MYOFIBRILLAR MYOPATHY IN WARMBLOOD HORSES: PROFILING THE ETIOPATHOLOGY AND ELUCIDATING MOLECULAR CHARACTERISTICS

By

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ABSTRACT

MYOFIBRILLAR MYOPATHY IN WARMBLOOD HORSES: PROFILING THE ETIOPATHOLOGY AND ELUCIDATING MOLECULAR CHARACTERISTICS

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Myofibrillar myopathy (MFM) is a protein aggregate disease that causes degeneration and loss of function of skeletal muscle and sometimes cardiac muscles. The clinical signs of this disease in people are heterogenous and can include distal limb weakness, atrophy, myalgia, loss of mobility, cardiomyopathy, and respiratory compromise. Approximately half of MFM cases have heritable etiologies that range from dominant to recessive. As many as eight genes have been found to cause MFM and eight additional genes have been implicated in protein aggregate myopathies similar to MFM. Classically, MFM is a progressive late onset disease affecting adults; however, juvenile forms do occur. Currently, there is no treatment for MFM. Research into the pathogenesis and potential treatments of MFM, as a whole, can be difficult due to variation in genetic causes, potential for environmental triggers, and disparity in clinical signs among patients. As a complex disease, a naturally occurring animal model may offer a unique perspective into the mechanisms of protein aggregation in skeletal muscle and present the opportunity for translational medicine and research.

MFM has been diagnosed in Warmblood (WB) horses based on the hallmark features of MFM in skeletal muscle: ectopic aggregation of a cytoskeletal protein called desmin coupled with myofibrillar disarray. WB with MFM were previously thought to have a glycogen storage disease and were placed on a recommended diet and exercise program to help manage horses with polysaccharide storage myopathies. However, recent studies found that WB diagnosed with

type 2 polysaccharide storage myopathy (PSSM2) did not have elevations in skeletal muscle glycogen. This finding led to the immunohistochemical staining for desmin and MFM diagnosis. It was unknown whether a diet recommended for a glycogen storage disease was beneficial for PSSM2 and MFM WB. Furthermore, the etiopathology of WB MFM was undetermined.

The overarching objective of this dissertation is to evaluate the responses of PSSM2/MFM WB to the recommended polysaccharide storage myopathy diet (PSSM) and further elucidate the etiopathology of MFM in WB by identifying candidate genes, proteins, and pathways that drive aberrant cellular responses and disease pathology. The first study used a retrospective questionnaire to determine owner perceived symptoms and responses of PSSM2/MFM WB to the PSSM diet. We determined that PSSM2/MFM WB had a decline in athletic performance and a reluctance to engage their muscles during work. Furthermore, we confirmed that PSSM2/MFM WB do not have elevations in skeletal muscle glycogen. The second study of this dissertation took a candidate gene approach to look for differential expression (DE) and coding sequence variants in 16 candidate MFM genes in MFM and non-MFM WB. Our results found there was no DE of the candidate genes and that no variants associated with the MFM disease phenotype. We next used transcriptomic and proteomic profiling to detect DE genes and proteins in MFM WB. Through pathway enrichment, we identified the Z-disc, mitochondrial complex I, and the extracellular matrix as key cellular components in MFM etiopathology. The identified new candidate genes and proteins using this multi-omic approach may serve as future diagnostic markers for MFM in WB. Overall, our results suggest MFM WB have aberrant Z-disc mechanosignaling resulting in sarcomere instability, which is further confounded by impaired redox homeostasis.

To my Mum and Dad, your support and love means more than words. Thank you.
To my big brother: what now, Brain?

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KEY TO ABBREVIATIONS

Ca^{2+}	Calcium
ACh	Acetylcholine
ACN	Acetonitrile
ACTA1	α-actin
ACTB	β–actin
AF	Allele frequency
Ankrd2	Ankyrin Repeat Domain 2
ATF3	Activating Transcription Factor 3
ATP	Adenosine triphosphate
BAG3	bcl-2-associated athanogene-3
BCS	Body condition score
CAT	Catalase
CHAC1	Glutathione specific gamma-glutamylcyclotransferase1
CPM	Counts per million
CRYAB	αβ-crystallin
CSA	Cross sectional area
cSNP	Coding single nucleotide polymorphism
CSRP3	Cysteine and glycine-rich protein 3
CT	Cycle threshold
Da	Dalton
DDT	D-dopachrome decarboxylase

DE	Differential expression
DES	Desmin
DNA	Deoxyribonucleic acid
DNAJB6	DNAJ/HSP40 homolog subfamily B, member 6
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid)
EM	Electron microscopy
EPM	Equine protozoal myeloencephalitis
ER	Exertional rhabdomyolysis
ETC	Electron transport chain
FDR	False discovery rate
FHL1	four-and-half LIM domain 1
FLNC	Filamin C
GAPDH	Glyceraldehyde phosphate dehydrogenase
GBED	Glycogen branching enzyme deficiency
GO	Gene ontology
GWAS	Genome wide association study
GYS1	Glycogen synthase 1
HBB	Hemoglobin subunit beta
HP	Haptoglobin
HSPB8	Heat-shock 22-kd protein 8
HYPP	Hyperkalemic periodic paralysis
IACUC	Institutional Animal Care and Use Committee
IMM	Immune mediated myositis

KCl	Potassium chloride
KLHL41	Kelch Like Family Member 41
kPa	kilopascal
KY	kyphoscoliosis peptidase
LMNA	Lamin A/C
M	Molar
MCal	Megacalorie
MFM	Myofibrillar myopathy
μg	Microgram
Mg^{2+}	Magnesium
MH	Malignant hyperthermia
MLP	Muscle LIM protein
mM	Millimolar
mRNA	Messenger RNA
MYOT	Myotilin
NCBI	National center for biotechnology information
NDUFA13	NADH:Ubiquinone Oxidoreductase Subunit A13
NEM	No evidence of myopathy
nM	Nanomolar
NMDL	Neuromuscular Diagnostic Lab
NSC	Nonstructural carbohydrate
OB	Other breeds
PAS	Periodic Acid Schiff's stain

PDLIM3	PDZ and LIM domain 3
PLEC	Plectin
PSSM	Polysaccharide storage myopathy
PSSM1	Type 1 polysaccharide storage myopathy
PSSM2	Type 2 polysaccharide storage myopathy
PYROXD1	pyridine nucleotide-disulfide oxidoreductase domain-containing protein 1
QH	Quarter Horse(s)
qRT-PCR	Quantitative real-time polymerase chain reaction
RIN	RNA integrity number/score
RNA	Ribonucleic acid
RNAseq	Ribonucleic acid sequencing
ROS	Reactive Oxygen Species
SQSTM	Sequestosome 1
STAT3	Signal Transducer & Activator of Transcription 3
SYNM	Synemin
SYNPO2	Synaptopodin 2/ myopodin
TB	Thoroughbreds
TEAB	Triethylamonium bicarbonate
TF	Transcription factor
TFMA	Transcription factor motif analysis
TIA1	Cytotoxic granule-associated RNA-binding protein
TMM	Trimmed mean on M-values
TMT	Tandem mass tag

UTR	Untranslated region
WB	Warmblood horse(s)
YBX1	Y-Box Binding Protein 1
ZASP	Z band alternatively spliced PDZ-containing protein
ZNF592	Zinc Finger Protein 592
μL	Microliter

CHAPTER 1

SKELETAL MUSCLE

There are three types of muscle: cardiac, smooth and skeletal. Together these tissues have essential roles in the cardiovascular, gastrointestinal, urogenital, pulmonary, and musculoskeletal systems. Skeletal muscle is necessary for posture, respiration, and locomotion. Unlike the other two types of muscle, it is under voluntary control (1).

