Diagnosis of atypical myopathy based on organic acid and acylcarnitine profiles and evolution of biomarkers in surviving horses

Déborah Mathis [a,b], Jörn Oliver Sass [c], Claudia Graubner [d], Angelika Schoster [e,*]

[a] University Institute of Clinical Chemistry, Inselspital, Bern University Hospital, University of Bern, Bern, Switzerland
[b] Department of Clinical Chemistry and Biochemistry, University Children's Hospital Zurich, Zurich, Switzerland
[c] Department of Natural Sciences & Institute for Functional Gene Analytics (IFGA), Bonn-Rhein-Sieg University of Applied Sciences, Rheinbach, Germany
[d] Institut Suisse de Médecine Equine, Vetsuisse Faculty, University of Berne, Länggassstrasse 124, 3012 Berne, Switzerland
[e] Clinic for Equine Internal Medicine, Equine Department, Vetsuisse Faculty, University of Zurich, Winterthurerstrasse 260, 8057 Zurich, Switzerland

ARTICLE INFO

ABSTRACT

Background: Atypical myopathy (AM), an acquired multiple acyl-CoA dehydrogenase deficiency (MADD) in horses, induce changes in mitochondrial metabolism. Only few veterinary laboratories offer diagnostic testing for this disease. Inborn and acquired MADD exist in humans, therefore determination of organic acids (OA) in urine and acylcarnitines (AC) in blood by assays available in medical laboratories can serve as AM diagnostics. The evolution of OA and AC profiles in surviving horses is unreported.

Methods: AC profiles using electrospray ionization tandem mass spectrometry (ESI-MS/MS) and OA in urine using gas chromatography mass spectrometry (GC–MS) were determined in dried blot spots (DBS, $n = 7$) and urine samples ($n = 5$) of horses with AM ($n = 7$) at disease presentation and in longitudinal samples from 3/4 survivors and compared to DBS ($n = 16$) and urine samples ($n = 7$) from control horses using the Wilcoxon test.

Results: All short- (C2-C5) and medium-chain (C6-C12) AC in blood differed significantly ($p < 0.008$) between horses with AM and controls, except for C5:1 ($p = 0.45$) and C5OH + C4DC ($p = 0.06$). In AM survivors the AC concentrations decreased over time but were still partially elevated after 7 days. 14/62 (23%) of OA differed significantly between horses with AM and control horses. Concentrations of ethylmalonic acid, 2-hydroxyglutaric acid and the acylglycines (butyryl-, valeryl-, and hexanoylglycine) were highly elevated in the urine of all horses with AM at the day of disease presentation. In AM survivors, concentrations of those metabolites were initially lower and decreased during remission to approach normalization after 7 days.

Conclusion: OA and AC profiling by specialized human medical laboratories was used to diagnose AM in horses. Elevation of specific metabolites were still evident several days after disease presentation, allowing diagnosis via analysis of samples from convalescent animals.

1. Introduction

Atypical myopathy (AM), also called seasonal pasture myopathy in North America, is a highly fatal form of non-exertional rhabdomyolysis in horses first described in 1939 in the UK [1,2].

Suggested causes of AM included ingestion of *Clostridium* species in the soil, or tar spot fungus on Acer trees [3,4], but the cause remained unknown until an acquired enzymatic deficiency of multiple acetyl-CoA dehydrogenases (acMADD) was shown to be present in affected horses [5]. In humans, acMADD (Jamaican vomiting sickness) is associated with the ingestion of unripe fruits of the Jamaican Ackee tree (*Blighia sapida*) and lychee tree (*Litchi chinesis*), which contain an amino acid called hypoglycin A [6,7]. A metabolite of hypoglycin A, methylenecyclopropylacetic acid (MCPA), is further metabolized to MCPA-CoA, which is a potent inhibitor of many dehydrogenases of the mitochondrial fatty acid beta-oxidation. The basic toxicological mechanism includes the interruption of fatty acid β-oxidation by inhibition of acyl-CoA dehydrogenases and enoyl-CoA hydratases [8]. This prompted the search for sources of hypoglycin A in the environment of horses. Despite early evidence that hypoglycin A was present in seeds from certain trees

---

of the acer species, a member of the same Sapindaceae family as the Jamaican ackee tree [9], it needed the link to MADD to discover that hypoglycin A from Acer trees was responsible for AM. Ingestion of hypoglycin A produced by leaves and seedlings from *Acer pseudoplatanus* (sycamore maple) in Europe [10] and *Acer negundo* (box elder tree) in North America [2] has now been shown to be the cause of AM.

The acquired disease observed in horses and humans due to hypoglycin A ingestion mimics the autosomal recessive disease multiple acyl-CoA dehydrogenase deficiency (MADD) observed in human, in which genetic mutations lead to electron transfer flavoprotein (ETF) or ETF-ubiquinone oxidoreductase deficiency [11]. The disease leads to disruption in mitochondria metabolism (fatty acid beta-oxidation, catabolism of branched-chain amino acids and lysine) by affecting many acyl-CoA-dehydrogenases and other dehydrogenases due to disturbance in electron transfer and inability to re-oxidize the cofactor FADH2 of the flavoproteins [12].

MADD diagnosis in human proceeds usually by the analysis of acylcarnitines in blood and organic acids in urine, in which increase of short and medium chain acylcarnitines and dicarboxylic acids, respectively, are the hallmark [11]. Similarly, the diagnosis in horses showing compatible clinical signs for acMADD can be made by determination of those same metabolites or by the presence of MCPA conjugates and hypoglycin A in blood and urine [13]. The presence of hypoglycin A in blood alone is not diagnostic, as it can be circulating in the blood of healthy horse grazing on pastures with sycamore maple, particularly in Europe where this tree is very widespread [14,15]. However, MCPA conjugates were only detected in AM affected horses [14,15].

