A GENOME-WIDE ASSOCIATION STUDY OF EQUINE METABOLIC SYNDROME AND PITUITARY PARS INTERMEDIA DYSFUNCTION

A Thesis

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ABSTRACT

Laminitis remains a poorly understood form of lameness in the horse despite being heavily researched. Endocrinopathic laminitis is an insidious form of laminitis that often results from insulin resistance (an indicator of Equine Metabolic Syndrome or EMS) or equine pituitary pars intermedia dysfunction (PPID). Both conditions occur in older horses with PPID having a later age of onset than EMS and horses are often characterized as having a "cresty" neck. EMS horses have persistent hyperinsulinemia while horses affected by PPID have elevated ACTH levels. Though no specific genetic predispositions have been identified for EMS or PPID, ponies are frequently observed to have both conditions. The objective of this study was to conduct a genome-wide association to identify candidate genes that may predispose individuals to EMS, PPID or both. A total of 65 horses, at least ten years old and of full or majority Arabian descent were divided into four categories based on previous endocrinology testing. Horses with ACTH levels greater than 70 pg/mL and insulin levels less than 40 uIU/mL were designated "PPID" while horses with insulin levels > 70 uIU/mL and ACTH levels less than 40 pg/mL were designated "EMS". Horses with ACTH and insulin levels < 40 pg/mL or uIU/mL, respectively, were considered "Normal" and those with both levels > 70 were grouped into the fourth category termed "Both". The Illumina Infinium® II Assay (Illumina Inc, San Diego, CA, USA) was performed on the EquineSNP 50 Genotyping BeadChip (Illumina Inc, San Diego, CA, USA) using DNA extracted from tail hair or blood samples. Data was analyzed using the PLINK v1.07 Whole genome association analysis toolset and JMP 8.0 (SAS Insitute Inc, Cary, NC, USA). Basic association (chi-square) tests were used to compare various groupings of disease cases and controls for a total of 15 qualitative associations. Quantitative associations were also performed for highest recorded ACTH (excluding August-October measurements) and insulin levels. An additional association was performed comparing horses with at least one episode of laminitis versus those with no recorded history of laminitis, regardless of disease category. SNPs with a missing genotype rate greater than 10% were excluded from analysis. SNPs in linkage disequilibrium ($r^2 > 0.99$) were pruned to a single SNP per haplotype block in all graphs. Any

SNPs exceeding the Bonferroni α threshold were mapped on the UCSC Genome Browser using the September 2007 assembly (EquCab2.0) of the horse genome. Candidate genes were identified within ~50 kb of each significant SNP. A total of 68 SNPs representing 59 different loci exceeded the significance threshold after correction for multiple testing. The most significant SNP, BIEC2-215377 (ECA 13) with a P-value of 4.34 e-7, was identified in the quantitative association with the highest ACTH level recorded for each individual. This SNP yielded one candidate gene, *XPO6*. Other SNPs that did not exceed the significance threshold but did vary from their expected P-value in a quantile-quantile plot include BIEC2-770354 (ECA3) and its neighboring SNP in linkage disequilibrium, BIEC2-770355 which yielded two candidate genes, *FTO* and *ATP5H*. This study resulted in the discovery of several good candidate genes that are worthy of fine mapping. However, epistatic effects may hamper the ability to identify all genetic predispositions. Improvements in diagnostic testing may allow for more specific classification of disease categories and refinement of the current data set. The present study is also limited by the relatively limited coverage of the Equine SNP50 chip. Expanded platforms with better coverage may allow for the discovery of more candidate genes.

BIOGRAPHICAL SKETCH

Cassy Streeter was born and raised in Syracuse, NY. She received her B.S. in Animal Science at Cornell University in January 2006. She is a Thoroughbred and Standardbred racing person. After this thesis she now considers herself an Arabian person and, after receiving many offers for free Arabians during the study, she may end up owning one in the future.

This thesis is dedicated to all of the owners of the horses that participated in this study.

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LIST OF ABBREVIATIONS

α-MSH – alpha-melanocyte-stimulating hormone

ACTH – adrenocorticotropic hormone

AIRg – acute insulin response to glucose

BCS – body condition score

DST – dexamethasone suppression test

ECD – equine Cushing's Disease

EMS – equine metabolic syndrome

FSIGTT – frequently sampled intravenous glucose tolerance test

POMC – proopiomelanocortin

PPID – pituitary pars intermedia dysfunction

SNP – single nucleotide polymorphism

CHAPTER 1

Review of Literature

Introduction

Laminitis remains a poorly understood form of lameness in the horse despite being heavily researched. While many studies have focused on the pathophysiology of acute onset laminitis due to experimental models like carbohydrate overload [1] and black walnut extract [2], attention is now being given to an insidious form of laminitis referred to as endocrinopathic laminitis [3]. Endocrinopathic laminitis is often a result of insulin resistance (associated with equine metabolic syndrome) or equine pituitary pars intermedia dysfunction (aka equine Cushing's Disease). Both equine metabolic syndrome and pituitary pars intermedia dysfunction show similar phenotypes and can be difficult to distinguish without diagnostic testing. There are currently no cures for either condition, though some management and drug therapies are available.

Equine Metabolic Syndrome

The term equine metabolic syndrome (EMS) was first coined in 2002 by Johnson to describe horses that show signs of chronic/previous laminitis in the absence of acute triggers such as carbohydrate overload or colic [4]. These horses are often obese and referred to as "easy keepers" because of their ability to maintain body weight with decreased caloric intake. The core characteristics of a horse affected by EMS are increased adiposity (often regional), insulin resistance (characterized by hyperinsulinemia) and a predisposition towards laminitis [4]. Johnson noted the age of onset to range from 8-18 years [4], though cases have occasionally appeared outside of this age range.

Obesity in horses is not as clearly defined as the body mass index designed for humans [5] because the popular Henneke scale [6] does not account for regional adiposity. While generalized obesity is often linked to insulin resistance in horses [7],

regional adiposity is now gaining recognition as a high risk factor for insulin resistance [8, 9]. The regional adiposity often seen in an EMS affected horse manifests as a "cresty neck" in which fat accumulates along the nuchal ligament on the dorsal side of the neck. Larger mean neck circumference has previously been shown to be associated with lowered glucose tolerance [8].This regional adiposity is comparable to the accumulation of fat around the waist in humans, a characteristic that is a risk factor for human metabolic syndrome [10]. Since the Henneke scale does not properly address this issue, an objective scoring system for quantifying the relative size of the crest of the neck has been developed with scores ranging from 0 to 5 [11]. Horses with scores greater than or equal to 3 are classified as having an excessively cresty neck. Horses in this category also tend to have regional fat accumulation in the supraorbital fat pads, tail head and the shoulder area [4].

Hyperinsulinemia, an indication of insulin resistance [8, 12, 13], is a key characteristic of EMS [4]. While the plasma insulin concentration in fasted, healthy horses is often less than 20 uIU/mL [14], insulin resistant horses can have levels in excess of 70 uIU/mL [15]. Ponies with a history of laminitis were observed to have higher levels of insulin when compared to non-laminitic ponies [16]. In addition, induction of hyperinsulinemia, while maintaining euglycemia, has been shown to induce laminitis in otherwise healthy ponies and horses [17, 18]. Though both of these studies used insulin concentrations in excess of the normal physiological range (>1000 uIU/mL), the onset of laminitis in the presence of an acute increase in insulin indicates that laminitis may also be brought on by prolonged or chronic hyperinsulinemia at physiological levels [17, 18]. It's possible that hyperinsulinemia alone does not bring about a laminitic episode; rather it predisposes insulin resistant horses to developing laminitis due to other causes. For example, acute ingestion of a large amount of

nonstructural carbohydrates in feed or pasture that leads to disruption of the microbial ecology of the intestine.

Hyperglycemia is often seen in humans as a result of insulin resistance and β cell failure [19]. However, hyperglycemia is rarely observed in horses with insulin resistance [16, 20], though isolated cases have been reported [21]. Place *et al.* (2010) observed significantly higher blood glucose levels in EMS affected horses than in controls, but the levels were still within the reference range [22]. It has been proposed that resistance to hyperglycemia in horses may be due to more robust β cell function than that of humans [20]. It is also possible that horses simply do not have a long enough lifespan for β cell failure (and subsequent hyperglycemia) to develop [20].

The repeated postprandial hyperinsulinemia that results from consumption of diets high in nonstructural carbohydrates may bring about the development of insulin resistance in the horse [23] and, subsequently, hyperinsulinemia. Alternatively, hyperinsulinemia may also be due to hepatic insulin resistance where insulin clearance from the liver is lower [24]. Insulin normally acts to stimulate glucose uptake in skeletal and adipose tissue by the recruitment of the GLUT4 glucose transport protein to the cell surface. In ponies, GLUT4 stimulation is lower than that observed in pigs and cows which may account for their susceptibility to insulin resistance [25]. Insulin resistant horses in general show decreased basal levels of active cell-surface GLUT4, though overall GLUT4 content remains the same [26]. Waller *et al.* used a biotinylated bis-mannose photolabeled technique that was able to distinguish active cell-membrane GLUT4 [26] while other studies simply used fractionation which only determines GLUT4 content. It may be worthwhile to repeat the work done by Duehlmeier *et al.* to see if pony breeds have decreased active GLUT4 when compared to horses.

Equine Metabolic Syndrome Diagnostic Testing

Many different diagnostic tests have been investigated to assess the degree of insulin sensitivity and insulin resistance in horses with metabolic syndrome. The resting plasma concentration of insulin has been shown to be a useful diagnostic test for insulin resistance [8]. Insulin and glucose responses to feeding are higher in the morning [27], so fasting insulin levels are preferred. Insulin concentrations do not appear to be influenced seasonally [22, 28]. The cutoff for hyperinsulinemia is variable among testing labs and researchers. Values ranging from 32 uIU/mL [29] to 70 uIU/mL [15] have been used to identify insulin resistance and EMS.

Eiler *et al.* developed the combined glucose insulin tolerance test in response to the need of practitioners to have a test that is easily applied to a clinical setting [30]. Horses are first fasted and baseline blood samples are drawn. Then, via catheter, dextrose followed by insulin infusions are administered and a second blood sample is drawn 45 minutes later. The blood samples are measured for both glucose and insulin levels and failure to return to baseline glucose levels by 45 minutes or the maintenance of elevated insulin levels indicates decreased insulin sensitivity [31]. The advantage of this test is that it takes less than one hour to collect all necessary samples and minimal endocrinology testing is required. However, this test may not be accurate when performed during a laminitic episode [31] and placement of catheters the night before the test is recommended.

A technique often used to assess insulin sensitivity in a research setting is the frequently sampled intravenous glucose tolerance test (FSIGTT) [32][13, 21, 26, 33, 34]. A minimal model analysis is used to determine the value of the acute insulin response to glucose (AIRg) and insulin sensitivity. Higher AIRg values have been linked with

lower insulin sensitivity in horses and ponies [35]. The FSIGTT involves injecting 100 mg/kg bodyweight dextrose followed by 20 mu/kg bodyweight insulin after 20 minutes [36]. Twenty-eight blood samples are then drawn at various times up to 180 minutes after the dextrose injection. Samples are measured for insulin and glucose plasma concentrations and the area under the curve for both insulin and glucose are calculated. The biggest drawback to this test is the number of samples needed and the length of time required to complete the test. However, this test is more specific to insulin sensitivity than the basal plasma insulin concentration, which only identifies the presence or absence of hyperinsulinemia. Due to its labor intensive nature, this diagnostic test is not often used in a clinical setting.

Equine Metabolic Syndrome Treatments and Therapies

Metformin is an oral biguanide used to improve insulin sensitivity and is the most widely prescribed drug for the treatment of type II diabetes in humans [37]. The efficacy of metformin in the horse is still being assessed. Vick *et al.* found that metformin was not effective long term for obese mares [38]. However, the higher dosage administered by Durham *et al.* showed improvement in insulin sensitivity up to 220 days but insulin resistance proxies failed to continue to decrease after two weeks [20]. As recurrent episodes of laminitis are linked to decreased insulin sensitivity [16], Durham *et al.* noted that metformin may be more effective prior to the first bout of laminitis [20]. The low bioavailability of metformin in the horse [39] may account for the lack of efficacy seen in previous studies and higher dosages may be needed to fully evaluate metformin as a treatment for EMS. Owners may also use chromium supplements for insulin resistant horses as it has been shown to increase mRNA

expression of insulin and GLUT4 receptors in the skeletal muscle of mice [40]. Pioglitazone, an insulin sensitizing drug used in humans, was recently investigated for use in horses, but no significant improvement in insulin sensitivity was detected [41].

Due to a lack of proven pharmaceutical therapies available for metabolic syndrome the most effective treatment for affected horses is intense management. As with humans, the best treatment to improve insulin sensitivity is through dietary restriction and increased exercise [42]. Limiting intake of feeds high in nonstructural carbohydrates will avoid sharp increases in plasma insulin concentrations and, therefore, the development or aggravation of insulin resistance [43]. This will also help shorten the insulin refractory time during which hyperglycemia is present [4]. Moderate exercise alone without concurrent adjustment to diet was shown to be ineffective in reducing insulin levels in obese individuals [33]. However, the combination of diet control and exercise was successful in lowering insulin levels and bodyweight [15], though results varied by individual.

Hypothyroidism and Thyroid Function in EMS horses

Horses affected by EMS are often incorrectly diagnosed as having hypothyroidism as these animals tend to have lower circulating thyroid hormone levels [44]. However, thyroidectomized horses do not show the clinical signs of regional adiposity and laminitis seen in EMS affected individuals [45, 46]. Levothyroxine is often prescribed for EMS affected horses to improve insulin sensitivity as pretreatment of horses with levothyroxine has been shown to prevent the development of endotoxemia-induced insulin resistance [47]. Levothyroxine may also be administered

to raise the basal metabolic rate and help "jump start" weight loss in horses that are currently affected by laminitis where an exercise regimen cannot be implemented.

Equine Pituitary Pars Intermedia Dysfunction (Equine Cushing's Disease)

Equine Pituitary Pars Intermedia Dysfunction (PPID), also commonly referred to as Equine Cushing's Disease [48], is the most common endocrinopathy in late middle-aged and geriatric horses [49]. PPID results in increased production of proopiomelanocortin (POMC) derived peptides due to the loss of dopaminergic inhibition from the hypothalamus [50-53]. POMC is further processed into adrenocorticotropic hormone (ACTH), corticotrophin-like intermediate lobe peptide (CLIP), melanocyte-stimulating hormone (MSH), β-endorphin and β-lipotropin [49]. Most PPID affected horses develop a pituitary adenoma or show signs of adenomatous hyperplasia [54, 55]. Originally, it was believed that the adenoma itself was rather inactive and it was the encroachment of the tumor on the hypothalamus that accounted for the clinical signs of Cushing's Disease [56]. However, it has been shown that this hyperplasia is responsible for increased circulating levels of ACTH [57] and β-endorphins [58]. These horses may also develop adrenal hyperplasia [55] and, in turn, hyperadrenocorticism. Though rare, some cases of pituitary independent Cushing's syndrome have been reported in the horse [59].

The pars intermedia, consisting of melanotropes, is under the direct inhibitory control of dopamine secreted by the periventricular neurons of the hypothalamus [60]. McFarlane *et al.*, using tyrosine hydroxylase staining, found that the expression of dopamine in PPID horses was only 20% of the levels expressed in younger and age-matched controls [53]. These results are in agreement with a previous study that found

dopamine levels to be decreased 88% in PPID affected horses [58]. Knockout mice deficient in dopamine D2 receptors (expressed in the pars intermedia) show selective proliferation of the melanotropes of the intermediate lobe which then double their POMC mRNA expression [61]. There is also alternative processing of POMC within melanotropes with increased expression of prohormone convertase 1 which, in turn, increases levels of ACTH [61]. Further, it was found that corticotropin-releasing factor, the releasing factor that stimulates corticotropes and melanotropes via the paraventricular nucleus [62], is not responsible for the increased POMC synthesis [61].

PPID shares many clinical signs with EMS including regional adiposity and a predisposition to laminitis [63]. The most obvious clinical sign of a PPID affected individual is abnormal hair coat which can manifest as hirsutism, delayed coat shedding or incomplete shedding [64, 65].Though the exact mechanism by which horses develop hirsutism is not understood, this sign has been shown to have a positive predictive value of 90% for PPID [66]. Mares may also display an abnormal estrus cycle [63]. PPID has a mean age of onset of 20 years [65, 67], though cases have been noted as young as 7 years [54]. A correlation has been shown between age and plasma cortisol and ACTH concentrations in healthy horses which suggests that age may be a predisposing factor for PPID [68]. Other signs of PPID include polyuria, polydipsia and muscle wasting [49].