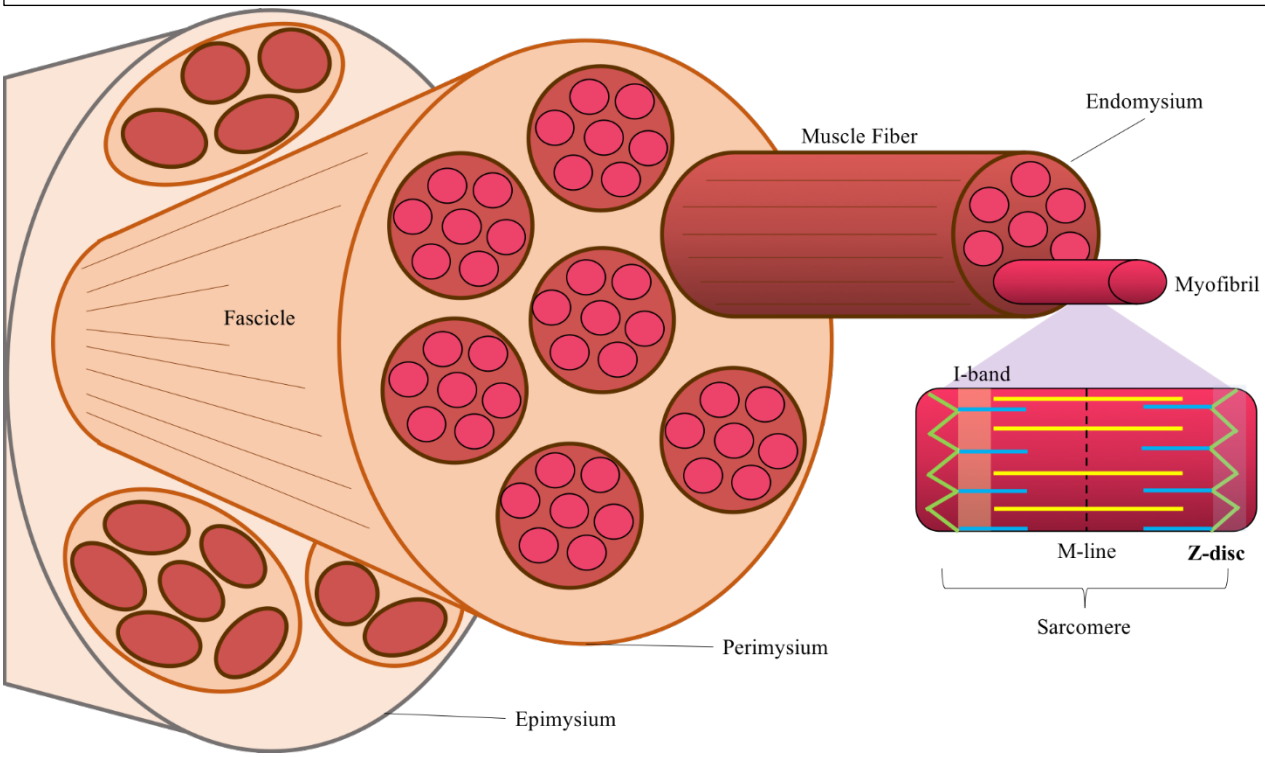
Skeletal muscle structure

Skeletal muscle is made of spindle shaped cells called myofibers with diameters ranging from 10 μm to 100 μm (1,2). Unlike most cells in the body, myofibers are multinucleated. The nuclei can be found evenly spaced along the cell's periphery. Centrally located nuclei are only present in developing or regenerating myofibers. Myofibers are surrounded by a plasma membrane referred to as the sarcolemma which invaginates into the myofiber forming transverse tubules, also known as T-tubules. The T-tubules allow for membrane propagation of electrical impulses into the myofiber. Membrane depolarization initiates at a neuromuscular junction where a myofiber is innervated by a motor neuron. Each myofiber is surrounded by a basement membrane and connective tissue called the endomysium which contains capillaries (1–3).

Myofibers are bundled together into fascicles surrounded by epimysium and the entire group of fascicles is encased in perimysium. The strategic orientation of muscle fibers and connective tissue allows muscle to generate immense power and motion while maintaining its structural integrity (Figure 1.1) (1,2).

Within each myofiber are parallel alignments of myofibrils composed of myofilaments composed of actin and myosin. These myofilaments are organized into contractile units called sarcomeres. Sarcomere boarders are defined from Z-disc to Z-disc (1–3) (Figure 1.1). The alignment of Z-discs across myofibrils gives skeletal muscle its striated appearance. Z-discs act to anchor actin filaments, but also maintain sarcomere organization and positioning of organelles, such as mitochondria, through intermediate filaments such as desmin (4). The Z-disc also acts as a mechanosensor to facilitate alterations in gene transcription for training and exercise adaptations (5). The M line is the central most part of the sarcomere and serves to anchor myosin filaments.

Figure 1.1: Skeletal muscle is composed of cells called myofibers. Myofibers contain contractile units called sarcomeres and surrounded connective tissue called endomysium. Myofibers are grouped together in fascicles which are surrounded by perimysium. Multiple fascicles are encased in epimysium.



Skeletal muscle contraction and relaxation

Skeletal muscle contraction is initiated by calcium (Ca^{2+}) mediated neuronal release of acetylcholine (ACh) at neuromuscular junctions. Ach receptors are located in the motor endplate on the sarcolemma and ACh binding depolarizes the sarcolemma causing propagation of an action potential down the t-tubules. This action potential causes a conformational change in the dihydropyridine receptor which then allows Ca^{2+} to be released from the sarcoplasmic reticulum by the ryanodine receptor (1,2,6).

Contraction occurs with the binding of myosin globular heads to actin thin filaments. Ca^{2+} is crucial for contraction because it binds to a subunit of troponin and causes a conformational change in tropomyosin to expose the binding site for myosin globular heads. The formation of a cross-bridge between actin and myosin allows the filaments to contract, thus shortening the sarcomere. However, the initial binding of myosin and actin can only slide the filaments a short distance. Hydrolysis of adenosine triphosphate (ATP) is needed to release the myosin head from the cross bridge so myosin can form a new cross-bridge with actin. This process of cross-bridge cycling allows for muscle fibers to contract in a ratchet like fashion. The process from action potential propagation to muscle contraction is called excitation-contraction coupling (1,2,6).

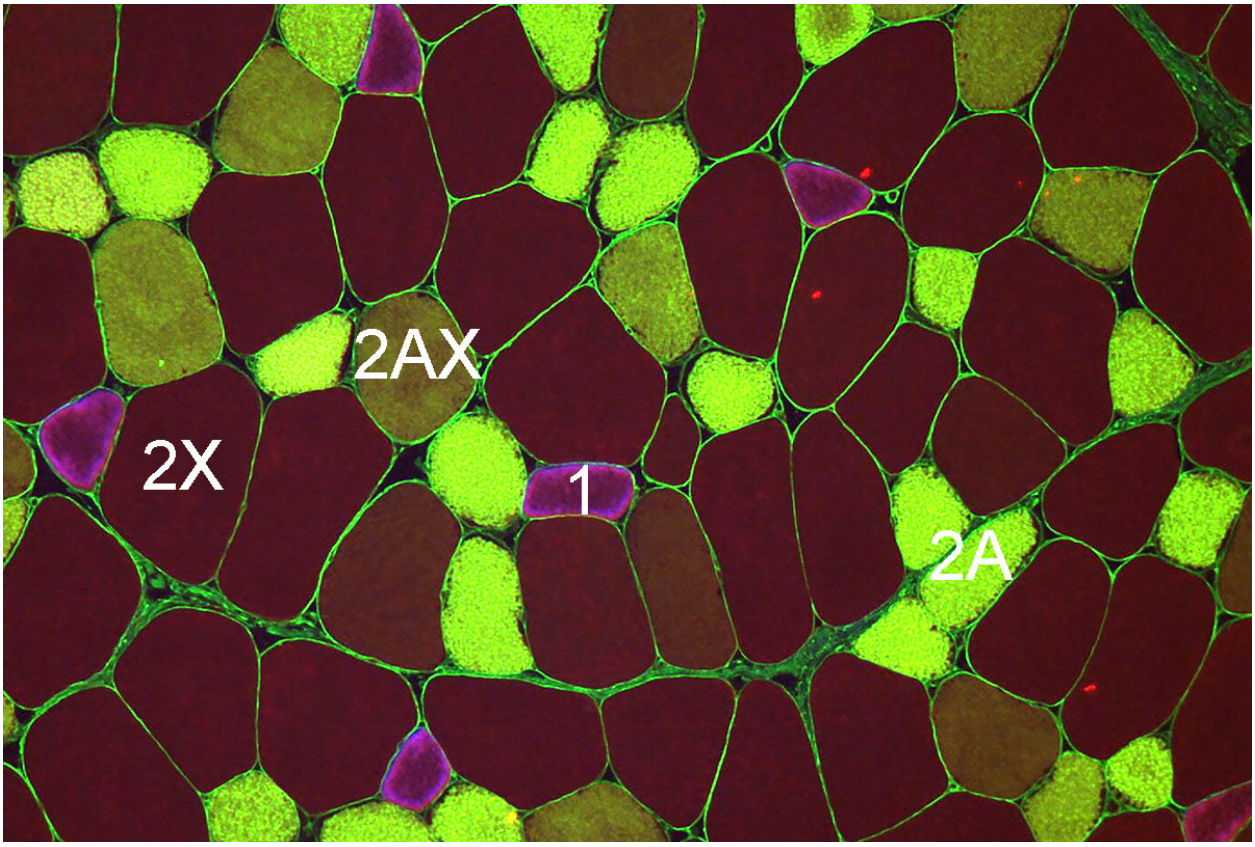
Relaxation occurs when the sarcolemma repolarizes, the ryanodine receptor closes preventing further Ca^{2+} release, and when the sarcoplasmic reticulum ATPase actively pumps myoplasmic Ca^{2+} back into the sarcoplasmic reticulum for sequestration. Without Ca^{2+}, troponin and tropomyosin resume their normal conformation and block the myosin binding sites on actin – preventing cross bridge formation (1,2,6).

ATP plays a crucial role in contraction and relaxation of skeletal muscle. It is needed to reestablish the resting membrane potential of the sarcolemma through the sodium/potassium ATPase pump, it plays a role in cross bridge cycling by binding and being hydrolyzed on myosin heads, and it also is required to sequester Ca^{2+} ions by the sarcoplasmic reticulum pump. ATP can be generated when creatine kinase takes a phosphate from creatine phosphate and adds it to adenosine diphosphate (ADP). The majority of ATP, however, comes from cellular metabolism. The ability to sustain contraction and resist muscle fatigue is dependent on the rate of ATP hydrolysis, determined by myosin ATPase expression, and the metabolic capacity of the fiber. This variation in contraction speed and metabolism is a defining feature of fiber types (1,2,6).

