healthy ponies and horses. *J. Vet. Intern. Med.* **19**, 217–222.

213. Bamford, N.J., Potter, S.J., Harris, P.A. and Bailey, S.R. (2014) Breed differences in insulin sensitivity and insulinemic responses to oral glucose in horses and ponies of moderate body condition score. *Domest. Anim. Endocrinol.* **47**, 101–107.

214. Jeffcott, L.B., Field, J.R., McLean, J.G. and O'Dea, K. (1986) Glucose tolerance and insulin sensitivity in ponies and Standardbred horses. *Equine Vet. J.* **18**, 97–101.

215. Geor, R.J. (2013) Endocrine and metabolic physiology. In: *Equine Applied and Clinical Nutrition: Health, Welfare and Performance*. pp 33–63.

216. Lindåse, S., Müller, C., Nostell, K. and Bröjer, J. (2018) Evaluation of glucose and insulin response to haylage diets with different content of non-structural carbohydrates in two breeds of horses. *Domest. Anim. Endocrinol.* . https://www.sciencedirect.com/science/article/pii/S0739724018300183.

217. Kronfeld, D. and Harris, P. (2003) Equine grain-associated disorders. *Compendium* **25**, 974–983. http://cp.vetlearn.com/Media/PublicationsArticle/PV_25_12_974.pdf.

218. Garner, H.E., Hutcheson, D.P., Coffman, J.R., Hahn, A.W. and Salem, C. (1977) Lactic acidosis: a factor associated with equine laminitis. *J. Anim. Sci.* **45**, 1037–1041.

219. Dou, L., Zhao, T., Wang, L., Huang, X., Jiao, J., Gao, D., Zhang, H., Shen, T., Man, Y., Wang, S. and Li, J. (2013) MiR-200s contribute to interleukin-6 (IL-6)-induced insulin resistance in hepatocytes. *J. Biol. Chem.* **288**, 22596–22606.

220. Meshkani, R. and Adeli, K. (2009) Hepatic insulin resistance, metabolic syndrome and cardiovascular disease. *Clin. Biochem.* **42**, 1331–1346.

221. Eder, K., Baffy, N., Falus, A. and Fulop, A.K. (2009) The major inflammatory mediator interleukin-6 and obesity. *Inflamm. Res.* **58**, 727–736.

222. Lecchi, C., Dalla Costa, E., Lebelt, D., Ferrante, V., Canali, E., Ceciliani, F., Stucke, D. and Minero, M. (2018) Circulating miR-23b-3p, miR-145-5p and miR-200b-3p are potential biomarkers to monitor acute pain associated with laminitis in horses. *Animal* **12**, 366–375.

223. Yang, W.-M., Min, K.-H. and Lee, W. (2016) MicroRNA expression analysis in the liver of high fat diet-induced obese mice. *Data Br.* **9**, 1155–1159. http://linkinghub.elsevier.com/retrieve/pii/S2352340916307417.

224. Trajkovski, M., Hausser, J., Soutschek, J., Bhat, B., Akin, A., Zavolan, M., Heim, M.H. and Stoffel, M. (2011) MicroRNAs 103 and 107 regulate insulin sensitivity. *Nature* **474**, 649–653.

225. Shao, Y., Ren, H., Lv, C., Ma, X., Wu, C. and Wang, Q. (2017) Changes of serum Mir-217 and the correlation with the severity in type 2 diabetes patients with different stages of diabetic kidney disease. *Endocrine* **55**, 130–138.

226. Sun, J., Li, Z.P., Zhang, R.Q. and Zhang, H.M. (2017) Repression of miR-217 protects against high glucose-induced podocyte injury and insulin resistance by restoring PTEN-mediated autophagy pathway. *Biochem. Biophys. Res. Commun.* **483**, 318–324.

227. Li, A., Qiu, M., Zhou, H., Wang, T. and Guo, W. (2017) PTEN, Insulin Resistance and Cancer. *Curr Pharm Des* **23**, 3667–3676.

228. Kato, M., Putta, S., Wang, M., Yuan, H., Lanting, L., Nair, I., Gunn, A., Nakagawa, Y., Shimano, H., Todorov, I., Rossi, J.J. and Natarajan, R. (2009) TGF-β activates Akt kinase through a microRNA-dependent amplifying circuit targeting PTEN. *Nat. Cell Biol.* **11**, 881–889.

229. Nakashima, N., Sharma, P.M., Imamura, T., Bookstein, R. and Olefsky, J.M. (2000) The tumor suppressor PTEN negatively regulates insulin signaling in 3T3-L1 adipocytes. *J. Biol. Chem.* **275**, 12889–95. http://www.ncbi.nlm.nih.gov/pubmed/10777587.

230. Li, Z.-Y., Na, H.-M., Peng, G., Pu, J. and Liu, P. (2011) Alteration of microRNA expression correlates to fatty acid-mediated insulin resistance in mouse myoblasts. *Mol. Biosyst.* **7**, 871–877.

231. Pando, R., Even-Zohar, N., Shtaif, B., Edry, L., Shomron, N., Phillip, M. and Gat-

Yablonski, G. (2012) MicroRNAs in the growth plate are responsive to nutritional cues: Association between miR-140 and SIRT1. *J. Nutr. Biochem.* **23**, 1474–1481.