Seasonal variation in plasma ACTH concentration have been shown with higher levels in the autumn [22, 28, 68], though the timing and duration of this elevation does not differ between PPID affected horses and healthy horses [69]. This seasonal increase is thought to help prepare the horse metabolically for lean winter months [70].

It has been reported that ponies have a lower plasma ACTH concentration than horses [51], but Donaldson *et al.* showed that there is no significant difference in ACTH levels between healthy horses and ponies [68].

Equine Pituitary Pars Intermedia Dysfunction Diagnostic Testing

A popular method for detecting PPID in horses is the dexamethasone suppression test (DST). This test requires plasma cortisol concentrations to be measured from blood samples drawn before and 19 hours after intramuscular administration of dexamethasone [48]. Horses affected by PPID will fail to suppress cortisol levels below 1 ug/dL [66]. Though it was first reported that the DST has 100% sensitivity and specificity [48], it has since been shown that the specificity may be closer to 76% and sensitivity to 65% [66]. The test is less accurate during the seasonal ACTH increase in the autumn, and the pituitary axis in older horses is less sensitive to dexamethasone administration [68]. There is also concern that the administration of dexamethasone can exacerbate laminitis in horses with current or previous bouts of laminitis [48, 49], although no laminitis was observed in a study using the DST [48]. Another complication of the dexamethasone suppression test is the need for multiple blood draws and the additional cost incurred by the owner. A cheaper option is needed, especially if there is a need for multiple testing throughout the year.

Another popular diagnostic test for PPID is to measure baseline plasma ACTH levels. ACTH is one of the POMC-derived peptides that is over expressed by melanotropes in PPID affected horses [61]. ACTH levels are elevated in PPID affected horses when compared to healthy controls [51, 67, 69]. This test requires a single blood draw [51] for plasma and is tested using a chemiluminescent ACTH immunoassay

validated specifically for equine plasma [71]. Reported reference values have varied between 35 pg/mL [28, 51], 50 pg/mL [72] and – 70 pg/mL [15], though the 35 pg/mL cutoff seems to be used most often in clinical practice [22]. Testing for endogenous ACTH also faces complications due to seasonal fluctuations as both PPID affected and normal horses reach their highest plasma concentrations in the autumn from August to October [28, 69], though the total percent increase in ACTH concentration is greater in PPID horses [28]. It has been proposed that a separate reference range for ACTH levels may be established for PPID affected horses in the autumn [69]. ACTH levels may also fluctuate throughout the year in response to stresses such as laminitis [73] and transportation [74]. However, baseline plasma ACTH levels are still advantageous over dexamethasone suppression tests, especially if repeated testing is needed to calibrate medication, because only a single blood draw and, therefore, a single veterinarian visit is required. While a single elevated endogenous ACTH level may be indicative of PPID, it may be more informative to test throughout the year to make an accurate diagnosis [75].

α-MSH, a POMC-derived peptide, has been investigated as another potential diagnostic marker for PPID [28]. αMSH suffers the same problem as ACTH in that levels are elevated during the autumn in both horses and ponies [76]. α-MSH shows a correlation with body mass index [77] and may not be an accurate diagnostic tool for horses that have lost body condition due to muscle wasting. There may also be a need for separate reference values based on breed as one study has shown an 11-fold seasonal increase in α-MSH in horses as compared to a 2-fold higher level in ponies [76]. Though it makes intuitive sense to measure cortisol concentrations to diagnose

PPID, cortisol levels are variable in PPID horses [48] and therefore does not make a reliable diagnostic test.

Treatments for PPID

Some of the most effective treatments currently available to owners of PPID affected horses are pergolide, cyproheptadine and trilostane [71, 78-80]. Pergolide is a dopamine agonist that acts to replace the action of the damaged dopaminergic neurons of the hypothalamus. Pergolide was evaluated as a treatment for Parkinson's Disease in humans [81]. In PPID affected horses, it reduces plasma ACTH concentrations [15, 71, 82]. Cyproheptaine, a serotonin antagonist, is less effective in terms of improving clinical signs of PPID when compared with pergolide [82]. A complication associated with pergolide is that dosing is difficult because severity of PPID may not be determined until post mortem examination of the pituitary and, even then, it is still not definitive [83]. Repeated baseline ACTH levels may need to be drawn to help assess the effectiveness of the treatment. It has yet to be determined if pergolide arrests the hyperplasia of the pituitary, though this is likely dose dependent.

Trilostane is a competitive inhibitor of the 3β-hydroxysteroid dehydrogenase. Widely used as a therapy for hyperadrenocorticism in dogs [84] and shown to be effective in horses [79], it acts to decrease circulating cortisol levels. It may not be the best treatment because, as mentioned above, cortisol levels are not always elevated in PPID affected horses. Also, trilostane does not control the hyperplasia of the pars intermedia. Pergolide is the better treatment because it likely also restores the balance of all POMC-derived peptides which may account for all of the symptoms where trilostane will only help control any phenotypic changes due to cortisol. PPID affected

horses would likely benefit from the same management of diet and exercise as EMS horses to help reduce obesity.

Identifying Genetic Predispostions

Though no specific genetic predispositions for EMS or ECD have been identified, breed differences have been noted in previous studies, with ponies frequently observed as particularly predisposed to both conditions [4, 65, 85-87]. Ponies have higher circulating levels of insulin when compared to horses [85, 88] and there is a notable difference in insulin sensitivity across breeds [88]. There may also be interplay between EMS and PPID, with PPID predisposing to hyperinsulinemia [15, 89, 90]. However Frank *et al.* has shown that mean insulin levels do not differ between PPID and normal horses and hyperinsulinemia was not observed after overnight stall confinement [69]. EMS horses have the same seasonal ACTH pattern as normal horses [22] which shows that EMS can develop independent of PPID. However, the overlap of clinical signs between these two conditions and the possible mechanisms that may predispose one condition to another warrants investigation of both conditions for possible genetic predispositions.

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CHAPTER 2

Original research

Background

The two most common conditions leading to endocrinopathic laminitis in the horse are equine metabolic syndrome (EMS) and equine Cushing's Disease (ECD), also known as pituitary pars intermedia dysfunction (PPID) [1]. Both of these conditions present similar clinical signs such as regional adiposity. It has been suggested that one condition may predispose to the other as high plasma concentrations of both insulin and ACTH in the same individual are frequently observed. Though no specific genetic predispositions to either condition have been identified, a difference in the prevalence of either condition across breeds suggests a genetic link.

PPID results from the loss of dopaminergic inhibition from the neurons of the periventricular nucleus [2] leading to increased production of proopiomelanocortin and, subsequently, ACTH. The mean age of onset for PPID is 20 years [3, 4] but cases have been observed in individuals as young as 7 years old [5]. An abnormal hair coat is the most obvious clinical sign of PPID [4] and is not observed in EMS affected horses. When diagnosing PPID the dexamethasone suppression test is considered the gold standard, though it is not reliable during the seasonal ACTH increase of autumn [6]. Endogenous plasma ACTH levels are preferable to the dexamethasone suppression test because only one plasma sample is needed, but it also is not a reliable measurement during the autumn [7, 8].

The key clinical signs of EMS (previously referred to as Peripheral Cushing's Disease) are regional adiposity, often presenting as a "cresty" neck, insulin resistance

characterized by hyperinsulinemia and a history of laminitis [9]. The average age of onset for EMS horses is slightly younger than that of PPID with ages often ranging from 8-18 years [9]. Diagnostic testing available for EMS include labor intensive methods such as the frequently sampled intravenous glucose tolerance test, the combined glucose insulin test and the euglycemic-hyperinsulinemic clamp technique. For clinical applications, it is simplest to draw a single blood sample and measure the fasted plasma insulin concentration to detect hyperinsulinemia.

Currently, there are few pharmacological options available to treat PPID or EMS affected horses. PPID is often successfully managed using the dopamine agonist pergolide, but it is usually given daily and dosage varies from 0.5 mg to 5 mg. At these dosages pergolide is an expensive option. Cyproheptadine, a serotonin antagonist, and trilostane, an inhibitor of β-hydroxysteroid dehydrogenase, are alternative treatments, but pergolide remains the most effective medication available. EMS horses have very limited pharmacological treatment options when considering insulin-sensitizing drugs. Responses to metformin treatment in EMS affected individuals have been mixed, possibly due to limited bioavailability of this drug in the horse.

The interaction between EMS and PPID has yet to be determined. Although PPID affected horses are often observed to also have hyperinsulinemia [10-12], Frank *et al.* showed that mean insulin levels do not differ between PPID and control horses and hyperinsulinemia was not observed after overnight stall confinement [13]. In addition, horses are able to maintain normal ACTH concentrations and seasonal patterns in the presence of hyperinsulinemia [7]. However, the frequent observation of concurrent

elevated plasma concentrations in both insulin and ACTH indicates that these conditions may predispose an individual to develop the other condition.

The differences observed in the frequency of either PPID or EMS across breeds indicates that there may be a genetic component to either condition. Treiber *et al.* suggest a dominant mode of inheritance for laminitis in individuals with EMS [14], though the research herd for that study consisted exclusively of ponies and the methods of pedigree analysis were not made available. Differences in the rate of elevated plasma concentrations of ACTH, an indication of PPID, and insulin, an indication of EMS, across different breeds shows that ponies, Morgans and Arabians have the highest frequencies of hyperinsulinemia while Thoroughbreds and Quarter Horses rank amongst the lowest frequencies (Streeter *et al.*, unpublished data). This is consistent with previous observations that ponies and Morgans are at a higher risk for EMS [15]. Arabians were chosen as the target breed for this study due to their moderate frequency of EMS and PPID as well as their large population and, consequently, ease of obtaining DNA samples in sufficient quantity for the study. Furthermore, any candidate genes identified in this study can be investigated in Arabian-derived breeds with low disease frequencies, such as the Quarter Horse and Thoroughbred that may have lost genetic predispositions to both conditions.

The objective of this study is to conduct a genome-wide association to identify candidate genes associated with EMS, PPID or both conditions within the Arabian breed using the EquineSNP50 chip. The EquineSNP50 chip has been used successfully to identify candidate genes for single gene diseases [16] as well as likely polygenic traits [17].

Materials and Methods

Horses – Horses were recruited for this study based on endocrinology records from Cornell University College of Veterinary Medicine's Animal Health Diagnostic Center. Owners were contacted and asked to submit a brief health history accompanied by a blood or hair sample. A total of sixty-five samples from Arabian horses were obtained for this study. All but five horses were confirmed to be of full Arabian ancestry based on registration records. Of the five individuals that were not confirmed to be full Arabians, all were anecdotally reported to be of full or a majority of Arabian descent although registration papers were not available. Ages, based on known ages at the time of the blood draw, ranged from 11 to 30 years. The mean ages for the disease categories Both, EMS, PPID, Normal and were 25.0, 18.6, 22.8, and 19.4 years, respectively. The group consisted of 32 geldings, 30 mares and 3 stallions.

Classification – Classification of horses was adapted from Walsh *et al*. into PPID (P), EMS (M), Normal (N) and an additional Both (B) group [11]. Horses with ACTH levels greater than 70 pg/mL and insulin levels less than 40 uIU/mL were designated "PPID" while horses with insulin levels > 70 uIU/mL and ACTH levels <40 pg/mL were designated "EMS". Horses with ACTH and insulin levels <40 pg/mL or uIU/mL, respectively, were considered "Normal" and those with both levels > 70 were grouped into the fourth category termed "Both". Individuals with levels between 40 and 70 were omitted as uncertainty in reference ranges made it difficult to assign those individuals to a disease category. Horses currently on medications were evaluated based on endocrinology results prior to starting medication. Horses with elevated levels for both insulin and ACTH were assigned to either the EMS or PPID groups if previous endocrinology testing was available to indicate which condition developed first. Levels taken between the months of August and October were not used to determine disease status due to the seasonal increase in plasma ACTH levels

[7]. The total number of individuals in each group for Both, EMS, PPID and Normal were 9, 15, 24 and 17, respectively.

DNA extraction and genotyping – Blood samples were extracted using the Gentra Puregene Blood Kit (Qiagen Inc, Valencia, CA, USA) using the manufacturer's protocol for whole blood. Hair samples were extracted using an adapted whole blood protocol starting with 15 hair bulbs incubated overnight at 65^{o}C in 100 µL of lysis buffer [1% Tween 20, 1X PCR buffer (Applied Biosystems, Foster City, CA, USA), 2.5mM $MgCl_2$] containing 2 µg Proteinase K. The extraction was completed following the manufacturer's protocol. A total of 500 ng of each DNA sample was sent for SNP genotyping at GeneSeek (Lincoln, NE, USA) where Illumina's Infinium® II Assay (Illumina Inc, San Diego, CA, USA) was performed on the EquineSNP 50 Genotyping BeadChip (Illumina Inc, San Diego, CA, USA) containing 54,602 SNPs. The EquineSNP 50 BeadChip SNPs were derived from the EquCab2.0 assembly with an average spacing of 43.2 kb across the genome.

Statistical Analysis – Statistical analyses were performed using the PLINK v1.07 Whole genome association analysis toolset [18] and JMP 8.0 (SAS Insitute Inc, Cary, NC, USA). Basic association (chi-square) tests were used to compare disease cases and controls. Since it is unknown whether there are common genetic predisposing factors between EMS and PPID or if they develop independently, various groupings of the disease and control categories were used to develop a total of 15 qualitative associations. Quantitative associations were also performed for highest recorded ACTH (excluding August-October measurements) and insulin levels. An additional association was performed comparing horses with at least one episode of laminitis versus those with no recorded history of laminitis, regardless of disease category. SNPs with a missing genotype rate greater than 10% were excluded from analysis. SNPs in linkage disequilibrium ($r^2 > 0.99$) were pruned to a single SNP per

haplotype block in all graphs. P-values unadjusted for multiple testing are reported and the Bonferroni corrected α level is shown where appropriate. Overall study α level is 5%. Any SNPs exceeding the significance threshold for multiple testing were mapped on the UCSC Genome Browser [19] using the September 2007 assembly (EquCab2.0) of the horse genome. Candidate genes were identified within ~50 kb of each significant SNP.

Results

A total of 68 SNPs representing 59 different loci exceeded the significance threshold after correction for multiple testing. When performing a quantitative association with the highest ACTH level recorded for each individual, SNP BIEC2-215377 (ECA 13) was the most significant SNP with a P-value of 4.34 e-7 (Figure 2.1). Two additional significant SNPs, BIEC2-377685 (ECA17) and BIEC2-1007280 (ECA 7) with P-values of 3.55 e-6 and 2.4 e-5, respectively, were also found. *XPO6* was the only candidate gene identified within 50 kb of SNP BIEC2-215377 (ECA13). Candidate genes identified for BIEC-1007280 (ECA7) include *OLFML1*, *SYT9* and *PPFIBP2*. No candidate genes were identified for BIEC2-377685 (ECA17).

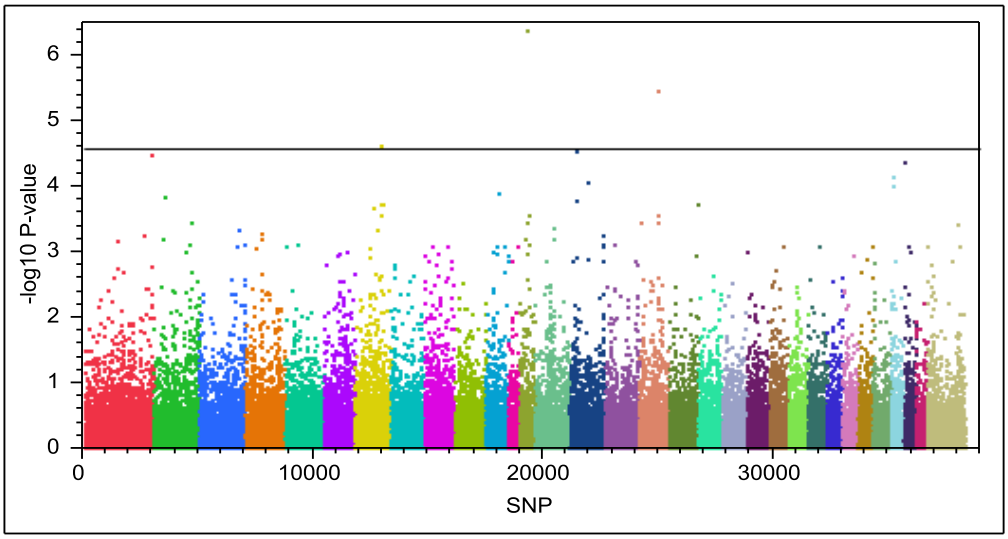

Figure 2.1 Quantitative association of highest recorded ACTH value. Association of 38,315 independent SNPS with $-\log_{10}$P-value on the y-axis and SNP, sorted and color-coded by chromosome, on the x-axis. Reference line included to show the significance threshold for multiple testing. Three significant SNPs are shown, including the most significant SNP, SNP BIEC2-215377 (ECA13) with a P-value of 4.34 e-7.

