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Metabogenomics reveals four candidate regions involved in the pathophysiology of Equine Metabolic Syndrome.

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Abstract

An analogous condition to human metabolic syndrome, Equine Metabolic Syndrome (EMS) is defined by several clinical signs including obesity, hyperinsulinemia, and peripheral insulin dysregulation (ID). Affected horses may also exhibit hypertension, hyperlipemia and systemic inflammation. Measures of ID typically comprise the gold-standard for diagnosis in veterinary care. Yet, the dynamic nature of insulin homeostasis and complex procedures of typical assays make accurate quantification of ID and EMS challenging. This work aimed to investigate new

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Conflicts of Interest Statement - Declarations of interest: none

Ethical Statements

Procedures in living animals were limited to the collection of blood by jugular venipuncture or hairs pulled from the mane or tail. All samples were voluntarily submitted to the Brooks Laboratory (Gainesville, FL; according to protocol approved by the University of Florida Institutional Animal Care and use Committee (protocol number 201408459).

Data Availability

The metabolomics dataset can be accessed at the NIH Common Fund's National Metabolomics Data Repository (NMDR) website, the Metabolomics Workbench, <https://www.metabolomicsworkbench.org> where it has been assigned Project ID PR000197. Genotyping data is hosted at Mendeley Data (<https://data.mendeley.com/datasets/zs5h47xsxc/1>) data with the Project DOI: 10.21228/M8XK51. This work was supported by NIH grant U2C-DK119886.

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strategies for identification of biochemical markers and correlated genes in EMS. To quantify EMS risk within this population, we utilized a composite score derived from nine common diagnostic variables. We applied a global liquid chromatography/mass spectroscopy approach (HPLC/MS) to whole plasma collected from 49 Arabian horses, resulting in 3392 high-confidence features and identification of putative metabolites in public databases. We performed a genome wide association analysis with genotypes from the 670k Affymetrix Equine SNP array utilizing EMS-correlated metabolites as phenotypes. We discovered four metabolite features significantly correlated with EMS score ($P < 1.474 \times 10^{-5}$). GWAs for these features results ($P = 6.787 \times 10^{-7}$, Bonferroni) identified four unique candidate regions ($r^2 > 0.4$) containing 63 genes. Significant genomic markers capture 43.52% of the variation in the original EMS score phenotype. The identified genomic loci provide insight into the pathways controlling variation in EMS and the origin of genetic predisposition to the condition. Rapid, feasible and accurate diagnostic tools derived from metabogenomics can be translated into measurable benefits in the timeline and quality of preventative management practices to preserve health in horses.

Keywords

Arabian horse; metabolic syndrome; metabogenomics; laminitis; horses

Introduction

One of the most impactful diseases in the modern world [1], obesity and metabolic syndrome are not exclusive to the human species. Many domestic horses suffer from Equine Metabolic Syndrome (EMS), a sum of risk factors leading to traumatic and sometimes fatal endocrinopathic laminitis (inflammation of the hoof lamellae). The consistent feature of this complex syndrome is insulin dysregulation (ID), a multifactorial condition that can present as insulin resistance (IR), resting and post-prandial hyperinsulinemia, and hyperglycemia, and is often accompanied by hypertriglyceridemia [2, 3].

Although hyperinsulinemia and peripheral insulin resistance are diagnostic hallmarks of EMS, affected horses may also exhibit obesity, hypertension, hyperlipemia and systemic inflammation. Measures of ID typically comprise the prevailing methods of diagnosis in veterinary care [3]. Yet, the dynamic nature of insulin homeostasis and the complex procedures required for definitive diagnosis of ID, like the *combined glucose-insulin test* (CGIT) and *frequently sampled intravenous glucose tolerance test* (FSIGTT), make accurate quantification of ID in the field challenging. The glucose and insulin response post *oral sugar test* (OST) results also differ by breed, therefore accurate diagnostic thresholds also require a breed-specific context [4-6]. Furthermore, heritability of metabolic traits was found to be high for plasma insulin post-OST in Welsh Ponies and adiponectin in Morgan horses, supporting the hypothesis of a breed-specific heritable component in EMS [7]. Individual horses may also possess values within standardized EMS-negative ranges, and yet present all the other hallmarks of EMS [8]. Finally, ID may result from underlying hormonal disfunctions; a consequence, rather than a cause, of the condition [9, 10].