232. Klöting, N., Berthold, S., Kovacs, P., Schön, M.R., Fasshauer, M., Ruschke, K., Stumvoll, M. and Blüher, M. (2009) MicroRNA expression in human omental and subcutaneous adipose tissue. *PLoS One* **4**.

233. Chartoumpekis, D. V., Zaravinos, A., Ziros, P.G., Iskrenova, R.P., Psyrogiannis, A.I., Kyriazopoulou, V.E. and Habeos, I.G. (2012) Differential expression of microRNAs in adipose tissue after long-term high-fat diet-induced obesity in mice. *PLoS One* **7**.

234. Zhu, H. and Leung, S.W. (2015) Identification of microRNA biomarkers in type 2 diabetes: a meta-analysis of controlled profiling studies. *Diabetologia* **58**, 900–911.

235. Yang, W.M., Jeong, H.J., Park, S.W. and Lee, W. (2015) Obesity-induced miR-15b is linked causally to the development of insulin resistance through the repression of the insulin receptor in hepatocytes. *Mol. Nutr. Food Res.* **59**, 2303–2314.

236. Liu, W., Bi, P., Shan, T., Yang, X., Yin, H., Wang, Y.X., Liu, N., Rudnicki, M.A. and Kuang, S. (2013) miR-133a Regulates Adipocyte Browning In Vivo. *PLoS Genet.* **9**.

237. Nakanishi, N., Nakagawa, Y., Tokushige, N., Aoki, N., Matsuzaka, T., Ishii, K., Yahagi, N., Kobayashi, K., Yatoh, S., Takahashi, A., Suzuki, H., Urayama, O., Yamada, N. and Shimano, H. (2009) The up-regulation of microRNA-335 is associated with lipid metabolism in liver and white adipose tissue of genetically obese mice. *Biochem. Biophys. Res. Commun.* **385**, 492–496.

238. Gallagher, I.J., Scheele, C., Keller, P., Nielsen, A.R., Remenyi, J., Fischer, C.P., Roder, K., Babraj, J., Wahlestedt, C., Hutvagner, G., Pedersen, B.K. and Timmons, J.A. (2010) Integration of microRNA changes in vivo identifies novel molecular features of muscle insulin resistance in type 2 diabetes. *Genome Med.* **2**.

239. Chen, G.-Q., Lian, W.-J., Wang, G.-M., Wang, S., Yang, Y.-Q. and Zhao, Z.-W. (2012) Altered microRNA expression in skeletal muscle results from high-fat diet-induced insulin resistance in mice. *Mol. Med. Rep.* **5**, 1362–8. http://www.ncbi.nlm.nih.gov/pubmed/22395471.

240. Massart, J., Katayama, M. and Krook, A. (2016) microManaging glucose and lipid metabolism in skeletal muscle: Role of microRNAs. *Biochim. Biophys. Acta* **1861**, 2130–2138. http://www.ncbi.nlm.nih.gov/pubmed/27183241.

241. Shan, Q., Zheng, G., Zhu, A., Cao, L., Lu, J., Wu, D., Zhang, Z.F., Fan, S., Sun, C., Hu, B. and Zheng, Y. (2016) Epigenetic modification of miR-10a regulates renal damage by targeting CREB1 in type 2 diabetes mellitus. *Toxicol. Appl. Pharmacol.* **306**, 134–143.

242. Calvier, L., Chouvarine, P., Legchenko, E., Hoffmann, N., Geldner, J., Borchert, P., Jonigk, D., Mozes, M.M. and Hansmann, G. (2017) PPARγ Links BMP2 and TGFβ1 Pathways in Vascular Smooth Muscle Cells, Regulating Cell Proliferation and Glucose Metabolism. *Cell Metab.* **25**, 1118–1134.e7.

243. Tijsen, A.J., Creemers, E.E., Moerland, P.D., Windt, L.J. De, Wal, A.C. Van Der, Kok,

W.E. and Pinto, Y.M. (2010) MiR423-5p as a circulating biomarker for heart failure. *Circ. Res.* **106**, 1035–1039.

244. Ortega, F.J., Mercader, J.M., Catalán, V., Moreno-Navarrete, J.M., Pueyo, N., Sabater, M., Gómez-Ambrosi, J., Anglada, R., Fernández-Formoso, J.A., Ricart, W., Frühbeck, G. and Fernández-Real, J.M. (2013) Targeting the circulating microRNA signature of obesity. *Clin. Chem.* **59**, 781–792.

245. Prabu, P., Rome, S., Sathishkumar, C., Aravind, S., Mahalingam, B., Shanthirani, C.S., Gastebois, C., Villard, A., Mohan, V. and Balasubramanyam, M. (2015) Circulating miRNAs of “Asian Indian phenotype” identified in subjects with impaired glucose tolerance and patients with type 2 diabetes. *PLoS One* **10**.