Fiber types

There are three types of muscle fibers in adult skeletal muscle. Slow-twitch fibers, also called type I fibers, hydrolyze ATP slower at their myosin heads and largely produce ATP through aerobic metabolism– known as oxidative phosphorylation. Their contractile speed and high number of mitochondria make them highly suitable for endurance exercise. Type IIA fibers are fibers with fast-twitch characteristics, but also a high oxidative capacity. Type IIB/IIX fibers are fast twitch and are often recruited at high speeds with lower oxidative and higher glycolytic capacity favoring anaerobic metabolism. These type IIX fibers can, however, increase their oxidative capacity– especially in trained athletes(2,7)(Figure 1.2).

Figure 1.2: Fiber types are categorized by their speed of contraction and metabolic abilities. Type I fibers are slow oxidative fibers, type IIA fibers are fast oxidative fibers, and type IIX fibers are fast glycolytic fibers. Immunofluorescence of equine gluteal muscle shows fiber type myosin expression for type I, type IIA, and type IIX fibers. Intermediate type IIAX fibers express myosin from both IIA and IIX fibers.



MYOFIBRILLAR MYOPATHY IN HORSES

Skeletal muscle and myopathies in horses

Throughout the years, horses have been bred for strength and speed – making their skeletal muscle adaptations highly selective. While most mammals have a muscle mass of 40-45% of their body weight, muscle can compose up to 55% of a horse's body weight (8). Unlike smaller mammals, which have a 3-5 fold difference in their maximum contractile shortening velocity between fiber types, horses have up to a 10 fold difference in contractile shortening velocity between 1 and 2X fiber types (7,9).

The selective breeding of horses and a strong founder effect have led to the propagation of several heritable monogenic myopathies. These include type 1 polysaccharide storage myopathy (PSSM1), glycogen branching enzyme deficiency (GBED), malignant hyperthermia (MH), hyperkalemic periodic paralysis (HYPP) and immune mediated myositis (IMM) (10–14). Beyond these monogenic myopathies are a plethora of other equine neuromuscular disorders that are likely far more complex in origin. These include recurrent exertional rhabdomyolysis (16), myotonia (17,18), and –recently– myofibrillar myopathy (MFM).

History of Equine MFM – formerly thought to be PSSM

Polysaccharide Storage myopathy (PSSM) was first reported in horses in 1992 (19). It was found in a variety of breeds including Quarter Horse (QH) and draft related breeds that had exertional rhabdomyolysis (ER) and dense amylase-resistant or amylase-sensitive polysaccharide aggregates observed in their skeletal muscle biopsies (19,20) (Figure 1.3). Management recommendations were developed for PSSM horses in 1997 and were targeted at decreasing

glycogen synthesis in skeletal muscle and promoting oxidative metabolism by providing fat as an alternative fuel source (21–23). The recommended PSSM diet consisted of < 20% of digestible energy as nonstructural carbohydrate (NSC) and approximately 15–25% of digestible energy as fat. Owners and trainers were advised to provide daily exercise for their PSSM horses (24). These management recommendations were found efficacious in PSSM horses through both controlled diet trials and retrospective questionnaires (24–28). Of the PSSM QH-related breeds that were on the diet and exercise regimen, 100% had a decrease in the frequency of ER with 71% of those horses having no further episodes of ER (28). In PSSM Warmblood horses (WB), 71% of horses also improved on the PSSM diet and exercise recommendations (27).

In 2008, advances in genome mapping allowed for the discovery of a dominant R309H gain of function mutation in glycogen synthase 1 (*GYS1*) in PSSM horses (29). This mutation was identified through a QH mapping study that only included PSSM phenotypes with amylase-resistant polysaccharide and >1.5 fold elevation in muscle glycogen concentrations (29). However, some horses diagnosed with PSSM, namely many of those with amylase-sensitive glycogen aggregates, did not have the *GYS1* mutation. Further analysis of over 800 horses diagnosed with PSSM by muscle biopsy in the United States found that 72% of PSSM QH and only 18% of PSSM WB had the *GYS1* mutation (11). Different breeds diagnosed with PSSM by muscle biopsy in the United Kingdom were also genetically tested and only 21% had the *GYS1* mutation (30). These findings coupled with the phenotypic criteria used to discover the *GYS1* mutation indicate that the *GYS1* mutation is much more likely to cause amylase-resistant polysaccharide aggregates than amylase-sensitive aggregates. This led to the categorization of PSSM into two types: type 1 PSSM (PSSM1) for horses with the *GYS1* mutation and type 2

PSSM (PSSM2) for horses with abnormal histologic muscle glycogen but no *GYS1* mutation (Figure 1.3). The specific etiologies for PSSM2 remain unknown.

Many of the previous PSSM diet studies likely included both PSSM1 and PSSM2 horses. Whether the improvements seen with diet and exercise were specific to PSSM1 versus PSSM2 horses was not ascertained. One study evaluated 13 PSSM2 WB, 14 PSSM1 WB, and 10 non-PSSM WB and found that, unlike PSSM1, PSSM2 WB did not have elevations in muscle glycogen concentrations (31). The clinical presentation also appears to differ from PSSM1 when examined further. Exertional rhabdomyolysis is a less common finding in PSSM2 WB than PSSM1 horses(31). PSSM2 WB were reported to be exercise intolerant and had a lameness not associated with any orthopedic causes (31). These findings suggest that although glycogen aggregation is observed on histopathology, PSSM2 WB may not actually have excessive glycogen storage. If PSSM2 in WB is not a glycogen storage disorder, it is possible that the PSSM recommendations for low NSC, fat supplemented diets are not optimal for PSSM2 WB (24,32).

Further research using immunohistochemical staining on muscle from PSSM2 WB horses revealed that several horses had ectopic aggregation of a cytoskeletal protein called desmin (33) (Figure 1.4). Desmin appears as granulofilamentous material under electron microscopy (34). These findings are a hallmark for a myopathy called myofibrillar myopathy (MFM), which is reported in humans.

Figure 1.3: PSSM in horses is diagnosed in skeletal muscle by the presence of abnormal aggregates of glycogen. A.) Periodic acid Schiff's (PAS) stain showing abnormal granular aggregates of glycogen in a PSSM1 gluteal muscle (arrows). B.) PAS staining after amylase digestion shows granular amylase resistant granular polysaccharide in PSSM1 (arrows). C.) PSSM2 biopsies show cytoplasmic glycogen aggregates and cytoplasmic pooling with PAS staining (arrows). D.) PSSM2 glycogen aggregates are amylase sensitive with a PAS-amylase stain (arrows).

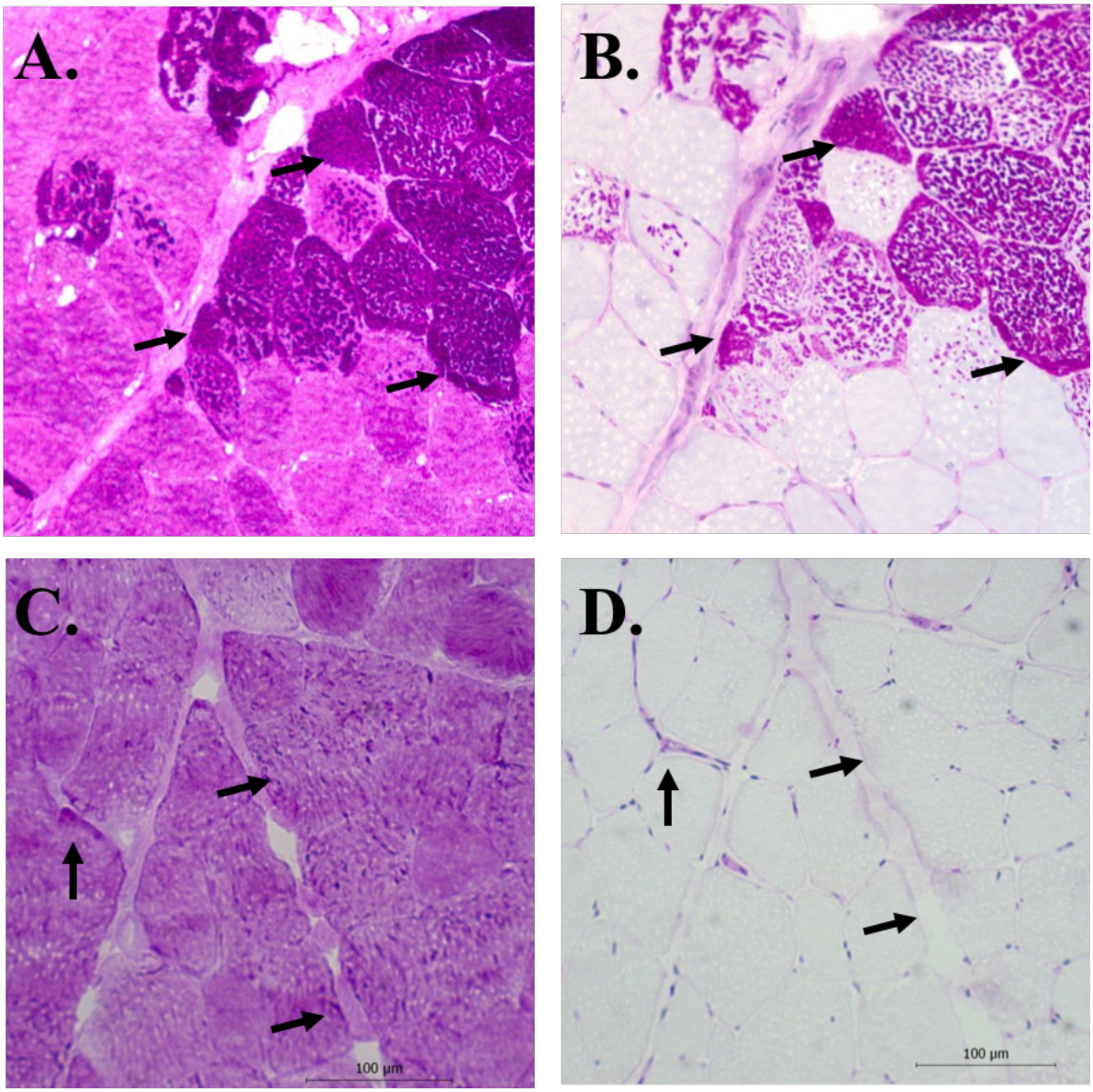
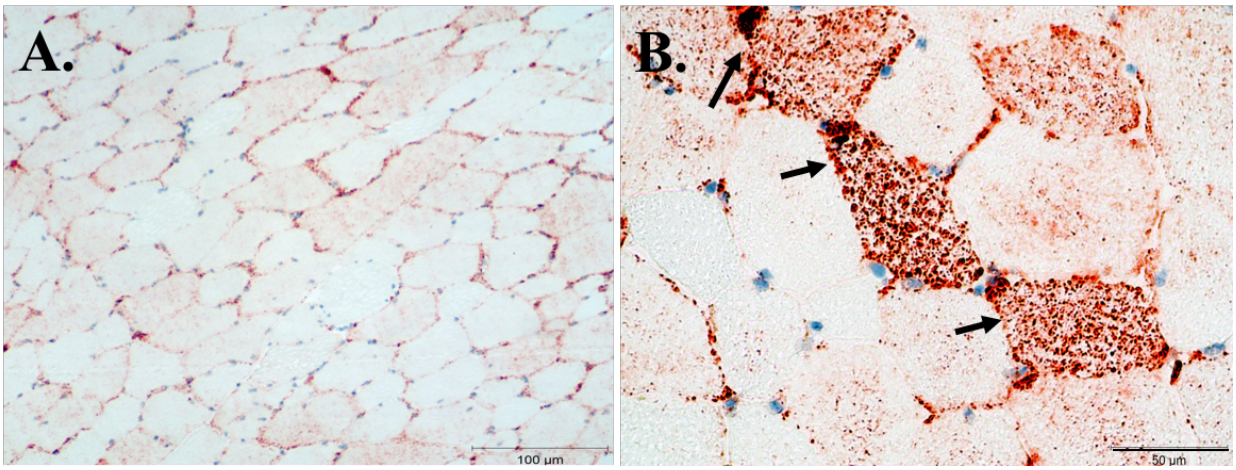


