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# Candidate gene expression and coding sequence variants in Warmblood horses with myofibrillar myopathy

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## Abstract

**Background:** Myofibrillar myopathy (MFM) of unknown aetiology has recently been identified in Warmblood (WB) horses. In humans, 16 genes have been implicated in various MFM-like disorders.

**Objectives:** To identify variants in 16 MFM candidate genes and compare allele frequencies of all variants between MFM WB and non-MFM WB and coding variants with moderate or severe predicted effects in MFM WB with publicly available data of other breeds. To compare differential gene expression and muscle fibre contractile force between MFM and non-MFM WB.

**Study design:** Case-control.

**Animals:** 8 MFM WB, 8 non-MFM WB, 33 other WB, 32 Thoroughbreds, 80 Quarter Horses and 77 horses of other breeds in public databases.

**Methods:** Variants were called within transcripts of 16 candidate genes using gluteal muscle mRNA sequences aligned to EquCab3.0 and allele frequencies compared by Fisher's exact test

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AUTHOR CONTRIBUTIONS

Z.J. Williams, D. Velez-Irizarry and S.J. Valberg contributed to study conception and design. Z.J. Williams and D. Velez-Irizarry performed statistical analyses. All authors contributed to data collection and data analysis and interpretation. Z.J. Williams, D. Velez-Irizarry, S.J. Valberg, C.J. Finno and J.L. Petersen contributed to manuscript preparation. All authors approved the final manuscript.

CONFLICT OF INTEREST

No competing interests have been declared.

OWNER INFORMED CONSENT

All muscle biopsies obtained for research purposes were done so with owner informed consent.

DATA ACCESSIBILITY STATEMENT

The data that support the findings of this study are openly available in NCBI Sequence Read Archive at https://www.ncbi.nlm.nih.gov/sra and the European Variation Archive and https://www.ebi.ac.uk/eva/, reference numbers SRR10997329 to SRR10997344 and PRJEB30116 and PRJEB28306 respectively.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

among MFM WB, non-MFM WB and public sequences across breeds. Candidate gene differential expression was determined between MFM and non-MFM WB by fitting a negative binomial generalised log-linear model per gene (false discovery rate <0.05). The maximal isometric force/cross-sectional area generated by isolated membrane-permeabilised muscle fibres was determined.

**Results:** None of the 426 variants identified in 16 candidate genes were associated with MFM including 26 missense variants. Breed-specific differences existed in allele frequencies. Candidate gene differential expression and muscle fibre-specific force did not differ between MFM WB (143.1 ± 34.7 kPa) and non-MFM WB (140.2 ± 43.7 kPa) ($P = .8$).

**Main limitations:** RNA-seq-only assays transcripts expressed in skeletal muscle. Other possible candidate genes were not evaluated.

**Conclusions:** Evidence for association of variants with a disease is essential because coding sequence variants are common in the equine genome. Variants identified in MFM candidate genes, including two coding variants offered as commercial MFM equine genetic tests, did not associate with the WB MFM phenotype.

### Keywords

horse; myopathy; RNAseq; skeletal muscle; contractility

## 1 | INTRODUCTION

A diagnosis of myofibrillar myopathy (MFM) in Arabian and Warmblood horses (WB) is based on histological features that overlap with human MFM, including desmin aggregates and myofibrillar disarray in skeletal muscle fibres.[1,2] Clinical signs associated with MFM WB are usually apparent by 11 years of age and include exercise intolerance, a reluctance to move forward under saddle and a mild lameness not attributable to an underlying orthopaedic cause.[1–3] In humans, clinical signs of MFM are often apparent after the 4th decade of life[4–8] and include progressive muscle atrophy and the potential for respiratory compromise, cardiomyopathy and death.[4,5,9,10] Cardiac impairment, the severity and progression of muscle atrophy as well as the extent of ectopic protein aggregation appear to be more marked in human patients with MFM than MFM-affected WB.[1,11,12]

In horses, a potential familial basis for MFM has been suggested by the presence of desmin aggregates in a 3-generation family of WB.[1] In humans, MFM often has a familial basis with autosomal dominant[5,13–17] inheritance more common than autosomal recessive, however, incomplete penetrance and variable disease severity are documented.[18] The majority of MFM patients have mutations in 1 of 8 genes associated with protein chaperones or the Z-disc including desmin (*DES*),[19–21] αβ-crystallin (*CRYAB*),[15,22,23] myotilin (*MYOT*),[16,24,25] Z band alternatively spliced PDZ-containing protein (*LDB3/ZASP*),[14,26] filamin C (*FLNC*),[13,27] bcl-2-associated athanogene-3 (*BAG3*),[7,28] kyphoscoliosis peptidase (*KY*)[29,30] and pyridine nucleotide-disulfide oxidoreductase domain-containing protein 1 (*PYROXD1*).[8,31] These mutations have been confirmed to cause MFM through segregation analysis by DNA sequencing of family members and functional studies in animal models.[14,16,31–41]

The use of high-throughput, next-generation sequencing has increased the number of genetic variants associated with MFM and atypical MFM phenotypes, which are protein aggregate myopathies with more diverse clinical signs than classic MFM.[31,42] These variants are found in four-and-half LIM domain 1 (*FHL1*),[17,43–45] DNAJ/HSP40 homolog subfamily B, member 6 (*DNAJB6*),[31,46,47] plectin (*PLEC*),[47–49] lamin A/C (*LMNA*),[50,51] alpha-actin (*ACTA1*),[31,52] heat-shock 22-kd protein 8 (*HSPB8*)[31,53] and digenic mutations in sequestosome 1 and cytotoxic granule-associated RNA-binding protein (*SQSTM1* and *TIA1*).[54] This brings the total to 16 genes associated with an MFM-like histological phenotype in humans. The challenge presented by next-generation sequencing is discrimination between possible causative variants and the thousands of unassociated variants present in any genome.[31] Segregation analysis in well-phenotyped individuals, modelling and functional studies are cited by the American College of Medical Genetics and Genomics as necessary means to determine that variants identified through sequencing are actually causative disease mutations.[55] Currently, variants in Z disc-associated genes *MYOT* and *FLNC* are being offered as commercial genetic tests for equine MFM, however, information on the variants or their validation has not been published in the peer-reviewed literature (https://www.centerforanimalgenetics.com/pssm2/, http://www.equiseq.com/learn_pssm2).