Recently, it was shown that certain fatty acid metabolites and acylcarnitines have a certain prognostic value for acMADD. Estimation of outcome can be based on three acylcarnitines (C2-, C10:2- and C18-carnitines) with a high sensitivity and specificity [16]. In 2020, Sander et al. reported the time course and metabolic effects of the consumption of canned ackee in one human volunteer. However, as far as we are aware of, such repeated sampling has never been shown in horses.

In this study, we aim to describe how the analysis of acylcarnitines in blood and also organic acids in urine available in human medical laboratories can be used for diagnosing AM in horses. Furthermore, we wanted to show the evolution of specific biomarkers in surviving horses affected by the disease in Switzerland.)

2. Methods

2.1. Animals and samples

Horses suspected to be affected by AM between 2014 and 2020 presented to the equine hospitals of the University of Bern (ISME) and University of Zurich were prospectively enrolled in this study. Horses were enrolled if clinical signs and laboratory changes were consistent with rhabdomyolysis and if a diagnosis of AM was suspected by the attending veterinarian. The relevant clinical signs included weakness or recumbency, tachycardia, myoglobinuria, and severe elevation of CK and AST without prior exercise. Horses with known exercise induced rhabdomyolysis were excluded. Native whole blood and urine were taken for routine measurements and only left-over material was used for this study; therefore, an animal use protocol was not necessary. Diagnosis was confirmed based on analysis of acylcarnitines in blood and urine, as described below.

To establish threshold values in control horses for acylcarnitines in blood and organic acids in urine, blood ($n = 16$) and urine samples ($n = 7$) from 18 horses examined at the clinic for equine internal medicine for reasons other than muscular disease of the University of Zurich were collected. As only left-over material was used, some horses had only blood and other only urine available. Demographic data, presenting complaint and diagnosis of these control horses is shown in Supplementary Information Table 1. An animal use protocol was not necessary,

since only left-over blood samples from routine hematocrit analysis and free catch urine samples were used.

A drop of native whole blood was spotted unto a filter paper card (Guthrie card, Whatman™ 903 paper, standard card for newborn screening, Druckerei Freytag AG, Zurich, Switzerland) and left to dry before shipment to the laboratory. Urine was frozen at −20 °C immediately after collection and shipped frozen to the laboratory.

2.2. Determination of acylcarnitines in dried blood spots (DBS) using electrospray ionization tandem mass spectrometry (ESI-MS/MS)

Acylcarnitine profiling were analyzed similar as described before [17]. One 1/8-in. circle of a dried blood spot (DBS) was punched out and placed into a 1.5 mL Eppendorf tube, in which 50 µL of extraction solution (methanol containing known concentrations of internal standards, d_3-acetylcarnitine, d_3-octanoylcarnitine, d_3-palmitoylcarnitine) were added. The mixture was let for 30 min at RT, after short vortexing the supernatant was pipetted in a second tube and dried at 50 °C for 40 min using a speed vacuum pump. 200 µL of acetonitrile/water (1:1; v:v) containing 0.1% of acetic acid were added and the sample was vortexed. 20 µL of the sample were injected using a Dionex UltiMate 3000 autosampler and analyzed by positive ESI-MS/MS using an AB SCIEX 4000 QTRAP. The solvent was acetonitrile/water (1:1; v:v) containing 0.1% of acetic acid and applied with a flow rate of 30 µL/min. Mass spectrometric data were acquired in precursor ion scan mode (product ion m/z = +85), and a range of m/z = 150 to 450 was scanned.

2.3. Determination of organic acids in urine

Urinary organic acids were determined by gas chromatography mass spectrometry with small modifications of the protocol published by Tanaka et al. [18] In short, 1 mL of urine sample (diluted to a concentration of 1 mmol/L creatinine) was added to 50 µL of the internal standard solution (3-methyladipic acid in water 40 mg/100 mL) in a glas tube. To this mixture, 50 µL of sodium hydroxide (6 M) and 100 µL of hydroxylamine-HCl (20 g/100 mL) were added and the mixture was incubated for 60 min at 60 °C to afford oximation of the ketone groups. 1 mL of a saturated sodium chloride solution was added, and the reaction was allowed to cool down for 10 min, afterward 100 µL of hydrochloric acid (12 M) was added. The solution was extracted 3 times with 3 mL of ethyl acetate, the supernatants transferred into an Erlenmeyer flask, in which sodium sulfate was added. After decantation, the dried solvent containing the organic acids was transferred into a fresh glass tube and evaporate under a gentle stream of nitrogen at 40 °C. Subsequently, the organic acids were derivatized with 8 µL trimethylsilyl-chloride (TMCS) and 40 µL *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA). The glass tube was sealed with a glass plug, mixed carefully by overturning the tubes and let incubate for 60 min at 60 °C. 5 µL of the derivatized sample was added to 100 µL of BSTFA in an autosampler vial. 1 µL of sample were injected in split mode (1:12) into the injector (250 °C) of the GC–MS machine mounted with a 30 m BGB-1701 column (0.32 mm; 0.45 mm; 0.25 µm). The carrier gas (helium) flow was set constant at 1.2 mL/min. The temperature gradient was as follow: 60 °C for the initial 13 min, increased temperature rate 5 °C/min for 38 min, hold final 250 °C for 5 min. Electron ionization source temperature was 230 °C at 70 V. Acquisition time started at 12 min for 33 min in positive ion and full scan mode, mass range 50–650 m/z. The organic acids were quantified using external calibration curves.