Figure 1.4: Desmin staining of a cross-section of gluteal muscle from a WB horse with MFM. A.) A normal equine gluteal muscle biopsy with minimal subsarcolemmal staining for desmin. 10X B.) MFM fibers contain abnormal aggregates of cytoplasmic desmin in MFM muscle fibers. 40X



Clinical signs and veterinary work up

MFM has been diagnosed in Arabian and WB breeds. The clinical presentation appears to be adult onset, with MFM diagnosed at the age of 11-15 years for Arabians and approximately 11 years of-age for WB horses (33,35). Arabian horses with MFM present with stiffness and ER (35,36). The principle clinical signs associated with MFM in WB are exercise intolerance, a reluctance to go forward under saddle and a mild lameness not attributable to an underlying orthopedic cause (31,33,37).

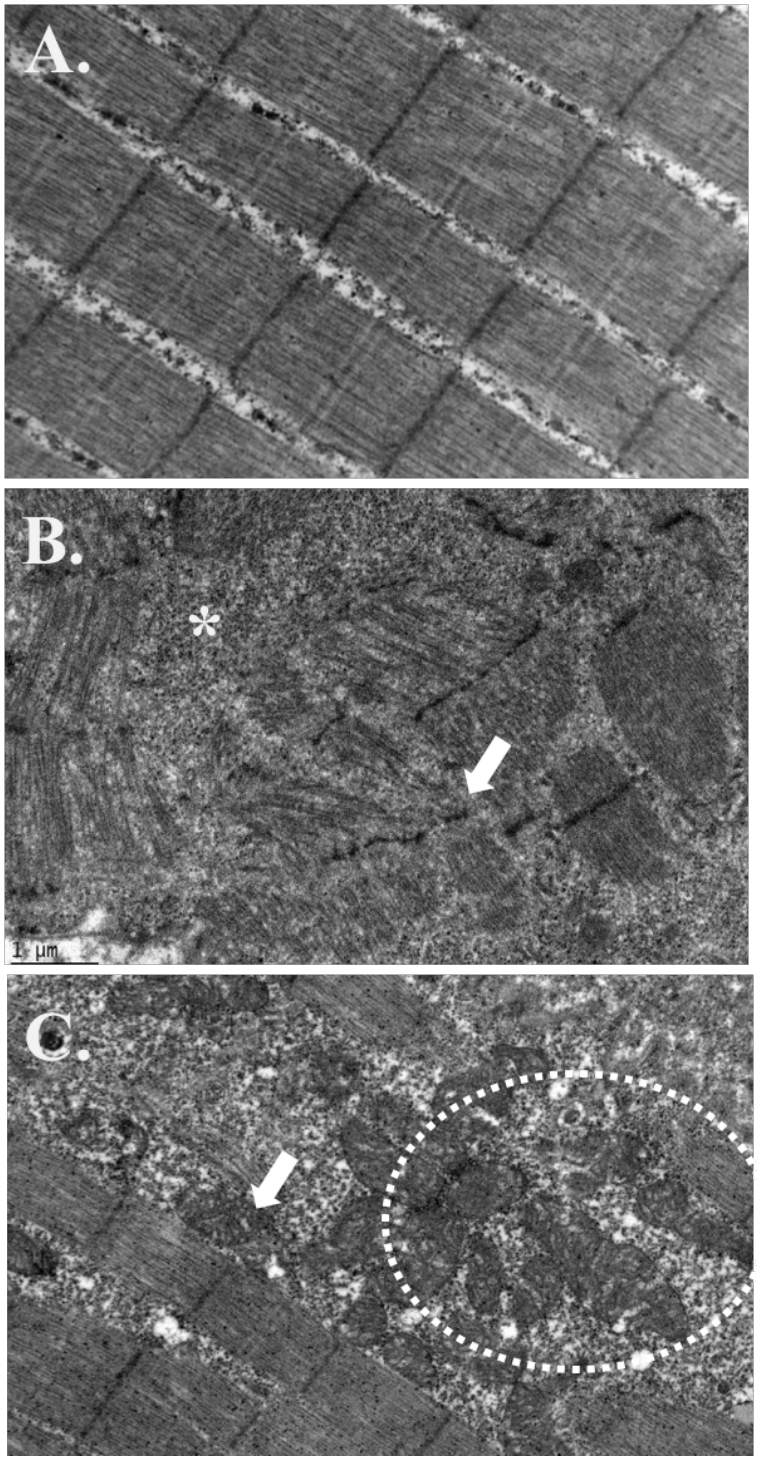
The current clinical practice of diagnosing MFM in WB is expensive, time consuming and often emotionally exhausting for owners and trainers. Many of the current cases are performance horses imported from Europe and are valued from $50,000 to more than $100,000. Currently, a complete veterinary diagnostic work-up is suggested to rule out other common causes of lameness and exercise intolerance (16). This work-up often includes multiple lameness exams, nerve blocks, nuclear scintigraphy, gastroscopy, dynamic upper airway endoscopy, exercise tolerance tests, dynamic ECG, ultrasounds and multiple blood tests. After these tests yield no remarkable findings, myopathies are often the next differential diagnosis. MFM in humans is diagnosed by advanced imaging to identify affected muscles for biopsy, immunohistochemistry on muscle biopsies, and exome sequencing of candidate genes (38–42). The use of magnetic resonance imaging on horses is not always feasible because of their large size (averaging 1000-1200 pounds), the expense of advanced diagnostics, and the requirement of general anesthesia. This makes the gold standard test for diagnosis of MFM in WB a muscle biopsy. Either *gluteal* or *semimembranosus* muscle biopsies are submitted to the Equine Neuromuscular Diagnostic Laboratory at Michigan State University for evaluation of myopathic changes.

Histopathology

Similar to human medicine, the equine diagnosis of MFM is founded on presence of desmin aggregation using immunohistochemical staining (33,43–48). We have observed that the degree of desmin aggregation varies between horses and between different stages of this disease. Desmin aggregation is typically present in type 2A fibers of WB (33). The most definitive diagnosis of MFM, however, is currently made with electron microscopy and the findings of myofibrillar disarray, Z-disc streaming, and aggregation of granulofilamentous material (33) (Figure 1.5).

Muscle biopsies of Arabian horses and WB horses with MFM have centrally located nuclei, myofibrillar disruption, and aggregation of both desmin and αβ-crystallin (49,50). However, their clinical presentation differs from one another. As seen in human MFM, this could represent either different etiologies or alternate clinical presentations of equine MFM.

