Pharmacodynamic Effects of Pioglitazone on High Molecular Weight

Adiponectin Concentrations and Insulin Response

after Oral Sugar in Equids

by

Rebecca Maria Legere

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Approved by

Anne Wooldridge, Chair, Professor, Department of Clinical Sciences

Debra Ruffin Taylor, Associate Professor Emerita, Department of Clinical Sciences

Robert Judd, Professor, Department of Anatomy, Physiology and Pharmacology

Abstract

Chronic insulin dysregulation is challenging to manage with pharmaceuticals in horses. Pioglitazone improves insulin sensitivity in humans, and the pharmacokinetics of pioglitazone have been evaluated in horses. The objectives of this study were to assess the pharmacodynamic effects of oral pioglitazone on morphometric parameters, hepatic enzyme activity and function, adipokines, and enteroinsular response to oral sugar. A prospective pilot study was performed using fifteen adult equids (8 ponies, 7 horses) to evaluate the effects of short-term pioglitazone administration (2 mg/kg PO q 24h, 28 days). Oral sugar tests (OST) were performed before and after treatment. Adipokines were measured at day 0, 14, and 28 of administration. Plasma drug concentrations were measured at day 14 and 28 of administration. The subjects were grouped into horses, ponies, and insulin dysregulated animals. Baseline values for all parameters were compared to values obtained at day 14 and 28 using one way or 2-way analysis of variance. Mild changes were noted in morphometric parameters and hepatic enzymes. No differences were found in leptin concentrations or the blood glucose response to the OST. Significant decreases were found in the insulin response to OST at 90 and 120 minute timepoints and the area under the curve post-pioglitazone treatment in the pony and ID groups. High-molecular-weight (HMW) adiponectin concentrations were significantly increased in all groups after pioglitazone treatment. Decreased insulin concentrations in response to oral sugar and increased HMW adiponectin concentrations indicate positive effects of pioglitazone for treatment of metabolic derangements in EMS, which warrant future clinical study.

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List of Abbreviations

ACN   Acetonitrile

ACTH   Adrenocorticotropic hormone

AMPK   Adenosine monophosphate-activated protein kinase

ACVIM   American College of Veterinary Internal Medicine

ANOVA   Analysis of variance

ATP   Adenosine triphosphate

AUC   Area under the curve

BCS   Body condition score

BW   Body weight

CHO   Carbohydrate

CGIT   Combined glucose-insulin test

CNS   Cresty neck score

DM   Dry matter

ECEIM   European College of Equine Internal Medicine

EEG   Equine Endocrinology Group

EL   Endocrinopathic laminitis

ELISA   Enzyme-linked immunosorbent assay

EMS	Equine metabolic syndrome
F	Intact female
G	Gelding
GFR	Glomerular filtration rate
GIP	Glucose independent insulinotropic polypeptide
GGT	Gamma-glutamyl transferase
GLP-1	Glucagon-like peptide-1
GLUT1	Glucose transporter type 1
GLUT2	Glucose transporter type 2
GLUT4	Glucose transporter type 4
H	Horse
H2O	Water
HMW	High molecular weight
HMW adipo	High molecular weight adiponectin
ID	Insulin dysregulation
IR	Insulin resistance
IRS	Insulin receptor substrate
IS	Internal standard
LMW	Low molecular weight

LOD	Limit of detection
LOQ	Limit of quantification
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MIRG	Modified insulin-to-glucose ratio
MRM	Multiple reaction monitoring
NEFA	Non-esterified fatty acid
NF-kβ	Nuclear factor kappa-light-chain-enhancer of activated B cells
NSC	Non-structural carbohydrate
OGT	Oral glucose test
OST	Oral sugar test
P	Pony
PAL	Pasture associated laminitis
PAEL	Pasture and endocrinopathy associated laminitis
PI3K	Phosphatidylinositol-3-kinase
PL	Previously laminitic
PO	Per os
PPAR-γ	Peroxisome proliferator activated receptor γ
PPID	Pituitary pars intermedia dysfunction

RIA   Radioimmunoassay

RISQI   Reciprocal of the square root of insulin

SDH   Sorbitol dehydrogenase

SBA   Serum bile acids

T1R2/3   Taste type 1 receptors 2 and 3

TNF-α   Tumor necrosis factor alpha

TRIG   Triglyceride

TZD   Thiazolidinedione

UPLC   Ultra-performance liquid chromatography

UPLIC-MS/MS Ultra-high performance liquid chromatography with tandem mass
spectometry

SBA   Serum bile acids

Chapter 1 – Introduction

Equine metabolic syndrome (EMS) is an important endocrine disorder, consisting of a collection of risk factors leading to the development of endocrinopathic laminitis. The most important risk factor is the clinicopathologic diagnosis of insulin dysregulation (ID), consisting of one or more of the following: fasting hyperinsulinemia, excessive insulin responses to carbohydrate feeding, decreased hepatic insulin clearance, and/or insulin resistance (IR). Other phenotypic characteristics include regional or generalized obesity, altered lipid metabolism, impaired reproductive functions, and low-grade inflammation. Certain breeds of horses (e.g. Morgans, Arabians, Tennessee Walking Horses) and certain breeds of ponies are at increased risk of EMS.[1] Current theories as to the epidemiology of EMS have identified a complex interplay between genetic predisposition and environmental influences (primarily diet and exercise), which culminate in development of EMS and subsequent endocrinopathic laminitis (EL).[1] Insulin dysregulation is considered the central pathophysiology of EMS as evidenced by the finding that severe hyperinsulinemia has been used to induce laminitis in otherwise clinically normal horses, and higher insulin responses to oral carbohydrate challenge are directly linked with increased risk of developing laminitis and severity of associated lameness in high-risk breeds.[2,3] Retrospective and prospective research to identify markers for high risk of developing EL within the EMS phenotype has shown a strong predictive value for EL associated with decreased circulating adiponectin concentrations.

Current treatment for EMS focuses on management changes, specifically diet and exercise. However, laminitis risk is still very high due to persistent hyperinsulinemia and inconsistency in management. Pharmacologic interventions could more effectively regulate hormonal functions, increase insulin sensitivity, and improve adipokine profiles. Identifying therapeutic interventions that can improve adiponectin secretion in horses with EMS and insulin dysregulation may help improve long-term management, especially for mitigating risk of EL.

Chapter 2 – Literature Review

Section 1: Equine Metabolic Syndrome and Risk for Endocrinopathic Laminitis

Equine Metabolic Syndrome (EMS) was first described in 2002, and was considered to be a cluster of phenotypic conditions known to predispose horses and ponies to pasture-associated laminitis (PAL). The initial definitions of EMS were in parallel with concurrent work in humans to define the metabolic and endocrinopathic derangements linked with increased risk of type 2 diabetes and coronary artery disease. The condition of PAL was described as a historically recognized link between access to pasture grass and development of acute and chronic, recurrent episodes of laminitis. Historically, seasonal increase in laminitic episodes was seen in spring and early summer, as well as with sudden introduction to lush pastures. These recurrent laminitic episodes were considered a distinct clinical entity from the acute laminitis secondary to severe systemic inflammation or to excessive loading of a support limb due to non-weight bearing lameness.

Early investigations into the risk factors and causes of laminitis identified increased risk of PAL in specific breeds, especially ponies.[4] Clinicopathologic investigations into the breed differences between Morgans, ponies, and thoroughbreds identified differences in glucose and insulin dynamics, as well as lipid regulation.[5] Increased risk within breeds led to researchers investigating the glucose and insulin dynamics of ponies with historical PAL as compared to age-matched ponies without laminitis.[6] The PAL-prone ponies had documented insulin insensitivity as compared to the normal ponies during intravenous insulin tolerance testing.

Interestingly, the authors noted the importance of seasonality during study design, as the insulin response test was performed in November and June because the authors identified much higher risk of laminitis during spring compared to the fall season in horses grazing pasture.[6] Differences in insulin sensitivity were more marked in this research population during the fall trial as compared to the spring trial in this investigation. This provided initial evidence for potential causation or at least correlation to clinical trends identified in equine practice. Concurrent investigation into systolic blood pressure during insulin administration indicated significant differences in the PAL-prone ponies, specifically increased mean arterial pressure and lower diastolic pressure, which the authors' considered linked to a potential vascular pathologic cause for laminitis.[6]

Further work investigated the glucose and insulin dynamics of phenotypic variations within a high-risk breed, using a small group of ponies (clinically normal, obese, and obese with history of PAL), and in comparison to normal Standardbred horses using oral glucose tolerance testing and intravenous insulin response testing.[7] Significant differences were seen in the oral glucose tolerance testing in both the obese and obese-PAL groups, indicating prolonged hyperglycemia, postprandial hyperinsulinemia, and failure to return to baseline glucose values compared to non-obese ponies and Standardbred horses. Furthermore, the obese and obese-PAL ponies exhibited hyperinsulinemia during the oral glucose tolerance testing, and failure to respond during an intravenous insulin response test (i.e. minimal reduction in circulating blood glucose after intravenous insulin administration)- both indicating likely insulin dysregulation in these populations.[7]

Clinical experience has shown that within large populations of horses exposed to pasture grazing during periods of high non-structural carbohydrate (NSC) content, only a small subset will develop PAL. This PAL-prone subpopulation appeared to have documented breed predispositions and altered insulin sensitivity within studied research populations, and a more chronic, recurrent history of laminitic changes seen in clinical cases.[8] It was also recently discovered that intravenous infusion of persistent, excessive quantities of insulin was able to repeatably induce laminitis in clinically normal horses and ponies.[9,10]

PAL is technically a pathologic outcome in horses introduced to, or kept on pasture, resulting in EL. Previous research has suggested that insulin and glucose homeostasis are important in the pathophysiology of PAL, however there are potentially other pathologic processes contributing to this condition. EL refers to a broader clinical outcome, of which PAL comprises a subset of cases, based on a well-described laminar pathology in horses with any combination of the following: demonstrated insulin dysregulation (ID), pituitary pars intermedia dysfunction (PPID), history of pasture grazing or introduction, and/or administration of exogenous corticosteroids.[11]

Several risk factors were identified during epidemiologic investigations of the incidence of EL. Early epidemiologic surveys in the United Kingdom and the United States identified laminitis while grazing pasture (PAL) to occur in 61% and 46% of laminitis cases, respectively.[8] In these studies, evidence for seasonal effects was also shown with higher prevalence in spring and summer. Retrospective research work

over 6 years in a large mixed horse and pony population on a UK rescue farm revealed annual incidence of PAL to range from 7.3-17.1%.[12] A positive correlation was identified between incidence and prevalence of PAL and hours of sunshine per day. This study also showed the highest incidence of PAL occurring between May and August, with the greatest prevalence during the month of May (2.6%). Within this group, the lower weight animals in the herd (i.e. ponies and pony mixes) had greater incidence and prevalence of PAL, as well as females.[12] No further work has shown a gender predisposition in PAL or EL.

Epidemiologic studies have identified EL as the most common form of laminitis to present to first opinion and referral equine practices.[13] A large case-control study combined the case definition of PAL and EL into a single clinical entity, referred to as pasture and endocrinopathy associated laminitis (PAEL).[14] Their survey of equine practitioners sought to identify risk factors associated with the development of PAEL, by matching each veterinary-reported case to a healthy control and lameness control from the respondent's practice. Season was significant, with more cases in the spring and summer, but age and gender were not significant.[14] The existence of a pre-existing endocrinopathy was considered a significant risk factor, although that was included in the case definition, and no further analysis was performed about type of endocrinopathy or diagnostic testing methods. Interestingly, exogenous administration of corticosteroids within 30 days was also a significant risk factor in this population, which may signify an increased susceptibility within this population as corticosteroid administration has repeatedly been shown to have no statistically significant link to induction of laminitis in the general equine population.[14-17] The vast

majority of epidemiologic work on PAL and EL have focused on acute episodes of laminitis, even though clinical experience has shown the importance of recurrent episodes in chronic EL. A recent prospective study evaluated the factors involved in recurrence of EL over a 2 year period of veterinary-diagnosed laminitis cases in the UK, Australia, and the US.[18] In this study, EL cases comprised the vast majority of reported cases (301/317 cases), and this data showed a recurrence rate of 34.1% in EL cases. Seasonality played a significant role, with greatest risk of recurrence in the summer. Gender and height were not found to be significant in this population. Within this population, EMS was present in 82% of EL cases; however the presence of EMS within this study population (as well as the presence of PPID, concurrent EMS and PPID, or PAL-type history) was not found to be a significant risk factor for recurrence of EL. Interestingly, for the equids affected by PPID, the rate of recurrence was also not affected by treatment with pergolide.[18]

Defining the EMS phenotype and risk factors for EL

Over time, the definition of EMS has evolved from a corollary of human metabolic syndrome to a more focused phenotypic description of risk factors for development of EL. Within the 2010 American College of Veterinary Internal Medicine (ACVIM) consensus statement, the three key components of the EMS phenotype were: (1) increased adiposity (regional or generalized); (2) insulin resistance, as characterized by hyperinsulinemia (either basal or excessively stimulated by carbohydrate administration); and (3) a predisposition towards laminitis (clinical or subclinical laminitis).[19] At that time, additional components of the EMS phenotype included dyslipidemia, hyperleptinemia, arterial hypertension, altered reproductive

cyclicity, mild hepatic enzyme elevation, and increased markers of inflammation. Diagnosis focused on clinical suspicion of EMS based on breed predisposition, young to middle age, presence of obesity, and clinical signs of EL, followed by documentation of insulin resistance and/or hyperinsulinemia in the absence of confounding conditions such as stress, pain, and systemic inflammatory response syndrome.[19]

Larger scale, prospective studies were subsequently performed to better characterize the EMS phenotype, based on the interaction between predetermined risk factors in combination with environmental factors.[20,21] This interaction, shown in Figure 1, is the integration and interaction between in-born predispositions (likely genetic and potentially epi-genetic) and environmental management factors (primarily diet and exercise).[1] The presence of obesity was thought to be a primary contributor to EMS pathophysiology, and therefore emphasis was placed on the digestible energy content of feeds leading to obesity, as opposed to NSC content of feeds.
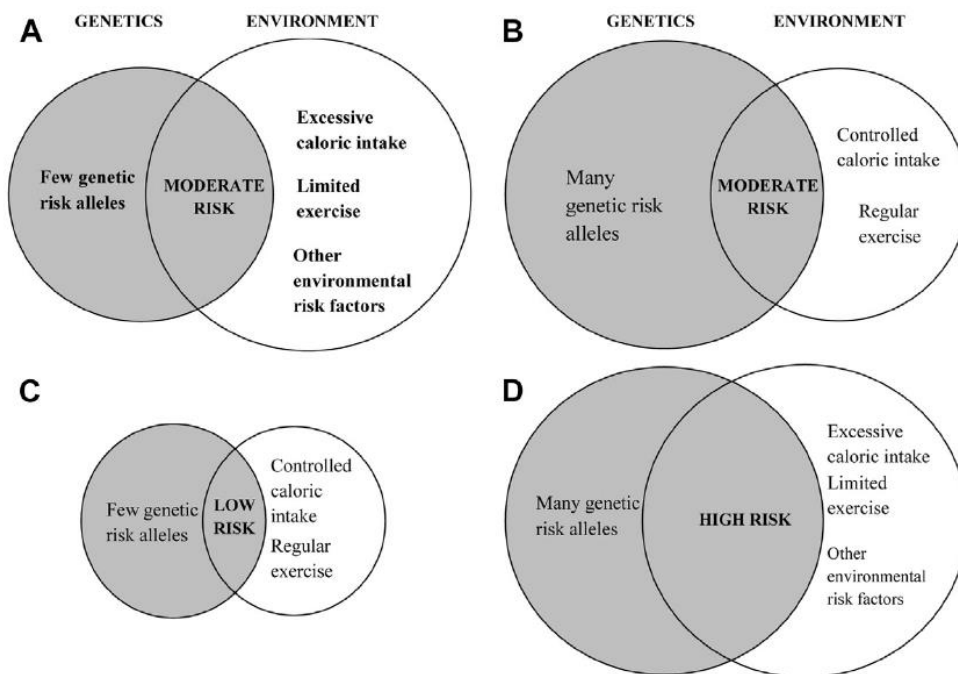
Figure 1. Interaction of EMS risk factors, both genetic and environmental, determines overall risk of EMS and subsequent laminitis.[1] "Depiction of four scenarios where the contribution of genetic and environmental factors that sum to low, moderate, or high risk. A and B, Horses with moderate genetic risk. C, Horse with low genetic risk. D, Horse with high genetic risk" [1]

Subsequent research and more refined understanding of the EMS pathophysiology prompted the 2018 European College of Equine Internal Medicine (ECEIM) consensus statement, where the central component of the EMS phenotype was insulin dysregulation, consisting of one or more of the following: fasting hyperinsulinemia, excessive insulin responses to carbohydrate feeding, decreased hepatic insulin clearance, and/or insulin resistance (discussed further in Chapter 2, Section 2).[3] This represented an expansion over past definitions, which focused largely on insulin resistance only. Obesity (either generalized or regional) was considered a "commonly associated feature" and likely to exacerbate existing pathology, but was no longer a central component of the diagnosis or considered to be the primary cause of metabolic derangements.[3] This definition allows for specific

subsets of EMS cases where ID has been shown to persist after weight loss as well as in the lean EMS phenotype. Additionally, the well characterized altered reproductive function associated with EMS was considered to be a more likely consequence of obesity than the true EMS phenotype. Dyslipidemia, adipokine dysregulation, and cardiovascular changes were considered inconsistent features of the EMS phenotype. In this consensus, laminitis was considered the ultimate clinical consequence of EMS, but presence of pre-existing laminitis was not essential for the diagnosis of EMS. Discussion of environmental exposures, such as high NSC feeding, lack of exercise, and exposure to endocrine disrupting chemicals (linked with high incidence of ID and EL), were limited to potential contributing factors but, again, the focus was primarily on ID as the chief problem driving EMS in the 2019 Consensus.[3]

Predispositions towards development of PAL and/or EL, identified both clinically as well as through epidemiologic studies, has prompted much research in order to better characterize these conditions and associated pathophysiology, with the ultimate goal of intervening prior to development of laminitis.

