UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE VETERINÁRIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

ASPECTOS ANATOMOPATOLÓGICOS E GENÉTICOS DE CASOS FATAIS DE CALCINOSE SISTÊMICA EM EQUINOS

GUILHERME CARVALHO SERENA

PORTO ALEGRE

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Orientador: Prof. Dr. Saulo Petinatti Pavarini

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ASPECTOS ANATOMOPATOLÓGICOS E GENÉTICOS DE CASOS FATAIS DE CALCINOSE SISTÊMICA EM EQUINOS

Aprovada em 2023.

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RESUMO

A calcinose sistêmica em equinos é uma doença rara, de fisiopatologia não completamente elucidada, com curso geralmente letal e lesões histopatológicas caracterizadas por inflamação e mineralização sistêmicas. Apresenta correlações com a miosite imunomediada, que é uma doença genética de origem inflamatória, relacionada com uma variante do gene *MYH1* que codifica a cadeia pesada da miosina 1. Apesar da falta de comprovação da influência genética no desenvolvimento de calcinose sistêmica, ambas as doenças apresentam curso clínico inicial semelhante, acometendo, principalmente, equinos da raça Quarto de Milha, e estão frequentemente associadas a históricos prévios de infecções bacterianas ou vacinação, que parecem servir de evento desencadeador das doenças. A miosite imunomediada diferencia-se da calcinose sistêmica por apresentar um bom prognóstico, causando atrofia muscular imunomediada com rápida recuperação, enquanto a calcinose sistêmica apresenta um curso grave de alta letalidade. Poucos estudos abordaram a calcinose sistêmica, sua relação com miosite imunomediada e com a mutação no gene *MYH1*. O objetivo deste estudo foi descrever os aspectos epidemiológicos, patológicos e moleculares de casos de calcinose em equinos submetidos à necropsia entre os anos de 2018 e 2021 nas regiões Sul e Centro-Oeste do Brasil. No período estudado, cinco casos histologicamente compatíveis com lesão muscular definida como calcinose sistêmica foram diagnosticados. Os equinos afetados eram da raça Quarto de Milha, tinham idade mediana de 15,4 meses e tiveram evolução da doença de 16,8 dias em média. As alterações macroscópicas eram caracterizadas por áreas de palidez acentuada na musculatura, com lesões microscópicas de necrose, calcificação, atrofia e regeneração muscular e manifestação em vários órgãos, com predomínio de processos inflamatórios e mineralizantes. Quatro dos cinco cavalos tiveram material submetido a avaliação molecular e todos apresentaram mutação para a variante patológica do gene *MYH1* em homozigose (n=1) ou heterozigose (n=3). Não havia outra mutação relacionada à doença muscular nos casos testados. Três equinos apresentaram marcação positiva contra antígenos de *Streptococcus equi* na avaliação imuno-histoquímica, especialmente, no trato respiratório. Os achados genéticos do presente estudam reforçam o envolvimento da variante *MYH1*_p.E321G no desenvolvimento da calcinose sistêmica em equinos QM e que esta apresenta consonância entre os achados clínicos e epidemiológicos da miosite imunomediada, diferenciando-se na manifestação histopatológica.

Palavras-chave: mineralização; doença muscular; *MYH1*; mutação genética; miosina; equino; diagnóstico.

ABSTRACT

Systemic calcinosis in horses is a rare disease with pathophysiology that is not completely elucidated, typically fatal course, and histopathological lesions characterized by systemic inflammation and mineralization. It presents deep correlations with immune-mediated myositis, which is a genetic inflammatory disease related to mutations in the *MYH1* gene that encodes the heavy chain of myosin. Despite the lack of evidence for genetic influence on the development of systemic calcinosis, both diseases present similar initial clinical courses, mainly affecting Quarter Horse breed horses, and are commonly associated with previous histories of bacterial infections or vaccination, which seem to serve as triggering events for the diseases. Immune-mediated myositis differs from systemic calcinosis in that it has a good prognosis, causing immune-mediated muscle atrophy with rapid recovery, while systemic calcinosis presents a severe course with high lethality. Few studies have addressed systemic calcinosis, its relationship with immune-mediated myositis, and the *MYH1* gene mutation. The aim of this study is to describe the epidemiological, pathological, and molecular aspects of cases of calcinosis in horses submitted to necropsy between 2018 and 2021 in the South and Midwest regions of Brazil. During the studied period, five histologically compatible cases with muscle injury defined as systemic calcinosis were diagnosed. The median age of affected horses was 15.4 months, all Quarter Horse breed, with an average disease progression time of 16.8 days. Macroscopic alterations were characterized by areas of pronounced paleness in the musculature with microscopic lesions of muscle atrophy with manifestation in various organs, with a predominance of inflammatory and mineralizing processes. Four of the five horses had material submitted to molecular evaluation and all presented a mutation for the pathological variant of the *MYH1* gene, one homozygous and three heterozygous horses. There was no other mutation related to muscle disease in the tested cases. Three horses showed positive marking against *Streptococcus equi* antigens on immunohistochemical evaluation, especially in the respiratory tract. The genetic findings of the present study indicate that the *MYH1* gene is involved in the development of systemic calcinosis and that it presents consistency between the clinical and epidemiological findings of immune-mediated myositis, differing in histopathological manifestation.

Keywords: Mineralization; muscle disease; *MYH1*; genetic mutation, myosin; horse; diagnosis.

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1. INTRODUÇÃO

As miopatias inflamatórias representam um conjunto de doenças de variada apresentação clínica com fisiopatologia definidas pela presença de inflamação muscular como atributo mais importante da doença (DALAKAS, 2020). Em humanos as formas mais prevalentes são de etiologias imunomediadas, onde o próprio sistema imune desencadeia a lesão (SHELTON, 2007). Na contraparte veterinária são bem caracterizadas na espécie canina, sendo a de maior prevalência a miosite do músculo mastigatório canina (EVANS; LEVESQUE; SHELTON, 2004; SHELTON, 2007). Nessa doença são produzidos autoanticorpos contra fibras musculares induzindo lesão atrófica e inflamatória (KENT et al., 2017).

Em equinos, a púrpura hemorrágica é a miopatia imunomediada mais estudada (citar refertência). Essa doença, usualmente, se desenvolve após processos infeciosos ou eventos vacinais, sendo o *Streptococcus equi* o agente mais associado (PUSTERLA et al., 2003). Complexos formados pela associação de antígenos e anticorpos depositados no tecido induzem o desenvolvimento de uma reação de hipersensibilidade tipo III (MALLICOTE, 2015), com extensa vasculite, hemorragias e infartos musculares (KAESE et al., 2005).

Processos imunomediados subsequentes a infecções ou outros tipos de estimulação antigênica, são frequentemente reportados em cavalos e estão associados, além da púrpura hemorrágica equina, com manifestações de rabdomiólise grave (SPONSELLER et al., 2005), anemia hemolítica imunomediada, miocardite (TRIMBLE et al., 2019) e miopatias crônicas autoimunes (PASOLINI et al., 2018). Em humanos, também são reportados processos semelhantes como a púrpura de Henoch-Schönlein (MALISKE; EDWARDS; SUNEJA, 2015) e a febre reumática (ARVIND; RAMAKRISHNAN, 2020).

Outras duas miopatias imunomediadas são descritas em equinos, a miosite imunomediada e a calcinose sistêmica (DURWARD-AKHURST; VALBERG, 2018). A miosite imunomediada provoca atrofia muscular severa associadas a inflamação (FINNO; SPIER; VALBERG, 2009). Sua patogênese está relacionada com mutações no gene *MYH1*, que codifica a cadeia pesada da miosina em fibras de contração rápida da musculatura esquelética (FINNO et al., 2018a; GIANINO et al., 2019). Equinos que apresentam a síndrome, comumente também têm histórico de infecções prévias (LEWIS; VALBERG; NIELSEN, 2007), Com 47% dos equinos Quartos de Milha com a variante patológica do gene *MYH1* apresentando histórico de vacinação, doença respiratória ou gastrointestinal, nos três meses antecedentes ao desenvolvimento de atrofia muscular (VALBERG et al., 2022).

