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Acquired multiple Acyl-CoA dehydrogenase deficiency in 10 horses with atypical myopathy

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Abstract

The aim of the current study was to assess lipid metabolism in horses with atypical myopathy.

Urine samples from 10 cases were subjected to analysis of organic acids, glycine conjugates, and acylcarnitines revealing increased mean excretion of lactic acid, ethylmalonic acid, 2-methylsuccinic acid, butyrylglycine, (*iso*)valerylglycine, hexanoylglycine, free carnitine, C2-, C3-, C4-, C5-, C6-, C8-, C8:1-, C10:1-, and C10:2-carnitine as compared with 15 control horses (12 healthy and three with acute myopathy due to other causes). Analysis of plasma revealed similar results for these predominantly short-chain acylcarnitines. Furthermore, measurement of dehydrogenase activities in lateral vastus muscle from one horse with atypical myopathy indeed showed deficiencies of short-chain acyl-CoA dehydrogenase (0.66 as compared with 2.27 and 2.48 in two controls), medium-chain acyl-CoA dehydrogenase (0.36 as compared with 4.31 and 4.82 in two controls) and isovaleryl-CoA dehydrogenase (0.74 as compared with 1.43 and 1.61 nmol min^{-1} mg^{-1} in two controls).

A deficiency of several mitochondrial dehydrogenases that utilize flavin adenine dinucleotide as cofactor including the acyl-CoA dehydrogenases of fatty acid β-oxidation, and enzymes that degrade the CoA-esters of glutaric acid, isovaleric acid, 2-methylbutyric acid, isobutyric acid, and sarcosine was suspected in 10 out of 10 cases as the possible etiology for a highly fatal and prevalent toxic equine muscle disease similar to the combined metabolic derangements seen in human multiple acyl-CoA dehydrogenase deficiency also known as glutaric acidemia type II.

Keywords: Horse; Multiple Acyl-CoA dehydrogenase deficiency; Myopathy; Lipid metabolism; Acylcarnitine; Organic acid; Glycine conjugate

1. Introduction

So-called atypical myopathy is an acute myopathy that appears in grazing horses [1–3]. To the authors' knowledge, the first case reports of myopathy in grazing horses concerned outbreaks that occurred in the autumn of 1939 in the North of Wales, UK [4]. Since the recognition of the syndrome, outbreaks of atypical myopathy have been reported in several European countries and case reports prior to the syndrome's identification suggest that the condition has also been encountered in Australia, Canada and the United States of America [2,5]. For example, in the autumn of 1995, over one hundred horses died from this condition in Northern Germany [1,2]. In autumn 2000, Belgium recognised its first cases of atypical myopathy

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and large outbreaks were recorded during cold periods in autumn and spring of the years 2002, 2004, and 2006 in Belgium and France. From 2004, the syndrome was recognised in more than ten European countries thereby suggesting its emerging nature. The syndrome is associated with a mortality rate of about 90% and death usually occurs within 72 h [2,3].

To date, a number of potential aetiological and contributory factors were considered, but both the exact aetiology and the pathophysiology have remained unresolved. Whatever the cause, particular weather conditions seem to trigger the appearance of the syndrome [2,5].

The main feature of this syndrome is the sudden onset of clinical signs characterized by acute generalised progressive myopathy. Serum biochemical abnormalities usually include markedly elevated muscle enzyme activities indicating severe muscle damage and at *post mortem* widespread myodegeneration are found in both skeletal muscle and myocardium [1–3]. It has been shown previously that in equine atypical myopathy predominantly type 1 muscle fibers were degenerated associated with the accumulation of neutral lipids [1].

The aim of the current study was to perform metabolic screening of lipid metabolism in horses with a tentative diagnosis of atypical myopathy.

2. Materials and methods

2.1. Horses

Five horses sampled during the autumn 2006 outbreak in the Netherlands and five Belgian cases from outbreaks in 2003, 2004, and 2006 with a tentative diagnosis of atypical myopathy were used. In nine out of these 10 horses (except case 5 which survived) the disease turned out to be fatal. The description and clinical course of these 10 horses (indicated as 1–10) is given in Table 1. Peak blood values in these horses are shown in Table 2. Inclusion criteria included access to pasture, no previous anaesthesia, nonexertional and nonrecurrent acute progressive rhabdomyolysis, absence of nonmuscular pathology and plasma creatine kinase activity >2000 IU L^{-1}.

Twelve clinically healthy warmblood mares belonging to Utrecht University housed in boxes and accustomed to frequent handling were used as healthy controls. These horses were 3.7–20.5 years of age (mean age ± SD, 9.6 ± 5.0 years) and weighed 470–758 kg (mean weight, 596 ± 84.7 kg). Their diet consisted of grass silage supplemented with concentrate feed and met nutrient requirements for maintenance and performance. The total diet contained 10% ash, 14.5% crude protein, 1.3% crude fat, 20% crude fiber, and 56.2% other carbohydrates. Water was provided ad libitum.

In addition, three horses suffering from acute myopathy due to other causes (including one case of post-anaesthetic myopathy (horse 11) and two cases of recurrent exertional rhabdomyolysis (horses 12 and 13)) without a tentative diagnosis of atypical myopathy were used as diseased controls. The description and clinical course of these three horses (indicated as 11–13) is also given in Table 1. Peak blood values in these horses are shown in Table 2.

2.2. Muscle pathology

Nine out of 10 horses (except case 5 which survived) with a tentative diagnosis of atypical myopathy were submitted for pathology. For histologic examination, muscle specimens were fixed in 4% (w/v) phosphate-buffered paraformaldehyde, sectioned at 5 μm, and stained with H&E. In addition, NADH, cytochrome *c* oxidase, succinate dehydrogenase, acid phosphatase, periodic acid Schiff, ATP-ase (pH 4.3 and 9.4), Sudan black B, and oil red O stains were performed on various muscle tissues frozen in isopentane that was precooled in liquid nitrogen, and stored at −80 °C.