246. Kameswaran, V., Bramswig, N.C., McKenna, L.B., Penn, M., Schug, J., Hand, N.J., Chen, Y., Choi, I., Vourekas, A., Won, K.J., Liu, C., Vivek, K., Naji, A., Friedman, J.R. and Kaestner, K.H. (2014) Epigenetic regulation of the DLK1-MEG3 MicroRNA cluster in human type 2 diabetic islets. *Cell Metab.* **19**, 135–145.

247. Locke, J.M., Silva Xavier, G. Da, Dawe, H.R., Rutter, G.A. and Harries, L.W. (2014) Increased expression of miR-187 in human islets from individuals with type 2 diabetes is associated with reduced glucose-stimulated insulin secretion. *Diabetologia* **57**, 122–128.

248. Frost, R.J.A. and Olson, E.N. (2011) Control of glucose homeostasis and insulin sensitivity by the Let-7 family of microRNAs. *Proc. Natl. Acad. Sci.* **108**, 21075–21080. http://www.pnas.org/cgi/doi/10.1073/pnas.1118922109.

249. Williams, M.D. and Mitchell, G.M. (2012) MicroRNAs in insulin resistance and obesity. *Exp. Diabetes Res.* **2012**.

250. Jiang, L.Q., Franck, N., Egan, B., Sjogren, R.J.O., Katayama, M., Duque-Guimaraes, D., Arner, P., Zierath, J.R. and Krook, A. (2013) Autocrine role of interleukin-13 on skeletal muscle glucose metabolism in type 2 diabetic patients involves microRNA let-7. *AJP Endocrinol. Metab.* **305**, E1359–E1366. http://ajpendo.physiology.org/cgi/doi/10.1152/ajpendo.00236.2013.

251. Lewis, S.L., Holl, H.M., Streeter, C., Posbergh, C., Schanbacher, B.J., Place, N.J., Mallicote, M.F., Long, M.T. and Brooks, S.A. (2017) Genomewide association study reveals a risk locus for equine metabolic syndrome in the Arabian horse. *J. Anim. Sci.* **95**, 1071–1079.

252. Erener, S., Marwaha, A., Tan, R., Panagiotopoulos, C. and Kieffer, T.J. (2017) Profiling of circulating microRNAs in children with recent onset of type 1 diabetes. *JCI Insight* **2**. https://insight.jci.org/articles/view/89656.

253. Herrera, B.M., Lockstone, H.E., Taylor, J.M., Ria, M., Barrett, A., Collins, S., Kaisaki, P., Argoud, K., Fernandez, C., Travers, M.E., Grew, J.P., Randall, J.C., Gloyn, A.L., Gauguier, D., McCarthy, M.I. and Lindgren, C.M. (2010) Global microRNA expression profiles in insulin target tissues in a spontaneous rat model of type 2 diabetes. *Diabetologia* **53**, 1099–1109.

254. Yang, W.M., Jeong, H.J., Park, S.Y. and Lee, W. (2014) Saturated fatty acid-induced

miR-195 impairs insulin signaling and glycogen metabolism in HepG2 cells. *FEBS Lett.* **588**, 3939–3946.

255. Karolina, D.S., Armugam, A., Tavintharan, S., Wong, M.T.K., Lim, S.C., Sum, C.F. and Jeyaseelan, K. (2011) MicroRNA 144 impairs insulin signaling by inhibiting the expression of insulin receptor substrate 1 in type 2 diabetes mellitus. *PLoS One* **6**.

256. Ying, W., Tseng, A., Chang, R.C.A., Wang, H., Lin, Y.L., Kanameni, S., Brehm, T., Morin, A., Jones, B., Splawn, T., Criscitiello, M., Golding, M.C., Bazer, F.W., Safe, S. and Zhou, B. (2016) MiR-150 regulates obesity-Associated insulin resistance by controlling B cell functions. *Sci. Rep.* **6**.

257. Esau, C., Davis, S., Murray, S.F., Yu, X.X., Pandey, S.K., Pear, M., Watts, L., Booten, S.L., Graham, M., McKay, R., Subramaniam, A., Propp, S., Lollo, B.A., Freier, S., Bennett, C.F., Bhanot, S. and Monia, B.P. (2006) miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. *Cell Metab.* **3**, 87–98.

258. Yi, F., Shang, Y., Li, B., Dai, S., Wu, W., Cheng, L. and Wang, X. (2017) MicroRNA-193-5p modulates angiogenesis through IGF2 in type 2 diabetic cardiomyopathy. *Biochem. Biophys. Res. Commun.* **491**, 876–882.

Table 17 Appendix 340 Equine microRNA primer sequences.