We hypothesised that potential pathogenic coding sequence variants that associated with the MFM phenotype could be identified in MFM candidate genes using RNA-seq analysis of skeletal muscle from MFM-affected and control WB that had precise clinical and histological phenotypes. Furthermore, because some MFM mutations have been shown to alter contractile force, we hypothesised that the contractile force of muscle fibres would be altered in MFM WB.[33,34]

Our objectives were to identify variants in 16 candidate genes and determine if they associated with the MFM phenotype by comparing allele frequencies of coding sequence variants among MFM WB, non-MFM WB and a variety of other breeds. Additional objectives were to determine whether differential expression of candidate genes or differences in specific isometric contractile force of permeabilised isolated fibres from gluteal muscle existed in WB MFM compared with healthy WB (non-MFM).

## 2 | MATERIALS AND METHODS

### 2.1 | Warmblood selection

The database of the Equine Neuromuscular Diagnostic Laboratory was searched to identify WB horses with both a history of poor performance and exercise intolerance that had also been diagnosed with MFM based on the presence of cytoplasmic aggregates of desmin in myofibres. From these horses, MFM WB were selected for inclusion in the study if they had snap-frozen samples of gluteus medius muscle available.[1,2] Control non-MFM WB with no evidence of myopathic changes or desmin aggregation in muscle biopsies were selected for inclusion if they had no history of exercise intolerance and had available snap-frozen samples of gluteus medius muscle. The 8 selected MFM WB and 8 non-MFM WB were of similar age and sex (Table 1).

Contractile force testing was performed on gluteus muscle samples of 4 MFM WB (3 castrated males and 1 female) and 4 non-MFM WB (1 male, 1 castrated male and 2 females). Fewer horses were included in this analysis because of limitations in tissue availability following histological analyses and RNA extraction.

### 2.2 | RNA isolation

Total RNA was isolated from flash-frozen gluteus medius samples using TRIzol (Thermo Fisher Scientific)/Chloroform extraction after homogenisation with a bead homogeniser (Bullet Blender Storm 24, Next Advance Inc.) as previously described.[56] Samples underwent DNase (RNase-free DNase I, New England BioLabs, Inc.) treatments on columns (EconoSpin, Epoch Life Science) according to manufacturer's instructions. Samples then underwent quantification with a fluorometer (Qubit, Thermo Fisher Scientific, RNA HS Assay Kit, Thermo Fisher Scientific) and RIN scores were measured with an Agilent 2100 Bioanalyzer (Agilent Technologies) and Agilent RNA 6000 Pico Kit (Agilent Technologies); samples with RIN scores >7.0 were sequenced.

### 2.3 | RNA library preparation and sequencing

All libraries were constructed by the Michigan State University Genomics Core using the Illumina TruSeq Stranded mRNA Library Preparation Kit (Illumina) per manufacturer's instructions. All libraries underwent quality control and were quantified using Qubit dsDNA High Specificity (Thermo Fisher Scientific) and Caliper LabchipGX High Specificity (Caliper Life Sciences) DNA assays. Libraries were then pooled in equimolar concentration for multiplex sequencing. Kapa Biosystems Illumina Library Quantification qPCR kit (Kapa Biosystems) was used to quantify the pooled libraries. Samples were then loaded onto 2 lanes of an Illumina HiSeq 4000 flow cell (Illumina). Sequencing was performed in a 150 base pair (bp) paired-end format using HiSeq 4000 SBS reagents (Illumina) for a target of 35–40 million reads for each sample. Base calling was done by Illumina Real Time Analysis (Illumina) (v2.7.7) and output of RTA was sorted and converted into fastq format with Illumina Bcl2fastq (Illumina) (v2.19.1) for analysis.

### 2.4 | Candidate genes

The literature and the database Online Mendelian Inheritance in Man (http://www.omim.org) were used to identify variants known to be associated with human MFM types 1–8 which included genes *DES*, *CRYAB*, *MYOT*, *LDB3/ZASP*, *FLNC*, *BAG3*, *KY* and *PYROXD1*.[7,8,14–16,19–21,23–26,28–31,36] In addition, the genes *FHL1*, *DNAJB6*, *PLEC*, *LMNA*, *ACTA1*, *HSPB8*, *SQSTM* and *TIA1* were selected as candidate genes because mutations in these genes create overlapping features of MFM that include desmin aggregation, myofibrillar disarray, progressive muscle weakness and atrophy.[31] Mutations in these genes are associated with the diseases scapuloperoneal myopathy, limb-girdle muscular dystrophy, Emery-Dreifuss muscular dystrophy, nemaline myopathy, Charcot-Marie-Tooth, Welander distal myopathy and MFM-like inclusion body myopathy.[17,31,43–54]

### 2.5 | Assembly, mapping and variant calling

FastQC (0.11.7)[57] and MultiQC (1.7)[58] were used to evaluate the quality of all 150 bp paired-end RNA-seq reads. Trimmomatic (v0.38)[59] was used to filter adapter sequences and ConDeTri (v2.0)[60] was used to filter low-quality reads. A quality threshold of 30 ($Q \geq 30$) was used to filter samples. Next, Bowtie2 (2.3.2)[61] was used to index EquCab 3.0 from the National Center of Biotechnology Information (https://www.ncbi.nlm.nih.gov/assembly/GCF_002863925.1/) and Tophat2 (v2.1.1)[62] was used to map all the reads. SAMTools (v1.9)[63] was used to retain all reads that were uniquely aligned (76%). Cufflinks (v 2.2.1)[64] was used to assemble the transcriptome. HTSeq (v0.11.1)[65] was used to count the number of normalised gene reads aligning to the genes of interest with expression abundance observed across all horses in this study. Coding SNP (cSNP) were called directly from the transcriptome using with SAMTools bcftools mpileup (v1.9.64).[66] Called variants were retained for downstream analysis if genotypes were called in at least 14 of 16 horses (≥88%) and had a Phred score of ≤30 and ≥10 reads. Based on founder effects within equine breeds, we assumed that causative mutations would be shared across MFM WB.[67] Therefore, if a mutation in a candidate gene had a low allele frequency in MFM WB compared with non-MFM WB and nonphenotyped WB, it likely would not be the cause of WB MFM. Codes written to perform the data analysis are available in the GitHub repository https://github.com/NMDL-MSU/MFM-Warmblood_CandidateGenes