2.3. Analysis of organic acids and glycine conjugates

Urine samples from all 10 cases with a tentative diagnosis of atypical myopathy were subjected to analysis of organic acids and glycine conjugates and results

Table 1
Breed, age, sex, and clinical progression of 10 horses with a tentative diagnosis of atypical myopathy (1–10) and three controls (11–13)

Horse number	Breed	Age	Gender	Clinical course
1	Fjord	2 years	Mare	Death within 3 days
2	Belgian draft	0.5 years	Mare	Death within 1 day
3	Standardbred	1.5 years	Mare	Death within 3 hours
4	Ardennais	0.5 years	Mare	Death within 10 hours
5	Draft crossbreed	10 years	Mare	Survived
6	Draft crossbreed	11 years	Mare	Death within 1 day
7	Pony	1 year	Mare	Death within 3 days
8	Friesian	10 months	Mare	Death within 1 day
9	Arabian	2 years	Stallion	Death within 1 day
10	Warmblood	3.5 years	Mare	Death within 2 days
11	Tinker	14 years	Gelding	Death within 2 days
12	Thoroughbred	4 years	Mare	Survived
13	Warmblood	10 years	Mare	Survived

Table 2
Peak blood values in 10 horses with a tentative diagnosis of acute myopathy (1–10) and three controls (11–13)

Horse number Reference range	CK <200 IU L^{-1}	AST <275 IU L^{-1}	LDH <600 IU L^{-1}	Lactate <1.0 mmol L^{-1}	Glucose <5.6 mmol L^{-1}	Total triglycerides <1.0 mmol L^{-1}
1	38,600	22,300	41,400	1.3	9.5	15.2
2	939,000					3.2
3	146,600		23,250	8.8	11.4	1
4	171,600	5060	1935	15.9	6.5	2.4
5	69,000	6017	26,160	2.5		
6	400,000	12,376	35,541	5.4	8.2	
7	409,400	2790		11.7	8.5	
8	>2000	>1000		14.7	12.4	
9	162,800	3701	8010			
10	410,000	11,522	36,350			
11	12,039	441	2681	5.3	7.1	
12	132,730	5091	14,318			
13	63,673	2721	8781			

Values include creatine kinase (CK), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH).

compared with urine samples from the 15 control horses (12 clinically healthy warmblood mares and three horses suffering from acute myopathy due to other causes without suspicion of atypical myopathy) by using the 95th percentile as the upper limit of the reference range based on the values obtained from the 12 healthy control horses. In addition, heparinised plasma samples from 8 cases with a tentative diagnosis of atypical myopathy were available for analysis of organic acids and results compared with samples from the 15 control horses (12 clinically healthy warmblood mares and three horses suffering from acute myopathy due to other causes without suspicion of atypical myopathy). Identification-analyses of organic acids and glycine conjugates in urine and plasma were carried out by gas chromatography–mass spectrometry (GC–MS) on a Hewlett Packard 5890 series II gas chromatograph linked to a HP 5989B MS-Engine mass spectrometer. Prior to this GC–MS analysis, the organic acids and glycine conjugates were trimethylsilylated with *N*,*N*-bis(trimethylsilyl)trifluoracetamide/pyridine/trimethylchlorosilane (5:1:0.05 v/v/v) at 60 °C for 30 min. The gas chromatographic separation was performed on a 25 m × 0.25 mm capillary CP Sil 19CB column (film thickness 0.19 mm) from Vrian/Chrompack, Middelburg, The Netherlands. The glycine conjugates were measured qualitatively only. Urinary concentrations are expressed as either organic acid: creatinine or glycine conjugate:creatinine ratios.

2.4. Analysis of free and acylcarnitines

Urine samples from all 10 cases with a tentative diagnosis of atypical myopathy were subjected to analysis of acylcarnitines and results compared with urine samples from the 12 clinically healthy control horses. In addition, heparinised plasma samples from 7 cases with a tentative diagnosis of atypical myopathy were available for analysis of acylcarnitines and results compared with samples from the 15 control horses (12 clinically healthy warmblood mares and three horses suffering from acute myopathy due to other causes without suspicion of atypical myopathy). Free carnitine and acylcarnitines in urine and plasma were analysed as their butyl ester derivatives by electrospray tandem mass spectrometry (ESI–MS–MS) on a Micromass Quattro Ultima system equipped with an Alliance HPLC system. Urinary concentrations are expressed as acylcarnitine:creatinine ratios.

2.5. Measurement of muscle dehydrogenase activities

Measurement of muscle dehydrogenase activities was performed in lateral vastus muscle tissue of only one horse (number 6) with a tentative diagnosis of atypical myopathy, collected immediately after euthanasia in liquid nitrogen and stored at −80 °C. Lateral vastus muscle tissue of two clinically healthy control horses was used for control measurements. The activities of medium-chain acyl-CoA dehydrogenase (MCAD), short-chain acyl-CoA dehydrogenase (SCAD), and isovaleryl-CoA dehydrogenase (IVD) were measured according to methods, which are based on the use of the substrate phenylpropionyl-CoA (for MCAD), butyryl-CoA (for SCAD) and isovaleryl-CoA (for IVD). Short-Chain Hydroxy Acyl-CoA Dehydrogenase (SCHAD) was used as a control enzyme in order to assess tissue viability. In brief, incubations were performed at 25 °C in a buffered medium, containing an aliquot of the muscle homogenate plus ferricenium hexafluorophosphate as the electron acceptor. After termination of the reactions by acidification, the acidified samples were centrifuged and the protein-free supernatants neutralized followed by HPLC-analysis to separate the different acyl-CoA esters.