Mature microRNA ID	Target microRNA Mature Sequence
eca-let-7a	ugagguaguagguuguauaguu
eca-let-7c	ugagguaguagguuguaugguu
eca-let-7d	agagguaguagguugcauaguu
eca-let-7e	ugagguaggagguuguauaguu
eca-let-7f	ugagguaguagauuguauaguu
eca-let-7g	ugagguaguaguuuguacaguu
eca-mir-1	uggaauguaaagaaguauguau
eca-mir-7	uggaagacuagugauuuuguugu
eca-mir-9a	ucuuugguuaucuagcuguauga
eca-mir-10a	uacccuguagauccgaauuugug
eca-mir-10b	uacccuguagaaccgaauuugug
eca-mir-15a	uagcagcacauaaugguuugug
eca-mir-15b	uagcagcacaucaugguuuaca
eca-mir-16	uagcagcacguaaauauuggcg
eca-mir-17	caaagugcuuacagugcagguag
eca-mir-18a	uaaggugcaucuagugcagauag
eca-mir-18b	uaaggugcaucuagugcaguuag
eca-mir-19a	ugugcaaaucuaugcaaaacuga
eca-mir-19b	ugugcaaaucuaugcaaaacuga
eca-mir-20a	uaaagugcuuauagugcagguag

eca-mir-20b	caaagugcucauagugcagguag
eca-mir-21	uagcuuaucagacugauguuga
eca-mir-22	aagcugccaguugaagaacugu
eca-mir-23a	aucacauugccagggauuucc
eca-mir-23b	aucacauugccagggauuacc
eca-mir-24	uggcucaguucagcaggaacag
eca-mir-25	cauugcacuugucucggucuga
eca-mir-26a	uucaaguaauccaggauaggcu
eca-mir-27a	uucacaguggcuaaguuccgc
eca-mir-27b	uucacaguggcuaaguucugc
eca-mir-283p	cacuagauugugagcuccugga
eca-mir-285p	aaggagcucacagucuauugag
eca-mir-29a	uagcaccaucugaaaucgguua
eca-mir-29b	uagcaccauuugaaaucaguguu
eca-mir-29c	uagcaccauuugaaaucgguua
eca-mir-30b	uguaaacauccuacacucagcu
eca-mir-30c	uguaaacauccuacacucucagc
eca-mir-30d	uguaaacaucccgacuggaag
eca-mir-30e	uguaaacauccuugacuggaag
eca-mir-31	aggcaagaugcuggcauagcu
eca-mir-32	uauugcacauuacuaaguugca
eca-mir-33a	gugcauuguaguugcauugca
eca-mir-33b	gugcauugcuguugcauugc
eca-mir-34	uggcagugucuuagcugguugu
eca-mir-92a	uauugcacuugucccggccugu

eca-mir-92b	uauugcacucgucccggccucc
eca-mir-93	caaagugcuguucgugcagguag
eca-mir-95	uucaacgggucuuuauugagca
eca-mir-96	uuuggcacuagcacauuuuugcu
eca-mir-98	ugagguaguaaguuguauuguu
eca-mir-99a	aacccguagauccgaucuugug
eca-mir-99b	cacccguagaaccgaccuugcg
eca-mir-100	aacccguagauccgaacuugug
eca-mir-101	uacaguacugugauaacugaa
eca-mir-103	agcagcauuguacagggcuauga
eca-mir-105	ucaaaugcucagacuccuguggu
eca-mir-106a	caaagugcuuacagugcagguag
eca-mir-106b	uaaagugcugacagugcagau
eca-mir-107b	agcagcauuguacagggcuauca
eca-mir-122	uggagugugacaaugguguuug
eca-mir-124	uaaggcacgcggugaaugcc
eca-mir-125a3p	acaggugagguucuugggagcc
eca-mir-125a5p	ucccugagacccuuuaaccuguga
eca-mir-125b	ucccugagacccuaacuuguga
eca-mir-1263p	ucguaccgugaguaauaaugcg
eca-mir-127	ucggauccgucugagcuuggcu
eca-mir-128	ucacagugaaccggucucuuu
eca-mir-129a3p	aagcccuuaccccaaaaaguau
eca-mir-129a5p	cuuuuugcggucugggcuugc
eca-mir-130a	cagugcaauguuaaaagggcau

eca-mir-130b	cagugcaaugaugaaagggcau
eca-mir-132	uaacagucuacagccauggucg
eca-mir-133a	uuuggucccuucaaccagcug
eca-mir-133b	uuuggucccuucaaccagcua
eca-mir-134	ugugacugguugaccagagggg
eca-mir-135a	uauggcuuuuuauuccuauguga
eca-mir-135b	uauggcuuuucauuccuauguga
eca-mir-136	acuccauuuguuuugaugaugg
eca-mir-137	uuauugcuuaagaauacgcguag
eca-mir-138	agcugguguugugaaucaggccg
eca-mir-1393p	ggagacgcggcccuguuggagu
eca-mir-1395p	ucuacagugcacgugucuccag
eca-mir-1403p	uaccacaggguagaaccacgg
eca-mir-1405p	cagugguuuuacccuaugguag
eca-mir-141	uaacacugucugguaaagaugg
eca-mir-1423p	uguaguguuuccuacuuuaugga
eca-mir-1425p	cauaaaguagaaagcacuacu
eca-mir-143	ugagaugaagcacuguagcuc
eca-mir-144	uacaguauagaugauguacu
eca-mir-145	guccaguuuucccaggaaucccu
eca-mir-146a	ugagaacugaauuccauggguu
eca-mir-146b3p	ugcccuagggacucaguucugg
eca-mir-146b5p	ugagaacugaauuccauaggcu
eca-mir-147b	gugugccgaaaugcuucugcua
eca-mir-148a	ucagugcacuacagaacuuugu

