Metabolomic study of dry blood spots in horses suffering from exertional rhabdomyolysis

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1 Introduction

Equine myopathies are a group of muscle disorders presenting with symptoms such as muscle pain, stiffness, lameness, weakness and exercise intolerance, among others, primarily due to rhabdomyolysis. Rhabdomyolysis, defined as the breakdown of skeletal muscle tissue, can result from exertional or non-exertional causes. Exertional rhabdomyolysis typically follows intense activity and includes both a classic phenotype with recurrent episodes and a non-classic form marked by weakness, ataxia, and gait abnormalities. Non-exertional myopathies arise from infectious, immune-mediated, nutritional, or toxic factors. Among these, atypical myopathy (AM, Figure 1) is a severe, often fatal condition linked to the ingestion of maple-derived toxins (hypoglyicn A - HGA, methylenecyclopropylglycine - MCPG) that impair fatty acid oxidation. Due to the overlap in clinical presentation with other conditions, such as colic, diagnosis can be challenging. This study aims to comprehensively characterize the profiles of amino acids, acylcarnitines (AC), organic acids, and acylglycines in dry blood spot (DBS) samples collected from horses with AM, a unique cohort with non-toxic-related myopathy, and healthy controls. The results may provide valuable insights into the pathophysiology of myopathy and may help to differentiate AM from other types of myopathy on the basis of their metabolic signatures.

Figure 1: Horse with atypical myopathy.

2 Materials and methods

The study included three groups of horses. The non-toxic myopathy group (ntM, n=7) showed no detectable HGA or its metabolite MCPA-carnitine. Based on history, clinical signs, and elevated creatine kinase (CK), horses were categorized into subtypes: exertional (EM), immune-mediated (IM), nutritional (NM), post-anesthetic (PM), and unknown cause (U). The AM group (n=15) consisted of various breeds with access to maple-contaminated pastures, elevated CK, and presence of HGA and MCPA-carnitine. The control group (CTRL, n=15) included clinically healthy horses from pastures without sycamore and/or box elder trees, with normal CK and no toxin detection. Whole blood was collected from the jugular vein into heparinized tubes. A drop of each sample was applied to newborn screening filter paper and dried at room temperature to prepare DBS.

Amino acid and acylcarnitine analysis
A 3.0 mm disc from each DBS was extracted using methanol containing internal standards. Concentrations of 31 acylcarnitines and 17 amino acids were determined by flow injection analysis tandem mass spectrometry (FIA; API 4000 triple quadrupole mass spectrometer; SCIEX).

Organic acid, acylcarnitine, and acylglycine analysis
For OA profiling, a 3.2 mm DBS disc was extracted with methanol containing butyrylcarnitine-d3 as internal standard. After incubation, samples were lyophilized and reconstituted in LC-MS water. Analysis was performed using an ExionLC™ coupled to a QTRAP® 6500+ mass spectrometer (SCIEX).

HGA and MCPA-carnitine analysis
A 3.2 mm DBS disc was extracted with methanol containing butyrylcarnitine-d3, deproteinized at –80 °C for 35 min, centrifuged and lyophilized. The residue was reconstituted in a mobile phase mixture (ammonium formate/formic acid in water and methanol, 1:4). The analysis of HGA and its metabolite, MCPA-carnitine, was based on LC-MS/MS.

Statistical analysis was performed in the R program using the Metabol package, SIMCA software and GraphPad Prism software. Natural logarithmic transformation and mean centering were applied to each datasets.

3 Results

Unsupervised PCA was performed to provide an overview of the metabolite profiles of the samples studied. In both the FIA (Fig. 2A) and OA (Fig. 2B) analyses, horses with myopathy were clearly distinguishable from the controls, with the most pronounced separation observed in the AM group. Similar to PCA, the OPLSDA completely separated the AM and ntM groups from the controls. OPLSDA S-Plot (Fig. 2C, D) was performed to identify and visualise metabolic differences between the AM and ntM groups. Elevation of short- and medium-chain AC was observed in FIA analysis for both groups. The most notable alterations were in C6, C8, C10, and C10:1, with increases of 27x, 26x, 27x, and 15x, respectively, compared to the ntM group. OA analysis revealed significant elevations in acylglycines, indicating a distinct metabolic pattern in AM. These changes reflect a toxin-induced disruption of fatty acid β-oxidation by HGA-derived MCPA-CoA and MCPG-derived MCPF-CoA, which inhibit key enzymes of this pathway. Accumulated acyl-CoAs are detoxified via conjugation with carnitine and glycine, forming the elevated AC and acylglycine profiles. The muscle type impairment may also contribute to the distinct metabolic patterns observed. Muscle fibres of the first type, which are rich in mitochondria and rely on fatty acid oxidation, the citrate cycle, and oxidative phosphorylation for energy production, are particularly affected in AM. In contrast, exertional rhabdomyolysis predominantly affects type II muscle fibres, which are specialized for rapid contractions but are more prone to fatigue. Type II fibres rely primarily on anaerobic metabolism reflecting their lower oxidative capacity.