2.6. Fluorescence microscopy

In order to clearly identify the presence of muscular lipid droplets, *M. vastus lateralis* specimens of one horse (number 6) with a tentative diagnosis of atypical myopathy were snap frozen in liquid nitrogen and cut on a Leica

CM3050 microtome. Lateral vastus muscle tissue of two clinically healthy control horses was used for control fluorescence microscopy. Thin cryosections (10 μm) were attached to Superfrost Plus slides and incubated in 0.02 μg/μl Bodipy 493/503 (Molecular Probes, Invitrogen, Breda, the Netherlands) for 15 min in a humidified environment in order to stain neutral lipids. Lipid droplets were visualised by a Leica DMR fluorescence microscope equipped with a Photometrics Coolsnap CCD digital photo camera. Images were processed using IP-lab image analyses software. For muscle fiber typing a monoclonal antibody was used specific for type 1 MyHC isoform kindly provided by prof. A.F.M. Moorman, Academic Medical Centre, Amsterdam, the Netherlands.

2.7. Riboflavin assay

Heparinised plasma samples from 7 cases with a tentative diagnosis of atypical myopathy were subjected to analysis of riboflavin and results compared with samples from 6 control horses. Riboflavin was assessed using HPLC. The detection limit of the HPLC was 13 nmol L^{-1}, and the intra-assay and inter-assay coefficients of variation were 1.86 and 8.97%, respectively.

2.8. Statistical analysis

Normality of the organic acids, acylcarnitines, and the glycine conjugates data was analysed using normal P–P Plot by means of Blom method and the Kolmogorov–Smirnov test. Given the fact that these data not always showed a normal distribution, the 95th percentile was calculated using the data from the 12 healthy control horses. As a consequence, the 95th percentile was always used as the upper limit of the reference range rather than the 95% confidence interval. The cut-off method used for acylcarnitines in urine was defined as a value above 5 mmol mol creatinine^{-1} following the subtraction of the 95th percentile from the average value of each acylcarnitine in urine.

The significance of differences between groups with reference to plasma riboflavin concentrations was assessed by the Mann–Whitney test (two-tailed). Values of $P < 0.05$ were considered significant. Results from riboflavin assay are reported as mean ± SD.

3. Results

Blood analysis supported the tentative diagnosis of acute myopathy in all 13 myopathy cases. The clinicopathological diagnosis of acute myopathy was confirmed *post mortem* in all 9 cases with a tentative diagnosis of atypical myopathy illustrated by pale, degenerated looking musculature in various muscles. Microscopic findings were floccular degeneration, necrosis and myolysis predominantly affecting type 1 muscle fibers, increase of internally located nuclei, subsarcolemmal vacuolation, subsarcolemmal accumulation of mitochondria, slight infiltrations with macrophages and neutrophils and histochemically in some type 1 fibers there was a slight increase of fat. More specific with reference to an 11-year-old Draft horse mare (number 6) suffering from fatal atypical myopathy, histopathological examination of a muscle biopsy from lateral vastus muscle showed myopathic changes with signs of regeneration and fibrotic areas. The extent and severity of the degenerative process varied between cases and muscles.

Fluorescence microscopy from lateral vastus muscle from the same horse (number 6) showed microvesicular lipidosis predominantly (Fig. 1). In addition, electron micrography of lateral vastus muscle from the same horse (number 6) showed subsarcolemmal accumulation of mitochondria (Fig. 2A) and absence of mitochondrial cristae associated with increased matrix density (Fig. 2B).

Metabolic screening of the horses with a tentative diagnosis of atypical myopathy (numbers 1–10) using urine obtained *ante mortem* revealed an increased excretion (above the 95th percentile) of ethylmalonic acid, 2-methylsuccinic acid, butyrylglycine, (*iso*)valerylglycine, and hexanoylglycine in all 10 cases and lactic acid in 9 of 10 as shown in Table 3a. Organic acid concentrations in plasma were not elevated except for lactic acid (Table 3b). Furthermore, the profile of acylcarnitines in urine showed a substantial elevation above the cut-off value for free carnitine, C2-, C3-, C4-, C5-, C6-, C8:1-, C8-, C10:1-, and C10:2-carnitine (Table 4a). Metabolic screening of plasma revealed similar results for C2-, C4-, C5-, C6-, C8:1-, C10:1-, and C10:2-carnitine in all 7 cases studied and free carnitine in 4 of 7 and C3- and C8-carnitine in 6 of 7 as shown in Table 4b.

Repeated metabolic screening of the only surviving horse (number 5) fifteen days later, revealed normal urinary excretion of lactic acid (6 mmol mol creatinine^{-1}), ethylmalonic acid (4 mmol mol creatinine^{-1}) and acylcarnitines without detectable glycine conjugates and 2-methylsuccinic acid associated with a normal acylcarnitine profile in plasma.

In comparison, metabolic screening of three diseased control horses suffering from acute myopathy without a tentative diagnosis of atypical myopathy (indicated as 11–13) revealed normal urinary excretion of ethylmalonic acid and 2-methylsuccinic acid as well as other organic acids (except for lactic acid) without detectable glycine conjugates (Table 3a). Organic acid concentrations in plasma were not elevated except for lactic acid (Table 3b). Metabolic screening of heparinised plasma revealed substantial elevation of free carnitine, C2-, C3-, and C4-carnitine in 2 out of 3 cases.