The SNP BIEC2-173131 (ECA12) achieved the second best P-value (1.48 e-6) of all associations in the quantitative association of the highest recorded insulin. This association also gave the greatest number of significant SNPs for a single association with a total of 38 SNPs representing 30 different loci (Figure 2.2). Candidate genes found for BIEC2-173131 include *ALX4* and *PRRX2*.

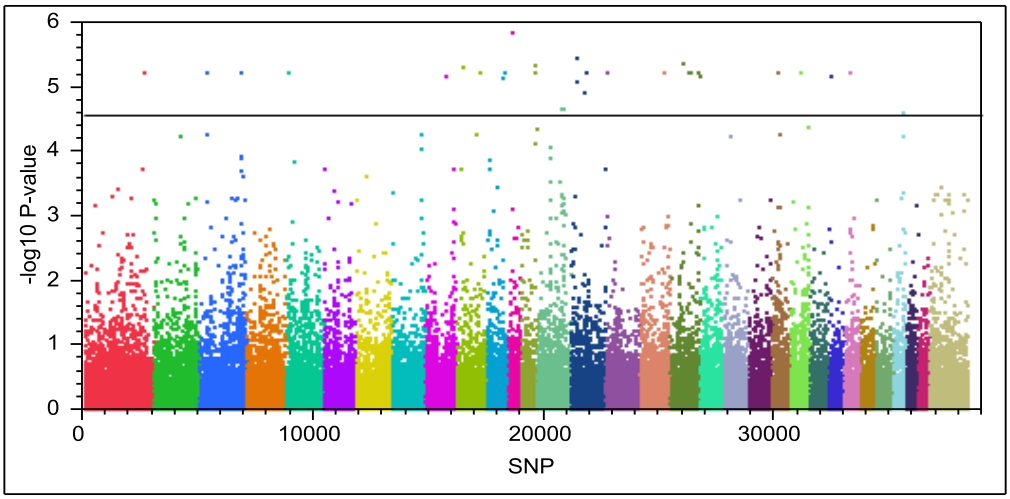

Figure 2.2 Quantitative association of highest recorded insulin value. Association of 38,343 independent SNPS with $-\log_{10}$P-value on the y-axis and SNP, sorted and color-coded by chromosome, on the x-axis. Reference line included to show the significance threshold for multiple testing. A total of 38 SNPs representing 30 loci exceeded the significance threshold.

Manhattan plots for each association and a table of all significant SNPs can be found in the Appendix. Any SNPs that do not exceed the significance threshold but deviate from expected P-values, as demonstrated by a quantile-quantile plot, may be worth further investigation. Also of particular interest are those SNPs with candidate genes that have been previously associated with obesity, diabetes and/or insulin resistance. A Manhattan plot of BM vs. N shows only one significant SNP, BIEC2-596175 (ECA22) (Figure 2.3). The SNP with the second smallest P-value, BIEC2-770354 (ECA3) approaches but does not exceed the significance threshold for multiple testing.

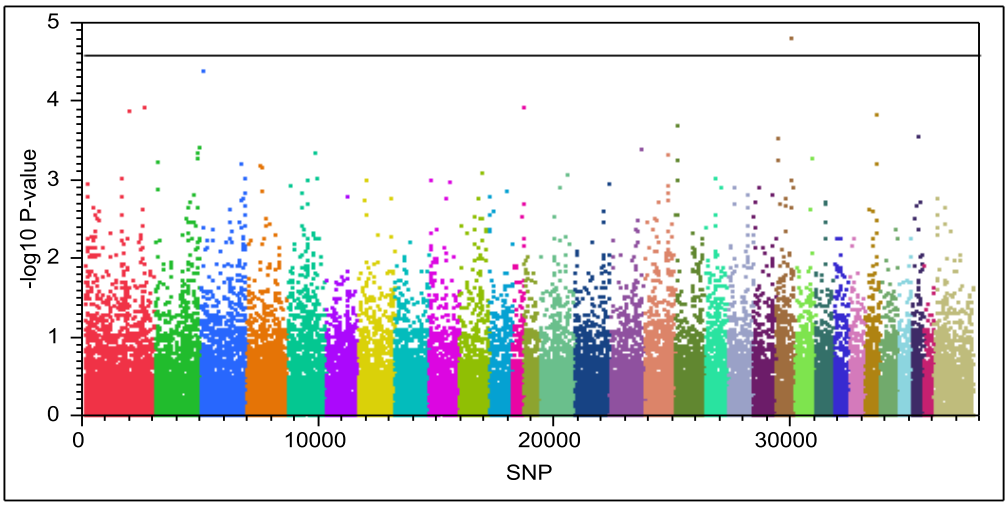

Figure 2.3 Qualitative association of hyperinsulinemia (B or M) vs. controls (N). Association of 37,647 independent SNPS with $-\log_{10}$P-value on the y-axis and SNP, sorted and color-coded by chromosome, on the x-axis. Reference line included to show the significance threshold for multiple testing.

Two candidate genes, *FTO* and *ATP5H*, were identified for BIEC2-770354 (ECA3) and its neighboring SNP in linkage disequilibrium, BIEC2-770355. Both SNPs are located within 500 bp of each other and are found in the intron of *FTO* in the human, mouse and cow. These two SNPs also deviate from expected $-\log_{10}$ P-values in the association BMP vs. N with a P-value of 1.03 e-4 (Figure 2.4).

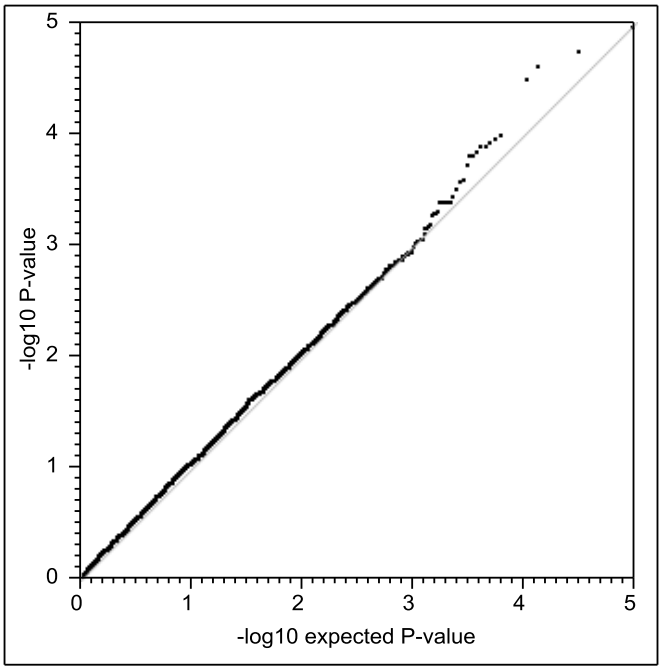

Figure 2.4 Quantile-quantile plot of qualitative association of all disease groups (B, M and P) vs. controls (N). The actual $-\log_{10}$ P-value is plotted on the y-axis with the expected $-\log_{10}$ P-value plotted on the x-axis.

Another candidate gene, attractin-like 1 (*ATRNL1*), was found in the association M vs. N. Two SNPs, BIEC2-7588 (ECA1) and BIEC2-7581 (ECA1), located within 7 kb of the first intron of *ATRNL1* did not exceed the significance threshold but did deviate from expected $-\log_{10}$ P-value with P-values of 1.01 e-4 and. 1.26 e-4, respectively.

Discussion

The lack of affordable and effective treatments for both EMS and PPID combined with the evidence of possible genetic predispositions to both conditions warrants further investigation into the heritable components of both disorders. Identification of the genetic underpinnings of EMS and PPID allows for a better understanding of how both conditions arise and if alternative therapies can be

developed. This genome-wide association study has identified several interesting candidate genes previously linked to obesity, diabetes and/or insulin resistance in humans and other animals. The most intriguing candidate is the fat mass and obesity associated gene or *FTO*. *Fto* was first identified in the rat by Peters *et al.* who designated it "fatso" due to its large, 250 kb sequence length [20]. This proved to be very serendipitous naming as *FTO* was first recognized as a predisposing factor to obesity in a genome-wide association study looking for genes associated with type II diabetes in humans [21]. Frayling *et al.* found that SNPs within the first intron of *FTO* were associated with type II diabetes through an effect on body mass index [21]. Since that original paper *FTO* variants have been associated with obesity in a wide variety of populations [22-24]. Though the function of *FTO* is not known, there have been many studies attempting to elucidate the mechanism of how *FTO* predisposes individuals to obesity. *FTO* expression is increased in the skeletal muscle of type II diabetic patients [25] as well as in the subcutaneous adipose tissue of obese individuals [26]. Murine *Fto* has been shown to increase feed intake and, therefore, increase obesity regardless of diet type [27]. Furthermore, this same study showed that a lack of *Fto* function results in a lean phenotype in mice [27].

FTO is an excellent candidate gene to investigate in both EMS and PPID affected horses due to the link with body mass index. The gene is highly conserved across all vertebrate species including teleost fish suggesting a history in vertebrate evolution of over 450 million years [28]. It is also worthy to note that, in the rat, *Fto* expression is detected in the paraventricular nucleus and is up regulated in the hypothalamus in response to 48 hours of food deprivation [28]. The exact mechanism

by which *FTO* regulates feed intake is unknown; therefore, if *FTO* is capable of regulating feed intake in PPID affected horses in the face of degeneration of the dopaminergic neurons of the paraventricular nucleus this may suggest a pathway independent of the innervation of the pars intermedia. *FTO* shows its best P-value in the association where all metabolic conditions are considered (p = 4.16 e-5, ECA3 6.5Mb). As the Both and EMS categories share this common locus, this suggests a genetic predisposition to EMS followed by PPID.

The SNP with the second most significant P-value, BIEC2-173131, yielded two candidate genes, *ALX4* and *PRRX2*. In a genome wide association study to identify loci associated with type II diabetes in humans, Sladek *et al*. identified *ALX4* as a candidate gene [29]. This study used stringent selection criteria to enrich for risk alleles associated with type II diabetes. The first cohort required individuals to have at least one affected first degree relative with type II diabetes. Individuals also had a lower body mass index to help select for alleles that predispose to insulin resistance through a pathway independent of obesity. A second cohort with relaxed inclusion criteria were then tested to confirm significant loci identified in the first stage. Though no SNPs were identified within *ALX4*, three SNPs were found within *EXT2* in the same haplotype block. *ALX4* is an interesting candidate gene as it was found independent of excessive body mass index in the first cohort. Due to insufficient information concerning weight and body measurements, it would be difficult to assess the relation of *ALX4* to hyperinsulinemia independent of obesity in the present study. However investigation of *ALX4* in an experimental herd of hyperinsulinemic and

control horses with varying levels of obesity may show an association similar to that found in humans.

Another top candidate gene from this dataset (p = 1.01 e-4) is attractin-like 1 or *ATRNL-1*. Attractin, a transmembrane protein product of the *Mahogany* gene [30], is believed to act as a co-receptor with the melanocortin 1 receptor (MC1R) for agouti, [31]. Attractin-like protein (ALP), unlike attractin, is able to interact directly with the MC4R C-terminus [32]. ALP has high expression in brain, kidney, heart lung and liver, overlapping with some of the same brain sites where *MC4R* mRNA is expressed [32]. It has been suggested that attractin has a protective role in the face of oxidative stress in the rat [33] and that attractin and ALP may have redundant roles [34]. This evidence makes a case for a possible role for *ATRNL-1* in protection of individuals from the oxidative stress associated with obesity and, in consequence, protection from the development of the neuronal degeneration associated with PPID. Notably, ALP null mice do not seem to show any abnormalities due to the possible redundancy with attractin [34], so any SNPs associated with *ATRNL-1* that result in loss-of-function would likely not result in obesity. However, if a SNP within *ATRNL-1* were associated with an altered form of ALP that antagonizes MC4R, then obesity may result.

MC4R has been extensively researched for its role in obesity in a variety of animals. MC4R normally acts to regulate energy homeostasis and food intake. Endogenous agonists for MC4R include the melanocyte-stimulating hormones derived from POMC, with β-MSH having the highest affinity [35]. Agouti-related protein (AgRP), an antagonist of MC4R, induces increased food intake [36] and prolonged antagonism of MC4R results in hyperphagia and obesity in rats [37]. MC4R has also

been associated with elevated insulin and glucose levels [38]. However, the obesity and metabolic syndrome associated with MC4R dysfunction was shown to be overcome through exercise in MC4R knockout mice [39]. This suggests that mutations of ATRNL-1 that affect its interaction with MC4R may possibly be overcome through environmental control rather than pharmacological intervention. MC4R itself failed to be significantly associated with either EMS or PPID. POMC would also be a likely candidate gene based on its association with PPID and MC4R mutations, but its closest SNP is located over 30 kb away and therefore may not be sufficiently covered by this panel of markers.

The results of this study are very encouraging with several quality candidate genes. However, further refinement of this study is needed to identify the strongest candidates. A major problem confronted by this study is the relatively limited coverage of SNPs across the horse genome. Comparable SNP chips for cows and humans represent over 500,000 and 1 million loci, respectively. As previously mentioned, several top candidate genes based on current literature, such as POMC, coverage was too sparse at those loci and significant associations in these genes may have been missed. This problem can only be overcome with the development of improved SNP chips with better coverage.

Another problem inherent in genome-wide association studies is that complex traits can be the result of many subtle genetic alterations that must come together to develop the disease. Individual alterations may confer a low to moderate risk of developing the disease, but it is the cumulative effect of these alterations that will bring about the phenotype of interest. A basic genome-wide association study,

however, is based on generating P-values for individual SNPs, so SNPs associated with low to moderate risk factors may not be distributed in the population in such a way that significance is detected. It is more likely that an individual that is considered unaffected will carry a mutation that confers a low to moderate effect if they do not carry other risk factors, therefore, the chi square statistic for that particular SNP will not be as extreme and significance will not be detected. Alternatively, a SNP conferring low to moderate risk may have a higher frequency in the diseased group and may exceed the significance threshold. However, this SNP may still need the cumulative effect of other risk factors to bring about the disease and, therefore, its predictive power will be overestimated. Complex statistical methods for deciphering patterns of epistasis are still in development and may clarify these relationships in the future.

A vital part of any genome-wide association study is the correct classification of the affected/disease cases versus the unaffected/control cases. Rare, monogenic diseases may be easier to assign a status to, as in the case of Lavender Foal Syndrome [16], but likely polygenic traits, such as EMS and PPID where several genes are interacting, are more difficult to properly assign to a category. Some of this difficulty lies in the ability to acquire complete health histories and uniform diagnostic testing. For example, the form of laminitis typically associated with both EMS and PPID is insidious and may not be recognized until postmortem examination. Some owners may not have noted a history of laminitis when in fact the horse was suffering from low grade laminitis without any clinical signs. Therefore, a lack of significant hits within the laminitis association is likely due to miscategorization of unaffected

individuals. This problem of incorrect assignment to a category may be overcome by increasing the number of horses in the study. In the case of Lavender Foal Syndrome, a monogenic disease, 36 individuals (6 affected and 30 unaffected) were needed to generate 14 significant SNPs. A complex trait with low to moderate risk factors will likely need more than a total of 65 samples in order to produce many significant hits. Adding more individuals will help to dilute the effect of incorrectly assigning a few individuals in the overall study.