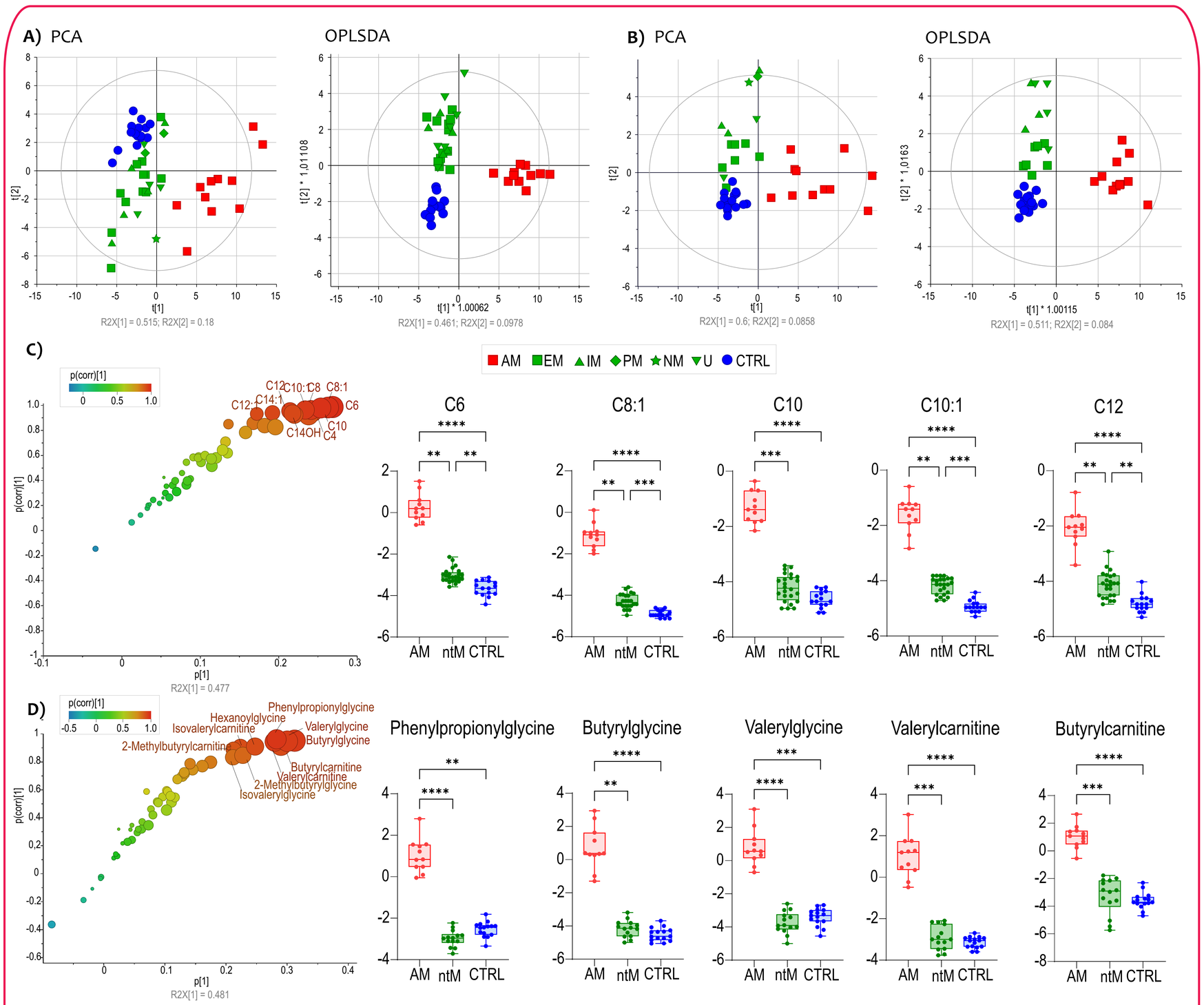

Figure 2: Unsupervised principal component analysis (PCA) and supervised orthogonal partial least square discriminant analysis (OPLS-DA) of acylcarnitine and amino acid (A) and organic acid (B) analyses. Due to the small number of samples, those from horses diagnosed with PM or NM were reclassified as myopathies of unknown aetiology (U) in the supervised analysis. OPLS-DA S-Plot of AM and non-toxic groups of myopathy (ntM) from (C) FIA analysis and (D) OA analysis. The point size reflects the VIP (variable importance for the projection) score and the colour of the points represents the p(corr)1 values. P-value in box plots was calculated using the Kruskal-Wallis test with Dunn's post-hoc analysis (*<0.05, **<0.01, ***<0.001, ****<0.0001).

4 Conclusion

The combined detection of HGA, MCPA-carnitine, and specific metabolite alterations offers a robust tool for diagnosing toxin-mediated myopathies and underscores the importance of integrating metabolic, clinical and environmental data.