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Metabolic perturbations in Welsh Ponies with insulin dysregulation, obesity, and laminitis

Sarah I. Jacob[1] | Kevin J. Murray[2] | Aaron K. Rendahl[2] | Raymond J. Geor[3] | Nichol E. Schultz[2] | Molly E. McCue[2]

[1]Michigan State University College of Veterinary Medicine, Large Animal Clinical Sciences, East Lansing, Michigan

[2]University of Minnesota College of Veterinary Medicine, Veterinary Population Medicine, St. Paul, Minnesota

[3]Massey University College of Sciences, Palmerston North, New Zealand

Correspondence
Sarah I. Jacob, Michigan State University College of Veterinary Medicine, 736 Wilson Road, Room D202, East Lansing, MI 48824
Email: jacobs67@msu.edu

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Background: Metabolomics, the study of small-molecule metabolites, has increased understanding of human metabolic diseases, but has not been used to study equine metabolic syndrome (EMS).

Objectives: (1) To examine the serum metabolome of Welsh Ponies with and without insulin dysregulation before and during an oral sugar test (OST). (2) To identify differences in metabolites in ponies with insulin dysregulation, obesity, or history of laminitis.

Animals: Twenty Welsh Ponies (mean ± SD; 13.8 ± 9.0 years) classified as non-insulin dysregulated [CON] (n = 10, insulin < 30 mU/L) or insulin dysregulated [ID] (n = 10, insulin > 60 mU/L) at 75 minutes after administration of Karo syrup, obese (n = 6) or nonobese (n = 14), and history of laminitis (n = 9) or no history of laminitis (n = 11).

Methods: Case-control study. Metabolomic analysis was performed on serum obtained at 0 minutes (baseline) and 75 minutes during the OST. Data were analyzed with multivariable mixed linear models with significance set at $P \leq .05$.

Results: Metabolomic analysis of 646 metabolites (506 known) detected significant metabolite differences. At baseline, 55 metabolites (insulin response), 91 metabolites (obesity status), and 136 metabolites (laminitis history) were different. At 75 minutes, 51 metabolites (insulin response), 102 metabolites (obesity status), and 124 metabolites (laminitis history) were different.

Conclusions and Clinical Importance: Use of metabolomics could have diagnostic utility for early detection of EMS and provide new knowledge regarding the pathophysiology of metabolic perturbations associated with this condition that might lead to improved clinical management.

KEYWORDS
equine metabolic syndrome, horse, hyperinsulinemia, insulin resistance, metabolomics, oral sugar test

1 | INTRODUCTION

Insulin dysregulation, generalized obesity, regional adiposity, and a predisposition for laminitis are central features of equine metabolic syndrome (EMS). Insulin dysregulation, defined as an abnormal resting insulin (hyperinsulinemia), abnormal insulin response to IV or oral glucose

Abbreviations: BCAA, branched-chain amino acid; EMS, equine metabolic syndrome; GC, gas chromatography; ID, insulin dysregulated; LASSO, least absolute shrinkage and selection operator; MS, mass spectrometry; NEFA, nonesterified fatty acid; OST, oral sugar test; PCA, principal components analysis; TCA, tricarboxylic acid; UPLC, ultraperformance liquid chromatography.

challenge, abnormal insulin response to feeding, or a combination of the listed perturbations, is a central pathophysiologic feature of EMS. Complex multifactorial disease processes such as human metabolic syndrome and EMS result from disruption of metabolic processes across multiple tissues that sum together to create clinical disease.[1] Due to the complex nature of EMS, measurement of glycemic and insulinemic responses to oral or IV glucose and insulin challenges is likely inadequate to distinguish between hyperinsulinemia caused by exaggerated pancreatic responses, tissue insulin resistance, or reduced insulin clearance.[2] Yet, except for studies addressing the lamina during experimentally induced laminitis[3,4] and dynamic assessment of insulin resistence,[5,6] few studies have attempted to identify the metabolic derangements of EMS at a tissue or cellular level. Our current understanding of EMS is based on clinical assays that do not directly assess the altered cellular and molecular pathophysiology within major metabolic tissues (muscle, adipose, and liver) and are therefore insufficient to unravel EMS pathophysiology.

Metabolomics, the study of molecules involved in cellular metabolism such as nucleotides, amino acids, fatty acids, and carbohydrates, refers to the global interrogation of the biochemical components in a biological sample such as serum, plasma, urine, saliva, or cerebrospinal fluid. Because metabolite abundance in the serum can provide information about disruption in metabolic processes across the tissues, evaluation of the serum metabolome is a logical place to start investigating the molecular perturbations relevant to EMS.[7] More than 4000 metabolites have been identified in human serum using high-throughput mass spectrometry and chromatography.[8] Several human studies have identified plasma metabolites and distinct metabolomic signatures associated with insulin resistance, glucose intolerance, obesity, and type-II diabetes mellitus.[9–11]

In addition to elucidating alterations in novel metabolic pathways implicated in disease development, serum metabolomics can be used to identify biomarkers that can effectively pinpoint animals at-risk for EMS and laminitis. In humans, metabolite biomarkers identified in cross-sectional data are useful for the detection of subclinical disease months to years before the onset of clinically identifiable insulin resistance.[12–16] Thus, serum metabolites hold promise as potential biomarkers that would allow timely identification of metabolic derangements in animals at-risk for insulin dysregulation.

Although metabolomic analysis is a potentially powerful tool to study the complex molecular pathophysiology of EMS, the measurement of metabolites is costly. Therefore, our objectives were to demonstrate the potential of serum metabolomics to explore the pathophysiology of metabolic dysregulation and to differentiate between individuals with and without evidence of insulin dysregulation, obesity, or history of laminitis by characterizing differences in the serum metabolome before and during an oral sugar test (OST) in a small cohort of Welsh Ponies.

2 | MATERIALS AND METHODS

2.1 | Animals

In a case-control study, 20 Welsh Ponies classified as non-insulin dysregulated [CON] ($n = 10$, insulin < 30 mU/L) or insulin dysregulated [ID] ($n = 10$, insulin > 60 mU/L) at 75 minutes after administration of Karo light corn syrup (ACH Food Companies Inc, Cordova, Tennessee) were used for this study. The cohort was comprised of client-owned ponies located on five different farms in Virginia, Maryland, Mississippi, Arkansas, and California that had previously participated in a large metabolic syndrome study conducted by the University of Minnesota.[17] Additional information on diet, exercise, management, body condition score,[18] laminitis history, and biochemical measures (nonesterified fatty acids [NEFAs], triglycerides, leptin, and adiponectin) was obtained for each pony. All ponies were examined by two authors (M.E. McCue and N.E. Schultz) to rule out clinical illness or clinical laminitis at the time of sampling. All methods were approved by the Institutional Animal Care and Use Committee at the University of Minnesota and Michigan State University.

2.2 | Oral sugar test

An OST was administered to all ponies as previously described.[19] Briefly, oral administration of commercially available corn syrup (Karo light) was given using a 60 cc catheter tip syringe (dose: 0.15 mL/kg bodyweight). Blood was collected via an indwelling jugular catheter at 0 minutes (baseline) and 75 minutes. The IV catheter was placed 1 hour before commencement of the OST after subcutaneous administration of lidocaine. Blood was centrifuged and serum separated and stored at −80°C until analysis.

2.3 | Determination of insulin and glucose measurements

Insulin concentrations were determined in duplicate by a radioimmunoassay (Coat-A-Count; Siemens Diagnostics, Los Angeles, California) previously validated for equids.[20] Intra-assay and interassay coefficients of variability (CV) were calculated for low and high equine serum controls (Intra: 4.9% [low] and 4.7% [high]; Inter: 6.0% [low]; and 3.9% [high]). Glucose concentrations were determined in duplicate via a membrane-based glucose oxidase method (YSI 2300 STAT Plus Glucose & Lactate Analyzer; YSI Incorporated Life Sciences, Yellow Springs, Ohio); intra-assay (1.2%) and interassay (1.5%) CVs were calculated.

2.4 | Determination of other hormonal and biochemical measurements

Nonesterified fatty acid concentrations were determined in duplicate using an in-vitro quantitative enzymatic colorimetric method assay (NEFA-HR; Wako Chemicals USA, Richmond, Virginia). Intra-assay and interassay CVs were calculated for low and high equine serum controls (Intra: 8.2% [low] and 3.4% [high]; Inter: 7.8% [low], and 3.1% [high]). Triglyceride concentrations were determined in triplicate using the Serum Triglyceride Determination Kit (TR0100; Sigma-Aldrich Company, St. Louis, Missouri); the intra-assay CV (6.0%) was calculated. Leptin concentrations were determined in duplicate using a radioimmunoassay (Multi-Species Leptin RIA; EMD Millipore Corporation, Billerica, Massachusetts); intra-assay CV (1.9%), and interassay CV (6.6%) were calculated. Adiponectin concentrations were determined in duplicate using the Human High Molecular Weight Adiponectin ELISA

(EMD Millipore Corporation, Billerica, Massachusetts) previously validated for equine serum.[21] Intra-assay CV (6.5%) and interassay CV (8.5%) were calculated.

