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RESEARCH ARTICLE

Genetic background of the Transylvanian endemic equine recurrent rhabdomyolysis

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ABSTRACT

To investigate the pathophysiology, prevalence and severity of equine exertional rhabdomyolysis in a mountainous region of Transylvania, Romania, this study considered genetic and histological factors. We determined the occurrence and frequency of a mutation in the glycogen synthase gene 1 (GYS1), associated with equine polysaccharide storage myopathy type 1 (PSSM1), in two adjacent populations, one with a significantly high prevalence of the disease (high altitude villages, HV) and the other with a rare prevalence (valley villages, VV). We genotyped GYS1 in 41 animals (HV = 31, VV = 10) and found a significant difference in the appearance of GYS1-positive animals in the HV region. Histological lesions of animals (n = 6) with and without muscle disorders of the two populations were examined. We demonstrated a significant association between the number of rhabdomyolysis episodes and GYS1 mutation (P = 0.03) and positive amylase-resistant PAS staining (P = 0.01). Nevertheless, the correlation between GYS1 mutation and PAS staining was weak (P = 0.06). These studies further confirm the versatile and complex nature of muscle disease in this region.

KEYWORDS

equine, rhabdomyolysis, polysaccharide storage myopathy, GYS1 mutation

INTRODUCTION

A severe form of recurrent rhabdomyolysis occurs endemically in a well-defined region of Transylvania, Harghita County, Romania. In the highest lying two settlements in Szeklerland, 800 m above sea level (Szentegyháza, Kápolnásfalu), the prevalence of equine rhabdomyolysis is much higher than in the neighbouring villages (Lövéte, Homoródfürdő) in the valley

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(Kósa et al., 2011, 2015, 2018, 2021). This specific rhabdomyolysis syndrome is generally associated with exertion, but clinical signs can also occur in horses without intense exercise (Kósa et al., 2011, 2018, 2021). Clinical signs include swollen, painful muscles, stiffness, weakness, rhabdomyolysis and myoglobinuria, generally shortly after the beginning of work. In more severe cases, dysphagia or recumbency may occur as well. In most affected horses, episodes are recurrent; in chronic cases, muscle atrophy is observed (Kósa et al., 2011, 2015, 2018, 2021).

Exertional rhabdomyolysis (ER) is a syndrome of muscle damage usually precipitated by exercise. Once considered a single disease entity, it is now understood to represent a common clinical presentation of several very distinct disease processes (Naylor et al., 2015a, 2015b; Valberg et al., 1999a). Although diagnosing ER is usually not difficult, categorising the disease by aetiology can be much harder. Both acquired and inherited causes should be considered in an animal with acute signs, whereas in a horse presenting after multiple episodes, an underlying genetic predisposition is more likely (Piercy et al., 2014). Several inherent equine skeletal muscle disorders can cause repeated episodes of exertional rhabdomyolysis: recurrent exertional rhabdomyolysis (RER), malignant hyperthermia (MH), polysaccharide storage myopathy type 1 (PSSM1), polysaccharide storage myopathy type 2 (PSSM2) and mitochondrial myopathy (MM) (Reed et al., 2004). Unfortunately, many cases contribute to a large idiopathic category, a situation familiar to the diagnosis of human ER (Landau et al., 2012).

Selecting horses for desired physical and performance traits often has the unintended consequence of increasing the frequency of heritable diseases. Focused research efforts over the past two decades have defined specific forms of heritable equine muscle disease through careful phenotyping of patient characteristics, physiological responses, muscle histopathology, biochemistry and genetic analysis. The currently known autosomal dominant equine muscle disorders, hyperkalaemic periodic paralysis (HYPP, gene: SCN4A), MH (gene: RYR1), myosin heavy chain myopathy (MYHM, gene: MYH1) and PSSM1 (gene: GYS1), intermittently negatively impact muscle function in adult horses. The currently known autosomal recessive equine muscle disorders, glycogen branching enzyme disease (GBED, gene: GBE1) and myotonia (gene: CLCN1), have a profound and persistent impact on muscle function in horses from a young age (Mickelson and Valberg, 2015; Valberg, 2020).

