

## ORIGINAL ARTICLE

# Absence of myofibrillar myopathy in Quarter Horses with a histopathological diagnosis of type 2 polysaccharide storage myopathy and lack of association with commercial genetic tests

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## Abstract

**Background:** Genetic tests for variants in *MYOT* (P2; rs1138656462), *FLNC* (P3a; rs1139799323 or P3b; rs1142918816) and *MYOZ3* (P4; rs1142544043) genes are offered commercially to diagnose myofibrillar myopathy (MFM) and type 2 polysaccharide storage myopathy (PSSM2) in Quarter Horses (QH).

**Objectives:** To determine if PSSM2-QH has histopathological features of MFM. To compare genotype and allele frequencies of variants P2, P3, P4 between control-QH and PSSM2-QH diagnosed by histopathology.

**Study design:** Retrospective cross-sectional.

**Methods:** The study includes a total of 229 healthy control-QH, 163 PSSM2-QH *GYS1* mutation negative. Desmin stains of gluteal/semimembranosus muscle were evaluated. Purported disease alleles P2, P3a, P3b, P4 were genotyped by pyrosequencing. Genotype, allele frequency and total number of variant alleles or loci were compared between phenotypes using additive/genotypic and dominant models and quantitative effects evaluated by multivariable logistic regression.

**Results:** Histopathological features of MFM were absent in all QH. A P variant allele at any locus was not associated ( $P > .05$ ) with a histopathological diagnosis of PSSM2 and one or more P variants were common in control-QH (57%) and PSSM2-QH (61%). Allele frequencies (control/PSSM2) were: 0.24/0.21 (P2), 0.07/0.12 (P3a), 0.07/0.11 (P3b) and 0.06/0.08 (P4). P3a and P3b loci were not independent ( $r^2 = 0.894$ ); and not associated with PSSM2 histopathology comparing the haplotype of both P3a and P3b variants to other haplotypes. A receiver operator curve did not accurately predict the PSSM2 phenotype (AUC = 0.67, 95% CI 0.62-0.72), and there was no difference in the total number of variant loci or total variant allele count between control-QH and PSSM2-QH.

**Main limitations:** P3a and P3b were not in complete linkage disequilibrium.

**Conclusions:** The P2, P3 and P4 variants in genes associated with human MFM were not associated with PSSM2 in 392 QH. Their use would improperly diagnose PSSM2/MFM in 57% of healthy QH and fail to diagnose PSSM2 in 40% of QH with histopathological evidence of PSSM2.

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## KEYWORDS

genomics, glycogen, horse, muscle disease, skeletal muscle, validation

## 1 | INTRODUCTION

The term type 2 polysaccharide storage myopathy (PSSM2) was derived to classify horses that lacked the glycogen synthase 1 (*GYS1*) mutation which causes type 1 PSSM (PSSM1) and yet had abnormal aggregates of amylase-sensitive or amylase-resistant polysaccharide in muscle fibres.<sup>1,2</sup> A definitive cause for PSSM2 has yet to be identified and clinical signs vary among breeds, suggesting there may be several aetiologies grouped under the histopathological descriptor PSSM2.<sup>3</sup> Ultrastructural and immunohistochemical evaluation of skeletal muscle recently identified ectopic accumulation of the cytoskeletal protein desmin, myofibrillar disarray and Z disc disruption, and novel proteomic and transcriptomic profiles in a subset of Arabian and Warmblood PSSM2 horses.<sup>4-7</sup> Based on the similarity of the histopathological findings in Arabian and Warmblood horses to a described myopathy in humans, the term myofibrillar myopathy (MFM) was applied to those horses in which abnormal desmin aggregates were identified.<sup>4,5,8-10</sup> The possibility exists that type 2 PSSM is an early indicator of MFM; however, this has not yet been substantiated. MFM has thus far not been described in Quarter Horses or similar stock breeds (eg Appaloosa or Paint Horses). Fifty percent of human patients diagnosed with MFM have one or more mutations in genes that encode proteins in the Z-disc [*DES*, *PLEC 1*, *CRYAB*, *FLNC*, *MYOT*, *ZASP*, *BAG3*, *FHL1* and *DNAJB6*], with eight other genes also found to cause MFM-like disorders.<sup>11</sup>