Laminitis is colloquially cited as the second leading cause of euthanasia in the horse and is therefore the most consequential complication of EMS [11]. Underlying endocrinopathy precipitates the majority of cases of laminitis in the US [12], comprising 89% of laminitis patients presenting to a large referral hospital [13]. Of these, EMS was diagnosed in 58% and Pituitary Pars Intermedia Dysfunction (*aka* PPID or Equine Cushings disease) in 31% [13]. In the South American Criollo, 44% of reported laminitis cases are secondary to EMS [14].

Due to the complex nature of EMS, a syndrome likely impacted by genetic background and diverse environmental components, there is notable advantage in utilization of novel technologies like global metabolomics to investigate this condition. The resulting “metabolomes” encompass a comprehensive spectrum of biochemical components, in contrast to the individual biomarkers commonly utilized in diagnostics. These individual diagnostic biomarkers often possess a breed/population, environment, lifestyle or age bias [6, 8, 15]. Shifts in the metabolome associated with a given physiologic state, known as a “metabotypes” can be causal or consequential of the pre-determined complex disease endpoint. Compared to the complex disease endpoint, metabotype or individual metabolite phenotypes provide detailed and precise biochemical phenotyping, increasing power in genome wide association analysis (GWAs) within relatively small populations [16, 17]. Usage of metabolite ratios as quantitative traits in a GWAs exponentially increases power by reducing dataset variance [18]. Metabolites can also improve our functional understanding of complex biological pathways and how environmental components correlate to the clinical phenotype [17, 18]. Thus, metabolites are excellent candidates for measuring endogenous alterations related to EMS.

A variety of applications for metabolomics techniques are under study in the horse, most often for detection of illicit use of performance enhancing drugs and hormones in equine athletes [19-21]. Indeed, in the horse, previous examination of athletic training-induced alterations included metabolic changes [22]. Preliminary examination of blood plasma by Nuclear Magnetic Resonance (NMR) before and after experimental induction of acute laminitis identified more than 20 candidate molecular markers implicated in regulation of circulating lipids and gut permeability [23]. However, this study was performed in a very small sample set of five horses, and no multiple testing corrections were performed.

Global metabolomics provides unmatched phenotypic information for characterizing biological changes related to endocrine conditions [24, 25]. Given the need for precise phenotyping in a complex syndrome as EMS, this work aimed to examine new strategies for identification of biochemical markers and their correlated genes for utilization in early diagnostics and assessment of susceptibility for EMS. Ultimately the rapid, feasible and accurate diagnostic tools developed from metabolomics could translate into measurable benefits in preventative management practices for EMS and endocrine laminitis.

Material and Methods

Sample Collection and Animals

Fifty registered Arabian horses were recruited for this study, (25 nonpregnant females and 25 neutered males), from 19 farms in north central Florida, with ages ranging from 8 to 34 years old (mean of 16.43 ± 6.1). We utilized the Arabian breed of horse, limiting variation in the genetic background and capitalizing on the relevance of this genetic resource given its contribution to the development of many other light horse breeds. The Arabian also possesses a moderate susceptibility to EMS, supplying an advantageous source of phenotypic diversity, rather than pony breeds many of which are likely fixed for common risk alleles [26-28]. Samples were collected from August to November 2014, between 6 am and 10 am. Horses were housed indoors or on pasture and participated in a diverse spectrum of activities including performance, breeding and/or pleasure. To limit potential carbohydrate interference on insulin and glucose measures, owners were instructed to withhold concentrated feed rations for 12 hours prior to sampling, yet horses were allowed their typical access to forage and water [26, 29].

We sampled blood from the jugular vein, using 10 mL EDTA BD Vacutainers (Becton, Dickinson and Company, Franklin Lakes, NJ) and processed as described in Lewis et al. (2018). In summary, we separated plasma from the cellular component of each blood sample on-site with a mobile centrifuge, within 15 minutes of collection. Once separated, the plasma was immediately flash frozen in a tank of liquid nitrogen and the cellular portion stored on wet ice within 45 minutes of blood draw. The flash frozen plasma samples were then stored at -80°C until processed for diagnostics or metabolomics. DNA extraction from white blood cell pellets was performed according to the manufacturer's directions in the Gentra PureGene Blood Core kit (Gentra Systems Inc., Minneapolis, MN). A comprehensive EMS score for each horse was generated in Lewis et al. (2018), using principal component analysis (PCA) to comprehensively describe variation in nine diagnostic criteria (age, neck circumference to height [NC/H], heart girth to height [HG/H], average body condition score [BCS], plasma-derived ACTH and leptin values, as well as cholesterol and triglycerides and a modified insulin-to-glucose ratio [MIRG]). In short, BCS values were averaged of independent measures by three researchers. Body measurements were collected on established landmarks utilizing a measuring tape [30]. Endocrinology assays were performed at University of Florida Veterinary Diagnostic Research Laboratory (potassium oxalate preserved plasma for glucose testing) and at the Cornell Animal Health Diagnostic Center (EDTA preserved plasma for ACTH, leptin, cholesterol and triglycerides testing). These measurement-based diagnostic criteria also reflect obesity in the horse [31].