Breed predispositions: Predisposition for EL has been historically recognized and confirmed through epidemiologic studies to be more prevalent in pony breeds, especially British northern breeds (Welsh, Dartmoor, and Shetland), as well as horse breeds developed under conditions of relative nutritional scarcity. These breeds include Morgan Horses, Arabians, Andalusians, Spanish Mustangs, Saddlebreds, Paso Finos, Tennessee Walking Horses, and warmblood horses.[8,11,22,23] These breeds have been associated with the "easy keeper" phenotype, where metabolic efficiency results

in maintenance of body condition with less digestible energy in the diet as compared to other light horse breeds, such as Thoroughbreds and Standardbreds. Quarter horses have been inconsistently identified as a high risk breed, however there has been some work that indicates Quarter horses may have heightened insulin sensitivity, especially in skeletal muscle, so this remains an area of active research.[24] Breed specific differences in insulin sensitivity have been noted since the early 1980's, and were recently well characterized. In this study, ponies and Andalusian horses (two high-risk breeds) were demonstrated to have decreased insulin sensitivity and increased postprandial insulin response (both peak and area under the curve) when compared to Standardbreds (low risk breed).[25] Breed differences have also been described in terms of incretin response to enteral carbohydrate challenge (described in greater detail in Chapter 2, Section 2). Within the same study population of ponies, Andalusians, and Standardbreds, higher postprandial insulin responses as well as higher postprandial incretin responses were noted in the ponies and Andalusians when compared to the Standardbreds.[26] This provided preliminary evidence for a possible contributing cause for breed-specific thriftiness. Heritability of the EMS phenotype has been identified in horses and ponies, indicating genetic predispositions even within high risk breeds.[1,27,28] Preliminary evidence has shown the potential impact of maternal nutritional status during pregnancy on glucose and insulin dynamics during the first year of life, suggesting environmental influences affecting heritable factors through complex epigenetic interactions that may have long-reaching implications for that individual as well as its offspring.[29,30]

Obesity: Obesity is a common problem in horses kept under modern housing and feeding conditions, where adipose tissue is accumulated due to excessive provisions of nutrients (both digestible energy and specifically non-structural carbohydrates) in combination with inadequate exercise. This results in uniform fat distribution throughout the body (generalized obesity), or fat may be selectively and disproportionately accumulated in specific regions (regional adiposity). These regions include the nuchal ligament of the neck, periorbital fossa, the tailhead, and premammary/preputial regions. Incidence rates of obesity have been reported in surveys to affect 10-51% percent of equids in developed countries.[31,32] Body weight is the most objective measure, and this can be estimated with some degree of accuracy using weight tapes when no scale is available. However, due to the marked variation of body size within the *Equus caballus* breeds and body types, subjective body condition scoring (BCS) has been used to described body fat mass across multiple breeds on a scale of 1-9, with 1 being emaciated, 9 being morbidly obese, and greater than 7 categorized as obese (see Figure 2A).[33] Another potential scoring for generalized obesity is the ratio of heart-girth to height, which has been advocated by some research groups due to its ease of measurement, objectivity, and ease of teaching to untrained owners for on-farm follow-up.[34] Regional adiposity has also been characterized by several methods. The most common method is assigning a cresty neck score (CNS) to provide a scale of 0-5 for assessment of adipose tissue accumulation along the nuchal ligament (See Figure 2B).[35] Horses with a CNS greater than 2/5 are considered to have significant regional adiposity. The ratio of neck circumference to height at the withers can be measured as a continuous, objective parameter, and this was used as a marker for regional adiposity in a population of

ponies for prediction of PAL (see Figure 2C).[34] Other researchers have utilized ultrasonographic measurement of fat pad thickness at the tailhead, and caliper measurements of the crest, to characterize regional adiposity in other studies.[36-38]
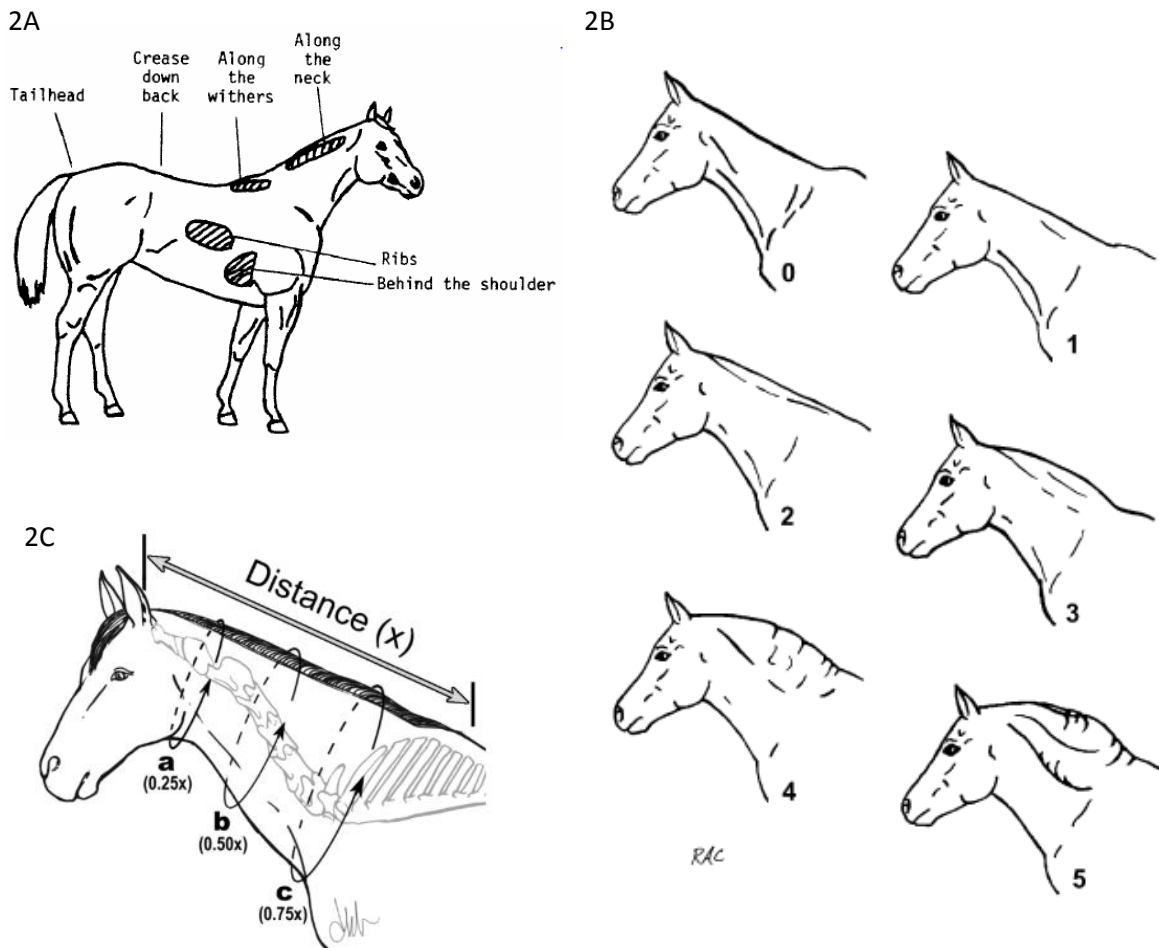
Figure 2. Methods for measuring body condition and obesity, both generalized and regional. Fig 2A. Body condition scoring averages findings over 6 specific body sites to assign an overall body condition score, with 1 being emaciated and 9 being extremely obese.[33] Fig 2B. Cresty neck score assigns a score to the regional adiposity over the nuchal ligament based on appearance and descriptions, with 1 being "ewe" necked and 5 having severe bulging, resulting in it being broken over to one side.[35] Fig 2C. Crest circumference measurements, later scaled to height at the withers, to provide objective measurements scaled to equid height.[34]

Obesity was considered a primary component of EMS until 2018, so various measurements have been compared in terms of predicting the onset of EL in high-risk populations. Earlier retrospective studies showed previously laminitic ponies were more likely obese with regional adiposity (largely focused on measures of crest adiposity) as compared to ponies without historical laminitis.[27,39-41] However, some argued that correlation was not necessarily causation and obesity may be consequence of existing disease instead of primary driver of laminitis.[19,21] Multiple retrospective and prospective studies have not shown a consistent link between measures of adiposity (BCS, CNS, and crest:height ratios) and EL.[20,21,34,37,39] Furthermore, several prospective studies specifically selected ponies with non-obese phenotypes to remove obesity as a confounder.[37,42-44] In those studies, measures of insulin dysregulation and adipokines (specifically adiponectin) were more predictive of developing laminitis under ideal management procedures in place to ensure appropriate body condition. However, a recent epidemiological study utilizing surveyed U.S. equine practitioners showed that obesity (did not clarify regional vs. generalized in survey questions) was a consistent risk factor for first time cases of PEAL compared to case controls in a large clinical population of horses and ponies, so it still may be playing a role in the pathogenesis of EL.[14] Proposed mechanisms included adipose tissue dysregulation and inflammation, as well as the purely mechanical aspect of increased weight-bearing strain on the laminae of the feet. This remains an area of active research, however the latest ECEIM consensus statement considers obesity and regional adiposity to be a secondary component of the EMS phenotype, whose presence leads to exacerbation of insulin dysregulation, and potentially (but not consistently) increased risk of EL.[3]

Insulin Dysregulation: There are four major components of insulin dysregulation- consisting of any one or more of the following conditions: fasting hyperglycemia, excessive insulin response to glucose, decreased hepatic clearance, and/or tissue insulin resistance. Researchers have used a variety of methods to describe the insulin dynamics within ponies and horses. Many researchers have measured endogenous insulin concentrations, some in fasted populations and some in pasture/forage fed equids.[2,20,39,40] These measurements have relatively low sensitivity for detection of ID, which was considered a limitation in these studies as ID diagnosis was likely underestimated.[2] To improve sensitivity, proxy measurements were developed from endogenous insulin and glucose concentrations to approximate measures of insulin sensitivity and insulin secretion that are usually measured with labor-intensive frequently sampled intravenous glucose tolerance testing and minimal model analysis. These included glucose/insulin ratio and RISQI (the reciprocal of the square root of insulin) as proxies for tissue insulin sensitivity, as well as the insulin/glucose ratio and MIRG (modified insulin-to-glucose ratio) as proxies for the acute insulin response from pancreatic beta cells to the glucose concentration.[45,46] The primary limitations of these methods were lack of usefulness in the face of profound hyperinsulinemia, as this results in falsely low and even negative values for some proxies, and incomplete validation.[45,47] Finally, dynamic testing has been utilized to more appropriately evaluate the interrelated components of ID. The combined glucose-insulin tolerance test (CGIT) evaluates both the immediate endogenous insulin response to intravenous glucose administration (beta-cell response), as well as the tissue response to exogenous insulin administration (tissue insulin

sensitivity).[47] Dexamethasone suppression testing was originally developed and utilized for evaluation of the pituitary-adrenal axis for PPID. However, it has also been used to diagnose insulin dysregulation concurrently, as the ID-affected equids had profoundly exaggerated insulin concentrations in response to dexamethasone administration.[44] However, the usefulness of this test has been challenged and it has largely fallen out of favor for dynamic testing that incorporates the enteroinsular axis.[43] These methods are primarily the oral glucose test (OGT) and oral sugar test (OST), in which the blood glucose and insulin concentrations are measured before and at various timepoints after a known bolus of non-structural carbohydrates is fed to the equid.[47] In the OGT, the NSC bolus consists of 0.75 – 1 g/kg of glucose, either administered via nasogastric tube or fed on a meal of bran or chaff, and insulin values are measured at 120 minutes after administration.[48] The OST typically uses an oral administration of 0.15 mL/kg of Karo corn syrup as the NSC bolus, however it has been adapted for use of 0.2 mL/kg of commercially available glucose syrup in Europe.[49,50] There has been some debate as to the optimal timing for insulin sampling in the OST, however 60 and 90 minutes post-administration are most commonly used.[47,51,52] These tests are considered safe and relatively easy to perform, while best approximating the post-prandial hyperinsulinemia conditions thought to be driving ID and subsequent EL.

Regardless of the methods used for evaluating ID, there has been a consistently strong link between insulin dysregulation and EL risk. In retrospective comparisons of previously laminitic (PL) ponies compared to those without laminitis history, measures of insulin resistance and hyperinsulinemia are consistently

associated with the PL pony group in both obese and non-obese states.[27,39,53-57] This is important because, as previously mentioned, there is evidence that the presence of obesity exacerbates ID in low-risk and high EMS risk breeds, especially when that obesity is induced by high starch feeding.[58] Prospective studies have also shown strong predictive value for varied measures of insulin dysregulation (hyperinsulinemia, dexamethasone suppression, proxies, OGT, and OST) and increased risk of EL.[20,21,40,43,44,59] Importantly, the severity of hyperinsulinemia in response to the OGT was predictive of increased risk and severity of subsequent laminitic episodes.[60] This is consistent with the hyperinsulinemic induction models for laminitis, where hyperinsulinemia with euglycemia over 48 hours duration in clinically normal ponies and horses induced pathology consistent with clinical EL.[9,10] Finally, the severity of hyperinsulinemia in response to dynamic testing was linked with increased risk of recurrence of EL within two years during a prospective cohort study of client-owned horses and ponies.[18]

Dyslipidemia: Dyslipidemia, or abnormal circulating lipid profiles within the bloodstream, has been proposed as a secondary component of the EMS phenotype, in parallel to the human metabolic syndrome. This largely consists of hypertriglyceridemia resulting from a lack of response to insulin in the liver and lipoprotein lipase in the adipose tissue, resulting in decreased uptake and utilization of these lipids and increased hepatic lipogenesis.[2,61] The degree of hypertriglyceridemia during basal conditions is thought to be relatively mild, however this can become life threatening during periods of critical illness and anorexia, resulting in hyperlipemia and associated consequences.[3] Elevated triglyceride

concentrations were considered significant predictors of previous laminitic episodes in an inbred pony population as well as an outbred pony population.[27,40] However, the relatively mild hypertriglyceridemia seen in these studies, and inconsistency in larger studies (potential seasonal effects that have not yet been explained) makes its use as a predictor of EL more challenging.[3,34,37] Similar inconsistencies have been seen in analysis of non-esterified fatty acid concentrations in EMS-affected populations, and their predictive role is considered low.[27,28,34,40] More specific research into lipid profiles and metabolomic analysis in EMS-affected ponies have identified preliminary differences in lipid metabolism that require further study before use of dyslipidemia as a predictive tool for EL within the EMS phenotype.[62,63]

Role of PPID: Pituitary pars intermedia dysfunction (PPID) is a known cause of EL, however laminitis does not occur in all cases of PPID. A recent case-control study in client-owned equids in the U.S. identified the presence of an endocrinopathy as an increased risk for incipient episode of EL, however this study did not separate PPID from EMS cases.[14] Evaluation of EL within the PPID affected population of horses have shown that ACTH levels do not predict laminitis risk.[59] Importantly, the diagnosis of insulin dysregulation within the PPID-affected population is linked to poor prognosis and increased risk of EL, and hyperinsulinemia is considered the most important risk factor for laminitis in PPID.[54,64-67] PPID may develop in EMS-affected equids, however this largely occurs by a separate pathophysiology than EMS, and is strongly associated with increased age and loss of dopaminergic inhibition of the pituitary pars intermedia.[64] In PPID, elevated ACTH secretion from the functional pituitary adenoma is thought to trigger hypersecretion of cortisol, which is antagonistic to the functions

of insulin, resulting in tissue insulin resistance.[64] However, the mechanisms driving ID in PPID-affected equids are likely multifactorial and incompletely described, as PPID has inconsistent effects on insulin sensitivity, inconsistent elevation of cortisol concentrations is seen in PPID-affected equids, and other dysregulated pituitary hormones in PPID can also impact insulin sensitivity.[64,66,68] In a recent prospective cohort study, the presence of concurrent EMS and PPID resulted in higher basal insulin concentrations, which implies greater risk of EL, than equids that had either EMS or PPID alone.[59] Furthermore, primary treatment of PPID with pergolide therapy may marginally improve laminitis risk, however it was recently shown that it did not lower rate of EL recurrence in a clinical cohort of affected equids.[3,18] Because of the importance of ID within the PPID affected populations, it is recommended to screen for ID during initial diagnosis of PPID as well as implement management strategies in ID-affected horses to improve insulin sensitivity and therefore lower subsequent laminitis risk.[3]

Adipose Dysregulation: Dysregulated adipose tissue in the EMS phenotype has been evaluated for its far-reaching effects as a paracrine and endocrine organ. Specific fat depots in the horse have been evaluated for altered metabolic functions, as seen in visceral adipose tissue in human metabolic syndrome.[69] Adipose tissue from the nuchal ligament fat depot of EMS-affected horses has demonstrated altered lipid metabolism and inflammatory cytokine secretion compared to subcutaneous and visceral fat depots, and compared to adipose tissue from healthy horses.[69,70] The role of macrophages within dysregulated adipose tissue in humans with metabolic syndrome has prompted investigation of inflammatory cytokine profiles in obese

horses and EMS-affected horses.[71] Some, but not all, of these studies have shown increased production of TNF-α in previously laminitic ponies as compared to those without laminitis, however, inflammatory profiles are influenced by many other factors, and not considered predictive for EL.[3,37,40,56,72]

Altered adipokine production from the adipose tissue of laminitic horses has also been evaluated, primarily leptin and adiponectin. Importantly, adipokines and lipids have key roles in insulin signaling and sensitivity. Leptin is an adipokine that controls appetite and metabolism through its effects on the brain, and circulating leptin concentrations are increased with body fat mass.[28,73] Early investigation into leptin as a potential biomarker showed it was increased in previously laminitic, obese ponies.[40] However, when evaluated in the lean EMS phenotype as well as in prospective studies, leptin had no predictive value for EL and was considered more likely to be a consequence of obesity.[3,20,21,28,74] Adiponectin is an adipokine with profound insulin sensitizing effects and has an inverse relationship with insulin and fat mass.[73,75] Circulating adiponectin consists of low molecular weight (LMW) trimers, middle molecular weight hexamers, and high molecular weight multimers (HMW), each of which has different biological activities that contribute to insulin sensitizing and anti-inflammatory functions.[76] The HMW fraction is the most insulin sensitizing, presumably from its greater affinity for adiponectin receptors.[75] Decreased adiponectin levels, both total and HMW fractions, have been consistently identified in laminitis-prone EMS horses and is considered a strong predictor of EL within the EMS phenotype.[3,20,21,28,37,42,74]

Cardiovascular Abnormalities: The importance of the cardiovascular outcomes of human metabolic syndrome and the potential link between microvascular pathology and laminitis has prompted much research in EMS-affected phenotypes. Hypertension has been identified in ponies with historical PAL, compared to ponies without laminitis.[39] This effect was more significant during the summer, presumably during times of increased hyperinsulinemia associated with pasture grazing. The pathophysiology is likely due to endothelial dysfunction, as hyperinsulinemia blocks nitric oxide production and associated vasodilation, while vasoconstriction through continued endothelin production.[61,77] Recent prospective research comparing cardiac structure and function between EMS and healthy horses showed mild echocardiographic changes in EMS-affected equids, presumably secondary to hypertension, however this was not evaluated in regards to risk of EL.[78] Cardiovascular abnormalities have not been a consistent finding in larger studies and have low predictive value for EL.[3]

While promising biomarkers and risk factors for development of EL have been identified in the EMS phenotype, the most consistent predictor for and primary pathophysiologic mechanism driving EL is insulin dysregulation, which has complex and multifactorial causes as well as far-reaching consequences on metabolism.