### 2.6 | Variant predictions

Ensembl's Variant Effect Predictor (v98)[68] (https://useast.ensembl.org/Tools/VEP) was used, referencing EquCab 3.0 to specify variant location, to predict the effect defined as synonymous, frameshift or missense based on the nucleotide and -associated amino acid substitution, and estimate the impact score defined as low, moderate or severe.

### 2.7 | Other breed populations

Whole-genome and RNA sequences obtained previously were compiled from 33 WB, 32 Thoroughbreds (TB), 80 Quarter Horses (QH) and 77 other breeds (OB) using data from the Equine Neuromuscular Diagnostic Laboratory collaborators, and data from the European Variation Archive (https://www.ebi.ac.uk/eva/) (accession numbers PRJEB30116, PRJEB28306).

The NCBI remap tool (https://www.ncbi.nlm.nih.gov/genome/tools/remap) was used to remap variants found in EquCab 3.0 (MFM and non-MFM data) to EquCab 2.0 (publicly available data) for sequencing data that were mapped to second version of the equine genome.

### 2.8 | Allele frequency and gene expression

Allele frequencies from all identified variants within candidate genes were compared between MFM WB and non-MFM WB using a Fisher's exact test, with *P* values adjusted using the Benjamini-Hochberg method (false discovery rate (FDR) <0.05). Allele frequencies of coding sequence variants of medium to high effects (missense and frameshift variants) were further compared among MFM WB (n = 8), non-MFM WB (n = 8) and public databases containing nonphenotyped WB (n = 33), TB (n = 32), QH (n = 80) and OB (n =

77) using a Fisher's exact test and the Benjamini-Hochberg method for multiple testing (FDR < 0.05) in R (v3.5.1). All significant results were followed by post hoc testing consisting of pair-wise comparison and corrected for multiple testing again with the Benjamini-Hochberg method (FDR < 0.05) in R (v3.5.1).

For the MFM WB and non-MFM WB, raw read counts per gene were normalised using the trimmed mean of M-values (TMM).[69] Differential expression was determined by fitting a negative binomial generalised log-linear model per gene with diagnosis of MFM as coefficient of interest using EdgeR (v3.24.3)[70] and corrected with the Benjamini-Hochberg method with an FDR < 0.05.

### 2.9 | Single myofibre force production

On the day of experiment, 6–7 single myofibres per horse were dissected from muscle biopsy specimens in a relaxing solution. Relaxing and activating solutions contained 4 mmol/L Mg-ATP, 1 mmol/L free $Mg^{2+}$, 20 mmol/L imidazole, 7 mmol/L EGTA, 14.5 mmol/L creatine phosphate and KCl to adjust the ionic strength to 180 mmol/L and pH to 7.0. The concentrations of free $Ca^{2+}$ were pCa ($-\log_{10}[Ca^{2+}]$) 9.0 mol/L (relaxing solution) and pCa 4.5 mol/L (activating solution). Myofibres were then individually attached between connectors leading to a force transducer and a lever arm system (model 1400A; Aurora Scientific). Sarcomere length was set to ≈2.50 μm and the temperature to 15°C. Fibre cross-sectional area (CSA) was estimated in $\mu m^2$ from the width and depth, assuming an elliptical circumference. The absolute maximal isometric force generation was calculated as the difference between the total tension in the activating solution (pCa 4.5) and the resting tension measured in the same myofibre while in the relaxing solution (pCa 9.0). Specific force was defined as absolute force divided by CSA (KPa). The specific force per myofibre was recorded and means were compared in MFM (n = 27 fibres) vs. non-MFM WB (n = 27 fibres) using an unpaired *t* test after normality was confirmed with the Shapiro-Wilk method using Prism (GraphPad) (v8.0).

### 2.10 | Fibre-type composition

Muscle fibre-type composition was determined on gluteus medius muscle that had been frozen in isopentane suspended in liquid nitrogen. Six-μm-thick sections were pre-incubated for 5 minutes at pH 4.4 and ATPase staining performed as previously described.[71] The percentage of type 1 and type 2 fibres in approximately 500 myofibres was calculated and compared between MFM and non-MFM WB by an unpaired *t* test in Prism (GraphPad) (v8.0). Fibre-type counts were not reported for 2 MFM horses due to insufficient sample remaining.

## 3 | RESULTS

### 3.1 | Variant identification and prediction

After filtering, 426 variants were found in the 16 candidate MFM genes of MFM WB and non-MFM WB. *LDB3/ZASP* had the most identified variants (n = 126) and *CRYAB* only had one identified variant (Figure 1). The identified variants had 3857 different predicted annotations depending on isoform expression. Of these variant annotations, 1161 were

synonymous (30.0%), 454 were missense (11.7%), 0 were frameshift and the rest were categorised as splice site, 5′ untranslated region (UTR), 3′ UTR, noncoding, intronic, upstream or downstream.

Ninety of the variants were annotated in exons of 13 genes *ACTA1*, *BAG3*, *DES*, *DNAJB6*, *FLNC*, *HSPB8*, *KY*, *LDB3/ZASP*, *LMNA*, *MYOT*, *PLEC*, *PYROXD1* and *SQSTM1*. No coding variants were found in *CRYAB*, *FHL1* or *TIA1*. Of the 90 coding variants, 62 were synonymous with a low predicted impact score, 2 were splice region variants with predicted low impact, 26 were missense with predicted impact of moderate and 0 had high predicted impacts. The 26 variants with moderate predicted effects were identified in 11of the 13 genes (not present in *ACTA1* and *HSPB8*) (Figure 1).