2.5 | Metabolomics

Forty serum samples, 0 minutes (baseline) and 75 minutes from each pony, were analyzed at Metabolon Inc using a combination of chromatography and mass spectrometry after sample preparation as described previously.[22]

2.6 | Sample preparation for global metabolomics

Samples were divided into five fractions: analysis by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS; positive ionization), analysis by UPLC-MS/MS (negative ionization), analysis by UPLC-MS/MS polar platform (negative ionization), analysis by gas chromatography–mass spectrometry (GC-MS), and one sample was reserved for repeat analysis. A targeted analysis utilized three types of controls in concert with the experimental samples: (1) samples generated from a small portion of each experimental sample of interest served as a technical replicate throughout the data set; (2) extracted water samples served as process blanks; and (3) a combination of standards spiked into every analyzed sample allowed instrument performance monitoring. Instrument variability was determined by calculating the median relative standard deviation for the standards that were added to each sample before injection into the mass spectrometers.

2.7 | Mass spectrometry analysis

The UPLC-MS/MS[23] platform used a Waters Acquity UPLC (Waters Corporation, Milford, Massachusetts) and a Thermo Scientific Q-Exactive (Thermo Fisher Scientific, Waltham, Massachusetts) high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization source and Orbitrap mass analyzer operated at 35,000 mass resolution. Three sample extracts were dried then reconstituted in acidic or basic liquid chromatography-compatible solvents. The first aliquot was analyzed using acidic, positive ion-optimized conditions (n = 254 metabolites), the second aliquot used basic, negative ion-optimized conditions (n = 284 metabolites), and the third aliquot was analyzed via negative ionization after elution from a hydrophilic interaction liquid chromatography (HILIC) column (n = 54 metabolites).

Gas chromatography–mass spectrometry[24] was performed with a Thermo-Finnigan Trace DSQ (Thermo Fisher Scientific, Waltham, Massachusetts) mass spectrometer with electron impact ionization. Samples were dried, derivatized, and separated on a fused silica column with helium as the carrier gas (n = 54 metabolites).

2.8 | Compound identification, quantification, and data curation

Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries that included retention time, molecular weight, preferred adducts, and in-source fragments as well as associated MS spectra and curated by visual inspection for quality control using software developed at Metabolon Inc.[25] Metabolon maintains a library of molecules based on authenticated standards that contain the retention time, mass to charge ratio, and chromatographic data. Identification of known chemical entities was based on comparison to metabolomic library entries of more than 3300 commercially available purified standards. Peaks were quantified using area-under-the-curve. Raw area counts for each metabolite in each sample were normalized to correct for variation resulting from instrument inter-day tuning differences. Subsequent quality control and curation processes were designed to ensure accurate, consistent identification, and to minimize system artifacts, misassignments, and background noise. Pathways were assigned for each metabolite, which allowed for examination of overrepresented pathways.

2.9 | Statistical analysis

Statistical analysis, performed in the statistical program R (R Core Team, Vienna, Austria), after log transformation to ensure normality included multivariable mixed linear models with sex as a covariate and examined metabolite differences between insulin dysregulated and non-insulin dysregulated ponies, obese, and nonobese ponies, and ponies with and without a history of laminitis. Correlations between metabolites and biochemical variables (basal glucose, basal insulin, NEFAs, triglycerides, leptin, adiponectin) were explored using mixed linear models. A simple *t*-test was performed to determine significance between insulin dysregulated ponies and non-insulin dysregulated ponies for each biochemical variable. Significance was set at $P \leq .05$. Network pathway interaction diagrams were generated using Metscape.[26]

Unsupervised principal components analysis (PCA) was performed to visualize the distribution of metabolic profiles within and between groups. Supervised least absolute shrinkage and selection operator (LASSO) penalized generalized linear models were fitted for optimal feature selection for classification of each group—insulin response, obesity status, and laminitis history. LASSO regressions were fitted using the "glmnet" R package. Model parameters were tuned using leave-one-out cross-validation and the optimal subset of features was selected from the model with minimal mean cross-validation error. No further model diagnostics could be performed because of an insufficient number of observations.

3 | RESULTS

3.1 | Animals

Non-insulin dysregulated ponies (mean ± SD; 13.8 ± 9.0 years) were a combination of mares (n = 6), geldings (n = 3), and stallions (n = 1) while the insulin dysregulated ponies (11.3 ± 6.1 years) were exclusively mares (n = 10). All ponies were in moderate to obese body condition (median [range]; CON: 5.5 [5.0–8.0] and ID: 6.8 [5.0–8.5] out of 9). Ponies did not have clinical laminitis at the time of testing; however, a history of laminitis was reported in both non-insulin dysregulated (n = 1) and insulin dysregulated ponies (n = 8).

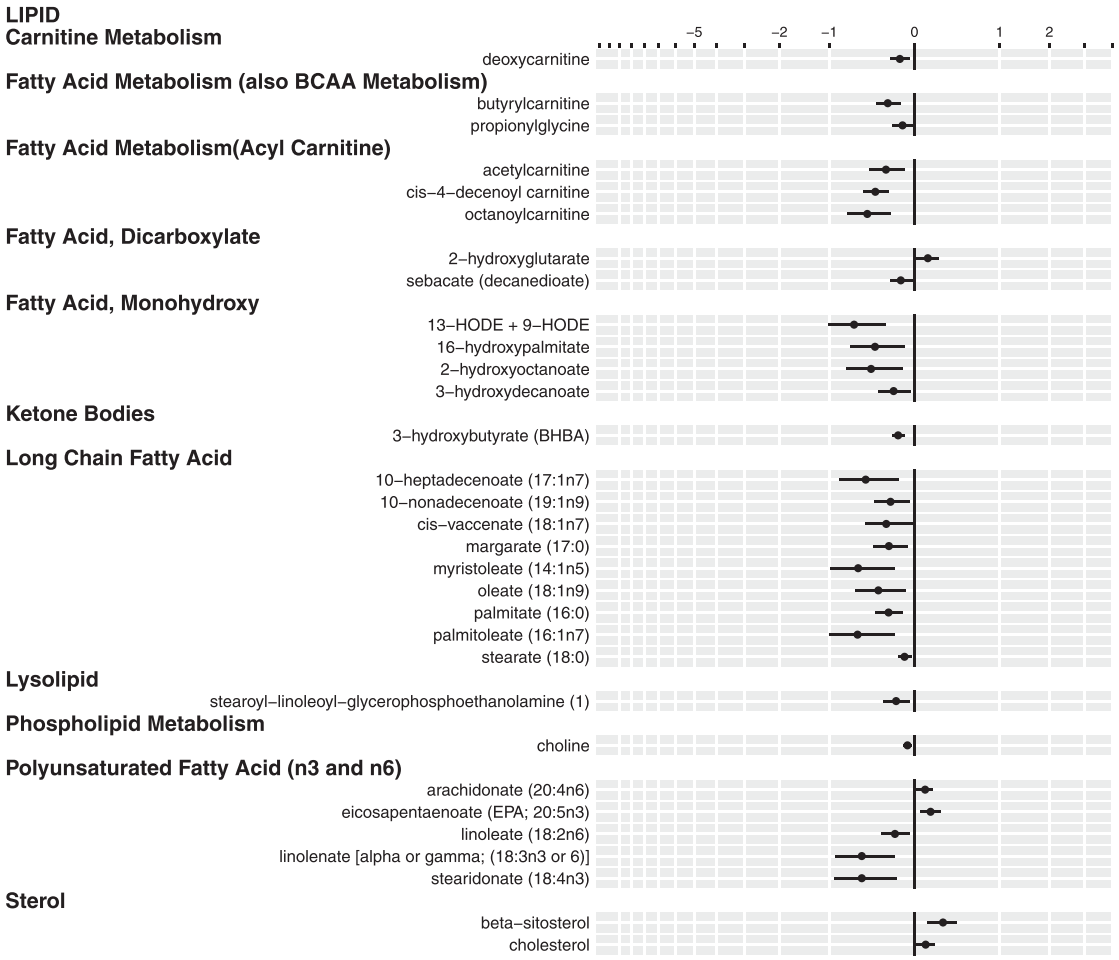

FIGURE 1 Significant ($P < .05$) metabolite differences across all ponies at 0 and 75 minutes during an OST are displayed for the lipid, amino acid, carbohydrate, cofactor and vitamin, energy, nucleotide, peptide, and xenobiotic pathways. Filled circles (•) represent the least squares (LS) means estimates and horizontal lines (—) represent the confidence interval around the LS means. For each metabolite, the LS means estimate for baseline is set to 0 (vertical line). Positive LS means indicate increases in metabolite abundance after administration of Karo light corn syrup relative to baseline, whereas negative LS means indicate decreases in metabolite abundance after administration of Karo light corn syrup relative to baseline. All data are represented on a log scale

3.2 | Insulin and glucose concentrations

Median insulin concentrations were significantly higher in insulin dysregulated ponies compared with non-insulin dysregulated ponies at 0 minutes (median [interquartile range]; ID: 15.1 [12.6–18.3] mU/L and CON: 4.5 [2.1–6.2] mU/L; $P < .001$) and at 75 minutes (ID: 100.2 [92.9–129.0] mU/L and CON: 15.8 [9.6–24.0] mU/L; $P < .001$) whereas median glucose concentrations did not differ between groups at 0 minutes (ID: 70.9 [66.5–77.2] mg/dL and CON: 72.8 [68.3–77.9] mg/dL; $P = .91$) or at 75 minutes (ID: 108.0 [100.3–117.6] mg/dL and CON: 109.8 [81.3–127.1] mg/dL; $P = .82$).