Sample Extraction

Plasma samples for metabolomics analysis were fast-frozen in liquid nitrogen for transportation, stored at -80°C until processing by the University of Florida Southeast Center for Integrated Metabolomics (SECIM) shortly after, as follows. One hundred microliters of plasma were spiked with twenty microliters of internal standards solution consisting of L-Tryptophan-D3, L-Leucine-D10, Creatine-D3, Acetoxybenzoic acid-D4, and Succinic acid-D4, Caffeine-D3 (CDN Isotopes, Quebec, Canada) and Salicylic Acid-D4

(Cerilliant, Round Rock, TX). Metabolite extraction was performed by protein precipitation, adding 800 μ L of extraction solution consisting of 8:1:1 Acetonitrile: Methanol: Acetone (individual solvents LC-MS, Optima grade from Fisher Scientific, San Jose, CA) to each sample. Samples were mixed thoroughly, incubated at 4°C for 30 min to allow for proteins to precipitate, and centrifuged at 20,000xg to pellet the proteins. After centrifugation, 750 μ L of supernatant was transferred into a clean tube and dried under a gentle stream of nitrogen at 30°C (Organomation Associates, Inc., Berlin, MA). Samples were then reconstituted with 100 μ L of LC-MS Optima Water with 0.1% formic acid (Fisher Scientific, San Jose, CA), mixed, and incubated at 4°C for 10-15 min. After, samples were centrifuged at 20,000xg and the supernatants transferred into LC-vials for LC-MS analysis.

Liquid chromatography–mass spectrometry (LC-MS) Analysis

Untargeted LC-MS metabolomic analysis was performed on a Thermo Q-Exactive Orbitrap mass spectrometer equipped with a Dionex UPLC system (Thermo, San Jose, CA). Separation was achieved on an ACE 18-pfp 100 x 2.1 mm, 2 μ m column (Mac-Mod Analytical, Inc., Chadsford, PA) with mobile phase A as 0.1% formic acid in water and mobile phase B as acetonitrile (Fisher Scientific, San Jose, CA). The gradient was run at a flow rate of 350 μ L/min and consisted of: 0-3 min, 0% B; 3-13 min, 80% B, 13-16 min, 80% B, 16-16.5 min, 0% B. The total run time was 20.5 min. The column temperature was set at 25°C. The injection volume was of 4 μ L for negative and 2 μ L for positive polarity. All samples were analyzed in positive and negative heated electrospray ionization with a mass resolution of 35,000 at m/z 200 as separate injections. The heated-electrospray conditions were: 350°C capillary temperature, 3.5 kV capillary voltage, 40 sheath and 10 arbitrary units auxiliary gas. LC-MS injection was done following a sequence of three blanks, neat QC, pooled QC, 10 randomized samples, blank, neat QC, pooled QC, 10 randomized samples, etc.

LC-MS Data Processing and Quality Control

The percent relative standard deviation of internal standard peak areas were calculated to evaluate extraction and injection reproducibility. The raw files were then converted to mzML using MS Convert (ProteoWizard, Palo Alto, CA). MZmine 2 [32] was used to identify features, deisotope, align features and perform gap filling to fill in any features that may have been missed in the first alignment algorithm. MZmine processing resulted in a total of 6950 detected features. All adducts and complexes were identified and removed from the dataset. The data was searched against SECIM internal retention time metabolite library.

Quality Control (QC) was then performed on the resulting log-transformed features utilizing as thresholds: Coefficient of Variation (CV) - top 10%, Blank Feature Filter (BFF) with 5000 threshold and criterion value of 100, and Bland-Altman (BA) plot utilizing the SECIM-Galaxy toolset [33].