Section 2: Pathophysiology of Insulin Dysregulation in the Horse

Glucose and insulin dynamics in the normal horse

Exocrine pancreatic physiology in the horse has not been as well characterized as in other domestic species due to anatomic inaccessibility for imaging and difficulty sampling (pre- and post-mortem).[29,79] However, the importance of the endocrine pancreatic functions, namely insulin dynamics, to EL and the EMS phenotype have made it the focus of greater research in recent years. Given the importance of ID to the development of EL, it is important to consider the dynamics of insulin and glucose in the normal horse.

Insulin is secreted from the β cells in the pancreatic islets of Langerhans. Histopathologic analysis of the equine pancreas has identified the beta cells within pancreatic tissue, however determination of the total beta cell mass within the equine pancreas is poorly characterized.[29] Typically, β cell function in horses is maintained even in the face of prolonged hyperinsulinemia.[2] Reports of beta cell exhaustion and failure, leading to inadequate insulin secretion and diabetes mellitus, are rare in horses.[80] Equine insulin has marked homology to that of other domestic species (including canine, feline, porcine, and bovine).[29] Insulin molecules are stored within cytoplasmic vesicles of the β cells, along with equimolar amounts of C-peptide (produced in a 1:1 ratio with insulin during post-translational processing of proinsulin).[81] The most important secretogogue for insulin is blood glucose, although secretion is also stimulated by circulating amino acids, glucagon, incretin hormones, and parasympathetic stimulation (in the post-prandial period).[29] Insulin secretion is suppressed by somatostatin and sympathetic stimulation.

A two-phase insulin secretion profile in response to meal feeding has been described.[29] In the post-prandial period, breakdown of dietary starches and simple sugars in the stomach and small intestine produces 6-carbon (hexose) sugars-glucose, fructose, and galactose, which are absorbed via diffusion (simple and facilitated with sodium), along with amino acids from the digested protein contents. This results in an influx of blood glucose to the portal system and then to circulating systemic blood. Insulin secretion is most immediately triggered by this post-prandial influx of glucose into the β cells via non-insulin dependent transmembrane proteins GLUT1 and GLUT2 (glucose transporters 1 and 2). This leads to increased adenosine triphosphate (ATP) production through glycolysis, changes in potassium flux, depolarization of the β cells, and subsequent influx of calcium ions.[29] This calcium influx triggers intracellular processes that end with fusion of the vesicles to the cell membrane and exocytosis of the contents. This component of post-prandial insulin release is considered to be highly responsive to circulating blood glucose concentrations.

The second phase of insulin secretion, which is slower but more sustained, is triggered by incretin (INtestinal seCRETion of INsulin) hormones that act on β cells to promote insulin secretion, while inhibiting glucagon secretion from α cells. Secretion of these hormones from specialized cells in the small intestine are triggered by intraluminal amino acids, fatty acids, and water soluble carbohydrates associated with the digestion of feed. The specific cells in horses responsible for their secretion have not yet been characterized, however secretory K cells and L cells within the

proximal and distal small intestine, respectively, have been described in other species.[82] The added insulin secretion seen after enteral carbohydrate loading as compared to intravenous carbohydrate loading is known as the incretin effect. This allows for a pre-emptive increase in circulating insulin concentrations after consumption of a meal prior to hyperglycemia, which promotes glucose uptake at insulin-sensitive tissues and more efficient utilization of the ingested carbohydrates. In horses, the incretin hormones best described are glucagon-like peptide-1 (GLP-1) and glucose-independent insulinotropic polypeptide (GIP).[82] This dynamic endocrine system for interplay between the intestinal tract and pancreas is also known as the enteroinsular axis. These hormones have several wide-reaching systemic effects outside of the pancreas, including effects on gastric emptying, gastric acid secretion, and appetite.

Upon fusion of the vesicles with the β cell membrane, both insulin and C-peptide are released into the extracellular space for transport into the pancreatic veins, which drain into the portal vein along with digested nutrients from the gastrointestinal tract. Circulating insulin is cleared by the liver via enzymatic processes, which has a very high first-pass clearance rate.[81] It can also be cleared from the blood by the kidneys in small amounts.[29]

Insulin has multiple functions within different tissues of the body, with the ultimate goal of regulating energy storage and metabolism and promoting utilization and storage of available nutrients. At target tissues, it binds to transmembrane insulin receptors, causing phosphorylation of the intracellular domain and recruitment

of insulin receptor substrate (IRS) proteins. These IRS proteins trigger two pathways-the mitogen-activated protein kinase (MAPK) pathway, which is unrelated to glucose metabolism, and the phosphatidylinositol-3-kinase (PI3K) pathway.[29,77] Through multiple intracellular signaling steps, the PI3K pathway leads to translocation of intracellular vesicles containing the GLUT4 (primary glucose transporter proteins) to the cell surface where GLUT4 are integrated into the cell membrane. The GLUT4 transporter proteins allow facilitated diffusion of glucose into the cell, until the insulin signal has ceased, and the GLUT4 is re-sequestered into vesicles. Glycolysis rapidly converts glucose to fructose 1,6-bisphosphonate, trapping the molecule in the intracellular space for production of ATP or storage.[83]

While insulin has effects on many body tissues, the three primary insulin-responsive tissues are adipose tissue, hepatic tissue, and skeletal muscle. At the skeletal muscle, insulin increases the cellular uptake of glucose and amino acids and synthesis of protein and glycogen as storage molecules. It also decreases protein breakdown and cellular output of amino acids. In adipose tissue, insulin increases fatty acid uptake and esterification and suppresses hormone sensitive lipase to decrease the mobilization of stored fats (conversion of triglycerides to fatty acids). At the liver, which is the major target of insulin, insulin stimulates glycolysis and intracellular storage of molecules through glyconeogenesis and fatty acid synthesis. Insulin also inhibits further production of cellular energy substrates through inhibition of gluconeogenesis, glycogenolysis, and ketogenesis. Finally, insulin stimulates lipoprotein lipase on the endothelium and in adipose tissue, to enhance the uptake of low density lipoproteins and promote breakdown of triglycerides.[29]

Insulin also has wide-reaching effects on vascular tone and the shift of potassium between the intra- and extra-cellular space. Its effects on vascular tone may play a role in vascular dysfunction associated with endocrinopathic laminitis.[84] Under normal conditions, insulin works at the endothelial cells to stimulate the release of nitric oxide (vasodilator) through the previously mentioned PI3K intracellular pathway, in balance with the production of endothelin-1 (vasoconstrictor) through the intracellular MAPK pathway.[29] In human metabolic syndrome, insulin resistance results in inhibition of the PI3K pathway (less vasodilation) and promotes the MAPK pathway (more vasoconstriction), resulting in inappropriate vasoconstriction and hypertension.[77] The role of this pathway in horses has not been fully characterized at this time.

Pathophysiology of Insulin Dysregulation

Earlier understanding of the EMS phenotype focused primarily on insulin resistance (IR), in which body tissues fail to respond appropriately to insulin signaling, resulting in inappropriate cellular glucose uptake and compensatory increase in blood insulin concentrations. Further characterization has described "insulin dysregulation" (ID) as a more appropriate description of the pathologic mechanisms associated with EMS and EL.[2] This condition of ID is comprised of any one or more of the following conditions: fasting hyperinsulinemia, excessive insulin response to glucose, decreased hepatic clearance, and/or tissue insulin resistance.[3] These four mechanisms are inter-related and can exacerbate each other to varying degrees (see Figure 3).
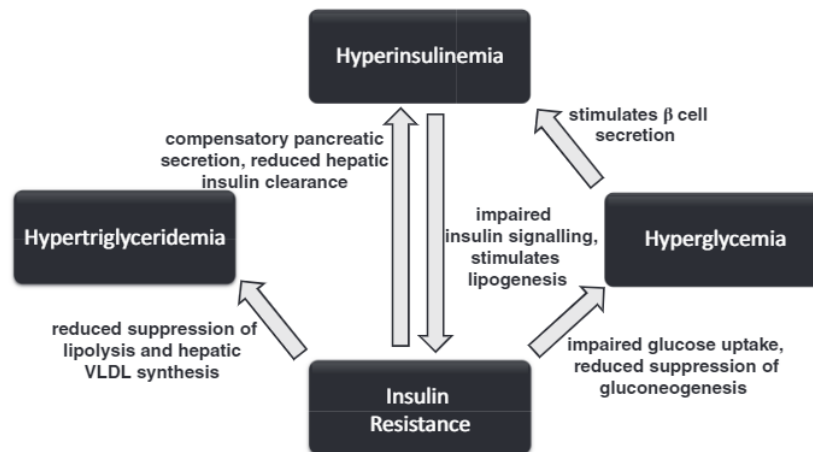
Figure 3. Interrelated components of insulin dysregulation.[3]

The primary trigger of ID has been difficult to identify, due to the interrelatedness of these pathways. However, current understanding of the EMS phenotype suggests an initial hyperinsulinemia in the post-prandial phase (likely due to genetically driven heightened incretin response) with compensatory insulin resistance that causes increased insulin secretion by the beta cells.[2] These changes worsen insulin resistance at the tissue level and decreased hepatic clearance of insulin. Obesity, untreated PPID, pregnancy, critical illness, exogenous corticosteroid administration, and other physiologic states drive worsening tissue insulin resistance and act as modifying conditions that worsen hyperinsulinemia, and therefore increase the risk of developing EL.[2,3,85]

Four major components of ID:

1. Excessive insulin release in response to oral glucose – This excessive insulin response to oral glucose is generally associated with exaggerated incretin response to feeds, leading to excessive postprandial insulin response and slowed gastric emptying.[2] In humans, this has been linked to increased incretin release at the enteroendocrine cells, increased sensitivity to incretins at the level of the pancreas, and/or decreased clearance of secreted incretin hormones, however this has not been fully characterized in equids at this time.[82,83] This exaggerated incretin effect has been demonstrated in high-risk EMS breeds (ponies and Andalusians), as well as in ponies diagnosed as ID based on OGT and horses diagnosed as ID by OST.[26,82,86] This is likely an inherited factor developed during times of sparse nutrient availability, where it was advantageous for insulin secretion to drive efficient utilization and storage of available energy substrates. However, under current management conditions (especially continuous grazing on lush pastures), this is especially problematic as continuous intake of feeds causes a persistent stimulation for insulin secretion.[86] This can be worsened by seasonal accumulations of starches and fructans within pasture grasses, which increase the glycemic index and post-prandial insulin response to grazing, and further increase risk of EL.[2] Chronically, this persistent stimulation for insulin secretion has been shown to drive beta cell proliferation and chronic hyperinsulinemia in rodents.[61] Similar physiology has been suspected in horses but not proven because evaluation of beta cell mass in horses is difficult.[83] When equine post-mortem pancreatic samples were evaluated by immunohistochemical staining for insulin within islets, no difference was seen in the

beta cell mass of IR horses compared to non-IR horses.[79] However, these methods were not sensitive enough to detect hypertrophy or hyperplasia of the beta cells, which could still be present as an adaptive response leading to excessive insulin secretion by existing beta cells caused by chronic adaptations.[79] Excessive insulin response to intravenous glucose administration has also been noted in ID-affected horses, potentially due to these adaptations or increased sensitivity to glucose at the beta cells, however post-prandial hyperinsulinemia secondary to the incretin effect is more important and applicable to daily living conditions of the horse.[2,83]

2. Tissue insulin resistance – This specifically refers to decreased responsiveness of insulin receptors at the body tissues, most importantly adipose tissue, skeletal muscle, and the liver. Chronic hyperinsulinemia is thought to induce tissue insulin resistance by downregulating insulin receptors at these tissues, as well as downregulating the intracellular signaling pathways triggered by insulin binding to its receptors.[2,83] Specific mechanisms have not been as well characterized in horses as in humans, however altered insulin receptor structure and quantity, as well as altered activity and serine phosphorylation of intracellular signaling proteins are considered likely.[61,77] Insulin resistance can also be induced or exacerbated by obesity, PPID, systemic inflammation, and pregnancy.[3] Corticosteroids, both endogenous and exogenous, can induce and worsen existing IR.[61] Inflammatory cytokines, in response to inflammatory triggers or resulting from dysregulated adipose tissue, can also contribute to IR.[71,87]

Horses tend to have compensated IR, in which acute amounts of insulin

released in the face of hyperinsulinemia will still result in glucose uptake to maintain normoglycemia, although several studies have noted that affected horses remain in the high end of the glucose reference interval.[2,3] Uncompensated IR occurs when hyperinsulinemia coexists with hyperglycemia, due to pancreatic insufficiency, and is less common.[61] When uncompensated IR develops, it can progress to glycosuria and diabetes mellitus, and this can be challenging to manage with exogenous insulin administration.[88]

The primary consequence of IR is decreased glucose uptake into body tissues, with or without subsequent hyperglycemia as mentioned previously. Tissue insulin resistance at the liver increases gluconeogenesis, as the action of insulin at the liver in normal individuals should suppress gluconeogenesis during times of hyperglycemia and hyperinsulinemia. Chronic periods of hyperglycemia result in glycosylation of plasma proteins (largely albumin) to form fructosamine, and increased circulating fructosamine concentrations are used diagnostically in other species to approximate average glycemic control over the duration of albumin's circulating half-life.[29,89] Fasting serum fructosamine concentrations were found to be significantly higher in a population of laminitic horses (primarily EL cases) as compared to non-laminitic horses, suggesting some degree of chronic hyperglycemia (either basal or episodic) in these horses.[90] Fructosamine concentrations were also significantly higher in horses with PPID and laminitis as compared to horses with PPID alone or controls.[89] Unfortunately, there was significant overlap between control horses and ID-affected horses in both studies, and its diagnostic value for ID is considered low in horses.[2,89,90]

Insulin resistance also results in peripheral lipolysis, leading to increased circulating non-esterified fatty acids (NEFA's) and increased lipoprotein secretion by the liver (normally suppressed by insulin).[61,83] This contributes to previously described lipid dysregulation, as well as ectopic lipid deposition in body tissues, which is thought to further contribute to tissue IR.[2]

3.   Decreased hepatic clearance – Insulin is cleared from the circulation by enzymatic breakdown within the liver, with an estimated roughly 70% clearance on the first pass through portal circulation.[61] Decreased hepatic clearance of insulin develops as a compensatory response to tissue insulin resistance, thereby shifting larger amounts of secreted insulin into systemic circulation to promote tissue glucose uptake and inhibition of lipolysis.[83] Renal clearance is responsible for removal of circulating insulin from the systemic circulation, and this rate is comparatively much slower than liver clearance and highly dependent on glomerular filtration rate (GFR).[83] Direct measurement of insulin clearance would require catheterization of the portal vein, so a proxy measurement used to evaluate hepatic clearance of insulin is the ratio between the insulin concentration and the concentration of C-peptide. C-peptide is released from beta cells in equimolar amounts to insulin but not cleared by the liver, therefore the C-peptide concentration reflects the quantity of insulin released by the pancreas and is an estimate of hepatic clearance of insulin.[47] A study in horses showed that insulin concentrations during IV glucose tolerance testing were 74% less than C-peptide concentrations, approximating the degree of first-pass removal of insulin by the liver.[81] In that study, obese horses with documented IR had only 69% less insulin than C-peptide, indicating decreased clearance by the liver.[81]

This is thought to be a relatively minor contribution to ID, however, it promotes hyperinsulinemia in circulating blood and contributes to enhanced risk of EL.[3]

4. Fasting hyperinsulinemia – Hyperinsulinemia persisting during fasting is due to hypersecretion from the beta cells, as well as increased beta cell mass. Hypersecretion is due to persistent stimulation of the beta cells, which has been linked to an intracellular enzyme shift from glucokinase to hexokinase, which still converts glucose to glucose-6-phosphate within the beta cell.[83] However, the kinetics of the hexokinase reaction leads to altered dynamics of glucose uptake to insulin release, ultimately resulting in lower glucose concentrations triggering insulin secretion. This is seen as an adaptation to IR in humans and murine models, and similar mechanisms are suspected in horses.[83] Beta cells are also driven to replicate and hypertrophy within islets in response to IR, leading to increased capacity for insulin secretion when triggered by glucose and other insulin secretogogues.[29] The resulting hyperinsulinemia during fasting is not present in all cases of ID, but is a common finding.[47] This persistent hyperinsulinemia reflects significant ID, as post-prandial insulin responses are additive on top of this elevated baseline, and represents a significantly elevated risk of EL.[2]

Section 3: The Role of Adipokines in Horses with ID

Altered metabolism and endocrine function of the adipose tissue, as well as system-wide altered lipid metabolism contributes to the pathophysiology of EMS. As previously discussed, dyslipidemia has far-reaching effects due to insulin resistance at the liver and adipose tissue, resulting in abnormally elevated circulating lipids within the blood. Adipose tissue also has paracrine and endocrine functions through the secretion of adipokines, which are protein hormones that are primarily or exclusively secreted by adipocytes. Secretion patterns of adipokines are significantly altered by obesity, adipose dysfunction, energy metabolism, and systemic inflammation, and feedback into further regulation of these processes.[76,91,92] Concentrations of adipokines have been used as biomarkers in evaluation of the human metabolic syndrome as well as evaluation of disordered eating behaviors.[76,93] The two best characterized adipokines in horses are leptin and adiponectin.