2.4. Statistical analysis

All acylcarnitines and organic acids in urine were compared between control horses and horses with AM with the Wilcoxon test (JMP 15). The Benjamin Hochberg Procedure was used to adjust *p*-values for false discovery rate (R). The threshold values (cut-offs) were determined by measuring samples of animals with no rhabdomyolysis and set to the

highest value obtained. As reference values of those methods were not established for animals, we choose to use threshold values, which is a more stringent criterion than reference values usually based on the 95% percentile. Descriptive statistics were used for the remaining data.

3. Results

3.1. Atypical myopathy in Swiss horses

Seven horses were suspected to have AM based on history, clinical signs and laboratory findings of rhabdomyolysis. The demographic data, relevant clinical findings as well as selected laboratory values are listed in Table 1. All horses presented for lethargy, weakness and a stiff gait or inability to rise, which were all confirmed on physical examination. All horses had increased CK and AST activities as well as lactate levels and were treated with intravenous fluid therapy, mild sedatives (acepromazine), pain medication (flunixin meglumine and butorphanol) and oral vitamin E as well as supportive care. Postmortem examination in all euthanized animals ($n = 3$) showed severe muscle cell degeneration particularly of type 1 muscle fibers, affecting mainly the respiratory and supporting muscles, which is compatible with AM. Dried spot blood samples of the day of disease presentation were available for all seven horses, whereas further follow-up samples were available for two horses only (Table 1). Urine samples of the day of disease presentation were available for five horses, with follow-up samples available for three horses. The number of samples available from each horse differs, due to variable progression of the disease and availability of left-over sample material.

Horse 1 presented in December 2014 at the age of 7 months to the Institute Suisse de Médicine Equine (ISME) of the University of Berne. The filly was bought 2 months before and kept on pasture with 6 other

equids of different breeds older than 7 months. During the next 10 days constant improvement of clinical signs and laboratory values (CK day 1: 30′500 IU/L, day 9: 314 IU) occurred and the filly was discharged from the clinic 10 days after admission.

Horse 2–7 were presented to the University of Zurich equine clinic. Horse 2 and 3 were weanlings from the same farm and presented within a week in November 2016. The farm had lost a yearling in 2015 due to similar clinical signs with a suspicion of AM. The acorn trees which were present on pasture had not been removed since. Horse 2 deteriorated within 12 h of presentation, became recumbent and was euthanized due to poor prognosis. Horse 3 remained stable, continued to be able to rise for short periods of time and laboratory values improved (CK: day 1: 384,600 IU/L, day 3 166,600 IU/L, day 4 19,000 IU/L, day 6 5500 IU/L, day 8 1800 IU/L). Urine was macroscopically normal by day 4 but still contained pigment on dipstick analysis, which was negative by day 8. Longitudinal blood and urine samples were collected for acylcarnitine and organic acid analyses. The horse was discharged after 8 days of hospitalization.

Horse 4 was presented in November 2018 due to progressive weakness and stiffness of 48 h duration. Three days prior to the onset of clinical signs the owner had collected leaves that contained acorn seeds from a nearby forest and used it as bedding. The horse improved clinically over the following days and longitudinal blood and urine samples were collected for acylcarnitine analysis and determination of organic acids. CK decreased from 132,200 IU/L on day 2 to 58,443 IU/L on day 3, 20,671 IU/L on day 4, 2763 IU/L on day 7. CK on day 1 could not be measured as it exceeded to the upper reference limit of 30′000 IU/L on emergency blood work. Myoglobinuria resolved on day 7 and the horse was discharged after 9 days of hospitalization.

Horse 5 was presented in May 2015. The exposure to acorn was unknown. Within 4 h of presentation the yearling became recumbent

Table 1
Signalment, clinical and laboratory findings of horses affected by AM.

Horse	H-1	H-2	H-3	H-4	H-5	H-6	H-7
Breed	Fell Pony	French Montagne	French Montagne	French Montagne	French Montagne	Paint	Dartmoor Pony
Sex	F	F	S	G	G	S	F
Age	7 m	8 m	6 m	14 y	1 y	6 m	1 y
Farm	F	A	A	D	E	C	B
Definitive acorn exposure	Tree on pasture	Tree on pasture	Tree on pasture	Forest leaves with acorn seeds as bedding	No	No	Tree on pasture
Presenting complaint	Weakness Stiffness Apathy						
Heart Rate Bpm Ref: 36–40	44	64	80	60	64	125	68
Respiratory rate Brpm Ref [8–12]	16	12	10	12	28	30	24
Mentation	Apathy						
Musculoskeletal	Standing Weak Stiff gait	Standing weak stiff gait	Standing weak stiff gait	Recumbent but able to rise with assistance	Standing weak stiff gait	Recumbent but able to rise with assistance	Recumbent but able to rise with assistance
Myoglobinuria	Yes						
CK (IU/L) ref.:112–305	30′500	924′000	384′600	132′200	692′064	550′600	35′586
AST(IU/L) Ref:229–393	na	25′000	16′928	12′192	38′955	12′615	28′743
cTNI (ng/mL) Ref: <0.03	na	0.01	0.28	1.25	0.27	0.2	na
Lactate mmol/L Ref < 1.0	2.5	5.4	4.5	11.4	5.0	16.4	2.3
Selenium ±µg/L Ref: 70–170	na					74	na
Vitamin E mg/L Ref >1	na					3	na
5 Panel Test	na					Negative	na
Blood for AC analysis	x	x	x (L)	x (L)	x	x	x
Urine for OA analysis	x (L)	x	x (L)	x (L)	x		
Survival	x		x	x			x

F: Female, G: gelding, S: Stallion, na: not available, bpm: beats per minute, brpm: breaths per minute, L: longitudinal samples available, 5 Panel Test: Genetic test for Quarter Horses for hereditary equine regional dermal agenesia (HERDA), glycogen enzyme branching deficiency (GBED), polysaccharide storage myopathy (PPSM), equine malignant hyperthermia (EMH), and hyperkalemic periodic paralysis (HYPP).

and unable to rise. The yearling deteriorated further clinically over the next 48 h and was euthanized due to poor prognosis.