Hipotetiza-se que a alteração morfológica induzida pela mutação, tenha semelhanças estruturais com epítopos de patógenos, ou que seja capaz de ativar vias de autoimunidade (FINNO; SPIER; VALBERG, 2009). A ativação de receptores Toll-like e a subsequente resposta inflamatória já foi demonstrada e pode ser capaz de promover lesões musculares e extra musculares (ZHANG et al., 2009).

A calcinose sistêmica, opondo-se a miosite imunomediada, tem prognóstico desfavorável e alta letalidade (TAN et al., 2010). Sua patogênese não é elucidada mas suspeita-se de um processo inflamatório semelhante ao da miosite imunomediada que evolui para manifestações sistêmicas de mineralização (FINNO; SPIER; VALBERG, 2009). Existem dois trabalhos relatando casos de calcinose sistêmica em equinos publicados, (SPONSELLER et al., 2022; TAN et al., 2010). Um com cinco cavalos, que apresentaram sinais clínicos de miopatia e em necropsia, após eutanásia, identificaram-se mineralizações teciduais sistêmicas (TAN et al., 2010). Outro relato com diagnóstico *ante-mortem* de um equino que sobreviveu a condição e tinha um diagnóstico inicial de miosite imunomediada mas que em exame histopatológico após biopsia possuía mineralização em pulmão, rins e musculatura (SPONSELLER et al., 2022).

Um terceiro trabalho relata um equino com sinais de afecção locomotora e com mineralização restrita em paredes de artérias torácicas, com diagnóstico de calcificação idiopática. associada a deposição uma matriz de aparência condroide, sugerindo um diagnóstico não conclusivo de desmopatia degenerativa equina e afastando o diagnóstico de calcinose sistêmica (FALES-WILLIAMS; SPONSELLER; FLAHERTY, 2008).

Dentre estes relatos, a genotipagem da variante *MYH1*_E321G foi realizada apenas no equino diagnosticado com calcinose sistêmica sendo homozigoto para a alteração (SPONSELLER et al., 2022).

A associação entre doenças musculares e alterações genéticas em equinos é alta. Um estudo que avaliou a presença de mutações em equinos com doença muscular clínica que tiveram biopsia submetida para avaliação histopatológica constatou que de 296 animais genotipados, 97 (33%) possuíam variantes associadas a doença com o índice subindo para 47% em animais com alterações histológicas no tecido muscular (ALEMAN et al., 2022). Outro estudo encontrou mutações em 299 (29%) dos 1031 equinos avaliados (TRYON et al., 2009).

As variantes mais prevalentes encontradas em genótipos causadores lesões musculares foram as associadas com o desenvolvimento de miopatias da cadeia pesada da miosina (MYHM), miopatia de armazenamento de polissacarídeo tipo 1 (PSSM1) e hipertermia maligna (MH) (ALEMAN et al., 2022).

A PSSM1 é uma doença autossômica dominante, caracterizada pelo aumento da atividade de glicogênese e consequente acumulação excessiva de polissacarídeos intracitoplasmáticos que interferem no metabolismo celular (MCCUE et al., 2008). Já a MH é uma doença autossômica dominante a que deriva de alterações de origem genética nos receptores de rianodina que comando o fluxo de cálcio em células musculares, na presença da mutação em associação a um estímulo, usualmente farmacológico, os canais são ativados em uníssono liberando grande quantidade de cálcio, levando a célula a um estado hipermetabólico e predispondo a processos de lise (ALEMAN; NIETO; MAGDESIAN, 2009).

Outras enfermidades musculares relevantes de origem genética são a paralisia periódica hipercalemica (HyPP) e a deficiência da enzima ramificadora do glicogênio (GBED) (FINNO; SPIER; VALBERG, 2009). A HYPP é uma doença autossômica dominante, em que os animais apresentam alterações dos canais de sódio que facilitam a ocorrência de despolarização celular sustentada causando estado de ativação anormal e fraqueza muscular subsequente (DELFIOL et al., 2015; FINNO; SPIER; VALBERG, 2009). GBED é uma enfermidade autossômica recessiva caracterizada por alteração no metabolismo no glicogênio e consequente deficiência na mobilização energética celular (VALBERG, 2020).

O impacto da mutação *MYH1* no desenvolvimento de lesão muscular é geralmente resumido em duas apresentações bem descritas: miosite imunomediada e rabdomiólise não-exercional. A miosite imunomediada é caracterizada por intensa atrofia muscular, principalmente, da musculatura dorsal e glútea, com início rápido e prolongada duração dos sinais clínicos. Fraqueza generalizada também é observada, e decúbito em cavalos mais afetados (DURWARD-AKHURST & VALBERG, 2018). A atrofia muscular se desenvolve rapidamente, com relatos de manifestação em três dias de progressão em 14 dos 23 cavalos estudados (LEWIS, VALBERG & NIELSEN, 2007).

A mutação *MYH1* também induz rabdomiólise grave sem correlação com exercícios. Essa apresentação está intimamente associada à miosite imunomediada, mas se distingue por apresentar uma evolução mais lenta da atrofia muscular, com um componente necrótico mais evidente do que um componente inflamatório. Mioglobinúria também pode estar presente em alguns casos (VALBERG, 2020).

Ambas as manifestações têm bom prognóstico, e 84% dos animais afetados apresentam recuperação após seis meses de acompanhamento com restabelecimento da massa muscular. Mesmo em casos de reaparecimento os índices de resolução atingem 90% (HUNYADI et al., 2017). Resultados similares foram observados em 13 de 23 cavalos que reverteram a atrofia muscular após cinco messes (LEWIS, VALBERG & NIELSEN, 2007), o

uso de terapia com corticosteroides está a associada com aceleração da recuperação (DURWARD-AKHURST & VALBERG, 2018).

A calcinose sistêmica é uma doença muscular inflamatória que apresenta intersecções clínicas e epidemiológicas com a miosite imunomediada, sugerindo que a variante patogênica do gene *MYH1* estejam envolvidas no desenvolvimento de ambas as patologias (VALBERG, 2020). Sendo assim, diante da existência de poucos relatos abordando a calcinose sistêmica em equinos, o objetivo desse trabalho, é descrever os aspectos epidemiológicos, patológicos, e moleculares de cinco casos fatais que apresentaram o diagnóstico de calcinose sistêmica em equinos na região sul e centro oeste do Brasil entre os períodos de janeiro de 2018 e dezembro de 2021.

Neste item é apresentado o artigo “ASPECTOS ANATOMOPATOLÓGICOS E GENÉTICOS DE CASOS FATAIS DE CALCINOSE SISTÊMICA EM EQUINOS”, redigido conforme as normas da EQUINE VETERINARY JOURNAL, a ser submetido após as contribuiçõe s da banca examinadora.

2. ARTIGO

ANATOMOPATHOLOGICAL AND GENETIC ASPECTS OF FATAL CASES OF SYSTEMIC CALCINOSIS IN HORSES

Abstract

This study aims to describe the epidemiological, pathological, and molecular aspects of systemic calcinosis with fatal outcome in horses submitted to necropsy between 2018 and 2021 in the Southern and Midwestern regions of Brazil. Five cases met the selection criteria, showing histopathological lesions compatible with systemic calcinosis. The mean age of affected horses was 15.4 months, all Quarter Horses, with an average disease evolution time of 16.8 days, and predominant clinical signs of apathy, generalized muscle swelling and stiffness. The macroscopic changes were characterized by pronounced pale areas in the skeletal muscles, which corresponded to necrotizing myositis, mineralization, and atrophy. In several organs, including lung, heart and kidney, there was an inflammatory and mineralizing manifestation. Four out of the five analyzed horses had samples submitted for molecular evaluation, which all showed a mutation for the pathological variant of the *MYH1* gene (1 homozygous and 3 heterozygous). The horses were homozygous wild type for other muscle disease (PSSM1, HM and HYPP). Three horses showed positive marking against antigens of *Streptococcus equi*, a known predisposing factor for immune disease development. The genetic findings of this study point to the involvement of the *MYH1* gene in the development of systemic calcinosis and its concurrence in physiopathology and epidemiology with immune mediated myositis, disease known to be caused by *MYH1* mutation.