Since 2016, a commercial company has been offering genetic tests with the claim that these tests are diagnostic of PSSM2 and/or MFM in horses. There are no data published by the company to show that a positive result with the commercial tests corresponds to a histopathological diagnosis, the means used to discover these diseases. The term “P variant” is used by the company and in this paper to define the alternate (non-reference) allele at each of the loci used to putatively diagnose PSSM2 or MFM. The inference from the developers of these commercial genetic tests is that cases of PSSM2 are an early stage of MFM.<sup>12</sup> Variants that are genotyped as part of these tests are in Z-disc-associated genes myotilin (P2 – *MYOT* rs1138656462), filamin C (P3a rs1139799323, P3b rs1142918816 *FLNC*) and myozenin 3 (P4 rs1142544043 *MYOZ3*). The utility of the P2, P3 and P4 genetic test variants to identify PSSM2 or MFM in Warmblood and Arabian horses was recently evaluated.<sup>13</sup> The commercial test variants, which are each reported to act in a dominant or semi-dominant manner, were common in both control and myopathic Warmblood and Arabian horses and were not significantly associated with a histopathological diagnosis of PSSM2 or MFM.<sup>13</sup> There are no published histopathological studies which have determined that MFM occurs in QH-related breeds, and no published data supporting the use of genetic testing in diagnosis of PSSM2/MFM in QH. This creates the potential for the available commercial genetic tests to be used by horse owners to “diagnose” MFM

and PSSM2 in QH and to make breeding, management, prepurchase and euthanasia decisions without scientific validation of their accuracy.

The first objective of our study was to determine if abnormal aggregates of desmin typical of MFM occur in muscle biopsies from QH diagnosed with PSSM2. The second objective was to compare genotype and allele frequencies of the commercial test variants designated as “P2” for the *MYOT* allele, “P3a and P3b” for the *FLNC* variant alleles and “P4” for the *MYOZ3* variant allele (Equiseq.com) between healthy control QH and those diagnosed with MFM or PSSM2 by muscle histopathology. The third objective was to determine if there was an association between a combination of the P variants and a histopathological diagnosis of MFM or PSSM2 in QH.

## 2 | MATERIALS AND METHODS

### 2.1 | Control and case selection

The database of the Neuromuscular Diagnostic Laboratory at Michigan State University was searched to identify healthy control horses of QH and QH-related breeds (Appaloosa, American Paint). The control samples had been previously acquired from several farms for the purpose of having a bank of muscle samples from healthy horses for research studies. Muscle histopathology was evaluated to ensure there was no evidence of a myopathy. These American stock horse breeds have a similar genetic background due to shared ancestry and continued interbreeding<sup>14</sup> and are defined in this study as QH. A search was also made of diagnostic submissions to select a similar number of QH and QH-related PSSM2 horses with a history of one or more of the following: poor performance, stiffness, sore muscles, atrophy, exertional rhabdomyolysis or an undiagnosed lameness. All control and PSSM2-QH horses were negative for the *GYS1* mutation,  $\geq 2$  years of age and had gluteal or semimembranosus muscle available for histopathology. Control horses had no history of muscle disorders or histopathological abnormalities in haematoxylin and eosin and no abnormal polysaccharide in periodic acid Schiff's (PAS) and amylase PAS stains. Diagnostic samples were also evaluated with the same stains and most cases evaluated additionally with modified Gomori trichrome, oil red O and nicotinamide adenine dinucleotide tetrazolium reductase. PSSM2 was diagnosed by the presence of aggregates or either amylase-resistant or amylase-sensitive polysaccharide in muscle fibres and a negative *GYS1* genetic test.<sup>2</sup>

### 2.2 | Desmin immunohistochemistry

To determine if PSSM2-QH was characterised by histopathology typical of equine MFM, desmin staining was performed retrospectively

on a subset of PSSM2-QH (N = 82) and control-QH (N = 188) samples as previously described.<sup>5</sup> The number of fibres with aggregates of desmin 50% the size of myonuclei was counted. Criterion for a presumptive diagnosis of MFM was a minimum of six mature muscle fibres with desmin aggregates  $\geq 50\%$  of the size of myonuclei.<sup>5</sup>

### 2.3 | DNA isolation and polymerase chain reaction

DNA was isolated from hair bulbs, buffy coat, whole blood or muscle tissue using Genra Puregene Kits and DNA quality/quantity evaluated using a Synergy H1 plate reader and Gene5 software (Biotek). Thermocycling was carried out using recombinant AmpliTaq<sup>5</sup> Gold polymerase in a PTC-200 thermocycler, and the PCR products run on a 1.0% agarose gel for visualisation.