Statistical Analyses and Metabolite Identification

We utilized the SECIM-Galaxy toolset to perform a linear regression between the EMS-score and the peak height for each of the 3392 metabolite features, and the Multiple-Testing adjustment tool for post-hoc correction for multiple comparisons [33, 34]. Significantly

correlated ($P < 0.05$) features were investigated utilizing the MetaboSearch tool [35] in four databases (HMDB, Metlin, MMCD and LipidMaps) by M/Z values with a defective parts-per-million (dppm) threshold of 15 (Suppl. table 1). Features exceeding Bonferroni threshold ($P < 1.474 \times 10^{-5}$) after MTA were individually analyzed for normal distribution, and the non-normally distributed (NEG_1088, POS_969) were natural log transformed in JMP® Pro 14.1.0 prior to GWAs.

Single Nucleotide Polymorphism Genotyping and Genome-Wide Association Analysis

The 49 horses successfully assayed for metabolites were genotyped for single nucleotide polymorphisms (SNPs) using the commercially available Affymetrics Equine 670k array (Affymetrics Inc.) by the genotyping service at GeneSeek, Inc. (Lincoln, NE) and retaining only those markers ranked in the poly-high resolution class. Genotypes were filtered for quality using PLINK version 1.90b3.39 (Purcell and Chang, <https://www.cog-genomics.org/plink/1.9>), retaining only samples with a genotyping rate above 95%, and SNPs with a minor allele frequency and missingness smaller than 5%. Linear regression was performed in PLINK using the `--linear` function. After pruning, a total of 286,013 SNPs and 49 individuals remained. Linkage disequilibrium (LD) based pruning was conducted in PLINK utilizing a 10 kb window size, with a step size of 5 and an r^2 threshold of 0.1, resulting in 73,667 independent markers. Bonferroni correction based on this number of independent markers corresponded to a cutoff of $P = 6.787 \times 10^{-7}$ for GWAs.

Linkage disequilibrium windows were calculated for the best associated SNP for each feature utilizing PLINK (`--chr [chromosome number] --ld-snp [marker name] --ld-window-r2 0 --ld-window 300`). Markers with $r^2 > 0.4$ to the lowest regional p-value defined the boundaries of candidate regions considered for further analysis.

Data Integration and Variant Analysis

Genes from LD-defined candidate regions were annotated utilizing the NCBI *Equus caballus* Annotation Release 103 (accession number GCF_002863925.1). A web-search of scientific literature for terminology and symptomatology related to EMS was performed on the genes and metabolites from the most strongly supported candidate region on ECA19. A gene ontology overrepresentation Fischer's exact test (PANTHER Overrepresentation Test) with Bonferroni correction for multiple testing was performed on resulting genes from individual candidate regions utilizing the PANTHER webtool on the GO Ontology database annotation (release 2019-12-09)[36].

Variant analysis was performed utilizing publicly available sequence data from 16 Arabian horses of diverse backgrounds aligned to EqCab2.0 and EqCab3.0 through the UCSC Variant Annotation Integrator tool [37-40]. To investigate specific feature correlations to the individual original nine diagnostic phenotypes utilized to build the EMS score [31], we employed an ANOVA in JMP® Pro 14.1.0 (SAS Institute Inc., 2017).

Results

Four metabolite features significantly correlate with EMS score

Linear regression detected four metabolite features significantly correlated with EMS score (Table 1). Pearson r correlation of the 74 significant features surpassing a Benjamini-Hochberg (BH) test illustrated relationships between the individual metabolites (Fig. 1). Among the four features significantly correlated to EMS scores (Fig. 2, Table 1), NEG_1088, NEG_3950 and POS_4105 are correlated to one another ($r^2 > 0.5$, cluster a), while POS_969 is correlated only with NEG_1088 (cluster b, $r^2 = 0.53197$) (Table 1).

The first putative metabolite (Table 1) for feature NEG_3950 is carbidopa, an extrinsic compound in humans and mice that inhibits DOPA decarboxylase, influencing the biosynthesis of dopamine and serotonin and increasing their biological availability [41]. The second putative metabolite identification for feature NEG_3950 is porphobilinogen, an intermediate metabolite in the biosynthesis of porphyrins, that derives natural metal complexes found in red blood cells (heme) [42].

