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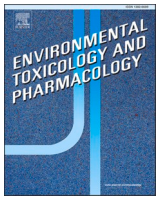

Large-scale study of blood markers in equine atypical myopathy reveals subclinical poisoning and advances in diagnostic and prognostic criteria

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ABSTRACT

Equine atypical myopathy (AM) is a severe rhabdomyolysis syndrome primarily caused by hypoglycin A (HGA) and methylenecyclopropylglycine protoxins. This study aimed to refine diagnostic and prognostic criteria for AM while exploring apparently healthy cograzers. Blood samples from 263 horses, including AM cases (n= 95), cograzers (n= 73), colic horses (n= 19), and controls (n= 76), were analyzed for HGA, its toxic metabolite, and acylcarnitines profile. Diseased horses exhibited alterations in acylcarnitines that strongly distinguished them from controls and colic horses. Regression analyses identified distinct acylcarnitines profiles among groups, with cograzers showing intermediate alterations. Age and gelding status emerged as protective factors against AM. Furthermore, serum acylcarnitines profiling was valuable in predicting AM survival, with isovaleryl-/2-methylbutyrylcarnitine (*i.e.*, C5 acylcarnitine) showing promise as both a diagnostic and prognostic marker. Subclinical alterations in cograzers underscore a novel aspect: the presence of subclinical cases of AM.

1. Introduction

Equine atypical myopathy, referred to as atypical myopathy (AM) in Europe (Votion et al., 2014) and as seasonal pasture myopathy (SPM) in the US (Valberg et al., 2013), is an often fatal rhabdomyolysis syndrome affecting equids kept at pasture. To date, two protoxins have been incriminated: hypoglycin A (HGA) and methylenecyclopropylglycine (MCPrG) (Bochnia et al., 2019). The toxicodynamic involves their activation into the toxic metabolites, methylenecyclopropylacetyl-CoA (MCPA-CoA) and methylenecyclopropylformyl-CoA (MCPF-CoA), respectively (Von Holt et al., 1964). These metabolites disrupt lipid metabolism by inhibiting the degradation of fatty acids through fatty acid ß-oxidation, leading to increased blood concentrations of various acylcarnitines in poisoned animals (Boemer et al., 2017; Lemieux et al., 2016; Westermann et al., 2008). These metabolites, once formed, exhibit high reactivity and readily form stable compounds through conjugation mainly with carnitine and glycine. The presence of these stable forms can be assessed in blood (Isenberg et al., 2015; Valberg et al., 2013), various tissues (Sander et al., 2023), and urine (Bochnia et al., 2019).

Currently, the provisional diagnosis of AM or SPM relies on acute signs of rhabdomyolysis, accompanied by pigmenturia as a common yet

Abbreviations: AM, atypical myopathy; CK, creatine kinase; MCPA, methylenecyclopropylacetyl acid; MCPF, methylenecyclopropylformyl; MCPrG, methylenecyclopropylglycine; SPM, seasonal pasture myopathy.

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not pathognomonic sign. Routine laboratory tests confirm extensive rhabdomyolysis through the assessment of serum creatine kinase (CK) activities, exceeding 10,000 IU/L (van Galen et al., 2012b; Votion et al., 2007).

The diagnostic confirmation involves blood testing to show the presence of at least one protoxin and its conjugated metabolites (Boemer et al., 2017). Various laboratories have developed analytical methods examining HGA and MCPA-conjugates in urine and/or blood (González-Medina et al., 2020; Rudolph et al., 2018; Sander et al., 2019). Serum or plasma HGA is considered an exposure marker (Baise et al., 2016; Renaud et al., 2022), while clinical poisoning is associated with significant quantities of both HGA and MCPA-carnitine in blood (Bochnia et al., 2015, 2016a; Karlíková et al., 2018; Sander et al., 2023).

Furthermore, based on the assumption that the acylcarnitines profile reflects energy deprivation, a statistical model integrating short, medium, and long-chain acylcarnitines has been proposed to estimate the chances of survival in clinically affected AM horses (Boemer et al., 2017).

One aspect of this study focuses on identifying blood markers useful for diagnosing AM and estimating the chances of survival in affected animals. The second aspect of this study focuses on the epidemiology of AM. In pastures close to toxic trees, all equids are exposed to the protoxins, including those that do not suffer from AM. These seemingly healthy equids have often been referred to as healthy cograzing animals (Baise et al., 2016; Bochnia et al., 2015; Kruse et al., 2024; Votion et al., 2007; Wimmer-Scherr et al., 2021). In the literature, a few demographic factors have been associated with an increased risk of poisoning (Votion et al., 2009) or, when intoxicated, an increased risk of lethality (Boemer et al., 2017; van Galen et al., 2012b). Notably, age has emerged as a key discriminant between healthy cograzers and clinically affected horses. Horses under 3 years old were described as primarily at risk. When diseased, the mean age of survivors was significantly higher than non-survivors. These results suggested an age-related resistance to intoxication. However, as the disease emerges over the years, the age distribution of cases seems to expand (Votion et al., 2020), making the age-related effect questionable.

Initial epidemiological studies suggested a higher risk of AM in intact males and a decreased risk in geldings (Votion et al., 2007); however, a later publication revealed age as a confounding factor for sex predisposition (Votion et al., 2009). Horses younger than 18 months were described as the age group with the highest risk of developing AM, however, castration is usually performed after the age of 18 months, therefore young intact males are overrepresented at pasture. Once these confounding factors were established, results of former studies were relativized, and no inherent sex predisposition for AM was considered.

This study aims to conduct a comprehensive analysis of clinical tools associated with risk factors, diagnosis, and prognosis related to AM. The objective is to refine diagnostic and prognostic criteria for AM. Additionally, we hypothesize that apparently healthy cograzers, sharing the pasture with equids suffering from atypical myopathy, may be better described as subclinical cases.

2. Material and methods

2.1. Animal inclusion and sampling

From autumn 2016 to autumn 2020, blood samples were systematically collected from horses referred to the equine hospital of the University of Liège (CVU-ULiège) with a tentative diagnosis of AM. Some horses with a presumptive diagnosis of AM were sampled outside of CVU-ULiège through collaboration with first-line equine practitioners, the equine hospital of Ghent University, and the equine clinic De Morette. Whenever possible, blood samples were collected from cograzers of suspected AM cases within the following 48 hours.

Blood samples were obtained via jugular venipuncture and stored at 4°C until separated serum was aliquoted within the following 12 hours. The aliquoted samples were then preserved at −80°C, until further analysis.

All procedures of this study are in accordance with both national and international guidelines for animal welfare. The Animal Ethics Committee of the University of Liege was consulted and since venipuncture is part of routine veterinary practice to establish a diagnosis or to prevent AM, an animal ethics approval was not required. Owners gave informed consent for their horses' inclusion in the study.

Data concerning medical history, pasture conditions, and clinical signs were obtained through online questionnaires and/or medical records. These collected data allowed the retrospective classification of the sampled horses into the following four groups:

I. **Diseased horses:** Horses included in the study were diagnosed with AM with a high probability based on a previously published diagnostic algorithm (van Galen et al., 2012a) and literature data (Boemer et al., 2017; González-Medina et al., 2017). These horses had no history of recurrent exertional rhabdomyolysis syndrome, had access to pasture, exhibited acute illness, and presented common clinical signs associated with AM. Additionally, they displayed serum CK activity levels over 10,000 IU/L and/or demonstrated signs of pigmenturia.

For the purposes of this study, certain analyses were conducted by dividing the group of Diseased Horses into two subgroups (survivors and dead horses).

I.a.**Survivors:** Horses within the affected group that survived the condition. Survival was assessed until the horse was discharged from the clinic. No follow-up was performed after discharge.

I.b.**Dead horses:** Horses among the diseased group that did not survive. This category includes horses that died naturally as well as those euthanised. Euthanasia was predominantly performed due to respiratory failure, with one exception (see further).

I. **Cograzers:** Clinically healthy horses with no clinical signs, within normal general examination parameters, that shared the same pasture as one of the diseased horses. The general examination consisted of an observational assessment from a distance, followed by a physical examination. Evaluation of the gingival mucosae included assessing moistness and confirming absence of icterus, hyperaemia, cyanosis, pallor, ulceration, and/or petechiae. Capillary refill time was determined by blanching the mucous membrane. Absence of submandibular lymphadenopathy was assessed through palpation of the intermandibular space. Examination of the lungs and heart was conducted through thoracic auscultation. Finally, a dynamic examination of the horses' locomotion was performed by observing their walk on a straight line.