PSSM1 in horses is associated with a variety of clinical signs, including intermittent exertional rhabdomyolysis, muscle fasciculations, gait abnormalities and paresis (Tyron et al., 2009; Valberg, 2020). Clinical signs occur most often in horses fed high-grain diets, exercised irregularly, with little turnout, or undergoing general anaesthesia (Valberg, 2020). Other signs of PSSM1 in draft horses, often homozygotes for the GYS1 mutation (P/P), include progressive weakness and muscle atrophy, resulting in difficulty rising, even with normal serum creatine kinase (CK) activity (Valberg, 2020; Firshman and Valberg, 2024). The dominant gain-of-function mutation in the glycogen synthase gene 1 (GYS1), which encodes glycogen synthase, results in the accumulation of amylase-resistant polysaccharide in a small percentage of fast-twitch muscle fibres (Valberg, 2020). It remains unclear the degree to which these signs are associated with the physical disruption of muscle fibre ultrastructure by the accumulations of glycogen or polyglucosan (Shea and Raber, 2009; Naylor, 2012), or with other disease mechanisms, such as diminished energy supply (Argov et al., 1987; Lewis et al., 1985, 1986; Naylor, 2012). The form of PSSM caused by the GYS1 mutation is now termed type 1 (PSSM1), whereas the form(s) of PSSM not associated with the GYS1 mutation are termed type 2 (PSSM2). Recent research has further subdivided PSSM2 into PSSM2-ER in quarter horses, myofibrillar myopathy-ER (MFM-ER) in Arabian endurance horses and myofibrillar myopathy in warmblood horses (MFM-WB) (Valberg, 2021, 2023; Firshman and Valberg, 2024). Both PSSM1 and PSSM2-ER are glycogen storage disorders, but other PSSM2 subtypes are not (Firshman and Valberg, 2024). It was also observed that MFM-WB rather presents with exercise intolerance rather than ER (Valberg et al., 2023; Firshman and Valberg, 2024), just as it was detected in a recent European study identifying a distinct exercise-associated myopathy phenotypic subtype termed non-classic exercise-associated myopathy syndrome (EAMS), consisting of horses with weakness, perceived ataxia, muscle pain, gait abnormalities and reluctance to go forward under saddle (McGee et al., 2024).

The clinical manifestations of Transylvanian endemic equine recurrent rhabdomyolysis syndrome mostly resemble those of a disorder related to abnormal glycogen metabolism, most likely PSSM1. The objective of our study was to clarify the genetic background, focusing on the role of the GYS1 mutation in high-prevalence rhabdomyolysis in this specific geographical region. We hypothesised that the GYS1 mutation is more common in the HV area, leading to a higher prevalence of ER.

MATERIALS AND METHODS

Ethical authorisation of animal experiments

The study was permitted by the bioethics commission of the Faculty of Veterinary Medicine, Cluj-Napoca (175/18.09.2019). Informed owner consent was signed for study participation for each animal and for every specific examination separately.

Animals in the study. The study was conducted in four villages in Transylvania: two villages from the affected region of Szentegyháza and Kápolnásfalu (high villages: HV) and two villages from the non-affected neighbouring valley region (valley villages: VV, Lövéte, and Homoródfürdő) (Fig. 1).

Enrolment criteria included an age range of 1–20 years and the absence of acute clinical signs of any disease at the time of sampling. Horses were selected from the attending veterinarian's database for these populations. An initial random selection was followed by the exclusion of animals that did not meet the age or health criteria.

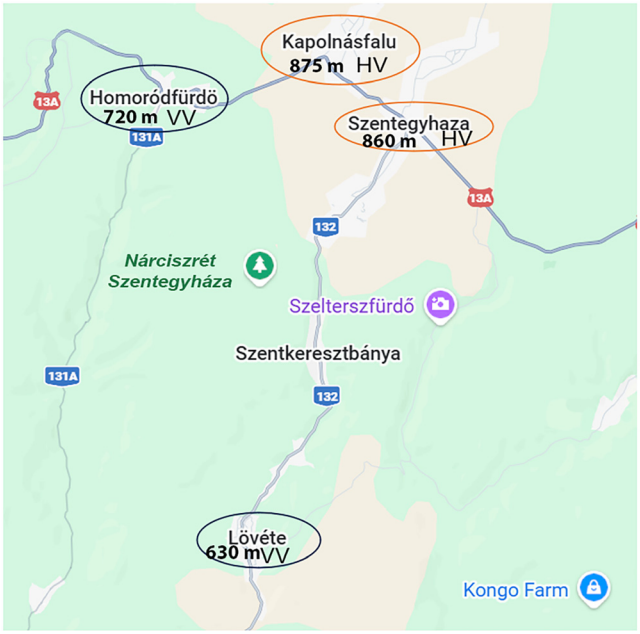

Fig. 1. Topographic map of the two mountain villages with rhabdomyolysis episodes (red circles) and two villages of the non-affected neighbouring valley region (blue circles) (Google maps)

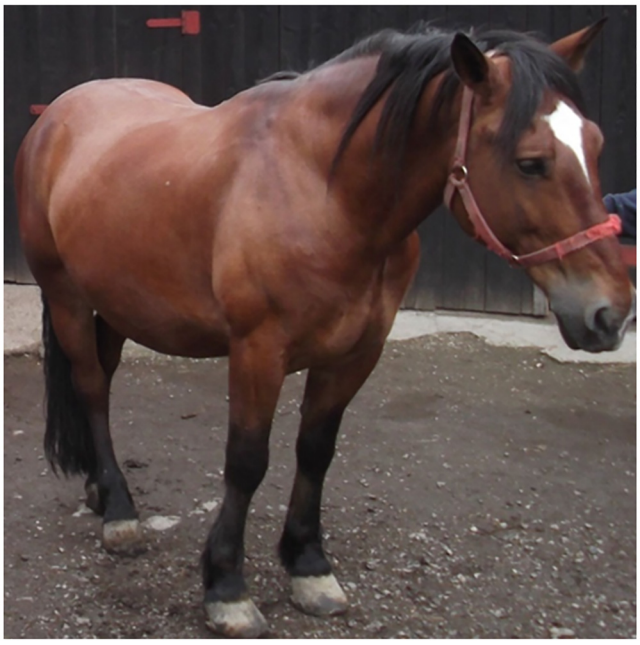

Fig. 2. One of the affected horses with the typical phenotype: a draft crossbred with muscular build, long hair on distal limbs