### 2.4 | Pyrosequencing

Primers (Table S1) were designed using the PyroMark Assay Design Software 2.5.8, based on the position of the alleles as reported by the company.<sup>15,16</sup> The reverse primer was biotinylated using Biotin-TEG and HPLC purification (IDT). The resulting biotin-labelled amplicons were annealed with the sequencing primer and relative expression of each allele determined by pyrosequencing as described in Kreutz et al.<sup>17</sup> Briefly, PCR products were diluted in a solution containing 4  $\mu$ l of streptavidin-coated Sepharose beads and 40  $\mu$ l of binding buffer (10 mM Tris-HCL, 2 M NaCl, 1 mM EDTA and 0.1% Tween<sup>TM</sup> 20 pH 7.6). Immobilised biotin-labelled amplicons were captured using a vacuum prep tool, washed and denatured to remove unbound primers and unbiotinylated strands using three solutions (ie 70% ethanol, denaturing solution containing 0.2 M NaOH and wash buffer containing 10 mM Tris-Acetate pH 7.6). Only the template strands remained bound after the washing steps. Sepharose beads with bound strands were diluted in annealing buffer (20 mM Tris-Acetate, 5 mM MgAc<sub>2</sub> pH 7.6) containing the sequencing primer and pyrosequenced (PyroMark Gold Q96 reagents and PSQ 96MA pyrosequencer, Biotage). Relative levels of each allele were quantified with the PyroMark AQ 2.5.8 software. All sample plates were analysed with positive controls consisting of horses with the alternate allele identified from RNA-seq data (GEO Series accession number GSE104388) and a template control containing no DNA. Resulting data were visually evaluated to determine genotype and allele frequencies. Genotypes for each P variant were verified by Sanger sequencing by evaluating one horse each that genotyped by pyrosequencing as homozygous reference, heterozygous or homozygous for each of the P2, P3a, P3b and P4 variants.

### 2.5 | Data analysis

Data analysis was performed at two separate universities (University of Nebraska, Lincoln and University of California, Davis) independent

from the author who generated the histopathological diagnosis (SJV). A Fisher exact test power analysis was performed using the R statmod package (v1.4.36)<sup>18</sup> to determine if our sample sizes (229 controls and 163 cases) were sufficient to detect a significant effect at  $\alpha = 0.05$  using a Cohen's W medium effect size of 0.3.<sup>19</sup> Power was calculated at 99%.

There were no QH identified with MFM by desmin staining and therefore no analyses were performed with respect to MFM. Allele frequencies were calculated from the genotype data. To compare control-QH versus PSSM2-QH, the count of horses with each genotype (homozygous reference versus heterozygous, homozygous alternate) for each of the four loci was evaluated using a Fisher's Exact Test; additive and dominant models were considered. Linkage disequilibrium ( $r^2$ ) was calculated between the two variants that comprise the P3 locus as well as between the P2 and P4 loci given they are both found on chromosome 14. Because the P3 alleles were not in complete linkage disequilibrium, haplotype analyses were also conducted. P3 haplotypes were determined from genotype data, assuming the reference allele at both loci comprises the most common haplotype, and when variant alleles at both P3a and P3b were present, they together comprise a haplotype. These analyses then considered P3 as having a variant allele only if both P3a and P3b variant alleles were present together. Also considering haplotypes at P3 as outlined above, the total number of variant loci across P2, P3 and P4 (maximum of 3), or total number of variant alleles (maximum of 6) was evaluated using a Fisher's Exact Test or in the case all cells had a value  $>5$ , a Chi-square Test. The total count of variant and reference alleles at each locus in control-QH vs PSSM2-QH was also compared using a Chi-square Test. Correction for multiple testing would have reduced the significance threshold (eg to 0.01, or 0.05/4 when testing four variants independently); however, we considered only a liberal, uncorrected *P* value of .05. The odds ratio, sensitivity, specificity and positive and negative predictive values of the genetic variants for PSSM2-QH were determined.

Regardless of non-significant univariable findings, multivariable models testing the association of a combination of genotypes with PSSM2 were generated with PSSM2 (0=non PSSM2, 1=PSSM2) as the outcome variable and P variant genotypes defined as dependent binary variables (0=homozygous reference, 1=heterozygous and 2=homozygous alternate alleles). Sex and age were included as variables, with sex as binary (male or female) and age as continuous. Any missing data (ie sex unknown) were set as missing. As the P3 loci were not independent, three models were evaluated: 1) outcome consisting of P2, P3a and P4 genotypes; 2) outcome including P2, P3b and P4 genotypes, and 3) the outcome defined as the P2, P4 and the colinear covariates P3a and P3b. Statistical significance was set at  $P < .05$  for the multivariable analysis. Area under the curve for the receiver operating characteristic curve (ROC AUC), 95% confidence intervals, negative predictive values and positive predictive values were calculated for each model.<sup>20</sup> Data were analysed using GraphPad Prism (version 9.0; GraphPad Software, [www.graphpad.com](http://www.graphpad.com)) and R (version 3.6.3).<sup>21</sup>