Figure 1.5: Electron microscopy (EM) of equine gluteal muscle. A.) Normal EM shows well aligned myofibrils with distinct electron dense Z-discs demarcating organized sarcomeres. B.) Gluteal muscle from MFM WB with areas of myofibrillar disarray, aggregation of granulofilamentous material (*) and Z-disc streaming (arrow). C.) MFM WB also have aggregation of mitochondria (dashed circle) and degenerate looking mitochondria with abnormal and enlarged cristae (arrow).



Etiologies

Currently the etiology of MFM in horses is unknown. It was suggested that there may be a hereditary component to MFM after a 3 generation family of WB with MFM was identified (33) This family of horses was in the same environment and had the same diet and management regimen. Therefore, it remains unclear if this disease is solely genetic in origin, of if the phenotypic expression is further impacted by environment, nutrition, and athletic demands.

To date, MFM has only been diagnosed in horses that reside in the U.S. One study concluded that European and U.S. performance horses had significant differences in their diet, specifically that European horses were fed less fat and more starch as an energy substrate (51). The significance of diet on MFM horses is unknown and warrants further investigation of its impact on this myopathy.

The NMDL previously investigated MFM in Arabian horses by utilizing transcriptomic and proteomic profiling. That study found decreased expression of the antioxidant enzyme peroxiredoxin 6 and altered expression of genes involved in cysteine synthesis. These findings suggested that MFM Arabian horses have a deficient thiol-based antioxidant capacity that predisposes them to oxidative stress and desmin aggregation (36).

MYOFIBRILLAR MYOPATHY IN HUMANS

Clinical signs

Myofibrillar myopathy (MFM) is a debilitating and progressive disease that causes myalgia, immobility, respiratory compromise, and sometimes heart failure in people (1– 3). MFM can affect both skeletal and cardiac muscle, but it remains unclear if smooth muscle can also be affected. There have been cases with reported malabsorption, gastrointestinal hypo-motility, and pseudo-obstructions in MFM (52). Clinical signs can be heterogeneous depending on the underlying genetic basis and can appear from infancy to late adulthood. The classical presentation is late onset with clinical signs appearing in the 4-5th decades of life (53,54).

Histopathology

MFM is uniquely defined as a protein aggregate myopathy causing sarcomere disorganization in skeletal and cardiac muscles (38,55,56). Fibrosis, degenerative and necrotic fibers, and fiber regeneration are evident in hematoxylin and eosin stains (38). With special staining, MFM is characterized by protein aggregates that may include cytoskeletal and myofibrillar proteins, intermediate filaments, oxidative stress proteins, nuclear proteins, chaperones, kinases, and ubiquitin-proteasome proteins (57). Aggregation of desmin, αβ -crystallin and myotilin are most commonly observed in immunohistochemical staining (53). The ultrastructural features of MFM include myofibril disarray, accumulation of granulofilamentous material between myofibrils and Z-disk alterations, loss, or streaming (40,53,54). Large degenerate mitochondria with abnormal cristae have also been identified in muscle from cases of MFM (58).

Etiologies

MFM in adult humans is often inherited as a late onset autosomal dominant disorder with clinical signs becoming apparent after the 4th decade of life (53,55,59–62). However, dominant or recessive forms of MFM can manifest in early childhood (47,53,54,63,64). Mutations in desmin, myotilin, αβ-crystallin, Z band alternatively spliced PDZ-containing protein, filamin C, bcl-2-associated athanogene-3, kyphoscoliosis peptidase, and pyridine nucleotide-disulfide oxidoreductase domain-containing protein 1cause MFM types 1-8 (40,65). There are also 8 more genes that are associated with ectopic protein aggregation with MFM-like histology and phenotypes (Table 1.1) (40,65). Mutations in these genes alter the structure and function of their resulting protein products (66). This results in protein aggregation and disruption of myofibrillar organization (Figure 1.6). The genes and proteins associated with MFM range from sarcomere stabilizing Z-disc proteins, I band proteins, chaperones, and heat shock proteins (67–69). It is estimated, however, that at least 50% of MFM cases have no identified etiology (38,53). The clinical and molecular variation observed in human MFM suggests the presence of complex components that affect the phenotypic expression of this disease (53,57).

Animal models

Many laboratory animal models have been established to investigate MFM. Non- mammalian models have used drosophila, zebra fish and medaka teleost fish to investigate MFM etiopathologies. A post-translational silencing of Z band alternatively spliced PDZ-containing protein *(ZASP)* in drosophila found that the knockdown of *ZASP* resulted in sarcomere disruption and locomotor disabilities (70). A zebrafish model of desmin (*DES*) knockouts and other aggregate forming mutations showed that DES aggregates are associated with abnormal

excitation-contraction coupling and that aggregates caused more pathology than desmin knock outs (71). A filamin C (*FLNC*) nonsense mutation, studied in Medaka teleost fish, showed that homozygous embryos have Z-disc destruction, myofibrillar and sarcolemmal detachment, and degeneration of myotendinous junctions (72). A zebrafish bcl-2-associated athanogene-3 (*BAG3)* P209L mutant showed that aggregates of the wild-type *BAG3* protein occur that, contrary to popular belief, were not the result of inhibition of autophagy (73). The *BAG3* mutant protein resulted in protein aggregation, but did not result in myofibrillar disorganization. Interestingly, knockdown of *BAG3* resulted in myofibrillar disorganization, but not protein aggregation. It was therefore proposed that the aggregation of BAG3 itself depletes its availability for proper function resulting in myofibrillar pathology (73). Thus, animal models of MFM reveal the complexity of the disease.

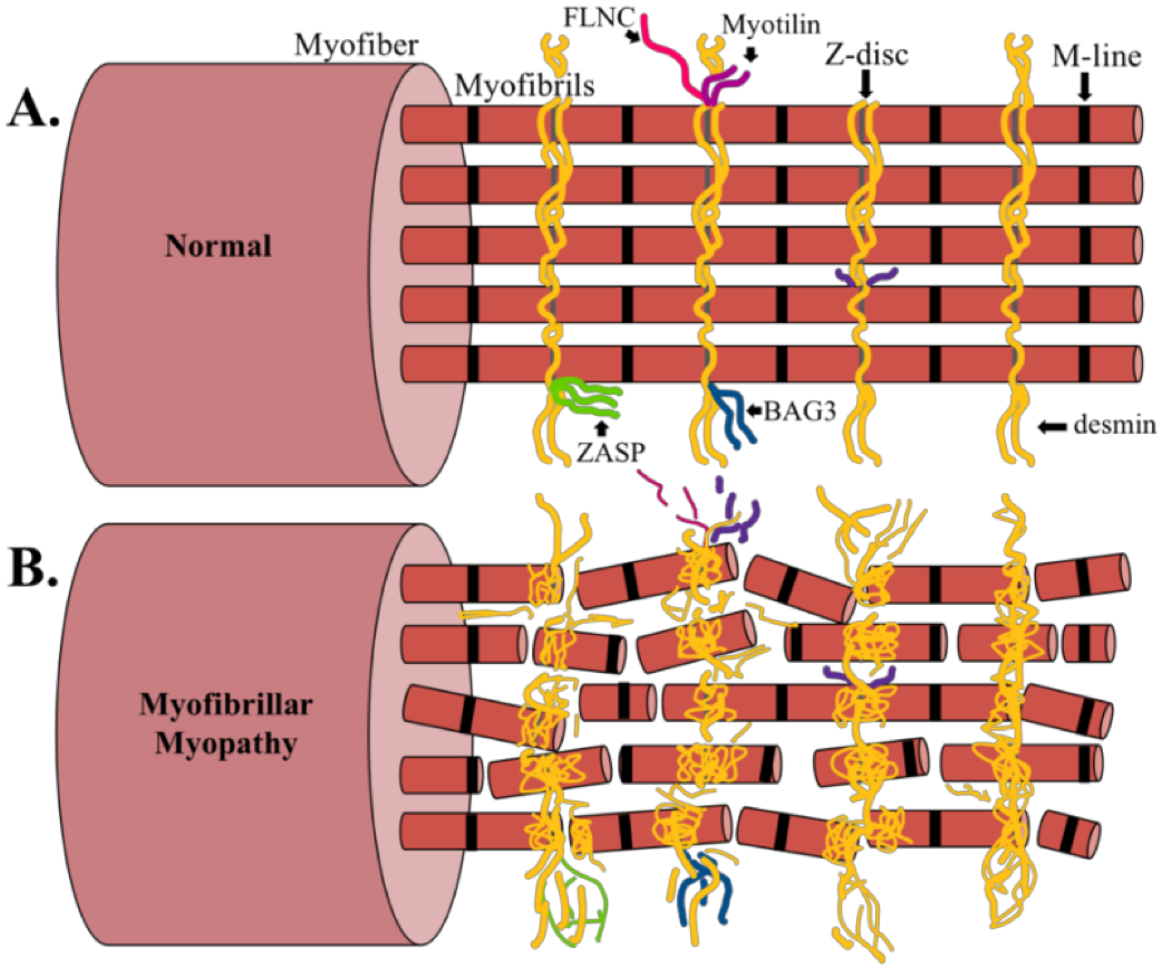
Mice serve as the primary mammalian model of skeletal and cardiac MFM. Transfected p.R406W and p.E413K *DES* mouse models have both resulted in myofibrillar disarray, protein aggregation and decreased contractile function (74). In contrast, p.L345P-HA *DES* mice have decreased muscle contractility, no protein aggregation, and vacuolization and swelling of mitochondria (75). This degenerative mitochondrial morphology has also been seen in *DES* p.R349P knock in mice which have age-dependent protein aggregate formation, myofibrillar disarray, decreased contractility, and increased cytochrome C oxidase activities (76). Other mouse models with W2710X *FLNC*, T57I myotilin, and p.R120G αβ-crystallin models have also shown myofibrillar disarray and protein aggregation (77–79).