II. **Colic horses:** Horses with access to pasture initially admitted with a tentative diagnosis of AM but later diagnosed, through ancillary examinations, as suffering from digestive colic. The diagnostic process included a general examination, digestive auscultation, transrectal palpation, nasogastric intubation, abdominal ultrasound and assessment of serum CK activities to exclude myopathy. To be included these horses had to have access to pasture.

III. **Control horses:** Horses at pasture, showing no clinical signs, with normal general examination parameters, and no detectable levels of HGA or MCPA-carnitine in their serum. It is noteworthy that the selection of control horses did not employ stratified sampling, which would have allowed for harmonization of age and sex with the groups of diseased horses or cograzers. Control horses' serum samples were obtained from an internal biobank at CVU-ULiège.

2.2. Hypoglycin A assay

Hypoglycin A assay was performed on serum according to a previously described methodology (Boemer et al., 2015; Votion, 2016). Quantification of HGA was carried out using aTRAQ® kit for amino acid analysis of physiological fluids. Hypoglycin A contained in samples was derivatised using an isotopic tag (mass *m/z* 121), while a second labelling reagent (mass *m/z* 113) allowed absolute quantification. Derivatised samples were introduced into a TQ5500 tandem mass spectrometer (Sciex, Framingham, MA, USA) using a Prominence AR HPLC system (Shimadzu). The lower limit of quantification associated to this method was measured at 0.090 µmol/l (Boemer et al., 2015).

2.3. MCPA-carnitine determination method

The separation and determination of MCPA-carnitine was conducted using ultra-performance liquid chromatography combined with subsequent mass spectrometry (UPLC-MS/MS) as previously described (Valberg et al., 2013).

2.4. Acylcarnitines determination method

Free carnitine and twenty-one acylcarnitines (C2, C3, C3-DC, C4, C5, C5:1, C5-OH, C5-DC, C6, C8, C8:1, C10, C10:1, C10:2, C12, C12:1, C14, C14:1, C16, C16:1, C18, and C18:1)(Boemer et al., 2017) were quantified in serum by tandem mass spectrometry (Chace et al., 2003). As described by Boemer et collaborators (2017),serum proteins were precipitated with a methanol solution with labelled internal standards. Supernatants were evaporated under nitrogen stream and derivatised with butanolic-HCl. The samples were then analysed with a TQ5500 mass spectrometer (Sciex, Framingham,MA, USA).

2.5. Statistical analysis

All statistical analyses and graphical representations were performed using GraphPad Prism 7 or SAS 9.4M7. The Kolmogorov-Smirnov test was employed to assess the assumption of normal distribution for continuous explanatory variables, and, when necessary, data were normalised through Log10 transformation. The following analyses were conducted in three steps:

(i) Logistic regression for risk assessment: Multiple logistic regressions were conducted in GraphPad Prism 7 to evaluate the trends of age and sex as risk and preventive factors associated with AM. Statistical significance was set at $p < 0.05$.

(ii) Group comparisons of blood markers: Comparison of means were undertaken with the aim of identifying serum acylcarnitines that can effectively differentiate the pre-established groups of horses. Partial Least Squares regressions (PLS) were employed to compare acylcarnitines profile, as these are correlated variables. Initially, PLS included Diseased horses, Cograzers Colics, and Control Horses to distinguish these groups based on their acylcarnitine serum profiles. Subsequently, the Colics group was excluded from further analysis due to specific considerations. In PLS iteration 3, the Diseased Horses group was subdivided into Dead Horses and Survivors to focus on prognostic aspects by comparing their acylcarnitine profiles with those of Cograzers and Controls. The PLS outcome is a two-dimensional projection that enables discrimination between the groups and provides an overview of relevant variables. The most relevant variables, *i.e.*, which have the strongest impact on the principal components of PLS, were extracted and analysed. The emphasis was placed on variables crucial for characterizing a group of animals, which were determined/ascertained using two metrics: variable importance in the projection (VIP) and centre parameter estimates. Acylcarnitines of primary interest have a VIP greater than 1 and an absolute value of the centre parameter estimates greater than 0.15 (Chong and Jun, 2005; Palermo et al., 2009).

(iii) Diagnostic and prognostic modeling: based on the most discriminative acylcarnitines identified from the PLS analysis, we established a prediction model using multiple logistic regression (GraphPad Prism 7). This model was evaluated serum acylcarnitine profile for its diagnostic and prognostic utility in predicting outcomes in AM cases.

3. Results

3.1. Samples collection and data of individuals

Data of the groups are presented in Table 1 and include a total of 263 analysed samples. Throughout the study period, 95 horses were suspected of having AM with a high probability, constituting the “diseased horses” group (van Galen et al., 2012a). Among these 95 horses, 32 either died naturally or were euthanised, forming the “dead horses” group, while 26 horses survived, forming the “survivors” group. The outcome of the disease remains unknown for 37 horses of this group. Most diseased horses (87/95) were sampled in the clinics previously mentioned and the remaining on the field by the first-line equine practitioners. All euthanised horses, except for one (horse D33), were put down due to general deterioration. This was characterised by severe dyspnoea, low arterial partial oxygen pressure non-responsive to oxygen therapy, and significant neurological impairments such as nystagmus, altered pupillary reflexes, and/ or convulsions. Horse D33 was euthanised for financial constraints and not for medical reasons.

For the remaining horses in the study: 73 were included as cograzers, 19 in the colic group, and 76 as control horses.

3.2. Age

The average age was similar between diseased horses (6.7±5.9 years old) and clinically healthy cograzers (5.1±2.9 years old) (Mann-Whitney test, $p = 0.8091$). However, a significant difference in age distribution between the two groups was noted (Kolmogorov-Smirnov test, $p = 0.0074$). Specifically, the age dispersion was greater for diseased horses, encompassing a wider range of ages, including more very young and older horses. Notably, 57 % of the diseased horses were either younger than 2 years or older than 10 years. In contrast, the age distribution for the cograzers was narrower, with 90 % of the horses falling between 2 and 10 years of age (Fig. A in the appendices).

No significant difference in mean or age distribution was observed between dead horses and survivors (Kolmogorov-Smirnov test, $p = 0.9865$; Mann-Whitney test, $p = 0.9195$) (Figure A in the appendices).

The multiple logistic regressions, considering confounding between the variables "age" and "sex," indicated that among horses exposed to the toxins, those over 10 years of age are less susceptible to develop the disease compared to younger animals (see Table 2). No significant impact of age on survival probability was observed.

The average age was higher for control horses compared to both the diseased horses (Mann-Whitney test, $p < 0.0001$) or the cograzers (Mann-Whitney test, $p < 0.0001$) (see Table 1).

3.3. Sex

The sexes of the included horses are listed in Fig. B in the appendices.

The variables “sex of the horse” and “health status” (*i.e.*, diseased horses *vs* cograzers) are independent (Chi-square test, $p = 0.3276$). The variables “sex of the horse” and “final outcome of the disease horses” (death or survival) are interdependent (Chi-square test, $p = 0.0233$).

The multiple logistic regressions, considering confounding between the variables "age" and "sex", indicated that among horses exposed to the toxin, geldings are less susceptible to develop the disease horses compared to intact males and females (see Table 2). No significant

Table 1
Demographic composition in the different studied groups.

	Diseased horses	Dead horses	Survivors	Cograzers	Colic horses	Control horses
Number of horses	95	32	26	73	19	76
Age Mean ± SD (years)	6.7 ± 5.9[a,b]	6.5 ± 5.5	6.6 ± 6.2	5.1 ± 2.9[a,c]	10.2 ± 7.7	13.2 ± 6.8[b,c]
Age minimum (years)	0.6	0.6	0.6	1.4	0.1	2.5
Age maximum (years)	22.5	20.0	21.0	13.9	24.8	31.4
Age median (years)	5.0	5.1	4.7	4.5	8.8	12.0
Ratio of:						
- intact male	15 %	13 %	25 %	8 %	5 %	7 %
- gelding	31 %	20 %	42 %	38 %	47 %	4 %
- female	55 %	67 %	33 %	53 %	47 %	79 %

SD = standard deviation
[a] Significant difference in age distribution, $p = 0.0074$
[b] Significant difference of average age, $p < 0.0001$
[c] Significant difference of average age, $p < 0.0001$

Table 2
Odds ratio estimate (95 % confidence interval) for age and sex.

	Diseased horses Vs Cograzers	Survivors Vs Dead horses
0–2 years	**0.19*** (0.24–0.91)	**1.61** (0.29–9.52)
3–10 years	**0.17*** (0.034–0.65)	**1.67** (0.39–7.57)
Intact male	**8.86*** (1.36–72.90)	**0.64** (0.12–3.18)
Female	**3.88*** (1.04–17.34)	**0.98** (0.26–3.78)

The class of age "over 10 years" was used as reference.
The category of sex "gelding" was used as reference.
* Significant results with $p < 0.05$

impact of sex on survival probability was observed.