3.3 | Other hormonal and biochemical concentrations

Mean NEFA concentrations (median [interquartile range]; ID: 0.5 [0.3-0.5] mEq/L and CON: 0.2 [0.2-0.3] mEq/L; $P = .31$), triglyceride concentrations (ID: 37.7 [29.3–124.0] mg/dL, and CON: 20.0 [16.7–25.2] mg/dL; $P = .18$), and leptin concentrations (ID: 5.0 [4.1–7.7] ng/mL, and CON: 4.4 [3.9-4.8] ng/mL; $P = .69$) did not differ between insulin dysregulated ponies and non-insulin dysregulated ponies. Adiponectin concentrations (ID: 2402.0 [1362.0–3251.0] ng/mL and CON: 8073.0 [6488.0–9866.0] ng/mL; $P < .001$) were significantly lower in insulin dysregulated ponies compared with non-insulin dysregulated ponies.

3.4 | Metabolomics

Metabolomic analysis revealed a total of 646 metabolites of which 506 were of known identity based on homology with human metabolites (Supporting Information item 1). The 506 known metabolites were classified into eight metabolic pathways (lipid, amino acid, carbohydrate, cofactors and vitamins, energy, nucleotide, peptide, and xenobiotics) and 71 subpathways based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway classification system (Supporting Information item 2).

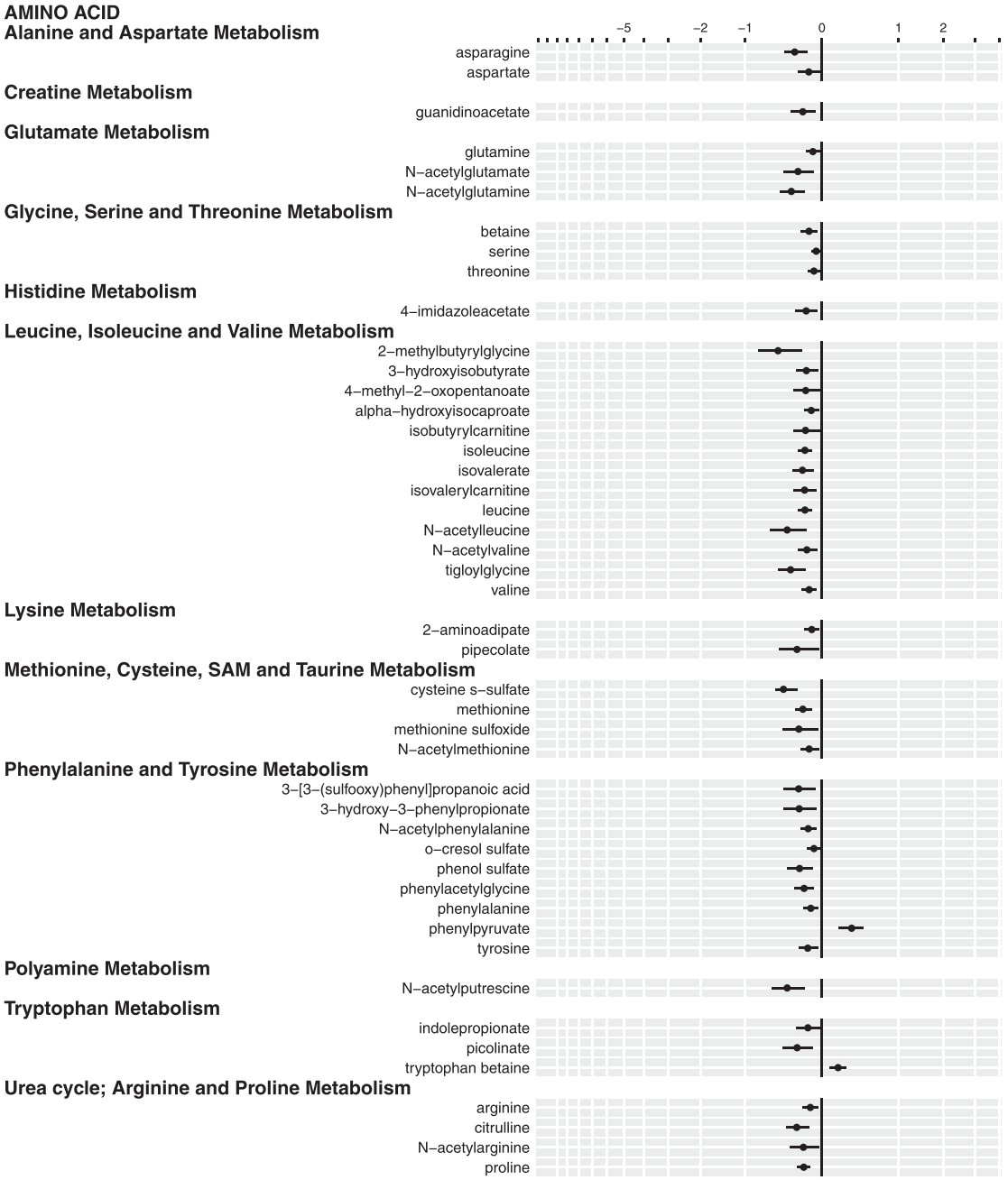

FIGURE 1 (Continued)

3.5 | Metabolite changes during an OST

To determine if the OST elicited metabolite alterations in addition to the expected glycemic and insulinemic responses, metabolites were compared at 0 minutes (baseline) and 75 minutes in all 20 ponies regardless of phenotype (insulin response, obesity status, and laminitis history). Seventeen metabolites had increased concentrations while 107 metabolites had decreased concentrations after administration of Karo light corn syrup when compared with baseline concentrations. Metabolites (n = 124) significantly different between these time points are listed by metabolic pathways and subpathways (Figure 1): lipid (n = 31), amino acid (n = 46), carbohydrate (n = 5), cofactors and vitamins (n = 6), energy (n = 3), nucleotide (n = 3), peptide (n = 9), and xenobiotics (n = 21). After administration of Karo syrup, glucose, fructose, and mannose increased, whereas lactate decreased relative to baseline. The tricarboxylic acid (TCA) cycle intermediates (alpha-ketoglutarate, malate, and succinate) were also significantly decreased at 75 minutes (Supporting Information item 3). Thirty-one lipid metabolites were significantly different between time points. Most lipid metabolites (26/31), including acylcarnitines, monohydroxy long-chain fatty acids, and polyunsaturated fatty acids, were decreased at 75 minutes indicating an overall decrease in fatty acid metabolism after administration of