A medical history database documenting previous episodes of rhabdomyolysis was available, and a clinical examination was performed at the time of sampling. Altogether, data from 45 horses were evaluated for the occurrence of rhabdomyolysis. 41 horses were genetically tested for PSSM1: 31 animals in the HV (average age: 8.54 years, SD: 4.52; 14 females and 17 males) and 10 horses in the VV area (average age: 11.4 years, SD: 5.9; 7 females and 3 males). The gold standard for the diagnosis of PSSM1 is the identification of the G to A base substitution in exon 6 of the GYS1, which causes an R309H substitution in glycogen synthase (Valberg, 2020). When the owner gave his/her consent (16 HV and 5 VV horses), a musculoskeletal biopsy for histological evaluation was taken. All horses were mixed-breed draft types, exhibiting cold-blooded phenotypic traits such as compact, heavy musculature and feathering on the lower limbs (Fig. 2). All these animals were working equids taking part in seasonal forestry work and were kept under similar conditions. When they were not used for work, they were most often kept in stables and had no daily turnout or regular training. Their daily ration consisted of 2.5% DMI of locally harvested pasture hay, to which no concentrate or mineral, electrolyte, or vitamin supplement was added. Some of these horses received ca. 250g of table sugar as an energy source before planned heavy work.

Clinical signalment and history of the horses were recorded, noting whether a horse was previously affected by rhabdomyolysis or not.

Genetic testing. To perform genetic testing for GYS1 mutation, either hair ($n = 30$) or blood ($n = 11$) samples were collected. All hair samples consisted of lots of clean hair and roots from the mane. Whole blood was collected from the jugular vein into sterile EDTA test tubes (Vacutest Kima®, Arzegrande, Italy). Samples were frozen within 12 h of collection and kept at −20 °C until processing. Genetic tests were performed by Animal Genetics (St. Austell, Cornwall, England). Semimembranosus muscle was sampled for histologic evaluation in 21 horses: $n = 16$ from the HV ($n = 12$ with previous rhabdomyolysis episode) and $n = 5$ from the LV group ($n = 1$ with previous rhabdomyolysis episode). After sedation with detomidin-hidroclorid (0.02 $mg^{*}bwkg^{-1}$ (Domosedan 10 $mg^{*}mL^{-1}$ suspension injectable, Orion Corporation, Espoo, Finland) and butorphanol 0.04 $mg^{*}bwkg^{-1}$ (Morphasol 10 $mg^{*}mL^{-1}$, aniMedica International GmbH, Frankfurt, Germany) and subcutaneous administration of 5–10 mL of local anaesthetic (Lidocaine hydrochloride, LidoBel 20 $mg^{*}mL^{-1}$ solution for injection, Bela-Pharma GmbH & Co. KB Vechta, Germany) by avoiding direct injection into the muscle layer, we prepared the skin for sterile open biopsy. We made a ca. 4 cm^3 incision (in the same orientation as the muscle fibres) in the skin and subcutaneous tissue in the middle of the semimembranosus muscle, exposing the underlying muscle. Approximately 1 cm^3 of muscle was collected, and the skin was closed with sutures. We pinned the piece onto a card and placed it in 10% formalin in a screw top container.

Histology and immunohistochemistry. For histologic examination, after fixation for 24 h, transverse and longitudinal sections were cut from the collected specimens and routinely processed. The paraffin-embedded samples were serial sectioned at 3 µm thickness and stained with haematoxylin and eosin (HE), periodic acid-Schiff (PAS), PAS following amylase (α-Amylase from porcine pancreas; Sigma-Aldrich, Darmstadt, Germany) pre-incubation for

5 min (PAS amylase) and antibodies recognising ubiquitin (rabbit polyclonal anti-Ubiquitin antibody (ab7780), dilution 1:50, Abcam, Cambridge, UK) and desmin (clone DE-R-11, ready-to-use antibody, Leica Biosystems, Newcastle, UK). For immunohistochemistry, the samples were automatically processed using Leica BondmaxTM Immunohistochemistry system (Leica Biosystems, Melbourne, Australia). The slides were examined by a board-certified pathologist (MT) and evaluated for the following microscopical features: myofibre size variation, degeneration, necrosis, atrophy, fibrosis, inflammatory infiltrates, regeneration/internal nuclei, presence of subsarcolemmal inclusions or deposits of PAS + glycogen (sensitive or resistant) (McGowan et al., 2009) and cytoplasmic expression of ubiquitin and desmin.