eca-mir-148b3p	ucagugcaucacagaacuuugu
eca-mir-149	ucuggcuccgugucuucacuccc
eca-mir-150	ucucccaacccuuguaccagug
eca-mir-1515p	ucgaggagcucacagucuagu
eca-mir-153	uugcauagucacaaaagugauc
eca-mir-154	uagguuauccguguugccuucg
eca-mir-155	uuaaugcuaaucgugauaggggu
eca-mir-181a	aacauucaacgcugucggugagu
eca-mir-181b	aacauucauugcugucgguggguu
eca-mir-182	uuuggcaaugguagaacucacacug
eca-mir-183	uauggcacugguagaauucacu
eca-mir-184	uggacggagaacugauaagggu
eca-mir-186	caaagaauucuccuuuugggcu
eca-mir-187	ucgugucuuguguugcagccgg
eca-mir-1883p	cucccacaugcaggguuugca
eca-mir-1885p	caucccuugcaugguggaggg
eca-mir-190	ugauauguuugauauauuaggu
eca-mir-190b	ugauauguuugauauuggguu
eca-mir-191	caacggaaucccaaaagcagcug
eca-mir-192	cugaccuaugaauugacagcc
eca-mir-193a3p	aacuggccuacaaagucccagu
eca-mir-193a5p	ugggucuuugcgggcgagauga
eca-mir-193b	aacuggcccacaaagucccgcu
eca-mir-194	uguaacagcaacuccaugugga
eca-mir-195	uagcagcacagaaauauuggc

eca-mir-196a	uagguaguuucauguuguuggg
eca-mir-196b	uagguaguuuccuguuguuggg
eca-mir-197	uucaccaccuucuccacccagc
eca-mir-199a3p	acaguagucugcacauugguag
eca-mir-199a5p	cccaguguucagacuaccuguuc
eca-mir-199b3p	acaguagucugcacauugguua
eca-mir-199b5p	cccaguguuuagacuaucuguuc
eca-mir-200a	uaacacugucugguaacgaugu
eca-mir-200b	uaauacugccugguaaugauga
eca-mir-200c	uaauacugccggguaaugaugga
eca-mir-204b	uucccuuugucauccuaugccu
eca-mir-205	uccuucauuccaccggagucug
eca-mir-206	uggaauguaaggaagugugugg
eca-mir-208a	auaagacgagcaaaaagcuugu
eca-mir-208b	auaagacgaacaaaagguuugu
eca-mir-211	uucccuuugucauccuuugccu
eca-mir-212	uaacagucuccagucacggcc
eca-mir-214	acagcaggcacagacaggcagu
eca-mir-215	augaccuaugaauugacagac
eca-mir-216a	uaaucucagcuggcaacuguga
eca-mir-216b	aaaucucugcaggcaaauguga
eca-mir-217	uacugcaucaggaacugauugga
eca-mir-218	uugugcuugaucuaaccaugu
eca-mir-2195p	ugauuguccaaacgcaauucu
eca-mir-221	agcuacauugucugcuggguuuc

eca-mir-222	agcuacaucuggcuacugggu
eca-mir-223	ugucaguuugucaaauacccca
eca-mir-224	caagucacuagugguuccguu
eca-mir-296	gaggguuggguggaggcuuucc
eca-mir-299	uaugugggaugguaaaccgcuu
eca-mir-301a	cagugcaauaguauugucaaagc
eca-mir-301b3p	cagugcaaugauauugucaaagc
eca-mir-302a	uaagugcuuccauguuuuaguga
eca-mir-302b	uaagugcuuccauguuuuaguag
eca-mir-302c	uaagugcuuccauguuucagugg
eca-mir-302d	uaagugcuuccauguuuuagugu
eca-mir-3233p	cacauuacacggucgaccucu
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eca-mir-3243p	ccacugccccaggugcugcugg
eca-mir-3245p	cgcauccccuagggcauuggugu
eca-mir-326	ccucugggcccuuccuccagc
eca-mir-328	cuggcccucucugcccuuccgu
eca-mir-329	aacacaccuaguuaaccucuuu
eca-mir-330	ucucugggccugugucuuaggc
eca-mir-331	gccccugggccuauccuagaa
eca-mir-335	ucaagagcaauaacgaaaaaugu
eca-mir-3373p	cuccuaugagaugccuuuccuc
eca-mir-3375p	gaacggcuucauacaggagcu
eca-mir-3383p	uccagcaucagugauuuuguug
eca-mir-3385p	aacaauauccuggugcugagug