3.4. Hypoglycin A

Laboratory findings for HGA quantification are presented in Table 3 and Fig. 1.

The mean serum concentration of HGA was significantly higher in diseased horses compared to cograzers (unpaired t-test, $p < 0.0001$).

When comparing horses that died from AM to surviving horses, no significant difference was observed regarding average serum HGA concentrations (unpaired t-test, $p = 0.0849$).

It must be noted that:

(i) only 2 out of 95 diseased horses had serum HGA concentrations under the limit of quantitation of 0.09 µmol/L, (horses D47 and D89);
(ii) only 2 out of 73 cograzers had serum HGA concentrations under the limit of quantitation of 0.09 µmol/L.

Among the 19 individuals in the colic horses' group, only 2 exhibited HGA levels over the limit of quantificationdetection (0.09 µmol/L).

None of the control horses exhibited HGA levels above the limit of detection, as this was part of their inclusion criteria.

3.5. MCPA-carnitine

Laboratory findings for MCPA-carnitine are presented in Table 4 and Fig. 2.

Mean serum concentration of MCPA-carnitine was significantly higher in diseased horses compared to cograzers (unpaired t-test, $p < 0.0001$).

When comparing dead horses to surviving horses, the mean serum MCPA-carnitine concentration was significantly higher in the first group (unpaired t-test, $p < 0.0001$).

Following additional observations were made:

(i) only 1 out of 95 diseased horses had serum MCPA-carnitine concentration below the limit of detection of 0.01 nmol/L (horse D89), and this horse is 1 of the 2 diseased horses with serum HGA concentration below the limit of detection;
(ii) only 7 out of 73 cograzers (9.6 %) had serum MCPA-carnitine concentrations below the limit of detection of 0.01 nmol/L.

None of the 19 individuals in the colic horses' group, exhibited MCPA-carnitine levels over the limit of detection (0.019 µmol/L).

None of the control horses exhibited MCPA-carnitine levels above the limit of detection, as this was part of their inclusion criteria.

3.6. Free serum carnitine and acylcarnitines profile

3.6.1. Mean comparisons

Laboratory findings are presented in Table C in the appendices, for both free carnitine and the serum acylcarnitines profile. The mean serum concentrations of free carnitine and the mean serum concentrations of 22 acylcarnitines (C2, C3, C3-DC, C4, C5, C5:1, C5-OH, C5-DC, C6, C8, C8:1, C10, C10:1, C10:2, C12, C12:1, C14, C14:1, C16, C16:1, C18, and C18:1) were compared among groups of horses. The mean serum concentrations of both free carnitine and all acylcarnitines, except for C18-

Table 3 –
Serum hypoglycin A concentrations in the different studied groups.

Group	Diseased horses	Dead horses	Survivors	Cograzers	Colics horses	Control horses
Mean ± SDM (µmol/l)	5.16 ± 0.55*	7.22 ± 1.21	4.15 ± 0.74	1.41 ± 0.18*	< LOQ	< LOQ
Minimum (µmol/l)	< LOQ	< LOQ	< LOQ	0.01	< LOQ	< LOQ
Maximum (µmol/l)	25.20	25.20	14.40	7.32	0.25	< LOQ

SDM = Squared deviations from the mean
< LOQ = below the limit of quantification of 0.09 µmol/L
* Significant difference in mean serum concentration of HGA, $p < 0.0001$

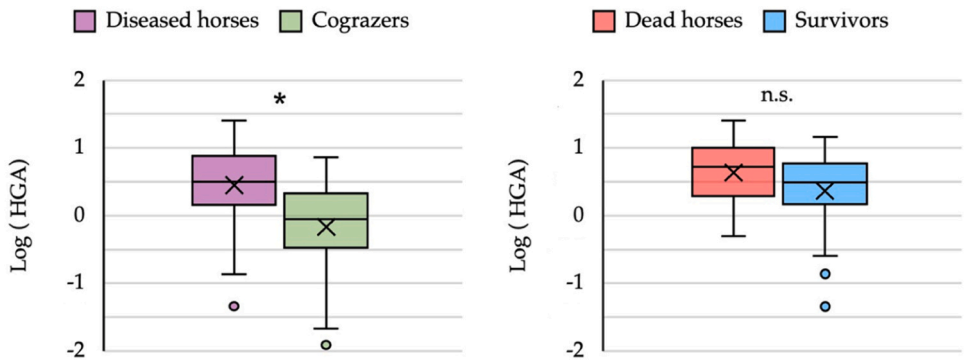

Fig. 1. Serum hypoglycin A concentrations Boxes range from the 25th to 75th percentiles. Medians are represented by horizontal lines in the box and means are symbolised by a cross. Box plot whiskers were established using the Tukey method. * stands for significant difference of mean serum HGA concentration, $p <$ 0.0001 n.s. stands for no significant difference of average serum HGA, $p = 0.0849$.

Table 4 –
Serum methylenecyclopropylacetylic-carnitine (MCPA-carnitine).

Group	Diseased horses	Dead horses	Survivors	Cograzers	Colic horses	Control horses
Mean + SDM* (nmol/L)	443.28 ± 84.08[a]	751.83 ± 198.05[b]	126.16 ± 39.74[b]	8.44 ± 1.40[a]	< LOD	< LOD
Minimum (nmol/L)	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Maximum (nmol/L)	4480.00	4480.00	1040.00	56.10	< LOD	< LOD

< LOD = below the limit of detection of 0.01 nmol/L
* SDM = Squared deviations from the mean
[a] Significant difference in mean serum concentration of MCPA-carnitine, $p < 0.0001$
[b] Significant difference in mean serum concentration of MCPA-carnitine, $p < 0.0001$

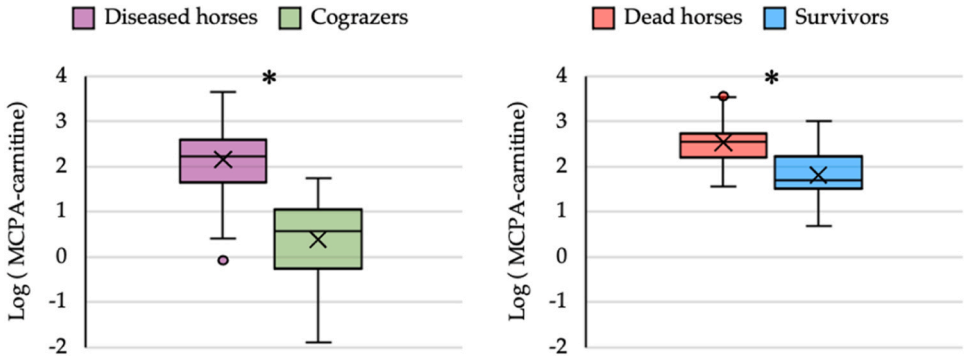

Fig. 2. Serum methylenecyclopropylacetyl-carnitine (MCPA-carnitine) observations Boxes range from the 25th to 75th percentiles. Medians are represented by horizontal lines in the box and means are symbolised by a cross. Box plot whiskers were established using the Tukey method. *Significant difference in mean serum concentration of MCPA-carnitine, $p < 0.0001$.

carnitine, were found to be higher in diseased horses compared to control horses (exceeding the 99th percentile of the control horse values). The mean serum concentration of C18-carnitine was the only one below the 99th percentile of the control horse values.

For the cograzers, 3 of the 23 variables (C4, C5 et C10:1) have a mean serum concentration exceeding the 99th percentile of the control horse values.

For the group of the colic horses, only 2 of the 22 variables (C10:1 et C14:1) have a mean serum concentration exceeding the 99th percentile of the control horse values.

3.6.2. Partial least squares regressions

Based on these 22 correlated variables, comprising free carnitine and the 22 acylcarnitines, 3 partial least squares regressions were performed, and their visual outcomes are depicted in Fig. 3. The key observations are as follows:

- there is a significant distinction between the group of diseased horses and the other groups;
- the group of cograzers exhibits differences from the other groups, albeit with some internal variability. Within the cograzers group, certain horses profile aligns with the one of diseased horses, while others align with the profile of control horses.

In regression 1 (Fig. 3-a), the colic group displays a noticeable difference from the diseased horses and is represented close to the control horses.