Transgenic animal models serve to demonstrate that specific genetic mutations can result in an MFM phenotype (43,45,47,80,81). However, tightly controlled laboratory environments coupled with the small size and reduced life expectancy of these models make it difficult to assess the environmental and lifestyle variables that impact the expression of MFM in humans (82). Therefore, a naturally occurring model of MFM would be beneficial to further evaluate the complex mechanisms causing myofibrillar disruption and protein aggregation. There has been only one case of MFM reported in a dog (83), but numerous cases of MFM have now been diagnosed in horses (33,35).

Table 1.1: Genes that cause MFM types 1-8 in humans and genes associated with MFM-like myopathies, corresponding diseases and mode of inheritance (53,65).

Genes that cause MFM			
Gene Symbol	**Name**	**Disease**	**Inheritance**
DES	desmin	MFM1	Autosomal dominant and recessive
CRYAB	αβ-crystallin	MFM2	Autosomal dominant
MYOT	myotilin	MFM3	Autosomal dominant
ZASP	Z band alternatively spliced PDZ-containing protein	MFM4	Autosomal dominant
FLNC	filamin C	MFM5	Autosomal dominant
BAG3	bcl-2-associated athanogene-3	MFM6	Autosomal dominant
KY	kyphoscoliosis peptidase	MFM7	Autosomal recessive
PYROXD1	pyridine nucleotide-disulfide oxidoreductase domain-containing protein 1	MFM8	Autosomal recessive
Genes associated with MFM			
FHL1	four-and-half LIM domain 1	Scapuloperoneal Myopathy	X-linked
DNAJB6	DNAJ/HSP40 homolog subfamily B, member 6	limb-girdle muscular dystrophy	Autosomal dominant
PLEC	Plectin	limb-girdle muscular dystrophy, epidermolysis bullosa simplex with muscular dystrophy	Autosomal recessive
LMNA	lamin A/C	limb-girdle muscular dystrophy type 1B, Emery-Dreifuss Muscular Dystrophy	Autosomal dominant
ACTA1	alpha-actin	Nemaline Myopathy 3, Scapuloperoneal Myopathy	Autosomal dominant and recessive
HSPB8	heat-shock 22-kd protein 8	Charcot-Marie-Tooth disease, distal motor neuropathy	Autosomal dominant
SQSTM	sequestosome 1	MFM-like inclusion body myopathy	Autosomal dominant and recessive
TIA1	cytotoxic granule-associated RNA-binding protein	Welander distal myopathy, MFM-like inclusion body myopathy	Autosomal dominant and recessive

Figure 1.6: A.) Muscle is made up of myofibers which are composed of myofibrils. Myofibrils contain functional contractile units called sarcomeres that are bordered by Z-discs and centered around M-lines. B.) Pathogenic variants in proteins associated with the Z-disc, such as desmin, ZASP, BAG3, Myotilin, and FLNC, cause sarcomere instability, myofibrillar disorganization and protein aggregation.



Mechanisms proposed

With the wide number of genes and different mutations that cause an MFM phenotype– several mechanisms have been proposed as the basis for protein aggregation (57). Protein aggregation in transgenic murine models and transfected cell lines is thought to be a direct result of protein misfolding due to dysfunctional protein chaperones (78,84–89). For example, studies found that a R120G *CRYAB* mutation caused desmin aggregation due to abnormal desmin folding from a mutation in *CRYAB* (90,91).

Alternatively, some MFM studies have propose that impaired proteosomal degradation of protein aggregates (92), may play a role in the clinical phenotype. These studies include a cardiac desminopathy mouse model that showed mutant desmin impaired ubiquitin-proteasome proteolytic activity –specifically dysfunctional protein transport into the 20S proteasome (80). The molecular chaperone clusterin has also been proposed to play a role in MFM pathology from its association with other protein aggregate diseases (93). This protein has been identified in abnormal protein aggregates of *MYOT* and *CRYAB* related MFMs, but hasn't been investigated further (94). Mutations in ubiquitin may also play a role in the decreased clearance of MFM aggregates and it has been identified in both *MYOT* and *DES* causes of MFM (95). Another protein known as p62 has been shown to play a role in the actual formation of protein aggregates (96) and is found in MYOT MFM, but not DES MFM (95). Transcription factors such as cold shock domain containing protein A and pro-apoptotic protein FRAP-related protein 1 have also been found in desminopathies (69), but their significance is unknown due to the limited investigation of transcription factors and their roles in MFM (57).

Oxidative and nitrosative stress is one other proposed mechanism thought to contribute to the MFM phenotype and has been shown to be present in both *MYOT* and *DES* MFM (97). Desmin has been shown to be oxidized in *DES* and *MYOT* MFM (48). The source of oxidative stress has not been established, however, MFM is accompanied by abnormalities in mitochondrial staining patterns, dysfunction of complex I respiratory chain and evidence of oxidative stress (58,98–100) (58,99,101,102), Complex I is known to produce robust amounts of reactive oxygen species (103) and thus may be involved in generation of oxidative stress in MFM.

Potential treatment

Currently, there is no successful treatment for MFM. Multiple therapeutics, however, have been proposed that promote autophagy/mitophagy, decrease protein aggregate formation, and help ameliorate mitochondrial dysfunction (104). Proposed treatment includes geranylgeranylacetone that induces the heat shock protein response and reduces protein aggregation while improving cardiac function *CRYAB* mouse models (105). 4-phenylbutyrate, a chemical chaperone, has been shown to reduce desmin aggregation in plectin deficient mice (106). Administration of doxycycline is another potential treatment for MFM developed in a transgenic *CRYAB* mouse model of desmin related cardiomyopathy which was shown to reduce protein aggregation (107). Exercise alone has also been shown to decrease protein aggregation by promoting protein turn over (91).

To combat the effects of dysfunctional mitochondria, AMP-activated protein kinase agonists have been used in murine models of muscular dystrophy to successfully eliminate degenerative mitochondria (108) but have yet to be investigated in MFM. Recently the use of antioxidants has

been investigated in MFM. One study showed that antioxidant supplementation resolved desmin aggregation by 75% in cell culture (109). Specifically, N-acetyl-L-cysteine has been shown to prevent oxidative stress induced protein aggregation (110,111). This has been further supported in mouse models of muscular dystrophy and MFM where supplementation of N-acetylcysteine significantly decreased protein aggregation (111,112).

USING OMICS TO INVESTIGATE DISEASE

Discovery based science has been used to create molecular profiles of diseases by elucidating key driver genes and proteins that participate in the cellular response to pathology (113). Through the generation of "big data", multi-omic approaches offer a unique opportunity to study the cellular response to disease on a global scale and lay the foundation for further hypothesis driven science. The comprehensive integration of multi-omic data, particularly of complex diseases, has promoted precision medicine by identifying new biomarkers and allowing for tailored treatments based on the specific pathways that make up the cellular response to disease pathology (114–117).

Omics is a broad term encompassing genomic sequencing of DNA, transcriptomic analysis with RNA sequencing and gene expression, proteomic analysis of protein expression, metabolomic measurements of metabolic intermediates, and microbiomic analysis of microbe population and abundance. Individually, these modalities provide a limited view into the cellular regulation and response to disease. However, the integration of two or more of these modalities has led to the rapid expansion of personalized and precision medicine of both common and rare diseases ranging from monogenic to complex (117). While many genes have been associated with human MFM, it is estimated that 50% of cases still do not have an identified etiology. Despite known causes of MFM, little is known about the cellular response to the pathology. Multi-omic approaches have been utilized to provide a better understanding of the cellular mechanisms of diseases by highlighting key pathways and responses that stretch beyond the predictive measures of genomic variation and causative mutations (115).

This integrative approach has yet to be implemented in human MFM. Transcriptomic and proteomic analyses have been used to investigate Arabian horses with MFM (36) and, now in the present study to elucidate the etiopathology of MFM in WB horses. The use of naturally occurring animal models is beneficial when investigating similar myopathies because the interindividual differences more closely resemble those between humans than inbred laboratory animals (118). Therefore, the use of these modalities will not only provide better identification of disease biomarkers and tailored therapy in the equine veterinary industry, but will also serve as a unique opportunity for translational medicine by identifying key regulatory genes and proteins that play a crucial role in the pathology of MFM.

RESEARCH SIGNIFICANCE AND OBJECTIVES

Significance

PSSM2 and MFM are the most common myopathies described in WB horses. The etiology of PSSM2 and MFM are unknown and therefore targeted treatments and management strategies have not been developed. Veterinarians cannot provide an accurate prognosis and horses owners are left not knowing if MFM is heritable, treatable or progressive. Furthermore, owners do not know if the pathogenesis of MFM relies on specific environmental or lifestyle factors which can be avoided. An insufficient understanding of the pathogenesis of MFM in WB horses means that there is a strong need to investigate this myopathy further to develop more sensitive and specific diagnostic markers and effective treatment interventions. Furthermore, identifying the etiopathology of MFM in WB could translate to a naturally occurring animal model that would provide insights into treatments for human MFM.