Statistics. Data were analysed using R (R Core Team, 2018), a language and environment for statistical computing (https://www.R-project.org/). Numeric variables were checked for normality with the Shapiro-Wilk test. Some factors were converted to numerical values for two-sided Pearson's product-moment correlation (yes/no, female/male, or positive/negative into 0/1; and N/N, P/N, P/P into 0, 1, and 2, respectively). All tests were conducted both with and without taking sex into account. Data of geldings and stallions were analysed together as "male". Only data from HV locations were included in the statistical analysis due to the extreme difference in the distributions of both variables and factors between HV and LV. Numeric variables (age, number of previous episodes) were analysed using Kruskal-Wallis rank-sum tests, with sex, GYS1 genetic test, PAS staining and signalment as factors. Two-sample proportion tests were used to determine the relationships between the following constants: sex (F/M), GYS1 (pos/neg), signalment (Y/N) and PAS (pos/neg). In pairs, the correlations between age, number of previous episodes, GYS1 and PAS were assessed using Pearson's product-moment correlation. Factors with insufficient sample size for a more detailed analysis were described using percentages and ratios. The probability of $P \leq 0.05$ was statistically significant.

RESULTS

In the HV region, 14 of 33 horses had the disease once and 13 more than once; the prevalence was 81.8% (27/33). The presence or absence of clinical signs was independent of age, sex, GYS1, or PAS positivity in the HV region. Still, among horses that had presented clinical signs, more episodes were detected in GYS1 heterozygous ($P = 0.04$) and PAS-positive animals ($P = 0.01$). In the VV area, the prevalence was only 8.3% (1 out of 12 horses developed the disease, but it occurred 3 times). The odds of getting the disease were 9.1 times higher in the HV area than in the VV area. Data on horses and results of genetic tests and histopathology are summarised in Table 1.

We have demonstrated a significant association between rhabdomyolysis episodes and GYS1 mutation ($P = 0.0355$) and positive amylase-resistant PAS staining ($P = 0.0106$). Nevertheless, the correlation between the GYS1 mutation and PAS staining was weak ($P = 0.0631$).

Histology and immunohistochemistry

Muscle biopsies showed features of PSSM1 in 6 horses out of 21 and consisted of myodegeneration, necrosis and intracytoplasmic pale grey, rod-like glycogen inclusions (Fig. 3A and B). The inclusions were PAS+ (Fig. 3C) and amylase-resistant (Fig. 3D). Ubiquitin was highly expressed in all myofibres containing amylase-resistant glycogen (Fig. 4A and B). Desmin was uniformly expressed in PSSM-negative horses (Fig. 4C), but aggregates of desmin were identified in scattered myofibres of two PSSM1/(GYS1+) horses (Fig. 4D). The presence of desmin aggregates in PSSM-affected horses may explain muscle regeneration or coexisting myofibrillar myopathy.

DISCUSSION

To prove that a genetic variant plays a role in disease pathogenesis, we must demonstrate its high frequency in an affected population and its low frequency in a healthy population (Valberg, 2020). Our study showed that the GYS1 mutation is more frequent in the HV area and accounts for the majority of ER cases. Furthermore, we observed a significant association between clinical signs consistent with ER and the presence of the GYS1 mutation or with positive findings on amylase-resistant PAS staining. However, the relationship between the GYS1 mutation and histopathological signs appeared weak, suggesting that other factors besides the GYS1 mutation may also contribute to the observed tissue changes.

There is no significant temperament, body type, or gender predilection for PSSM1 (Firshman et al., 2003, 2005; Johling et al., 2011; Reed et al., 2018; Firshman and Valberg, 2024). We found no significant differences in age or sex between horses with and without rhabdomyolysis. Horses with the GYS1 mutation begin to show clinical signs of muscle disease between 1 and 14 years of age (Firshman et al., 2005; Tyron et al., 2009), with PSSM2, between 8 and 11 years of age, while MFM is considered an adult-onset disease often evident in WB 7 years of age or older (Valberg et al., 2017; Reed et al., 2018; Valberg, 2020). In our study, the youngest horse with clinical signs was 1 year old with a positive GYS1 mutation.

The GYS1 mutation defining PSSM1 has been reported in numerous breeds worldwide, with particularly high prevalence in certain continental European draft breeds (Baird et al., 2010; Schwarz et al., 2011; Valberg, 2020). The highest prevalence of the mutation occurs in continental European breeds derived from the Belgian draft (36–90% prevalence), which also have a high prevalence of homozygous P/P horses (Firshman et al., 2005; Valberg, 2020). The origin of draft horses in the Carpathian Basin is traced to the wild horse (Equus robustus) that once lived in Belgium.

Table 1. Data of clinical and laboratory results (location of horse identification: HV-high villages, VV-valley villages; horse identification: age in years, gender: M-male, F-female; Mc-castrated male; clinical history: N - without clinical history, I -with clinical sign history; genetic results: P-positive, N-negative; histopathological results: P-positive, N-negative)