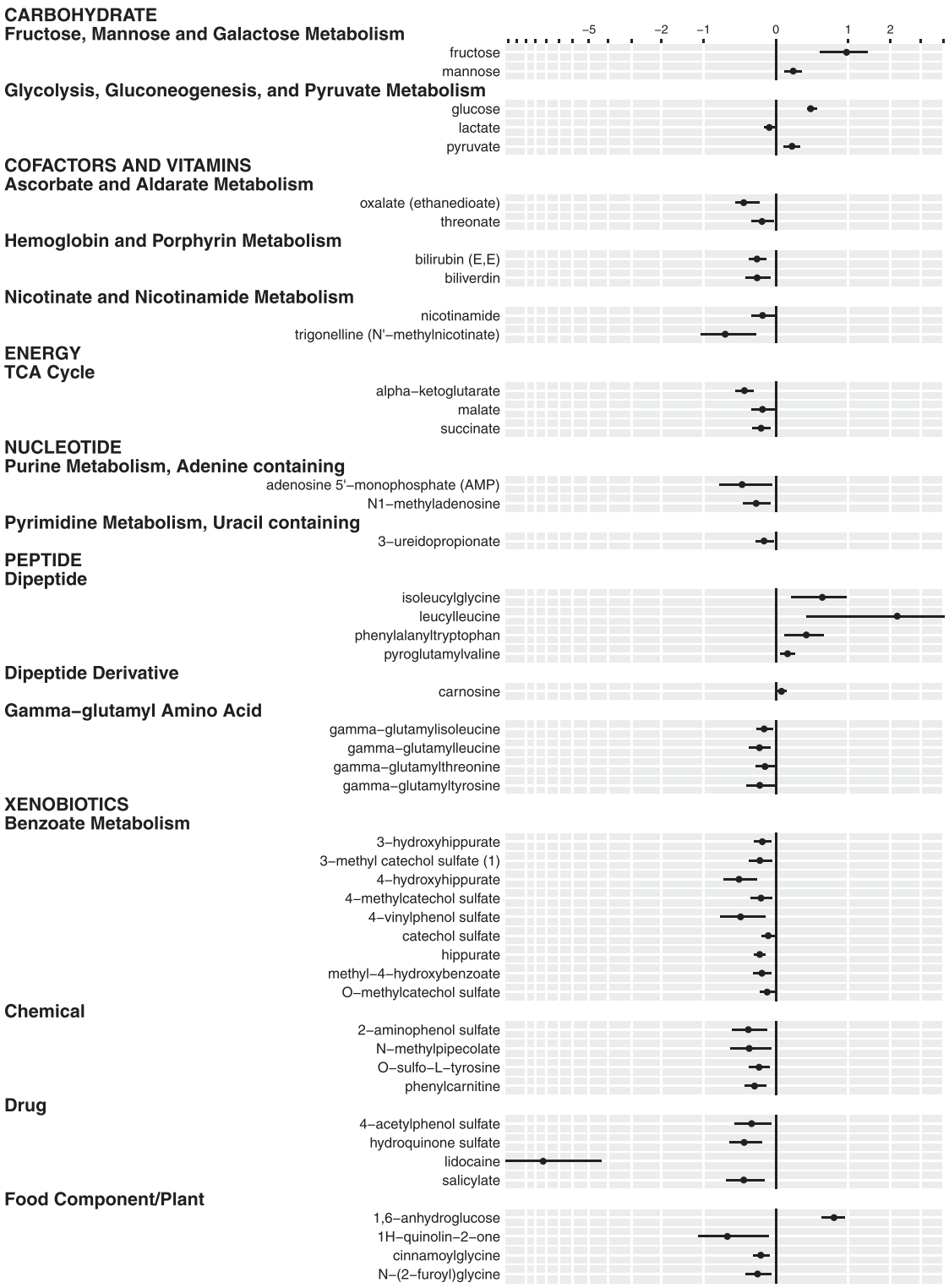

FIGURE 1 (Continued)

Karo syrup. Similarly, amino acid metabolism metabolites (44/46), including branched-chain amino acids, were reduced (Supporting Information item 4). The most dramatic decrease in metabolites from baseline to 75 minutes was a decrease in lidocaine from subcutaneous administration for catheter placement 1 hour before starting the test.

3.6 | Metabolite differences between insulin dysregulated and non-insulin dysregulated ponies

When comparing the insulin dysregulated ponies versus the non-insulin dysregulated ponies, 55 metabolites at 0 minutes (baseline) and

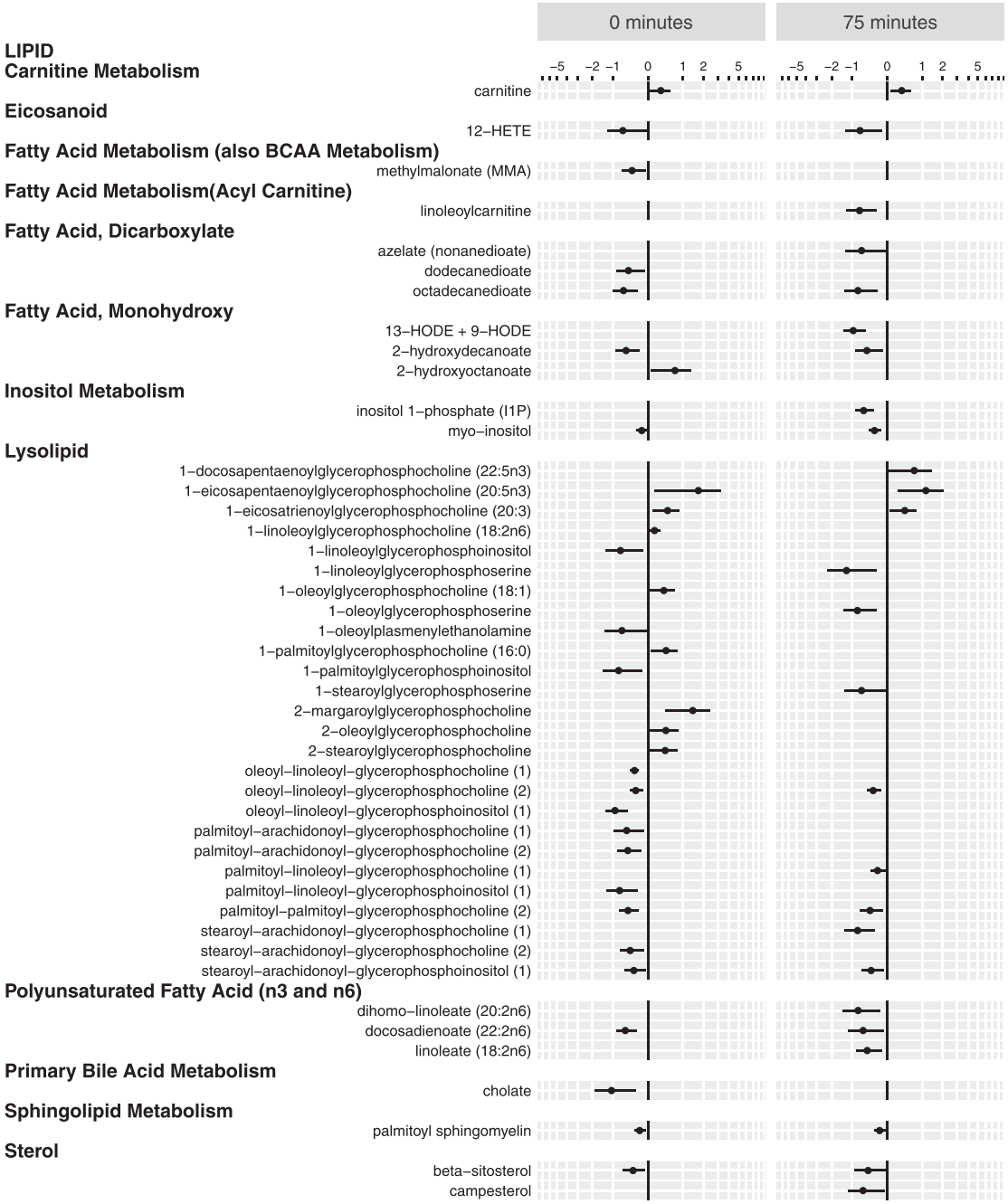

FIGURE 2 Significant ($P < .05$) metabolite differences in the lipid pathway for ponies with insulin dysregulation compared with non-insulin dysregulated ponies. Filled circles (•) represent the LS means estimates and horizontal lines (—) represent the confidence interval around the LS means for insulin dysregulated ponies. For each metabolite, the LS means estimate for non-insulin dysregulated ponies is set to 0 (vertical line). Positive LS means in insulin dysregulated ponies indicate increases in metabolite abundance relative to controls, whereas negative LS means in insulin dysregulated ponies indicate decreases in metabolite abundance relative to controls. All data are represented on a log scale

51 metabolites at 75 minutes were statistically different. Metabolites (n = 82) significantly different between insulin dysregulated and non-insulin dysregulated ponies are listed based on their metabolic pathways and subpathways: lipid (Figure 2; n = 45), amino acid (Figure 3; n = 16), carbohydrate, cofactors and vitamins, energy, nucleotide, peptide, and xenobiotics (Figure 4; n = 21). At baseline, 12 metabolites had increased concentrations while 43 metabolites had decreased concentrations in insulin dysregulated ponies compared to non-insulin dysregulated ponies. Further, at baseline, most metabolites with decreased concentrations were in the lipid (n = 22) and amino acid (n = 9) pathways. At 75 minutes, 7 metabolites had increased concentrations and 44 metabolites had decreased concentrations in insulin dysregulated ponies compared to non-insulin dysregulated ponies. Similar to baseline, insulin dysregulated ponies had decreased

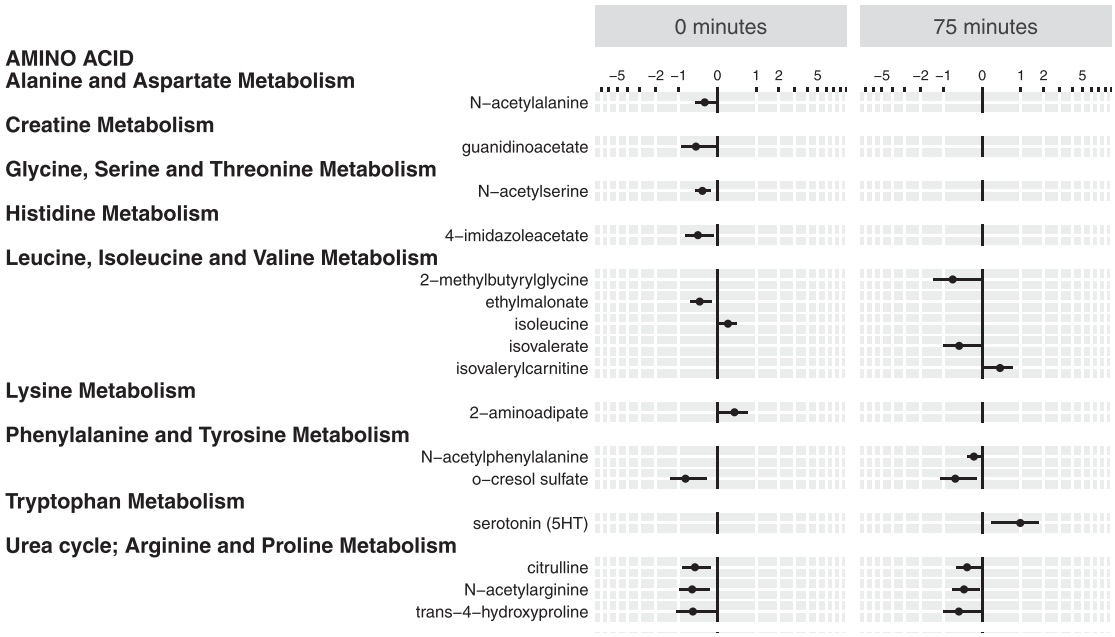

FIGURE 3 Significant ($P < .05$) metabolite differences in the amino acid pathway for ponies with insulin dysregulation compared with non-insulin dysregulated ponies. Filled circles (●) represent the LS means estimates and horizontal lines (—) represent the confidence interval around the LS means for insulin dysregulated ponies. For each metabolite, the LS means estimate for non-insulin dysregulated ponies is set to 0 (vertical line). Positive LS means in insulin dysregulated ponies indicate increases in metabolite abundance relative to controls, whereas negative LS means in insulin dysregulated ponies indicate decreases in metabolite abundance relative to controls. All data are represented on a log scale