NEG_1088 putative metabolites are biochemical components in the glyceroneogenesis, or glycerolipid (GL) metabolism. The GL metabolism is essential for diverse organism functions, from heat production to signaling molecules. Along with the free fatty acid in the glycerolipid/free fatty acid cycle (GL/FFA) provides a source of glucose during fasting [43]. Previous studies implicated disturbances in the GL/FFA cycle in human obesity [44, 45]

Putative metabolites for POS_969 are intermediates biomolecules in purine metabolism. In the murine model, diabetic conditions increase degradation of purine nucleotides, leading to a decrease of ATP and CTP availability in the liver [46]. The major final product of purine metabolism is serum/plasma uric acid, a component previously correlated to MetS in humans and modulated by hepatic insulin levels [47-49].

The 286,013 SNP markers surviving QC filtering revealed little to no population stratification as demonstrated by a multidimensional scaling plot of genomic relatedness (Supplemental Fig. 1). Genome Wide Association identified significant SNPs ($P < 6.787 \times 10^{-7}$) for features NEG_3950 (Fig. 3), POS_969 and NEG_1088 (Supplemental File 1). NEG_3950 shared a significant locus with NEG_1088. ECA 5 held multiple significant candidate regions, including two unique candidate loci on NEG_1088 (EqCab3 LD region, $r^2 > 0.4$, chr5:38312603-38754502 and chr5:39713988-40110108) and a third for feature POS_969 (Table 2, Supplemental Fig. 2).

Candidate Genes and Variant Analysis

Four unique genomic regions were identified by GWAs, containing a total of 63 genes according to the NCBI gene model annotation (Table 2). We chose to focus on the ECA19 candidate region given the strong and well-supported signal of association. Examination of publicly available whole-genome from Arabian horses sequence data found no coding sequence changes or gene-disrupting structural variants [37, 38, 40]. Gene ontology overrepresentation analysis using PANTHER identified significant terms in biological

processes for detection of chemical stimuli involved in “sensory perception of bitter taste” ($P = 7.18 \times 10^{-4}$), as well as “protein insertion into membrane” ($P = 1.61 \times 10^{-3}$).

The top significant SNP markers, representing the four candidate regions, capture 43.52% of the variation in the original EMS score phenotype. To better illustrate the specific diagnostic variables correlated to feature NEG_3950, we examined the relationships of the newly identified features to the original diagnostic criteria used to assemble the composite EMS score. Average BCS was the only significantly correlated criterion for NEG_3950 ($p = 0.0045$, ANOVA) (Supplemental Fig. 4). ACTH, MIRG and insulin are significantly correlated ($p < 0.05$, ANOVA) to feature POS_969 (Supplemental Figures. 2 and 3).

Discussion

Examination of the biochemistry at play has enabled improved understanding of EMS-like conditions in other species and identifies useful biomarkers for early detection of subclinical disease. Analysis of a targeted set of 140 metabolites in humans revealed three specific compounds significantly altered during the pre-diabetic state [50]. Furthermore, after a follow-up sampling, two of these metabolites proved to be predictive for development of type 2 diabetes (T2D) - a condition analogous to EMS - as early as seven years prior to diagnosis. An expanded, global analysis subsequently revealed 42 additional metabolites associated with T2D, only about half of which were previously linked to this condition in the published literature [51]. Utilization of metabolite correlations to phenotypes, or as endophenotypes, in analysis also yielded novel genes and pathways in a canine model for human metabolic syndrome (MetS) [52]. In a small study of 28 overweight (OW) Labrador Retrievers, plasma metabolomics identified lowered carnitine concentrations in the overweight dogs [53].

Combination of a quantitative EMS score with a high-throughput global metabolomics dataset lent better precision than the traditional case/control or group/treatment metabolomics approach, enabling detection of biologically meaningful correlations despite the small sample size of this preliminary study [17, 18]. This approach also increases sensitivity, as the classical methodology relies on qualitative grouping of phenotypes, whereas a quantitative score system captures variation within the population. Yet, some observed metabolites may be environmental byproducts, and could reflect differences within the diet or management of each horse [54]. In some cases, these components can be biologically pertinent to the disease condition, as for microbiome-derived metabolites found in patients at risk for T2D [55].