							Histopathology (staining)		
Location	ID	Age (years)	Gender	Clinical signs of rhabdomyolysis	Number of previous scenarios	Result of genetic test for GYS1	PAS amylase resistant	Ubiquitin	Desmin
HV	**1**	**10**	**M**	**N**	**0**	**N/N**	**–**	**–**	**–**
HV	**2**	**20**	**M**	**I**	**3**	**P/N**	**N**	**N**	**N**
HV	**3**	**9**	**F**	**I**	**3**	**–**	**–**	**–**	**–**
HV	**4**	**8**	**M**	**I**	**1**	**P/N**	**N**	**N**	**N**
HV	**5**	**15**	**F**	**I**	**1**	**N/N**	**–**	**–**	**–**
HV	**6**	**4**	**M**	**I**	**1**	**P/N**	**–**	**–**	**–**
HV	**7**	**4**	**M**	**N**	**0**	**P/N**	**–**	**–**	**–**
HV	**8**	**5**	**M**	**I**	**1**	**N/N**	**N**	**N**	**N**
HV	**9**	**6**	**Mc**	**I**	**1**	**N/N**	**N**	**–**	**N**
HV	**10**	**5**	**F**	**I**	**8**	**P/N**	**P**	**P**	**N**
HV	**11**	**6**	**F**	**I**	**3**	**P/N**	**–**	**–**	**–**
HV	**12**	**9**	**F**	**I**	**3**	**N/N**	**P**	**P**	**N**
HV	**13**	**8**	**F**	**I**	**4**	**N/N**	**N**	**N**	**N**
HV	**14**	**6**	**M**	**I**	**3**	**P/N**	**N**	**N**	**N**
HV	**15**	**10**	**Mc**	**I**	**3**	**P/N**	**P**	**P**	**P**
HV	**16**	**8**	**Mc**	**I**	**1**	**N/N**	**N**	**–**	**N**
HV	**17**	**10**	**M**	**I**	**8**	**P/N**	**P**	**P**	**N**
HV	**18**	**18**	**F**	**I**	**11**	**P/N**	**–**	**–**	**–**
HV	**19**	**7**	**Mc**	**N**	**0**	**–**	**N**	**N**	**N**
HV	**20**	**9**	**Mc**	**N**	**0**	**P/N**	**P**	**P**	**N**
HV	**21**	**9**	**Mc**	**I**	**1**	**N/N**	**N**	**–**	**N**
HV	**22**	**9**	**Mc**	**I**	**5**	**P/N**	**P**	**P**	**N**
HV	**23**	**12**	**F**	**I**	**2**	**N/N**	**N**	**–**	**N**
HV	**24**	**7**	**F**	**I**	**1**	**N/N**	**–**	**–**	**–**
HV	**25**	**8**	**F**	**I**	**1**	**P/N**	**–**	**–**	**–**
HV	**26**	**8**	**F**	**I**	**1**	**P/P**	**–**	**–**	**–**
HV	**27**	**1**	**F**	**N**	**0**	**P/N**	**–**	**–**	**–**
HV	**28**	**20**	**Mc**	**I**	**4**	**N/N**	**–**	**–**	**–**
HV	**29**	**11**	**F**	**I**	**1**	**N/N**	**–**	**–**	**–**
HV	**30**	**2**	**F**	**N**	**0**	**N/N**	**–**	**–**	**–**
HV	**31**	**7**	**Mc**	**I**	**1**	**N/N**	**–**	**–**	**–**
HV	**32**	**5**	**F**	**I**	**1**	**N/N**	**–**	**–**	**–**
HV	**33**	**5**	**M**	**I**	**1**	**P/P**	**–**	**–**	**–**
VV	**1**	**18**	**F**	**N**	**0**	**N/N**	**N**	**–**	**N**
VV	**2**	**12**	**F**	**N**	**0**	**N/N**	**–**	**–**	**–**
VV	**3**	**8**	**M**	**N**	**0**	**N/N**	**–**	**–**	**–**
VV	**4**	**20**	**F**	**N**	**0**	**N/N**	**–**	**–**	**–**
VV	**5**	**5**	**M**	**N**	**0**	**N/N**	**–**	**–**	**–**
VV	**6**	**8**	**F**	**N**	**0**	**N/N**	**–**	**–**	**–**
VV	**7**	**15**	**F**	**N**	**0**	**N/N**	**–**	**–**	**–**
VV	**8**	**5**	**Mc**	**N**	**0**	**N/N**	**N**	**N**	**N**
VV	**9**	**19**	**F**	**N**	**0**	**N/N**	**–**	**–**	**–**
VV	**10**	**4**	**F**	**I**	**3**	**P/N**	**N**	**N**	**N**
VV	**11**	**2**	**M**	**N**	**0**	**–**	**N**	**N**	**N**
VV	**12**	**2**	**M**	**N**	**0**	**–**	**N**	**N**	**N**

In their books describing the Szekler-horse breed in the early 1900s, Béla Hankó (1943) and Imre Bodó (2021) wrote that local Szekler-horses had been bred with stallions imported from Mezőhegyes and Fogaras, bringing English, Arabian, Nonius, Gidran, Lipizzaner and Hungarian half-breed stallions to Szekler land. By the 1970s, the original local Szekler horse breed had disappeared and cold-blooded horses became the primary foundation of draft horse breeding in the region (Kósa et al., 2018; Bodó et al., 2021). The higher incidence of the genetic disorder in these draft horses is probably due to evolutionary factors (Mickelson and Valberg, 2015). It is hypothesised that poor quality feed and harsh agricultural work have contributed to the development of the GYS1 mutation. This has had a detrimental effect on