concentrations of lipid metabolites (n = 22) and amino acid metabolites (n = 7) after administration of Karo syrup. In addition, at 75 minutes, insulin dysregulated ponies had lower concentrations of TCA cycle intermediates (citrate, fumarate, and malate) compared with non-insulin dysregulated ponies. Insulin dysregulated ponies had a significantly higher concentration of serotonin (5HT) relative to non-insulin dysregulated ponies.

3.7 | Metabolite differences between obese and nonobese ponies

To determine if the same or similar metabolites were different in obese (body condition score ≥ 7.0) ponies (n = 6) versus nonobese ponies (n = 14), metabolite measurements were compared in these two groups. Overall, 91 metabolites at 0 minutes (baseline) and 102 metabolites at 75 minutes were statistically different between these groups. Metabolites (n = 145) significantly different between groups are listed based on their metabolic pathways and subpathways: lipid (Supporting Information item 5; n = 79), amino acid (Supporting Information item 6; n = 41), carbohydrate, cofactors and vitamins, nucleotide, peptide, and xenobiotics (Supporting Information item 7; n = 25). At baseline, 65 metabolites had increased concentrations while 26 metabolites had decreased concentrations in obese ponies compared with nonobese ponies. Obese ponies compared to nonobese ponies had significantly higher baseline concentrations of long-chain fatty acids (n = 8) and acylcarnitines (n = 8). Obese ponies also had significantly higher baseline concentrations of branched-chain amino acids (isoleucine, leucine, and valine). At 75 minutes, 57 metabolites had increased concentrations and 45 metabolites had decreased concentrations in obese ponies compared with nonobese ponies. Similar to baseline, obese ponies had increased concentrations of several acylcarnitines and branched-chain amino acids compared with nonobese ponies after administration of Karo syrup. Obese ponies also had significantly lower concentrations of polyunsaturated fatty acids at 75 minutes. The most dramatic metabolite increases in obese ponies compared with nonobese ponies was 1,5-anhydroglucitol.

3.8 | Metabolite differences between ponies with and without a history of laminitis

To determine if the same or similar metabolites were different because of laminitis status, ponies with a history of laminitis (n = 9) were compared with ponies without a history of laminitis (n = 11). One hundred and thirty-six metabolites at 0 minutes (baseline) and 124 metabolites at 75 minutes were statistically different between previously laminitic and nonlaminitic ponies. Metabolites (n = 182) significantly different between these groups are listed based on their metabolic pathways and subpathways: lipid (Supporting Information item 8; n = 91), amino acid (Supporting Information item 9; n = 50), carbohydrate, cofactors and vitamins, energy, nucleotide, peptide, and xenobiotics (Supporting Information item 10; n = 41). At baseline, 62 metabolites had increased concentrations while 74 metabolites had decreased concentrations in ponies with a history of laminitis compared with ponies without a history of laminitis. At baseline, long-chain fatty acids (n = 10), polyunsaturated fatty acids (n = 7) and monoacylglycerols (n = 8) were consistently higher in previously laminitic compared with nonlaminitic ponies. At 75 minutes, 60 metabolites had increased concentrations and 64 metabolites had decreased concentrations in ponies with a

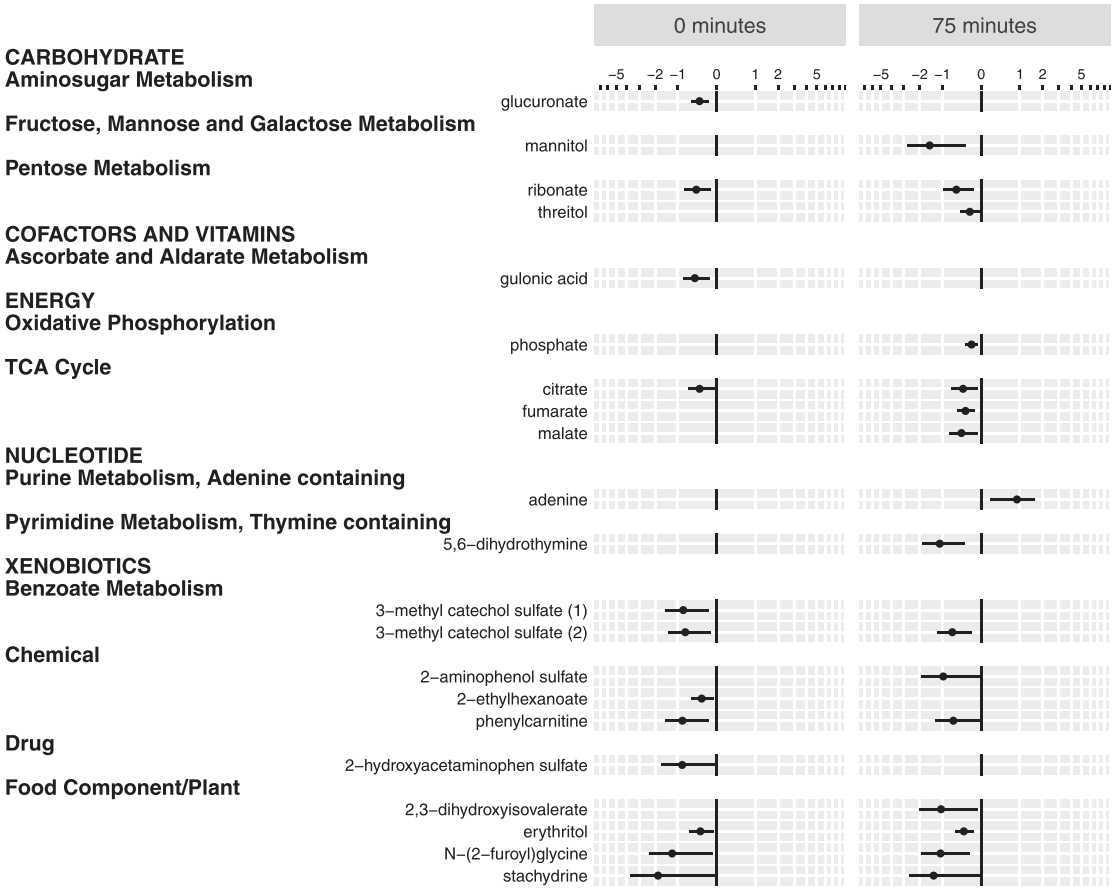

FIGURE 4 Significant ($P < .05$) metabolite differences in the carbohydrate, cofactor and vitamin, energy, nucleotide, and xenobiotic pathways for ponies with insulin dysregulation compared with non-insulin dysregulated ponies. Filled circles (•) represent the LS means estimates and horizontal lines (—) represent the confidence interval around the LS means for insulin dysregulated ponies. For each metabolite, the LS means estimate for non-insulin dysregulated ponies is set to 0 (vertical line). Positive LS means in insulin dysregulated ponies indicate increases in metabolite abundance relative to controls, whereas negative LS means in insulin dysregulated ponies indicate decreases in metabolite abundance relative to controls. All data are represented on a log scale

history of laminitis compared with ponies without a history of laminitis. These differences were characterized by greater concentrations of polyunsaturated fatty acids (n = 6) and monoacylglycerols (n = 6) in previously laminitic compared with nonlaminitic ponies.

3.9 | Metabolite similarities between insulin response, obesity status, and laminitis history

The similarities between the significant metabolites identified when ponies were parsed by insulin response, obesity, or laminitis are depicted in Figure 5. A total of 6 metabolites were shared in all three analyses. Twenty-five metabolites overlap when ponies were parsed by obesity status or laminitis history, 5 metabolites overlap when ponies were parsed by laminitis history or insulin response, and 3 metabolites overlap when ponies were parsed by obesity status or insulin response.