Furthermore, quantitative biochemical measurement of dehydrogenase activities in lateral vastus muscle from one horse (number 6) with a tentative diagnosis of fatal atypical myopathy indeed showed a deficiency of short-chain acyl-CoA dehydrogenase (SCAD; 0.66 as compared with 2.27 and 2.48 nmol min^{-1} mg^{-1} in two controls), medium-chain acyl-CoA dehydrogenase (MCAD; 0.36 as

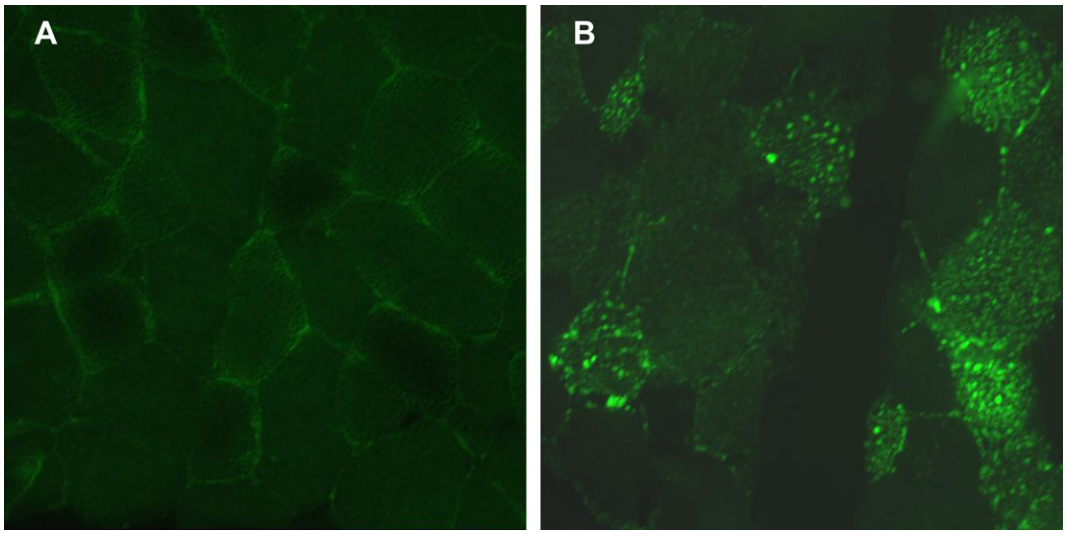

Fig. 1. Neutral lipid staining of lateral vastus muscle from an 8-year-old control warmblood gelding (A) and an 11-year-old Draft horse mare suffering from fatal atypical myopathy showing microvesicular lipidosis (B).

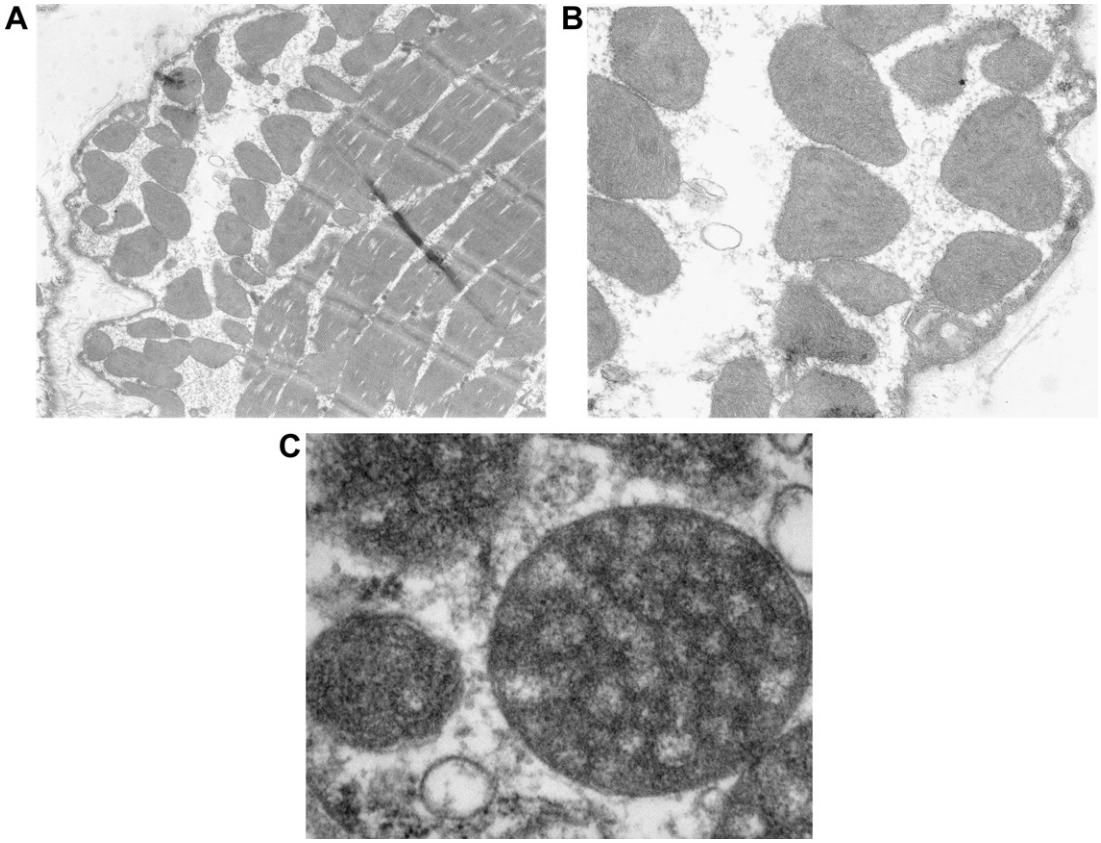

Fig. 2. Electron micrograph from lateral vastus muscle from an 11-year-old Draft horse mare suffering from fatal atypical myopathy showing subsarcolemmal accumulation of mitochondria (A) and absence of mitochondrial cristae associated with increased matrix density (B) as compared with mitochondria in a 9-year-old control warmblood mare (C).

compared with 4.31 and 4.82 nmol min^{-1} mg^{-1} in two controls) and isovaleryl-CoA dehydrogenase (IVD; 0.74 as compared with 1.43 and 1.61 nmol min^{-1} mg^{-1} in two controls). Short-chain hydroxy acyl-CoA dehydrogenase showed similar results in all three animals (343 as compared with 387 and 307 nmol min^{-1} mg^{-1} in two controls) indicating similar tissue viability.