Further complicating the matter of correctly assigning individuals to disease categories is the influence of environmental factors such as diet and exercise. Individuals with genetic predispositions that put them at a low risk of developing EMS may develop the disease if they are put on a diet high in nonstructural carbohydrates. Diets high in nonstructural carbohydrates can lead to repeated bouts of postprandial hyperinsulinemia and, subsequently, insulin resistance [40, 41]. While general dietary information was provided for the individuals in this study, estimation of total nonstructural carbohydrates intake was difficult to determine. Some individuals had switched diets after being diagnosed with EMS and dietary information leading up to diagnosis was not available. Many individuals in this study were not routinely exercised and were kept on pasture at all times. A lack of physical activity can put individuals with low genetic predispositions at a higher risk for developing insulin resistance [42]. Another factor affecting miscategorization is the age of onset, especially for PPID. With the mean age of onset for PPID being 20 years, it may not be appropriate to use younger horses as controls as they may still yet develop the disease. The mean age of the control group in this study was within the mean age range of the

disease categories, but older controls may be more appropriate to use to minimize miscategorization. In addition, some EMS horses may go on to develop PPID and be assigned to the Both category. However, it is more beneficial to identify the first condition that develops to help separate genetic predispositions to one disease or the other. Any common genetic predispositions between EMS and PPID can be found by combining the two groups as one disease category in basic associations.

It is also important to assess the practical applications of this study for the common horse owner. While the mechanisms of the development of both PPID and EMS are of interest to researchers, the value for horse owners will be derived from new preventative management practices. As quoted from the ACVIM Consensus Statement "EMS is a complex disorder for which there are more questions than answers at present" [43]. As information becomes available to researchers about how to properly characterize EMS and PPID, these developments need to be transferred to owners to help identify more affected individuals and increase awareness of both disorders.

The prevalence of obesity in horses is difficult to estimate as studies conducted through owner-based reporting show bias towards lower obesity scores [44]. Stephenson *et al.* found a prevalence of obesity in leisure horses in the UK of 20.6%, which is less than the 45% reported in a study of horses in Scotland [45]. This same study also found that when 15 randomly selected horses from the study were visited by researchers trained in assessing body condition scores, the mean score assigned by researcher was significantly higher than that assigned by the owner. Further, eight owners assigned a body condition score a full point below that of the researcher. When

this error rate was extrapolated to the questionnaire data the percentage of obesity more than doubled to 54.1%. It may be that owners are not properly trained to assign body condition scores or, more likely, owners have misconceptions of what constitutes a "healthy" weight for a horse. The muscle wasting seen in PPID affected horses can be confused with the loss of body condition sometimes seen in older horses [46]. In an attempt to avoid weight loss in geriatric horses owners will supplement the diet of their horse above their nutritional needs which may result in obesity and, consequently, insulin resistance and laminitis. It is necessary to educate owners on how to properly assess and maintain an appropriate body condition, especially in the geriatric horse that is at a higher risk of EMS and PPID.

Early detection is very important to avoid laminitis associated with both PPID and EMS. However, early detection is complicated by the fact that clinical signs of both EMS and PPID may be seen much later than the actual onset of either condition. Older horses show decreased sensitivity to the dexamethasone suppression test in the absence of other clinical sign of PPID [6]. Therefore, it can be difficult to determine the precise onset of PPID in the geriatric horse. It is unknown what extent of pituitary hyperplasia or duration of endogenous ACTH elevation is required to bring about clinical signs of PPID as the mechanisms of most of these signs remain unknown. There is considerable overlap of clinical and histological findings between PPID affected and healthy aged controls [47, 48]which makes correct diagnosis of PPID, especially early on in the disease, difficult for clinicians.

The identification of genetic predispositions to both EMS and PPID may allow horses to be used as models for the development of metabolic syndrome and

Parkinson's disease in humans. Parkinson's disease results from the degeneration of dopaminergic neurons of the substantia nigra [49]. Pergolide, the most effective treatment available for PPID, is still being investigated as a treatment for Parkinson's disease [50]. The horse offers a model for spontaneous dopaminergic neurodegeneration with PPID. By identifying the genetic predispositions associated with PPID in horses, researchers may be able to look for these same predispositions in humans and elucidate the subtle pathways that lead to Parkinson's in humans.

PPID is also very important to study because it cannot be cured at present. Pergolide is an effective pharmacological intervention, but it has not yet been shown to shrink or even arrest the hyperplasia of the pars intermedia. Medication must be administered for the rest of the horse's life. Surgery is not a practical solution for most horse owners, so control of environmental factors or being able to breed the predisposition out of the horse would be more effective. The late onset of PPID also means that owners are less likely to invest in costly solutions unless that particular horse still has value for breeding or performance.

The horse also poses as an interesting model for metabolic syndrome in humans as horses normally lack hyperglycemia in the presence of hyperinsulinemia. There may be genetic protective measures in the horse that prevents failure of the pancreatic β cells or it may simply be due to the horse's shortened lifespan relative to humans. The characteristic regional adiposity that confers a higher risk factor for developing laminitis is similar to the accumulation of abdominal fat seen in humans developing metabolic syndrome. Again, the ability to selectively breed against predispositions in the horse can be beneficial for breeders, but EMS may be controlled

by environmental factors. Although insulin resistance may be reversible through intense management, it is difficult to implement a program on previously laminitic individuals. The identification of genetic predispositions will allow owners to identify high risk individuals at birth and manage their diet and exercise regimen appropriately before the onset of disease.

The identification of genetic predispositions to EMS and/or PPID is a necessary step toward developing better diagnostic tests for at risk individuals and better therapies for those affected. The candidate genes identified in this study, especially those previously associated with obesity and insulin resistance, are worthy of further fine mapping. These candidate genes should be investigated in both Arabians and Arabian-derived breeds with low disease frequencies, such as the Thoroughbred and Quarter Horse. The addition of more individuals, both affected and older controls, will also help strengthen the power of the associations and identify more candidate genes. The dataset can also be improved with expanded marker panels (SNP chips) with better coverage across the genome. This study is limited by the information currently available concerning diagnosis and pathophysiology of EMS and PPID. The development of more diagnostic tests for other parameters will help classify affected individuals into their appropriate disease category. Finally, identification of genetic predispositions to EMS and PPID will allow owners to breed these predispositions out of their herds and provide better treatment options to those individuals already affected by either condition.

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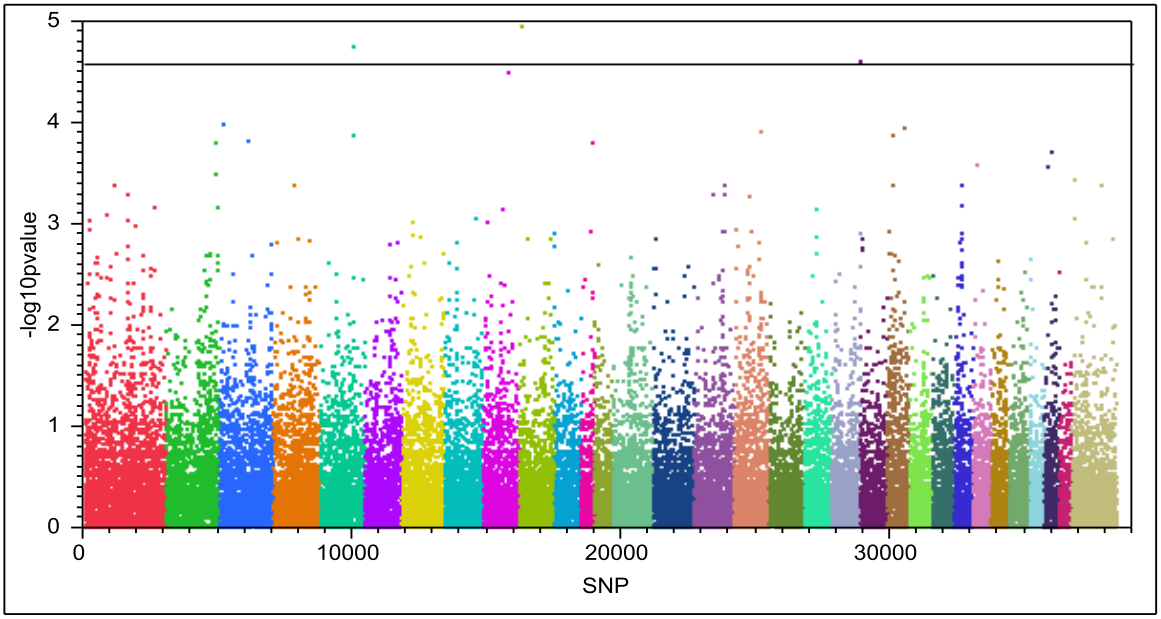
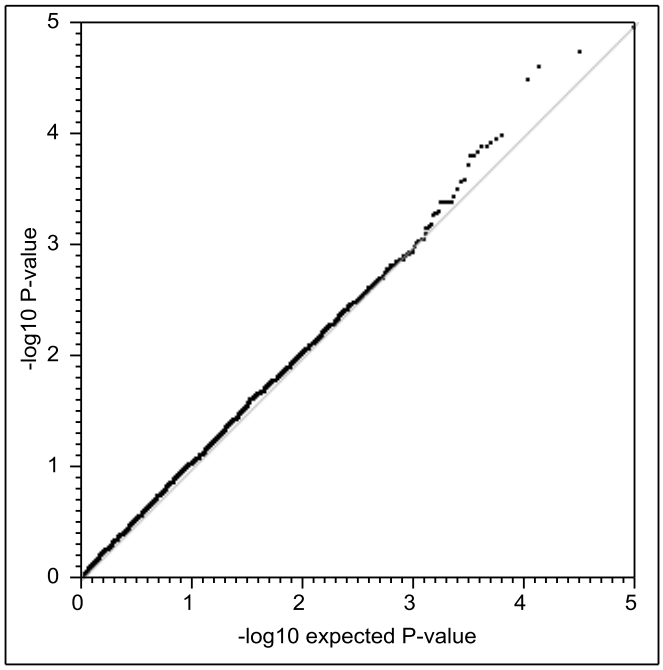
APPENDIX

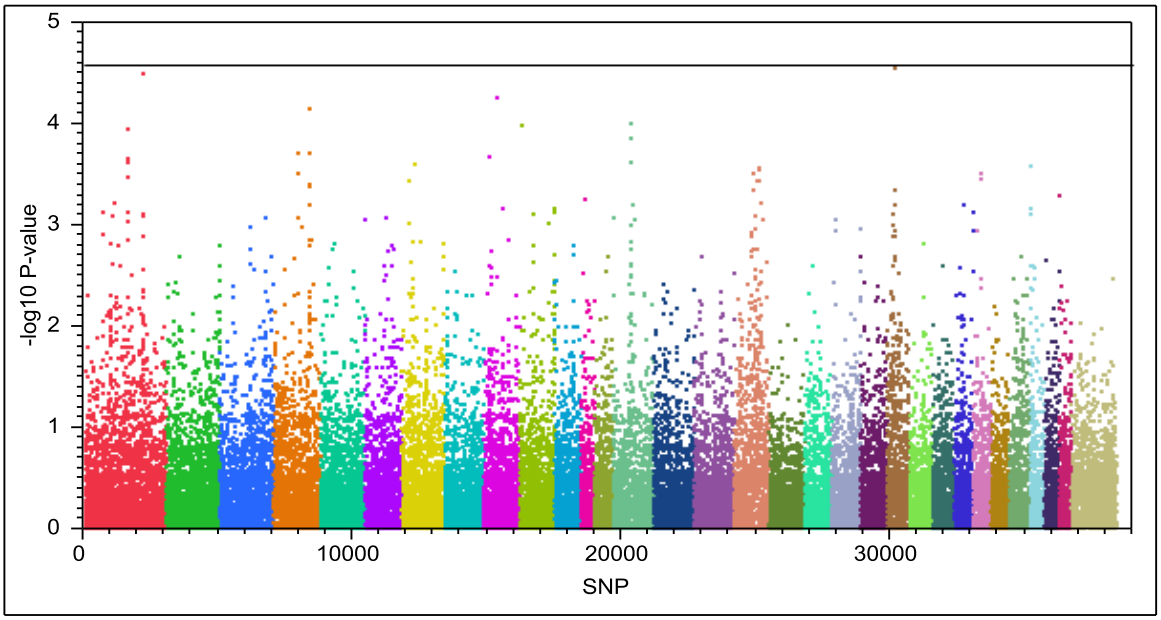
For each phenotypic classification association statistics were calculated. Notable associations were discussed in the main body of this work. For comparison, results from all calculations are shown below. All Manhattan plots have a reference line to show the significance threshold for multiple testing.



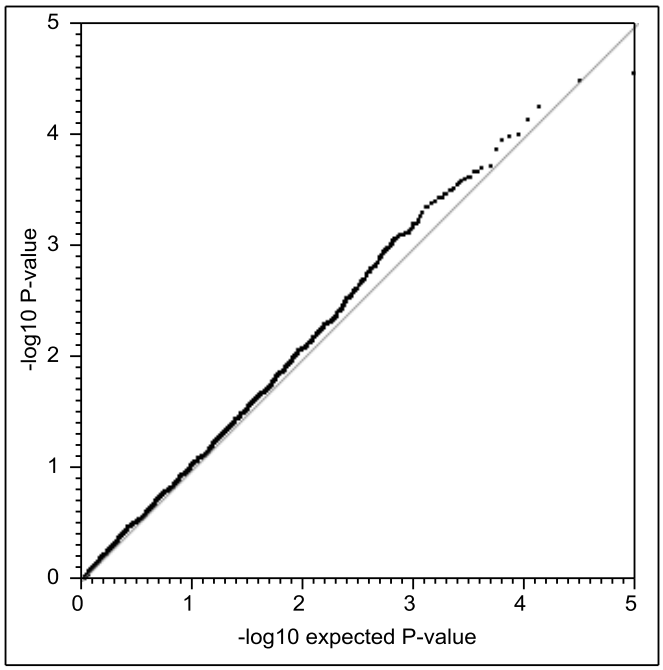
Supplemental Figure 1: Manhattan Plot of Qualitative Association of BMP (48 horses) vs. N (17 horses). Horses with any diagnosis compared to normal, healthy horses.



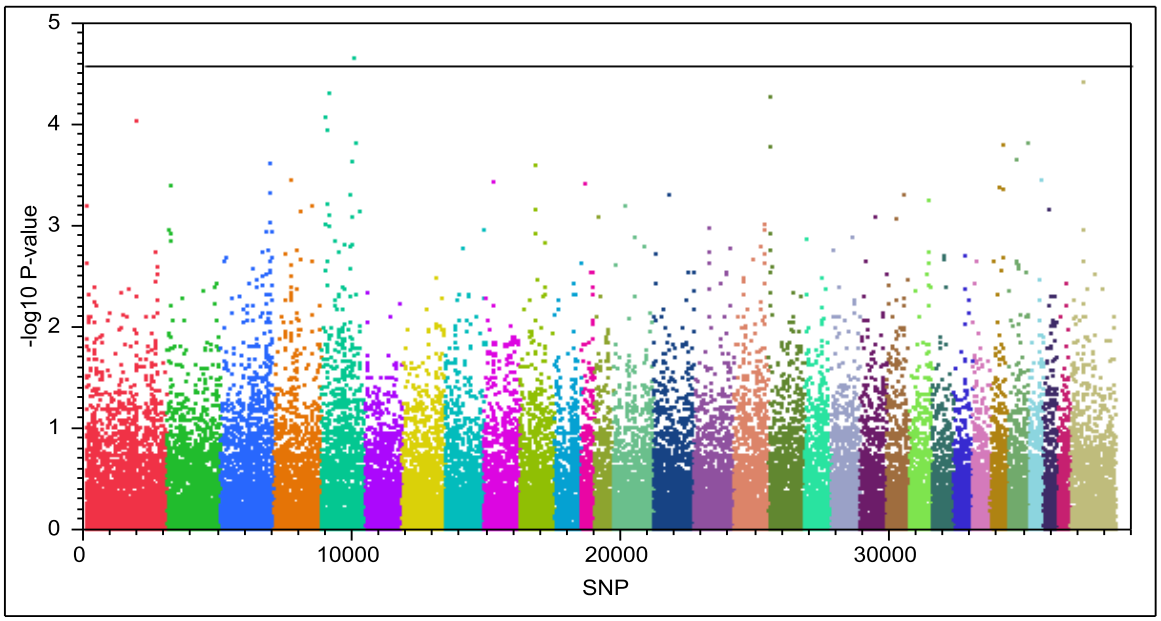
Supplemental Figure 2 Quantile-quantile Plot of Qualitative Association (48 horses) vs. N (17 horses)



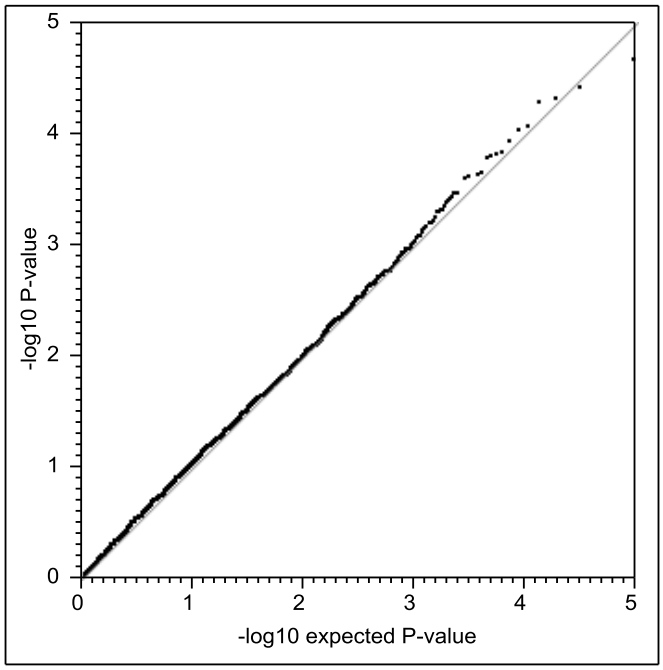
Supplemental Figure 3 Manhattan Plot of Qualitative Association of BP (33 horses) vs. MN (32 horses). Horses with elevated ACTH levels compared to those without elevated ACTH levels.