### 3 | RESULTS

#### 3.1 | Horses

The study included 163 PSSM2-QH ( $8.4 \pm 4.3$  years, range 2-22 years; 84 mares, 73 geldings and 6 stallions) and 229 control-QH ( $8.6 \pm 4.9$  years, range 2-26 years; 179 mares, 45 geldings, 3 stallions and 2 unspecified). Age was not significantly different between PSSM2-QH and control-QH (T test,  $P = .66$ ). The greater proportion of female control-QH was due to the sampling of breeding farms. Breed distribution for PSSM2-QH was 137 QH, 17 Paints, 9 Appaloosas and for control-QH was 218 QH and 11 Paints.

#### 3.2 | Amylase-PAS and desmin staining

No QH was diagnosed with MFM based on the presence of desmin aggregates in six or more muscle fibres. All 163 of the PSSM2-QH had abnormal aggregates of polysaccharide in the PAS stain of which 23 had amylase-resistant polysaccharide. Four control-QH and four PSSM2-QH had one fibre/biopsy with a desmin aggregates that were either subsarcolemmal or <50% of the size of myonuclei.

#### 3.3 | Genotypes and allele frequencies

All four loci were successfully genotyped in each sample. No significant differences (all  $P > .05$ ) were found comparing the frequency

of any of the four P variants between PSSM2-QH and control-QH considering either the count of horses with each genotype, or the total number of variant alleles per locus (Tables 1-2, Tables S2-S3). The P2 variant occurred in 37.4% of PSSM2-QH and 42.4% of control-QH with an overall allele frequency of 0.21 in PSSM2-QH and 0.24 in controls (Table 1). There was no evidence of linkage disequilibrium between the P2 and P4 loci ( $r^2 < 0.001$ ). The P3a and P3b variants were in high, but not complete linkage disequilibrium with  $r^2 = 0.894$  overall (0.854 in PSSM2-QH and 0.937 in control-QH). The P3a variant occurred in 22.1% of PSSM2-QH and 14.4% of control-QH, with an overall allele frequency of 0.12 and 0.07, respectively (Table 1). The P3b occurred in 20.2% of PSSM2-QH and 14.0% of control-QH with an overall allele frequency of 0.11 in PSSM2-QH and 0.07 control-QH (Table 1). Considering locus P3 as a haplotype, 19.6% of PSSM2-QH and 14% of control-QH had a variant haplotype (both P3a and P3b present); these frequencies were not significantly different between phenotypes (Table S2). The P4 variant was present in 14.7% of PSSM2-QH and 12.2% of control-QH, with overall allele frequencies of 0.08 in PSSM2-QH and 0.06 in controls (Table 1). No statistical significance was found considering total number of variant alleles across all 3 loci, considering the two P3 loci independently or as a haplotype (Table 3; Table S3).

Overall, 60.7% of PSSM2-QH and 56.8% of control horses had one or more of the P variants, which was not statistically significant (Table 2, Figure 1). Multivariable logistic regression also failed to identify a significant contribution of any locus/loci to the phenotype but did identify a significant association between the P3a and P3b

TABLE 1 Count of horses with each genotype for the four P variant loci. P-values resulting from Fisher's Exact Tests are given considering both a genotypic and dominant model

	N	Homozygous reference	Heterozygous variant	Homozygous variant	% Horses with variant	Variant allele frequency	P-value for number of horses with variant allele	
							Genotypic	Dominant
<b>P2</b>								
Control-QH	231	132	83	14	42%	0.24	0.6	0.3
PSSM2-QH	163	102	53	8	37%	0.21		
MFM-QH	0							
<b>P3a</b>								
Control-QH	231	196	32	1	14%	0.07	0.1	0.06
PSSM2-QH	163	127	34	2	22%	0.12		
MFM-QH	0							
<b>P3b</b>								
Control-QH	231	197	32	0	14%	0.07	0.07	0.1
PSSM2-QH	163	130	31	2	20%	0.11		
MFM-QH	0							
<b>P4</b>								
Control-QH	231	201	27	1	12%	0.06	0.8	0.5
PSSM2-QH	163	139	23	1	15%	0.08		
MFM-QH	0							