Keywords: mineralization; muscle disease; *MYH1*; genetic mutation, myosin; horse; diagnosis; *Streptococcus equi.*

1. Introduction

Inflammatory myopathies represent a group of diseases with various clinical presentations and defined by the presence of muscle inflammation as a main attribute of the disease pathophysiology (DALAKAS, 2020). In horses, two classic myopathies are currently linked to an alteration of the *MYH1* gene, which encodes the heavy chain of fast-contracting skeletal muscle myosin (FINNO; SPIER; VALBERG, 2009; GIANINO et al., 2019), the nonexertional rhabdomyolysis and the immune-mediated myositis.

Systemic calcinosis is a third disease that is closely related with immune-mediated myositis, both are correlated with immune system disturbance and have similar epidemiological presentation (DURWARD-AKHURST; VALBERG, 2018; VALBERG, 2020). Although, genetic association between systemic calcinosis and *MYH1* mutation has only been only reported once (SPONSELLER et al., 2022).

Epidemiologically, the development of *MYH1* gene associated diseases are preceded by infectious processes or vaccination events, with *Streptococcus equi* being the most frequently reported agent (LEWIS; VALBERG; NIELSEN, 2007), with 47% of Quarter Horse carrying the pathological variant of the *MYH1* gene had a history of vaccination, respiratory or gastrointestinal disease in the three months prior to the development of muscle atrophy (VALBERG et al., 2022).

It is hypothesized that the morphological alteration induced by the mutation has structural similarities with pathogen epitopes, or that it is able to activate autoimmune pathways (FINNO; SPIER; VALBERG, 2009). Activation of Toll-like receptors and subsequent inflammatory response has been shown to be capable of promoting muscle and extra-muscular lesions (ZHANG et al., 2009).

The immune-mediated myositis causes atrophic muscle lesions associated with inflammation (DURWARD-AKHURST; VALBERG, 2018). The cause of immune-mediated myositis (IMM), characterized by recurrent, rapid-onset muscle atrophy in Quarter Horses (QH), is unknown. The histopathologic hallmark of IMM is a lymphocytic inflammatory infiltration in myofibers as well as autoimmune disease (FINNO et al., 2018; GIANINO et al., 2019). The prognosis of immune-mediated myositis is favorable with most animals surviving and regaining muscle after a few months (LEWIS, VALBERG, AND NIELSEN, 2007). Systemic calcinosis, unlike immune-mediated myositis, has an unfavorable prognosis and high lethality (TAN et al., 2010). Its pathogenesis is not elucidated but it is suspected to be a similar

inflammatory process to immune-mediated myositis, sharing early clinical signs and muscle atrophy development but evolving into a characteristic systemic manifestations of tissue mineralization (FINNO, SPIER, AND VALBERG, 2009).

Only two reports of horses with definitive systemic calcinosis diagnosis have been published, one with five horses with clinical signs of myopathy with mineralization identified during necropsy, the *MYH1* gene mutations was not searched for this animals (TAN et al., 2010). And another one of a homozygous horse for *MYH1* mutation, that developed muscle wasting suggestive of immune-mediated myositis but had at biopsy muscle, lung and kidney mineralization, there for receiving the diagnostic of systemic calcinosis (SPONSELLER et al., 2022).

The existence of few studies addressing systemic calcinosis, histopathology, and epidemiology together with the lack of evaluation of the genetic impacts of *MYH1* gene mutations on the lesion's development, substantiate the objective of this study. This work aims to describe the epidemiological, pathological, and molecular aspects of five fatal cases that presented the diagnosis of systemic calcinosis in horses in the South and Midwest regions of Brazil between January 2018 and December 2021.

2. Material and methods

2.1. Case selection

Post-mortem records were reviewed for all horses with a cause of death compatible with systemic calcinosis, submitted to the Service of Veterinary Pathology at the Universidade Federal do Rio Grande do Sul (SPV - UFRGS) and to the Anatomic Pathology Laboratory at the Universidade Federal of Mato Grosso do Sul (LAP - UFMS) from January 2018 to December 2021.

All information related to the animal's origin, epidemiology, clinical history, pathological and microbiological findings were analyzed, along with immunohistochemistry and genetic evaluations, to help characterize the disease and understand its pathogenesis and progression.

2.2. Sampling, histopathology, and microbiology analyses

In the retrospective review, five horses were found, three from UFRGS (Horses 1, 2, and 4) and two from UFMS (Horses 3 and 5). The macroscopic lesions in each case were photographed and described in the necropsy reports.

Microbiology evaluation was performed in two reports (Horses 2 and 4), with an attempt to isolate bacteria from lung (Horses 2 and 4), central nervous system, and lymph node tissue (Horse 4). The samples were streaked on blood agar and MacConkey agar for Gram-negative bacteria and were incubated in an aerobic environment at 37º Celsius for 72 hours with daily evaluations (QUINN; QUINN, 2011).

All the pathology findings presented in the necropsy reports were re-evaluated, characterized by location, size, and severity (mild, moderate, and accentuated). Samples of all organs, including different skeletal muscles (pelvic limbs, thoracic limbs, lumbar muscles, and diaphragm) were collected, fixed in a 10% formaldehyde solution, embedded in paraffin, and cut into 3 μm sections for histopathological examination.

When lesions compatible with necrosis, fibrosis, or tissue mineralization were found, additional histochemical staining was performed to further characterize the pathological process, and to determine the severity and extent of the lesions. Von Kossa staining was used to search for tissue mineral deposits, Masson's trichrome for collagen and Picro-Sirius for collagen fibers type I and III, Gram staining (modificated Brown-Hopps) for bacteria and periodic acid-Schiff (PAS) and Alcian Blue for acidic myxoid extracellular accumulation or, in case of PAS, intracellular glycogen degeneration.

2.3. Immunohistochemistry

After the histological analysis, tissue sections, primarily from the retropharyngeal lymph nodes and lung samples of Horses 2, 4, and 5, which showed signs of bacterial infection such as suppurative inflammation with necrosis and the presence of basophilic granular material suggestive of cocci bacteria, were subjected to anti-*Streptococcus equi* immunohistochemistry (IHC).

The IHC was performed using a non-commercial rabbit primary polyclonal antibody anti-*Streptococcus equi* at a dilution of 1:500 in phosphate-buffered saline (PBS). Antigen retrieval was performed with EDTA buffer under pressure heat of 98ºC for 40 minutes, followed by the use of MACH 4 Universal HRP-Polymer. Reactions were revealed with 3-Amino-9-Ethylcarbazole chromogen (AEC) and slides were counterstained with hematoxylin. Positive control slides from known cases of *Streptococcus equi* in horses (BIANCHI et al., 2020) were used as positive controls, while the primary antibodies were replaced by Universal Negative Control Serum (BioCare Medical, California) in randomly selected sections as negative controls.

2.4. Molecular analysis

Genetic analyses were conducted by extracting genetic material from frozen muscle tissue samples of horses 1, 2, 4, and 5. For horse 3, as no frozen tissue was available, the extraction was attempted on paraffin-embedded blocks of liver and muscle. Twenty 10-µm thick slices were taken from each block and immediately placed into sterile microtubes. The microtome blade was changed, and the equipment was cleaned to prevent DNA cross-contamination.

DNA was extracted using the QIAamp DNA FFPE tissue kit from Qiagen, following the manufacturer's instructions, excluding the xylene step. DNA purity was evaluated using the Nanodrop (Thermo Scientific) by calculating the A260:A280 and A260:A230 ratios. DNA was quantified using the Qubit (Life Technologies).

Polymerase chain reaction (PCR) was performed at Molecular Biology Laboratory of the Veterinary Clinics of the São Paulo State University/Brazil database (LBMCV-UNESP) using specific primers that were previously described (DE ALBUQUERQUE et al., 2022; DELFIOL et al., 2015; ZANZARINI DELFIOL et al., 2018) for each mutation (HyPP, PSSM1, HM and *MYH1*) as illustrated in Table 1.

The PCR reaction (25 µL) contained 2.5 µL of template DNA, 0.3 µM of each forward and reverse primer, 12.5 µL of GoTaq Green PCR Master Mix (Promega, Madison, WI, USA), and 8.5 µL of nuclease-free water. A non-template control reaction was also performed to detect possible contamination in the PCR preparation.