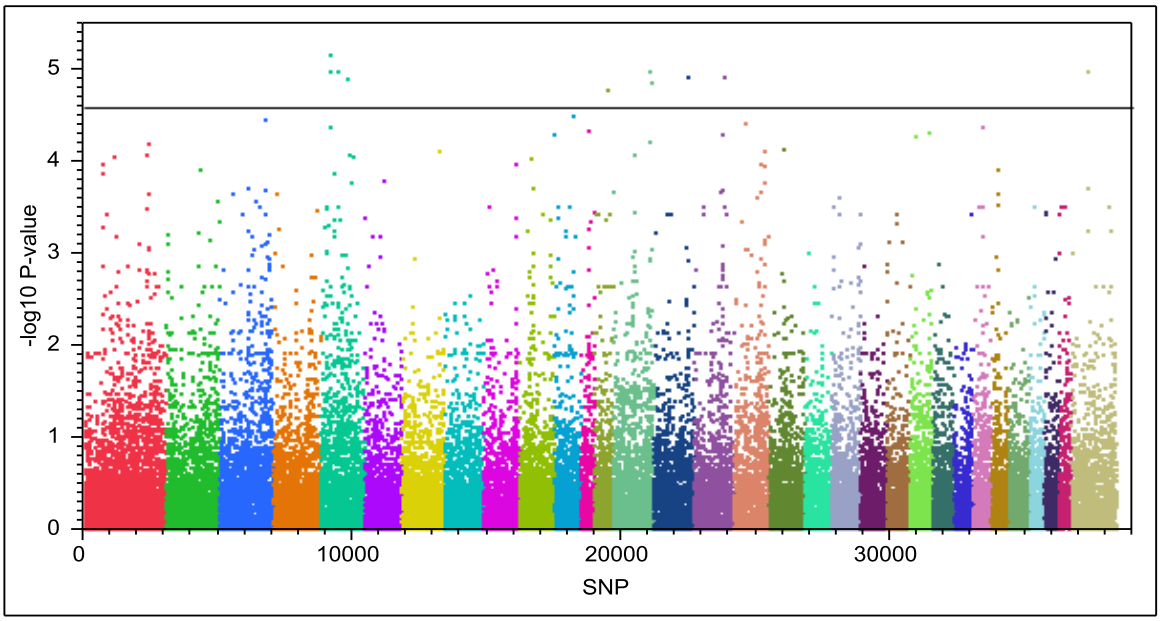
Supplemental Figure 4 Quantile-quantile Plot of Qualitative Association BP (33 horses) vs. MN (32 horses).



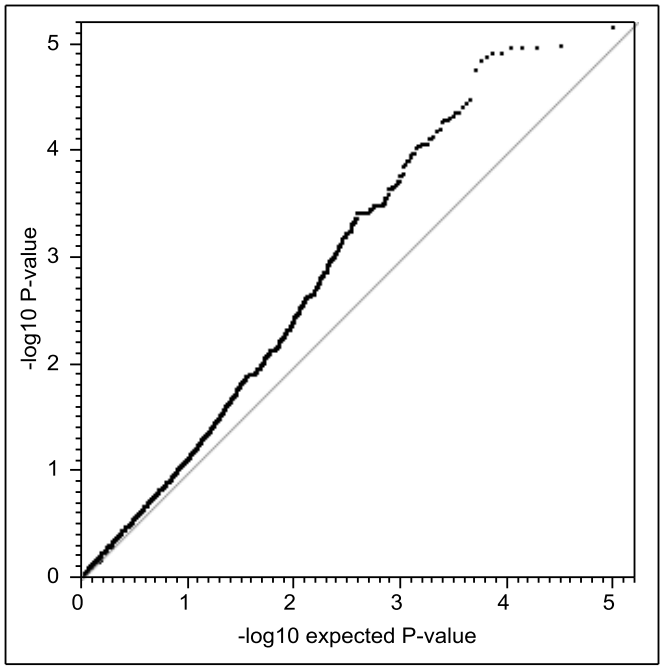
Supplemental Figure 5 Manhattan Plot of Qualitative Association of BM (24 horses) vs. PN (41 horses). Horses with elevated insulin levels compared to those without elevated insulin levels.



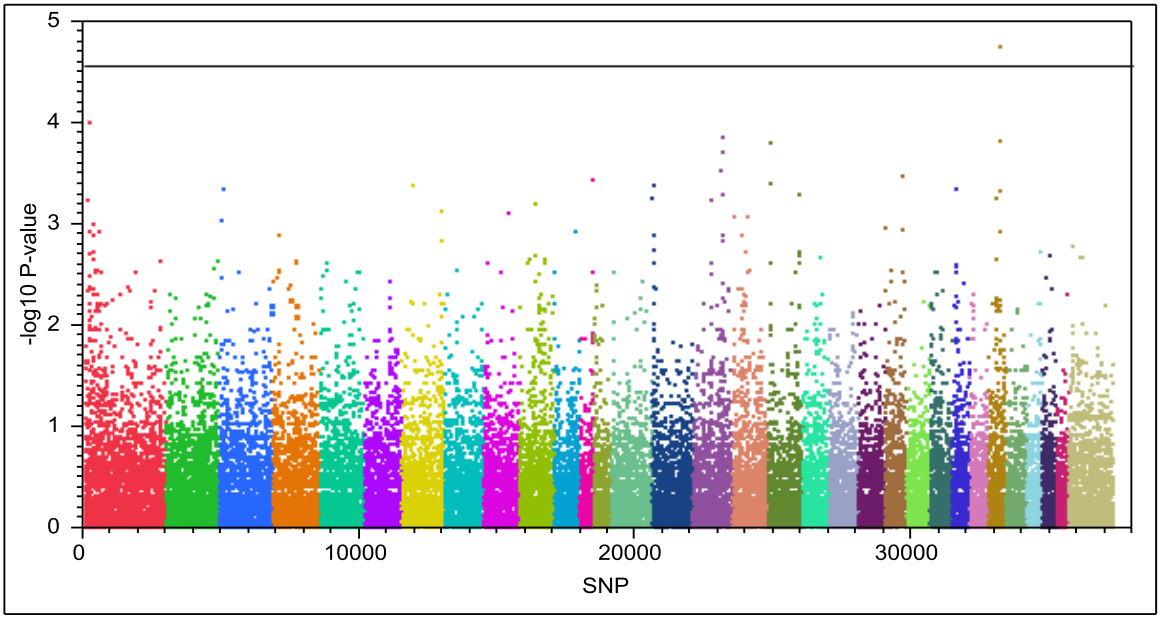
Supplemental Figure 6 Quantile-quantile Plot of Qualitative Association BM (24 horses) vs. PN (41 horses).



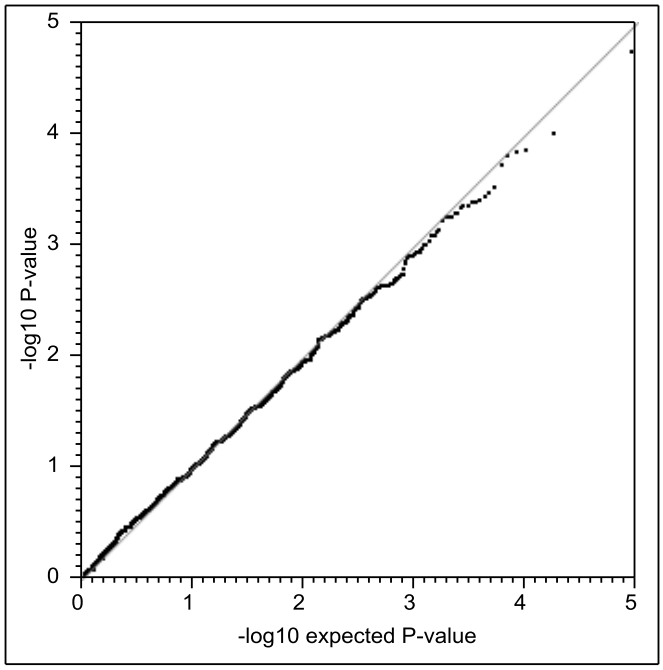
Supplemental Figure 7 Manhattan Plot of Qualitative Association of B (9 horses) vs. PMN (56 horses). Horses with elevated levels of ACTH and insulin compared to normal, healthy controls and those with either ACTH or insulin levels elevated.



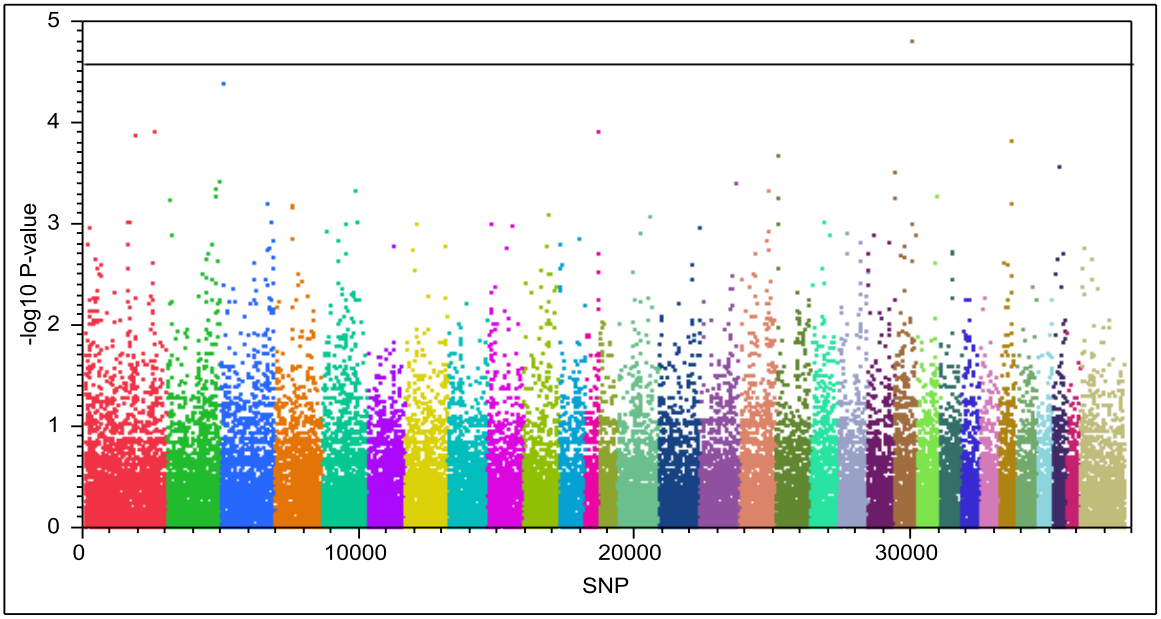
Supplemental Figure 8 Quantile-quantile Plot of Qualitative Association B (9 horses) vs. PMN (56 horses).



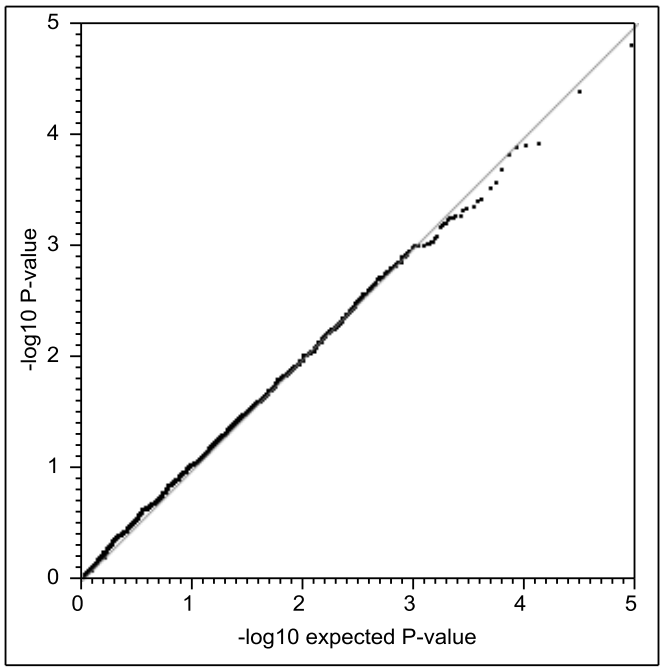
Supplemental Figure 9 Manhattan Plot of Qualitative Association of M (15 horses) vs. N (17 horses). Horses with elevated insulin levels only compared to normal, healthy controls.



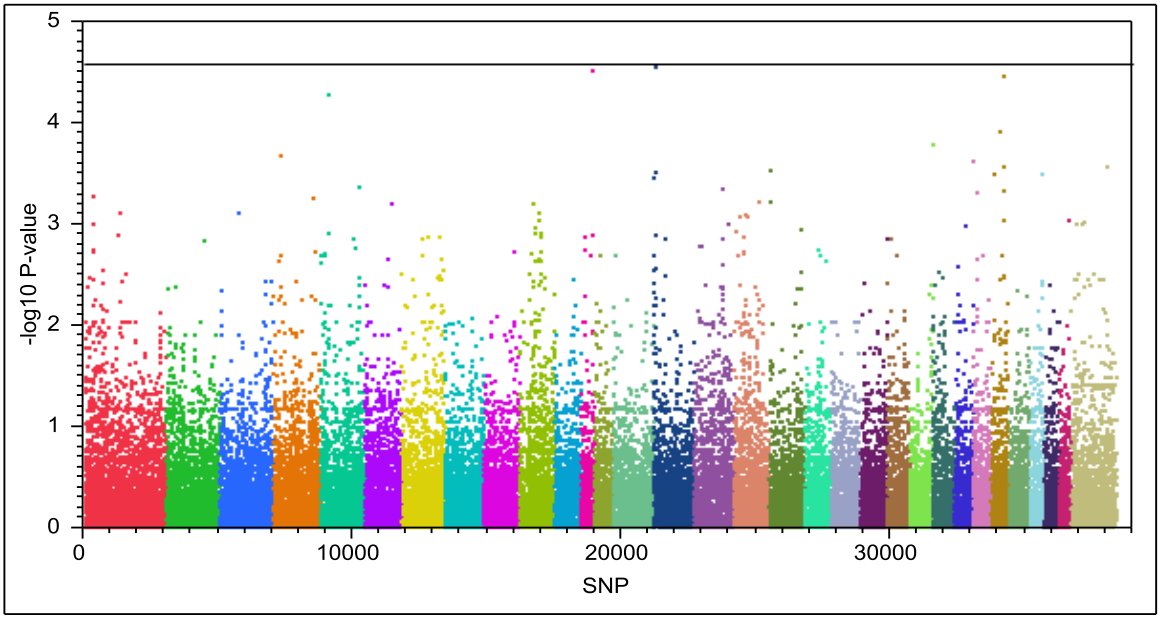
Supplemental Figure 10 Quantile-quantile Plot of Qualitative Association M (15 horses) vs. N (17 horses).