The transcription of leptin is regulated by energy storage within the adipocyte, and circulating concentrations are proportional to body fat content.[92] There are mild fluctuations in leptin documented in response to circadian rhythms in horses, with higher concentrations overnight and nadir during the daytime, however this may be impacted by husbandry and meal-feeding.[76] Leptin concentrations are documented to fluctuate throughout the estrus cycle in humans and in response to certain sex hormones in humans and potentially in horses.[76,92] A transient decrease in circulating concentrations occurs from parturition through early lactation, which is thought to drive increased feed consumption by the mare

and avoidance of a negative energy balance.[76] Leptin's primary biological effect is on the hypothalamus. After crossing the blood-brain barrier and binding to receptors on specialized cells, leptin signals a positive energy balance, drives satiety, decreases appetite, and increases thermogenesis in brown fat (via secondary sympathetic stimulation).[92,94] Its signaling of the body's nutritional status is also used to drive reproductive functions and estrus in cattle during positive energy balance, as well as suppression of reproductive functions during periods of starvation.[76] Complete lack of leptin receptors results in morbid obesity in rodent models, due to lack of satiety and dysregulated signaling for body energy content, however these defects are rare in humans and not described in horses.[76,94] In human obesity, a partial leptin resistance has been described, in which decreased biologic responses to leptin binding lead to hyperleptinemia. This occurs by multiple mechanisms, including leptin receptor saturation, down-regulation of leptin receptors, and modification of downstream signaling pathways.[92,94] Leptin resistance has not yet been described in horses.[76] In horses, leptin is typically measured using a radioimmunoassay (RIA). Initial studies utilized a multispecies RIA and now an equine-specific RIA has been validated in horses.[76]

In horses, circulating leptin concentrations have been strongly correlated to body fat mass.[3,73,95,96] However, this has not always been consistent, as others found only 35% of obese mares had elevated leptin concentrations, and this portion of the group also had elevated fasting insulin concentrations.[97] There were similar findings when a group of obese horses (mares and geldings, all with BCS >7.5/9) was evaluated and hyperleptinemia was associated with documented ID.[98] This was

initially considered a potential link between ID and hyperleptinemia, potentially through leptin resistance.[76] Initial retrospective evaluations into the EMS phenotype found some correlations between hyperleptinemia, ID, and previous laminitic episodes in ponies.[1,40,99] However, these subjects were obese, which was likely was the cause of the high leptin concentrations and a confounding issue. In a subsequent prospective study in a closed herd of ponies, hyperleptinemia had some predictive value for development of EL, however elevated BCS and CNS were also important components of this model and obesity was considered to be driving this hyperleptinemia.[40] More recent larger prospective studies have not shown a correlation between hyperleptinemia and risk of EL. In a prospective study of 446 ponies without history of laminitis, 72% of the study population was obese (BCS > 6/9) but neither leptin concentration nor measures of obesity (BCS, crest height, or weight) were predictive of development of an incipient case of EL over 3 years.[20] In a matched case-control study of 610 horses and ponies, leptin concentration was correlated to measures of obesity (BCS, neck:height, and girth:height ratios) but was not predictive of laminitis status.[28] Leptin concentration is considered to have low predictive quality for development of EL in cases of EMS.[3]

Adiponectin is an adipokine, which is produced as a protein primarily by differentiated adipocytes in white adipose tissue. It undergoes significant post-translational modifications prior to secretion, and then forms trimers, hexamers, or high molecular weight (HMW) multimers within circulating blood.[92] These different forms have different biologic activities, however HMW adiponectin is considered the most potent and most insulin-sensitizing form of adiponectin.[100] Adiponectin has

three primary receptors: AdipoR1 (primarily in skeletal muscle), AdipoR2 (primarily in the liver), and T-cadherin (in endothelial and smooth muscle cells).[92,101] Binding to these receptors triggers multiple intracellular pathways that promote insulin sensitivity and improved metabolic processing (shown in Figure 4A). It directly increases insulin sensitivity by increasing GLUT4 translocation to the cell membrane for improved glucose uptake, promotes intracellular glycolysis by phosphofructokinase and glucose trapping, and promotes intracellular insulin signaling pathways associated with the PI3K pathway.[94,100] Additional metabolic functions include increased oxidation and utilization of intracellular fatty acids by increased signaling through Peroxisome proliferator-activated receptor alpha (PPAR-α) and adenosine monophosphate-activated protein kinase (AMPK), vasodilation, improved oxygen delivery in adipose tissue by stimulating nitric oxide production, and decreased inflammation by inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kβ).[92,100] The combined effects of adiponectin directly counteract multiple causative factors and consequences of insulin resistance (shown in Figure 4B).

Measurement of adiponectin has presented challenges in the horse. Total adiponectin concentration in equine serum was initially measured with radioimmunoassay, but concentrations were much lower than those in other species.[76] An enzyme-linked immunosorbent assay (ELISA) method has been validated for measurement of HMW adiponectin in the horse, although ELISAs tested for measurement of total adiponectin were unsuccessful in the same study.[75] Concentrations of HMW adiponectin measured by the validated ELISA were more in

line with other species.[75] A recent study validated the use of a human immunoturbidometric assay for total adiponectin concentration in horses, but the tested ELISA assays (different companies and ELISAs from the other study by Wooldridge et al.) did not perform adequately in that study.[102]

In equids, total and HMW adiponectin concentrations are inversely related to body fat mass and negatively correlated to insulin concentrations.[73,75,95] No evidence is seen for effects of season, circadian rhythms, or short-term exercise on adiponectin concentrations in horses.[42,76] Obesity and concurrent ID induced by high carbohydrate diet lowered total adiponectin concentrations; whereas obesity induced by high fat diet did not significantly affect adiponectin concentrations.[74] In human and rodent models of metabolic syndrome, with subjects matched by measures of obesity, lower adiponectin concentrations were associated with increased risk of type 2 diabetes and cardiovascular disease.[76,94] Furthermore, in humans, decreased polymerization of adiponectin was seen in type 2 diabetes prior to decreased total adiponectin concentration, therefore HMW adiponectin concentration is considered a more sensitive biomarker for metabolic dysfunction.[93,94] Low adiponectin concentration has also recently emerged as a strong predictor of EL in retrospective and prospective studies of laminitis independent of obesity status.[20,21,28,37] The importance of adiponectin in EMS and the possible link between hypoadiponectinemia and the laminitis-prone phenotype in EMS makes adiponectin an exciting therapeutic target. However, exogenous administration is not feasible, so increasing endogenous production of adiponectin may be a powerful tool to improve management of EMS.
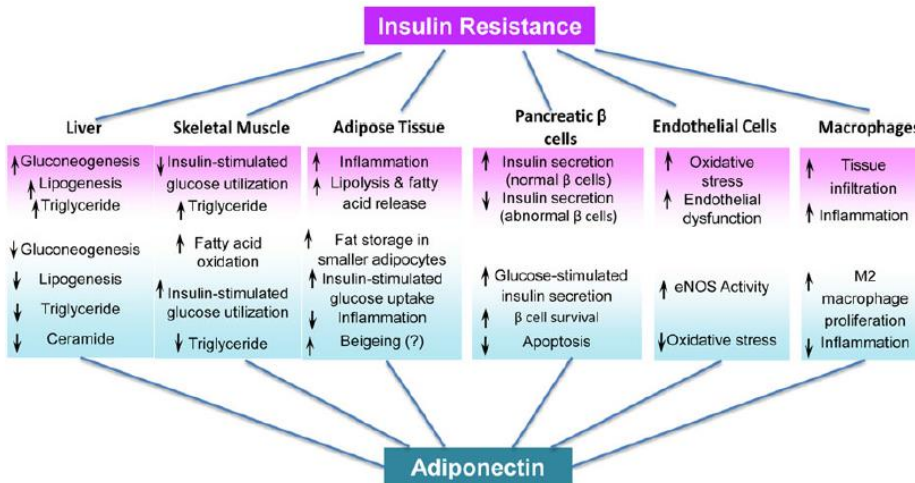
4A.
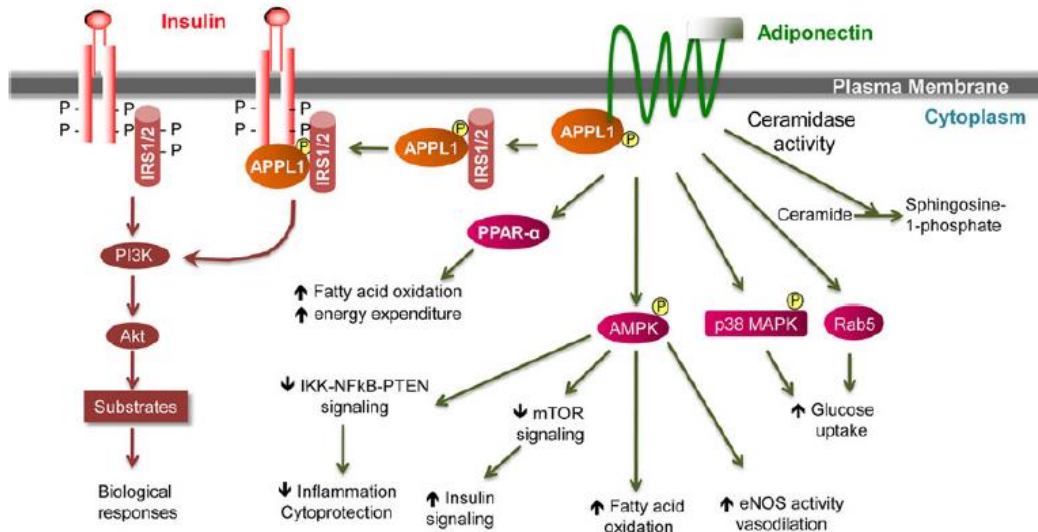
4B.

Figure 4. Adiponectin's effects on energy metabolism and insulin sensitivity.[100] Fig.4A. Intracellular pathways impacted by adiponectin binding to its specific membrane receptors. Fig. 4B. Effects of adiponectin to counteract causes for and consequences of insulin sensitivity.

Section 4: Current Treatments for EMS

There are two primary therapeutic objectives for EMS. The first and most important goal is to improve insulin regulation. Lower insulin concentrations overall will decrease risk for EL. The second objective is to decrease obesity, which improves insulin and lipid regulation, while decreasing the mechanical load on the laminae.[3] Management changes are critical to achieving long term success and cannot be overlooked. These primarily include dietary modifications and exercise; however long-term client compliance can be difficult to achieve, and some equids are resistant to these changes. A recent prospective study determined that there was no change in laminitis recurrence even when owners had implemented veterinary recommended management changes.[18] There are likely more management-resistant cases than previously realized, and these patients would benefit from therapeutic medications to help manage ID and mitigate risk of EL in conjunction with management.[103]

Dietary management primarily focuses on reducing digestible energy and decreasing intake of non-structural carbohydrates (NSC). These changes reduce daily caloric intake, body fat mass, post-prandial glycemic response, and accompanying post-prandial insulin responses.[104,105] Feed restriction largely focuses on eliminating excess concentrates, limiting forage intake to measured quantities based on desired body weight, and eliminating (or at least limiting) access to pasture grazing.[104,105] To ensure a lower glycemic response to forages, hay should be evaluated for NSC content, and a total NSC content of approximately 10% NSC on dry matter (DM) basis should be targeted.[3] This can be achieved through soaking hay in water for 1-2 hours to remove water soluble carbohydrates, recognizing that mold growth and

leaching of essential nutrients may occur.[104,106] Pasture access should ideally be eliminated during initial dietary management, although grazing muzzles can be used to help limit intakes when alternative housing is unavailable.[3,105] Pasture grazing should be avoided during times of high NSC and fructan content. These times are seasonally during spring/fall, after frosts, as well as during late afternoon and evenings.[107] It is important to use ration balancers to ensure that appropriate nutrient requirements are met, especially for protein, vitamins, and minerals that may not be present in adequate amounts when dietary restrictions and/or hay soaking are in place.[3,105] The lean EMS phenotype presents a challenge, where regional adiposity or no significant adiposity exists on a lean horse with documented ID. These horses require careful management to provide appropriate nutrients and digestible energy without exacerbating existing ID. Digestible fat sources and fiber sources for hind gut fermentation (such as beet pulp) can be especially useful for providing additional calories without raising the postprandial glycemic response.[3,104]

Exercise has also been a crucial intervention that expends calories and decreases body fat mass, thereby reducing the endocrine and inflammatory impact of excess adipose tissue, while independently increasing tissue insulin sensitivity.[104,105] There are many recommended protocols, however evidence has shown that exercise sessions (> 5 times per week) are required to see beneficial effects.[104,105] The recent ECEIM consensus statement on EMS provides a baseline exercise protocol to implement, using targeted heart rates achieved during walk and trot, as well as clinical experience to prescribe exercise protocols for special circumstances (e.g. during recovery from laminitis).[3] Implementing a consistent

exercise program with sufficient intensity to improve insulin sensitivity and achieve measurable, consistent weight loss can be difficult or even impossible if the horse is actively dealing with laminitis, or laminar integrity is questionable.[3] Exercise protocols can be tailored to the individual patient based on their current fitness level and laminar stability. It is important to coordinate exercise protocols in a safe, slow introduction to not cause further damage to the laminae. Researchers have described the successful use of tailored exercise protocols paired with dietary modifications and supportive podiatry measures (removing weight-bearing from the perimeter hoof wall and placing in supportive boots) for chronic cases of EL.[108]

Frequently, horses are resistant to management changes and remain overweight, or they achieve weight loss but maintain ID status and associated metabolic dysfunctions. These patients often benefit from pharmacologic aids to achieve appropriate weight loss when exercise is not safe or feasible, and attain more consistently improved insulin and metabolic regulation. Levothyroxine supplementation has been a useful adjunct therapy to temporarily increase metabolic rate and promote weight loss while the patient is unable to exercise due to laminitis or other health restrictions.[3,88] Its use has been relatively safe, without documented adverse effects at recommended dosing protocols, but its use is exclusively recommended for short term administration (less than 6 months).[109,110] Its secondary effects on improved insulin sensitivity are likely from decreased adiposity.[3,88] Metformin, a biguanide medication used to decrease blood glucose by inhibiting hepatic gluconeogenesis in humans, has been evaluated in horses and used clinically.[3,88] Despite initial promising results of decreased basal insulin concentrations

in ID equids, metformin has poor oral bioavailability in horses and inconsistent systemic effects on tissue insulin sensitivity.[111-114] Its proposed mechanism of action in horses is to directly reduce the absorption of NSC in the small intestine and thereby reducing post-prandial glycemic and insulin responses, and so its administration must be timed for 30-60 minutes prior to meal feeding.[3,115] It has had inconsistent clinical benefit in horses, however it is well tolerated and can help in some horses[88]. Efficacy in an individual case can be evaluated by performing a dynamic oral carbohydrate challenge test (OST or OGT) after administration of metformin, or measuring insulin concentrations at 2 and 4 hours post-prandially at baseline and after administration of metformin.[3] Pergolide, a dopamine agonist, has been useful in the management of clinical signs associated with PPID, and its use has been considered to commonly improve ID when diagnosed clinically along with PPID.[3,88] In a retrospective study comparing treatment modalities for PPID, the administration of pergolide in 20 clinical patients resulted in significant reduction in clinical laminitis, however this has not been repeated in larger studies.[116-118] At this time, there is no published evidence that pergolide administration improves ID in studies of PPID-affected equids, or decreases laminitis recurrence in PPID cases.[18,118] Unpublished reports of pergolide improving insulin sensitivity in cases of ID unrelated to PPID have been reported in the ECEIM consensus statement, however this requires further investigation before clinical implementation.[3] Currently, there are no consistently beneficial therapeutic options for management of ID in horses, and this is a significant need for the industry.

There are two potential medications being evaluated in active research for future use in EMS cases. Velagliflozin, a sodium glucose cotransporter 2 inhibitor,

decreases reuptake of glucose from glomerular filtrate and promotes wasting of glucose in the urine. Its use in human type 2 diabetes has been beneficial, however it has reported adverse effects of hypoglycemia and urinary tract infections.[119] When tested in ID ponies, velagliflozin administration for 3 weeks prior to high NSC diet challenge reduced the glycemic and insulin response to diet challenge, and no adverse effects were noted.[119] Importantly, velagliflozin reduced the onset of painful EL in response to 18 days of the high NSC diet challenge (14/37 controls developed laminitis whereas none of the 12 treated ponies developed laminitis).[119] One limitation was that this study did not take into account pre-existing chronic or subclinical laminitis present in the ID ponies selected for the study, and glucose was not measured in the urine. A follow-up study evaluated velagliflozin efficacy in ID ponies for 16 weeks duration, and similar reductions in insulin response to high NSC diet and lack of adverse effects were seen.[120] No changes were noted in insulin sensitivity between velaglifozin-treated ponies and placebo-treated ponies, as evaluated by CGIT performed at baseline, 8 and 16 weeks of drug administration, and at 4 weeks after discontinuing medications. The drug's efficacy was more likely associated with reduction of post-prandial hyperinsulinemia, as the treated ponies had significantly lower maximum insulin concentration during OGT at 8 and 16 weeks of drug administration, compared to control ponies.[120] Interestingly, there was no significant effect of drug treatment on the serum glucose concentrations after OGT.[120] Before implementation in clinical practice, further investigation into its efficacy in clinical patients (as opposed to controlled high NSC diet challenge), and thorough investigations for adverse effects should be pursued.