The obtained PCR products were analyzed using 1.5% agarose gel electrophoresis, purified, and subjected to direct Sanger sequencing. The sequencing was performed using 10 µL of purified PCR product, 5 µL of the forward primer, and the Big Dye® Terminator Cycle Sequencing Kit (Life Technologies™, CA, USA) on the ABI 3500 Genetic Analyzer (Life Technologies™ CA, USA). The obtained sequences and electropherograms were analyzed using the Geneious® software (Biomatters©, Auckland, New Zealand).

3. Results

3.1. Epidemiological and clinical findings

After analyzing the *post-mortem* records of all horses whose primary cause of death was diagnosed as systemic calcinosis. Five necropsy reports were found, three from UFRGS (horses 1, 2 and 4) and two from UFMS (horses 3 and 5). Of the three horses that were necropsied at UFRGS's pathology laboratory two came from cities in the state Rio Grande do Sul (horse 4 from

Nova Santa Rita, and horse 1 from Portão) and one came from Mato Grosso do Sul (horse 2 from Campo Grande). The horse from UFMS were one from the state of Goiás (horse 5 from Rio Verde) and the other from Mato Grosso do Sul (horse 3 from Campo Grande). The epidemiology data and the original locations of the horses are shown in Table 2 and in Figure 1.

All animals were received for necropsy between November 2019 and October 2020 and the diagnoses were made randomly during Brazil's spring and summer seasons. There were not enough cases to establish any pattern of occurrence. The age range of the affected horses varied from 11 months to 24 months, with a mean age of 15.4 months and a median and mode of 13 and 11 months, respectively. All animals were of the Quarter Horse breed, with two females and three males. The horses were in good body condition with no signs of emaciation, with horses 4 and 5 having a recent history of respiratory disease, and no information regarding their vaccination status was obtained.

The severity and progression of the disease varied among the animals, with a generally short time from onset to death. The average duration of the clinical progression was 16.8 days with a median of 18 days, lasting between 6 to 30 days. Apathy was a common symptom in all horses, while locomotor signs such as stiffness, difficulty in movement, and claudication were observed in 3 out of the 5 horses (1, 2, and 5). Intense and generalized muscle swelling was observed in cases 1 and 4. Out of the 5 animals, 4 rapidly developed sustained lateral recumbency, except for horse 1, which had the fastest progression (6 days).

Fever was recorded in two horses (numbers 3 and 4), and respiratory clinical signs, such as nasal discharge, were observed in horses 3 and 5. Polyuria and dark urine were seen in horses 4 and 1, respectively. The clinical signs of all five horses worsened progressively despite treatment, leading to their eventual death.

Regarding treatment, the necropsy reports of 3 out of the 5 horses provided information on the clinical management of the affected animals. Horse 2 was initially diagnosed with tetanus and treated with antitetanic serum and an undisclosed antibiotic. Horses 3 and 4 were treated with penicillin, gentamicin and ceftiofur antibiotic therapy combined with corticosteroids such as dexamethasone and prednisolone, respectively. All horses also received supportive therapy, but their conditions did not improve, and the disease eventually led to their death.

3.2. Pathological findings

3.2.1. Gross lesions

All five horses presented macroscopic lesions during necropsy, as indicated in Table 3,

affecting multiple organ systems with distinct morphologies and severity levels, depending on the duration of the disease progression.

The skeletal muscle was the most affected system in terms of consistency and intensity. Out of the 5 horses, 4 had noticeable edema in their limbs, primarily in the posterior region, which was characterized by the presence of yellowish gelatinous material in the subcutaneous tissue (Figure 2A) and interwoven with the muscle and was closely related to areas of hemorrhage.

The musculature of the gluteal, lumbar, pelvic, thoracic, and cervical regions, as well as the limb muscles, presented pale areas in all horses (Figure 2B and 2C). Three out of five cases (horses 2, 4, and 5) had discernible macroscopic mineralization, with whitegrey granular deposits that squeaked when cut and were intermixed with muscle tissue (Figure 2D). Areas of damaged muscle were usually marginated by a well-defined white strip. Unfortunately, due to a lack of accurate descriptions in the necropsy reports, it is not possible to evaluate the involvement of each muscle individually.

Pulmonary involvement, characterized by inflammatory infiltration, was noted in 4 out of 5 equines (horses 2, 3, 4, and 5). The affected lungs demonstrated marked areas of consolidation, evidenced by dark red atelectatic lobules and increased stiffness of the lung parenchyma, in addition to edema and fluid exudation. Equine 4 exhibited, in association to pulmonary lesions, abscessation of the retropharyngeal lymph nodes with accentuated cavitation formation filled with friable yellowish purulent material. Equines 2 and 3 displayed macroscopic signs of mineralization in their lungs, which were non-collapsible, rigid, hypocrepitant with multifocal areas of pale discoloration that crackled upon incision (Figure 3A).

Severe mineralization, similar to the lesions observed in the skeletal muscle tissue, was also noted in 2 out of 5 cases (horses 2 and 3) on their heart, with multifocal areas of an irregular deposition of a white granular material primarily on the myocardium, endocardium (Figure 3B). Alongside the mineralization, well-circumscribed pale areas were present in all horses regardless of measurable macroscopic mineralization.

The kidneys of 3 out of 5 cases (horses 3, 4, and 5) showed depressed, wedge-shaped, pale areas of infarction with varied extension. All horses, except horse 5, had multiple, white, irregular stripes diffusely distributed on the cortex and renal medulla, suggesting mineralization of the kidney (Figure 3C). Additionally, horse 3 had evident mineralization in urinary bladder wall, which had a bulging that was diffusely white and hardened when cut.

Oral cavity ulceration was observed in 2 out of 5 animals (horses 3 and 5), characterized by circular, depressed regions of loss of mucosal tissue with deposition of a yellow, diphtheritic

membrane in association with a halo of hyperemia. The ulcers were multiple, located near the teeth, inside the cheeks, and under the tongue. In horses 2, 4 and 5, ulcers were found in the stomach mainly in the aglandular region, with deposition of greenish material and evidence of hemorrhage, with horse 2 exhibiting the most severe presentation with accentuated diffused white striped thickening of the stomach wall, with deep extensive areas of ulceration (Figure 3D).

3.2.2. Histological lesions

The microscopic examination of the muscle tissue in five animals revealed lesions of variable severity and presentation. All animals had some degree of muscle compromise, showing different stages of necrosis and regeneration of the muscular fibers, varying in relation to its chronicity and macroscopic presentation.

Animal 1, which died the quickest, had predominantly necrotic myofibers with caryolysis, loss of sarcoplasmic stippling, and intense cytoplasmatic hyalinization (Figure 4A). This led to progressive derangement of the polygonal cell structure and myotubular conformation, characterized by cell flocculation and vacuolization, some muscle fiber showed punctuated intracytoplasmic basophilic granules of mineral accumulation, readily observed on Von Kossa staining. Despite the acute lesion, early signs of regeneration were observed, including intense infiltration of necrotic tissue by cleaning macrophages, discrete satellite cell migration and proliferation. There was also a secondary inflammatory component present, with lymphocytes marginating around blood vessels and near muscle cells. Few neutrophils intermixed with the necrotic areas were also noted.

The other 4 out of 5 animals (horses 2, 3, 4, and 5) exhibited lesions consistent with a chronic evolution, with substantial replacement of necrotic contractile cells with fibrosis and mature collagen deposition (Figure 4B), evident on Masson's trichrome and Picro-Sirius stains.

Horse 3 still had large areas of necrotic tissue and a vascular component to the microscopic presentation, characterized by accentuated fibrin exudation and hemorrhage. The inflammation was milder in these four animals compared to horse 1. Significant signs of polyphasic regeneration were seen in 3 out of 5 cases (horses 3, 4, and 5), with some muscle cells infiltrated with tissue-cleaning macrophages and intense satellite cell mitosis forming cells with multiple nuclei aligned in the center of the fiber, with little myofibril formation and loss of orientation. Animals 3, 4 and 5 showed accentuated atrophy of muscle fibers with severe reduction in its size, affecting areas with or without necrosis, fibrosis, or inflammation.

Accentuated mineralization of the muscle tissue was seen in 3 out of 5 animals (horses 2, 3, and 4), with deposition of intense basophilic granular material in the cytoplasm of muscle fibers (Figure 4C) that was positive on Von Kossa stain (Figure 4D). In contrast to horse one that had only granular mineral formation, these three animals showed entire cells mineralized. In horses 2 and 4, the deposition was severe and diffuse, while in animal 3 it was more meager. No animal showed signs of extracellular myxoid accumulation or glycogen degeneration on PAS or Alcian Blue staining.