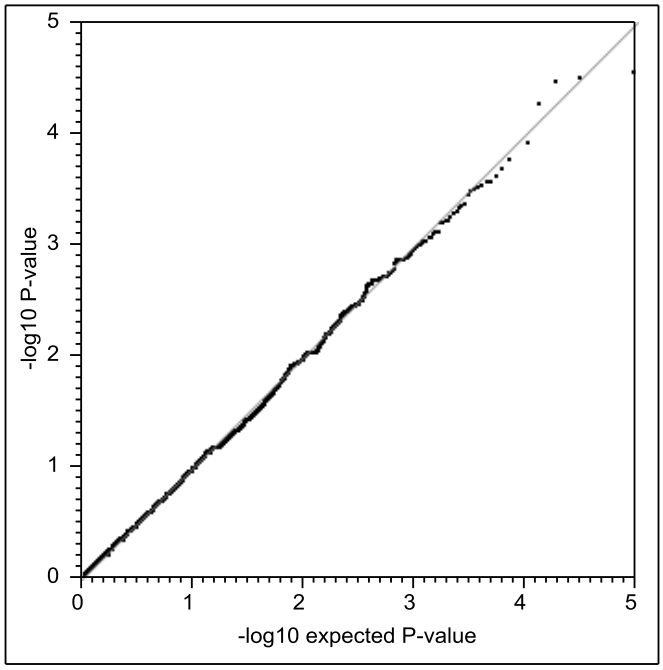
Supplemental Figure 11 Manhattan Plot of Qualitative Association of BM (24 horses) vs. N (17 horses). Horses with elevated insulin levels compared to normal, healthy controls.



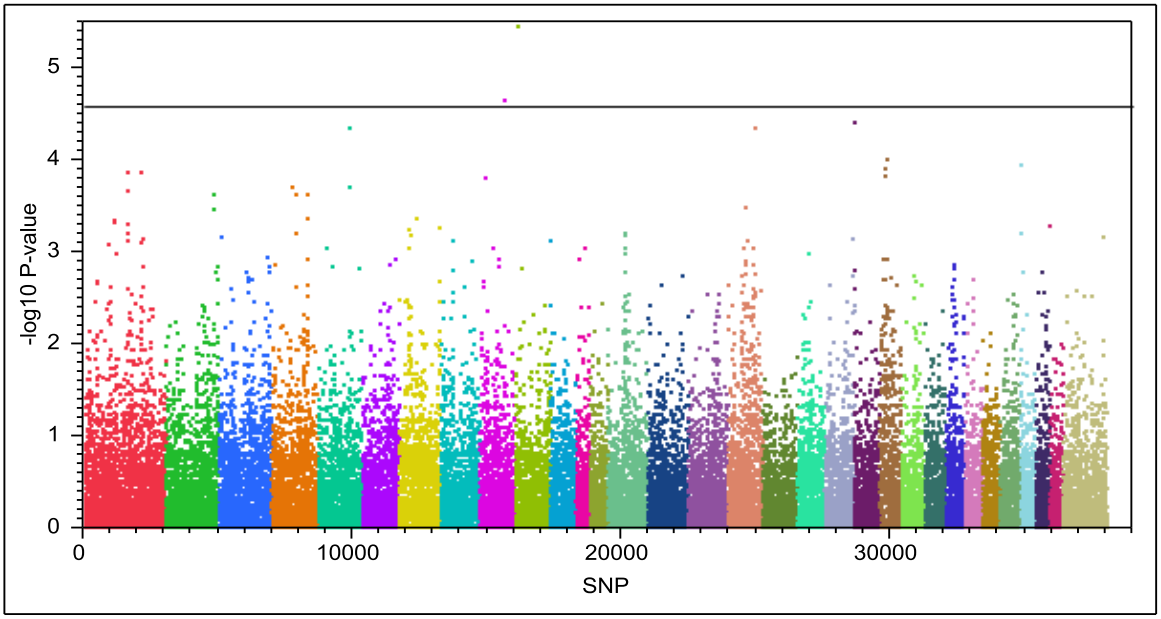
Supplemental Figure 12 Quantile-quantile Plot of Qualitative Association BM (24 horses) vs. N (17 horses).



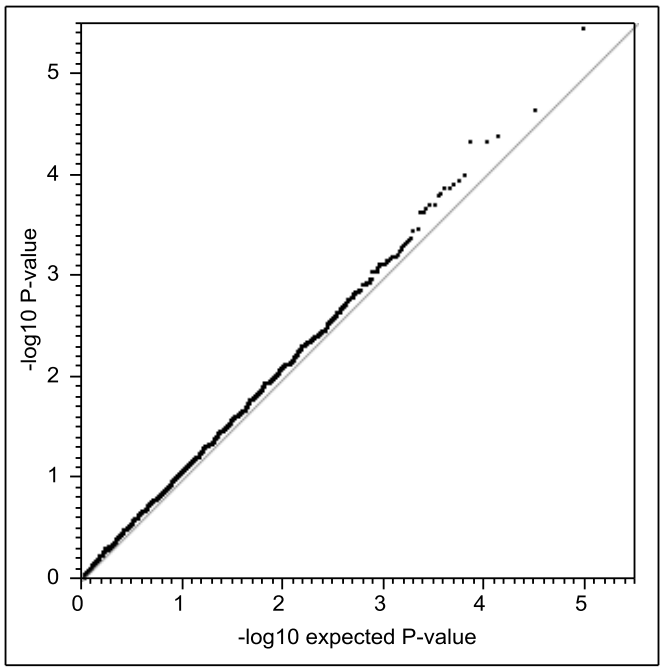
Supplemental Figure 13 Manhattan Plot of Qualitative Association of M (15 horses vs. BPN (50 horses). Horses with elevated insulin levels only compared to healthy horses and those with elevated ACTH levels.



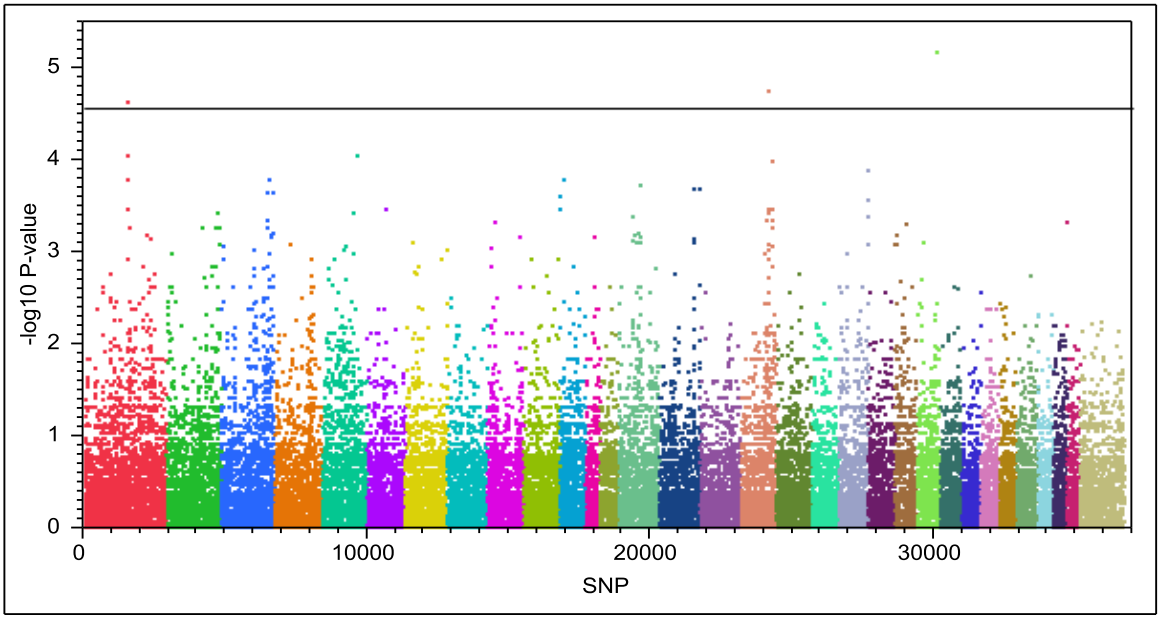
Supplemental Figure 14 Quantile-quantile Plot of Qualitative Association M (15 horses vs. BPN (50 horses).



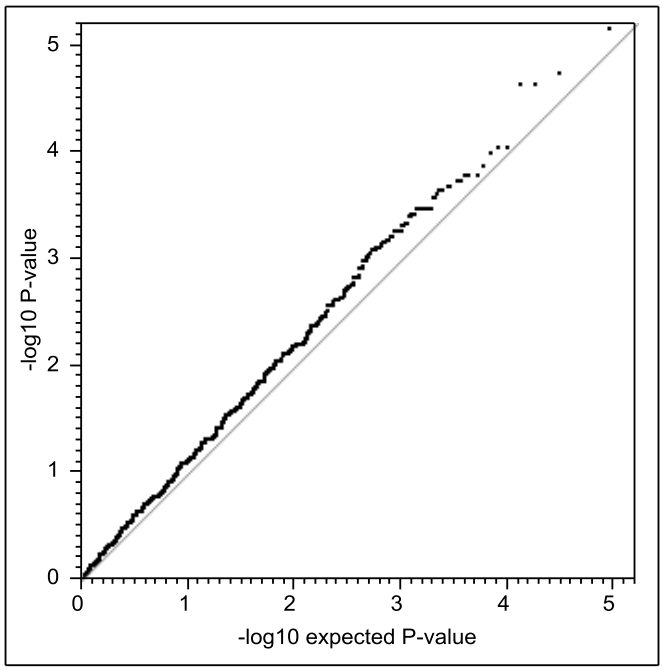
Supplemental Figure 15 Manhattan Plot of Qualitative Association of BP (33 horses) vs. N (17 horses). Horses with elevated ACTH levels compared to normal, healthy controls.



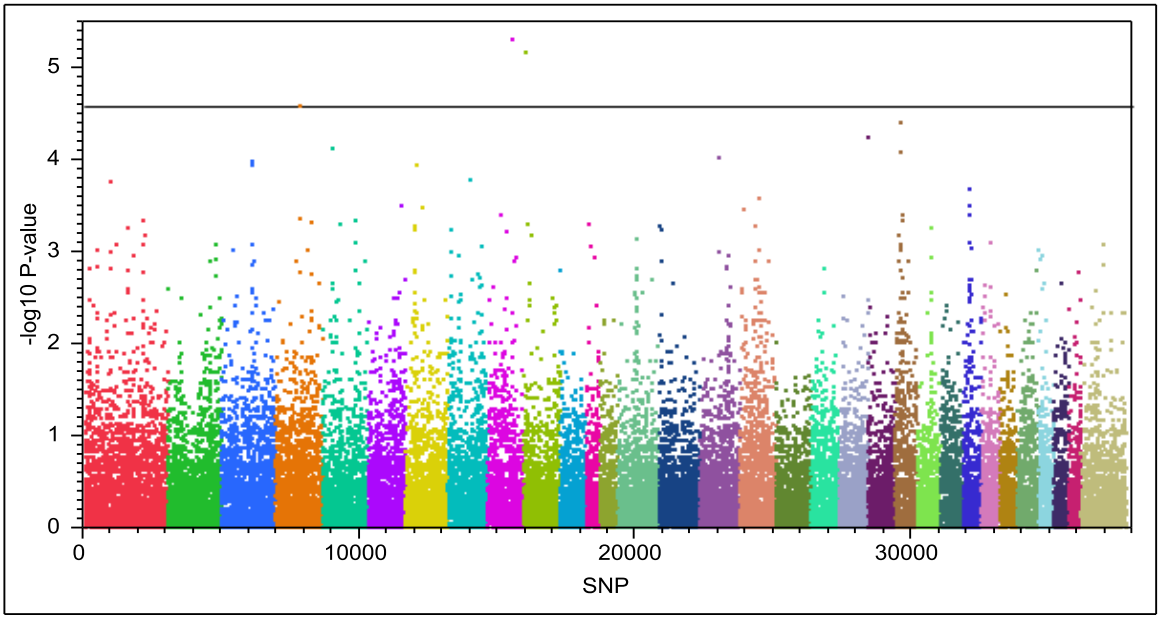
Supplemental Figure 16 Quantile-quantile Plot of Qualitative Association BP (33 horses) vs. N (17 horses).



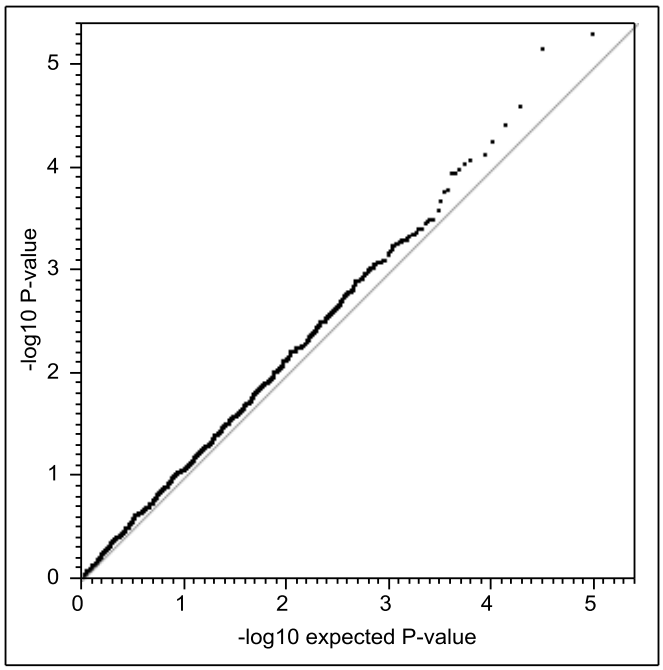
Supplemental Figure 17 Manhattan Plot of Qualitative Association of B (9 horses) vs. N (17 horses). Horses with elevated levels of ACTH and insulin compared to normal, healthy controls.



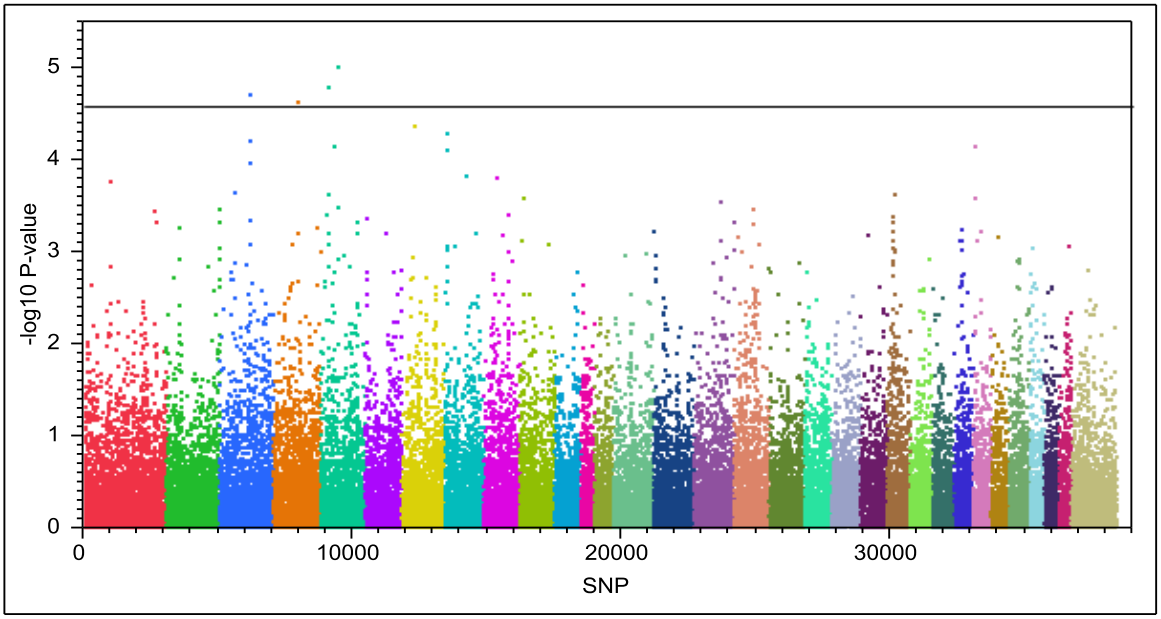
Supplemental Figure 18 Quantile-quantile Plot of Qualitative Association B (9 horses) vs. N (17 horses).