A second regression was conducted, excluding the group of colic horses (Fig. 3-b). In this second regression, the differentiation between the remaining groups is enhanced and follows the same trends as previously described, with the cograzers positioned between the

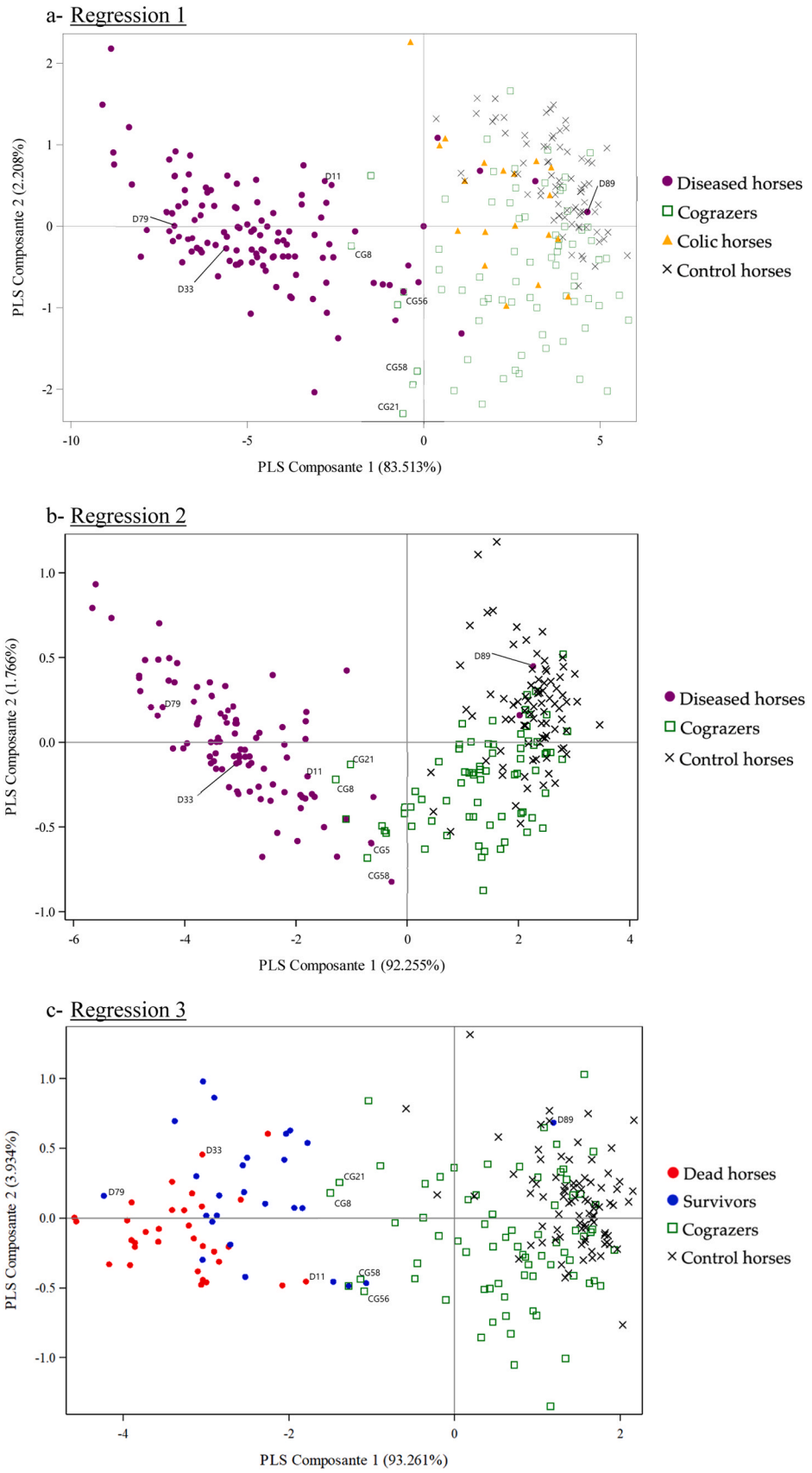

Fig. 3. a,b,c – Partial least squares regression between groups using free carnitine and 22 acylcarnitines.

diseased and control horses.

In regression 3, the diseased horses were further categorised into two groups: dead horses and survivors. This regression, still incorporating all 22 correlated variables, compares the groups of dead horses, survivors, cograzers, and control horses. The associated graphical representation can be observed in Fig. 3-c.

The horizontal component 1 accounts for 93 % of the difference between the groups. This trend, also observed in the previous regressions, is accentuated in this third PLS. The serum acylcarnitines profile of the sampled animals appears to oscillate between two extremes: the severely altered profile of animals that died from AM on the left side of the graph, and, on the right of the graph, control animals which were not exposed to the toxin. Survivors had an acylcarnitines profile close to that of the dead horses but appeared to be grouped closer to clinically healthy horses than the latter. Cograzers have an intermediate acylcarnitines profile between the diseased horses and the control horses.

Based on the analysis of these three regressions, with a particular focus on their primary horizontal component that accounts for most of the variation between the groups, certain individuals can be identified as outliers utilizing the ROUT method (Motulsky and Brown, 2006).

In Regression 2 (Fig. 3-b), two outliers were identified within the cohort of diseased horses: horses D89 and horse D47.

In Regression 3 (Fig. 3-c), within the survivor group, Horse D89 continues to stand out as an outlier while it is important to note that horse D47 no longer features in this projection, as it is classified within the subset of horses whose ultimate disease outcome is undetermined.

Nevertheless, in addition to the two aforementioned outliers, several animals with distinctive profiles can be identified in the regression analyses (Fig. 3):

- four cograzers, horses CG8, CG21, CG56 and CG58, display acylcarnitines profile that overlap with those of the diseased horses. These horses were selected as being part of the 5 % of the cograzers with the greatest acylcarnitines increase.
- within the cohort of dead horses, there is one individual, D11 whose acylcarnitines profile exhibit the least deviation from those of survivors and cograzers. This individual falls within the 95th percentile of the dead horses;
- among survivors, one standout case is D79, which exhibits a component 1 value lower than the 0.5 percentile of the survivor group;
- finally, one horse D33 was euthanised due to budgetary constraints.

Based on regression 3 (Fig. 3-c), but excluding both horses D47 and D89, it was possible to identify the variables with the most significant impact on distinguishing among the four groups: dead horses, survivors, cograzers, and control horses. Six variables had VIP scores exceeding 1 and negative central parameter estimates: C2, C3, C3DC, C4, C5, and C10:1 acylcarnitines (Table D). These variables were considered relevant and have been selected for subsequent analysis, with the aim of verifying if any of them could, on its own, serve as a reliable diagnostic and prognostic indicator. The sensitivity and specificity of these six markers in the differentiation of diseased horses (excluding horses D47 and D89), cograzers, dead horses, and survivors are represented by a ROC curve (Fig. E - appendices). The area under the curves (A) and associated standard error (SE) are also included in the same Figure E. In order to establish a predictive model, we sought discriminant markers useful for both diagnostic and prognostic purposes, including the 6 above-mentioned acylcarnitines, HGA, MCPA-carnitine, and CK, as it is one of the markers easily obtainable in the field. (Fig. E - appendices). The C5 carnitine emerges as the best candidate, with the largest area under the curve observed in both ROC curves comparing diseased horses to their cograzers (A=0.9983; SE=0.005754) and survivors to dead horses (A=0.8713; SE=0.04827).

4. Discussion

In this study, the demographic profile of horses at risk for AM has been refined and factors that enable the differential diagnosis with digestive colics have been identified. This study highlights the significance of acylcarnitines profiling to prognosticate the chance of survival but also to document the existence of subclinical cases.

4.1. Update on demographic profile of diseased horses

In previous studies, age was identified as a confounding variable concerning the risk factors associated with the sex of the animal (Votion et al., 2009). However, this new study highlights that age exceeding 10 years and the gelding status serve as protective factors against the onset of the disease in horses exposed to the toxins. This observation is likely linked to the most recent epidemiological observations which suggest a gradual shift in the age distribution of horses affected by AM over the years. Over a 20-year period, the age group targeted by the disease has undergone significant change. Initially, nearly seventy percent of affected equids were less than 3 years old. However, presently, this age group constitutes less than forty percent of affected individuals (Votion et al., 2020). No factors related to age or sex were found to be indicative of chances of survival.

4.2. Update on the diagnostic blood markers

In this study, the observed serum concentrations of HGA (mean ± SDM = 5.16 ± 0.55 µmol/L) and MCPA-carnitine (mean ± SDM = 443.28 ± 82.84 nmol) in the diseased horses are consistent with the descriptions of equids suffering from AM, whose blood was tested using the same methods (Baise et al., 2016; Boemer et al., 2015; Høffer et al., 2016). There was variability in serum HGA observations within the group of the diseased horses (median = 3.17 µmol/L; min = 0.00 µmol/L; max = 25.20 µmol/L), and it is worth noting that two affected animals did not have measurable HGA in their blood (horses D47 and D89).