Another potential therapy under investigation is the inhibition of sweet taste receptors. These were recently evaluated for their role in the post-prandial insulin response in horses. The taste type 1 receptors 2 and 3 (T1R2/3) are located on the tongue as well as in the K and L enteroendocrine cells within the small intestine, and their role is in detection of NSC within digesta.[121] Triggering of these receptors is associated with pre-emptive insulin secretion with ingestion of NSC, as well as incretin release from the small intestine and heightened post-prandial insulin response.[121] Evaluation of two T1R2/3 inhibitor substances (lactisole and *Gymnema sylvestre*) was first performed with in vitro intestinal explants, to evaluate the effect of incubation with each inhibitor on the uptake of 2-deoxyglucose by the intestinal sample, as measured by gas chromatography-mass spectrometry.[121] After indicating reduction of glucose uptake in vitro, these two inhibitor substances were evaluated in vivo at three escalating doses each, to evaluate effects on the glucose and insulin response to OGT in a group of otherwise healthy ponies. They were well tolerated, but no effect was seen from lactisole.[121] There was a moderate reduction in the glucose and insulin responses to the OGT after single dose administration of *Gymnema sylvestre.*[121] While promising, this requires further investigation to better characterize its role in insulin response with continued administration, potential adverse effects, and clinical efficacy in reduction of ID and subsequent development of ID. Many nutraceuticals have been investigated but none have shown clinically significant benefit and cannot be recommended at this time.

Section 5: Thiazolidinediones

Thiazolidinediones (TZD) are a class of antidiabetic medications that improve insulin sensitivity in humans by stimulating the peroxisome-proliferator-activated receptor-γ (PPAR-γ) nuclear receptor within adipocytes. This results in upregulation of adiponectin transcription and secretion by adipocytes.[122] As previously described, increased adiponectin secretion is also associated with insulin sensitizing effects at multiple tissue types, improved lipid metabolism, anti-inflammatory effects and improved microvasculature functions.[100] An added benefit of TZD administration over other antidiabetic medications such as biguanides in humans is that TZD's have no effect on beta cell secretion of insulin, and therefore do not cause clinically significant hypoglycemia seen with other antidiabetic medications.[123] Members of the TZD class include troglitazone, rosiglitazone, and pioglitazone. All three medications have resulted in marked increase in adiponectin secretion and improved insulin sensitivity in murine models and type 2 diabetic human patients.[124,125]

Importantly, this class of medications has been associated with adverse effects in people including fluid retention, cardiovascular disease, increased bone fractures, and hepatotoxicity.[124,126] Troglitazone did not cause significant hepatotoxicity in initial clinical trials, however it was pulled from the market by the FDA in 2000 due to severe hepatotoxicity, primarily an idiosyncratic hepatic necrosis resulting in cirrhosis, hepatic failure, and death.[127,128] Rosiglitazone has been associated with increased risk of myocardial infarction and death, and its use has been restricted in the US and pulled from the market in several countries.[124,129]

Pioglitazone has been thoroughly evaluated for safety and shown to improve long term cardiovascular disease outcomes in multiple systematic reviews.[130-132] Its safety profile is considered much greater than the other members of the TZD class. Adverse effects include fluid retention (resulting in weight gain and congestive heart failure), osteoporosis, inconsistent risk of bladder neoplasia, and very rare hepatotoxicity, however the FDA considers the clinical benefits to outweigh these adverse effects.[123,124,126,131-133]

Pioglitazone binds to PPAR-γ, which results in proliferation of smaller, more insulin-sensitive adipocytes and increased production of adiponectin, both of which have powerful effects on improving insulin sensitivity.[134,135] Genetic polymorphism has been identified in the human PPAR-γ genes, and it has been suggested to play a role in altered pharmacokinetics and pharmacodynamics associated with pioglitazone in people.[136] This has not been evaluated in veterinary species. Pioglitazone consistently increases production of total and HMW adiponectin in healthy and insulin resistant humans, and improves long term cardiovascular health.[137,138] It has also been shown to selectively increase HMW adiponectin and the HMW adiponectin:total adiponectin ratio, resulting in improved hepatic insulin sensitivity in humans with ID.[139,140] Pioglitazone has also been evaluated for its anti-inflammatory functions, especially its antagonism against TNF-α mediated inflammatory cascades, which are considered a secondary benefit in management of human metabolic syndrome due to the secretion of TNF-α from adipose-embedded macrophages in obese states.[100,123,141]

Pioglitazone has been evaluated in several veterinary species. In dogs, it has been evaluated in models of type 2 diabetes, where insulin sensitivity was markedly improved, and in models of experimentally induced osteoarthritis, where inflammatory markers and cartilaginous lesions were reduced in a dose-dependent manner.[142,143] No hepatotoxicity was reported in these studies. In a prospective pharmacodynamic study in obese cats, pioglitazone was well tolerated and significantly improved insulin sensitivity and serum triglyceride concentrations.[144] Thiazolidinediones have also been evaluated in ruminants for beneficial effects on peripartum glucose and insulin dynamics, and on lipid metabolism and marbling within muscle.[145-147] Pharmacokinetics of pioglitazone have been established in sheep, although pharmacodynamics were not evaluated.[148]

Pioglitazone had been proposed in horses as a potential insulin sensitizing and anti-inflammatory agent, and it has been evaluated in three equine studies at this time. Much of the knowledge on PPAR-γ in horses comes from its role in transcription of adipocytes in stem cell research, however it is considered to have similar functions as in human adipocytes.[149] An initial pharmacodynamic study was performed to evaluate multiple oral dosing in horses.[150] In this study, a dose of 1 mg/kg per os every 24 hours was empirically derived from experimental studies in rodents, swine, monkeys, and humans. It was initially evaluated over a 5 day course in a single fasted horse with intragastric administration. This was well tolerated, and pharmacokinetics were favorable. This was followed by an 11-day course of the same dose,

administered by oral syringe in six non-fasted horses.[150] Pharmacokinetic analysis revealed it to be well tolerated and orally absorbed, with no significant difference between day 1 and day 11 pharmacokinetic parameters. True bioavailability could not be calculated as no intravenous drug administration was performed in comparison to oral administration. Peak drug concentrations and the area under the curve of plasma concentrations in the horses were lower than therapeutic levels in humans, suggesting lower absorption, faster elimination, or higher volume of distribution in horses compared to humans. Plasma drug concentrations showed day-to-day variability between and within individuals, likely associated with non-fasting state and variations in gastric fill and protein binding of the drug. The authors suggested that further investigation in horses should use higher doses or increased frequency of administration to achieve higher plasma concentrations, although there was no established therapeutic concentration in horses.[150]

Initial pharmacodynamic research for pioglitazone in horses evaluated its role in insulin sensitization and inflammation. The effects of 12 days of pioglitazone administration (1 mg/kg per os every 24 hours) prior to a lipopolysaccharide-induced model of acute insulin resistance in horses was evaluated.[151] It did not show clinical benefit for improving insulin sensitivity, evaluated by frequently sampled intravenous glucose tolerance test, prior to LPS administration or after LPS-induced insulin resistance. Pioglitazone administration did not significantly alter protein expression of insulin receptors or glucose cotransporters, although mild changes in RNA transcription were noted and the authors suggested greater changes may have been seen over longer duration or higher dosing of administration.[151] In another study from

the same research group, pioglitazone administration for 14 days prior to LPS administration did not have a statistically significant impact on circulating inflammatory cytokines, clinical parameters, hematologic abnormalities, or adipose tissue inflammatory markers.[152] Again, the authors suggested that the 1 mg/kg per os every 24 hours dosing may not have achieved clinically effective concentrations. Additionally, this was not an ideal analogue for the chronic insulin dysregulation seen with EMS and PPID, and the authors recommended evaluation with a more clinically appropriate model. Triglyceride concentrations and leptin transcript abundance in adipose tissue did not change after 12 days of pioglitazone treatment in healthy horses.[151,152] However, peak concentrations of pioglitazone in these studies were lower than therapeutic doses in humans, indicating an increased dose may be necessary. Pioglitazone's effect on circulating adiponectin concentrations or chronic insulin dysregulation has not been investigated in horses.

Section 6: Justification of the Study

Endocrinopathic laminitis, caused by any combination of EMS, PPID, and/or pasture associated laminitis, is ultimately driven by insulin dysregulation. Primary treatment goals to mitigate the risk of EL are focused on management changes, primarily diet and exercise, to reduce obesity and improve insulin regulation. However, long term outcomes have not been as promising, with clinical experience showing challenges with owner compliance, as well as recent studies showing that recurrence of EL was unchanged despite implementation of these management changes.

Pharmaceutical management of EL and EMS has been investigated to augment current management practices, as well as provide therapeutic options for treatment-resistant ID. Adipokines, specifically adiponectin, are promising therapeutic targets to improve insulin sensitivity and adipose dysfunction in the lean EMS phenotype. Pioglitazone has been shown to be bioavailable and well-tolerated in horses, however initial studies utilized an acute lipopolysaccharide induction model and a sub-therapeutic dose. Higher dosing and longer duration of administration was needed for pilot study to evaluate effect on tissue insulin sensitivity in a more chronic ID model. This pilot study aimed to evaluate the effects of pioglitazone administration (2mg/kg PO q24 hr X 28 days) on obesity, adiponectin and leptin concentrations, liver enzymes and function, triglycerides, and insulin and glucose responses to oral sugar.

Chapter 2 – Statement of Hypotheses and Objectives

Specific Aim 1: Evaluate pharmacodynamic effects of oral pioglitazone in horses

A. Hypothesis Aim 1: Orally administered pioglitazone (2 mg/kg q24 hours for 28 days) in clinically normal horses and ponies will result in significant decreases in morphometric measures of obesity, decreased leptin concentrations, increased adiponectin concentrations, and decreased insulin response to oral sugar.

B. Objectives Aim 1:

   a. Evaluate effect of pioglitazone administration on morphometric measures relevant to the EMS phenotype, namely BCS and CNS.

   b. Evaluate effect of pioglitazone administration on leptin and adiponectin concentrations.

   c. Evaluate effect of pioglitazone administration on the glucose and insulin response to oral sugar through the oral sugar test.

Specific Aim 2: Evaluate safety of oral pioglitazone in horses

A. Hypothesis Aim 2: Orally administered pioglitazone (2 mg/kg q24 hours for 28 days) in clinically normal horses will result in no significant adverse effects.

B. Objectives Aim 2:

   a. To describe adverse effects observed in study population during the course of 28 days of daily administration of the medication.

   b. Evaluate effects of pioglitazone administration on hepatic enzyme activity and hepatic function.

Chapter 3 – Publication

Introduction

Several risk factors have been identified for the development of endocrinopathic laminitis (EL) in horses with equine metabolic syndrome (EMS). In several studies, strong predictive value for laminitis was found with elevated insulin concentrations and low adiponectin concentrations, but measures of obesity and leptin had no predictive value.[20,28,37,74] Correcting insulin dysregulation (ID) is an important therapeutic goal, and adiponectin has an important role in increasing insulin sensitivity.

Current therapeutic guidelines for EMS focus primarily on management changes, specifically diet and exercise, however pharmacologic interventions may be required in refractory cases. Metformin has been employed in clinical practice, however it has shown inconsistent benefits.[111-113] Pioglitazone has been utilized in management of human ID, acting as an agonist for the peroxisome proliferator activated receptor γ (PPAR-γ), which results in proliferation of smaller, more insulin-sensitive adipocytes and increased production of adiponectin, both of which have powerful effects on improving insulin sensitivity.[134,135] Pioglitazone consistently increases production of total and HMW adiponectin in healthy and insulin resistant humans, and improves long term vascular health.[137,138] Pharmacokinetic analysis of pioglitazone of 1 mg/kg orally every 24 hours for 11 days in healthy horses resulted in plasma concentrations slightly below therapeutic concentrations in people.[150] Prior investigations into the use of pioglitazone in horses have used a model of acute, transient ID, and peak concentrations of pioglitazone using the 1 mg/kg dose were

lower than therapeutic concentrations in humans, indicating an increased dose and more chronic ID model may be necessary.[150,151]

The objectives of this study were to assess the pharmacodynamic effects of oral pioglitazone in healthy equids on (1) morphometric parameters; (2) plasma concentrations of high molecular weight adiponectin and leptin; and (3) enteroinsular response to oral carbohydrate challenge, as measured by oral sugar tests. An additional objective was to document any adverse effects from administration of a higher dose for longer duration than previously studied.

Materials and Methods

Animals and Criteria

The study population consisted of fifteen adult equids (7 horses, 8 ponies) from the teaching herd of the Auburn University Large Animal Teaching Hospital. The horses were all geldings, whose average body weight was 610 kg (range, 508 – 667 kg) and age was 12.6 years (range, 7 – 17 years), and consisted of a mixture of breeds (2 Warmbloods, 1 Arabian, 1 Tennessee Walking Horse, 1 Thoroughbred, and 2 Draft crosses). The mixed breed ponies consisted of 7 geldings and 1 mare, and the average weight and age were 185 kg (range, 101 – 249 kg) and 13.8 years (range, 5 – 22 years), respectively. Complete blood count and serum biochemistry profile evaluated at baseline were normal. Endogenous plasma ACTH concentrations were measured by a commercial, validated immunoassay (Immulite 1000, Siemens Healthcare Diagnostics, Cornell University Animal Health Diagnostic Center). All animal research was performed in the summer months (June and July) in the

Southeast United States. Subjects were housed as groups in paddocks with sparse grass, and with free choice access to coastal Bermuda grass hay, water, and mineral blocks. They received one concentrate meal per day at 6:00 – 6:30 AM, which consisted of 1.4 kg per horse and 0.23 kg per pony of a 12% protein, 5% fat, and 25% non-structural carbohydrate pellet. This study was approved by the Institutional Animal Care and Use Committee of Auburn University.

Drug Administration

Each subject received 28 consecutive days of oral pioglitazone administration at the same time each morning (6:00 – 6:30 AM), starting 24 hours after the initial oral sugar test, and the final administration was 24 hours prior to the final oral sugar test. Pioglitazone tablets (45 mg tablets, TEVA Pharmaceuticals, North Wales, PA, USA) were dosed at 2 mg/kg, crushed and dissolved in 30 mL water. Medications were administered per os to all subjects within 20 minutes of dissolution in water. Drug was administered in conjunction with concentrate feeding in all subjects to facilitate handling.

Outcome Assessments

A schematic of the overall experimental protocol is shown in Figure 5.
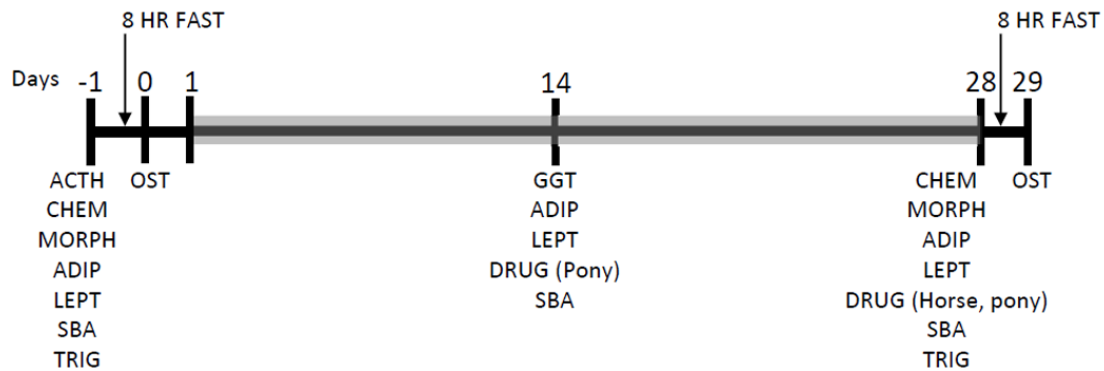
Figure 5. Schematic of the overall experimental protocol for drug administration and outcomes assessment. The grey bar indicates the pioglitazone administration period at 2 mg/kg q24h.

Abbreviations: CHEM, serum biochemistry profile (which included SDH and GGT); MORPH, morphometric measurement; ADIP, HMW adiponectin concentration; LEPT, leptin concentration; SBA, serum bile acid concentration; TRIG, triglyceride concentration; OST, oral sugar test; GGT, gamma glutamyl transferase activity; SDH, sorbitol dehydrogenase activity; DRUG, pioglitazone concentration.

Morphometric Evaluation

All subjects were evaluated for morphometric parameters on day -1 and 28. Body weights were measured with a calibrated scale utilized for large animal clinical patients. Body condition scores (BCS) and cresty neck scores (CNS) were assigned by experienced evaluators (RML and AAW). Investigators were not blinded, but did not re-examine the baseline scores prior to performing the final evaluations. Body condition scores (BCS) were assigned on a scale of 1-9, according to the Henneke body condition scoring criteria for horses.[33] Cresty neck scores were assigned on a scale of 1 – 5, according to a standardized scoring system.[35]

Hepatic Enzymes and Function

Hepatic enzyme activity and hepatic function were assessed on day -1, 14, and 28, in a non-fasted state. Blood samples were acquired by jugular venipuncture into vacutainer blood tubes (lithium heparin anticoagulant used for plasma samples and tubes with no additives for serum samples), and samples were submitted for analysis within 15 minutes of sampling to the Clinical Pathology Laboratory at Auburn University College of Veterinary Medicine. On days -1, 14, and 28, measured parameters were serum bile acid concentrations, plasma gamma glutamyl transferase (GGT) activities and plasma sorbitol dehydrogenase (SDH) activities. On days -1 and 28, plasma triglyceride concentrations were measured. All parameters were measured with an automated chemistry analyzer (Cobas 4000 Analyzer, Roche Diagnostics, Indianapolis, IN).

Leptin Measurement

Serum leptin concentrations were measured on day -1, 14, and 28 in a non-fasted state. Blood was sampled by jugular venipuncture into vacutainer tubes with no additives and allowed to clot at room temperature for 30 minutes. Serum was collected after centrifugation and immediately frozen at -80 C. Samples were shipped and analyzed as a batch using a commercial radioimmunoassay at the Cornell University Animal Health Diagnostic Center.

High Molecular Weight Adiponectin Measurement

Serum HMW adiponectin concentrations were measured on day -1, 14, and 28 in a non-fasted state. Blood was allowed to clot at room temperature for 30 minutes, and serum was collected after centrifugation and immediately frozen at -80°C. Samples were batch analyzed in duplicate with a human sandwich ELISA assay (EMD Millipore Corporation, St. Louis, MO) that has been validated for measurement of HMW adiponectin in horses.[75] The assay was performed according to the manufacturer's instructions with a few modifications as previously described.[75] Linearity under dilution for pooled equine serum had an R2 value of 0.95, and the intraassay coefficient of variation was 11.4%.