Feature NEG_3950 had two suggested putative metabolites. Carbidopa, a DOPA decarboxylase inhibitor, has some noteworthy biological effects. The DOPA decarboxylase inhibition activity of carbidopa is utilized to improve the efficacy of levodopa in the treatment of Parkinson's Disease by increasing the quantity of levodopa that is available to cross the blood-brain barrier [56, 57]. There is no published information suggesting horses naturally produce carbidopa, although other intrinsic amino acid decarboxylase enzymes were previously implicated in pasture-induced laminitis [58]. Carbidopa was also previously proposed as a part of a compound drug for laminitis treatment, by reducing amino acid

decarboxylation in the hind gut, a process implicated in peripheral vasoconstriction, though this putative treatment was not investigated [59].

Porphobilinogen, the second putative metabolite for NEG_3950 is a known biomolecular marker utilized as screening test for human acute porphyria and cutaneous porphyria from human urine and plasma. Porphyria refers to a collection of porphyrin disorders, a biological chemical essential for the functioning of the heme complex [60]. In human patients diagnosed with metabolic syndrome (MetS), 76.7% also possess porphyrin metabolic disturbances, lowered porphyrin fractions, as well as increased porphyrin precursors [42]. An association between *porphyria cutanea tarda* (PCT), a disease that causes late-in-life photosensitivity and T2D is well-recognized, but the basis for this relationship is still unclear [61].

Interestingly, horses with more severe disease as measured by higher EMS scores had lower plasma concentrations of the NEG_3950 metabolite. Based on human PCT disease models, higher concentrations of NEG_3950 would be expected in EMS horses. However, in human diabetes, inhibition of the δ -Aminolevulinatase (δ -ALA-D) enzyme leads to impairment of the heme pathway, also causing porphyria [62, 63]. In mice, high fat diets lower δ -ALA-D activity, induce oxidative stress and increase iron absorption, impairing porphobilinogen synthesis [64]. In horses, high-fat diets possess an oxidative effect and increase obesity risk, although no significant effect was observed on resting insulin levels, and the impact of a high-fat diet on iron absorption and the heme pathway were not examined [65, 66]. Thus, both putative identifications of NEG_3950 offer novel and relevant hypotheses for EMS, and further work is needed to definitively identify this key metabolite.

Feature POS_969 putative metabolites are correlated to the oxidation of purines, primarily from endogenous sources, which generates uric acid as final biochemical product [67]. Elevated serum concentrations of uric acid contribute to the development of insulin resistance in humans by inducing oxidative stress within the mitochondria [49]. Furthermore, uric acid also decreases circulating adiponectin levels by triggering oxidative stress within adipocytes, the cell type responsible for adiponectin synthesis [68]. In horses, low adiponectin concentrations are correlated with increased risk of laminitis, obesity and insulin dysregulation [69-71]. Interestingly, decomposing the EMS score into the original diagnostic variables illustrates that the POS_969 metabolite correlates specifically to MIRG, ACTH and insulin values. Due to the biological functions within the purine oxidation pathway containing the most likely feature identifications, feature POS_969 warrants further research to conclusively identify this metabolite.

GL re-esterification of free fatty acids during a low-carb diet or fasting causes obesity and increased body weight, while still maintaining normoglycemia and normoinsulinemia [43, 72]. Lipolysis and GL/FFA is also enhanced during insulin dysregulation [73]. As our sampling did not require fasting or feeding low-carb diets, the feature NEG_1088 metabolite is more likely due to either obesity or insulin dysregulation leading to increased GL [74].

In this study, GWAs of feature NEG_3950 and NEG_1088 resulted in a shared candidate region supported by ten SNPs and spanning six genes on ECA19. These six genes offer a

number of interesting functional hypothesis regarding EMS. *STGAL1* (ST6 beta-galactosamide alpha-2,6-sialyltransferase 1), contributes to human pancreatic beta-cell function in T2D among South Asian-ancestry populations, and contains methylation sites correlated to fat-mass index in European preschool children [75, 76]. Mannan-binding lectin serine peptidase 1 (*MASPI*), also lies within the ECA19 candidate region and was previously associated with the prediabetic state in a human proteomics cohort. The risk of developing prediabetes increased by 77% if *MASPI* protein concentration was elevated in plasma [77]. Transcriptomics of human plasma also significantly correlated prediabetes and obesity to upregulation of *MASPI* mRNA [78]. Essential for lectin pathway activation, and possessing relatively non-specific binding affinity, *MASPI* is hypothesized to influence proinflammatory signaling, particularly in adipose tissue [78, 79]. Due to its binding affinity for sugars, *MASPI* protein is at higher concentrations in plasma during a hyperglycemic state [80]. As *MASPI* plays a role in both inflammation and monosaccharide-binding in hyperglycemia, it is a strong candidate for EMS related processes and deserves further investigation.