Horse 6 presented in October 2018 after being purchased and transported for 6 h from Germany to Switzerland the day before. The weanling tested negative on a standard 5-panel Quarter horse genetic test before purchase (Table 1). There was no known history of acorn exposure at the farm of origin, however the premise was not inspected. Within 12 h of presentation the weanling became unable to rise. CK increased to 1,111,900 IU/L and cTNI increased to 8.14 ng/mL. Due to the clinical progression and worsening of laboratory values the weanling was euthanized after 48 h of hospitalization.

Horse 7 was presented in November 2016 at the same time as a 2-year-old Conemara Pony from the same farm. Both ponies were kept together on pasture with acorn trees and were last checked 7 days ago. On the day or presentation, the owner found one pony recumbent and the other standing but weak. The year before two yearlings were found dead on the same pasture, and an undetermined number of horses kept on that pasture had died or been weak in the preceding years. The accompanying pony died upon arrival and was therefore not included in this case series. The yearling improved clinically over the following days, myoglobinuria resolved and the pony was discharged after 27 days of hospitalization. Longitudinal samples were not collected.

3.2. Urinary organic acids in atypical myopathy

Determination of organic acid profile (>100 metabolites) in urine of horses with AM resulted in specific increase of fatty-acid beta-oxidation metabolites and amino acids catabolites. Of the 113 tested metabolites, 51 (45%) were not detected in any control horses or horses with AM (Supplementary Table 2). Of the remaining 62 metabolites 14 (23%) differed significantly between control horses and horses with AM (p-values based on a Wilcoxon test are presented in Supplementary Information Table 2). To assess increase of organic acids in horses, threshold values were previously determined by analysis of control urine samples from horses not suffering from muscle disease ($n = 7$). The highest value measured in control horses were set as threshold. It is to note that many organic acids are not detected in urine of controls or horses with AM and exclusively serve as biomarker for other specific diseases. Organic acid summary data of controls horses and horses with AM including p-values to assess significant differences are presented in Supplementary Information Table 2.

A selection of organic acids important in the diagnosis of acMADD with the data obtained from the present study is displayed in Table 2. Ethylmalonic acid, 2-hydroxyglutaric acid and the acylglycines: butyrylglycine, valerylglycine, and hexanoylglycine were strongly elevated in the urine of all horses affected with AM at the day of disease presentation. Those biomarkers were also much higher in Horse 2 and 5, which later succumbed to the disease. In Horses 1, 3 and 4, those metabolites decreased during disease remission to approach normalization after 7 days in Horse 4 (Table 2). Lactate, pyruvate, 2-methylsuccinic acid and further dicarboxylic acids (glutaric, adipic, suberic) were elevated in the urine of the majority of the horses at the day of disease presentation (see Table 2 for details).

3.3. Acylcarnitines in DBS in atypical myopathy

Determination of acylcarnitines in DBS of horses with AM resulted in increased concentrations of short-, medium- and long-chain acylcarnitines in comparison to the control cohort ($n = 16$). Of 32 analyzed acylcarnitines, 7 were elevated above the threshold in all 7 horses with AM (C4, C5, C6, C8:1, C8, C10:1, C10) on the day of hospitalization (Table 3).

All short- (C2-C5) and medium-chain (C6-C12) acylcarnitines in blood differed significantly between control horses and horses with AM, except for C5:1. Of the long-chain acylcarnitines (C14-C18) only C14, C14:1, C14:2, C14OH, C14:1OH, C16OH and C18OH differed between

Table 2
Data of selected organic acids in urine of horses with AM and their progression in surviving animals.

Selected organic acids	Threshold value (mmol/mol creatinine)	H-1 Day 1	H-1 Day 4	H-2[a] Day 1	H-3 Day 1	H-3 Day 2	H-3 Day 3	H-3 Day 4	H-4 Day 1	H-4 Day 5	H-4 Day 6	H-4 Day 7	H-5[a] Day 1
Metabolites of mitochondrial fatty-acid beta-oxidation													
Butyrylglycine (C4)	n.d.	385	17	334	117	25	87	25	132	32	27	2	415
Ethylmalonic acid	<20	76	<20	188	113	66	65	45	70	36	23	<20	174
2-Methylsuccinic acid	<25	43	<20	91	31	<20	<20	24	27	n.d.	<20	<20	23
Valerylglycine (C5)	n.d.	36	n.d.	107	16	6	6	9	9	n.d.	n.d.	n.d.	26
Hexanoylglycine (C6)	<3	655	<10	370	60	6	56	4	86	11	12	n.d.	124
Adipic acid (C6-di)	n.d.	37	n.d.	37	<20	n.d.	n.d.	n.d.	20	n.d.	n.d.	n.d.	62
Suberic acid (C8-di)	n.d.	15	n.d.	<10	<10	n.d.	n.d.	n.d.	18	n.d.	n.d.	n.d.	23
Metabolites of amino acid catabolism													
2-methylbutyrylglycine	n.d.	12	n.d.	28	2	2	1	2	n.d.	n.d.	n.d.	n.d.	n.d.
Isovalerylglycine	<20	59	<20	102	<20	<20	<20	<20	<20	<20	<20	<20	54
2-Hydroxyglutaric acid	<20	39	<20	128	24	<20	<20	<20	40	<20	<20	<20	51
Glutaric acid (C5-di)	n.d.	33	n.d.	37	<10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	33
Metabolites of the glycolyse													
Lactate	<110	<50	<50	1500	629	108	<50	<50	<50	<50	<50	<50	322
Pyruvate	<20	22	<20	262	47	<20	<20	<20	<20	<20	<20	<20	44