### 3.2 | Variant allele frequencies

None of the 426 identified variants, regardless of location within the candidate gene or predicted effects, significantly associated with the MFM WB phenotype when comparing the MFM WB to the non-MFM WB. The allele frequencies of the 26 missense variants with moderate predicted effects were not significantly different among the MFM WB, non-MFM WB and the nonphenotyped WB from publicly available data (Figure 2, Table S1).

### 3.3 | Breed effect

When comparing WB, TB, QH and OB in a pair-wise fashion, 8 of the missense variants with moderate predicted effects had significantly different AF after multiple test correction. This included *LDB3* (1 variant), *PYROXD1* (2 variants), *PLEC* (3 variants), *MYOT* (1 variant) and *KY* (1 variant) (Table 2). There was an insufficient number of quality reads for *DES* chr6:8696183 in the nonphenotyped WB (n = 33), QH (n = 80) and OB (n = 77) for evaluation. Additionally, some variants did not have enough data to meet read count requirements and quality thresholds for certain horses within a breed population. *PLEC* chr9:84703610 and *KY* chr16:71665337 only had data in one horse in the nonphenotyped WB (n = 33).

### 3.4 | Differential gene expression

All candidate genes passed quality control and filtering criteria for differential expression analyses. The normalised coverage for all of the candidate genes averaged 13.64 counts per million ± 0.28 for the non-MFM WB and 13.65 ± 0.28 counts per million for MFM WB (Figure 3, Table S2). There was no significant differential expression for any of the 16 MFM candidate genes between MFM WB and non-MFM WB with $\log_2$-fold changes ranging from −0.3 to 0.3 and $P_{adj}$ ranging from .3 to .9 (Figure 3, Table S2).

### 3.5 | Fibre types and contractile force

Muscle fibre-type composition did not differ ($P = .2$) between MFM (type 1: 26% ± 11%, type 2: 74% ± 11%) and non-MFM WB (type 1: 32% ± 6%, type 2: 68% ± 6%) (Table 1). Mean CSA of membrane-permeabilised muscle fibres was not significantly different ($P = .5$) between MFM WB (3365.4 ± 992.4 $\mu m^2$) and non-MFM WB (3570.1 ± 1099.0 $\mu m^2$). The coefficient of variation (CV) for fibre CSA ranged from 24.1% to 29.0% for MFM WB and 27.3% to 40.5% for non-MFM WB. Specific myofibre force was normally distributed and

there was no significant difference in specific myofibre force generated ($P = .8$) between MFM WB (143.1 ± 34.7 kPa) and non-MFM WB (140.2 ± 43.7 kPa) (Figure 4). The CV for specific force generated by muscle fibres within the same horse ranged from 10.3% to 29.5% for MFM WB and 15.5% to 42.3% for non-MFM WB (Figure 4). The power to detect a 50% difference in force generation using our sample size of 27 fibres per group, $\sigma$ of 0.40 and $\alpha$ of 0.05 was 0.44 and to determine an 80% difference in force the power was 0.82.

## 4 | DISCUSSION

Following a clinical and histopathologic diagnosis of MFM in humans, exome sequencing of candidate genes is one approach to identify potential causative mutations.[72,73] RNA-seq has also been used to examine coding variants in expressed transcripts in diseases ranging from muscular dystrophy to acute myeloid leukaemia.[74–76] In the absence of exome sequencing for equine myopathies, we used RNA-seq data from skeletal muscle of MFM WB to identify potential causative variants in candidate genes that were selected based on histopathologic features of desmin aggregate myopathies. The American College of Medical Genetics and Genomics has guidelines for the interpretation of DNA sequence variants that recommends a process for classification of variants as ‘pathogenic’, ‘likely pathogenic’, ‘uncertain significance’, ‘likely benign’ and ‘benign’ based on criteria derived from clinical, population, computational, functional and segregation data.[55] As this nomenclature does not exist for equine medicine, variants identified in MFM and non-MFM horses were classified using Ensembl’s variant effect predictor. Our investigation included 2 coding variants in *FLNC* (chr4: 83738769 EquCab 2.0; chr4: 83840299 EquCab 3.0) and *MYOT* (chr14: 38519183 EquCab 2.0; chr14: 37818823 EquCab 3.0) that are commercially offered as a genetic test for equine MFM and type 2 polysaccharide myopathy (PSSM2) (patent # WO2017165733A1). Data regarding the predictive value or functional impact of these variants thus far have not been made available in a peer-reviewed publication.

We identified over 400 variants in the 16 candidate genes, including 26 missense variants with “moderate” impact scores and we found allele frequencies differed significantly among breeds. The abundance of variants, including missense variants, detected in healthy and diseased horses in the present study agrees with a recent report that found 23.5 million single nucleotide variants in whole-genome sequencing data from 88 horses of different breeds.[77] Combined, these findings emphasise the importance of validating purported disease-causing variants using appropriate breeds as control populations before inferring pathogenicity.

We examined allele frequencies to determine whether a significant association existed between a variant and the MFM phenotype. None of the 426 identified variants in our study associated with the MFM WB phenotype. For coding variants with moderate to high predicted effects, at most 5 of 8 MFM horses were heterozygous or homozygous for 1 of 5 variants, however, between 3 and 7 of the 8 non-MFM WB also were heterozygous or homozygous for these same variants. Heterozygosity or homozygosity is assuming an absence of allele-specific expression. Analysis of 33 additional nonphenotyped WB found no difference in AF for any of the coding sequence variants with moderate to high predicted

effects among MFM, non-MFM and other WB. Thus, the identified variants did not significantly associate with MFM WB. To date, the genetic myopathies identified in horses have been monogenic and given equine founder effects and the structure of horse breed types, it is reasonable to hypothesise that horses sharing disease pathology also share the same causative variant.[67] Polygenic inheritance is certainly possible, however, inspection of the heat map generated for variants in 16 MFM candidate genes in all horses did not identify a consistent pattern in which a set of multiple variants were present in MFM WB and not present in controls (Figure 2).