eca-mir-3405p	uuauaaagcaaugagacugauu
eca-mir-3423p	ucucacacagaaaucgcacccgu
eca-mir-3425p	aggggugcuaucugugauugag
eca-mir-3455p	gcugacuccuaguccagugcuc
eca-mir-346	ugucugcccgcaugccugccucu
eca-mir-3613p	ucccccaggcgugauucugauuu
eca-mir-3615p	uuaucagaaucuccagggguac
eca-mir-3623p	aacacaccuauucaaggauuca
eca-mir-3625p	aauccuuggaaccuaggugugagu
eca-mir-363	aauugcacgguauccaucugua
eca-mir-365	uaaugccccuaaaaauccuuau
eca-mir-367	aauugcacuuuagcaaugguga
eca-mir-3693p	aauaauacaugguugaucuuu
eca-mir-3695p	agaucgaccgugucauauucgc
eca-mir-370	gccugcuggguggaaccuggu
eca-mir-3713p	aagugccgccauuuuuugagugu
eca-mir-3715p	acucaaacuguggggcacu
eca-mir-374a	uuauaauacaaccugauaagug
eca-mir-374b	auauaauacaaccugcuaagug
eca-mir-376a	aucauagaggaaaauccacgu
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eca-mir-376c	aacauagaggaaauuccacgu
eca-mir-377	aucacacaaaggcaacuuuugu
eca-mir-378	acuggacuuggagucagaagg
eca-mir-379	ugguagacuauggaacguagg

eca-mir-380	uauguaauaugguccacgucuu
eca-mir-381	uauacaagggcaagcucucugu
eca-mir-382	gaaguuguucgugguggauucg
eca-mir-383	agaucagaaggugauuguggcu
eca-mir-384	auuccuagaaauuguucaca
eca-mir-4093p	gaauguugcucggugaaccccu
eca-mir-4095p	agguuacccgagcaacuuugcau
eca-mir-410	aauauaacacagauggccugu
eca-mir-411	uaguagaccguauagcguacg
eca-mir-412	uucaccugguccacuagccg
eca-mir-421	ggccucauuaaauguuuguug
eca-mir-4233p	agcucggucugaggccccucagu
eca-mir-4235p	ugaggggcagagagcgagacuuu
eca-mir-424	cagcagcaauucauguuuugaa
eca-mir-429	uaauacugucugguaaugccg
eca-mir-431	ugucuugcaggccgucaugcagg
eca-mir-432	ucuuggaguaggucauugggugg
eca-mir-433	aucaugaugggcuccucggugu
eca-mir-448	uugcauauguaggaugucccau
eca-mir-449a	uggcaguguauuguuagcuggu
eca-mir-450a	uuuugcgauguguuccuaauau
eca-mir-450b3p	uugggaacauuuugcauccaua
eca-mir-450b5p	uuuugcaauauguuccugaaua
eca-mir-451	aaaccguuaccauuacuguguu
eca-mir-454	uagugcaauauugcuuauagggu

eca-mir-4853p	gucauacacggcucuccucucu
eca-mir-4855p	agaggcuggccgugaugaauuc
eca-mir-4863p	cggggcagcucaguacaggau
eca-mir-4865p	uccuguacugagcugccccgag
eca-mir-487a	aaucauacagggacauccaguu
eca-mir-487b	aaucguacagggucauccacuu
eca-mir-488	uugaaaggcuauuucuugguc
eca-mir-489	gugacaucacauauacggcggc
eca-mir-4903p	caaccuggaggacuccaugcug
eca-mir-4905p	ccauggaucuccaggugggu
eca-mir-4913p	cuuaugcaagauucccuucuac
eca-mir-4915p	aguggggaacccuuccaugagg
eca-mir-492	aggagcugcgggacaagauucuu
eca-mir-493b	ugaaggucuuccgugugccagg
eca-mir-494	ugaaacauacacgggaaaccuc
eca-mir-495	aaacaaacauggugcacuucuu
eca-mir-496	ugaguauuacauggccaaucuc
eca-mir-497	cagcagcacacuguggguuugu
eca-mir-4993p	aacaucacagcaagucugugcu
eca-mir-4995p	uuaagacuugcagugauguuu
eca-mir-500	uaauccuugcuaccugggugaga
eca-mir-501	auccuucgucccugggugaga
eca-mir-5023p	aaugcaccugggcaaggauuca
eca-mir-5025p	auccuugcuaucugggugcua
eca-mir-503	uagcagcgggaacaguacugcag

eca-mir-504	agacccuggucugcacucuauc
eca-mir-505	cgucaacacuugcugguuuccu
eca-mir-507	auuggcaccucuuagagugaa
eca-mir-5083p	ugauugucaccuuuuggaguaga
eca-mir-5085p	uacuccagagggugucauucaca
eca-mir-5095p	uacugcagacaguggcaauca
eca-mir-514	auugacaccucugugagugga
eca-mir-5323p	ccucccacacccaaggcuugca
eca-mir-5325p	caugccuugaguguaggaccgu
eca-mir-539	ggagaaauuauccuugcugugu
eca-mir-541	uggugggcacagaauccagucu
eca-mir-5423p	ugugacagauugauaacugaaa
eca-mir-5425p	cucggggaucaucaugucacga
eca-mir-543	aaacauucgcggugcacuucuu
eca-mir-544b	auucugcauuuuuaacaaguuc
eca-mir-545	ucaacaaacauuuauugugugc
eca-mir-551a	gcgacccacucuugguuucca
eca-mir-551b	gcgacccauacuugguuucag
eca-mir-568	auguauaaauguauacacac
eca-mir-5825p	uuacaguuguucaaccaguuacu
eca-mir-5823p	uaaccgguugaacaacugaacc
eca-mir-5905p	gagcuuauucauaaaaguacag
eca-mir-5903p	uaauuuuauguauaagcuagu
eca-mir-592	uugugucaauaugcgaugaugu
eca-mir-598	uacgucaucguugucaucguca