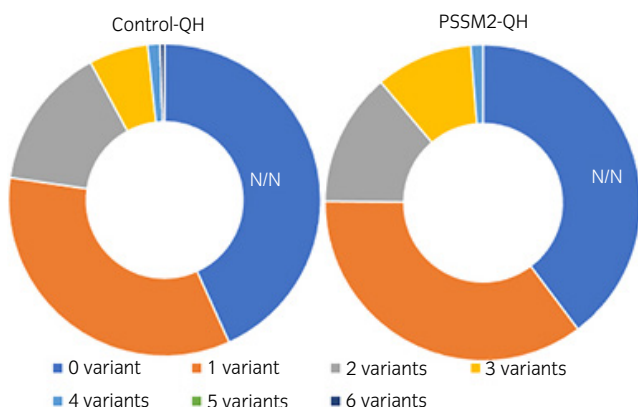
Abbreviations: PSSM2, Polysaccharide storage myopathy type 2; MFM, Myofibrillar Myopathy.

**TABLE 2** Count of horses that had none, one or more than one variant allele or locus considering each of the four loci as independent. Analysed using a  $3 \times 2$  Chi Square

	N	Zero variants	Percent with No variants	One variant allele/locus	% One variant allele/locus	> 1 variant alleles/loci	% >1 P variant allele/Locus	P-value (allele/locus)
Control-QH	231	99	43.2	78/90	34.0/39.3	52/40	22.7/17.5	0.7/0.5
PSSM2-QH	163	64	39.3	57/63	35.0/38.7	42/36	25.8/22.1	

**TABLE 3** Count of horses with none, one or two variant haplotypes at the P3 locus. A variant haplotype was defined as having both the P3a and P3b variant alleles. P-values were calculated using a  $3 \times 2$  Fisher's Exact Test

	N	No variant haplotypes (N/N, P3a/N, N/P3b)	Heterozygous variant (one haplotype P3a/P3b)	Homozygous variant (P3a/P3b, P3a/P3b)	% Horses with variant haplotype	Variant haplotype frequency	P value	
							Genotypic	Dominant
Control-QH	229	197	32	0	14%	7%	0.1	0.2
PSSM2-QH	163	131	30	2	20%	10%		

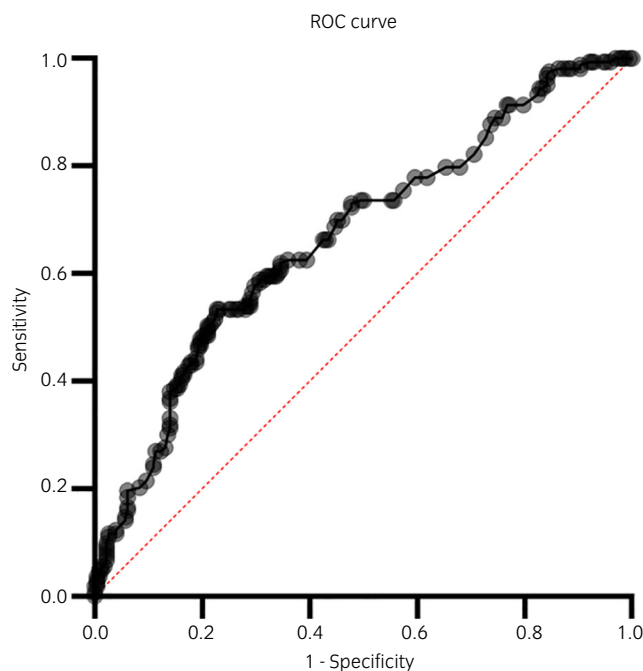


**FIGURE 1** The percentage of horses with no P variants (NN), 1 variant allele for any of P2, P3a, P3b or P4 (orange), 2 P variant alleles (grey), 3 variant alleles (yellow), 4 variant alleles (light blue), 5 variant alleles (green), or 6 variant alleles (navy) in 229 Quarter Horse controls and 163 Quarter Horses diagnosed with PSSM2 by muscle histopathology. There was no significant difference in the prevalence of any of the individual P variants, or a significant difference in the total number of variant alleles or loci between control and PSSM2/MFM

loci ( $R^2 = 0.89$ ) corresponding to the linkage disequilibrium between those loci. For the models including (1) either P3a and P3b genotypes (ie Models 1 and 2) or (2) both P3a or P3b genotypes (Model 3), the AUC for the receiver operator curve was 0.67 (95% CI), with a positive predictive value of 68% and negative predictive value of 63% (Figure 2).