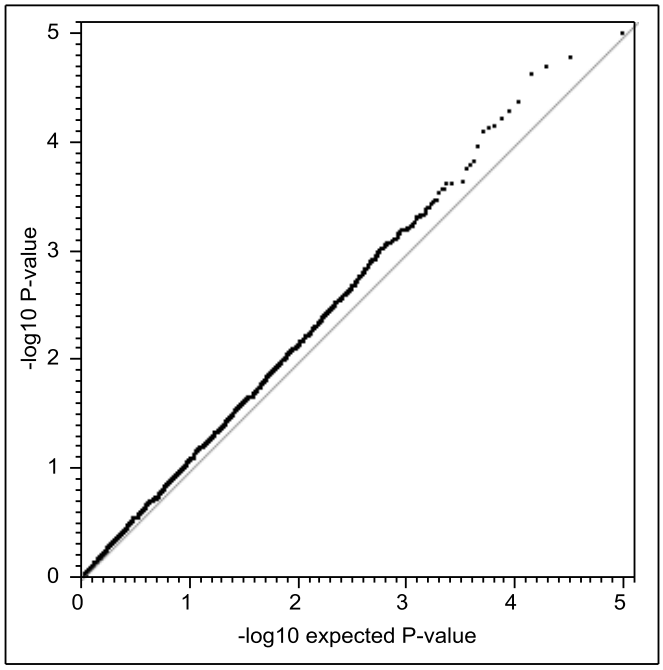
Supplemental Figure 19 Manhattan Plot of Qualitative Association of P (24 horses) vs. N (17 horses). Horses with elevated ACTH levels only compared to normal, healthy controls.



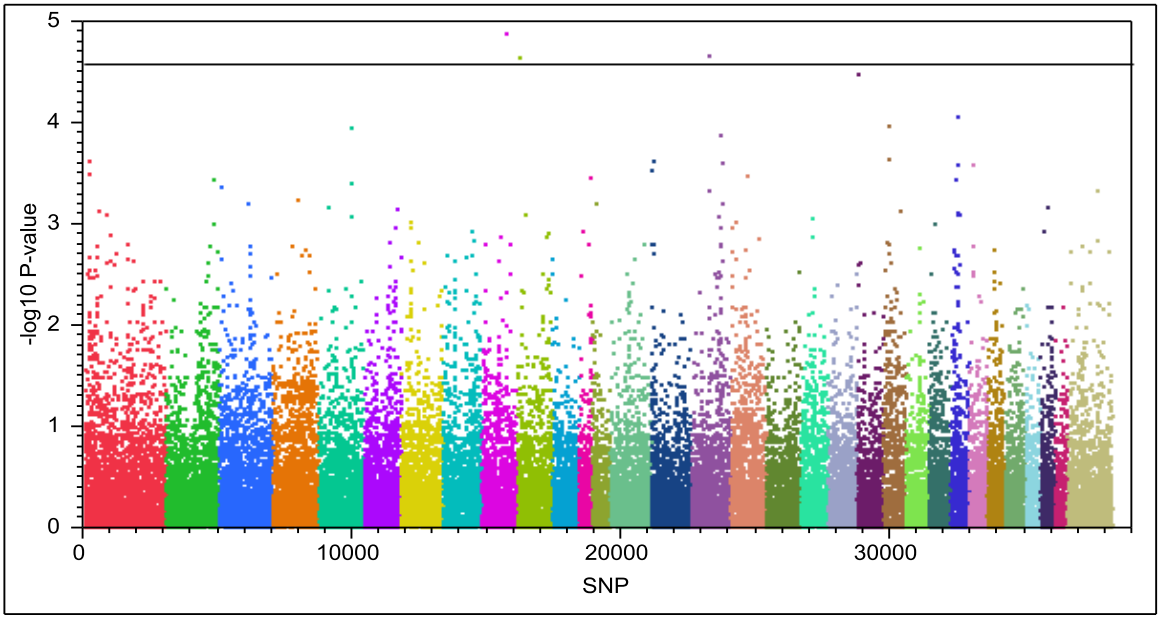
Supplemental Figure 20 Quantile-quantile Plot of Qualitative Association P (24 horses) vs. N (17 horses).



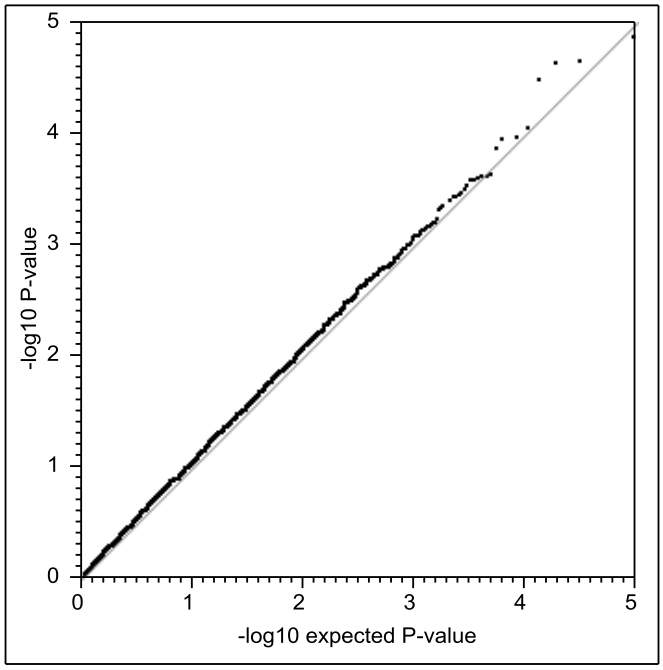
Supplemental Figure 21 Manhattan Plot of Qualitative Association of P (24 horses) vs. BMN (41 horses). Horses with elevated ACTH levels only compared to normal, healthy controls and those with elevated insulin levels.



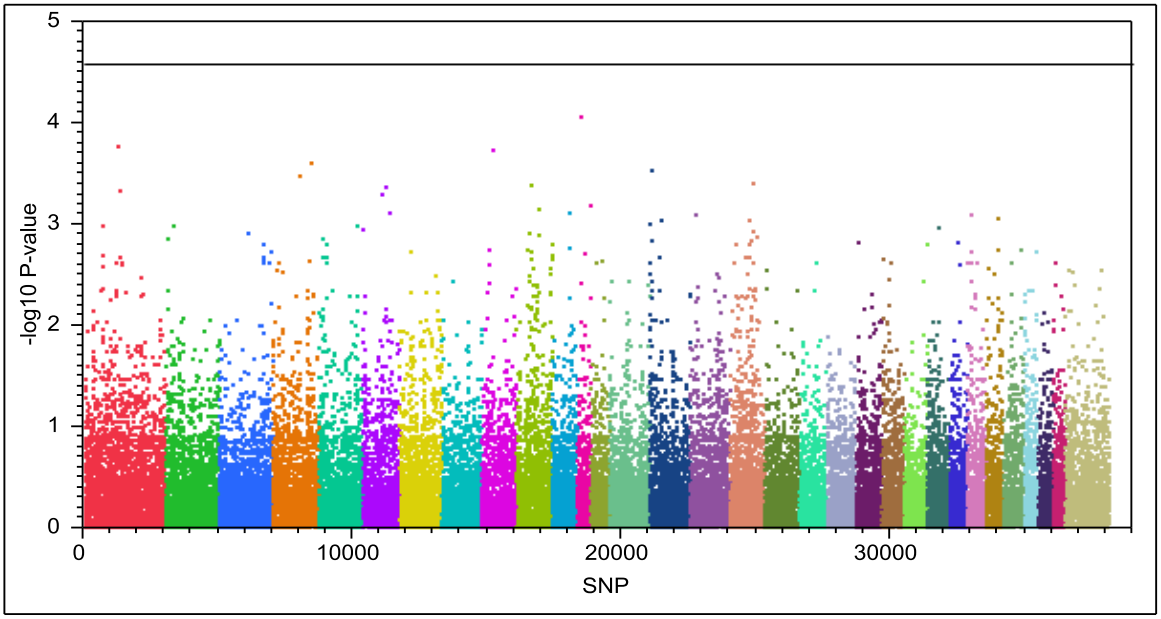
Supplemental Figure 22 Quantile-quantile Plot of Qualitative Association P (24 horses) vs. BMN (41 horses).



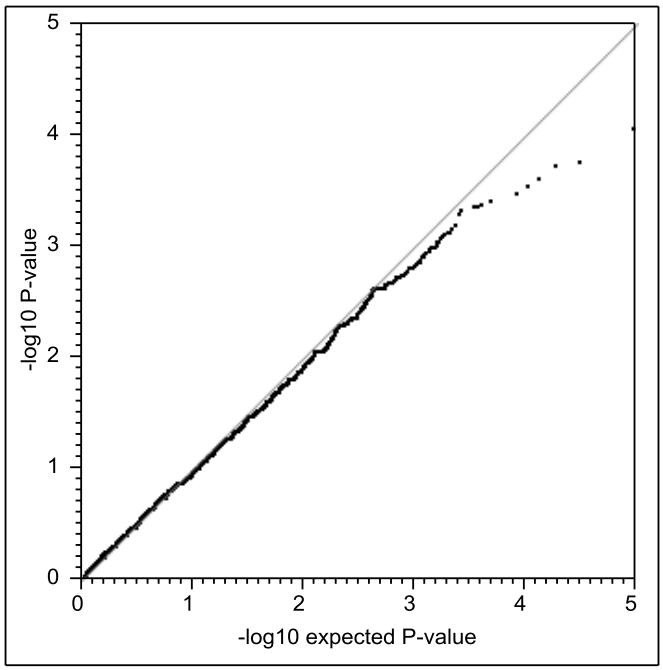
Supplemental Figure 23 Manhattan Plot of Qualitative Association of PM (39 horses) vs. N (17 horses). Horses with either elevated ACTH or insulin levels only compared to normal, healthy controls.



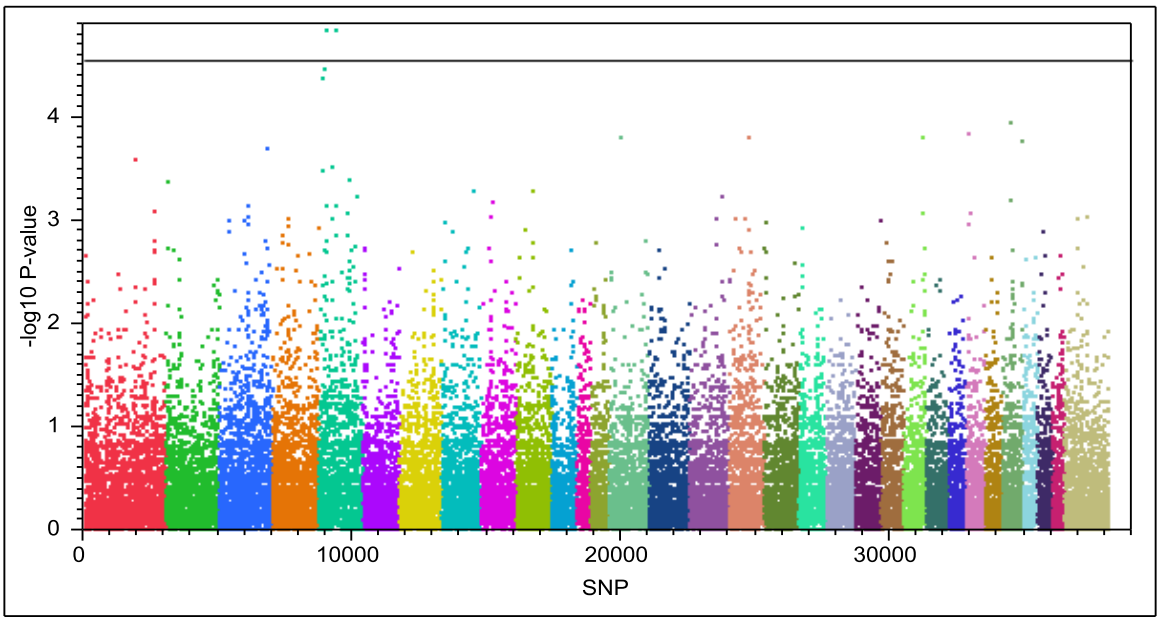
Supplemental Figure 24 Quantile-quantile Plot of Qualitative Association PM (39 horses) vs. N (17 horses).



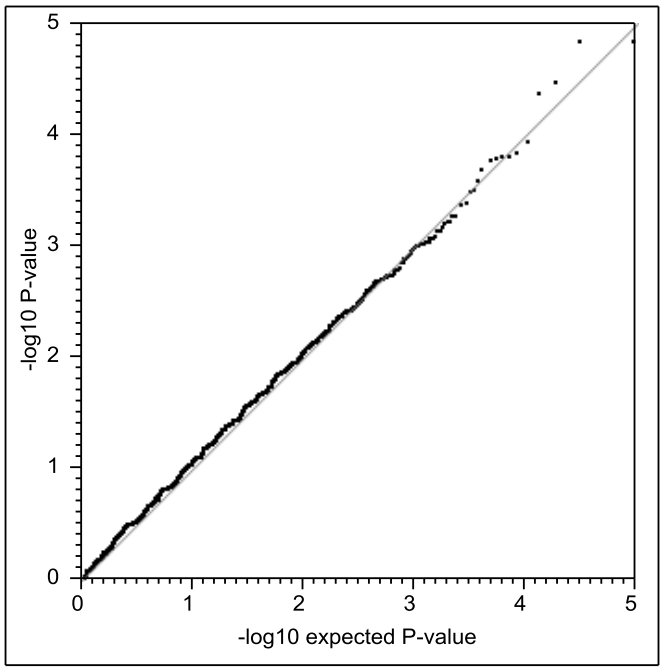
Supplemental Figure 25 Manhattan Plot of Qualitative Association of BP (33 horses) vs. M (15 horses). Horses with elevated ACTH levels compared to those with elevated insulin levels only.



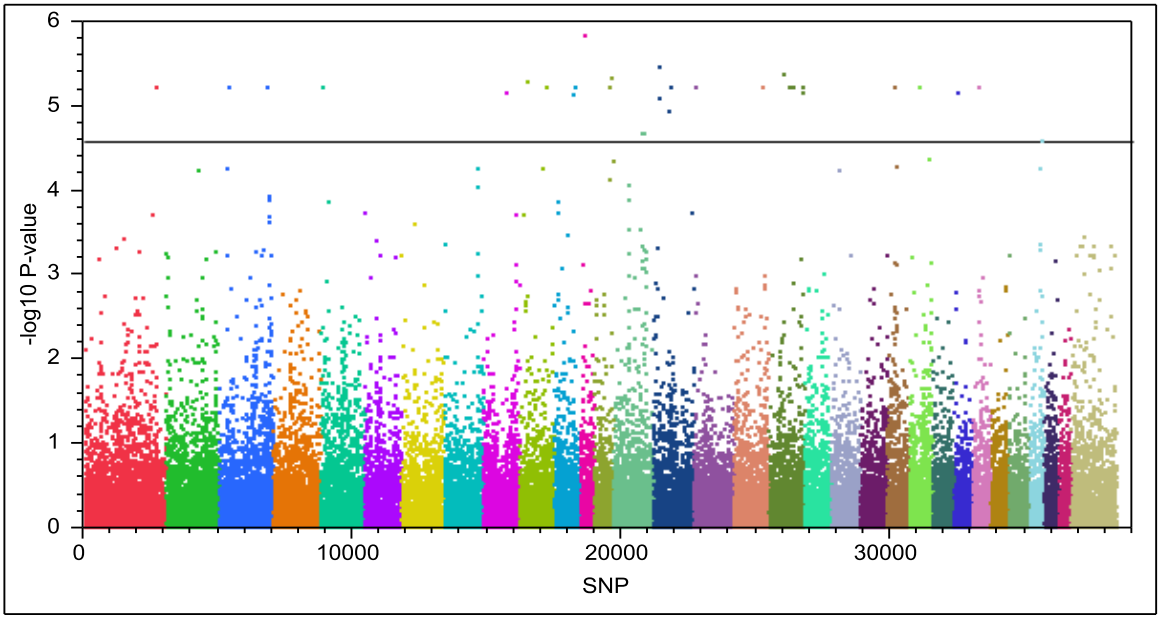
Supplemental Figure 26 Quantile-quantile Plot of Qualitative Association BP (33 horses) vs. M (15 horses).



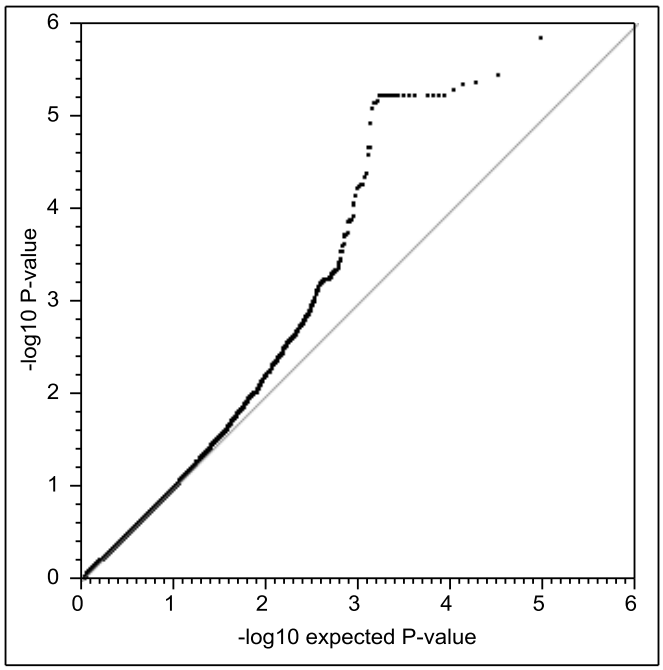
Supplemental Figure 27 Manhattan Plot of Qualitative Association of BM (24 horses) vs. P (24 horses). Horses with elevated insulin levels compared to those with elevated ACTH levels only.