Spots of mineralization were seen in the heart of 2 out of 5 animals, with extensive compromise of the myocardium in horse 3, with areas of associated fibrosis, and with a more discrete mineral deposition in horse 4, accompanied by areas of necrotic cardiomyocytes. Although lacking mineralization, myocardium necrosis was an important aspect of horse 5.

Similar mineralization was present in the kidney of 4 out of 5 animals (horses 2, 3, 4, and 5), with moderate tubular mineralization affecting random areas of the parenchyma in all of them. However, horse 3 had a significantly more severe process, extending diffusely and compromising the glomeruli (Figure 5A). This same horse also had mineral deposits in the urinary vesicle wall, accompanied by discrete neutrophil inflammation and severe bladder wall fibrosis.

Necrosis was seen in the kidneys of four out of five horses, being discrete in horse 2 and moderate in horses 1, 3, and 4. Horse 3 also had a kidney infarction area with well-delineated, extensive cell loss. The coagulative necrosis was closely associated with mineralization, except in horse 1, which had moderate multifocal necrosis with no discernible mineralization but had intense intratubular protein accumulation and thrombosis of the glomeruli capillary network. This same horse also had the adrenal gland with diffuse hemorrhage, suggesting shock as a component of the outcome. No relevant presentation of kidney inflammation was seen in any of the horses.

The gastrointestinal system had, in 3 out of 5 animals (horses 2, 3, and 4), mineralization of the stomach wall extending throughout the mucosa, with the most severe compromise being on the isthmus and column of the stomach glands in horses 2 and 3. The mineralization process was also seen in the intestine wall of horses 2 and 4. Horse 5 also had stomach ulceration with loss of lining epithelium and exposition of submucosal tissue (Figure 5B).

In the lung examination, edema and severe congestion were present in all 5 horses, with 3 out of 5 animals (horses 1, 3 and 5) having moderate and multifocal areas of quantifiable hemorrhage. Fibrin exudation was seen in 4 out of 5 cases (horses 2, 3, 4, and 5), with horses 2 and 4 having histological alterations of thrombosis with fibrinoid necrosis of the vascular wall affecting large portions of the parenchyma.

Mineralization was also evident in horses 2, 3, and 5, affecting the alveoli septum and bronchiolar smooth muscle wall (Figure 5C). The deposition was usually associated with discrete neutrophil influx to the tissue. Horses 3 and 5 showed signs of chronic lung tissue damage with accentuated multifocal and coalescing fibrosis and mineralization (Figure 5D), loss of viable alveolar lumen, marked proliferation of type two pneumocytes, and syncytia formation in lining epithelial cells.

Aside from horse 1, all horses had discernible infiltration of inflammatory cells, with predominantly mononuclear appearance except for horses 4 and 5, which also had extensive neutrophilic inflammation. Horse 4 and 5 manifested areas of suppurative bronchopneumonia (Figure 5E) with horse 4 also showing retropharyngeal lymph nodes with discernible Gram-positive coccoid bacteria, an associated suppurative inflammation, and extensive tissue necrosis. Other organs exhibit less pronounced lesions, which are summarized in tables 4 through 7.

3.3. Microbiological and Immunohistochemistry Evaluation

Microbiological evaluation was mentioned to have been performed in only two cases (horses 2 and 4), but all bacterial growth was classified as contamination regardless of the tissue sample or streaking method used. As a result, no significant findings were obtained.

At the IHC evaluation, positive immunostaining for *Streptococcus equi* antigen was observed in three animals (horses 2, 4, and 5), with the intensity of the immunohistochemical staining varying from discreet (in horse 5) to accentuated (in horses 2 and 4).The labeling was seen freely in necrotic areas (Figure 5F) and inside the cytoplasm of macrophages in the pulmonary lesions of horses 2, 4, and 5, in the retropharyngeal lymph node of animal 4 near regions of suppuration, the same animal also showed positive staining inside the blood vessels of the skeletal muscle and the central nervous system.

3.4. Molecular findings

Biological samples from all 5 animals were submitted for molecular analysis. The analysis was possible in 4 out of 5 animals; however, one horse (number 3), whose DNA extraction was performed on formalinized tissue blocks, did not yield any viable amplification of target genes.

All 4 cases showed positive PCR results for the *MYH1* gene, which encodes the myosin heavy chain protein. Only horse 2 was homozygous (*M*y/*M*y) for the mutated variant, while horses 1, 4 and 5 presented a heterozygous genotype (*M*y/N). None of the horses had any

mutations in the PSSM1, MH, or MYPP genes, expressing only the wild type (WT), which is not correlated with disease development. Results are shown in table 8.

4. Discussion

The histopathological finding of muscle necrosis and mineralization of muscle and other tissues in this case series are suggestive of systemic calcinosis, a poorly described disease that is closely associated with the common form of immune-mediated myositis with a similar initial clinical presentation and inflammatory reaction, but with a progressive worsening systemic disturbance, and a fatal outcome (VALBERG, 2020).

In this report there were five horses, two females and three males. A previous study failed to show that the animal's sex was a statistically relevant factor in the development of the immune-mediated myositis (Lewis et al., 2007). Horses diagnosed with systemic calcinosis in literature were three males out of five horses (TAN et al., 2010), another report was also of a male horse (SPONSELLER et al., 2022).

In a series of five cases of horses diagnosed with systemic calcinosis, all were younger than 9 years old, with a mean age of 3.58 years, three were Quarter horses, and two were Paint horses. All animals showed initial signs of myopathy that instead of improving became more intense, and all were euthanized up to 30 days from the start of symptoms (TAN et al., 2010). The other reported horse was a 9 years old Quarter horse (SPONSELLER et al., 2022). In this report, although all animals were Quarter horses, there for confirming the predisposition of the race to develop systemic calcinosis and immune-mediated myositis (DURWARD-AKHURST; VALBERG, 2018), the age distribution was slightly different, with younger animals being affected, with a mean age of 15.4 months, also been inside the most affect age range reported for immune-mediated myositis, beneath 8 years old or above 17 years (DURWARD-AKHURST; VALBERG, 2018). The onset of the disease in younger animals can be associated with the codominant aspect of mutation of the *MYH1* gene, which was observed in most of the animals in the present study (FINNO et al., 2018).

Previous genomic studies have shown a high prevalence of mutations in genes related to muscle function and physiology in horses diagnosed with muscle disease, with values reaching 47% (ALEMAN et al., 2022). The same study also showed that Quarter horses were at an

increased risk, belonging to this breed 90% of the animals with genetic mutations associated with muscle diseases.

This current study found, through molecular analysis, that four out of five horses had genetic mutations in the *MYH1* gene. Horse 3 was not evaluated due to technical limitations. The wild type of variant was the only present in the evaluation of the PSSM1, HM, and HYPP associated genes. Published reports of genetic evaluation of animals with systemic calcinosis were negative for polysaccharide storage myopathy (PSSM1) and malignant hyperthermia mutation (MH) (TAN et al., 2010), with only one out six animals been tested and positive for *MYH1*mutation (SPONSELLER et al., 2022). The presence of *MYH1*alterations and the absence of other majors' mutations associated with muscle disease is an indicative that the gene responsible for codify the heavy chain of the muscle fibers can be involved in the pathogenies of, not only immune-mediated myositis and nonexertional rhabdomyolysis, but also with systemic calcinosis.

The *MYH1* gene encodes the myosin heavy chain, a protein associated with muscle contraction in fast-contracting 2X muscle fibers. Its pathological form has variable penetrance and is inherited in an autosomal codominant manner, with homozygosity being related to more intense manifestation (VALBERG, 2020). The missense E321G mutation predisposes to a morphological alteration in the myosin globular head by changing a glutamic acid (E) amino acid to a glycine (G) at a site related to ATP interaction (FINNO et al., 2018a).