Mean heparinised plasma riboflavin concentrations in seven horses with a tentative diagnosis of atypical myopathy did not differ significantly from those in six

Table 3a
Organic acids (mmol mol creatinine^{-1}) and glycine conjugates (mmol mol creatinine^{-1}) in urine from 10 horses with a tentative diagnosis of atypical myopathy (1–10) and three diseased controls (11–13)

Horse number	1	2	3	4	5	6	7	8	9	10	P95	11	12	13
Lactic acid	66	4824	2086	2093	182	173	3157	2720	3772	143	133	3375	138	9
Glycolic acid	16	38	23	17	24	23		55	64	12	22	25	17	21
Pyruvic acid	38	143	72	49	49	29	85			42	9	76	3	3
2-OH-butyric acid	1	18	2	2	1		22	38	12	3	5	25		
3-OH-butyric acid	44	74	30	16	22	12	383	144	295	43	131	216	3	4
3-OH-isobutyric acid	20	49	21	23	18	17	210	74	141	25	127	82	11	12
3-OH-isovaleric acid	15	23	4	5	1	34	16	76	42	2	34	5	4	3
Methylmalonic acid	2	9	2	1	3	2	15	9	13	4	7		4	2
Ethylmalonic acid	74	194	76	122	324	231	381	270	491	604	5	4	3	2
2-Methylsuccinic acid	39	69	32	66	90	98	78	132	93	105	–	–	–	–
Succinic acid	3	2	1	2	6	5	5	116	4		10		2	3
Glutaric acid	6				57	39		170			122	7	4	
Fumaric acid	6	1	4	5	8		19		11	8	12	6		
2-Oxoglutaric acid	10	11	3	12	4	3	16	74	29	5	56			
Unsaturated suberic acid	39	18	9	12	33	31	35	63	23	36	10	2	1	1
Suberic acid	39	20	8	13	23	21	30	78	18	33	6		1	1
Homovanillic acid	2	2	1	6	1	1	5	5	3	25	37	1	8	7
Adipic acid			3			39	289	16		401	4	4	1	1
Butyrylglycine	+	+	+	+	+	+	+	+	+	+	–	–	–	–
(*iso*)valerylglycine	+	+	+	+	+	+	+	+	+	+	–	–	–	–
Hexanoylglycine	+	+	+	+	+	+	+	+	+	+	–	–	–	–

The glycine conjugates were measured qualitatively only and their presence (+) or absence (−) indicated. Percentile 95 was used as the upper limit of the reference range as not all values obtained from the healthy control horses were normally distributed.

Table 3b
Organic acids (μmol) in heparinised plasma from eight horses with a tentative diagnosis of atypical myopathy (3–10) and three diseased controls (11–13)

Horse number	3	4	5	6	7	8	9	10	P95	11	12	13
Lactic acid	606	24831	1112	5778	6861	6242	8484	7941	2162	9356	1260	1488
2-OH-butyric acid	14	14	2	38	66	105	32	69	24	63	6	6
3-OH-butyric acid	15	103	182	313	375	275	327	317	379	202	227	154
3-OH-isobutyric acid	20	44	18	25	54	48	68	79	107	37	39	21
3-Oxobutyric acid		52	5	40	82	106	90	70	11			
Cis-4-decenoic acid		1		5	4	9	2	4	4			
Glutaric acid	4	22		37	26	56	19	16	5			
Lauric acid	2	9	1			17		3	1			
Myristic acid	6	16	2	15	11	50	9	19	1	10		
Palmitoleic acid	21	30	3	44	17	89	13	49	1	40	3	
Palmitic acid	133	105	29	154	114	414	95	258	5	93	22	13
Oleic acid	76	80	34	142	97	418	77	252	3	97	16	11
Linolic acid	121	80	17	105	109	309	95	201	4	33	15	20
Stearic acid	68	33	26	34	192	184	146	120	4	16	12	5

Percentile 95 was used as the upper limit of the reference range as not all values obtained from the healthy control horses were normally distributed.

clinically healthy control horses (78.9 ± 17.6 versus 68.3 ± 11.8 nmol L^{-1}).

4. Discussion

The 10 horses with a tentative diagnosis of atypical myopathy in this study suffered from a rapidly progressing muscular disease with a mortality rate of 90% associated with degeneration and necrosis primarily affecting type 1 muscle fibers in agreement with the findings on this disease as reported earlier [2]. The signs of regeneration and fibrotic areas as seen on histopathology in only one case were similar to a previously described case (case 7 in [2]) and might suggest some subclinical chronicity of the syndrome.

In the body, fat serves as an efficient source of tissue fuel as compared with carbohydrates. The central role of the mitochondrion is immediately apparent, since it acts as the focus and cross-roads of carbohydrate, lipid, and amino acid metabolism. In particular, it houses the enzymes of the citric acid cycle, of the respiratory chain and ATP synthase, of β-oxidation of fatty acids, and of ketone body production [6]. The main function of muscle mitochondria is oxidative phosphorylation using fatty acids (besides carbohydrates) as the chief substrate to concentrate potential energy. The type 1 fibers have cytochemical features that indicate a mainly aerobic-oxidative metabolic profile associated with numerous mitochondria [7].