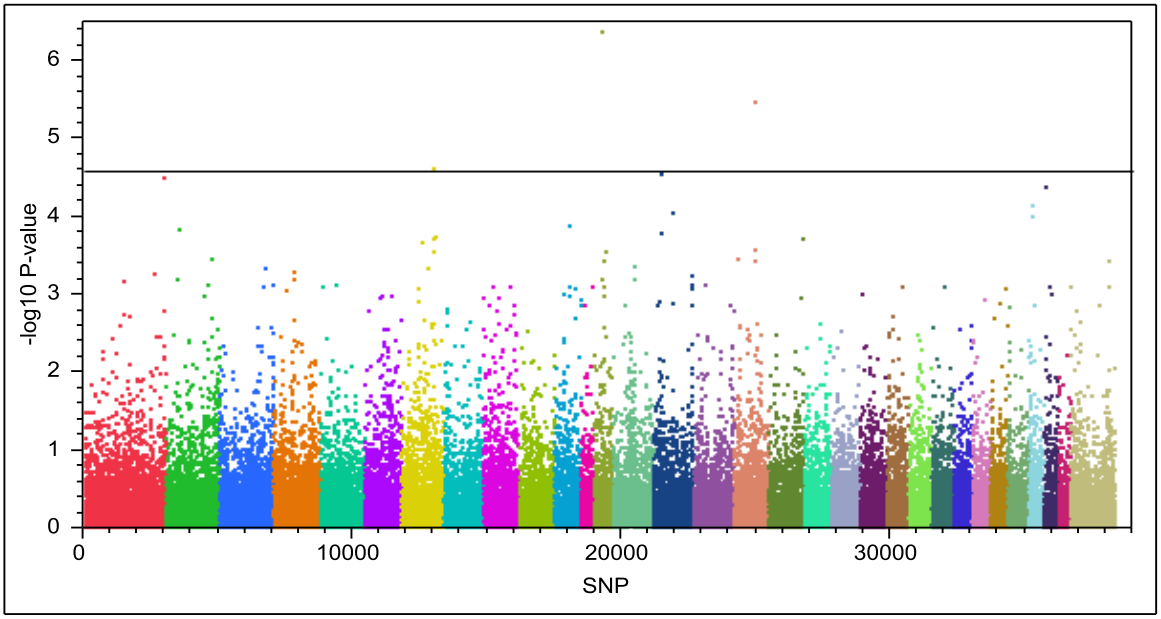
Supplemental Figure 28 Quantile-quantile Plot of Qualitative Association BM (24 horses) vs. P (24 horses).



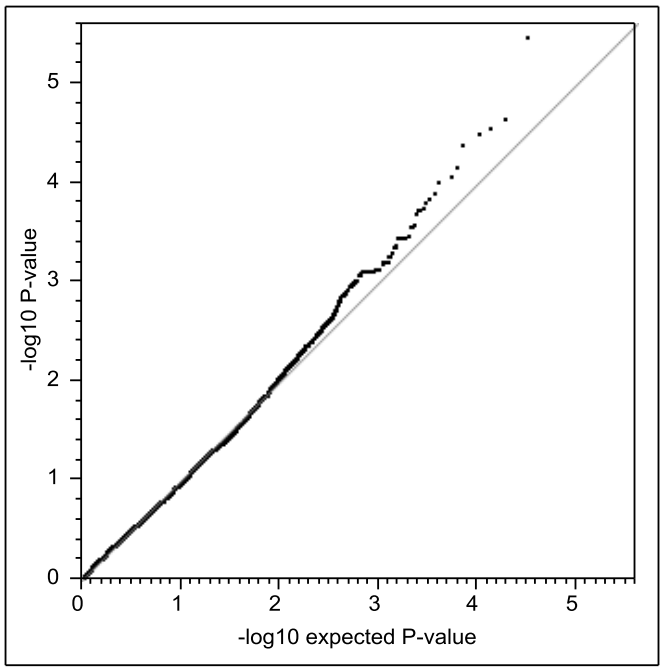
Supplemental Figure 29 Manhattan Plot of Quantitative Association with Highest Recorded Insulin.



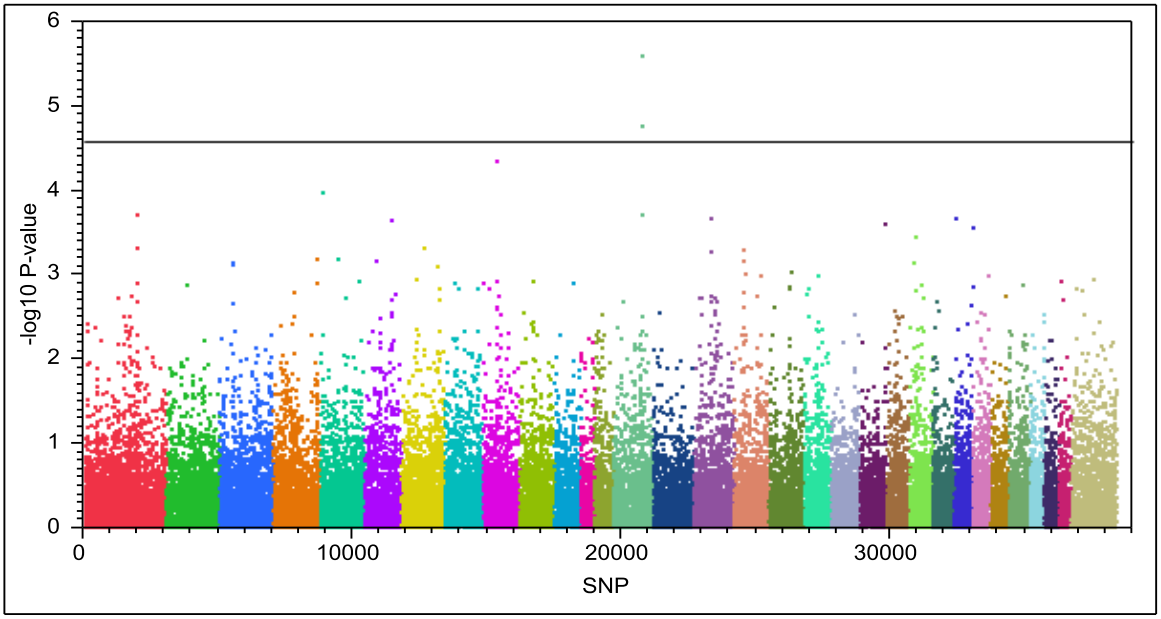
Supplemental Figure 30 Quantile-quantile Plot of Quantitative Association with Highest Recorded Insulin.



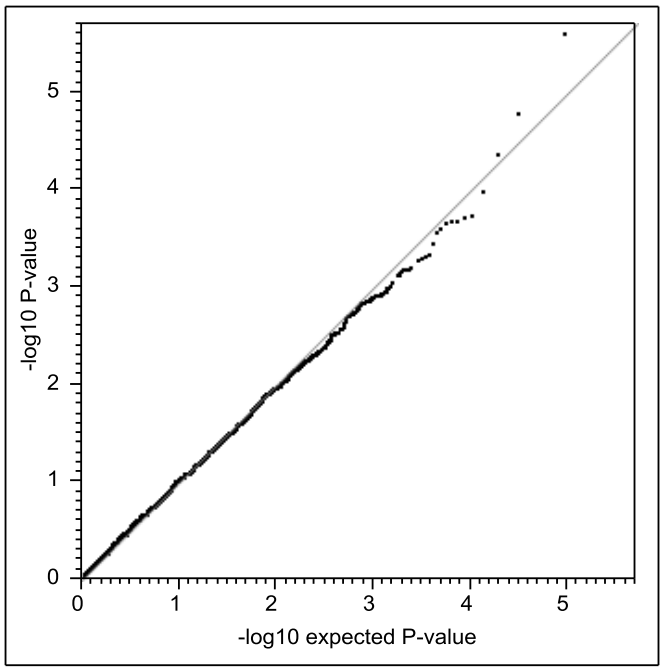
Supplemental Figure 31 Manhattan Plot of Quantitative Association with Highest Recorded ACTH.



Supplemental Figure 32 Quantile-quantile Plot of Quantitative Association with Highest Recorded ACTH.



Supplemental Figure 33 Manhattan Plot of Qualitative Association with History of Laminitis (26 cases, 39 controls).



Supplemental Figure 34 Quantile-quantile Plot of Quantitative Association with History of Laminitis (26 cases, 39 controls).

Supplemental Table 1 Table of Significant SNPs from All Associations

Association	N cases/ controls	# Loci/ Locus info	Total sig SNPs	Most significant	Best P-value	Nearby Genes
BPMvN	48/17	3	4			
		ECA5 73.1Mb	2	BIEC2-918417	1.83 e-5	HFM1
		ECA10 4.6Mb	1	BIEC2-95500	1.13 e-5	PEPD
		ECA21 1.7Mb	1	BIEC2-547188	2.53 e-5	Eps1511 CALR3 CHERP
BPvMN	33/32	0	0			
BMvPN	24/41	1	1			
		ECA5 76.2Mb	1	BIEC2-919693	2.1 e-5	No gene
BvPMN	9/56	10	10			
		ECA5 22.6Mb	1	BIEC2-898132	7.11 e-6	HMCN1 AGPAT2
		ECA5 22.9Mb	1	BIEC2-898144	1.09 e-5	No gene
		ECA5 39.7Mb	1	BIEC2-906312	1.1 e-5	CD1D
		ECA5 65.0Mb	1	BIEC2-914202	1.34 e-5	CXorf40B
		ECA13 33.3Mb	1	BIEC2-232141	1.75 e-5	CLEC16A CG12753
		ECA14 87.3Mb	1	BIEC2-273090	1.08 e-5	AP3B1
		ECA14 92.1Mb	1	BIEC2-276910	1.47 e-5	No gene
		ECA15 78.7Mb	1	BIEC2-321447	1.26 e-5	No gene
		ECA16 65.0Mb	1	BIEC2-356433	1.24 e-5	DAZL
		ECAX 44.8Mb	1	BIEC2-1123081	1.11 e-5	HUWE1 MIRLET7F2 MIR98
MvN	15v17	1	1			
		ECA27 28.1Mb	1	BIEC2-713528	1.83 e-5	No gene
BMvN	24/17	1	1			
		ECA22 38.8Mb	1	BIEC2-596175	1.6 e-5	BCAS4 PARD6B
MvBPN	15/50	0	0	N/A	N/A	N/A
BPvN	33/17	2	2			

		ECA9 58.8Mb	1	BIEC2-1097793	2.26 e-5	SFT2D1
		ECA10 4.6Mb	1	BIEC2-95500	3.63 e-6	PEPD
BvN	9/17	4	4			
		ECA1 99.6Mb	1	BIEC2-42874	2.38 e-5	No gene
		ECA1 99.6Mb	1	BIEC2-42897	2.38 e-5	No gene
		ECA17 63.4Mb	1	BIEC2-382291	1.82 e-5	GPC6
		ECA23 48.8Mb	1	BIEC2-626348	7.01 e-6	No gene
PvN	24/17	3	3			
		ECA4 57.8Mb	1	BIEC2-867420	2.58 e-5	SKAP2
		ECA9 58.8Mb	1	BIEC2-1097793	5.11 e-6	SFT2D1
		ECA10 4.6Mb	1	BIEC2-95500	7.07 e-6	PEPD
PvBMN	24/41	4	4			
		ECA3 68.9Mb	1	BIEC2-789598	2.03 e-5	No gene
		ECA4 57.8Mb	1	BIEC2-867420	2.36 e-5	SKAP2
		ECA5 18.9Mb	1	BIEC2-897199	1.69 e-5	CACNA1E
		ECA5 39.7Mb	1	BIEC2-906312	1.01 e-5	CD1D
PMvN	39/17	3	3			
		ECA9 58.8Mb	1	BIEC2-1097793	1.37 e-5	SFT2D1
		ECA10 4.6Mb	1	BIEC2-95500	2.32 e-5	PEPD
		ECA16 40.8Mb	1	BIEC2-342954	2.23 e-5	LZTFL1 SLC6A20 CCR9
BPvM	33/15	0	0	N/A	N/A	N/A
BMvP	24/24	2	2			
		ECA5 18.9Mb	1	BIEC2-897199	1.47 e-5	CACNA1E
		ECA5 39.7Mb	1	BIEC2-906312	1.49 e-5	CD1D
Highest Insulin	N/A	30	38			

		ECA 1 164.2Mb	2	BIEC2-78264	6.10 e-6	No gene
		ECA3 17.1Mb	1	BIEC2-773422	6.10 e-6	TK2 CKLF CMTM1 CMTM2 BEAN1
		ECA3 106.8Mb	2	BIEC2-808839	6.10 e-6	LDB1 LDB2
		ECA5 6.1Mb	1	BIEC2-890054	6.10 e-6	SELP SELL
		ECA9 53.2Mb	2	BIEC2-1094996	6.92 e-6	TMEM74
		ECA10 16.4Mb	1	BIEC2-104865	5.18 e-6	PGLYRP1 CCDC61 NOVA2 EBAG9
		ECA10 65.9Mb	1	BIEC2-130168	6.10 e-6	DCBLD1 GOPC
		ECA11 44.7Mb	1	BIEC2-155077	7.28 e-6	VPS53
		ECA11 48.9Mb	1	BIEC2-157582	6.10 e-6	No gene
		ECA12 9.3Mb	1	BIEC2-173131	1.48 e-6	ALX4 PRRX2
		ECA13 37.7Mb	1	BIEC2-235337	6.10 e-6	No gene
		ECA13 39.8Mb	1	BIEC2-235746	4.67 e-6	FLYWCH1 KREMEN2 PAQR4 PKMYT1 CLDN6
		ECA14 69.3Mb	1	BIEC2-263524	2.18 e-5	No gene
		ECA14 75.6Mb	1	BIEC2-266578	2.15 e-5	No gene
		ECA15 14.9Mb	1	BIEC2-289514	3.58 e-6	ANAPC1
		ECA15 15.0Mb	1	BIEC2-289674	8.41 e-6	MERTK
		ECA15 32.2Mb	1	BIEC2-301455	1.20 e-5	TGFA
		ECA15 40.6Mb	1	BIEC2-304398	6.10 e-6	BCL11B
		ECA16 4.6Mb	2	BIEC2-328336	6.10 e-6	TMEM40 RAF1

		ECA17 65.2Mb	1	BIEC2-382723	6.10 e-6	ABCC4
		ECA18 35.0Mb	2	BIEC2-410728	4.33 e-6	No gene
		ECA18 48.1Mb	1	BIEC2-412288	6.10 e-6	No gene
		ECA18 55.2Mb	2	BIEC2-414366	6.10 e-6	FUCA1
		ECA18 78.7Mb	2	BIEC2-421249	6.10 e-6	No gene
		ECA18 81.4Mb	1	BIEC2-421894	7.11 e-6	No gene
		ECA22 16.4Mb	1	BIEC2-583538	6.10 e-6	No gene
		ECA23 28.6Mb	1	BIEC2-622442	6.10 e-6	PPIB
		ECA25 8.2Mb	1	BIEC2-657342	7.15 e-6	ZFP933
		ECA26 12.3Mb	2	BIEC2-684068	6.10 e-6	ROBO2
		ECA29 26.8Mb	1	BIEC2-761473	2.57 e-5	SFMBT2
Highest ACTH	N/A	3	3			
		ECA7 76.9Mb	1	BIEC2-1007280	2.40 e-5	OLFML1 SYT9 PPFIBP2
		ECA13 20.2Mb	1	BIEC2-215377	4.34 e-7	XPO6
		ECA17 50.8Mb	1	BIEC2-377685	3.55 e-6	No gene
Laminitis	26/39	2	2			
		ECA14 69.1Mb	1	BIEC2-263373	1.73 e-5	FAM174A
		ECA14 69.3Mb	1	BIEC2-263524	2.56 e-6	No gene