The pathophysiology of the *MYH1* mutation-associated myopathies is complex and not fully understood, but several studies have shown a correlation between a history of previous infection by *Streptococcus equi*, other infections, or vaccination as a trigger for lesion development. Previous descriptions reported that 39% of horses with immune-mediated myositis had a history of recent infection or vaccination, mainly for influenza, herpesvirus-1, or *Streptococcus equi*. The animals developed the muscular disease three to four weeks after antigen stimulation (FINNO et al., 2018b), a claim supported by other studies (ALEMAN et al., 2022). As for systemic calcinosis, from all literature cases researched, five out six horses presented a previous history of respiratory or bacterial infections, with one report of immune modulation therapy for sarcoid anteceding the disease also being mentioned (SPONSELLER et al., 2022; TAN et al., 2010).

In this case series, three out of five horses (60%), had positive immunolabeling for *Streptococcus equi* antigen and showed histopathologic alterations of suppurative inflammation and bronchopneumonia, with two animals presenting a history of respiratory disease or strangle. One animal also had lymphadenitis with abscess formation and Gram-positive bacteria. The

relationship between *Streptococcus* sp. infections and immune-mediated disorders is well established in horses. Infarctive purpura hemorrhagica is an immune-mediated type III hypersensitivity that can develop after a streptococcal or other bacterial infection (PUSTERLA et al., 2003). The immune complexes formed during the chronic infection deposit on the vascular wall, inducing systemic vasculitis and thrombus formation (MALLICOTE, 2015). In humans, rheumatic fever represents another mechanism of immune disease development through interaction with streptococcal antigens. Bacterial proteins, such as M protein, can induce the formation of antibodies that can induce a humoral response against host proteins, especially against cardiac muscle tissue, inducing immune-mediated injury (ARVIND; RAMAKRISHNAN, 2020).

In immune-mediated myositis, it is postulated that an initial aggression, such as a *Streptococcus equi* infection or direct tissue lesion, leads to muscle cell destruction and liberation of cytoplasmic proteins. The mutant form of myosin heavy chain then induces activation of the immune system by interacting with Toll-like receptors or by sharing similarities with previous sensitized antigens, inducing an adaptive inflammatory response, mainly Th1 and Th2, producing chronic tissue damage and mononuclear inflammatory cell infiltration that mediates tissue destruction (VALBERG, 2020; ZHANG et al., 2009). No IgG production targeting autoantigens were detected in horses with immune-mediated myositis (LEWIS; VALBERG; NIELSEN, 2007).

The inflammation can lead to high production of cytokines that induce muscle catabolism (DURWARD-AKHURST; VALBERG, 2018), and promote the abnormal sarcolemma expression of major histocompatibility complex (MHC) class I and class II, usually absent in normal muscle cells. This contributes to inducing immune cell damage by presenting autoantigens to inflammatory cells inducing muscle tissue destruction and fiber atrophy (DURWARD-AKHURST et al., 2016). While not well described, the pathogenesis of the early muscle lesion of systemic calcinosis is thought to be similar of the one found in immune-mediated myositis (SPONSELLER et al., 2022; VALBERG, 2020).

The histopathological presentation had a remarkable association with the time of evolution of the disease. Animal 1, which had the shortest period, with only six days between the start of the clinical signs and death, manifested acute lesions with predomination of intense necrosis of myofibers with lymphocytic perivascular and peri-muscular inflammation, hallmarks of immune-mediated myopathy in association with spots of mineralization that characterize systemic calcinosis (DURWARD-AKHURST et al., 2016). Tissue cleaning macrophages were present, but regeneration was minimal.

Kidney necrosis was also found in the same animal, with areas of thrombosis and presence of eosinophilic protein cylinders inside the tubules. The renal injury is closely associated with muscle destruction; rhabdomyolysis promotes the liberation of myoglobin that is filtered in the urine, promoting its accumulation of the heme pigment that is visible on light microscopy (FOGO et al., 2014). The clinician also reported that the animal showed episodes of dark-red urine, probably associated with these phenomena.

The renal necrosis can be induced by myoglobin tubular toxicity through oxidative damage. The loss of fluid promoted by the edema formation on limbs, in the subcutaneous tissue, and in intra-alveolar spaces, as seen in horse 1, helps activate the renin-angiotensin system and leads to sympathetic system up-regulation, reducing renal blood flow, allowing ischemia to ensue, and further intensifying the edema formation (BOSCH, POCH, & GRAU, 2009).

The vascular disturbance and loss of fluid also promote hemoconcentration in association with immune mediators inducing a prothrombotic state (HOWIE, 2020). These events can be responsible for the thrombus and infarction seen in the kidneys of animals 1 and 3 and in the lungs of animals 2 and 4. In systemic calcinosis with secondary renal failure with disturbance in electrolyte and circulatory balance probably resulted in circulatory shock, with a loss of fluid from circulation to adjacent tissues (HOWIE, 2020; MUNDAY, 2017).

The other four cases, had a more prolonged evolution than horse 1, varying from 10 to 30 days of clinical course before death, with a mean of 19.5 days and a median of 19 days. Clinical progression may be associated with disease severity, development of permanent lateral recumbency and treatment. The My/My genotype did not manifested substantial difference in clinical evolution and the only survived animal reported in literature was homozygous (SPONSELLER et al., 2022).This longer evolution resulted in a substantial difference in the pathological manifestation of the disease. All these four horses had chronic muscle lesions characterized by fibrosis and regeneration instead of necrosis, and they all also had severe mineral deposition in various organs.

At histopathology, all animals presented a mononuclear inflammatory component, with severe fibrosis present in four out of five animals, three horses had substantial polyphasic regeneration of muscle cells. The muscle fibrosis usually happens in lesions that myotubular structure is compromised, there for removing the guide for satellite cell migration, polyphasic regeneration is also an important indicative of the disease pathogenesis because, for its manifestation is necessary repeated injuries with cycles of necrosis and regeneration, excluding single aggressor events (GASPAR; VASISHTA; RADOTRA, 2019; MUNDAY, 2017).

Pulmonary lesions of inflammatory neutrophilic infiltrate, type 2 pneumocytes

hyperplasia and fibrosis are compatible with chronic injuries induced by mineralization. (HONDA et al., 2003). Similar processes of neutrophil influx and fibrosis associated with mineralization were seen in other organs, such as the urinary bladder and the kidney.

Histological characterization of tissue mineralization described in the present study is consistent with prior descriptions of systemic calcinosis with the majority of animals showing mineral deposits in the lung and kidney with skeletal muscle and heart following (SPONSELLER et al., 2022; TAN et al., 2010). A detailed comparison between literature reports and the cases of systemic calcinosis in this article is provided in Table 9.

Mineralization is the distinctive hallmark that allow to differentiate between systemic calcinosis and immune-mediated myositis, because necrosis, muscle atrophy and the mononuclear inflammatory component are shared between the diseases, suggesting an initial common physiopathology (SPONSELLER et al., 2022; VALBERG, 2020).In humans mineralization is associated with kidney dysfunction, the synthesis of vitamin D and the metabolism of calcium is jeopardized, resulting in secondary hyperparathyroidism, which mobilizes calcium reabsorption from bones, increasing its circulating levels and promoting its deposition on dermal tissue (GALLO MARIN et al., 2023).

Metastatic calcification shares the same mechanism and is also associated with chronic renal failure and secondary hyperparathyroidism. The uremic state induced by renal insufficiency can also alter tissue proteins, making them more susceptible to calcification, and the reduced phosphate glomerular excretion promotes its deposition in association with calcium (BELÉM et al., 2014).

Although in this case series all animals had some kind of renal disturbance this is probably not a determinant to tissue calcification. In horses, the impact of chronic renal failure on calcium metabolism is different. Secondary hyperparathyroidism is not common because horses absorb high concentrations of dietary calcium that are later filtered and eliminated in the urine. When glomerular excretion is reduced, calcium levels can rise due to less excretion (TORIBIO, 2011), while parathyroid hormone levels remain unchanged (BROBST et al., 1982).

Tissue mineralization due to high levels of calcium derived from renal failure is poorly reported in horses (SCHOTT, 2007). In addition, during kidney disease, it is more common for horses to develop hypophosphatemia instead of hyperphosphatemia that is seen in other species (SCHOTT, 2007).