### 3.4 | Sensitivity of commercial genetic test variants

The sensitivity for testing ranged from 0.39 (P3a) to a maximum of 0.44 (P2) (Table 4). Odds ratios ranged from 0.59 (P3a) to 1.23 (P2) and were not significant (Table 4).



**FIGURE 2** Receiver operator curve for multivariable logistic regressions with PSSM2 (yes/no) as the outcome variable and P variant genotypes defined as dependent binary variables (0 = homozygous reference, 1 = heterozygous and 2 = homozygous alternate alleles), including sex (as binary, male or female) and age (as continuous) as covariates. There was no significant contribution of any locus/loci to the phenotype. For the model including (1) both P3a and P3b genotypes or (2) either P3a or P3b genotypes, the area under the curve for the receiver operator curve was 0.67 (95% CI), with a positive predictive value of 68% and negative predictive value of 63%

## 4 | DISCUSSION

The first objective of our study was to determine if QH diagnosed with PSSM2 based on abnormal polysaccharide inclusions in myofibres also have myofibre desmin aggregates resembling those seen in

**TABLE 4** The values and confidence intervals (CI) for odds ratio (OR), sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for the P2, P3 and P4 variants in PSSM2-QH. None of the calculated values reached statistical significance ( $P < .05$ )

Variant	OR		Sensitivity		Specificity		PPV		NPV	
		CI		CI		CI		CI		CI
P2	1.23	0.82-1.84	0.44	0.37-0.50	0.61	0.54-0.69	0.63	0.55-0.70	0.42	0.36-0.49
P3a	0.59	0.36-1.1	0.39	0.34-0.45	0.48	0.37-0.59	0.78	0.71-0.84	0.14	0.11 to 0.20
P3b	0.64	0.38-1.08	0.40	0.35-0.45	0.49	0.38-0.61	0.80	0.73-0.85	0.14	0.10-0.19
P4	0.84	0.47-1.52	0.41	0.36-0.46	0.55	0.42-0.67	0.85	0.79-0.90	0.13	0.09-0.18

*Abbreviations:* CI, Confidence interval; NPV, negative predictive value; OR, odds ratio; PPV, positive predictive value.

Arabian and Warmblood horses with MFM.<sup>4,5</sup> Notably, not a single QH was identified as having MFM based on desmin staining, which was the means used to discover MFM in horses.<sup>4</sup> To ensure horses in the present study were old enough to have developed desmin aggregates, we included horses with a wide range of ages, with 33% of PSSM2-QH and 35% of control-QH  $\geq 10$  years of age and some as old as 26 years.<sup>5</sup> One fibre in two PSSM2-QH and two control-QH had subsarcolemmal desmin or a few small desmin aggregates, which were not considered abundant enough to be consistent with a diagnosis of MFM. If QH had been found to have significant desmin aggregates, electron microscopy and immunohistochemical stains for other ectopic proteins would have been indicated to confirm the diagnosis of MFM. These were not performed, however, because based upon our sample, MFM appears to be rare to nonexistent in QH. It is possible that if many additional QH were evaluated a case of MFM could have been identified.

Based on the lack of desmin aggregation in QH, and assertion that these P variants are predictive of disease, it was surprising that P variants were relatively common in the control horses. Given these genotype data, well over half (56.8%) of control horses had at least one P variant. The P2 and P3 variants in the tests offered as diagnostic of MFM are located in genes that have been associated with MFM in humans, but the gene location of the variants differs from the known human mutations responsible for MFM.<sup>11</sup> The location of a variant in a gene and the specific alteration in the amino acid sequence that may result is important because different amino acid substitutions have varying effects on the encoded protein's function and potential to cause disease. The impact of an amino acid substitution depends on whether the specific region of the protein that the amino acid occupies has any functional or structural significance. From our data, the amino acid substitutions resulting from the P variants do not cause desmin aggregation. The common presence of these P variants, combined with the lack of evident desmin aggregates, suggests that P variants are amongst the 400,000+ genetic variants found thus far in the equine genome<sup>22</sup> that change an amino acid sequence without having any apparent ill-effects such as desmin aggregation. As reported by Valberg et al,<sup>13</sup> the P2, P3 and P4 variants are found across many breeds, are hundreds (P4) to thousands (P2, P3a, P3b) of years old, and P2 was even present in one-third of 18 Przewalski horses which represent a feral population that does not share direct ancestry with the modern domestic horse.<sup>23</sup>