Horse D47 had no detectable HGA in its blood at the time of sampling but had a low level of MCPA-carnitine (2.50 nmol/L) compared to other affected horses (MCPA-carnitine median = 155.00 nmol/L; min = 0.00 nmol/L; max = 4480.00 nmol/L, 1st percentile = 2.35 nmol/L). Its serum acylcarnitines profile was within the median range among affected horses and corresponds to the 65th percentile of observations (Fig. 3b). Based on this severe alteration in the serum acylcarnitines profile, we consider D47 to be effectively suffering from AM, despite the absence of protoxins and the low levels of metabolites in its blood. To explain these atypical findings, our hypothesis is that blood markers could have been modified by intravenous fluid therapy in this case, as the anamnestic elements do not allow us to determine whether venipuncture was performed before or after fluid initiation. Interestingly, the presence of MCPA-carnitine in the blood of this horse while the serum HGA level is already below the limit of detection, could be related to the differences in elimination kinetics. The literature on the dynamics of HGA and MCPA-carnitine in blood is limited (Bochnia et al., 2016b; Jahn et al., 2022). In a study of one horse suffering from MA, Jahn and collaborators (2022) observed a decrease in plasma concentrations of HGA and MCPA-carnitine. Hypoglycine A was detected in the blood of the affected horse for a longer time than its metabolites. The MCPA-carnitine, however, was no longer detectable 4 days post-onset of clinical signs. Another case study suggests a faster decrease of HGA compared to its metabolite: Bochnia and collaborators (2016) describe a fast metabolism of HGA into toxic metabolites with a subsequent increase in serum MCPA-carnitine levels after onset of clinical signs. Indeed, for these two cases, as well as for our study, the issue of timing before sampling arises, along with the variation that can exist between

the onset of clinical signs and the moment they are observed. To our knowledge, there is no standardised study on horses intoxication with HGA. Nevertheless, Sander and collaborators (2020) describe that, in a human individual, the renal excretion of unmetabolised HGA is higher than that of MCPA-carnitine. Furthermore, HGA renal excretion was described as proportional to its serum concentration. Therefore, increasing glomerular filtration via fluid therapy may increase the elimination of HGA more than that of MCPA-carnitine. This hypothesis could explain the atypical profile of horse D47, which presented MCPA-carnitine in its blood but no longer had HGA (Sander et al., 2020).

Horse D89 on the other hand, was the only animal included as diseased horse which had neither HGA toxin nor measurable amounts of MCPA-carnitine in his blood. Furthermore, D89 exhibits a serum acylcarnitines profile that was inconsistent, according to our results, with that of a horse suffering from AM. Indeed, D89's serum acylcarnitines profile overlapped with the ones of the control group (Fig. 3). This suggested an error in the tentative diagnosis of AM, which was later confirmed. Four months after his hospitalization, D89 underwent a genetic test which revealed a mutation in the glycogen synthase 1 enzyme, leading to the diagnosis of polysaccharide storage myopathy type 1. Despite meeting all our inclusion criteria, including pigmenturia and a serum CK activity exceeding 10,000 UI/L, D89 was not suffering from AM. This highlights the diagnostic potential of serum acylcarnitines, suggesting their role in potentially distinguishing between various types of myopathies.

As previously documented in existing literature, the levels of various serum acylcarnitines significantly increase in animals afflicted with AM. Nevertheless, previous publications fail to reach a consensus regarding the most affected group of acylcarnitines. These groups encompass free carnitine (van der Kolk et al., 2013), short-chain acylcarnitines (C2-C5) (Bochnia et al., 2019; Karlíková et al., 2016; Sponseller et al., 2012; van der Kolk et al., 2013; Westermann et al., 2008, 2007), medium-chain acylcarnitines (C6-C12) (Bochnia et al., 2019; Karlíková et al., 2016; Sponseller et al., 2012; van der Kolk et al., 2013; Westermann et al., 2008, 2007), long-chain acylcarnitines (C14-C20), very long-chain acylcarnitines (C22-C26), and unsaturated chain acylcarnitines (Karlíková et al., 2016).

Our study's findings indicate the following:

(i) The modification of the serum acylcarnitines profile is severe, resulting in an increase in all acylcarnitines, irrespective of their chain length. This increase is significant when comparing diseased horses to their healthy pasture companions, with the exception of C18-carnitine.

(ii) For the first time, this significant alteration was compared to another pathology, specifically horses with colic signs and another myopathy, initially suspected to have AM. Analysis of the serum acylcarnitines profile revealed a clear distinction between horses suffering from colic and those afflicted with AM (regression 1). This tends to highlight the value of the serum acylcarnitines profile in the diagnosis of AM. It allows a clear differentiation between horses suffering from AM and those suffering from abdominal colic, and seems to allow differentiation between horses suffering from AM and other types of myopathies. However, it's important to note that this study only included a single case of another myopathy, which is discussed further below. Recognizing the limitations of this study, further investigations, particularly comparative studies of acylcarnitines profile among cases of AM and various other myopathies, are warranted to elucidate broader diagnostic applications.

(iii) Relying solely on HGA and/or MCPA-carnitine does not enable effective differentiation between diseased horses and their pasture companions. Given the inability to discriminate between cograzers and affected horses using HGA and MCPA-carnitine alone, our observations underscore the diagnostic significance of serum acylcarnitines. In addition, exceptionally, a case may be negative for HGA.

(iiii) Concerning CK, which is a marker that can be easily investigated even as a first-line test, the results of this study show a significant specificity of CK in distinguishing Diseased Horses vs. Control (Figure E.g - appendices). The interpretation of this result remains debatable since the severe increase in CK to more than 10,000 IU/L was part of the inclusion criteria for Diseased Horses based on van Galen et al. (2012).

4.3. Update on the prognostic blood markers

Blood samples in this study were collected upon the admission of diseased animals to the clinic. We explored the possibility of using these early blood samples to predict the chances of the animal's survival. This would provide owners and the medical team with valuable information about the risks that must be considered before initiating financially and emotionally demanding therapies.

A similar approach was undertaken in the study by Boemer and colleagues in 2017, resulting in an estimation of outcomes based on three acylcarnitines (C2, C10:2, and C18-carnitines). This calculated prognosis was applied to the 32 survivors and the 28 dead horses in the study. This allowed us to generate a ROC curve (Figure E.j - appendices) with an area under the curve of 0.736. When applied to our samples, the test, as described by Boemer et al., was associated with a negative predictive power of 62 % and a positive predictive power of 67 % in distinguishing between diseased horses and cograzers (calculated prognosis cutoff at 0.44).

This test seems insufficiently specific for clinical practice. Among the blood markers studied in the present study, C5 carnitine appears to be the most suitable for developing a simple diagnostic and prognostic tool. Using C5 carnitine, we created two ROC curves, depicted in Figure E.e (in the appendices).

To estimate the chances of survival of a diseased animal based on C5 carnitine, the area under the ROC curve is 0.871. According to this model, a serum concentration of C5 carnitine would allow a correct identification of a diseased animal likely to die in 76 % of cases (negative predictive power with C5 cutoff at 12.21 μmol/L). Using the same test, a survivor would be correctly identified in 81 % of cases (positive predictive power with C5 cutoff at 12.21 μmol/L). A rapid on-site or clinic admission test could, therefore, provide a relatively reliable estimation of the disease outcome.

To distinguish between healthy and diseased horses, the area under the ROC curve is 0.988. According to this new model, a serum concentration of C5 carnitine would allow a correct identification of a diseased horse from a cograzer in 92 % of cases (negative predictive power with C5 cutoff at 3.04 μmol/L). Using the same test, a cograzer could be correctly identified in 97 % of cases (positive predictive power with C5 cutoff at 3.04 μmol/L). A rapid test could, therefore, confirm or exclude a diagnosis of AM in horses in pasture, exposed to sycamore maple trees during the risky seasons. The same continuum of serum C5 carnitine values could also help identify cograzers in which lipid catabolism is most affected, thereby providing insight into the animals at the highest risk of developing the disease.

It is interesting to note that neither the blood levels of HGA toxin, nor those of the MCPA-carnitine metabolite, nor those of CK can distinguish between horses that will survive and those that will die. In this study, the analyses were performed at a single and relatively early point in the clinical course of the disease. As described by Jahn and *al.* (2022), the blood parameters HGA, MCPA-carnitines, and CK evolve during the first hours of the diseas. A longitudinal study focusing on the blood kinetics of these markers seems warranted to definitively conclude on their utility. This hypothesis has already been proposed by Dunkel et al. (2018), who observed that increasing CK over time worsened the prognosis. (Dunkel et al., 2018).