n.d.: not detected, threshold value: urine of 7 control horses were used to determine the threshold value (highest value obtained).
Bold numbers are above the threshold value.
[a] Horse died.

Table 3

Data of acylcarnitines in dried blood spots of horses with AM at the day of disease presentation. The concentration factor marked in red were above the threshold for all horses, and thus could be used as diagnostic markers for acquired multiple acetyl dehydrogenase deficiency (acMADD).

Abb.	Name	Threshold value (μmol/L)	H-1	H-2[†]	H-3	H-4	H-5[†]	H-6[†]	H-7	Concentration factor from threshold (range)
C0	Free carnitine	<32.06	22.1	**38.10**	18.80	30.40	10.10	20.30	11.90	0.3-1.2
C2	Acetylcarnitine	<5.85	5.5	**24.90**	**12.30**	**45.00**	**14.20**	**12.60**	5.70	1.0-7.7
C3	Propionylcarnitine	<0.49	0.42	**2.59**	**0.75**	**0.61**	**0.87**	**1.25**	0.36	0.7-5.3
C4	n-/iso-Butyrylcarnitine	<0.77	**3.89**	**27.11**	**5.25**	**5.43**	**8.33**	**7.83**	**1.75**	**2.3-35.2**
C5:1	Tiglyl-/Methyl-Crotonylcarnitine	<0.02	0.01	**0.04**	0.01	<0.01	0.01	0.01	0.01	0.5-2.0
C5	n-/iso-Valerylcarnitine	<1.39	**2.52**	**30.87**	**3.84**	**4.84**	**6.24**	**6.52**	**1.87**	**1.3-22.2**
C4OH+C3DC	OH-But.-/Malonic carnitine	<0.07	0.04	**0.56**	**0.09**	**0.21**	**0.17**	**0.20**	0.06	0.9-8.0
C6	Hexanoylcarnitine	<0.04	**0.6**	**5.27**	**0.98**	**0.99**	**1.48**	**0.96**	**0.26**	**6.5-131.8**
C5OH+C4DC	Succinylcarnitine and isobaric acylcarnitines	<0.23	0.09	**0.46**	**0.26**	**0.27**	**0.28**	0.15	0.15	0.7-2.0
C5DC+C6OH	Glutarylcarnitine	<0.05	**0.1**	**0.36**	**0.10**	**0.06**	**0.09**	**0.07**	0.03	0.6-7.2
C8:1	Octenoylcarnitine	<0.02	**0.28**	**2.05**	**0.40**	**0.27**	**0.32**	**0.27**	**0.09**	**4.5-102.5**
C8	Octanoylcarnitine	<0.05	**0.47**	**1.51**	**0.53**	**0.40**	**0.64**	**0.36**	**0.10**	**2.0-30.2**
C6DC	Adipoyl-/Methylglutaric carnitine	<0.02	**0.04**	**0.09**	**0.03**	**0.04**	**0.04**	**0.04**	0.02	1.0-4.5
C10:1	Decenoylcarnitine	<0.02	**0.16**	**0.46**	**0.19**	**0.12**	**0.21**	**0.16**	**0.08**	**4.0-23.0**
C10	Decanoylcarnitine	<0.05	**0.37**	**0.46**	**0.29**	**0.36**	**0.24**	**0.21**	**0.06**	**1.2-9.2**
C8DC	Suberylcarnitine	<0.01	**0.05**	**0.06**	**0.02**	0.01	**0.02**	0.01	<0.01	0.0-6.0
C12:1	Laureoylcarnitine	<0.04	**0.15**	**0.10**	**0.07**	**0.10**	**0.07**	0.04	0.02	0.5-2.5
C12	Lauroylcarnitine	<0.03	**0.15**	**0.16**	**0.11**	**0.11**	**0.06**	**0.10**	0.02	0.7-5.3
C14:2	Myristdienoylcarnitine	<0.03	0.03	0.02	**0.04**	**0.04**	0.02	0.02	<0.01	0.0-1.3
C14:1	Myristeoylcarnitine	<0.05	**0.17**	**0.13**	**0.10**	**0.21**	**0.08**	**0.06**	0.02	0.4-4.2
C14	Myristoylcarnitine	<0.06	**0.14**	**0.29**	**0.19**	**0.08**	**0.12**	**0.08**	0.04	0.7-4.8
C14:1OH	OH-Myristeoylcarnitine	<0.03	0.03	**0.07**	0.03	**0.05**	**0.04**	0.03	0.01	0.3-2.3
C14OH	OH-Myristoylcarnitine	<0.01	**0.04**	**0.10**	**0.07**	**0.02**	**0.03**	**0.04**	<0.01	0.0-10.0
C16:1	Palmiteoylcarnitine	<0.1	0.08	**0.20**	**0.13**	**0.16**	0.08	0.09	0.02	0.2-2.0
C16	Palmitoylcarnitine	<2.25	0.19	1.54	1.16	1.53	1.92	0.67	0.66	0.3-0.9
C16:1OH	OH-Palmiteoylcarnitine	<0.13	0.03	**0.16**	0.09	0.12	0.09	0.08	0.05	0.4-1.2
C16OH	OH-Palmitoylcarnitine	<0.02	0.02	**0.14**	**0.08**	**0.04**	0.02	**0.04**	0.01	0.5-7.0
C18:2	Linoleoylcarnitine	<0.2	0.03	0.15	0.13	0.11	0.07	0.07	0.03	0.2-0.8
C18:1	Oleoylcarnitine	<1.5	0.16	0.79	0.81	1.33	0.88	0.31	0.28	0.2-0.9
C18	Stearoylcarnitine	<1.24	0.07	0.83	0.77	0.88	0.98	0.37	0.46	0.3-0.8
C18:1OH	OH-Oleoylcarnitine	<0.05	0.02	**0.07**	0.04	0.04	0.02	0.03	0.01	0.2-1.4
C18OH	OH-Stearoylcarnitine	<0.02	0.01	0.02	0.01	0.01	0.01	0.01	<0.01	0.5-1.0