Overarching objective

The overarching objective of this dissertation is to evaluate the responses of PSSM2/MFM WB to the recommended PSSM diet and to further elucidate the etiopathology of MFM in WB by identifying candidate genes, proteins, and pathways that drive the aberrant cellular responses of this disease. Elucidating the etiopathology of MFM in WB will allow for the development of tailored veterinary care and management recommendations that will help ameliorate the effects of this disease. This research project combines hypothesis driven research and discovery-based *omics* to identify candidate genes, proteins and pathways involved in the MFM pathogenesis of WB horses.

Aim 1 PSSM2 WB clinical signs and response to PSSM diet and exercise

Hypothesis: PSSM2 WB have lower skeletal muscle glycogen concentrations compared to PSSM1 WB. Therefore, a significant improvement in clinical signs of PSSM2 WB will not occur with the same low starch and fat supplemented diet shown to benefit PSSM1 horses.

Objectives:

1. Compile owner reported symptoms of PSSM2 WB before implementing treatment of management regimens.
2. Determine if PSSM2 WB owner reported symptoms improved with PSSM1 diet.
3. Determine if PSSM2 WB gluteal muscle glycogen concentrations differ when compared with PSSM1 and control WB.

Aim 2 Candidate MFM genes in horses

Hypothesis: Genes associated with human MFM and MFM-like protein aggregate myopathies are candidates for WB MFM. Therefore, MFM WB will have differential gene expression and/or an associated coding variant in one or more of the candidate MFM genes when compared to non-MFM WB controls.

Objectives:

1. To determine the differential expression of MFM candidate genes in the gluteal muscle-derived mRNA from 8 MFM WB and 8 non-MFM WB.
2. To identify variants and compare allele frequencies in 16 MFM/MFM-like candidate genes in MFM WB vs. non-MFM WB.

3. To determine if variant allele frequencies vary between different breeds using publicly available RNAseq and whole genome sequencing data.

Aim 3 Transcriptomic and proteomic profiling and transcription factor motif analysis of MFM WB

Hypothesis: MFM WB have specific genes, proteins and transcription factors that drive the aberrant cellular response and have key roles in the etiopathology of MFM WB.

Objectives:

1. To perform electron microscopy on MFM WB to further document the ultrastructural changes related to MFM pathology.
2. To identify differentially expressed genes and proteins in gluteal muscle of MFM and non-MFM WB using transcriptomic and proteomic analyses.
3. To determine enriched pathways for differentially expressed genes and differentially expressed proteins using gene ontology analyses.
4. To employ an amalgamated approach to elucidate key aberrant pathways and identify candidate genes for MFM in WB.
5. To identify candidate transcription factors using a transcription factor motif enrichment analysis of DE genes and DE proteins.

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CHAPTER 2

MUSCLE GLYCOGEN CONCENTRATIONS AND RESPONSE TO DIET AND EXERCISE REGIMES IN WARMBLOOD HORSES WITH TYPE 2 POLYSACCHARIDE STORAGE MYOPATHY

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ABSTRACT

Type 1 polysaccharide storage myopathy (PSSM1) is a glycogen storage disorder of known cause whereas the basis for type 2 PSSM (PSSM2) is unknown. The same diet and exercise regime prescribed for PSSM1 is recommended for PSSM2; however, the benefit of these recommendations for PSSM2 is undocumented. The objectives of this study were to determine traits of PSSM2 Warmblood horses (WB), determine the changes in exercise responses that occur with a recommended low-starch/fat-supplemented diet and exercise regime, and determine if glycogen concentrations correspond to the severity of signs. Owners of PSSM2 WB (2008 - 2016), completed a retrospective questionnaire regarding their horse. Glycogen concentrations ([glycogen])were analyzed in skeletal muscle of PSSM2 WB (n = 36) obtained prior to recommendations and in control WB with no evident myopathy (n = 23). Chi-square, Fisher's exact, McNemar's tests with Bonferroni correction and Mann Whitney testing were utilized. Abnormal exercise responses reported by owners, began at approximately 6 years of age and included a decline in performance, a reluctance to collect and reluctance to go forward in over 50% of horses. With the recommended diet and exercise regime, 80% of PSSM2 WB owners reported an overall improvement with significant decreases in the proportion of horses showing a decline in performance and rhabdomyolysis. However, 53% of PSSM2 WB were still not advancing as expected with reluctance to go forward and collect persisting in approximately one third of horses. Median muscle [glycogen] did not differ between PSSM2 WB and WB with no evident myopathy. PSSM2 WB with the highest [glycogen] were significantly more likely to show a decline in performance than those with lower glycogen concentrations. In conclusion, diet and exercise recommendations ideal for PSSM1 improve but do not eliminate the decline in performance and reluctance to go forward under saddle characteristic of PSSM2.

INTRODUCTION

In 1992, Polysaccharide Storage Myopathy (PSSM) associated with chronic ER was discovered in Quarter Horse-related breeds based on the hallmark feature of amylase-resistant polysaccharide in skeletal muscle biopsies (1). Aggregates of amylase-sensitive glycogen and subsarcolemmal lakes of muscle glycogen were later added as potential diagnostic features which expanded the range of breeds affected by PSSM (2). Sixteen years after the original discovery of PSSM, a dominant R309H gain of function mutation was identified in glycogen synthase 1 (*GYS1*) through a Quarter Horse mapping study that used phenotypic criteria of amylase-resistant polysaccharide and >1.5 fold elevations in muscle glycogen concentrations (3). Analysis of over 830 horses of a variety of breeds in the US determined that 72% of Quarter Horses and 18% of WB diagnosed with PSSM by the presence of aggregates of amylase-sensitive or resistant glycogen in muscle samples possessed the *GYS1* mutation (4). In the United Kingdom, 21% of horses diagnosed with PSSM by muscle biopsy were found to have the *GYS1* mutation (5). Because some horses diagnosed with PSSM by muscle histopathology did not possess the *GYS1* mutation, two terms were applied; type 1 PSSM (PSSM1) was used to denote horses with the *GYS1* mutation and type 2 PSSM (PSSM2) to denote horses that had abnormal histologic muscle glycogen yet lacked the *GYS1* mutation. The specific cause or causes for PSSM2 remain unknown.

The management recommendations for PSSM provided to horse owners since 1997 have been similar for PSSM1 and PSSM2 and directed at reducing muscle glycogen synthesis, promoting oxidative metabolism and providing fat as an alternative fuel (6–8). Recommendations include a diet that provides < 20% of digestible energy as nonstructural carbohydrate (NSC), 15–25% of

digestible energy as fat and regular daily exercise (9). These recommendations were developed and found to be efficacious using a herd of Quarter Horses that had on average 1.8 times higher muscle glycogen concentrations than normal horses and, when tested in retrospect, had PSSM1 (9; 10). The efficacy of the diet and exercise recommendations for PSSM has been evaluated in clinical cases through retrospective studies performed prior to identification of the *GYS1* mutation (11–13). These studies, which likely included both PSSM1 and PSSM2 horses, found that in Quarter Horse-related breeds 100% of horses had a decrease in the frequency of ER and 71% had no further ER episodes (11). In WB, clinical signs of PSSM also improved in 71% of PSSM horses (12). Whether these improvements were specific to PSSM1 vs PSSM2 horses could not be ascertained from these early studies.

Recently, a retrospective study found that, unlike PSSM1, mean muscle glycogen concentrations in 13 PSSM2 WB horses were not above normal reference ranges (14). If PSSM2 is not a glycogen storage disorder, it is possible that the current recommendations for low NSC, fat supplemented diets are not optimal for PSSM2 WB (9; 15). To determine how well PSSM2 WB respond to current recommendations, we performed a retrospective study to determine the specific exercise responses and behaviors owners attributed to PSSM2 in WB and the perceived changes owners recognized following receipt of PSSM diet and exercise recommendations. We also determined muscle glycogen concentrations in the respondent's horses prior to changing diet and exercise to determine whether horses had excessive glycogen storage and if there was a relationship between muscle glycogen and response to recommendations.

METHODS

To determine the effect of diet and exercise regimes on the traits and exercise responses associated with PSSM2, a questionnaire was designed for horses diagnosed with PSSM2 by the Neuromuscular Diagnostic Laboratory (NMDL). Validation of the questionnaire was achieved using previously established principles (16, 17). The questionnaire was designed with the purpose of describing the range of traits exhibited by PSSM2 horses and to determine if exercise responses improved or declined after institution of diet and management recommendations. The target group identified was owners of WB horses previously diagnosed with PSSM2 by muscle biopsies that had been evaluated by the Neuromuscular Diagnostic Laboratory. Questions used in previous epidemiological studies of equine myopathies were selected for the initial questionnaire and modified to elicit information on specific traits, exercise responses, diet and exercise regimens (11, 12). The questionnaire was previewed and screened by two veterinarians, an equine nutritionist, and three horse owners to validate the content and clarity of the questionnaire and to allow for revisions before it was opened for responses. A pilot test was run on owners with PSSM2 affected horses of non-Warmblood breeds. The questionnaire was distributed to 109 owners of PSSM2 WB in June 2016 and responses were collected through October 2016. Signalment, traits, exercise responses, management and improvement or decline in responses was assessed by the owner comparing the horse's condition before to after institution of PSSM management recommendations.