To evaluate all potential missense variants in 16 candidate genes, we used RNA-seq analyses of 8 well-phenotyped MFM horses that had numerous desmin-positive fibres. This sample size was limited due to cost and the availability of snap-frozen muscle samples. Future analysis of other proposed putative mutations for MFM could include targeted sequencing of genomic DNA in a larger number of MFM-affected horses. The possibility remains that the MFM phenotype is strongly influenced by environment and post-translational modifications.

The *FLNC* and *MYOT* coding variants commercially offered as a genetic test for equine MFM and PSSM2 did not associate with the MFM WB phenotype. The two variants were present in similar allele frequency in MFM WB (13% for *FLNC*, 19% *MYOT*), non-MFM WB (0% for *FLNC*, 13% *MYOT*) and 33 other WB horses (8% for *FLNC*, 8% *MYOT*). The V238A *MYOT* variant is predicted to be “tolerated” by SIFT classification and the same position in human *MYOT* encodes 1 of 3 different amino acids (N238, T238 and Y238) depending on isoform expression.[78,79] Thus, this region is either not conserved across species or there are other isoforms not yet annotated in the current version of the equine genome. The results of the present study demonstrate that these variants do not associate with MFM in WB and do not support the use of these commercial tests to identify horses at risk of MFM. Therefore, at the present time, skeletal muscle biopsies remain the gold standard to identify MFM in horses.

The expression of the 16 MFM candidate genes in skeletal muscle did not differ between MFM WB and non-MFM WB. In human MFM, mutations in *DES, FLNC* and *LDB3/ZASP* have been shown to increase gene expression, whereas mutations in human *MYOT* and mice W2710X *FLNC* have no impact on gene expression when compared with healthy/wild-type controls.[80,81] A similar variable effect of mutations on gene expression is reported for equine genetic myopathies with increased *GBE1* expression in glycogen branching enzyme deficiency and no change in *GYS1* expression in type 1 polysaccharide storage myopathy.[82,83] Our gene expression results alone did not rule out any of the candidate genes as a whole as causative of MFM because genetic mutations can still impact protein translation or protein function without necessarily altering the abundance of mRNA transcript expression.

Limitations to using RNA-seq data to investigate candidate genes include evaluation of only annotated transcripts expressed in the tissue sampled (skeletal muscle) at the time of sample acquisition, a variability in the depth of coverage achieved, reduced ability to quantify lowly expressed genes and difficulty in mapping short-read data to repetitive regions. The normalised coverage of the 16 genes evaluated in the present study was more than adequate, met all filtering criteria and did not identify any variants associated with the MFM WB

phenotype. However, the other regions in these genes cannot be ruled out due to the limitations of RNA-seq. Whole-genome sequencing could be used to assess intronic regions in WB MFM horses. A genome-wide association study would ideally be used to identify a genomic locus for WB MFM prior to candidate gene sequencing. However, more horses with muscle biopsy phenotypes than were available for the present study would be required to achieve the statistical power needed for genome-wide association.[84] Genetic studies in WB are complicated by the fact that many WB breed registries do not have a closed stud book and horses of a variety of breeds may be registered as WB. The MFM WB in the present study came from a wide variety of WB registries or were crossbred. At this time, a heritable basis for WB MFM has yet to be definitively established.

A preliminary investigation into the absolute isometric force generated by muscle fibres was undertaken in order to determine whether MFM has a major impact on myofibre contractile function in WB. Mouse models of muscular dystrophies, nemaline myopathies and centronuclear myopathies have shown a decrease in specific force generated in affected mice using 22–38 isolated muscles or isolated fibres per disease/control group.[85–87] Similarly, mouse models of MFM have shown decreased absolute force and maximum specific isometric force generation such that a *MYOT* mutation decreased whole muscle-specific force by 24%.[33,34] Our preliminary study of 27 fibres from MFM and 27 fibres from non-MFM WB did not identify an effect of MFM on absolute maximal isometric force generated per CSA among samples with similar muscle fibre-type compositions. The power to detect difference in our study was, however, limited and power calculations indicate that the sample size available would only have detected a large effect size. Thus, our results did not rule-out a more subtle effect of MFM on specific fibre force or an isolated effect of MFM on fibres with desmin aggregates. An ideal study would have evaluated contractile force in a larger number of fibres of known fibre type, particularly those that contained desmin aggregates. It would have been impossible, however, to evaluate enough fibres to perform such a study due to limited percutaneous needle biopsy sizes available and the very small number of type 2 fibres in MFM gluteal muscle that contain desmin aggregates.[1]

In conclusion, the detection of numerous variants, including coding sequence variants, within the 16 candidate genes examined underscores the importance of validating potential disease-causing variants by comparing variant allele frequencies between well-phenotyped affected and control populations. In our study, none of the identified variants in candidate genes associated with the MFM WB phenotype including two variants offered commercially as equine genetic tests for MFM; these variants had low allele frequencies of only 25% and 13% in MFM WB horses respectively.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ETHICAL ANIMAL RESEARCH

All research was approved by Michigan State University IACUC AUF # 04/16-045-00.