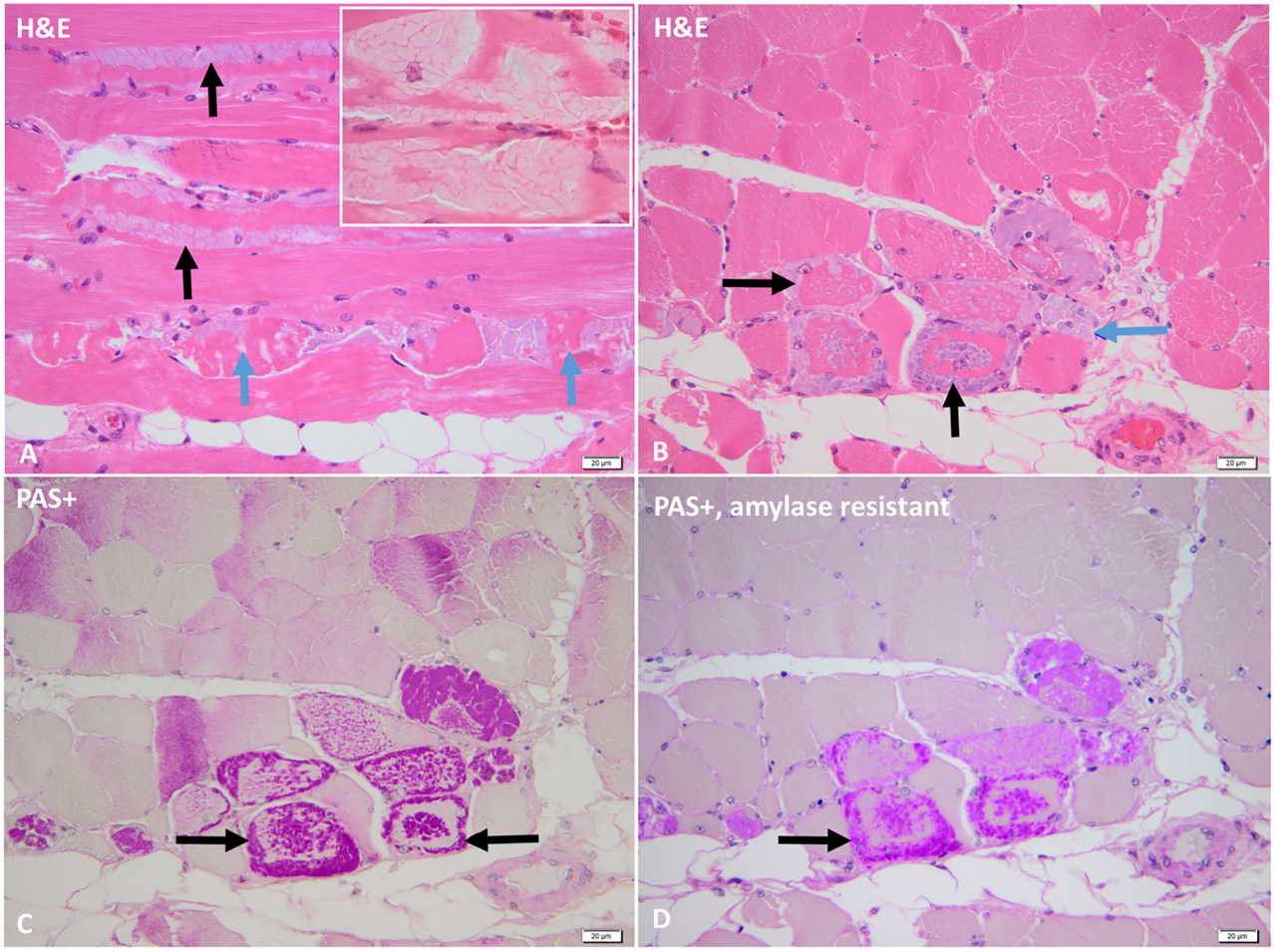

Fig. 3. Histological features of skeletal muscle affected by polysaccharide storage myopathy type 1 (PSSM1)
A) and B): The semimembranosus muscle showing intracytoplasmic subsarcolemmal intracytoplasmic pale grey, rod-like glycogen inclusions (black arrows and the inset) associated with myofiber degeneration (A: blue arrows, and macrophages containing glycogen (B: blue arrow), H&E stain; bar = 20 µm
C): The intracytoplasmic glycogen is PAS positive (black arrows), PAS; bar = 20 µm
D): The intracytoplasmic polysaccharide is resistant to digestion with amylase (black arrow). PAS and amylase; bar = 20 µm

horses fed a high-carbohydrate diet and working at variable intensities (Mickelson and Valberg, 2015). Given their phenotypic characteristics, pedigree and exposure to variable work intensity, the clustering of exertional rhabdomyolysis (ER) cases in the Transylvanian HV herd is not unexpected. Our study demonstrated that the GYS1 mutation occurred more frequently among horses living in the affected regions, which may partly explain the higher incidence of rhabdomyolysis observed in these areas. The widespread presence of the GYS1 mutation in the two high-altitude settlements is likely due to inbreeding practices adopted by local horse owners to address financial constraints and limited access to diverse breeding stock.