A known cause of soft tissue mineralization in horses is hypervitaminosis D, due to ingestion of plants containing the active form of vitamin D, such as *Solanum glaucophyllum*, *Nierembergia veitchii1* and *Nierembergia rivularis*, or even by iatrogenic administration

(ODRIOZOLA et al., 2018). Intoxication leads to increased calcium intestinal absorption and subsequent tissue deposition. None of horses in this analysis had any history of contact with sources of vitamin D toxicity, and all animals were from different regions of the country.

Since renal failure, toxicity, other diseases can be excluded as pathophysiological origin for the mineralization found in horses with systemic calcinosis, some have proposed inflammation as a mechanism that can lead to mineral deposition.

Early inflammatory events induced by a trigger or resembling those described for immune-mediated myositis can induce systemic calcinosis (SPONSELLER et al., 2022). The production of inflammatory mediators can promote bone resorption and induce osteoclast formation, up regulate its activity, leading to abnormal serum levels of calcium and its tissue deposition (VALBERG, 2020). This pathway is illustrated by the interaction between TNF-alpha and RANKL (the ligand for the receptor activator of NF-kappaB), which can induce osteoclastogenesis (LAM et al., 2000), smooth muscle cells transformation into an osteogenic phenotype can also be presuppose (VALBERG, 2020).

Although the origin of the mineralization in systemic calcinosis is not clearly understood, its effects on tissue histopathology are. The deposition of minerals usually occurs in sites that secrete hydrogen ions, producing an alkaline environment that reduces calcium solubility and predisposes it to precipitation (BELÉM et al., 2014).

This process is illustrated in this case series by the pronounced mineralization of the kidney, stomach, and lung, all sites of ion exchange. The lung, being a site of CO2 removal, has a more alkaline microenvironment that facilitates calcium deposition (CHAN et al., 2002), and the same can be said of the kidney, which loses hydrogen ions in the urine (MCTAVISH; SHARMA, 2018).

In two out of three horses that had calcification on the gastric wall, there was more affected area of mineralization comprised by the isthmus and column of the stomach glands. This can be explained by the presence of HCl producing cells that secrete cations to stomach lumen and increase the pH of the area (ZACHARY, 2017).

The reduction of renal function, either from direct organ damage or from reduced circulating volume, may explain the ulceration found on the stomach in horses 2, 4, and 5, and on the oral cavity in horses 3 and 5. With declining renal excretion, urea concentration increases and bacterial present in the oral cavity convert urea into ammonia. Both compounds can cause tissue damage and lead to ulceration (ZACHARY, 2017). Reduced elimination of gastrin in urine, combined with an active sympathetic system due to fluid loss, exacerbates gastric acid production and contributes to ulcer formation (SCHOTT, 2007).

5. Conclusion

For conclusion, in horses, systemic calcinosis is a poorly described disease that is closely associated in its epidemiology and clinical manifestation with the immune-mediated myositis, but usually with a fatal outcome usually affect young horses, with mean age of reported in this article of 15.4 years, varying from 11 months to 24 months. The evolution time is seen to be closely correlated with the manifestation of the disease. Four out five animals in this report showed muscle lesions with signs of a chronic response, which were absent in horse 1 which had a faster evolution. All four horses, that were analyzed, had mutations in the *MYH1* gene which suggested that the gene is evolved in disease development, with three horses having an associated trigger Streptococcus infection. Additionally, all animals presented inflammatory lesions similar to those seen in immune-mediated myositis, which supports the probable relationship between systemic calcinosis and immune-mediated myositis pathophysiology also correlated with *MYH1* mutation.

Conflict of interest

The authors declare no existence of conflict of interest regarding the research, authorship, and publication of this article.

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Table 1: PCR and sequencing primer sets used in this study for equine muscle genetic diseases.

Primer sets	Primer sequences	Product (bp[1])	Melting
HyPP_Forward[2]	5'- ACGAAGCAGGTGTTCGACAT -3'	441	59 ºC
HyPP_Reverse[2]	5'- ATTCACGTGTGTGCAGGCAA -3'		
PSSM1_Forward[3]	5'- AAGTGAAACATGGGACCTTCTCCC -3'	279	59 ºC
PSSM1_Reverse[3]	5'- ACTCAGCCATTGTTCTGACGC -3'		
HM_Forward[3]	5'- AGCTGGGCGTCCTAGAGTTA-3'	208	72 ºC
HM_Reverse[3]	5'-ATCTGCAGAGGGAGGCTGATGAT-3'		
MYH1_Forward[4]	5'- CCAGCTAAAGGCGGAAAGAA -3'	713	62 ºC
MYH1_Reverse[4]	5'- GGGCAGAGTAGGAGTGAGTAA -3'		

[1]Base pairs, Primers previously described by [2]Delfiol et al., 2015; [3]Delfiol et al., 2018; [4]Albuquerque et al. 2021.

Table 2: Epidemiological data on cases of equine systemic calcinosis in Brazil.

Animal	1	2	3	4	5
Age (in months)	13	11	24	11	18
Gender	Female	Female	Male	Male	Male
Race	Quarter Horse	Quarter Horse	Quarter Horse	Quarter Horse	Quarter Horse
Origem	Portão, RS	Campo Grande, MS	Campo Grande, MS	Nova Santa Rita, RS	Rio Verde, GO
Clinical evolution time (in days)*	6	10	18	20	30
Clinical signs**	apathy, generalized muscle swelling, stiffness and dark urine	apathy, stiffness, sustained decubitus	apathy, fever, nasal discharge, susteined decubitus	apathy, fever, polyuria, sustained decubitus, generalized muscle swelling	apathy, stiffness, sustained decubitus, nasal discharge
Comorbidities	-	Streptococcus infection	-	Streptococcus infection	Streptococcus infection

*Interval between onset of symptoms and death

** As reported by the veterinary clinician

Table 3: Anatomical distribution and severity of macroscopic lesions in the most affected organs of horses with systemic calcinosis.

Organ system and lesions	Animal				
	1	2	3	4	5
General evaluation					
Body condition score	4/5	4/5	3/5	4/5	4/5
Pale mucous	+ + +	\	+ + +	+ +	+ +
Hydrothorax	\	\	\	+	\
Hydroperitoneum	\	\	+	+ + +	\
Subcutaneous edema of limbs	+ +	\	+ +	+ + +	+
Musculoskeletal system					
Pale areas	+ + +	+ +	+ + +	+ + +	+ +
hemorrhage	+	\	+ +	+	+
Macroscopic mineralization signs	\	+ + +	+ + +	+ + +	\
Lungs					
Pulmonary edema	+ +	+	+ +	+ + +	+
Areas of lung consolidation	\	+ +	+ + +	+	+
Macroscopic mineralization signs	\	+ + +	+ + +	\	\
Heart					
Myocardium pale areas	+ +	+	+ + +	+ +	\
Macroscopic mineralization signs	\	+ + +	+ + +	\	\
Hydropericardium	\	\	+	+ +	+
hemorrhage	+	\	\	\	\
Urinary system					
Kidneys Infarct	\	\	+ +	+	+
Pale areas on Kidneys	\	\	+ +	+ + +	\
Kidneys, macroscopic mineralization	+ +	+	+ + +	+ + +	\
Bladder, macroscopic mineralization	\	\	+ + +	\	\
Gastrointestinal system					
Oral cavity, ulceration	\	\	+ + +	\	+ +
Stomach, ulceration	\	+ + +	\	+	+
Intestinal hemorrhage	\	\	+	+	\
Hepatic					
Congestion	+ +	\	\	\	\
Lymphopoietic system					
lymph node, suppurative exsudate	\	\	\	+ + +	\

Lesion severity was classified as absent (\), mild (+), moderate (++) and marked (+++).

Table 4: Summary of the main histopathological lesions in musculoskeletal tissue in cases of systemic calcinosis.

Musculoskeletal tissue lesions	Animal				
	1	2	3	4	5
Mineralization	+	+ + +	++	+ + +	\
Necrosis	+ + +	+	+ + +	+	+
Polymorphonuclear infiltrate	+	\	\	\	\
Mononuclear infiltrate	+ + +	+	+	+ +	+ +
Fibrin exudation	\	\	+ + +	\	+
Fibrosis	\	+ +	+ + +	+ + +	+ + +
Hemorrhage	\	\	+ + +	+	+ + +
Vacuolation of muscle fibers	+	\	\	\	+ + +
Muscle atrophy	\	+	+ + +	+ + +	+ + +
Regeneration	+	\	+ + +	+ + +	+ + +

Lesion severity was classified as absent (\), mild (+), moderate (++) and marked (+++).