Table 4a
Acylcarnitines in urine (mmol mol creatinine^{-1}) from 10 horses with a tentative diagnosis of atypical myopathy (1–10)

Horse number	1	2	3	4	5	6	7	8	9	10	P95
Free carnitine	369.41	250.29	328.65	131.08	339.43	318.12	196.64	327.88	289.81	762.91	12.5
C2-carnitine	379.53	151.60	104.79	68.78	226.74	128.33	177.14	442.19	210.28	385.91	1
C3-carnitine	7.89	13.58	10.30	4.75	17.51	12.79	26.41	22.12	29.77	22.08	0.1
C4-carnitine	28.29	167.66	25.91	78.74	587.63	240.03	98.31	199.72	288.98	164.96	1
C5:1-carnitine	0.12	0.16	0.11	0.10	0.08	0.17	0.27	0.55	0.22	0.20	0.01
C5-carnitine	17.64	341.07	23.03	73.57	388.86	226.67	233.19	395.46	676.69	250.61	0.1
C4:3-carnitine	7.87	1.85	0.70	0.95	1.11	1.81	2.56	6.37	3.45	2.50	0
C6-carnitine	2.28	44.12	7.47	11.40	72.19	53.07	14.95	25.68	23.70	17.81	0.02
C5-OH-carnitine	0.31	2.81	0.94	0.84	4.42	2.82	2.71	9.21	5.15	1.69	0.1
C8:1-carnitine	0.49	19.43	3.96	11.48	23.53	21.46	11.37	32.53	28.24	16.49	0.01
C8-carnitine	0.66	21.04	5.38	10.27	29.30	20.19	9.40	30.83	16.11	14.05	0.03
C10:2-carnitine	0.34	29.71	5.00	14.40	25.78	16.11	13.87	23.53	26.42	14.86	0.02
C10:1-carnitine	0.26	7.19	2.84	4.72	11.16	10.21	6.78	11.18	9.44	7.43	0.01
C10-carnitine	0.40	5.76	2.60	3.21	1.81	1.61	4.12	8.64	3.80	3.16	0.02
C4DC-carnitine	0.11	0.24	0.36	0.15	0.44	0.37	0.24	0.50	0.40	0.28	0.4
C5DC-carnitine	0.95	5.29	4.24	6.81	11.35	6.97	2.15	5.19	6.13	2.49	0.5
C12:1-carnitine	0.14	0.47	0.39	0.28	0.14	0.13	0.51	0.96	0.39	0.55	0.01
C12-carnitine	0.05	0.14	0.08	0.10	0.04	0.05	0.11	0.23	0.12	0.11	0.04
C6-DC-carnitine	0.43	0.43	0.42	0.16	0.96	0.74	0.51	1.61	0.67	0.62	0.1
C14:2-carnitine	0.01	0.03	0.02	0.02	0.06	0.05	0.04	0.09	0.06	0.08	0
C14:1-carnitine	0.02	0.03	0.04	0.04	0.06	0.10	0.04	0.04	0.10	0.11	0.01
C14-carnitine	0.04	0.05	0.06	0.03	0.16	0.19	0.09	0.18	0.12	0.10	0.03
C8-DC-carnitine	0.13	0.20	0.18	0.08	0.29	0.25	0.24	0.58	0.28	0.25	0.05
C14-OH-carnitine	0.04	0.02	0.03	0.02	0.04	0.03	0.03	0.07	0.05	0.02	0.01
C16:1-carnitine	0.02	0.02	0.02	0.01	0.03	0.04	0.03	0.04	0.04	0.02	0
C16-carnitine	0.03	0.03	0.05	0.03	0.14	0.16	0.09	0.14	0.11	0.14	0.02
C10-DC-carnitine	0.05	0.07	0.06	0.07	0.09	0.09	0.10	0.22	0.10	0.10	0.02
C16:1-OH-carnitine	0.01	0.02	0.01	0.01	0.03	0.03	0.04	0.04	0.05	0.01	0
C16-OH-carnitine	0.03	0.02	0.03	0.03	0.08	0.08	0.04	0.10	0.06	0.05	0.01
C18:2-carnitine	0.02	0.04	0.02	0.03	0.01	0.01	0.01	0.02	0.01	0.02	0
C18:1-carnitine	0.01	0.02	0.01	0.01	0.01	0.02	0.03	0.04	0.03	0.04	0
C18-carnitine	0.06	0.05	0.04	0.04	0.08	0.08	0.08	0.16	0.07	0.09	0
C18:2-OH-carnitine	0.00	0.00	0.00	0.00	0.01	0.00	0.02	0.01	0.01	0.00	0
C18:1-OH-carnitine	0.01	0.01	0.01	0.01	0.02	0.02	0.03	0.04	0.03	0.01	0
C16-DC-carnitine	0.00	0.01	0.01	0.01	0.02	0.01	0.01	0.03	0.01	0.01	0
C18:1-DC-carnitine	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0

Percentile 95 was used as the upper limit of the reference range as not all values obtained from the healthy control horses were normally distributed.

Based on the characteristic urinary profiles of organic acids (ethylmalonic acid and 2-methylsuccinic acid), glycine conjugates ((*iso*)valerate, butyrate and hexanoate) and predominantly short-chain acylcarnitines (acylgroups less than 10 carbon atoms) in all 10 horses with a tentative diagnosis of atypical myopathy, a deficiency of several mitochondrial dehydrogenases that utilize flavin adenine dinucleotide (FAD) as cofactor was suspected. Several mitochondrial dehydrogenases utilize FAD as cofactor including the acyl-CoA dehydrogenases of fatty acid β-oxidation, and enzymes that degrade the CoA-esters of glutaric acid, isovaleric acid, 2-methylbutyric acid, isobutyric acid, and sarcosine (a precursor of glycine)[6]. Decreased activity of many FAD-dependent dehydrogenases results in the combined metabolic derangements seen in human multiple acyl-CoA dehydrogenase deficiency (MADD) also known as glutaric acidemia type II (GA-II) [8,9].