3.10 | Metabolite correlations to clinical variables

Additional analysis revealed subsets of measured metabolites correlated ($r \geq .5$) to the clinical variables commonly measured in equids with suspected metabolic dysfunction (basal glucose, basal insulin, NEFAs, triglycerides, leptin, and adiponectin; Tables 1 and 2). Seven metabolites were correlated to basal glucose, 85 metabolites were correlated to basal insulin, 7 metabolites were correlated to NEFAs, 13 metabolites were correlated to triglycerides, 54 metabolites were correlated to leptin, and 12 metabolites were correlated to adiponectin. Thirty compounds were correlated to more than 1 variable. Regardless of the variable, most of the metabolites arise from the lipid and amino acid pathways.

3.11 | Metabolites as potential biomarkers

To show the utility of serum metabolites to distinguish between ponies grouped by insulin response, obesity, or laminitis, unsupervised PCA was performed. The first two principal components depicted capture ~34.2% (principal component 1: 22.8%, principal component 2: 11.4%) of the variation in the data and separate the ponies into two groups. Plots of the first two dimensions from unsupervised PCA allow for visualization of the relationships between the metabolic profiles of each group when ponies are labeled by insulin dysregulation (Figure 6A), obesity status (Figure 6B), or laminitis history (Figure 6C). The optimal

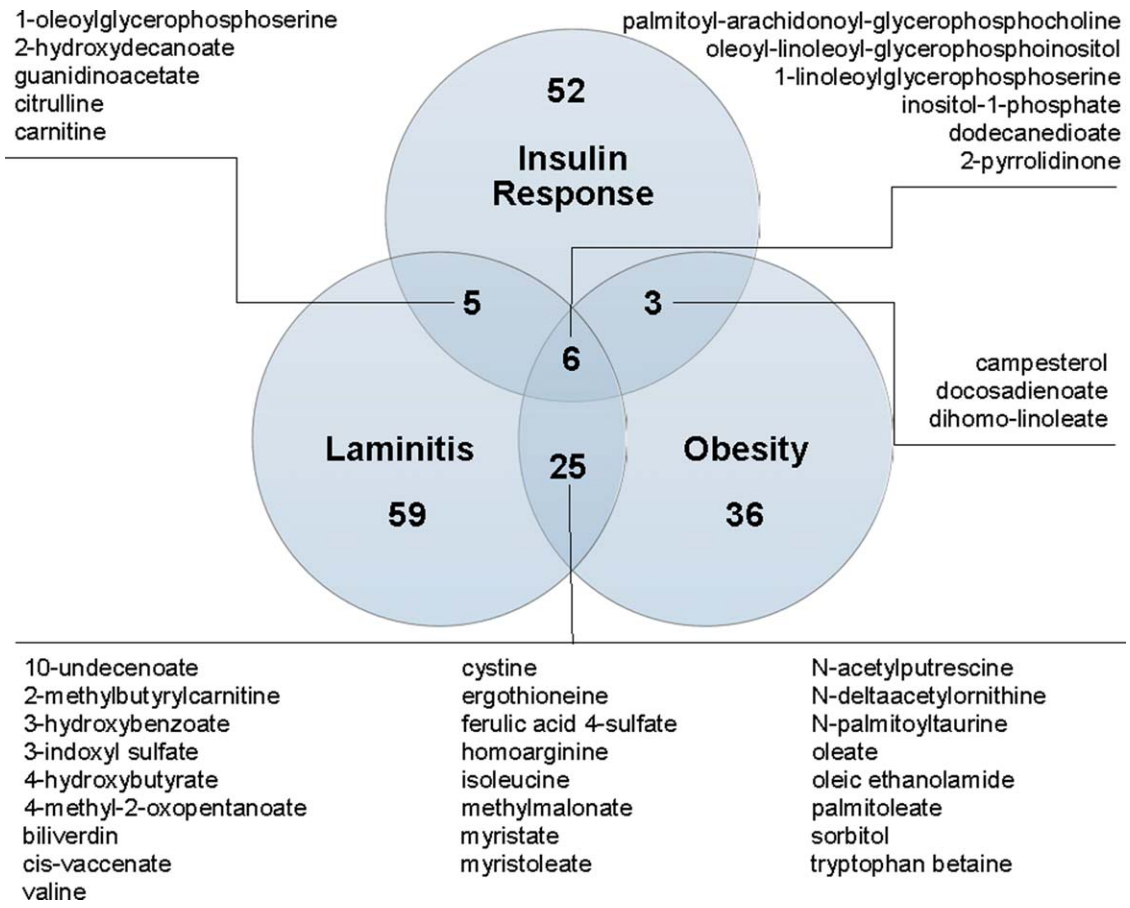

FIGURE 5 Relationship between different phenotypes (insulin response, obesity, and laminitis) and metabolites

number of metabolites (ie, biomarkers) necessary to distinguish individuals based on insulin response, obesity status, and laminitis history was determined using LASSO regression. The number of metabolites needed to differentiate ponies based on insulin response (n = 23), obesity status (n = 14), and laminitis history (n = 21) are listed in Table 3. Minimal overlap was observed between these metabolite lists, with only three metabolites (2-margaroyl-GPC, oleoyl-linoleoyl-glycerophosphocholine, and phenylcarnitine) shared between the insulin response and laminitis history biomarker lists.

4 | DISCUSSION

For complex diseases that span multiple tissues, metabolomics from biologic fluid samples provide an opportunity to obtain additional quantitative biologic information. Here, we demonstrate the utility of serum metabolomics to contribute to our understanding of EMS pathophysiology by: (1) establishing the power of serum metabolomics to give insight into the metabolic responses to an OST beyond measurement of glucose and insulin concentrations; (2) identifying a list of metabolites that differentiates between the three EMS phenotypes (insulin response, obesity status, and laminitis history), (3) identifying metabolites that correlate to other typical measures of metabolic dysfunction (basal glucose, basal insulin, NEFAs, triglycerides, leptin, and adiponectin), and (4) pinpointing metabolites that can potentially be used as biomarkers for disease.

The OST is a relatively simple test used in horses and ponies to provide an indication of glycemic and insulinemic responses to an oral sugar bolus. Similar to an oral glucose tolerance test performed in humans, an OST should provide a physiologic stimulus that results in metabolite flux through specific metabolic reactions/pathways. Comparable to findings in humans, the metabolite concentrations in ponies (regardless of phenotype) indicate a switching from a relatively catabolic state (baseline/fasting) to an anabolic state after administration of Karo syrup. Many of these changes can be attributed to four key areas of insulin action—an increase in glycolysis, and decreases in lipolysis, ketogenesis, and proteolysis.[27,28] First, after administration of an oral sugar bolus, the cytosolic pathways of glucose disposal are overloaded as demonstrated by significant increases in glucose, fructose, and mannose. Second, increases in pyruvate concentrations above baseline values indicate increases in glycolysis.[27] In humans, a switch to glycolysis is also indicated by an increase in lactate, which occurs ~30 minutes after peak insulin values[27]; however, lactate did not significantly increase at 75 minutes in our study. Insulin peaks between 60 and 90 minutes after Karo syrup administration[17,29]; therefore the 75 minute sample might have been too early to detect increases in lactate associated with insulin action. The switch to glycolysis from beta-oxidation is also supported by changes in acylcarnitines. Carnitine conjugation of

TABLE 1 Uphill (positive) correlations ($r \geq .5$) between metabolites and clinical variables (basal glucose, basal insulin, NEFAs, triglycerides, leptin, and adiponectin)

Basal glucose	Basal insulin	NEFAs	Triglycerides	Leptin	Adiponectin
	$r = .7$	$r = .5$	$r = .7$	$r = .7$	$r = .5$
	Hydroquinone sulfate	3-[3-(Sulfooxy)phenyl] propanoic acid	Cinnamoylglycine	Mannitol	1-Linoleoyl glycerophosphocholine
	Arginine	Asparagine	Phenylcarnitine	Tartronate (hydroxymalonate)	2-Stearoyl glycerophosphocholine
	$r = .6$	Stearoyl-arachidonoyl-glycerophosphoinositol	$r = .6$	*N*-acetylvaline	Adenine
	N-acetylleucine		2-Ethylhexanoate	*N*-(2-furoyl)glycine	Arabinose
	Cyclo (leu-pro)		Taurolithocholate	Tricarballylate	Cytidine
	N-(2-Furoyl)glycine		Taurolithocholate 3-sulfate	Hydroquinone sulfate	Isovalerylcarnitine
	Citrulline		$r = .5$	4-Allylphenol sulfate	Oxalate (ethanedioate)
	4-Imidazoleacetate		*N*-oleoyltaurine	$r = .6$	
	Guanidinoacetate		Taurohyodeoxycholic acid	Xylose	
	Indoleacetate		13-HODE + 9-HODE	1-Dihomo-linoleoyl glycerophosphocholine	
	$r = .5$		2-Arachidonoyl glycerophosphocholine	Cyclo (gly-pro)	
	Methionine sulfoxide		N-acetylglycine	1-Palmitoylglycerophosphoinositol	
	Asparagine		2-Ethylphenylsulfate	1H-quinolin-2-one	
	Dihydroferulic acid			1-Linoleoylglycerophosphoserine	
	3-(3-Hydroxyphenyl)propionate			1-Linoleoylglycerophosphoinositol	
	Salicylate			*N*-acetylleucine	
	Gentisate			*N*-methylpipecolate	
	1-Linoleoylglycerophosphoinositol			*N*-acetylasparagine	
	Pyroglutamine			Indolin-2-one	
	2-Stearoylglycerophosphoinositol			Propionylglycine	
	Dodecanedioate			Isoleucylaspartate	
	1-Palmitoylglycerophosphoinositol			Isoleucylglycine	
	Hippurate			$r = .5$	
	N-Acetylalanine			Benzoate	