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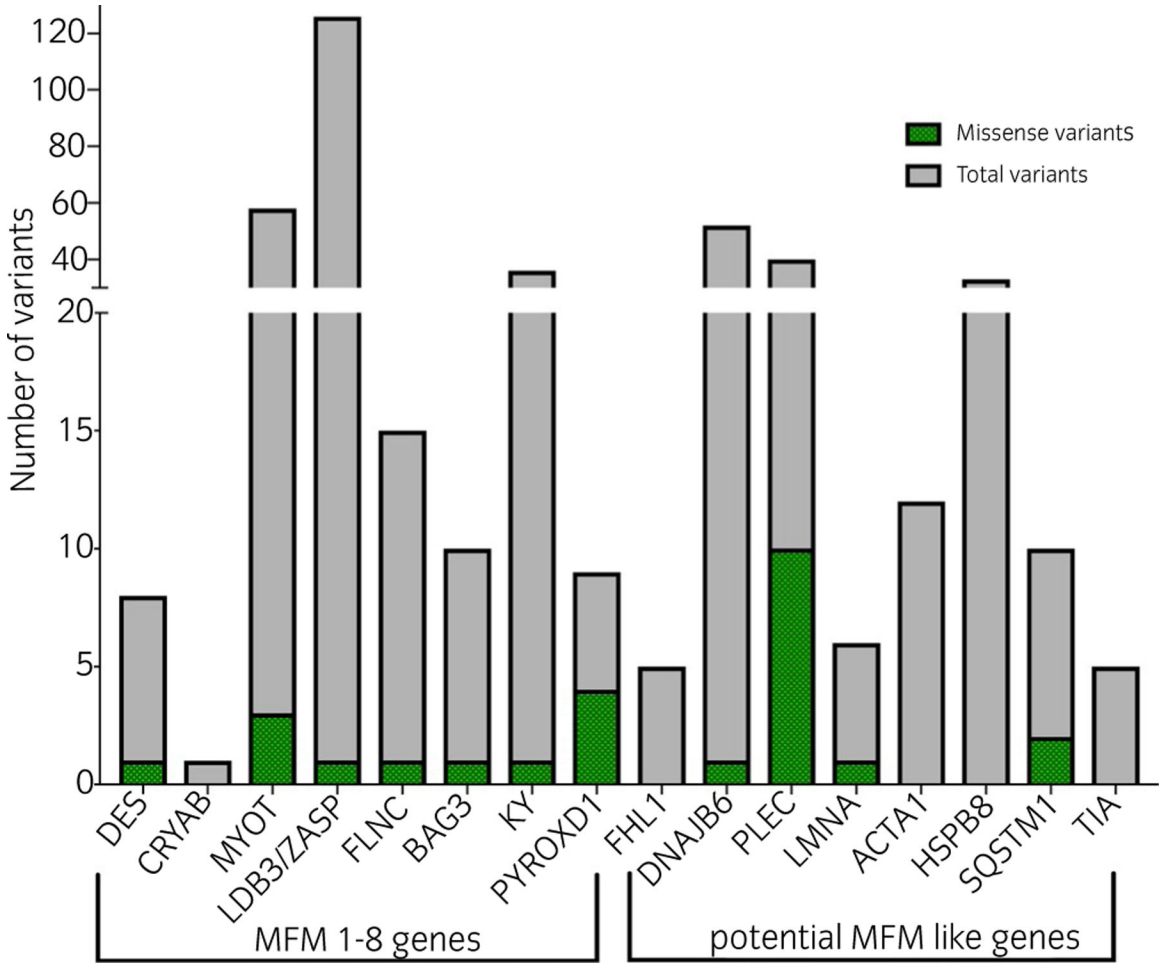


**FIGURE 1.**

The total number of identified variants and missense coding variants found in the 16 candidate genes from RNA-seq data of gluteal muscle from 8 MFM WB and 8 non-MFM WB. No statistically significant association was found between any of the variants and the MFM phenotype

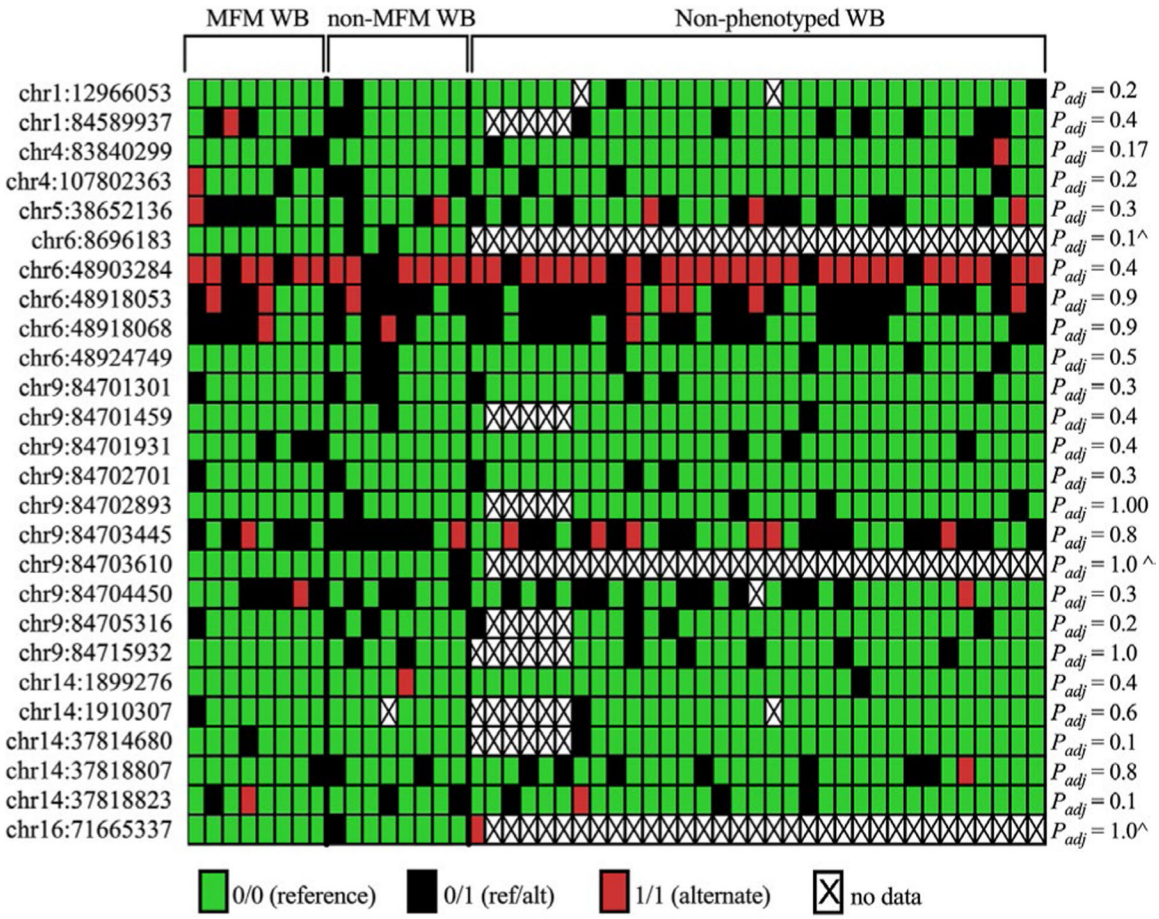


**FIGURE 2.**

A heat map of the 26 detected coding sequence variants of moderate-to-high effects for MFM, non-MFM and non-phenotyped WB shown for individual horses. Each identified variant is noted by its chromosomal location per row and each column references a horse grouped with its phenotype. There were no significant differences among WB groups. ^Indicates that there were minimal data for comparison in the nonphenotyped WB group