4.4. Subclinical cases of HGA poisoning

One of the strengths of this study is its extensive inclusion of cograzers (n=73). These clinically healthy animals, which shared pastures with diseased horses, were sampled within 48 hours of the onset of clinical signs of AM cases. At the time of sampling, cograzers had not

been removed from toxic exposure yet, maximizing the likelihood of obtaining a blood profile that closely mirrored their status at the onset of clinical signs in affected horses.

Additionally, it is worth noting that the presence of HGA in apparently healthy cograzers has been previously documented in the literature, both in inferior (Bochnia et al., 2015; Gröndahl et al., 2015) and in equivalent amounts to those found in affected horses (Baise et al., 2016; Bochnia et al., 2016a; Boemer et al., 2015). Our observations in this study reveal that most cograzers not only had HGA (97 % of cograzers) but also MCPA-carnitine (90 % of cograzers) in their blood.

Furthermore, while there is significant variability in protoxin and metabolite serum levels, in contrast to previous publications (Sander et al., 2016), it appears that the quantity of ingested toxin is not the sole factor determining whether an animal becomes ill as both HGA and MCPA-carnitine concentrations largely overlapped between diseased and cograzers horses. These new findings also challenge the hypothesis that the combined elevation of both blood parameters, HGA and MCPA-carnitine, coincides with typical signs of AM (Bochnia et al., 2016). Moreover, many cograzers not only displayed the presence of the protoxin and its metabolite in their blood but also showed elevated acylcarnitines level. These alterations overlap the range observed in control and diseased horses, suggesting that fatty acid catabolism of cograzers is already altered despite the absence of clinical signs. Therefore, it is appropriate to describe these cograzers as subclinically intoxicated animals rather than healthy pasture companions. It is noteworthy that a similar subclinical animal profile has been previously suggested in herbivores other than equids (Renaud et al., 2022).

From a practical standpoint, when a horse is suffering from AM, this study emphasizes the need to manage not only the affected animal but also the other equids in the pasture. The top priority is to remove these animals from toxin exposure.

4.5. Limitations of the Study

Limits of this study include several important considerations.

Firstly, the ideological framework of our investigation was constrained by markers already identified in previous studies as either protective or indicative of risk. Additionally, while the overall sample size was substantial, the group-specific and sex-specific sample sizes were comparatively small, limiting the statistical power of our observations.

Secondly, regarding the diagnostic marker aspect, our study focused primarily on markers previously suggested in the literature. Future research could benefit from including horses afflicted with other myopathies to further validate the initial findings of this study.

Thirdly, a longitudinal study assessing a larger cohort would be beneficial to definitively evaluate the kinetics of the toxin, its metabolites, CK levels, and the serum acylcarnitines profile. Such a study would provide more robust conclusions regarding their respective significance.

Lastly, from a practical standpoint, the rapid measurement of plasma C5, which could confirm diagnosis and estimate survival chances, is currently not easily accessible to veterinary practitioners. Improved accessibility to this diagnostic tool would greatly enhance its clinical utility.

5. Conclusion

Our findings highlight the complexity and variability within horses exposed to HGA. We observe that the serum concentrations of HGA and MCPA-carnitine alone do not provide a clear distinction between affected horses and their healthy pasture companions. Instead, it is the alteration of the serum acylcarnitines profile that stands out as a key diagnostic indicator. This study highlights that the acylcarnitines profile undergoes significant changes in horses suffering from AM, with an increase in nearly all acylcarnitines, regardless of their chain length. This differentiates affected animals from control horses and horses with colic signs or with other myopathies.

Additionally, the present study makes advancements in prognostic markers for AM, with a specific focus on C5 carnitine. We found that C5 carnitine holds great potential to become a rapid on-site or clinic admission test to confirm or exclude an AM diagnosis and provide critical information about the likelihood of survival. Development of such a test could aid veterinarians and horse owners in making informed decisions about treatment and management strategies in cases of AM. Additional studies are necessary to assess the feasibility and potentially develop a simple and rapid diagnostic tool based on those findings.

Furthermore, this study introduces a critical dimension to the AM landscape by addressing HGA subclinical intoxication. This novel notion suggests the need for early management of both the affected horse and its pasture companions. It would be prudent to reduce toxin exposure by removing cograzers from affected pastures.

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Institutional Review Board Statement

Ethical review and approval were waived for this study. The whole procedure is part of routine veterinary practice to establish a diagnosis in case of disease.

Informed Consent Statement

Not applicable.

CRediT authorship contribution statement

Benoît Renaud: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Caroline-J. Kruse:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Géraldine Luis:** Writing – review & editing, Methodology, Investigation. **François Boemer:** Writing – review & editing, Methodology, Investigation. **Dominique-Marie Votion:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Pascal Gustin:** Writing – review & editing. **Carla Cesarini:** Writing – review & editing, Resources. **Anne-Christine François:** Writing – review & editing, Methodology, Conceptualization. **Katrien Palmers:** Resources. **Gunther van Loon:** Writing – review & editing, Resources.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used Chatgpt in order to improve language After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Votion Dominique-Marie reports financial support was provided by Wallonie agriculture SPW. Votion Dominique-Marie reports financial support was provided by Les Fonds Spéciaux pour la Recherche (FSR) of Liege University (Liège, Belgium). If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendices

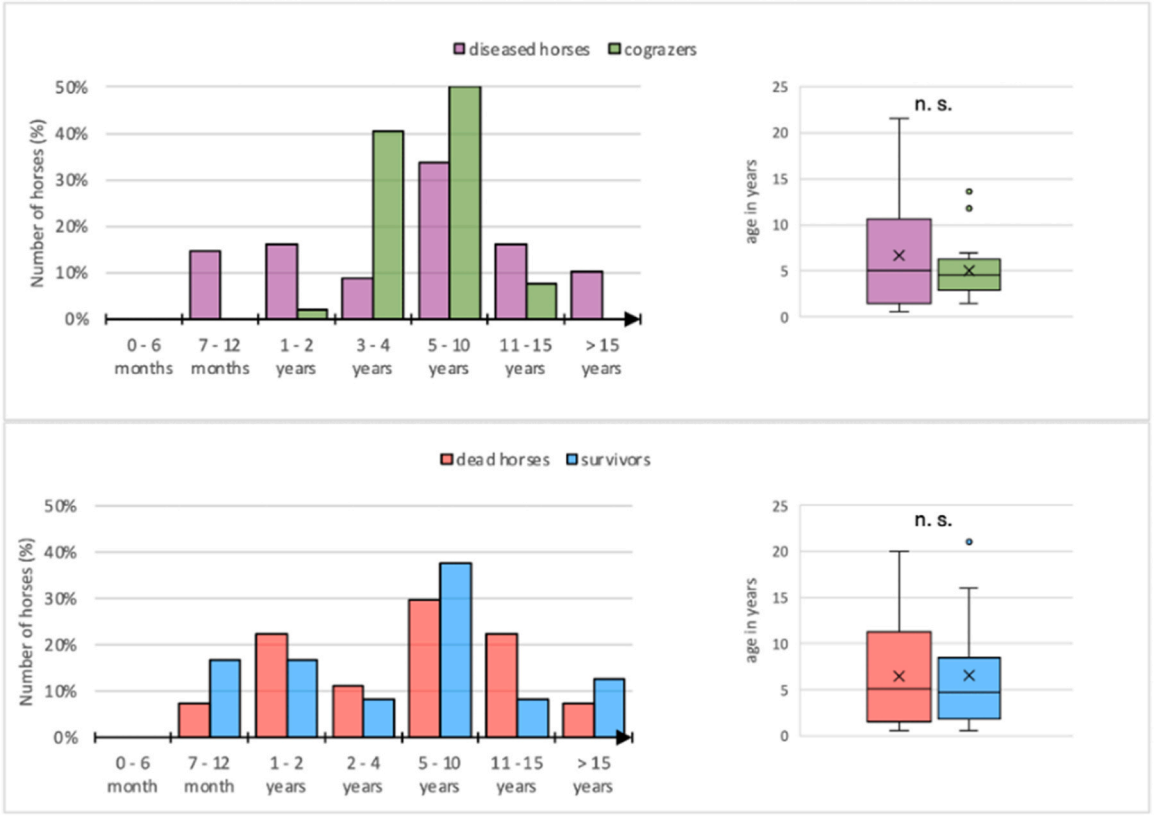


Fig. A. – Age distribution between groups: histograms and boxplots Boxes range from the 25th to 75th percentiles. Medians are represented by horizontal lines in the box and means are symbolized by a cross. Box plot whiskers were established using the Tukey method. n.s. stands for no significant difference of average age,