Two members of the receptor transporter protein family, *RTP1* and *RTP4*, may play a role in feeding behavior. *RTP1* is a chaperone of odorant receptor proteins, promoting their expression on the cell surface [81]. *RTP4* also increases the expression of genes within the bitter taste receptor family (*TAS2R*) [82]. Horses can distinguish between sweet and bitter substances as evidenced by behavioral data: administration of bitter substances was accompanied by mouth opening and tongue protrusion (gape), while sweet flavors led to head nodding [83]. Altered function of this gene could result in differential presentation of taste receptors and preference for saccharide-based feeds, or a lack of distinction between flavors leading to indiscriminate feeding behavior that ultimately contributes to over-consumption and obesity.

We did not discover any variants within the coding sequence of annotated genes in the ECA19 candidate region. Thus, a polymorphism altering gene regulation likely underlies the EMS phenotype. Notably, just outside of the LD-defined ECA19 candidate region lies the coding sequences for *Adiponectin* on the 5'-end and *Somatostatin* on the 3'-end. Produced by adipose tissue, adiponectin hormone levels are inversely correlated to IR and body fat composition. Plasma adiponectin is considered a sensitive marker for metabolic disease and obesity in humans, and lowered circulating concentrations may indicate risk for laminitis in horses [70, 84]. Somatostatin is a peptide hormone that plays a role in the conversion of proinsulin to insulin. A decrease in plasma somatostatin increases circulating insulin levels, leading to hyperinsulinemia and possibly IR [85]. Variation disrupting the action of a distal enhancer or other regulatory element within the candidate region, altering the expression of either/both of these logical candidate genes, could underly the risk for development of EMS. In humans, a 3' UTR variant (*rs6773957*) in the *ADIPOQ* gene was correlated to higher serum adiponectin levels [86]. High molecular weight adiponectin is recommended as a biomarker for risk and diagnosis of EMS [3]. Availability of transcriptomics data released through the Functional Annotation of Animal Genomes (FAANG) consortium will enable investigation of this hypothesis in the future [87].

One of the strengths of a multi-“omics” approach is the integration of large and hypothesis-neutral datasets to aid in identification of novel and relevant trends among the many possible genetic and environmental components. However, the substantial number of variables resulting from global metabolomics when applied to a small sample set results in a situation where $p > N$, and a consequential increased risk of Type I error. Yet, employing multiple testing corrections and significance thresholds in a small sample set increases chances of a Type II error increase. This is a frequent problem of medical studies, where the number of patients or cases is restricted [88, 89]. While we acknowledge the limitations of this study design, we also utilized the multi-omics approach as a tool to improve identification of biologically relevant metabolites, including strict multiple-testing statistical corrections in each step of the analysis. Resulting putative metabolites and genomic candidate regions are evidence of the success of this approach. Yet, due also to breed-specific components in EMS, further studies in other breeds unrelated to the Arabian, as well as in larger populations of horses are recommended, in addition to improved metabolomics techniques to accurately identify metabolites.

Conclusion

We identified four metabolite features significantly associated with EMS score. No specific identification for feature NEG_3950 and feature POS_969 could be determined from public databases, however, their putative metabolites offer interesting new hypotheses regarding EMS etiology. Genetic markers significantly correlated with feature NEG_3950 spanned a ~576Kb region on ECA19, overlapping six coding genes, four of which contribute to T2D and/or obesity in humans. Thus, through a metabogenomics approach we identified metabolic and genetic components correlated to EMS and provide excellent targets for future EMS research. While promising for the Arabian breed, these newly identified metabolites are candidates for further investigation utilizing a larger sample set and encompassing additional breeds of horse or following individuals through a longitudinal study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights:

- Description of a combination of a quantitative Equine Metabolic Syndrome (EMS) score with a high-throughput global metabolomics dataset and high-density SNP array, enabling detection of biologically meaningful correlations.
- We identified four metabolite features significantly associated with EMS score and four candidate regions and loci providing novel hypothesis into pathways controlling EMS.
- Genetic markers significantly correlated with one of the metabolite features overlap four genes that contribute to type 2 diabetes and/or obesity in humans, providing excellent targets for future EMS research.