[†]horse died, Threshold value: DBS from 16 control horses were used to determine the threshold value (highest value obtained was set as threshold), see Supplementary Table 1.

control animals and horses with AM. Free carnitine (C0) was not significantly different between cases and controls (p-values based on a Wilcoxon test are presented in Supplementary Information Table 3).

In survivors the concentrations of acylcarnitines decreased over time but were still partially elevated after 7 days in Horse 4 (Figure 1, Supplementary Information Table 4). Whether values had normalized depended on the initial degree of elevation.

4. Discussion

This study shows that analysis of organic acids in urine and acylcarnitines in blood by specialized human medical laboratories can be used to diagnose AM in horses. The study further shows that elevation of organic acids in urine and acylcarnitines in blood were still evident

several days after disease presentation, allowing submission of samples from convalescent animals feasible for diagnosis.

4.1. Diagnosis of atypical myopathy using human assays

The metabolite of the toxin hypoglycin A (MCPA-CoA) present in *A. pseudoplatanus* ingested by horses, inhibits many mitochondrial dehydrogenases that induce elevation of specific metabolites. Determination of those metabolites in horse urine and blood can serve as biomarkers to diagnose AM. In agreement with prior studies [2,5,19], we observed an elevation of the acylcarnitines (C4-C14) measured in blood and the acylglycines (butyryl-, valeryl-, and hexanoylglycine) as well as ethylmalonic acid, 2-methylsuccinic acid and the dicarboxylic acids (succinic, adipic, and sebacic acid) in urine, arising from inhibition
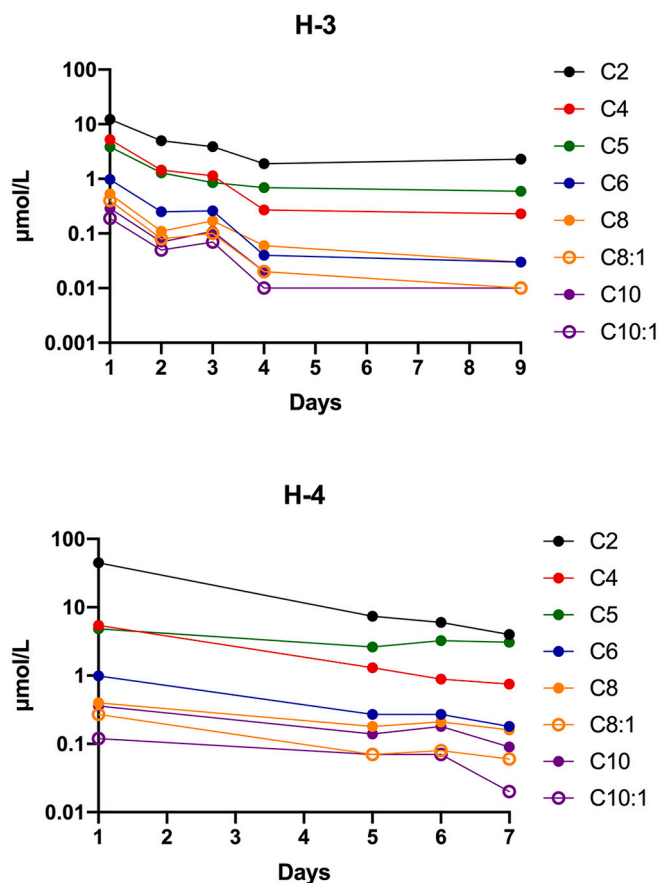
Fig. 1. Evolution of selected acylcarnitines in DBS during remission of acquired multiple acetyl dehydrogenase deficiency (acMADD) in two survivors (H3 and H4, Logarithmic scale). Acetylcarnitine (C2), Butyrylcarnitine (C4), valerylcarnitine (C5), hexanoylcarnitine (C6), octanoylcarnitine (C8), octenoylcarnitine (C8:1), decanoylcarnitine (C10), decenoylcarnitine (C10:1).

of the mitochondrial fatty acid beta-oxidation. Also, like prior studies [2,19], elevation of 2-methylbutyrylglycine, isovalerylgylcine and 2-hydroxyglutaric acid was observed, arising from inhibition of the amino acid catabolism. As the energy supply from fatty acids oxidation decreases in diseased horses, energy needs are replaced by glycolysis and lactate and pyruvate increase [11]. As previously described and in contrast to humans, glutaric acid is not a good biomarker for the disease, as glutaric acid was not found to be elevated in all affected horses [3].