We also found some conflicting results: from the 17 horses which have never had an episode of rhabdomyolysis, 11 did not carry the mutant gene (3 of these healthy horses were positive on genetic testing and 3 were not tested) but from the 28 animals previously affected by rhabdomyolysis and tested for GYS1 mutation only 14 was diagnosed as a homo- (12/14) or heterozygote (2/14). To have positive horses without clinical episodes is not surprising, as it has previously been described that many animals with the GYS1 mutation are asymptomatic (Valentine et al., 2001; Naylor et al., 2012; Schroder et al., 2015; Reed et al., 2018; Valberg, 2020; Firshman and Valberg, 2024). On the other hand, it was less expected that horses with the typical phenotype and clinical presentation would prove harmful in genetic testing. Other genetic factors likely contribute to rhabdomyolysis. Still, to date, no other DNA variants that explain other forms of PSSM (for example, PSSM2) have been published (Firshman and Valberg, 2024). In addition to the gold-standard genetic test, muscle biopsy provides a means to diagnose PSSM1 in horses over 2 years of age (Valberg et al., 1992; De La Corte et al., 2002; Valberg, 2020). The distinctive features of PSSM1 in muscle biopsy samples are numerous subsarcolemmal vacuoles and dense, crystalline PAS–positive, amylase-resistant inclusions in fast-twitch fibres (Valberg et al., 1992; Valberg, 2020).

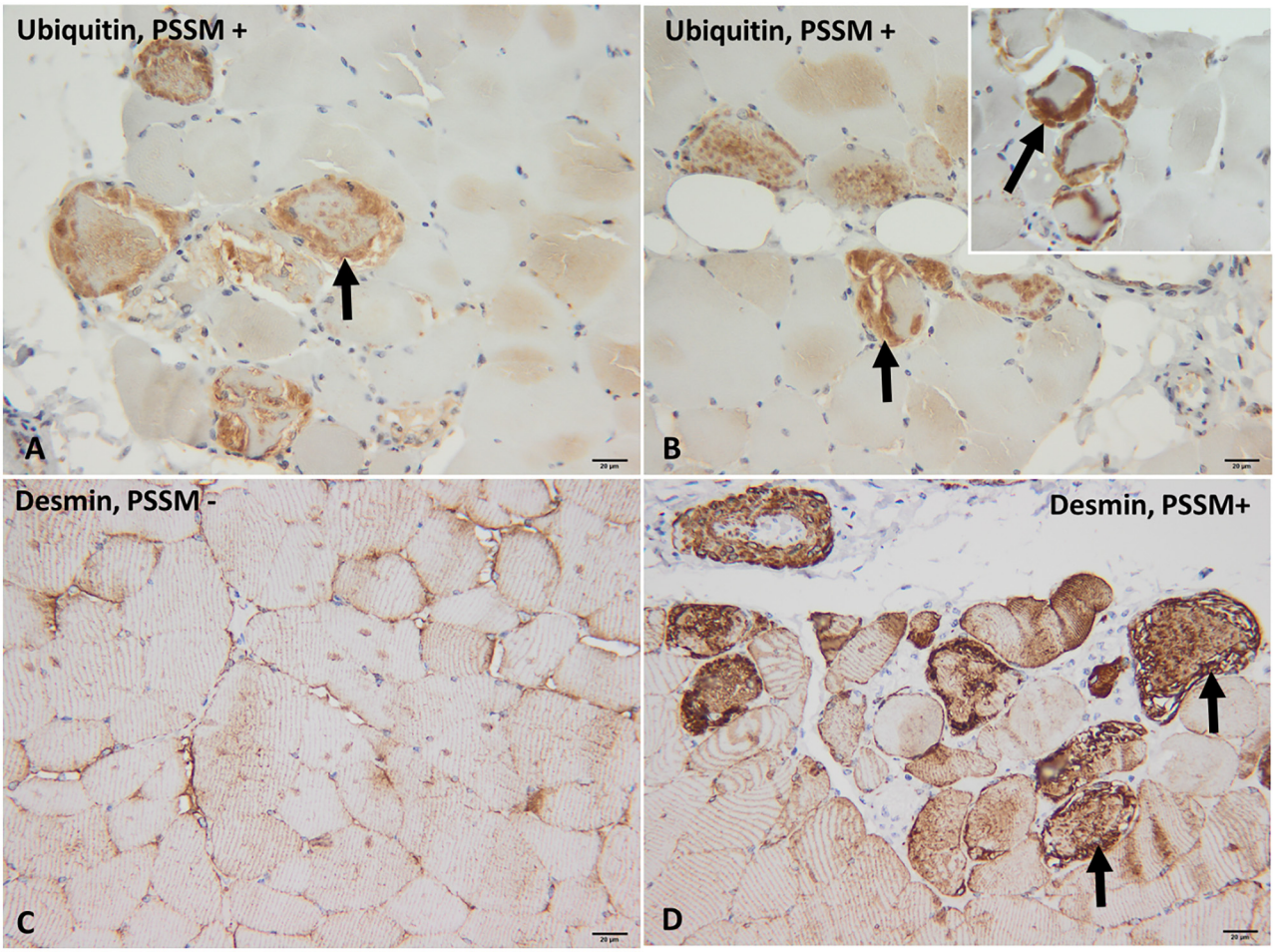

Fig. 4. Immunohistochemical analysis for ubiquitin and desmin in horses with polysaccharide storage myopathy type 1 (PSSM1)
A) and B): Groups of myofibers showing intense ubiquitin expression in the subsarcolemmal amylase-resistant polysaccharide (black arrows and the inset)
C): GYS1 negative horse with a normal pattern of desmin staining
D): Cross-section of semimembranosus muscle from a GYS1 positive horse demonstrating desmin positive aggregates in the subsarcolemmal region; IHC and Mayer's haematoxylin counterstain; bar = 20 µm