Oral Sugar Tests

Oral sugar tests were performed on all subjects on day 0 and day 28 in a fasted state, according to established methodology.[49] All subjects were removed from pasture and placed in stalls with free choice Bermuda grass hay at 5 PM on

the evening prior to OST, and all feed was removed at 10 PM on the evening prior to OST. Free choice water access was maintained until the start of the OST. Jugular venous catheters (14 gauge) were placed prior to the start of the OST. Some subjects required sedation with alpha-2 agonists (xylazine 0.4 mg/kg IV, or detomidine 0.01 mg/kg IV) to facilitate catheter placement, which was performed 12 hours prior to starting the OST to minimize any potential residual effects on the OST results. Baseline sampling was performed within 5 minutes prior to the oral administration of Karo® light corn syrup, dosed at 0.15 mL/kg body weight. Blood was subsequently sampled via the catheter (after removal of 10 mL of waste blood) at 30, 60, 75, 90, and 120 minutes after oral sugar administration. After conclusion of the OST, jugular catheters were removed and all subjects were immediately returned to the paddocks, where feeding protocols were resumed.

Whole blood was immediately analyzed for blood glucose with a validated hand-held point-of-care glucometer (Alphatrak 2, Abbott Pharmaceuticals, Parsippany, NJ) and then placed into untreated vacutainer blood tubes and allowed to clot on ice until the completion of the OST.[153] Serum was separated after centrifugation and immediately frozen at -80°C. Serum samples were batch analyzed for insulin concentrations by a commercial radioimmunoassay at the Cornell University Animal Health Diagnostic Center.

Serum Pioglitazone Concentrations

Convenience samples of stored plasma from previous sampling time points for biochemical parameters were analyzed retrospectively for plasma drug concentrations. For the ponies, this occurred at 6 hours post drug administration on day 14 and at 9 hours post drug administration on day 28. For the horses, samples were collected on day 14 and at 7 hours post drug administration on day 28. Data for the horses on day 14 were not included as the time of blood collection relative to drug administration was unclear.

Plasma concentrations of pioglitazone were measured by ultra-high performance liquid chromatography with tandem mass spectrometry (UPLIC-MS/MS). Pioglitazone hydrochloride reference standard was purchased from Fisher Scientific. Rosiglitazone maleate (3 ng/mL) was used as the internal standard (IS) and was purchased from Cayman Chemical. Reference and internal standards were prepared in 60:40 (v / v) of acetonitrile (ACN) / water (H_2O). Samples were prepared by combining 100 µL of plasma, 100 µL internal standard (3 ng / mL) and 300 µL of ACN in 1.8 -mL microcentrifuge tubes. Each sample was vortexed for 5 minutes before being centrifuged at 16,100 x g for 5 minutes. The resulting supernatant solution (250 µL) was diluted 1:1 by adding 2% (v/v) aqueous formic acid, vortexing to mix, then centrifuging at 16,100 x g for 10 minutes.

Samples were separated using a Waters H-Class ultra-performance liquid chromatography (UPLC) system with a C18 column (Waters Acquity UPLC HSS T3,

100 mm length x 2.1 mm ID x 1.8 µm) and matching guard column (Waters

Acquity UPLC HSS T3 VanGuard Pre-Column, 5 mm length x 2.1 mm ID x 1.8 µm).

Five microliters of sample was injected using an autosampler maintained at 8 °C.

Mobile phase A consisted of 1% (v/v) aqueous formic acid, and mobile phase B

consisted of 1% (v / v) formic acid in ACN. The gradient elution program was as

follows: time 0: 80 / 20% A/ B; time 2.5 min: 2 / 98% A/ B; time 3.5 min: 2 / 98%

A/ B; time 3.51 min: 80 / 20% A/ B; time 5.25 min: 80 / 20% A/ B. Flow rate was

0.4 mL/min. Drug was detected using a triple-quadrupole mass spectrometer

(Waters Xevo TQD) operated in positive-ion mode using multiple reaction

monitoring (MRM). The specific tuning parameters for each analyte, the parent and

product ion transitions used in the MRM mode analysis, and the mass spectrometer

parameters used for the detection of pioglitazone are shown in Appendix 1.

Calibration curves were linear (R^2 > 0.99) within a range of 1–200 ng/mL.

Calibration curves were used to determine pioglitazone concentration in samples

based on the sample / IS ratio. Pioglitazone concentrations above the range of the

calibration curve were diluted 1:10 from the original sample extract with 30:70:1

ACN/H2O/FA (v/v/v). The coefficient of determination (R^2) for all curves was

>0.99, and all standard values were within ±12% of the expected range. Using this

method, the limit of detection (LOD) was 0.4 ng / mL, as determined by the signal-

to-noise ratio. The limit of quantification (LOQ) was 1 ng/mL, determined as the

lowest concentration found to be linear on the calibration curve.

Data analysis

Sample size was determined using G*Power (Version 3.1.9.2) for power analysis, based on published data from pioglitazone's effect on total and high molecular weight adiponectin concentrations in healthy human male population.[138] Power analysis was calculated for two-tailed difference between two independent means (baseline vs. post pioglitazone treatment effects on adiponectin), at significance level (alpha) of 0.05, power (beta) of 0.95, and effect size of 4.5.[138] Power analysis for this data showed sample size of 6 based on human data, however a population of 7 horses and 8 ponies was used to provide sufficient sample size within breed group, and maximize power if the equine response to pioglitazone was not as marked as the results seen in this human study.

All subjects served as their own controls. The horses and ponies were analyzed as separate groups. Within the population, equids were identified as insulin dysregulated, based on the results of baseline oral sugar test, and this cohort was analyzed as a sub-group. Subjects were considered insulin dysregulated if serum insulin concentration was greater than 65 μU/mL at 60 minutes or later after Karo® syrup administration during the initial oral sugar test.[47,49]

All data were evaluated for normality by the Shapiro-Wilk test and presented as mean ± SD or median (range), as appropriate. The Wilcoxon rank sums test was used for categorical data (BCS, CNS). Body weight, enzyme activities, serum bile acids, and triglycerides were analyzed by either a paired t-test or repeated measures

ANOVA, depending on the number of time points. HMW Adiponectin and leptin were analyzed with a 2-way repeated measures ANOVA with Sidak's multiple comparison test for the interactions between horse vs. pony and study time point (baseline, midpoint, and final). Leptin and adiponectin in ID versus non-ID groups for all time points were compared using an unpaired t-test. Glucose data (normally distributed) and insulin data (natural log transformed) from the OSTs were analyzed by 2-way repeated measures ANOVA with Sidak's multiple comparison test to compare time points within the OST and baseline versus final study time points. Area under the curve (AUC) for insulin OST curves at baseline and final time points were calculated by the trapezoidal method, and compared using a paired t-test. A commercial software package (Graphpad Prism 6, La Jolla, CA) was used for all statistical analysis, and a P value < 0.05 was considered statistically significant.

Results

All horses tolerated the medication well, and no adverse effects were noted. One pony developed colic signs associated with presumptive ileal impaction the night before the final OST, which resolved with conservative medical therapy. The final OST was not performed on this subject because of stress and analgesic administration, so that pony's OST data were excluded from baseline versus final OST comparisons. All horses and ponies returned to their herds at the completion of the study. Results are summarized in Tables 1 and 2.

Table 1. Demographic and outcome data for the horse and pony groups at the Baseline (day -1 or 0), Midpoint (day 14), and Final (day 28 or 29) timepoints. All continuous data noted as mean ± standard deviation, and all ordinal data (BCS, CNS) represented as median (range).

Time point	Horses (n=7)				Pony (n=8)				P-value, Horse vs. Pony
	Base line	Mid point	Final	*P*-value, Baseline vs. Final	Base line	Mid point	Final	*P*-value, Baseline vs. Final	
Age (yrs)	14.0 ± 3.6	-	-	-	14.8 ± 5.1	-	-	-	0.3962
Sex	7 G	-	-	-	7 G, 1 F	-	-	-	-
BCS (1-9)	8 (5-8)	-	6.5 (4-8.5)	0.1003	6 (4.5-8)	-	6.5 (4-8.5)	0.1003	-
CNS (1-5)	4 (2-4)	-	3 (1-3.5)	*0.0156*	4 (2-5)	-	3.75 (1.5-5)	0.0898	-
BW (kg)	610 ± 56	-	604 ± 55	0.3068	184 ± 45	-	186 ± 45	0.3068	-
ACTH (pg/mL)	30.0 ± 29.0	-	-	-	32.5 ± 16.0	-	-	-	0.8267
[Pioglit] (ng/mL)	-	-	87.2 ± 75.0 (7 hr)	-	-	399.5 ± 351.8 (6 hr)	135.6 ± 101.1 (9 hr)	-	-
Insulin 0 min (µU/mL)	13.2 ± 7.7	-	13.9 ± 4.57	-	15.0 ± 7.7	-	15.6 ± 6.8	-	-
Insulin 60 min (µU/mL)	39.4 ± 25.1	-	35.8 ± 19.9	-	67.5 ± 47.6	-	70.4 ± 52.4	-	-
Insulin AUC	3692 ± 2410	-	3788 ± 1938	0.3132	8037 ± 4610	-	5989 ± 3894	*0.0316*	*0.0604*
SDH (U/L)	1.7 ± 1.4	-	5.2 ± 2.1	*0.0066*	2.6 ± 1.0	-	5.5 ± 3.4	*0.0288*	0.1739
GGT (U/L)	11.7 ± 3.0	11.1 ± 2.4	10.7 ± 2.3	0.1563	18.3 ± 6.9	16.0 ± 4.2	14.3 ± 2.9	*0.0477*	*0.0303*
SBA (µmol/L)	4.1 ± 1.3	4.4 ± 1.3	5.1 ± 1.8	0.9713	6.4 ± 3.1	5.9 ± 2.3	5.3 ± 2.4	0.9713	0.1785
TRIG (mg/dL)	39.3 ± 18.3	-	36.1 ± 8.0	0.9698	39.8 ± 12.5	-	39.5 ± 13.8	0.6568	0.9546
Leptin (ng/mL)	17.6 ± 16.5	15.8 ± 13.8	20.1 ± 17.0	0.2177	18.4 ± 12.9	16.1 ± 11.1	20.6 ± 16.7	0.2177	0.2966
HMW Adipo (µg/mL)	2.5 ± 1.0	3.5 ± 1.5	4.9 ± 2.9	*< 0.01*	1.6 ± 2.6	2.4 ± 3.2	3.3 ± 4.4	*< 0.05*	0.4057

Abbreviations: G, gelding; F, intact female; BCS, body condition score; CNS, cresty neck score; BW, body weight; SDH, Sorbitol dehydrogenase; GGT, Gamma glutamyl transferase; SBA, serum bile acids; TRIG, triglyceride; adipo, adiponectin; [pioglit], pioglitazone concentrations at timepoint post administration

Table 2. Demographic and outcome data for the ID and Non-ID groups at the

Baseline (day -1 or 0), Midpoint (day 14), and Endpoint (day 28 or 29) timepoints.

All continuous data noted as mean ± standard deviation, and all ordinal data (BCS,

CNS) represented as median (range).

Timepoint	Insulin Dysregulated (n=7)			Non-Insulin Dysregulated (n=8)			*P*-value, Baseline ID vs. Non-ID
	Baseline	Final	*P*-value, Baseline vs. Final	Baseline	Final	*P*-value, Baseline vs. Final	
Age (yrs)	14.1 ± 2.9	-	-	14.6 ± 5.5	-	-	0.6900
Sex	6G, 1F	-	-	8 G	-	-	-
Type	4P, 3H	-	-	4P, 4H	-	-	-
BW (kg)	361 ±201	358 ±199	0.1671	403 ± 257	400 ± 251	0.5505	0.7342
BCS (1-9)	8 (6-8)	7 (5.5-8.5)	0.5000	5.5 (4.5-8)	4.75 (4-8.5)	0.5156	*0.0137*
CNS (1-5)		3.5 (3.5-4)	0.3750	3 (2-5)	1.75 (1-5)	*0.0313*	0.1282
ACTH (pg/mL)	41.0 ± 29.2	-	-	22.8 ± 7.8	-	-	0.1613
Insulin 0 min (µU/mL)	20.9 ± 4.8	18.8 ± 5.0	-	7.8 ± 2.5	10.6 ± 2.1	-	-
Insulin 60 min (µU/mL)	84.9 ± 39.2	74.7 ± 46.5	-	28.2 ± 13.7	31.5 ± 24.3	-	-
Insulin AUC	8984.9 ± 3723.1	6604.4 ± 3479.8	*0.0119*	3013.7 ± 1445.8	3172.9 ± 1664.4	0.2557	*0.0019*
SDH (U/L)	2.0 ± 1.2	5.6 ± 3.2	*0.0189*	2.3 ± 1.3	5.1 ± 2.5	*0.0112*	-
GGT (U/L)	16.6 ± 4.7	13.7 ± 2.7	*0.0465*	14.0 ± 7.1	11.6 ± 3.3	0.1017	-
SBA (µmol/L)	4.8 ± 1.3	5.8 ± 2.2	0.9713	5.8 ± 3.5	4.7 ± 2.0	0.9713	-
Trig (mg/dL)	46.3 ± 15.2	40.4 ± 7.9	0.4087	33.6 ± 12.6	35.8 ± 13.6	0.7480	-
Leptin (ng/mL)	27.7 ± 14.2	30.2 ± 18.3	0.2177	9.50 ± 6.90	11.7 ± 7.7	0.2177	*<0.0001*
HMW Adipo (µg/mL)	1.0 ± 0.8	2.3 ± 1.8	*0.0272*	2.9 ± 2.4	5.6 ± 4.4	*0.0186*	*0.0039*

Abbreviations: G, gelding; F, intact female; P, pony; H, horse; BCS, body condition score; CNS, cresty neck score; BW, body weight; SDH, Sorbitol dehydrogenase; GGT, Gamma glutamyl transferase; SBA, serum bile acids; Trig, triglyceride; HMW adipo, high molecular weight adiponectin;

Endocrine Status and Morphometric Data

No lameness at a walk or divergent growth rings on the hooves were present in any subjects at any time during the study. Of the horses, 4/7 were considered obese by BCS ≥ 7/9, and 4/7 had evidence of regional adiposity with CNS = 4 at baseline. Two subjects (one horse and one pony) had endogenous ACTH concentrations greater than or equal to 50 pg/mL, indicative of pars pituitary intermedia dysfunction (PPID, Table 1). Both had some clinical signs of PPID including long guard hairs under the mandible and regional adiposity (horse), and hypertrichosis and regional adiposity (pony). The endogenous ACTH concentration in the horse was 95 pg/mL (age, 17 years) and 63 pg/mL in the pony (age, 15 years). An ID group was identified based on the results of baseline OST, consisting of 3/7 horses and 4/8 ponies. The two equids with PPID were also in the ID group (Table 2). The morphometric characteristics (BCS, CNS, etc.) of all groups are presented in Table 2. No significant differences were observed between baseline (day -1) and final (day 28) body weights or BCS in all groups (ponies, horses, ID, and non-ID animals) (Tables 1 and 2). For ID versus non-ID equids, there were no significant differences between baseline values for body weight (P=0.7342) or CNS (P=0.1282), but baseline BCS (P=0.0137) was significantly higher in ID equids (Table 2). In horses and non-ID animals, final CNS was significantly lower than baseline CNS (P= 0.015 and 0.0313, respectively).

Hepatic Enzymes and Function

Hepatic enzyme activities (GGT and SDH), and serum bile acid concentrations remained within normal limits in all subjects throughout the study (Table 1 and 2).

In all groups evaluated, a statistically significant increase in SDH activity was present after pioglitazone treatment (Table 1 and 2). A statistically significant decrease in GGT activity was observed after pioglitazone treatment in ponies and the ID equids. No significant differences were observed in triglyceride concentrations when compared between baseline (day -1) and final (day 28) in ponies, horses, or ID equids (Table 1 and 2).

Adipokines

No significant differences were observed in leptin concentrations between horses and ponies (P=0.2966), between study time points (days -1, 14, and 28; p=0.9713), or the interaction between these parameters (P=0.9999) (Table 1). When all time points and subjects were combined, the leptin concentrations for the ID group (27.64 ± 14.13 ng/ml) were significantly higher than the non-ID group (9.76 ± 6.8 ng/ml) (P<0.0001).