Despite the great similarity between human acMADD and equine AM, several important differences are present. Horses usually show hyperglycemia and increased serum free carnitine concentrations, which was also observed in our study [5,19,20], while humans show hypoglycemia and decreased free carnitine concentrations [21,22]. This is likely due to species specific differences in energy metabolism and should be kept in mind for interpretation of results and comments obtained from human laboratories.

In our cohort, both assays showed specific pattern for the diagnosis of acMADD. In urine organic acid analysis, the best biomarkers were ethylmalonic acid, the acylglycines and 2-hydroxyglutaric acid. Among the acylcarnitines, the best biomarkers were the short- and medium-chain acylcarnitines, C4, C5, C6 and C8, C8:1, C10 and C10:1, where concentration factors from cut-off were higher than one in all horses at the day of disease presentation. It has been shown that 2/3 horses suffering from acute myopathies other than AM also had increased short chain acylcarnities (C2-C4) in blood [5]. Therefore, a diagnosis should not be blindly based on acylcarnitine profile, but also consider the corresponding history, clinical signs and measurement of urinary

organic acids. Hypoglycin A and MCPA-conjugates in urine and blood could be considered for diagnosis confirmation, although this is not essential, as demonstrated by this study.

4.2. Prediction of survival

In our small cohort, the acylcarnitines that were diagnostic and which were consistently higher than the threshold, were also higher in the horses that succumbed to the disease. In one study with 11 survivors and 40 non-survivors it has been shown that estimation of survival may be based on evaluation of three acylcarnitines. Horses that had C2 > 17.3 µmol/L or C10:2 > 0.11 µmol/L or C18 > 0.21 µmol/L had a > 80% increased risk of dying [16]. In our study C4 and C5 were the best predictors of survival, however this is based on a very small case number and further studies using larger cohorts should be conducted. In our study acetylcarnitine (C2) was not a good predictor marker as surviving horses had higher C2 values as some horses that later died of the disease. In the organic acid as well, the specific organic acids for acMADD were higher in the horses that died at the day of disease presentation. Thus, apart from diagnostic, those analyses could be used as prediction tool for a possible remission.

4.3. Following progression of disease

To our knowledge, no information on biomarker progression in horses during disease remission was available in the literature. In our study the biomarkers in blood and urine correlate with the disease remission.

4.4. Limitations

The number of cases presented in this study was small and the number of surviving animals with longitudinal samples available was even smaller. Furthermore, daily sampling in survivors was not feasible. A larger cohort should be studied with more frequent sampling in order to further improve our understanding of the evolution of acylcarnitines and urinary organic acid concentrations in this disease. Despite the lack of analyses for hypogylcin A and or MCPA-carnitine conjugates, the signalment, history, clinical signs, progression and typical changes in acylcarnitines and urinary organic acids make a false diagnosis very unlikely. The control horses presented to the hospital for signs of diseases other than muscle disease and cannot therefore be considered healthy. An additional group of horses with muscle diseases other than AM should also be included in future studies.

4.5. Conclusions

In conclusion, determination of acylcarnitine and organic acid profiles by special assays available in human medical laboratories were able to clearly distinguish between controls and horses affected with AM. Ethylmalonic acid, 2-hydroxyglutaric acid, butyrylglycine and hexanoylglycine in urine and the acylcarnitine C4-C10 in blood were the best biomarkers for diagnosis. Furthermore, the initial concentration of C4 and C5 acylcarnitines could be used as predictor marker in acMADD.

Funding

Declaration of Competing Interest

The authors have no competing interest to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymgmr.2021.100827.

References

[1] D.M. Votion, G. van Galen, L. Sweetman, F. Boemer, P. de Tullio, C. Dopagne, L. Lefere, A. Mouithys-Mickalad, F. Patarin, S. Rouxhet, G. van Loon, D. Serteyn, B.T. Sponseller, S.J. Valberg, Identification of methylenecyclopropyl acetic acid in serum of European horses with atypical myopathy, Equine Vet. J. 46 (2014) 146–149.

[2] S.J. Valberg, B.T. Sponseller, A.D. Hegeman, J. Earing, J.B. Bender, K.L. Martinson, S.E. Patterson, L. Sweetman, Seasonal pasture myopathy/atypical myopathy in North America associated with ingestion of hypoglycin A within seeds of the box elder tree, Equine Vet. J. 45 (2013) 419–426.

[3] J.H. van der Kolk, I.D. Wijnberg, C.M. Westermann, L. Dorland, M.G. de Sain-van, L.C. der Velden, M. Kranenburg, J.A. Duran, J.J. van der Dijkstra, R.J. Lugt, E. Gruys Wanders, Equine acquired multiple acyl-CoA dehydrogenase deficiency (MADD) in 14 horses associated with ingestion of maple leaves (Acer pseudoplatanus) covered with european tar spot (Rhytisma acerinum), Mol. Genet. Metab. 101 (2010) 289–291.

[4] L. Unger-Torroledo, R. Straub, A.D. Lehmann, F. Graber, C. Stahl, J. Frey, V. Gerber, H. Hoppeler, O. Baum, Lethal toxin of Clostridium sordellii is associated with fatal equine atypical myopathy, Vet. Microbiol. 144 (2010) 487–492.

[5] C.M. Westermann, L. Dorland, D.M. Votion, M.G. de Sain-van der Velden, I.D. Wijnberg, R.J. Wanders, W.G. Spliet, N. Testerink, R. Berger, J.P. Ruiter, J.H. van der Kolk, Acquired multiple Acyl-CoA dehydrogenase deficiency in 10 horses with atypical myopathy, Neuromuscul. Disord. 18 (2008) 355–364.