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Fig. 2.
Hierarchical clustering of study horses based on plasma concentrations of the four EMS correlated metabolites recapitulates traditional diagnostic categories of EMS-suspect vs. the Healthy and PPID-suspect conditions

Fig. 3.

Genome Wide Association of metabolite feature NEG_3950 to EMS score. (A) GWAs identified 10 significant markers on chromosome 19 ($P = 2.01 \times 10^{-7}$, Bonferroni = solid red line) (B) Quantile-quantile plot demonstrating a genomic inflation factor (λ) of 1.15. (C) SNP markers, color coded by r^2 value, from the chromosome 19 candidate region and annotated genes (NCBI release 103 accession number GCF_002863925.1)

Table 1.

Metabolite features significantly correlated with EMS scores, dppm threshold and respective putative metabolites.

Feature	P-value	Dppm	Putative Metabolites
NEG_3950^a	1.522 x 10 ⁻⁶	1.349	Carbidopa Porphobilinogen
POS_4105^a	3.42 x 10 ⁻⁶	0.607	Porphobilinogen Carbidopa
NEG_1088^{a,b}	1.03 x 10 ⁻⁵	7.673	2-(alpha-D-Galactosyl)-sn-glycerol 3-phosphate 2-(beta-D-glucosyl)-sn-glycerol-3-phosphate 2-(beta-D-Glucosyl)-sn-glycerol 3-phosphate alpha-D-Galactosyl-(1,1')-sn-glycerol 3-phosphate 1-(sn-glycero-3-phospho)-1D-myo-inositol sn-glycero-3-Phospho-1-inositol
POS_969^b	1.33 x 10 ⁻⁵	1.974	5-Ureido-4-imidazole carboxylate 4-Ureido-5-imidazolecarboxylic acid

Correlated features ($r^2 > 0.5$) are noted by superscript letters (a, b).

Table 2.

Candidate Region Annotation for EMS-significant metabolite features.

FEATURE				
	NEG_3950 & NEG_1088	POS_969	NEG_1088	
EqCab2.0				
	chr19:24871458-25447414	chr5:78226651-80159121	chr5:41701370-42144464	chr5:43105321-43502407
EqCab3.0				
	chr19:27276058-27851899	chr5:75169395-77101830	chr5:38312603-38754502	chr5:39713988-40110108
GENES	<i>ST6GAL1</i>	<i>CCN2</i>	<i>IQGAP3</i>	<i>KCNN3</i>
	<i>RTP1</i>	<i>DDAH1</i>	<i>MEF2D</i>	<i>ADAR</i>
	<i>MASP1</i>	<i>BCL10</i>	<i>C1orf61</i>	<i>UBE2Q1</i>
	<i>RTP4</i>	<i>SYDE2</i>	<i>MIR9-1</i>	<i>TDRD10</i>
	<i>SSC</i>	<i>WRD63</i>	<i>RHBG</i>	<i>SHE</i>
	<i>BLC6</i>	<i>C1orf52</i>	<i>CCT3</i>	<i>CHRNA2</i>
		<i>MCOLN3</i>	<i>GLMP</i>	<i>CHRNA3</i>
		<i>MCOLN2</i>	<i>TMEM79</i>	<i>UBE2Q1-AS1</i>
		<i>LPAR3</i>	<i>PMF1-BHLAP</i>	<i>IL6R</i>
		<i>SSX2IP</i>	<i>UBQLN4</i>	<i>ATP8B2</i>
		<i>Ctbs</i>	<i>LAMTOR2</i>	<i>HAX1</i>
		<i>SPATA1</i>	<i>LMNA</i>	<i>UBAP2L</i>
		<i>GNG5</i>	<i>SLC25A44</i>	<i>SNORA58</i>
		<i>RPF1</i>	<i>SMG5</i>	<i>C1orf43</i>
		<i>DNASE2B</i>	<i>PAQR6</i>	<i>C1orf189</i>
		<i>UOX</i>	<i>CCT3</i>	
		<i>SAMD13</i>	<i>TSACC</i>	
		<i>PRKACB</i>	<i>PMF1</i>	
		<i>TTLL7</i>	<i>SEMA4A</i>	
			<i>ARHGEF2</i>	
			<i>MEX3A</i>	
			<i>RAB25</i>	
			<i>SSR2</i>	