No significant differences were observed in HMW adiponectin concentrations between horses and ponies (P=0.4057). Study time point was significant (P=0.0002), with both ponies and horses having higher HMW adiponectin concentrations at the final timepoint versus their own baseline (Table 1, Figure 6). When all time points and subjects were combined, the HMW adiponectin concentrations for the ID group (1.708 +/- 1.423 µg/ml) were significantly lower than the non-ID horses (4.145 +/- 3.406 µg/ml) (P=0.0039). HMW adiponectin concentrations were also higher than baseline at final time point in the ID group (P= 0.0272) and in the non-ID group (P=0.0186) (Table 2, Figure 6).
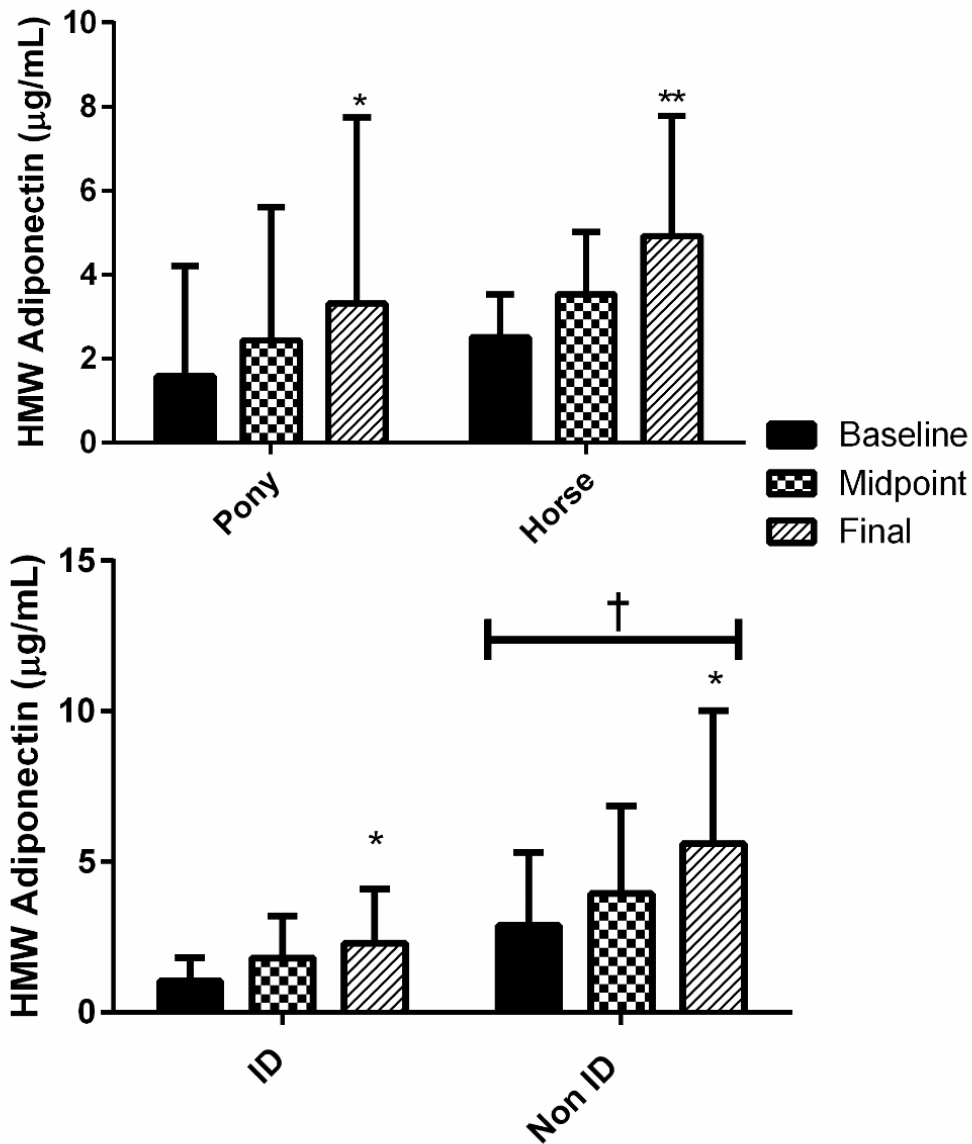
Figure 6. HMW Adiponectin concentrations measured at day -1 (Baseline, black bar), 14 (Midpoint, checkered bar), and day 28 (Final, slash bar) after pioglitazone administration. Significant differences denoted by asterisk. Fig 6a. HMW adiponectin was significantly increased between final timepoint and baseline in pony group and in horse group, *P<0.05, **P<0.01. Fig 6b. †Indicates HMW adiponectin was significantly higher in the non-ID group compared to the ID group overall, P<0.01. *Indicates that HMW adiponectin concentrations were significantly increased between final timepoint and baseline in ID group and in the non-ID group, P<0.05.

Oral Sugar Tests

No significant differences between study baseline and final time points were observed in the glucose response to oral sugar for ponies (P=0.3699), horses (P=0.5304), or the ID group (P=0.1774) (Figure 7). There were no significant differences in the insulin response to oral sugar between horses and ponies (P=0.4990). AUC for the insulin responses were also not different between horses and ponies (P=0.0604). In the horses, no significant differences were noted in the insulin response to oral sugar for baseline versus final at any time point in the overall model (P=0.5340) or for AUC (P=0.3132) (Figure 8). In the ponies, there was a significant interaction between insulin response at time points after sugar and in the overall study time point (baseline versus final, P=0.0096). Post-hoc comparison revealed insulin values at two OST time points [90 minutes (P<0.01) and 120 minutes (P<0.001)] were significantly lower at the final OST compared to the baseline OST in the pony group. Insulin AUCs for the ponies were significantly lower in the final curves compared with the baseline curves (P=0.0316). In the ID group, there was a significant interaction between insulin response at time points after sugar and in the overall study time point (baseline versus final) (P=0. 0109). Post-hoc comparison revealed insulin values at two OST time points [90 minutes (P<0.001) and 120 minutes (P<0.0001)] were significantly lower at the final OST compared to the baseline OST in the ID group. Insulin AUCs for the ID group were significantly lower in the final curves compared with the baseline curves (P=0.0119). In the non-ID group, there was no difference in the AUC for the insulin OST curves between baseline and final time point (P=0.2557). The insulin response to oral sugar improved in all ID equids tested. Based on the previously mentioned criteria, the insulin

response to oral sugar in 2 of the 4 ID ponies and 1 of the 3 ID horses was no longer classified as ID after pioglitazone treatment.

Serum Drug Concentrations

On day 14, plasma samples from ponies collected 6-7 hours after drug administration had drug concentration of 399.49 +/- 351.78 ng/mL. The samples from horses were not measured due to a sample handling issue.
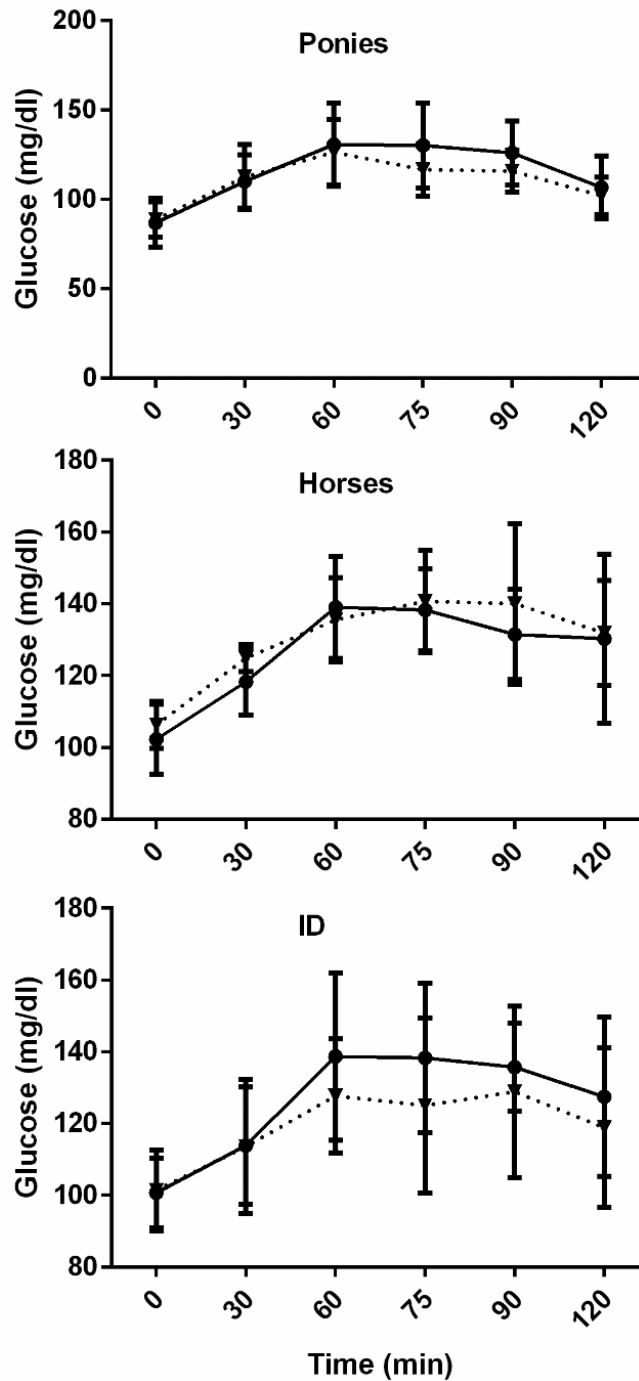
Figure 7. Blood glucose concentrations measured during the OST at day 0 (Baseline, black bars) and day 29 (Final, gray bars) in horses, ponies, and the insulin dysregulated (ID) group. Significant differences were observed over the time-course of the OST, however no significant differences were seen between baseline and final OST after pioglitazone treatment.
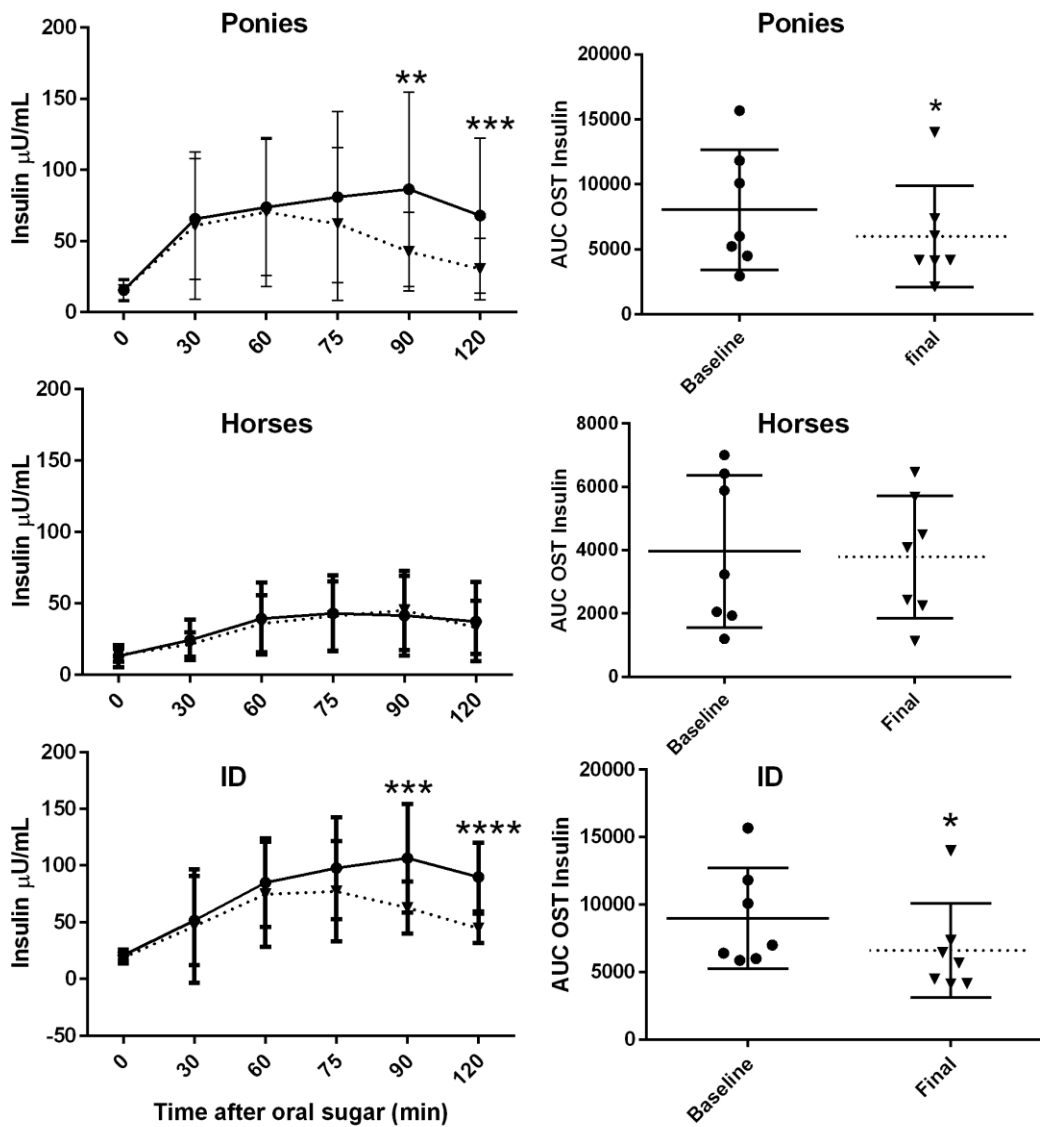
Figure 8. Blood insulin concentrations measured during the OST at day 0 (Baseline, black bars and black circles) and day 29 (Final, gray bars and gray squares) in ponies, horses, and the insulin dysregulated group. Significant differences denoted by asterisks. In all groups, there was a time-course difference in the insulin concentration over the OST sampling period. Fig 8a & 8d. In the pony group, significantly lower insulin concentrations were observed at 90 and 120 minutes of the OST after pioglitazone therapy, $**P<0.01$, $***P<0.001$. The AUC of the insulin curves were also significantly lower in the pony group after pioglitazone therapy, $*P<0.05$. Fig 8b & 8e. In the horse group, no significant differences were observed in response to pioglitazone therapy, at individual time points during OST or in the AUC of the insulin curve. Fig 8c & 8f. In the ID group, significantly lower insulin concentrations were observed at 90 and 120 minutes of the OST after pioglitazone therapy, $***P<0.001$, $****P<0.0001$ The AUC of the insulin curves were also significantly lower in the ID group after pioglitazone therapy, $*P<0.05$.

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Discussion

This pilot study represents the first evidence in equids for pioglitazone affecting plasma adiponectin concentrations and the dynamic insulin response to oral sugar, both of which have been identified as important predictive factors for the development of endocrinopathic laminitis[20,28,37,74]

Current therapeutic options for ID in the horse are limited for long-term efficacy for improving ID and reducing the risk of EL. Strict, life-long management to reduce caloric intake and non-structural carbohydrate content within the feeds and increase exercise remain the most consistently recommended strategies for addressing EMS and ID; however some cases are refractory to these changes or cannot be exercised due to lameness.[2] Short-term supplementation of thyroxine is often used as an adjunct therapy to increase basal metabolic rate and reduce body fat mass, especially when laminitis precludes safe exercise regimens.[2] Velagliflozin, an inhibitor of sodium-glucose cotransporter 2, promotes renal wasting of glucose and has been shown to lower the glucose and insulin response to enteric carbohydrates, and reduced the incidence of EL in ponies experimentally challenged with a high carbohydrate diet.[119] Metformin has been utilized in many clinical cases, however its limited oral absorption and varied response in horses has created a market gap for a safe, effective chronic pharmaceutical option.[111-113] The potential of pioglitazone to affect both insulin secretion in response to oral sugar and HMW adiponectin secretion makes it a promising option to address multiple pathologic mechanisms that contribute to EMS and development of EL.

Initial evaluation of pioglitazone in horses focused on pharmacokinetics of oral administration after multi-day dosing at 1 mg/kg, as well as validation of laboratory measurement of plasma drug concentrations.[150] Dosing had been extrapolated from trials in multiple species, however plasma concentrations and total drug exposure achieved at this dosing scheme were considered to be lower than human therapeutic drug concentrations.[150] Further work by the same research group evaluated pharmacodynamic effects of a 12 day course of pioglitazone on transient insulin resistance, as induced by intravenous infusion of lipopolysaccharide (LPS). No effect was seen on peripheral insulin sensitivity, as measured by frequently sampled intravenous glucose tolerance test; however there were noted alterations in transcript abundance of insulin receptors in muscle and nuchal ligament fat samples.[151] The authors attributed this lack of effect on inadequate dosing quantity or duration, based on concerns raised in the initial pharmacokinetic paper. Additionally, this IR induction model more closely mimics pathophysiology associated with acute laminitis induced during systemic inflammatory response syndrome, which is distinctly different from the pathophysiology associated with chronic insulin exposure seen in endocrinopathic laminitis.[154] Based on this prior work, a dosing scheme of 2 mg/kg for 28 days duration was utilized in the current study, and pioglitazone was considered likely to be more beneficial in chronic management of insulin dysregulation associated with EMS. The plasma drug concentrations measured 6 hours after 2 mg/kg pioglitazone administration on day 14 of multidosing in the ponies from the current study were higher than the 6 hour concentrations from normal horses after 11 days of dosing at 1 mg/kg in a previous study.[150]

The goal of the study was to look for evidence of pharmacodynamic effects of pioglitazone that could justify a future placebo-controlled clinical trial for treatment of refractory cases of EMS. We were concerned that a crossover design with a washout period between drug and control for the current study could have confounding effects of season and grass availability, especially with a small sample size, so this pilot study was limited to describing potential pharmacodynamic effects of short term pioglitazone administration to justify a larger study in the future. In order to magnify potential effects of the drug with a small sample size, the study did not exclude horses with evidence of EMS or PPID, although no horses had clinical signs of EL. The drug was well tolerated by all subjects. Troglitazone, an earlier drug in the same thiazolidinedione class as pioglitazone, was removed from the human market due to severe hepatotoxicity.[128] Pioglitazone has been associated with very rare hepatotoxicity in humans, and more common adverse effects in humans include weight gain, heart failure (secondary to water retention), and increased risk of bone fractures (due to osteoporosis). There was no evidence of hepatotoxicity in any subjects, nor any other adverse effects noted during the 28 day administration of pioglitazone in this study. Mild, statistically significant changes were noted in the two measured hepatic enzyme activities in response to pioglitazone, but these were not considered to be clinically significant.

After pioglitazone therapy, there was no apparent effect on body weight or subjective scoring for overall generalized obesity (BCS). This was not surprising, as pioglitazone is intended to alter the functions of existing adipose tissue and promote smaller, more insulin-sensitive adipocytes within existing adipose tissue, and not to

reduce the total body fat mass.[135] In humans, thiazolidinedione administration has been associated with mild weight gain, largely due to water accumulation as well as accumulation of fat, however this effect is mitigated by dietary caloric restriction, and is likely to be less significant in horses when managed by current EMS treatment recommendations to limit caloric intake.[133] Additionally, the 28 day duration of treatment may not have been adequate to cause statistically significant changes in overall BCS, however with more long-term therapy there may be greater differences in obesity. Likewise, the leptin concentrations were not impacted by the administration of pioglitazone, which fits with the lack of change in the total body fat mass.[28] There was a significant decrease in regional adiposity (CNS) seen in the horses and non-ID group, which may have been an early sign of improved fat distribution and metabolism, as indicated by the significant increase in adiponectin in these groups, or may just be a consequence of the relative subjectivity of CNS scoring.