eca-mir-6155p	gggggucccggugcucggauc
eca-mir-6153p	uccgagccuggucucccucuc
eca-mir-628a	augcugacauauuuacuagagg
eca-mir-632	gugccuguuuccuguggga
eca-mir-652	aauggcgccacuaggguugug
eca-mir-653	guguugaaacaaucucugcug
eca-mir-655	auaauacaugguuaaccucuuu
eca-mir-656	aauauuauacagucaaccucu
eca-mir-660	uacccauugcauaucggaguug
eca-mir-664	uauucauuuaucuccuagccuaca
eca-mir-670	gucccugaguguauguggugaa
eca-mir-6715p	aggaagcccuggaggggcuggag
eca-mir-6713p	uccgguucucagggcuccacc
eca-mir-672	ugagguugguguacuguguguga
eca-mir-6745p	ggugcucacuuguccuccu
eca-mir-6743p	aggaggccauaguggcaacugu
eca-mir-675	uggcgcggagagggcccacagug
eca-mir-684	aguuuucccuucaauucag
eca-mir-703	aaaaccuucagaaggaaagga
eca-mir-708	aaggagcuuacaaucuagcuggg
eca-mir-711	gggacccagggagagacguaag
eca-mir-758	uuugugaccugguccacuaacc
eca-mir-761	gcagcagggugaaacugacaca
eca-mir-763	ccagcugggaggaaccaguggc
eca-mir-7675p	ugcaccaugguugucugagcaug

eca-mir-7673p	ucugcucauacuccaugguuccu
eca-mir-7695p	ggagaccucuggguucugagcu
eca-mir-7693p	cugggaucucgggggucuugguu
eca-mir-769b	ggaaaccucuggguucugagcu
eca-mir-770	agcaccacgugucugggccaug
eca-mir-802	caguaacaaagauucauccuugu
eca-mir-872	aagguuacuuguuaguucagg
eca-mir-873	gcaggaacuugugagucuccu
eca-mir-874	cugcccuggcccgagggaccga
eca-mir-8765p	uggauuucuuugugaaucacca
eca-mir-8763p	ugguggugguuuacaaaguaauuca
eca-mir-8855p	uccauuacacuacccugccucu
eca-mir-8853p	aggcagcggguguaguggaua
eca-mir-889	uuaauaucggacaaccauugu
eca-mir-1179	aagcauucuuucauugguugg
eca-mir-1180	uuuccggcucgagugggugugu
eca-mir-1185	agaggauacccuuuguauguu
eca-mir-1193	uaggucacccguuugacuauc
eca-mir-1197	uaggacacauggucuacuucu
eca-mir-1204	ucguggccuggucccacuau
eca-mir-1244	gagugguugguuuguaugagaugguu
eca-mir-1248	uccuucuuguauaagcacugugcuaaa
eca-mir-1255b	cggauaagcaaagaaaagugguu
eca-mir-1261	guggauuaggcuuuggcuu
eca-mir-1264	caagucuuauuugagcaccuguu

eca-mir-1271	cuuggcaccucguaagcacuca
eca-mir-1282	agugguugguuuguaugagaugguu
eca-mir-1289	uggaguccaggaaucugcauuuu
eca-mir-1291a	uggcccugacugaagaccagcagu
eca-mir-1291b	aggcccugaaucaagaccagcagu
eca-mir-1296	uuagggcccuggcuccaucucc
eca-mir-1298	uucauucggcuguccagaugua
eca-mir-1301	uugcagcugccugggagugauuuc
eca-mir-13021	uugggacauacuuauacuaaa
eca-mir-1302b2	uugggacauacuuauacuaga
eca-mir-1302d4	uugggacauacuuaugcuaaa
eca-mir-1302e6	uugggauauacuuauacuaaa
eca-mir-1302e7	uugggauauacuuauacuaaa
eca-mir-1302c5	uugcgacauacuuauacuaaa
eca-mir-1461	aucucuacggguaaguguguga
eca-mir-1468	cuccguuugccuguuuugcug
eca-mir-1597	ugaggagcucugcgagcaugua
eca-mir-1839	aagguagauagaacaggucuug
eca-mir-1842	uggcucugugaggucggcuca
eca-mir-1892	auuuggggugggggaugggga
eca-mir-1898	aggucaagguucacaggggauc
eca-mir-1902	agaggugcaguaggcaugacuu
eca-mir-1905a	caccacgagcccuaccacgcgguag
eca-mir-1905b	caccagccccacuacgcgguag
eca-mir-1905c	caccaccagccccaccacgcgguag

eca-mir-1912	uacccagagcgugcagugugaa

Table 18 Appendix: Significantly different microRNA and their associated diseases, in humans. DIANNA Pathway analysis.