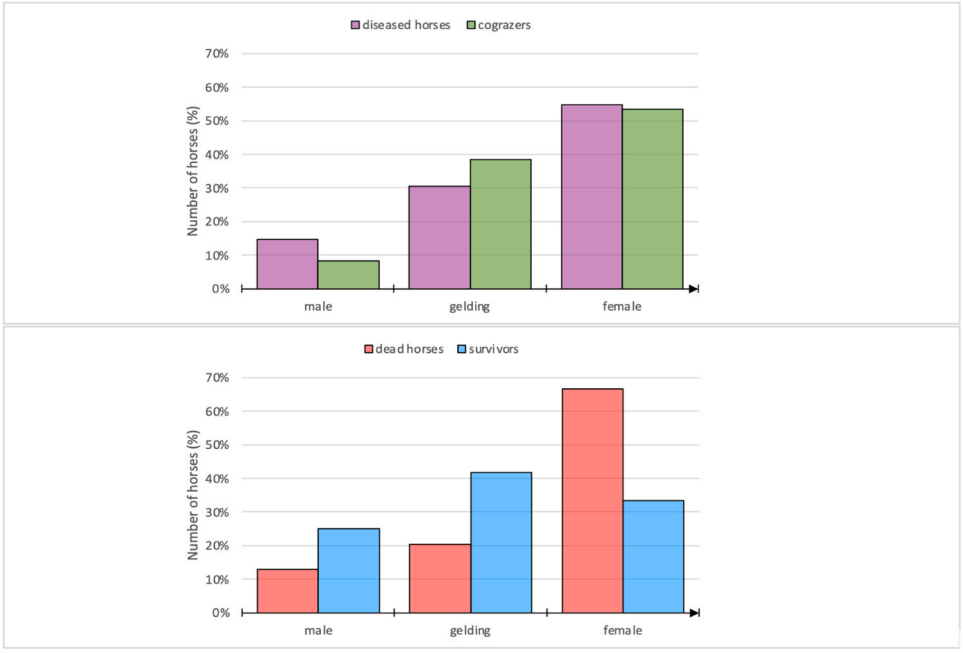


Fig. B. – Sex distribution between groups: histograms.

Table C
– Serum (mean ± standard deviation) concentrations of free carnitine and acylcarnitines (μmol/L)

	Diseased horses	Dead horses	Survivors	Cograzers	Colic horses	Control horses
Free Carnitine	89.69 ± 8.11*	90.28 ± 11.32*	49.92 ± 4.30	29.08 ± 1.20	29.44 ± 4.53	26.27 ± 1.16
C2	33.61 ± 2.06*	39.32 ± 3.21*	23.37 ± 2.14*	6.83 ± 0.32	13.26 ± 2.22	7.35 ± 0.39
C3	2.29 ± 0.16*	3.01 ± 0.33*	1.48 ± 0.14	0.74 ± 0.05	0.72 ± 0.18	1.00 ± 0.06
C3-DC	0.34 ± 0.04*	0.48 ± 0.10*	0.14 ± 0.02*	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
C4	17.99 ± 1.40*	24.95 ± 2.55*	11.58 ± 1.61*	1.33 ± 0.15*	0.41 ± 0.07	0.60 ± 0.02
C5	15.90 ± 1.23*	22.19 ± 2.07*	8.02 ± 1.28*	1.00 ± 0.11*	0.27 ± 0.07	0.25 ± 0.01
C5-OH	0.61 ± 0.08*	0.87 ± 0.21*	0.27 ± 0.04*	0.07 ± 0.00	0.11 ± 0.03	0.06 ± 0.01
C5-DC	1.71 ± 0.15*	2.07 ± 0.25*	0.90 ± 0.11*	0.21 ± 0.02	0.19 ± 0.03	0.14 ± 0.01
C6	3.96 ± 0.42*	5.37 ± 0.82*	2.46 ± 0.48*	0.15 ± 0.02	0.05 ± 0.01	0.04 ± 0.00
C8	1.54 ± 0.16*	1.95 ± 0.34*	1.08 ± 0.17*	0.09 ± 0.02	0.03 ± 0.01	0.03 ± 0.00
C8:1	1.06 ± 0.11*	1.40 ± 0.19*	0.59 ± 0.10*	0.04 ± 0.01	0.03 ± 0.00	0.02 ± 0.00
C10	0.47 ± 0.04*	0.51 ± 0.05*	0.42 ± 0.06*	0.03 ± 0.01	0.03 ± 0.00	0.02 ± 0.00
C10:1	0.39 ± 0.03*	0.45 ± 0.05*	0.30 ± 0.04*	0.05 ± 0.01*	0.07 ± 0.04*	0.01 ± 0.00
C10:2	0.72 ± 0.06*	0.98 ± 0.12*	0.50 ± 0.08*	0.05 ± 0.01	0.02 ± 0.00	0.01 ± 0.00
C12	0.25 ± 0.02*	0.26 ± 0.02*	0.20 ± 0.02*	0.03 ± 0.01	0.04 ± 0.00	0.02 ± 0.00
C12:1	0.20 ± 0.01*	0.21 ± 0.02*	0.17 ± 0.02*	0.01 ± 0.00	0.04 ± 0.01	0.01 ± 0.00
C14	0.22 ± 0.01*	0.24 ± 0.02*	0.17 ± 0.02*	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
C14:1	0.34 ± 0.03*	0.32 ± 0.03*	0.27 ± 0.04*	0.02 ± 0.00	0.06 ± 0.01*	0.01 ± 0.00
C16	0.61 ± 0.03*	0.62 ± 0.07*	0.50 ± 0.04*	0.14 ± 0.02	0.14 ± 0.02	0.13 ± 0.01
C16:1	0.11 ± 0.01*	0.25 ± 0.04*	0.17 ± 0.02*	0.02 ± 0.00	0.04 ± 0.00	0.01 ± 0.00
C18	0.27 ± 0.02	0.29 ± 0.03*	0.19 ± 0.02	0.05 ± 0.01	0.07 ± 0.01	0.06 ± 0.01
C18:1	0.63 ± 0.04*	0.66 ± 0.09*	0.44 ± 0.04*	0.07 ± 0.01	0.10 ± 0.01	0.05 ± 0.01

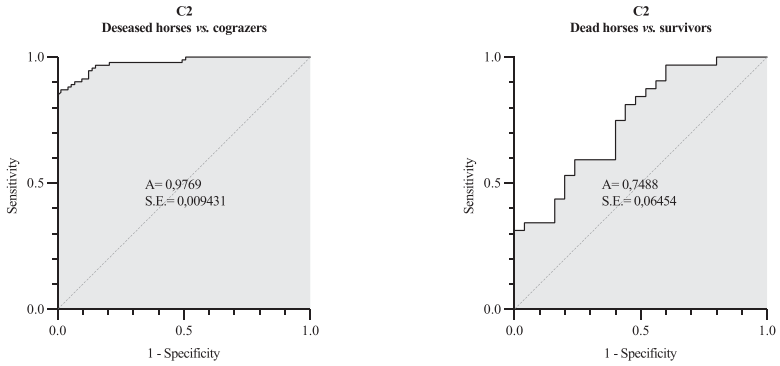
* Mean value above the 99th percentile of the reference range obtained from control horses

Table D
– Regression coefficients to select variables

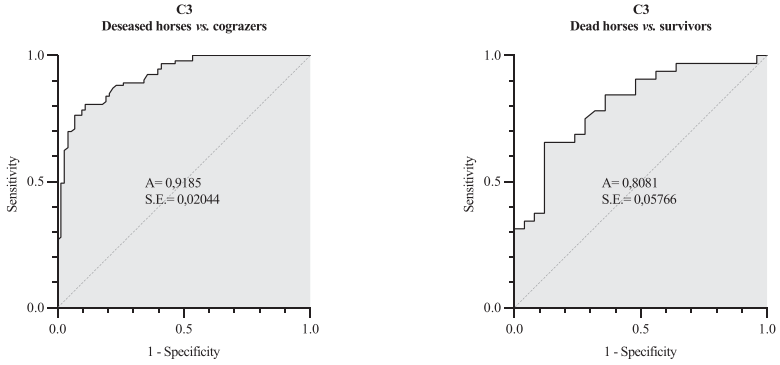
Variable	VIP	Center/Scaled parameter estimates
C5*	**1,14**	−0,44
C3DC*	**1,14**	**0,19**
C10:1*	**1,13**	−0,19
C14:1	1,07	−0,09
C2*	**1,06**	−0,19
C12:1	1,04	0,03
C4*	**1,04**	−0,27
C12	1,03	−0,09
C10	1,02	0,04
C3*	**1,02**	**0,30**
C14	1,00	−0,05
C18:1	0,98	−0,20
C8	0,98	0,13
C18	0,95	0,01
C5DC	0,95	−0,15
C10:2	0,94	−0,08
C16:1	0,94	0,08
C8:1	0,94	0,08
C16	0,92	−0,13
C5OH	0,90	0,12
C6	0,90	0,03
Free carnitine	0,83	0,11