In the current study, treatment with pioglitazone had significant effects on secretion of HMW adiponectin in all subjects, including the non-ID equids. This was indicative of improved adipocyte metabolism and adipokine secretion, and clinically significant as HMW adiponectin is the most insulin sensitizing form of adiponectin. Baseline HMW adiponectin concentrations in the ID group were lower than non-ID group, consistent with past papers indicating abnormal metabolism associated with obesity and EL.[28,75] Importantly, significant increases were identified in the affected group (ID) as well as the unaffected group (non ID), indicating uniform drug effects and indications of improved metabolism, even in those without significant changes to

OST. In addition to improving insulin sensitivity, adiponectin has other benefits, such as anti-inflammatory effects and effects on vascular health, which could benefit EMS patients with EL long term.[135]

The glucose response to OST was unaffected by pioglitazone treatment, which was expected as equine glucose regulation is relatively stable despite ID, and hyperglycemia is relatively uncommon. The more clinically relevant outcome during the OST is the enteroinsular response to the oral sugar. Excessive insulin response to oral carbohydrates is a cornerstone of ID in horses, potentially caused by excessive response of incretin to non-structural carbohydrate intake and absorption at the level of the small intestine.[82] Certain EL-prone breeds have documented excessive enteroinsular responses to the OST when compared to less EL-prone breeds, and this is thought to be a component of the "easy keeper" phenotype.[82] In this study, pioglitazone was shown to reduce the insulin response to oral sugar challenge in the ID group as well as in the pony group. It did not have any effect on the horses or on the non-ID group during this study. This was significant because this indicates reduction in the insulin response to oral carbohydrates in the subsets of the study population most at risk for EL, pony breeds and the ID group.[13] In high-risk ponies diagnosed with ID, it has been shown that lower insulin response to oral carbohydrate challenge has been correlated with decreased laminitis risk.[60] After pioglitazone treatment, every equid in the ID group had improved insulin response to the final OST, and 3 of the 7 were no longer classified as ID.

Conclusions

In conclusion, after 28 days of pioglitazone at 2 mg/kg, insulin concentrations in response to oral sugar were decreased compared to day 0, and HMW adiponectin concentrations were increased compared to day 0. These are clinically important outcomes, and indicate effective improvements in some of the strongest phenotypic predictors for development of EL. Further assessment of pioglitazone utilizing placebo controlled clinical trial is needed to evaluate its effects on predictive factors for EL, as well as its ultimate ability to mitigate the development of naturally occurring EL in clinical patients. Pioglitazone could be an important addition to the arsenal for treatment of ID in the horse.

Chapter 4 – Summary

This project was the first known effort to evaluate the effect of a thiazolidinedione on secretion of adiponectin in the horse, and these results suggest a similar mechanism in horses as that seen in humans, where the stimulation of the adipocyte nuclear receptor PPARγ increased secretion of HMW adiponectin. A limitation here was that total adiponectin was not measured, however HMW adiponectin is considered the most biologically active and most potent insulin sensitizing form, and therefore the primary outcome of interest for the ID central to the EMS phenotype.[100] Adiponectin's potent role in promoting insulin sensitivity throughout many body tissues, improving secondary metabolic disturbances, and reducing risk of EL within the EMS phenotype makes this an important finding.

This project focused on two primary objectives—to evaluate the effects of oral pioglitazone administration in horses on adiponectin concentrations and the insulin response to oral sugar, while monitoring for safety and adverse effects at a higher dose and longer duration than previously utilized in equine trials. Based on published data on pioglitazone use in humans, this administration protocol was hypothesized to impact the components of EMS most strongly linked with EL risk, specifically serum adipokine concentrations and the enteroinsular response to carbohydrate challenge.[123] These have been identified as the most consistent predictors for the development of EL within the EMS phenotype in multiple retrospective and prospective studies.[20,21,28,37] Importantly, pioglitazone's ability to increase HMW adiponectin secretion regardless of obesity and ID status represents

a powerful mechanism to impact early or subclinical cases of EMS and equids with the lean EMS phenotype. Also, because adiponectin is shown to be decreased prior to the development of EL, pioglitazone may be an important tool for intervention prior to the development of EL in cases with mild ID or strong suspicion of EMS based on phenotype and risk factors.[20] Another potential benefit from increasing adiponectin regardless of obesity and ID status is that pioglitazone may potentially may play a role in improving ID in other conditions where hyperinsulinemia increases risk of EL, such as in PPID.[66]

Morphometric measurements were largely unchanged, as the only significant difference noted was a reduction in CNS within the horse group and the non-ID group. However, this was likely due to the relatively short duration of therapy and lack of management changes recommended to reduce obesity (namely reduction of digestible energy and increased aerobic exercise). Measures of adiposity, both generalized and regional accumulations, are not strong predictors of EL and likely result secondary to genetic predisposition and management factors instead of driving pathology of EMS.[2,3] In parallel to this static obesity status, measurement of serum leptin in this population remained unchanged in response to the administration of pioglitazone. This was likely tied to overall body fat mass, as the ID group had significantly higher BCS and significantly higher leptin concentrations. Leptin concentration had low predictive value for EL within the EMS phenotype, so this finding was neither surprising nor a primary goal for reducing risk of EL. However, longer term administration of pioglitazone may impact leptin concentrations if improved insulin sensitivity reduces obesity, especially when

paired with the recommended management changes that are considered standard of care in management of EMS.[3]

Despite the lack of significant change in evaluated measures of obesity, adiponectin concentrations were increased in all sub-populations in response to pioglitazone administration. This was especially noteworthy as it indicates intended pharmacodynamic effects of the pioglitazone on adiponectin secretion from adipocytes in both ID and non-ID equids, suggesting improved lipid metabolism regardless of body fat mass. This mirrors findings in human diabetes research, where a large placebo-controlled clinical trial found that pioglitazone increased adiponectin concentration and insulin sensitivity in humans, regardless of severity of hypoadiponectinemia and insulin resistance at the start of the 2.5 year study.[155] The specific increase of HMW adiponectin in the equids in this study indicates an especially powerful pharmacodynamic effect, as this form of adiponectin is considered the most potent insulin sensitizing multimer.[100] Importantly, classically obese but treatment-resistant patients, and patients with lean body condition and persistent ID are primary candidates for pharmacotherapy in EMS. Although this project is small in scale and intended as a pilot study, the positive impact of pioglitazone administration on adiponectin secretion regardless of obesity and ID status indicates this may be an important potential adjunct therapy for these patients.

Because ID remains the central risk factor for EL, assessing the effects of pioglitazone administration on glucose and insulin dynamics was a critical component of this study. Administration of pioglitazone resulted in significant improvement in the insulin response to oral carbohydrate challenge through the oral sugar test, while having no effect on the glucose response. Both non-ID and ID subjects had appropriate blood glucose response curves prior to administration, indicating compensated IR even in the ID subjects, and the insulin sensitizing effects of pioglitazone were not intended or reported to affect blood glucose response in normoglycemic human patients.[124] However, reduction of the insulin response to enteral carbohydrate challenge in ID equids and ponies, measured both at individual time points during the OST as well as the area under the insulin curve, represents an exciting and potentially powerful pharmacodynamic effect. The severity of hyperinsulinemia following enteral carbohydrate challenge in EMS ponies and horses has been strongly correlated with increased risk for EL, severity of lameness, and risk of recurrence. In this study, administration of pioglitazone was not only able to reduce the insulin response during the OST in ID equids and ponies, the two subgroups considered at greatest risk for EL, but it also reclassified 3 out of the 7 ID equids as no longer being ID. Although this was studied in a small population, if this pharmacodynamic effect is replicated in larger populations, it represents an opportunity to intervene and improve ID. Incorporating pioglitazone as an adjunct therapy in ID equids, in addition to recommended management changes for diet and exercise, as well as pharmaceutical management with pergolide in cases with concurrent PPID, may have great potential to mitigate risk of EL development and recurrence, which would greatly improve equine welfare.

Utilization of the higher dose and longer duration for pioglitazone administration necessitated close monitoring for adverse effects within the research population. This was especially important to monitor for evidence of hepatocellular toxicity, which was observed in people with another thiazolidinedione, troglitazone, and it was subsequently removed from the human market.[128] Hepatotoxicity associated with pioglitazone in humans is rare, and has not been reported in previous equine trials. After 28 days of administration, there were mild statistically significant, but clinically insignificant, increases in two hepatic enzyme activities, but all values remained within normal limits and no significant effect on measures of hepatic function were seen. This will be important to monitor during longer term administration to larger populations, especially with the reported mild increases in some EMS-affected equids on these enzyme activities due to proposed, but not confirmed, ectopic lipid accumulation within the liver.[3] It will also be important to specifically monitor for additional adverse effects associated with longer term administration of pioglitazone in humans, namely weight gain (largely due to water retention), water retention (in some cases leading to cardiac failure), and osteoporosis (in some cases leading to bone fractures).[123] Increased weight gain would be counterintuitive for management of EMS patients, as it would add to mechanical stress on the lamina and may potentiate laminitic changes. Additionally, chronic laminitis can result in pedal osteitis and decreased density and impaired integrity of the coffin bone. It is important to note that these adverse effects have not been reported in equine trials thus far, and marked differences in the metabolic syndrome phenotypes between humans and horses could mean that these adverse

effects are unlikely to develop. It will be important to monitor for these outcomes, as well as any additional equine-specific changes, through active surveillance during a future clinical trial.

There were several limitations in this study, some of which were inherent to its intended purpose as a pilot study. The overall sample size was relatively small in number, however it was calculated to have statistical power within both the pony and horse groups, based on a human study on the short term effects of pioglitazone administration on adiponectin secretion in healthy men with documented normal glucose tolerance.[138] The duration of the trial was also intended to mimic this documented short term administration, which had been demonstrated in human and murine models to isolate the specific effects of pioglitazone on upregulated adiponectin secretion from more long-term, multimodal effects.[138] As previously noted, this is likely to have been too short to generate significant changes in markers of regional or generalized obesity, especially when no dietary changes or exercise regimens were implemented. However, documented improvements in adipokines, as indicated by increased adiponectin secretion in all subjects after pioglitazone, may suggest that improvements in obesity could be seen after longer term administration.

Selection of the research population was partially limited by availability of subjects within the research herd at Auburn University. The population used in this study was considered representative of average "apparently healthy" middle-aged

to elderly equine populations, in which physical examination and routine bloodwork were apparently normal; however there were underlying endocrinopathic abnormalities identified after batch analysis of stored samples. The pony population were all mixed breeds, which likely did not affect EMS genetic risk as all ponies are considered at greater risk for EMS. Within the horse group, the breeds selected were intentionally considered low to moderate risk of developing EMS. Quarter horses were specifically excluded due to potential confounding based on potential concern regarding altered insulin sensitivity in Quarter horses.[47] Subsequently, limited numbers of available horses and ponies matching the inclusion criteria negated the possibility of developing a concurrent breed- and age- matched placebo control group. While this would have certainly added to the external validity of results, due to the marked influence of breed and age on measures of insulin sensitivity, an appropriate control group would have required this type of matching to be considered acceptable for comparison. Furthermore, a crossover design would bring concerns about seasonal affects due to the need for a washout period. The diet, housing, and weather remained the same through the study and all measurements were taken in one (summer) season, so we considered the use of animals as their own control appropriate.

The selected research population had apparently normal physical examinations, and were intentionally chosen to have no outward evidence of EL and normal baseline bloodwork. Two of the subjects (one horse and one pony) had clinical signs and baseline ACTH concentrations consistent with PPID. Additionally, equids with non-laminitic clinical signs of EMS were not excluded. This allowed for

inclusion of pre-laminitic ID equids, which were only identified in retrospective insulin measurements to inadvertently lead to blinding during evaluation for subjective morphometric scoring. Inclusion of several subjects with documented endocrinopathies generated a less homogenous study population, however we may have elicited more significant effects of the pioglitazone within subgroups since affected animals were included. The two equids with PPID were both classified as ID during the initial OST, which places them at high risk of future development of EL. Even though they were both classified as ID at the endpoint of this trial, their insulin values during the second OST were both improved, indicating reduced risk of future EL after pioglitazone administration even without primary treatment of PPID with pergolide.

Insulin dysregulation was evaluated in this study using the oral sugar test because it allowed for dynamic evaluation of the enteroinsular response to carbohydrate challenge. Measurement of fasting basal insulin concentration is considered a low-sensitivity screening test for clinical patients, but it would be inappropriate for evaluation of insulin dynamics within a population containing apparently normal equids.[2] Postprandial hyperinsulinemia has been identified as the ultimate trigger for development of EL in EMS, therefore it was considered essential to evaluate the effect of pioglitazone on the enteroinsular response. The OST was selected for this purpose, due to increased sensitivity and repeatability, simplicity and uniformity of testing protocols, and relevance to clinical practice.[3] Decreased hepatic insulin clearance has been documented in ID equids in the research setting, although its contribution to ID is considered to be relatively minor. Hepatic insulin

clearance is evaluated through measurements of C-protein:insulin concentrations within the blood as a proxy. The performance of this parameter in predicting laminitis did not perform as well as measurements of tissue insulin resistance or postprandial hyperinsulinemia.[47,81] Therefore, this was not evaluated in the current study but could be considered for future research specifically on the pharmacodynamic effects of pioglitazone on laboratory analysis of equine ID. Furthermore, measurement of tissue insulin sensitivity was not performed during this study. Insulin sensitivity was likely affected by pioglitazone, however its role in the insulin dynamics documented in the OST procedures is unknown. This could have been more directly evaluated using the CGIT or insulin tolerance test, however dynamic evaluation of the enteroinsular response was prioritized and this could again be considered for future work on the specific pharmacodynamic effects of pioglitazone on the different components of ID.[47]

An interesting finding was that serum drug concentrations were highly variable and lower than expected, despite dosing pioglitazone at twice the dosage used in previous studies. Caution should be taken in over-interpretation of these limited data points, especially as they were retrospective and utilizing convenience samples as opposed to rigidly designed, prospective sampling to appropriately characterize pharmacokinetics. Wide variation in plasma drug concentrations between individual subjects was noted in previous pharmacokinetic work as well.[150] Timing of administration and lack of fasting may have played a role in the wide variation in drug concentrations, due to variations in gastrointestinal fill, however the animals were intentionally not fasted to recreate natural husbandry conditions

during this study. The pioglitazone product used in this study was a generic tablet (45 mg tablets, TEVA Pharmaceuticals, North Wales, PA, USA), as opposed to the innovator product (Actos®, 45 mg tablets; Takeda Pharmaceuticals North America, Inc., Deerfield, IL, USA) used in prior equine studies. Therefore, potential exists for altered pharmacokinetic parameters in this population due to differences in pharmaceutical formulation and manufacturing. Drug concentrations were significantly lower in the single time point measured in the horses, compared to the two time points measured in the ponies. To the authors' knowledge, there is no existing evidence that demonstrates altered drug absorption in ponies compared to horses. Without further data points, the consistency of this difference is unknown and the cause for this discrepancy remains unclear. Despite these limitations, significant increases in adiponectin concentrations were identified in all subjects and subgroups, indicating at least partial pharmacodynamic effects on adipokine secretion and adipocyte metabolism in response to pioglitazone treatment.

Future research on pioglitazone use in equids should utilize larger populations and longer durations of treatment, in order to more fully evaluate its effects on metabolic parameters and patient safety as a long-term medication, necessitated by the chronic, life-long nature of EMS. Ideally, this would include populations of equids that are clinically affected by EMS, as well as a uniform population of truly normal animals without documented endocrinopathy, where more homogenous study populations may clearly elucidate clinical effects of drug administration. Additionally, further prospective investigation into the pharmacokinetics of pioglitazone and pharmacodynamics in the horse should be

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investigated to characterize the differences between innovator and generic drugs, breed differences (horses versus ponies, high-risk vs. low-risk horse breeds), and the effects of timing of administration compared to meal feeding.

In humans and dogs, pioglitazone administration has been identified to reduce certain markers of inflammation and improve blood lipoprotein profiles.[123,142] While the role of low-grade inflammation and blood lipoprotein dysregulation has been better characterized in human metabolic syndrome than EMS, this may be worthy of further investigation in the EMS phenotype. Initial work on the effects of pioglitazone on markers of LPS-induced inflammation indicated mild changes, and there is potential for greater benefit at the higher dose and longer duration used in this study.[152] Additionally, evidence for genetic polymorphism in the human pharmacodynamic response to pioglitazone also raises the possibility for similar findings in horses.[136] To the authors' knowledge, no evidence currently exists in horses regarding this, however characterization of PPARγ in the horse is rapidly expanding due to its role in adipocyte derived equine stem cell research and this may warrant future investigation if pioglitazone moves towards commercialization and implementation in larger equine practice.

In conclusion, the prediction of EL within the EMS phenotype has been repeatedly and strongly linked with measures of ID (especially postprandial hyperinsulinemia) and decreased adiponectin concentrations (total and HMW). Current standard of care is based largely around management changes to reduce body fat mass and improve insulin sensitivity, however many equids are resistant

to these changes and remain ID, even when weight loss is achieved. These equids would benefit from pharmacotherapy options to improve insulin sensitivity and secondary metabolic disturbances, and there is currently no effective long-term pharmaceutical options for this purpose. In this pilot study, pioglitazone resulted in decreased insulin concentrations in response to oral sugar and increased HMW adiponectin concentrations. These are clinically important outcomes, and indicate effective improvements in some of the strongest predictors for development of EL. Further assessment of pioglitazone in a clinical trial, utilizing a larger population of equids for longer term administration, is needed to evaluate its effects on predictive factors for EL, as well as its ultimate ability to mitigate the development of naturally occurring EL in clinical patients. Current therapeutic options for ID are limited by lack of owner compliance, long term efficacy, and consistent effects within the population. Within this study population, pioglitazone has been demonstrated to improve lipid metabolism in all subjects and insulin dynamics within the ID-affected group, and could be an important addition to the arsenal for treatment of ID in the horse.

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Appendix 1

Supplemental Tables for Pioglitazone Detection Assay in Serum

Supplemental Table 1.

MRM transitions and specific mass spectrometry tuning parameters for the quantification of pioglitazone.

Analyte	Parent Ion (amu)	Product Ion (amu)	Cone Energy (V)	Collision Energy (eV)	Quant/Qual Transition
Pioglitazone	357.1	134.1	52	26	Quantifier
	357.1	119.0	52	46	Qualifier 1
	357.1	106.2	52	42	Qualifier 2
Rosiglitazone	358.2	135.0	46	24	Quantifier
	358.2	119.0	46	56	Qualifier 1
	358.2	106.6	46	40	Qualifier 2

Supplemental Table 2.

Mass spectrometer tuning parameters for the detection of pioglitazone.

Parameter	Value
Capillary (kV)	0.6
Cone (V)	52
RF (V)	2.50
Extractor (V)	3.00
Source Temperature (°C)	150
Desolvation Temperature (°C)	600
Cone Gas Flow (L/Hr)	10
Desolvation Gas Flow (L/Hr)	1000