A diagnosis of PSSM1 can be made regardless of diet or the proximity of sampling to recent episodes of rhabdomyolysis (Valberg, 2020). Typical findings of myodegeneration, necrosis and intracytoplasmic pale inclusions in 6 of 21 horses affected by rhabdomyolysis were sampled. Ubiquitin was highly expressed in all myofibres containing amylase-resistant glycogen in our samples. Since ubiquitin is only known to recognise abnormal proteins, ubiquitination associated with the development of amylase resistance is unlikely to be related to the carbohydrate component. The presence of abnormal protein structures is supported by the detection of desmin aggregates in some PSSM-affected horses. The presence of desmin aggregates may be explained by muscle regeneration or coexisting myofibrillar myopathy (Valberg, 2018). On the other hand, desmin accumulation is a typical feature of muscle regeneration. It is a non-specific feature of most myopathies characterised by muscle damage and elevated serum CK activity (McGee et al., 2024). We had 1 case, where histopathology was positive on abnormal glycogen and the horse showed signs of rhabdomyolysis but tested negative for GYS1 mutation. GYS1-negative horses with ER and a true glycogen storage disease based on histopathology are now termed as PSSM2-ER cases. Research into PSSM2-ER has focused on quarter horses, but this form of ER is likely prevalent in other breeds (Firshman and Valberg, 2024). Polyglucosan inclusions occur with various enzyme defects involving carbohydrate metabolism. In some people (Goebel et al., 1992; Miladi et al., 2005) and in horses with polysaccharide storage myopathy (Valberg et al., 1999b; Annandale et al., 2004), no enzyme defect has been identified. It appears that the PSSM1 variant does not explain all cases of excessive abnormal glycogen accumulation in the muscle. These findings suggest that multiple pathways can lead to the formation of polyglucosan inclusions within skeletal muscle fibres (Valentine et al., 2006).

These findings also underline the roles of other genetic disorders, except the GYS1 mutation (cases categorised as PSSM2) and of promoting factors such as exercise and diet. We also found 10 cases in which histopathology was negative despite recurrent clinical signs and 4 of them were

positive on the genetic test. A false-negative diagnosis of PSSM1 by muscle biopsy can occur if biopsy samples are small or if horses are less than 2 years of age (De La Corte et al., 2002; Valberg, 2006, 2020). We lacked frozen samples due to logistic constraints, so all analyses were performed on formalin-fixed sections, which can also affect histopathologic results. These horses had no regular exercise but did occasionally perform hard forestry work, so some cases of rhabdomyolysis could have been related to simple overexertion and to training and nutritional failures. That would also explain why having a history of rhabdomyolysis was neither associated with GYS1 mutation nor with PAS staining results. Still, the number of episodes demonstrating the recurrence of clinical signs did correlate.

In metabolic myopathies, exploring patients' clinical signs in relation to the timing and type of exercise will provide a strong clinical clue. Another clue to a metabolic diagnosis is that signs are present in all skeletal muscles. In glycogen metabolism disorders, signs are induced within minutes by isometric muscle contraction (such as weight pulling), intense maximal exercise (such as galloping), or within a few minutes of aerobic physical activity (such as walking). By contrast, in disorders of fatty acid metabolism, symptoms occur only after more prolonged (over 45 min and often after several hours) aerobic exercise (Scalco et al., 2015). Based on the clinical description of the Transylvanian endemic cases, disorders of glycogen metabolism have to be suspected in the background in most cases.

Apart from genetic abnormalities in the background, we usually find other promoting factors in cumulative cases. Variable activity with long periods of limited motility and hard pulling work was observed in both HV and VV horses. Although the daily ration was very similar in the two groups, our previous study on antioxidant availability found that selenium was significantly lower in HV horses' feed (Kósa et al., 2018). Also, horses in the affected regions had significantly lower serum selenium levels and glutathione peroxidase activity than those in non-affected areas (Kósa et al., 2018). Previously, Valentine (2005) suggested that horses with PSSM and also with selenium or vitamin E deficiency may exhibit more frequent and/or more severe signs of exertional rhabdomyolysis owing to oxidative injury incited by the release of free radicals from damaged muscle cell membranes. On the other hand, low antioxidant levels can also result from abnormal muscle glycogen metabolism, in which reactive oxygen species levels are increased (Barrey et al., 2009).

In conclusion, genetic mutations in glucose metabolism are likely to be the primary cause for the high prevalence of exertional rhabdomyolysis in the affected villages. Still, other significant risk factors, such as diet and exercise that lead to low antioxidant levels and overexertion, must also be considered. A complex plan of genetic selection, the introduction of a low (water-soluble) carbohydrate diet with antioxidant supplementation and regular aerobic training would be suggested to reduce losses associated with exertional rhabdomyolysis episodes.

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