(Continues)

TABLE 1 (Continued)

Basal glucose	Basal insulin	NEFAs	Triglycerides	Leptin	Adiponectin
	3-Hydroxybenzoate			Salicylate	
	4-Hydroxyhippurate			Picolinate	
	Phenol sulfate			Gentisate	
	N-Acetylglutamate			3-Phenylpropionate (hydrocinnamate)	
				3-Hydroxy-3-phenylpropionate	
				2-Hydroxydecanoate	
				Equol glucuronide	
				4-Hydroxybutyrate (GHB)	
				4-Acetamidophenol	
				3-Hydroxybenzoate	
				Hippurate	
				2-Pyrrolidinone	
				3-(3-Hydroxyphenyl) propionate	
				Prolylglycine	
				N-Acetylglutamine	
				2-Aminophenol sulfate	

TABLE 2 Downhill (negative) correlations ($r \geq -.5$) between metabolites and clinical variables (basal glucose, basal insulin, NEFAs, triglycerides, leptin, and adiponectin)

Basal glucose	Basal insulin	NEFAs	Triglycerides	Leptin	Adiponectin
$r = -.6$	$r = -.7$	$r = -.5$	$r = -.5$	$r = -.5$	$r = -.6$
Valine	Carnitine	2-Hydroxypalmitate	Betaine	10-Heptadecenoate	Allantoin
$r = -.5$	Homocitrulline	Corticosterone	N1-methyladenosine	10-Nonadecenoate	Asparagine
3-Methyl-2-oxobutyrate	β-alanine	cortisol		10-Undecenoate	$r = -.5$
Cortisone	$r = -.6$	Transurocanate		2-Aminobutyrate	Cholate
N1-methyladenosine	Erucate			Alanine	Hypotaurine
Transurocanate	1-Eicosapentaenoylglycerophosphocholine			Carnitine	Octadecanedioate
Betaine	10-Nonadecenoate			Cholestanol	
Riboflavin (Vitamin B2)	Eicosenoate			Dihomo-linolenate	
	Beta-hydroxyisovaleroylcarnitine			Docosahexaenoate (DHA)	
	Oleic ethanolamide			Docosapentaenoate	
	Eicosapentaenoate (EPA)			Linolenate	
	5-Dodecenoate			Lysine	
	Cholestanol			Margarate	
	Isovalerylcarnitine			Oleate	
	$r = -.5$			Palmitoleate	
	1-Margaroylglycerophosphocholine			Stearidonate	
	Phosphate				
	Myristoleoylcarnitine				
	10-Heptadecenoate				
	16-Hydroxypalmitate				
	Sphinganine				
	Palmitate				
	Stearidonate				
	Indole-3-carboxylic acid				
	Margarate				
	15-Methylpalmitate				
	4-Methyl-2-oxopentanoate				
	1-Palmitoylglycerophosphocholine				
	Urate				
	Cis-vaccenate				

(Continues)

TABLE 2 (Continued)

Basal glucose	Basal insulin	NEFAs	Triglycerides	Leptin	Adiponectin
	Isoleucine				
	7-Alpha-hydroxy-3-oxo-4-cholestenoate				
	Myristoylcarnitine				
	1-Linolenoylglycerophosphocholine				
	2-Margaroylglycerophosphocholine				
	1-Palmitoleoylglycerophosphocholine				
	Glutarate (pentanedioate)				
	Palmitoleate				
	Dihomo-linolenate				
	Myristate				
	2-Hydroxyisobutyrate				
	2-Palmitoleoylglycerophosphocholine				
	Palmitoylcarnitine				
	1-Oleoylglycerophosphocholine				
	1-Eicosatrienoylglycerophosphocholine				
	Stearate				
	5-Alpha-pregnan-3 alpha, 20 beta-diol disulfate 1				
	2-Arachidoylglycerophosphocholine				
	2-Methylbutyrylcarnitine				
	Oleate				
	3-Hydroxydecanoate				
	2-Aminobutyrate				
	Alpha-Hydroxyisovaleroyl carnitine				
	3-Methyl-2-oxovalerate				
	Docosatrienoate				
	Stearoylcarnitine				
	Oleoylcarnitine				
	N-delta-acetylornithine				
	Linolenate				
	Ornithine				
	N-stearoyltaurine				

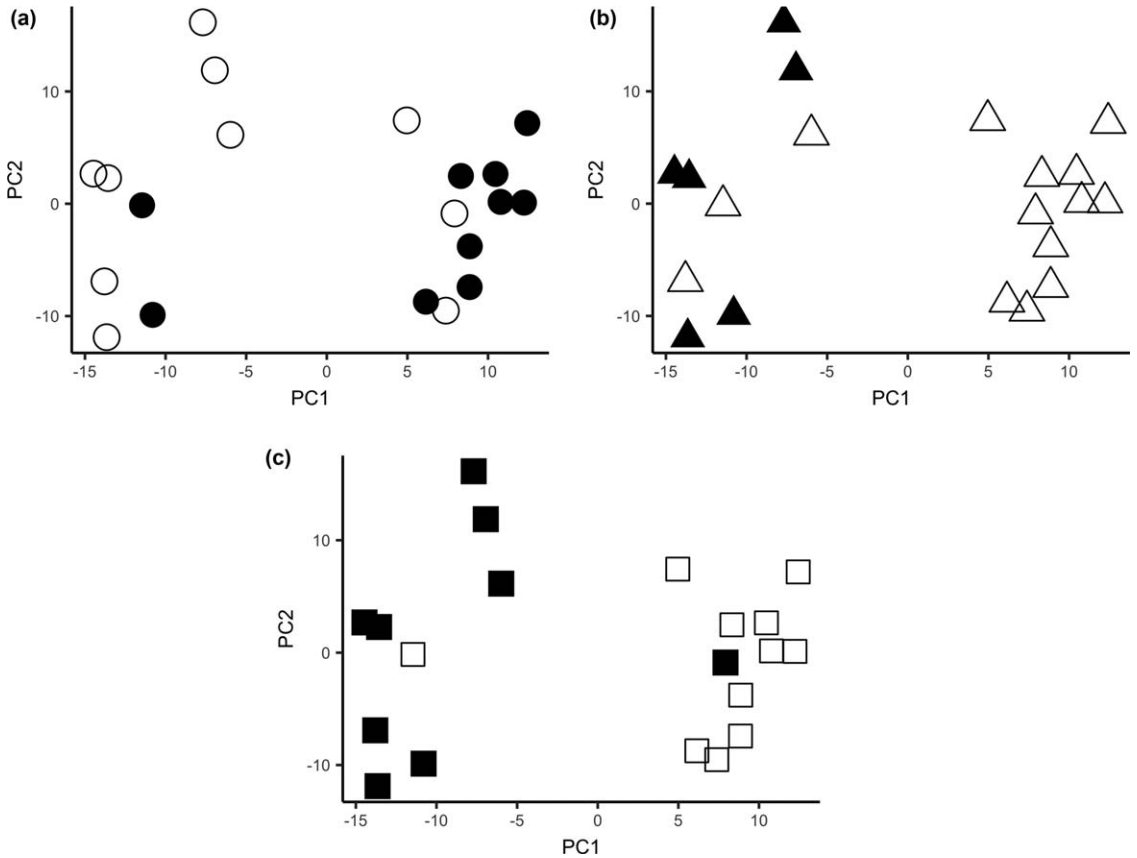

FIGURE 6 PCA plots of metabolic profiles for different phenotypes: (a) insulin dysregulated (○) and non-insulin dysregulated (●), (b) obese (Δ) and nonobese (▲), and (c) history of laminitis (□) and no history of laminitis (■)

long-chain fatty acids is a required step for import into the mitochondria before beta-oxidation and acylcarnitine accumulation. Acylcarnitine release into the plasma reflects substrate flux through beta-oxidation; decreases in acylcarnitines at 75 minutes relative to baseline suggest a decrease in beta-oxidation.[30] Decreases in saturated and monounsaturated long-chain fatty acids and polyunsaturated fatty acids indicate an inhibition of lipolysis.[30] Inhibition of ketogenesis and decrease TCA cycle flux is evidenced by decreases in ketones (3-hydroxybutyrate) and TCA cycle intermediates (alpha-ketoglutarate, malate, and succinate). Lastly, a decrease in amino acid concentrations including branched chain amino acid concentrations after administration of Karo syrup indicates an inhibition in proteolysis and possible usage for protein synthesis.