Riboflavin is a precursor in the synthesis of flavin mononucleotide (FMN) and FAD. Both products are the prosthetic groups of numerous enzymes (called flavoproteins) that catalyze the various electron-transferring reactions in energy-producing, biosynthetic, detoxifying, and electron-scavenging pathways. Most of these flavoproteins are found in mitochondria [10,11]. Mean plasma riboflavin concentrations in horses with a tentative diagnosis of atypical myopathy did not differ significantly from those in control horses indicating no riboflavin deficiency.

Multiple acyl-CoA dehydrogenase deficiency is a severe inborn error of metabolism, which can lead to early death in human patients. This autosomal recessive disease was first reported in 1976 by Przyrembel and co-workers [12]. The clinical presentation of MADD is very heterogeneous and ranges from neonatal death to late-onset myopathy. In the majority of human cases, MADD is caused by mutations in the genes encoding the α- or β-subunit of electron transfer flavoprotein (ETF) or ETF-dehydrogenase (ETF-DH) [13–15]. Based on the epizootic occurrence of the disease, we hypothesize that the MADD seen in these horses may be caused by an exogenous factor predominantly affecting SCAD, MCAD, and IVD directly or indirectly via FAD as cofactor.

Table 4b
Acylcarnitines in plasma (μmol/l) from seven horses (3, 4, and 6–10) with a tentative diagnosis of atypical myopathy and three diseased controls (11–13)

Horse number	3	4	6	7	8	9	10	P95	11	12	13
Free carnitine	27.46	44.84	54.40	25.66	75.11	32.75	131.06	44	111.91	70.23	37.43
C2-carnitine	7.09	11.92	21.30	9.63	26.97	10.88	46.98	5.6	50.54	21.89	3.47
C3-carnitine	0.51	1.62	1.66	1.66	3.44	1.94	3.13	0.95	1.53	1.19	0.42
C4-carnitine	2.09	27.74	41.55	13.69	60.84	17.60	31.05	0.7	1.82	0.78	0.30
C5:1-carnitine	0.01	0.03	0.06	0.05	0.15	0.06	0.08	0.02	0.04	0.04	0.03
C5-carnitine	2.64	34.57	29.47	23.98	92.77	39.06	30.44	0.5	0.92	0.59	0.26
C4:3-OH-carnitine	0.08	0.50	0.58	0.38	1.53	0.59	0.81	0.03	0.66	0.10	0.03
C6-carnitine	0.43	5.72	5.87	1.56	16.90	1.94	6.66	0.02	0.30	0.01	0.01
C5-OH-carnitine	0.07	0.35	0.44	0.36	1.54	0.37	0.44	0.04	0.03	0.01	0.01
C8:1-carnitine	0.06	1.20	1.00	0.60	4.89	1.12	0.30	0.02	0.12	0.02	0.02
C8-carnitine	0.01	1.64	1.67	0.57	5.38	0.77	1.72	0.02	0.11	0.03	0.02
C10:2-carnitine	0.07	1.43	1.21	0.65	3.54	0.81	0.16	0.02	0.10	0.04	0.03
C10:1-carnitine	0.05	0.55	0.75	0.41	2.03	0.39	0.43	0.02	0.06	0.03	0.04
C10-carnitine	0.01	0.76	0.93	0.30	2.06	0.30	0.67	0.03	0.13	0.04	0.04
C4DC-carnitine	0.01	0.08	0.13	0.04	0.09	0.05	0.14	0.05	0.15	0.07	0.06
C5DC-carnitine	0.13	1.87	0.88	0.19	0.96	0.45	0.71	0.06	0.10	0.06	0.07
C12:1-carnitine	0.01	0.26	0.22	0.12	0.36	0.08	0.07	0.02	0.13	0.05	0.05
C12-carnitine	0.01	0.63	0.22	0.13	0.39	0.14	0.16	0.02	0.12	0.06	0.05
C6-DC-carnitine	0.04	0.06	0.10	0.06	0.39	0.07	0.15	0.02	0.08	0.05	0.06
C14:2-carnitine	0.01	0.06	0.07	0.05	0.14	0.05	0.14	0.02	0.04	0.01	0.02
C14:1-carnitine	0.04	0.40	0.25	0.14	0.34	0.11	0.09	0.03	0.17	0.03	0.02
C14-carnitine	0.01	0.78	0.24	0.13	0.30	0.13	0.12	0.02	0.05	0.02	0.01
C8-DC-carnitine	0.01	0.03	0.04	0.03	0.13	0.04	0.04	0.02	0.02	0.01	0.01
C14-OH-carnitine	0.01	0.14	0.04	0.04	0.06	0.06	0.02	0.01	0.01	0.01	0.01
C16:1-carnitine	0.01	0.53	0.22	0.09	0.21	0.11	0.07	0.02	0.08	0.01	0.01
C16-carnitine	0.06	1.54	0.56	0.35	0.61	0.34	0.29	0.02	0.08	0.02	0.02
C10-DC-carnitine	0.01	0.06	0.03	0.02	0.07	0.03	0.07	0.01	0.02	0.01	0.01
C16:1-OH-carnitine	0.01	0.25	0.09	0.10	0.16	0.17	0.04	0.01	0.01	0.01	0.01
C16-OH-carnitine	0.01	0.23	0.05	0.04	0.07	0.09	0.03	0.01	0.02	0.01	0.01
C18:2-carnitine	0.25	0.33	0.16	0.13	0.26	0.19	0.03	0.01	0.02	0.01	0.02
C18:1-carnitine	0.05	1.19	0.68	0.36	0.71	0.38	0.08	0.02	0.15	0.02	0.03
C18-carnitine	0.04	0.30	0.28	0.25	0.33	0.29	0.18	0.01	0.03	0.01	0.01
C18:2-OH-carnitine	0.01	0.11	0.05	0.07	0.06	0.13	0.01	0.01	0.01	0.01	0.01
C18:1-OH-carnitine	0.01	0.13	0.04	0.08	0.12	0.14	0.03	0.01	0.01	0.01	0.01
C16-DC-carnitine	0.01	0.01	0.01	0.01	0.04	0.01	0.01	0.01	0.01	0.01	0.01
C18:1-DC-carnitine	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01