Table 5: Summary of the main histopathological lesions in the most affected organs in cases of systemic calcinosis.

Organ system and lesions	Animal				
	1	2	3	4	5
Lungs					
Mineralization	\	+ +	+ + +	\	+ + +
Fibrinoid vascular necrosis	\	+ + +	\	+ +	\
Thrombosis	\	+ + +	\	+ +	\
Edema	+ + +	+ +	+ +	+	+ +
Fibrin exudation	\	+ + +	+ +	+ +	+ +
Congestion	+ + +	+ + +	+ +	+ + +	+ +
Hemorrhage	+ +	\	+ +	\	+ +
Polymorphonuclear infiltrate	\	+ +	+	+ +	+ + +
Mononuclear infiltrate	+	+ +	+	+ +	+
Fibrosis	\	\	+ + +	\	+ + +
Proliferation of type 2 pneumocytes	\	\	+ +	\	+ +
Heart					
Mineralization	\	\	+ + +	+	\
Necrosis	\	\	\	+	+ +
Fibrosis	\	\	+ +	\	\
Kidney					
Tubular mineralization	\	+ +	+ + +	+ +	+
Vascular mineralization	\	\	+ +	\	\
Necrosis	+ +	+	+ +	+ +	\
Polymorphonuclear infiltrate	\	\	\	+	\
Mononuclear infiltrate	\	\	+	+	\
Tubular protein cylinders	+ + +	\	+	\	\
Thrombosis and infarction	+ + +	\	+ + +	\	\

Lesion severity was classified as absent (\), mild (+), moderate (++) and marked (+++).

Table 6: Summary of the histopathological lesions in the secondary affected organs in cases of systemic calcinosis.

Organ system and lesions	Animal				
	1	2	3	4	5
Urinary bladder					
Mineralization	\	\	+ + +	\	\
Polymorphonuclear infiltrate	\	\	+	\	\
Fibrosis	\	\	+ + +	\	\
Stomach					
Mineralization	\	+ + +	+ + +	+	\
Ulceration	\	+ + +	\	+	+
Fibrin exudation	\	+ + +	\	\	\
Intestine					
Mineralization	\	+ +	\	+	\
Necrosis	\	+ + +	\	\	\
Hemorrhage	\	\	\	\	\
Mononuclear infiltrate	\	+ +	\	\	\
Lymph node					
Necrosis	\	\	\	+ + +	\
Edema	\	+ + +	\	+	\
Thrombosis	\	+ + +	\	\	\
Polymorphonuclear infiltrate	\	\	\	+ + +	\
Coccoid bacteria	\	\	\	+ + +	\
Central nervous system					
Vacuolation	\	+	\	+	\
Edema	\	+	\	\	\
Hemorrhage	\	\	\	\	+ +
Adrenal gland					
Hemorrhage	+ + +	\	\	\	\

Lesion severity was classified as absent (\), mild (+), moderate (++) and marked (+++).

Table 7: Microscopic mineralization evaluation in cases of systemic calcinosis.

Organ system	Animal				
	1	2	3	4	5
Skeletal muscle tissue	+	+ + +	++	+ + +	\
Von Kossa staining on muscle	Positive	Positive	Positive	Positive	\
Lungs	\	+ +	+ + +	\	+ + +
Heart	\	\	+ + +	+	\
Kidney	\	+ +	+ + +	+ +	+
Stomach	\	+ + +	+ + +	+	\
Intestine	\	+ +	\	+	\
Urinary bladder	\	\	+ + +	\	\

Lesion severity was classified as absent (\), mild (+), moderate (++) and marked (+++).

Table 8: Genetic evalutation of horses with systemic calcinosis.

Genetic variant	Protein affected	Animal				
		1	2	3*	4	5
MYHM	Myosin heavy chains	*My/N*	*My/My*	\	*My/N*	*My/N*
PSSM1	Glycogen synthase 1	WT	WT	\	WT	WT
HM	Ryanodine receptor 1	WT	WT	\	WT	WT
HYPP	Sodium channel	WT	WT	\	WT	WT

*Genetic analysis could not be performed (\)

Table 9: Comparison between cases of systemic calcinosis in the literature and those presented in this article.

Animal	Race	Age	Evolution till death	Sex	Comorbidities	Mineralized organs	Outcome	Genetic mutation
Present article								
1	Quarter Horse	13 months	< 15 days	F	\	Muscle	Natural Death	*My/N*
2	Quarter Horse	11 months	< 15 days	F	Streptococcus infection	Muscle, Lung, Kidney, Stomach, Intestine	Natural Death	*My/My*
3	Quarter Horse	24 months	< 15 days	M	\	Muscle, Lung, Heart, Kidney, Stomach, Bladder	Natural Death	\
4	Quarter Horse	11 months	15-30 days	M	Streptococcus infection	Muscle, Heart, Kidney, Stomach, Intestine	Natural Death	*My/N*
5	Quarter Horse	18 months	15-30 days	M	Streptococcus infection	Lung, Kidney	Natural Death	*My/N*
SPONSELLER et al., 2022								
1	Quarter Horse	9 years	\	M	\	Muscle, lungs and kidney *1	Survived	*My/My*
TAN et al., 2010 *2								
1	Paint Horse	6 years	15-30 days	M	Anaplamosis	Muscle, Lung, Kidney	Euthanized	N/ MH and GYS1
2	Quarter Horse	14 months	15-30 days	M	Salmonelosis	Muscle, Heart, Lung, Kidney	Euthanized	N/ MH and GYS2
3	Paint Horse	8 months	< 15 days	M	Respiratory disease	Heart, Lung, Liver, Kidney	Euthanized	N/ MH and GYS3
4	Quarter Horse	9 years	< 15 days	M	Respiratory disease, use of immunomodulator	Heart, Large vasels, Lung, Intestine, Kidney	Euthanized	N/ MH and GYS4
5	Quarter Horse	12 months	< 15 days	F	Respiratory disease	Kidney	Euthanized	N/ MH and GYS5
FALES-WILLIAMS; SPONSELLER; FLAHERTY *3								
1	Paint Horse	6 years	> 30 days	M	\	Thoracic arteries	Euthanized	\

*1: Assessed by biopsy.

*2: All animals were negative for mutations in the MH and GYS1 genes, the only ones evaluated.

*3: The animal did not receive a diagnosis of systemic calcinosis by the authors and presented distinct lesions from the other horses.

CONCLUSÕES

Entre os anos de 2018 e 2021, cinco casos compatíveis com calcinose sistêmica foram diagnosticados. A calcinose sistêmica é uma miopatia de base inflamatória caracterizada pela mineralização tecidual em diversos órgãos associada a necrose, inflamação e atrofia muscular, a doença apresenta semelhanças epidemiológicas, clínicas e em sua patogenia com a miosite imunomediada, que por sua vez é causada por mutação dominante no gene *MYH1*.

Doenças musculares de origem genética representam grande importância devido aos processos de seleção em que rebanhos estão induzidos, fixando genes patológicos antes desconhecidos, animais da raça Quarto de milha estão sobre representados na casuística de doenças musculares imunomediadas de origem genética.

A idade média dos animais afetado pela calcinose sistêmica foi de 15,4 anos, variando de 11 a 24 meses, e três dos cinco animais apresentaram infecção por *Streptococcus equi*, reforçando a relação existente entre estimulação antigênica e desenvolvimento da doença.

As lesões na musculatura variaram de necróticas e inflamatórias a alterações de fibrose e mineralização, distúrbios vasculares também foram notados em todos os animais, com formação de edema a e congestão pulmonar exuberante. Quatro de cinco animais apresentaram lesões de mineralização distribuídas por diversos órgãos induzidas e associadas com processos inflamatórios.

Na avaliação molecular foi constatada a presença de mutação no gene *MYH1* em quatro animais avaliados, com um homozigoto e três heterozigotos, a distribuição alélica não pareceu impactar a apresentação da doença.

Dessa forma, deve-se considerar incluir a avaliação do gene *MYH1* em suspeitas de doença muscular com caráter inflamatório ou mineralizante, tendo consciência das repercussões sistêmicas das doenças.

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