Comparison of ponies with and without insulin dysregulation primarily identified differences in lysolipids, TCA cycle intermediates, and urea cycle metabolites. Several glycerophosphocholines, such as oleoyl-linoleoyl-glycerophosphocholine, were decreased in insulin dysregulated ponies at both time points. In humans, individuals with low concentrations of oleoyl-linoleoyl-glycerophosphocholine develop glucose intolerance and type-II diabetes mellitus.[31] In addition, decreases in the TCA cycle intermediates (citrate, malate, and fumarate) in insulin dysregulated ponies mirror decreased TCA cycle intermediates in type-II diabetes mellitus patients.[27,28,32] Similar to humans with insulin resistance and type-II diabetes mellitus, decreases in urea cycle metabolites, including the amino acid citrulline and other urea cycle intermediates were present in insulin dysregulated ponies.[31,32] Finally, decreases in polyunsaturated fatty acids (linoleate) and bile acids (cholate), which were both decreased in insulin dysregulated ponies, are important biomarkers of insulin resistance in humans.[31,33] Altered fatty acid metabolism in tissues contributes to insulin resistance[34] in humans and the findings of our study suggests that similar alterations might be present in insulin dysregulated ponies.

In humans, branched-chain amino acids, NEFAs, acylcarnitines, and phospholipids have been identified as potential biomarkers for obesity.[35–37] For some of the metabolites, group differences were evident at baseline, while for others the difference was only evident after an OST, which is a similar finding in human studies.[27,28] Analogous to obese humans, obese ponies compared with nonobese ponies have increased serum concentrations of several long-chain fatty acids at baseline.[38] However, unlike obese humans, long-chain fatty acid concentrations were not different between obese and nonobese ponies at 75 minutes.[39] Obese ponies also had increased concentrations of several long-chain acylcarnitines (C16, C18, C18:1) at both time points when compared with nonobese ponies. These findings parallel findings in obese humans, and might indicate lipid oversupply resulting in saturation of the mitochondrial capacity for beta-oxidation and incomplete long-chain fatty acid oxidation.[38] Obese ponies also had higher carnitine concentrations at baseline

TABLE 3 Metabolites that distinguish between control and disease status for each variable (insulin response, obesity status, and laminitis history) as determined by LASSO analysis

Insulin response	Obesity	Laminitis
13-HODE + 9-HODE	1-Eicosadienoyl-GPC	1-Dihomo-linolenylglycerol
1-Eicosatrienoyl-GPC	1-Linoleoyl-GPS	1-Oleoylglycerol
1-Linoleoyl-GPC	Arabitol	2-Docosahexaenoyl-GPC
2-Margaroyl-GPC	Betaine	2-Ethylphenylsulfate
3-(3-Hydroxyphenyl)propionate	Carnosine	2-Margaroyl-GPC
3-Hydroxyisobutyrate	Chenodeoxycholate	2-Palmitoylglycerol
4-Imidazoleacetate	Dodecanedioate	2-Pyrrolidinone
5-Hydroxyindoleacetate	Equol glucuronide	4-Acetylphenyl sulfate
Asparagine	Ferulic acid 4-sulfate	4-Hydroxybutyrate
Cinnamoylglycine	Methylmalonate	Benzoate
Glucuronate	*N*-6-trimethyllysine	Carnitine
Imidazole propionate	Octanoylcarnitine	Homocitrulline
Indole-3-carboxylic acid	Palmitoyl-arachidonoyl-glycerophosphocholine	*N*-delta-acetylornithine
Isovalerylcarnitine	Ursodeoxycholate	Oleoyl-linoleoyl-glycerophosphocholine
N-palmitoyltaurine		Orotate
Octadecanedioate		Phenylcarnitine
Oleoyl-linoleoyl-glycerophosphocholine		Propionylglycine
Oxalate (ethanedioate)		Quinolinate
Palmitoyl-linoleoyl-glycerophosphocholine		Serotonin
Phenylcarnitine		Tartarate
Sphingomyelin		Tauro-alpha-muricholate
Stearoyl-arachidonoyl-glycerophosphoinositol		

and 75 minutes, which has been associated with increased body mass index and waist circumference as well as insulin resistance and increased triglycerides in humans.[40] Similar to findings in obese humans,[41] obese ponies had lower lysolipid concentrations relative to nonobese ponies after administration of an oral sugar bolus. In addition, obese ponies had higher concentrations of branched-chain amino acids (isoleucine, leucine, valine) compared with nonobese ponies, indicating a delayed suppression of branched-chain amino acid oxidation after an OST.[39–41] The most dramatic metabolite increases in obese ponies compared with nonobese ponies was 1,5-anhydroglucitol, a metabolite that competes with glucose for filtration and elimination by the kidneys. This metabolite is a recognized marker of postprandial glucose control in humans.[42]

The largest number of significant differences in metabolites were identified when ponies with a history of laminitis were compared with ponies without a history of laminitis; however, with the exception of increases in monoacylglycerols and polyunsaturated fatty acids at baseline and 75 minutes, many of the differences in previously laminitic compared with nonlaminitic ponies overlapped with important metabolic differences in the insulin dysregulation and obesity analyses, or overlapped between all three phenotypes. The metabolite overlap between phenotypes is not surprising given that these phenotypes often occur concurrently in EMS. Elevated circulating free fatty acids and hyperinsulinemia have been reported in obese horses with insulin resistance[43] and triglyceride accumulation in muscle rather than adipose tissue[44] was seen in healthy horses challenged with super-physiologic levels of insulin. Metabolites involved in fatty acid metabolism and amino acid metabolism were increased in insulin dysregulated ponies, obese ponies, and ponies with a history of laminitis. In addition, the TCA cycle was less efficient in insulin dysregulated ponies and ponies with a history of laminitis as citrate, malate, and fumarate, metabolites associated with the mitochondrial use of pyruvate, were lower in these phenotypes after administration of oral sugar suggesting that the mitochondria were less able to remove acetyl-CoA equivalents through energy production. Levels of homoarginine were lower in obese and previously laminitic ponies when compared with nonobese or nonlaminitic ponies. In humans, low homoarginine levels are a risk factor for cardiovascular, cerebrovascular, and renal diseases potentially because of effects on nitric oxide and cellular energy metabolism.[45]

The overlap in metabolite changes between these three phenotypes is also evident in the PCA. In this analysis, more than 30% of the variation was captured by the first two principal components and plotting the first versus the second principal component separated the ponies into two clusters. However, the two clusters did not align completely with any of the three clinical phenotype groups (insulin response, obesity status, and laminitis history), suggesting that these clinical phenotypes alone are inadequate to separate metabolic differences between the ponies.

Much focus has been directed towards identification of animals at-risk for EMS and the identification of biomarkers that can provide prognostic information about laminitis risk. An ideal diagnostic test for EMS would be based on measurements at a single time point, would not be impacted by environmental variables, would be minimally confounded by individual factors (sex, age, breed, and genetics), and would accurately classify horses and predict laminitis risk, allowing appropriate early intervention and disease prevention. In humans, serum metabolites have been identified that predict metabolic diseases up to a decade before clinical onset. In our study, LASSO analysis yielded a subset of compounds that differentiate between disease and healthy individuals that could play a role as diagnostic tests. Identification of differing metabolites between the non-insulin dysregulated and insulin dysregulated phenotypes in a baseline sample might eliminate the need for a dynamic challenge test in the future. In addition, the ability to define a metabolomic signature might reveal specific biomarkers that predict and diagnose insulin dysregulation leading to a better understanding of disease processes that might help identify new therapeutic targets.

This study has provided evidence that metabolomic profiling is a relevant approach for further defining metabolic alterations because of insulin dysregulation and obesity in horses. Examination of the serum metabolome of this Welsh Pony cohort demonstrated significant differences in metabolites primarily derived from the lipid and amino acid pathways when comparing ponies grouped by each EMS phenotype (insulin response, obesity status, and laminitis history). Further, examination of the metabolite list against currently used metabolic dysregulation measurements (basal glucose, basal insulin, NEFAs, triglycerides, leptin, and adiponectin) revealed a strong correlation to leptin and triglycerides, suggesting that metabolites might be useful for linking obesity and insulin dysregulation to other components of the EMS phenotype. However, despite the parallel between the findings from our study and findings in humans with insulin resistance, type-II diabetes mellitus, and obesity, results from this cohort should be interpreted with caution. First, this is a small cohort restricted to a single breed. Second, ponies were initially included based on insulinemic responses to an OST; thus, there is significant overlap between the insulin dysregulation, obesity, and laminitis groups limiting our analyses. When ponies were parsed by the three phenotypes (insulin response, obesity status, laminitis history) metabolomics showed that there were similarities and distinct differences, which coincides with our understanding that EMS is complex. The results presented here should be confirmed in a large cohort of animals that will allow for metabolite differences because of pathologic factors such as insulin dysregulation and obesity and physiologic factors such as age, sex, and breed to be differentiated. Despite these limitations, our results clearly demonstrate the potential of serum metabolomics to provide insight into molecular pathophysiology and to define a metabolomic signature for EMS.

CONFLICT OF INTEREST DECLARATION

The authors declare that they have no conflict of interest with the contents of this article.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

All methods were approved by the IACUC at the University of Minnesota and Michigan State University.

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ORCID

Sarah I. Jacob http://orcid.org/0000-0002-8028-4685

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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