Although variation with a subtle negative impact on phenotype was likely perpetuated prior to domestication, predicted deleterious variants in samples of ancient horses were found at low frequencies, and were likely recessive in their mode of action<sup>24</sup>; variants with a truly deleterious impact on fitness, particularly dominant variants, are unlikely to have been perpetuated across many generations, especially prior to modern management. The absence of histopathological evidence of MFM in QH, coupled with the common presence of P variants in healthy control-QH, strongly indicates that P variants are not diagnostic of MFM, a finding in agreement with a previous study of Warmblood and Arabian horses with PSSM2 or MFM.<sup>13</sup>

In Warmblood and Arabian horses, the link between MFM and PSSM2 appears to be that the breaks that occur in myofibrils of MFM horses provide space for regional pooling of cytoplasmic glycogen.<sup>4,5</sup> Total concentrations of muscle glycogen are not elevated in Warmblood or Arabian horses with MFM.<sup>3-5</sup> Glycogen concentrations have yet to be reported in PSSM2-QH. The lack of desmin aggregation in PSSM2-QH in the present study, but presence in Arabians and Warmbloods, however, suggests a different aetiology exists for PSSM2-QH. In support of this, PSSM2-QH have a distinct clinical presentation of exertional rhabdomyolysis, whereas PSSM2 in Warmbloods is characterised by exercise intolerance with rare episodes of exertional rhabdomyolysis.<sup>3,5</sup> Clinical signs of exertional rhabdomyolysis are observed in Arabians with MFM; however, they often present with muscle stiffness and myoglobinuria after 50-100 km endurance rides whereas fulminant rhabdomyolysis and inability to move occurs after very little exercise in PSSM2-QH.<sup>4</sup>

The second objective of our study was to determine whether the commercial P variant genetic tests accurately reflected a diagnosis of PSSM2 in QH. The company website suggests that PSSM2 is an earlier form of MFM and claims that the presence of any one of the P variants indicates a susceptibility for PSSM2/MFM inherited in a dominant or semi-dominant manner. To determine this, we compared genotypes and allele frequencies between healthy control-QH and PSSM2-QH diagnosed by the means used to discover PSSM, PAS-positive glycogen aggregation.<sup>1</sup> To prevent bias in analysis, data were evaluated, analysed and interpreted by researchers at two universities independent of the laboratory performing the histopathological diagnosis. In contrast to the high prevalence of a putative causative variant in affected animals versus controls in validation studies of hyperkalemic periodic paralysis (100% of HYPP

affected horses had the suspect variant and 0% of controls)<sup>25</sup> and PSSM1<sup>26</sup> (78% of affected horses and 4% controls had the variant), there was no difference in the proportion of PSSM2-QH with one or more P variants compared with the controls (61% PSSM2-QH: 57% control-QH). Further, considering each locus independently, there were no statistically significant differences between the genotypic or allele frequencies of P2, P3a, P3b or P4 between the PSSM2-QH and control-QH. Although no linkage disequilibrium was found between the P2 and P4 loci, the two loci classified as P3, designated a and b in our study, were in high, but not complete, linkage disequilibrium; the lack of complete linkage disequilibrium was also found in public data queried in Valberg et al.<sup>13</sup> Because it was not clear how the two P3 loci are used in genetic test reports and because they are not in complete linkage disequilibrium in this sample, we analysed P3a and P3b independently as well as by considering the two loci as a single haplotype. No matter how P3 was considered, no analyses showed a significant association of that locus with PSSM2-QH even when using a liberal P value with no correction for multiple testing.

To investigate the possibility that more than one of the P variants or a combination of the P2, P3a, P3b or P4 variants contributes to PSSM2-QH, and to consider the impact of the combined, P3a/P3b genotype, we performed multivariable logistic regression analysis. Regardless of the model studied, the ROC failed to identify a quantitative impact of these alleles on PSSM2 as an ROC <0.7 is not considered an acceptable diagnostic test.<sup>27</sup> Thus, this further supports that these loci are not of value to accurately diagnose PSSM2-QH. If a combination of variants did contribute to the phenotype, the specific impact of each variant on phenotype (eg proportion of phenotypic variation explained) should be clearly reported in order to use this information for breeding or management decisions.