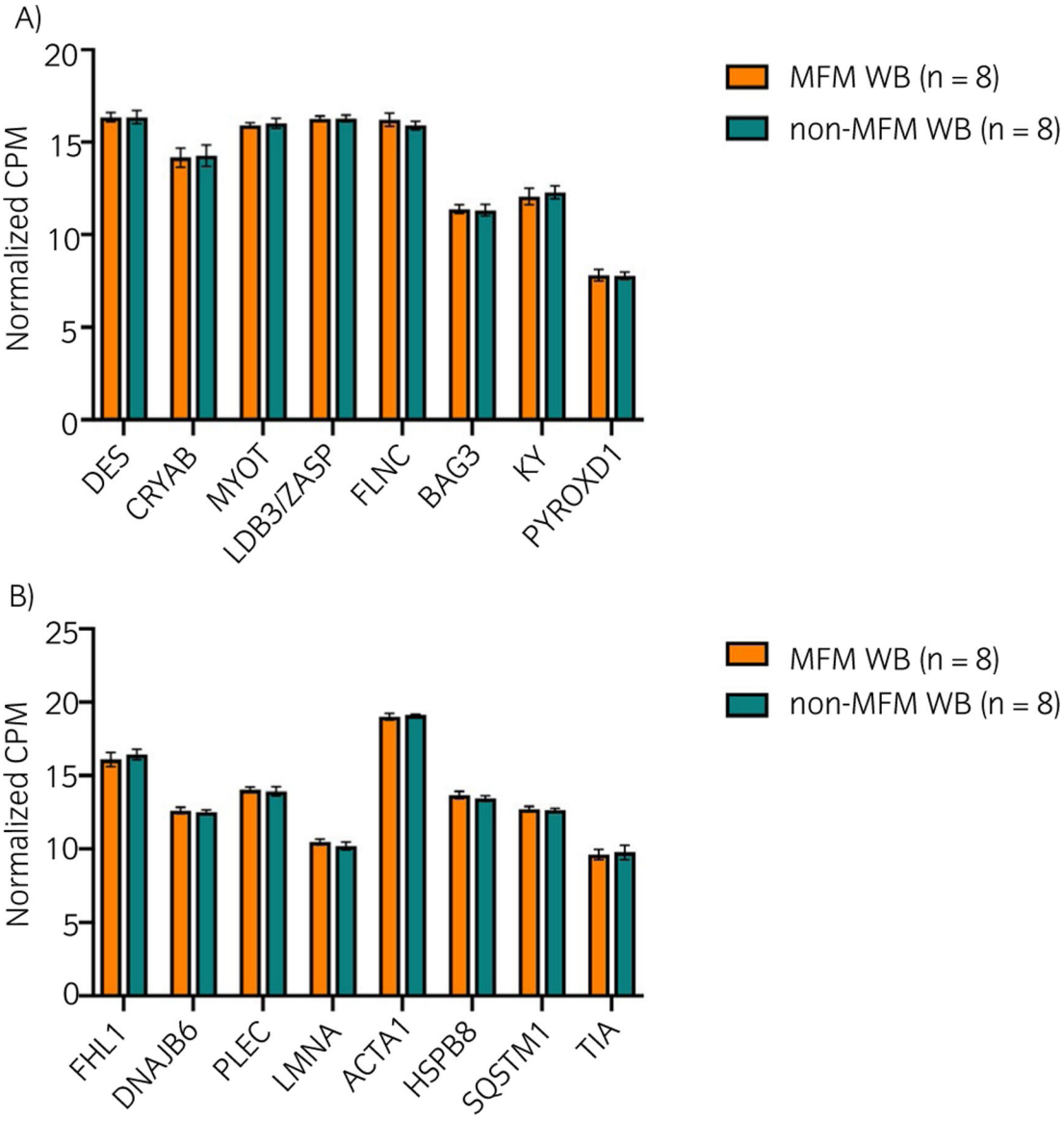


**FIGURE 3.**

A, Expression of the 8 genes known to cause MFM types 1–8 reported by normalised counts per million reads (CPM). B, Expression of the 8 additional genes known to have MFM-like features reported by normalised CPM. There were no significant differences in gene expression between MFM WB and non-MFM WB