* Acylcarnitines with a variable importance in the projection scores greater than 1 and a center parameter lower than 0
VIP stands for variable importance in the projection

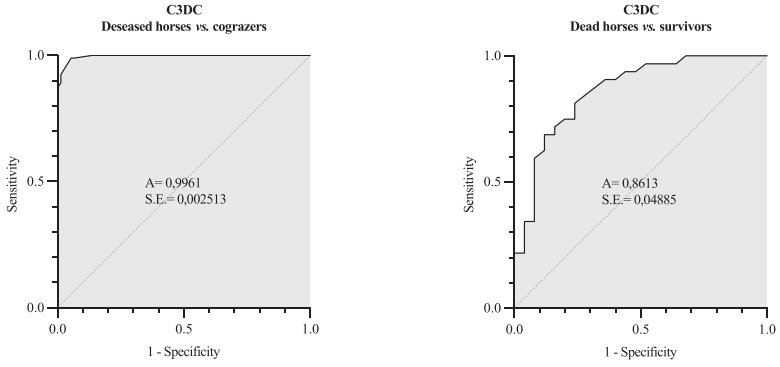
a- acetyl carnitine (C2)



b- propionyl carnitine (C3)



c- Malonylcarnitine (C3-DC)



d- butyryl-/isobutyrylcarnitine (C4)

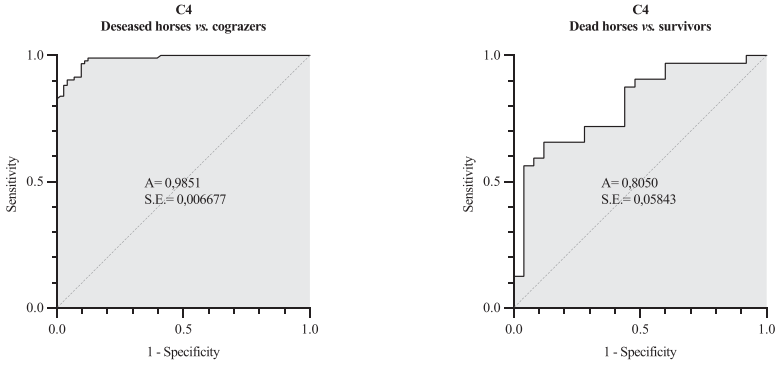
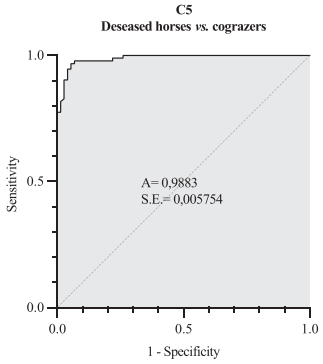
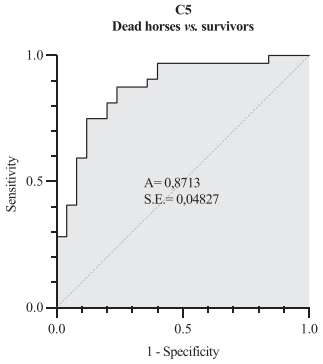


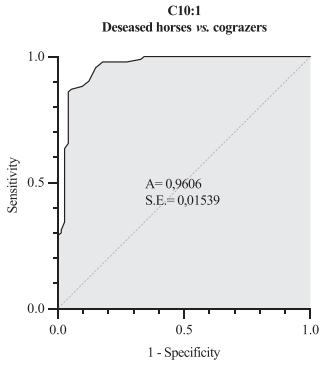
Fig. E. a-j – ROC curves with area under the curve (A) and standard error (S.E.). Excluding horses D47 and D89.

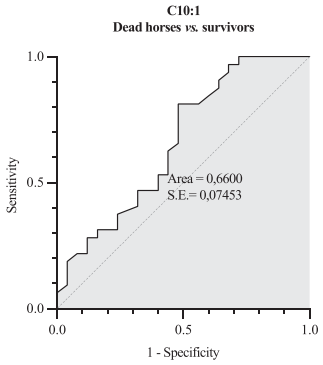
e- isovaleryl-/2-methylbutyrylcarnitine (C5)



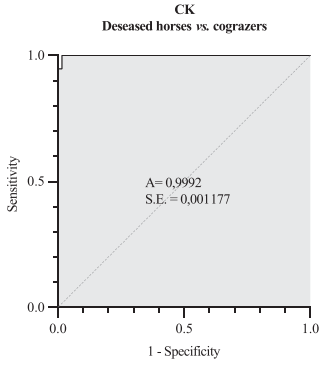


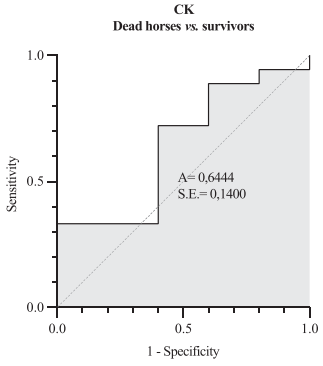
f- decenoylcarnitine (C10:1)



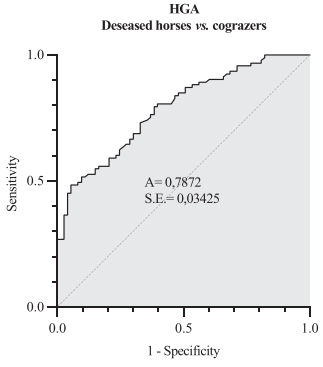


g- Serum creatine kinase activity (CK)





h- Hypoglycin A



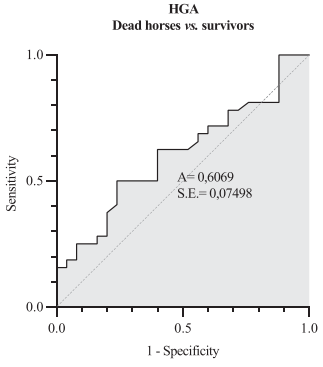
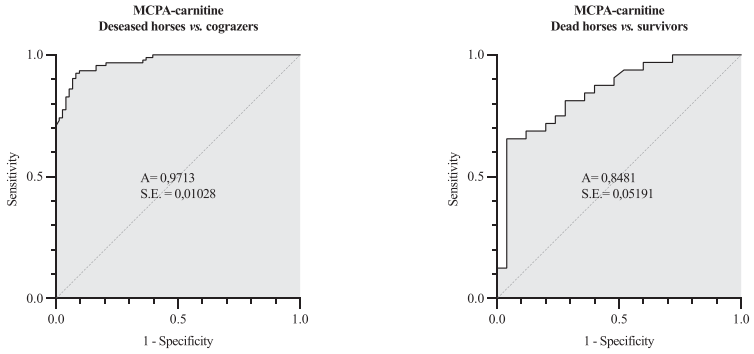


Fig. E. (*continued*).

i- MCPA-carnitine



j- Survival prognosis calculated according to Boemer and *al.* (2017)

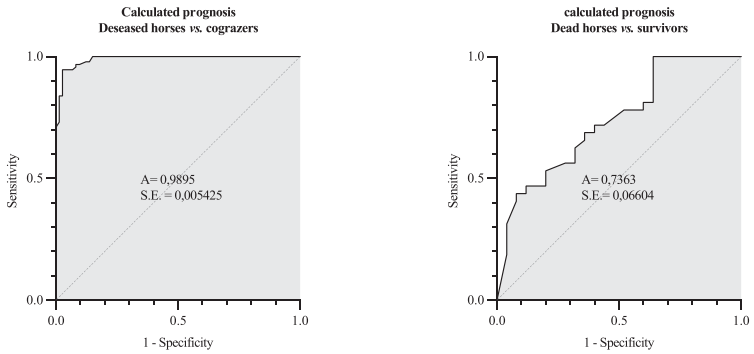


Fig. E. (*continued*).

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