microRNAs in Horses	T2D	MetS	Hyperins	Hyperglyc	Obesity	Ins Resist
mir-200b	Yes	Yes	Yes	Yes	No	Yes
mir-421	Yes	No	Yes	Yes	No	No
mir-216b	No	No	No	No	No	No
mir-103	No	No	No	No	No	No
mir-217	No	No	No	No	No	No
mir-7	No	No	No	No	No	No
mir-507	No	No	No	No	Yes	No
mir-140-3p	Yes	No	No	No	Yes	No
mir-539	Yes	No	No	No	Yes	No
mir-544b	No	No	No	No	No	No
mir-1597	No	No	No	No	No	No
mir-433	Yes	No	No	No	Yes	No
mir-30e	Yes	Yes	Yes	Yes	Yes	No
microRNAs in Ponies	**T2D**	**MetS**	**Hyperins**	**Hyperglyc**	**Obesity**	**Ins Resist**
mir-1291a	No	No	No	No	No	No
mir-15b	Yes	Yes	No	Yes	Yes	No
mir-133a	Yes	No	Yes	Yes	No	No
mir-10a	Yes	Yes	No	No	Yes	No
mir-324-5p	Yes	No	Yes	Yes	No	No
let7a	Yes	No	No	No	Yes	No
let7d	Yes	No	No	No	Yes	Yes
mir-187	No	No	No	No	No	No
mir-331	No	Yes	No	No	No	No
mir-423-5p	Yes	No	Yes	Yes	No	No
mir-200c	Yes	Yes	Yes	Yes	No	Yes
mir-323-3p	Yes	No	No	No	Yes	No
mir-204b	No	No	No	No	No	No
mir-23b	Yes	No	No	No	Yes	Yes
mir-195	No	No	No	No	No	No
microRNAs Combined	**T2D**	**MetS**	**Hyperins**	**Hyperglyc**	**Obesity**	**Ins Resist**
let7d	Yes	No	No	No	Yes	Yes
mir-331	No	Yes	No	No	No	No
mir-1291a	No	No	No	No	No	No

let7a	Yes	No	No	No	Yes	No
mir-15b	Yes	Yes	No	Yes	Yes	No
mir-200b	Yes	Yes	Yes	Yes	No	Yes
mir-193a-5p	Yes	No	Yes	Yes	Yes	No
mir-23b	Yes	No	No	No	Yes	Yes
mir-150	No	No	No	No	Yes	No
mir-122	No	No	No	No	No	No

Type 2 diabetes (T2D), metabolic syndrome (MetS), hyperinsulinism (Hyperins), hyperglycemia(Hyperglyc), insulin resistance (Ins Resist).

Table 19 Appendix: Significantly different microRNA and their associated diseases, in mouse. DIANNA Pathway analysis.

microRNAs in Horses	T2D	MetS	Hyperins	Hyperglyc	Obesity	Ins Resist
mir-200b	Yes	No	Yes	Yes	No	Yes
mir-421	Yes	No	Yes	Yes	No	No
mir-216b	No	No	No	No	No	No
mir-103	No	No	No	No	No	No
mir-217	No	No	No	No	No	No
mir-7	No	No	No	No	No	No
mir-507	No	No	No	No	No	No
mir-140-3p	No	No	No	No	No	No
mir-539	Yes	No	No	No	Yes	No
mir-544b	No	No	No	No	No	No
mir-1597	No	No	No	No	No	No
mir-433	No	No	No	No	No	No
mir-30e	Yes	Yes	Yes	Yes	Yes	No
microRNAs in Ponies	**T2D**	**MetS**	**Hyperins**	**Hyperglyc**	**Obesity**	**Ins Resist**
mir-1291a	No	No	No	No	No	No
mir-15b	Yes	Yes	No	Yes	Yes	No
mir-133a	Yes	No	Yes	Yes	No	No
mir-10a	Yes	Yes	No	No	Yes	No
mir-324-5p	Yes	No	Yes	Yes	No	No
let7a	Yes	No	No	No	Yes	Yes
let7d	Yes	No	No	No	Yes	Yes
mir-187	No	No	No	No	No	No
mir-331	No	Yes	No	No	No	No
mir-423-5p	Yes	No	Yes	Yes	No	No
mir-200c	Yes	Yes	Yes	Yes	No	Yes
mir-323-3p	Yes	No	No	No	Yes	No
mir-204b	No	No	No	No	No	No

mir-23b	Yes	No	No	No	Yes	Yes
mir-195	No	No	No	No	No	No
microRNAs Combined	**T2D**	**MetS**	**Hyperins**	**Hyperglyc**	**Obesity**	**Ins Resist**
let7d	Yes	No	No	No	Yes	Yes
mir-331	No	Yes	No	No	No	No
mir-1291a	No	No	No	No	No	No
let7a	Yes	No	No	No	Yes	Yes
mir-15b	Yes	Yes	No	Yes	Yes	No
mir-200b	Yes	No	Yes	Yes	No	Yes
mir-193a-5p	No	No	No	No	No	No
mir-23b	Yes	No	No	No	Yes	Yes
mir-150	No	No	No	No	Yes	No
mir-122	No	No	No	No	Yes	No

Type 2 diabetes (T2D), metabolic syndrome (MetS), hyperinsulinism (Hyperins), hyperglycemia(Hyperglyc), insulin resistance (Ins Resist).