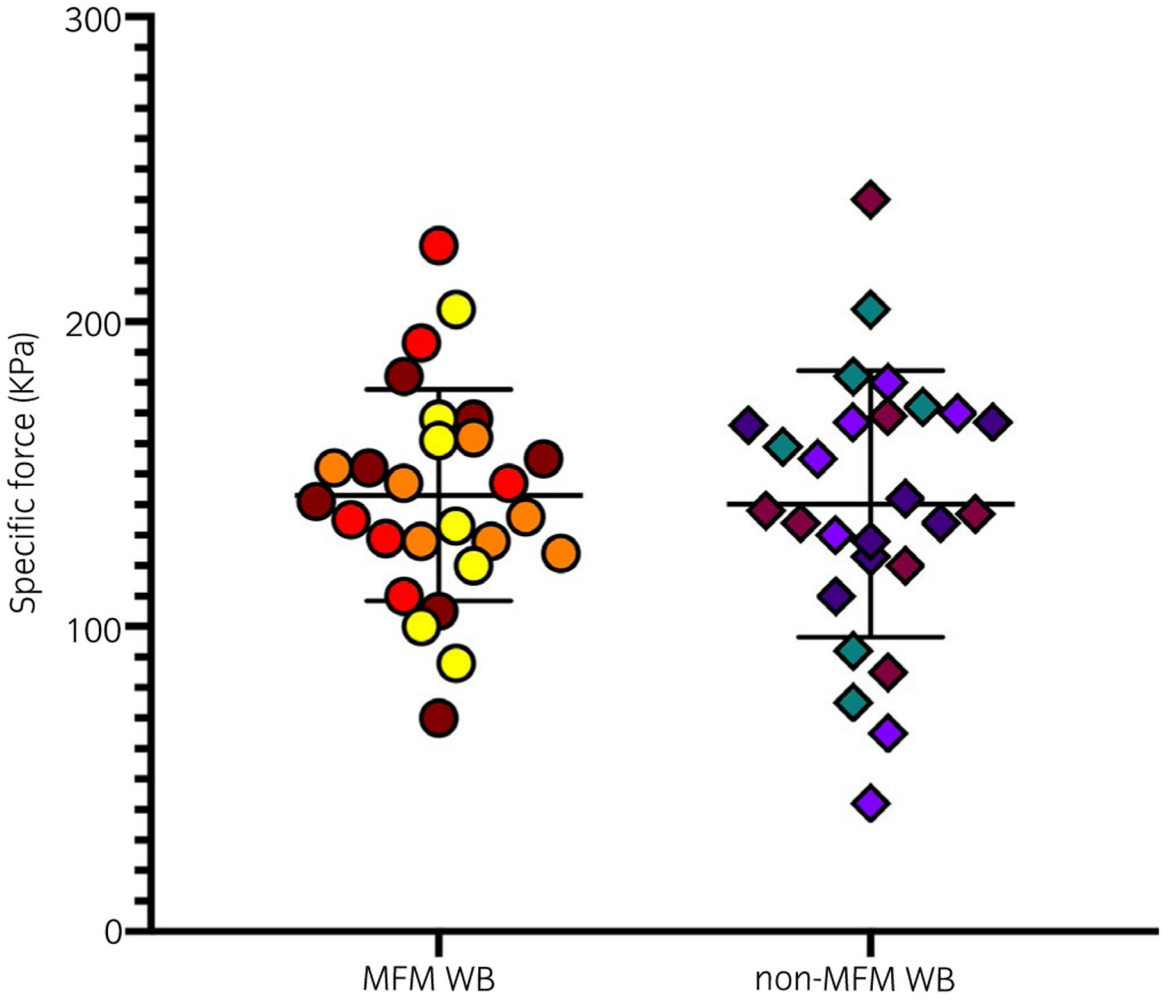


**FIGURE 4.**

Specific force for individual muscle fibres from MFM and non-MFM WB. The specific force (kPa) was not different between MFM and non-MFM WB ($P = .8$). Each point corresponds to single permeabilised fibre. Fibres from the same horse are indicated by the same colour

## TABLE 1

Breed, sex, age and muscle fibre-type composition of individual horses and their use for muscle fibre contractile force studies

| | Sex | Age (y) | % type 1 fibres | % type 2 fibres |
|---|---|---|---|---|
| MFM WB (n = 8) | 4 MC, 3 F, 1 M | 12 ± 4 | 26 ± 10 | 74 ± 10 |
| non-MFM WB (n = 8) | 4 MC, 3 F, 1 M | 13 ± 4 | 32 ± 6 | 68 ± 6 |

Abbreviations: WB, Warmblood; MFM, myofibrillar myopathy; M, stallion; MC, male castrate; F, female.

**TABLE 2**

Missense variant allele frequencies that were significantly different between breeds. Allele frequencies did not differ among MFM WB, non-MFM WB or publicly available sequences of nonphenotyped WB. There were significant differences in allele frequencies when comparing WB groups with Thoroughbreds (TB), Quarter Horses (QH) and other breeds (OB)

| Gene | Variant | Breed with highest alternate allele frequency | Breed comparison | *Padj* |
|---|---|---|---|---|
| *LDB3/ZASP* | chr1: 84589937 T/C | MFM WB (n = 8) | TB (n = 29) | .01 |
| | | Nonphenotyped WB (n = 28) | TB (n = 29) | .02 |
| | | OB (n = 71) | nonphenotyped WB (n = 28) | .03 |
| | | QH (n = 66) | TB (n = 29) | .01 |
| | | OB (n = 71) | TB (n = 29) | <.001 |
| | | OB (n = 71) | QH (n = 66) | .01 |
| PYROXD1 | chr6: 48918068 G/A | Nonphenotyped WB (n = 33) | QH (n = 79) | .01 |
| | | QH (n = 79) | OB (n = 77) | <.001 |
| PLEC | chr9: 84702893 A/G | OB (n = 70) | TB (n = 29) | .03 |
| | | OB (n = 70) | QH (n = 66) | .03 |
| | chr9: 84704450 T/C | MFM WB (n = 8) | TB (n = 32) | .03 |
| | | MFM WB (n = 8) | QH (n = 41) | .03 |
| | | Nonphenotyped WB (n = 32) | QH (n = 41) | .04 |
| | | OB (n = 75) | QH (n = 41) | .03 |
| | chr9: 84715932 C/A | Nonphenotyped WB (n = 27) | OB (n = 52) | .06 |
| SQSTM1 | chr14: 1899276 G/A | Non-MFM WB (n = 8) | OB (n = 76) | .06 |
| | | QH (n = 80) | OB (n = 76) | .06 |
| MYOT | chr14: 37818807 A/G | QH (n = 41) | TB (n = 32) | .01 |
| | | OB (n = 77) | TB (n = 32) | .01 |
| KY | chr16: 71665337 C/T | QH (n = 19) | MFM WB (n = 8) | <.001 |
| | | OB (n = 9) | MFM WB (n = 8) | <.001 |
| | | QH (n = 19) | non-MFM WB (n = 8) | <.001 |
| | | OB (n = 9) | non-MFM WB (n = 8) | <.001 |
| | | QH (n = 19) | TB (n = 28) | <.001 |
| | | OB (n = 9) | TB (n = 28) | <.001 |