Percentile 95 was used as the upper limit of the reference range as not all values obtained from the healthy control horses were normally distributed.

Since not all patients suffering from human MADD have mutations in the genes encoding the α- or β-subunit of ETF or ETF-DH, other as yet unidentified genes are predicted to be involved as well. Because all affected mitochondrial flavoproteins in MADD have FAD as a prosthetic group, the underlying defect in these patients may be due to a thus far undisclosed disturbance in the metabolism of FAD. Since a proper mitochondrial flavin balance is maintained by a mitochondrial FAD transporter [9], a defect of this transporter could also cause a MADD-like phenotype.

Here we identify the biochemical defect in 10 out of 10 horses leading to fatal atypical myopathy based on the characteristic urinary profiles of organic acids, glycine conjugates, and predominantly short-chain acylcarnitines as well as additional quantitative biochemical measurement of dehydrogenase activities in lateral vastus muscle in a single case as a deficiency of several mitochondrial dehydrogenases that utilize FAD as cofactor. As a consequence, atypical myopathy in grazing horses reflects a MADD-like phenotype. With reference to future cases, the characteristic urinary profiles of organic acids, glycine conjugates, and acylcarnitines might be helpful in the diagnosis of atypical myopathy. However, glutaric acid excretion was not elevated possibly due to the fact that glutaric acid appears to be a normal constituent in equine urine in contrast to man [16]. We have no clear explanation for the observation that the equine patients had elevated concentrations of glutarate in plasma, but not in urine.

The disease is characterized by a very high mortality rate of about 90% and death usually within 72 h due to acute generalised progressive myopathy. As the exact pathophysiology of the disease was unknown, effective treatments were not available. Although the mean plasma concentration of riboflavin found in diseased horses was not different from control horses this does not rule out riboflavin as a possible treatment option given the possibility that there might be competition between riboflavin and a toxic compound. Our findings could aid in developing effective treatments by improving efficacy of carbohydrate metabolism rather than fat metabolism in diseased horses. As mentioned before, carbohydrates and fatty

acids are the main metabolic fuels for skeletal muscle. Glucose transport in equine muscle is mediated by the glucose transporter 4 protein, which is stimulated by insulin and muscle contraction [17]. Hence, we suggest intravenous administration of fluids enriched with insulin (in addition to glucose given the presence of hyperglycaemia upon admission) to be most likely candidates for potential treatments of the condition via increased cellular glucose uptake due to enhanced glucose transporter 4 protein translocation. With reference to improvement of efficacy of lipid metabolism, intravenous carnitine might be a likely candidate.

In humans, MADD is an autosomal recessive inherited disorder. However, this seems to be unlikely in equine atypical myopathy given the epizootic occurrence and the recovery of a 10-year-old Draft horse mare following hospitalisation. It should be realized also that humans with fatty acid oxidation genetic defects may have normal biochemistry between episodes of decompensation. As a consequence, the equine disease might be considered as an acquired (rather than inherited) model of the human counterpart. Despite several experiments in order to obtain mice or rats with MADD like diseases, there are, as yet, no mutations that might be considered as a model of human MADD. To the authors' knowledge, MADD has not been diagnosed in species other than man, except for two case reports in horses not associated with atypical myopathy [16]. It might be of importance to apply new therapeutic approaches of possible benefit to man in horses with atypical myopathy.

As mentioned previously, particular weather conditions seem to trigger the appearance of the syndrome [2,5] suggesting that an exogenous factor may play an important role in the pathogenesis of this disease. It has been hypothesized that (myco)toxins might be regarded as potential aetiological factors [2]. Interestingly, on a pasture with a severe outbreak of atypical myopathy in the Netherlands fungi of the species Cortinarius type Dermocybe uliginosus were found. The subsarcolemmal vacuolation seen at histopathology also suggests toxic muscular changes given the absence of glycogen storage disorders. Furthermore, a new myopathy syndrome affecting humans has recently been attributed to fungi of the group of Tricholoma equestre. Repeated consumption of this wild mushroom may cause fatal myopathy [18,19]. In Japan, another mushroom, Russula subnigricans, has also been reported as being myotoxic [20]. In addition, toxic alkaloidal substances in the seeds of *Galeopsis ladanum* via ingestion of quail also caused rhabdomyolysis in man [21]. To the authors' knowledge, neither histopathology for lipid storage nor metabolic screening of lipid metabolism has yet been performed in these human toxic myopathies.

With reference to these potential aetiological factors and possible public health consequences further research is necessary. The possibility that the catalytic efficiency of several mitochondrial dehydrogenases that utilize FAD as cofactor is impaired due to the attachment of (myco)toxins thereby causing atypical myopathy might be of help in the potential isolation of these (myco)toxins. Future studies might reveal the exact aetiology of the condition in horses as proceeded by its elucidated pathophysiology.

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