[6] H.A. Meda, B. Diallo, J.P. Buchet, D. Lison, H. Barennes, A. Ouangre, M. Sanou, S. Cousens, F. Tall, P., Van de perre, epidemic of fatal encephalopathy in preschool children in Burkina Faso and consumption of unripe ackee (Blighia sapida) fruit, Lancet 353 (1999) 536–540.

[7] R. Joskow, M. Belson, H. Vesper, L. Backer, C. Rubin, Ackee fruit poisoning: an outbreak investigation in Haiti 2000–2001, and review of the literature, Clin. Toxicol. (Phila.) 44 (2006) 267–273.

[8] S.L. Isenberg, M.D. Carter, L.A. Graham, T.P. Mathews, D. Johnson, J.D. Thomas, J.L. Pirkle, R.C. Johnson, Quantification of metabolites for assessing human exposure to soapberry toxins hypoglycin A and methylenecyclopropylglycine, Chem. Res. Toxicol. 28 (2015) 1753–1759.

[9] L. Fowden, H.M. Pratt, Cyclopropylamino acids of the genus Acer: distribution and biosynthesis, Phytochemistry 12 (1972) 5.

[10] L. Unger, A. Nicholson, E.M. Jewitt, V. Gerber, A. Hegeman, L. Sweetman, S. Valberg, Hypoglycin a concentrations in seeds of Acer pseudoplatanus trees growing on atypical myopathy-affected and control pastures, J.Vet.Int.Med. 28 (2014) 1289–1293.

[11] M. Mereis, R.J.A. Wanders, M. Schoonen, M. Dercksen, I. Smuts, F.H. van der Westhuizen, Disorders of flavin adenine dinucleotide metabolism: MADD and related deficiencies, Int. J. Biochem. Cell Biol. 132 (2021), 105899.

[12] B.J. Henriques, R.Katrine Jentoft Olsen, C.M. Gomes, P. Bross, Electron transfer flavoprotein and its role in mitochondrial energy metabolism in health and disease, Gene 776 (2021), 145407.

[13] R. Karlikova, J. Siroka, P. Jahn, D. Friedecky, A. Gardlo, H. Janeckova, F. Hrdinova, Z. Drabkova, T. Adam, Equine atypical myopathy: a metabolic study, Vet. J. 216 (2016) 125–132.

[14] M. Bochnia, W. Scheidemann, J. Ziegler, J. Sander, S. Vollstedt, M. Glatter, N. Janzen, M. Terhardt, A. Zeyner, Predictive value of hypoglycin A and methylencyclopropylacetic acid conjugates in a horse with atypical myopathy in comparison to its cograzing partners, Equine Vet .Educ 30 (2018) 24–28.

[15] M. Bochnia, J. Ziegler, J. Sander, A. Uhlig, S. Schaefer, S. Vollstedt, M. Glatter, S. Abel, S. Recknagel, G.F. Schusser, M. Wensch-Dorendorf, A. Zeyner, Hypoglycin A content in blood and urine discriminates horses with atypical myopathy from clinically normal horses grazing on the same pasture, PLoS One 10 (2015), e0136785.

[16] F. Boemer, J. Detilleux, C. Cello, H. Amory, C. Marcillaud-Pitel, E. Richard, G. van Galen, G. van Loon, L. Lefere, D.M. Votion, Acylcarnitines profile best predicts survival in horses with atypical myopathy, PLoS One 12 (2017), e0182761.

[17] M.S. Rashed, P.T. Ozand, M.P. Bucknall, D. Little, Diagnosis of inborn errors of metabolism from blood spots by acylcarnitines and amino acids profiling using automated electrospray tandem mass spectrometry, Pediatr. Res. 38 (1995) 324–331.

[18] K. Tanaka, A. West-Dull, D.G. Hine, T.B. Lynn, T. Lowe, Gas-chromatographic method of analysis for urinary organic acids. II. Description of the procedure, and its application to diagnosis of patients with organic acidurias, Clin. Chem. 26 (1980) 1847–1853.

[19] B.T. Sponseller, S.J. Valberg, N.E. Schultz, H. Bedford, D.M. Wong, K. Kersh, G.D. Shelton, Equine multiple acyl-CoA dehydrogenase deficiency (MADD) associated with seasonal pasture myopathy in the midwestern United States, J. Vet. Intern. Med. 26 (2012) 1012–1018.

[20] D.M. Votion, A. Linden, C. Saegerman, P. Engels, M. Erpicum, E. Thiry, C. Delguste, S. Rouxhet, V. Demoulin, R. Navet, F. Sluse, D. Serteyn, G. van Galen, H. Amory, History and clinical features of atypical myopathy in horses in Belgium (2000-2005), J. Vet. Intern. Med. 21 (2007) 1380–1391.

[21] W. Onkenhout, V. Venizelos, P.F. van der Poel, M.P. van den Heuvel, B.J. Poorthuis, Identification and quantification of intermediates of unsaturated fatty acid metabolism in plasma of patients with fatty acid oxidation disorders, Clin. Chem. 41 (1995) 1467–1474.

[22] C.G. Costa, L. Dorland, U. Holwerda, I.T. de Almeida, B.T. Poll-The, C. Jakobs, M. Duran, Simultaneous analysis of plasma free fatty acids and their 3-hydroxy analogs in fatty acid beta-oxidation disorders, Clin. Chem. 44 (1998) 463–471.