Based on our data, the use of any of the reportedly semi-dominant or dominant P2, P3 or P4 variant genotypes for diagnostic purposes would fail to identify a histopathologically confirmed diagnosis of PSSM2 in 40% of true cases. The odds ratios for diagnostic value of the P variants were not statistically significant. Based on these results, the most accurate means to diagnose PSSM2 and differentiate the many causes of exertional myopathies in horses remains a careful history, physical examination, serum creatine kinase activity, PSSM1 genetic test, and, in the absence of PSSM1, muscle histopathology.<sup>28,29</sup> There are some limitations, however, to a histopathological diagnosis of PSSM2. A risk of a false negative diagnosis of PSSM2 exists if there is a delay in shipping fresh muscle samples because this provides time for metabolism of glycogen during shipping. We eliminated the risk of a false negative PSSM2 diagnosis in the control-QH studied by freezing those samples immediately after they were collected via biopsy. If one was to argue that the control horses could develop PSSM2 after the biopsy, the fact that nearly 40% of horses confirmed to have PSSM2 with a histopathological diagnosis did not have any of the P variants further bolsters the lack of utility of P variants as diagnostic.

It is of particular concern that if purchase or breeding decisions were made based upon the results of the commercial tests, approximately 57% of QH would be eliminated by chance alone

(as determined by the allele frequencies in the control horses and considering P3 as variant if both P3a and P3b are present together). Removing any horse from breeding simply due to having a variant at any of these three loci could have a tremendously negative impact on the QH industry. To put the frequencies of these P variants into context, the most common genetic muscle disease reported to date in QH is PSSM1, which impacts <10% of the QH population.<sup>30</sup> It is therefore highly unlikely that nearly 60% of the healthy QH population would have PSSM2.

The equine genetics community has released a statement supporting peer-reviewed publications and outlining recommendations for steps required for test validation.<sup>31</sup> Peer-reviewed, published validation in this manner has been performed for equine muscle diseases such as hyperkalemic periodic paralysis,<sup>25</sup> glycogen branching enzyme deficiency,<sup>32,33</sup> PSSM1<sup>26</sup> and myosin heavy chain myopathy,<sup>34</sup> but is lacking for the P variants offered as diagnosis for PSSM2/MFM. The results of the present study clearly show that the first criterion for validation, a high frequency of the variant in affected and low frequency in controls, is not achieved for P variants reported to be diagnostic of PSSM2 and MFM in QH. Further, the second step in validation, demonstration that a variant has a functional effect conducive to developing the disease is also lacking as not only were the variants not associated with PSSM2 but no horses in the study, regardless of genotype, had MFM.

In conclusion, MFM is rare to nonexistent in QH based on muscle histopathology. That finding itself refutes the use of P variant testing as diagnostic of MFM in QH. Further, our results show that P variants, considered individually or in combination, lack a significant association with PSSM2 and thus are neither themselves causative nor diagnostic of PSSM2. Based upon these data, nearly 57% of QH are expected to have at least one P variant. A healthy horse or a horse with lameness or poor performance is therefore more likely than not to have a P variant. Thus, submission of a commercial genetic test will often yield a supposed explanation for the horse's problem. It is important, however, that it is understood that our research data confidently demonstrate that a "positive" result from the commercial genetic tests is not associated with the prevalence of or susceptibility to PSSM2. This outcome mirrors that of Valberg et al<sup>13</sup> with respect to MFM and PSSM2 in Warmblood and Arabians. In summary, based on our research there is no support for the use of commercial P variant tests in breeding and selection decisions, prepurchase examination or for diagnosis of a myopathy.

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#### CONFLICT OF INTERESTS

SJ Valberg runs the Neuromuscular Diagnostic Laboratory at Michigan State University that processes muscle biopsies through the Michigan State Veterinary Diagnostic Laboratory. She has no personal financial interest in muscle biopsy submissions. SJ Valberg with other colleagues license commercial laboratories to

perform type 1 PSSM testing and personally receives royalties. Some horses in the present study may have been tested by their owners in licensed PSSM1 laboratories. Other authors report no competing interests.

### ETHICAL ANIMAL RESEARCH

An Institutional Animal Care and Use Committee and Animal Use Form exemption for archived samples was in place for this study.

### INFORMED CONSENT

Materials were obtained from archives resulting from previous research studies and samples taken for clinical purposes. Specific owner consent for the current study was not stated.

### AUTHORSHIP

SJ Valberg contributed to data collection. ML Henry, D. Velez-Irizarry, K. Herrick and SJ Valberg contributed to laboratory analyses. CJ Finno and JL Petersen contributed to data analysis and interpretation. JL Petersen, SJ Valberg and CJ Finno contributed to manuscript preparation. All authors approved the final manuscript.

### PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1111/evj.13574>.

### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on reasonable request from the corresponding author.

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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