This retrospective study obtained informed consent of all participants prior to data collection and previously obtained muscle biopsies were analyzed. An Institutional Animal Care and Use Committee and Animal Use Form exemption was obtained for this study. Because the research

goal of the study was to obtain information regarding the horse's response to exercise and diet management and not to perform research directed at investigating the owners of the horses, an Institutional Animal Care and Use Committee (IACUC) Administrator at Michigan State University did not deem it necessary to obtain Institutional Review Board approval.

Case selection

Records of the NMDL database were searched to identify horses diagnosed with PSSM2 based on accumulation of aggregates of either amylase-sensitive or amylase-resistant polysaccharide. If PSSM2 horses did not have a negative R309H *GYS1* genotype on file, frozen muscle biopsies were retrospectively tested. Cases were selected from 2008 to 2016 to enhance accurate owner recall, to avoid overlap with a previous epidemiologic study of PSSM and to provide a minimum of 5 months for horses to have followed the diet and exercise recommendations (12). Out of 452 PSSM2 cases, telephone, email or home addresses were on file for 260 horse owners and 155 additional cases had up to date contact information for veterinary clinics that was used to seek owner contact information.

To determine how common the behaviors and exercise responses were in the region where participants were located, participants were asked to identify another horse in their barn that had no previous history of a neuromuscular disorder or, if not in their barn, owned by a friend that had a horse in their geographic area. Horses in this baseline population were the same breed type, approximate age, gender and level of work as PSSM2 horses.

Recommendations

Each PSSM2 horse owner had been provided with written recommendations included with the original muscle histopathology report which included minimizing stress, providing regular routines and maximizing daily turn out (Supplement 2.1). Dietary recommendations included a range of digestible energy for maintenance to moderate exercise from 16.4–24.6 MCal/day and directions to provide less than 20% of digestible energy as NSC, 15–20% of digestible energy as fat and 697–836 g of protein per day. In 2014, additional recommendations for horses with PSSM2 included providing a prolonged warm up with long and low stretching, as well as rest periods during exercise to allow muscle relaxation. For those horses that exhibited muscle atrophy, the addition of an amino acid or protein supplement was recommended (Supplement 2.1 and 2.2).

Questionnaire

Telephone calls, emails, and postal delivery were used to encourage owners to answer a questionnaire with up to three solicitations per owner. A description of the study and an electronic link to a questionnaire were provided by email and, for non-respondents, in paper format. The questionnaire had closed ended questions with the option for additional written responses and included sections on signalment, results of PSSM1 genetic testing, general health history, performance history, lameness history, exercise responses, traits, diet before and after the diagnosis of PSSM2, exercise and turnout regimes before and after the diagnosis of PSSM2, and changes in traits and responses after diagnosis of PSSM2.

Owners of horses in the baseline group answered an abbreviated questionnaire that contained the same sections as the PSSM2 questionnaire, but omitted questions related to alterations made following diagnosis of PSSM2. Rather than provide information with regard to the time prior to the diagnosis of PSSM2, owners provided current information extending up to the year prior to answering the questionnaire.

Signalment

Age, breed, gender, information on current ownership, relationship to the horse (owner/rider/trainer), length of relationship, and alive or deceased status of the horse was ascertained. If the horse was deceased or re-homed, participants were asked to answer questions with regard to the months preceding death or rehoming.

History

The horse's performance discipline, highest level of training or competition and any change in performance was ascertained. Additionally, appetite, body condition score (BCS) and breeding history were obtained. Appetite was scored on a Likert-scale from 1–5, with 1 defined as poor and 5 defined as excellent (18). Pre-existing conditions of pituitary pars intermedia dysfunction, equine metabolic syndrome, shivers, stringhalt, equine protozoal myelitis (EPM), laminitis, colic, heaves, and gastrointestinal ulcers were reported along with any assessment of serum vitamin E status. The type and age of onset of abnormal exercise responses and behaviors relating to PSSM2, circumstances leading to these responses, frequency and severity were recorded.

Participants were asked to indicate if a lameness was present within the 6 months prior to the muscle biopsy, if a lameness examination was performed by a veterinarian, which leg and structures were affected, the cause/diagnosis established for the lameness and whether a bone scan was performed. Participants described any treatments for lameness, if treatment resolved the lameness satisfactorily, and if the lameness still affects the horse's performance or participant's expectations of the horse.

Diet

The PSSM2 WB horses' diet prior to muscle biopsy and diet at the time of the questionnaire was ascertained from a list of commonly fed feeds such as fresh grass, dried forages, complete feeds, low starch ration balancers and supplements. Owners were asked to indicate changes made to the diet in terms of specific feeds, portion size, feeding frequency, feeding times, protein sources, supplements and pasture grazing. Additionally, participants were asked if they felt that the diet change helped improve abnormal exercise responses.

Exercise

The number of days without work, the frequency and duration of work under saddle and turn out time prior to muscle biopsy (PSSM2) and upon receiving the questionnaire was documented. Extent and type of warm-up as well as number of breaks during a ride were recorded. Owners indicated if they changed the horse's discipline or exercise regime after the PSSM2 diagnosis. Changes to the duration and intensity warm up and work under saddle, use of a long and low frame and amount of turn out were recorded. The participants' impression of whether the horse improved was reported as well as the timeframe over which any improvements occurred.

Overall improvement

Participants evaluated their horse's overall change in performance as a percentage increase or decrease from the horse's initial performance level prior to muscle biopsy. A list of abnormal exercise responses, traits and behaviors was provided and participants indicated if they saw improvement, no change or decline in those signs. Open ended questions were also included to allow expansion on the severity and frequency of any current abnormal exercise or behavioral responses or further treatments.

Muscle histopathology

Staining of frozen muscle biopsy specimens at the time of submission included hematoxylin and eosin, modified Gomori trichrome, periodic acid Schiff (PAS), amylase-PAS and oil red O. Desmin immunohistochemistry was performed retrospectively. Sections from questionnaire respondent's horses were re-evaluated and scored for the presence of internalized myonuclei, anguloid atrophy, inclusions in Gomori trichrome, subsarcolemmal glycogen, cytoplasmic PAS positive aggregates, amylase-resistant polysaccharide and desmin positive cytoplasmic aggregates. Findings were summarized using a previously described scoring system that used 20x magnification to grade the above features based on: 0 = not present, 1 = present in approximately 10% of fibers in the biopsy, 2 = present in approximately 11–25% of fibers in the sample, 3 = present in more than 25% of fibers in the sample (19). A desmin score was assigned based on: 0 = not present, 1 = present in 1–2 fibers, 2 = present in 3–6 fibers, 3 = present in 7–10 fibers, 4 = present in >10 fibers (19). The proportion of horses with a score > 1 was determined for each histopathologic characteristic except desmin where a score > 2 was used.

Muscle glycogen concentrations

Muscle glycogen concentrations were analyzed in those respondent's PSSM2 WB horses that had enough frozen skeletal muscle tissue available for analysis (n = 32 shipped, 4 snap frozen out of 42 PSSM2 WB). Samples were either snap frozen in liquid nitrogen at the time of biopsy or shipped fresh on ice packs and received in the laboratory within 24–48 h. Fresh samples shipped in a similar fashion from 10 WB and 11 Quarter Horses with no histopathologic evidence of a myopathy and 14 WB and 7 Quarter Horses that possessed the R309H *GYS1*mutation were used as negative and positive controls, respectively for fresh shipped samples. Snap frozen samples from 9 healthy WB and 11 healthy Quarter Horses were used as controls for snap frozen samples. Glycogen concentrations were assayed fluorometrically as glucose residues using approximately 4 mg wet weight muscle as previously described (20).

Statistical analysis

Responses were compiled in a spreadsheet (Microsoft Excel®), and the diagnosis of PSSM2 by the NMDL was verified by cross referencing NMDL records. Not all respondents answered each question so results were calculated relative to the total number of responses for each question. To determine whether exercise responses or behaviors responded to recommendations, responses for post-PSSM2 diagnosis were compared to answers for pre-PSSM2 diagnosis. Responses were analyzed with Chi-square and Fisher's exact tests (GraphPad Prism 7®). All paired continuous data were analyzed with a generalized McNemar's test (21). To reduce the risk of an alpha error, Bonferroni correction ($p = 0.05/n$) was performed for related responses. This included responses pertaining to exercise/performance ($n = 4$; $p \leq 0.013$), traits associated with a neuromuscular disorder ($n = 8$; $p \leq 0.006$) and behavior ($n = 4$; $p \leq 0.013$). Skeletal muscle glycogen

concentrations in shipped PSSM2 samples were not normally distributed (Shapiro-Wilk normality test) and median values were compared to PSSM1 and to NM using a Mann-Whitney U test (GraphPad Prism 7®). PSSM2 WB were divided into those with glycogen values above the median and those with glycogen values below the median. Above and below median glycogen groups were analyzed for differences in responses before and after the recommendations using Chi-square. Significance was set at $p \leq 0.05$.

RESULTS

Responses to questionnaire

In total, contact was made with 89 owners by email and for those that did not respond 20 surveys were mailed. A total of 42 completed questionnaires (38.5% response rate) were received from PSSM2 WB horse owners. WB was defined as horses identified as a type of Warmblood (n = 33), draft cross (n = 5) or WB cross (n = 4). Not all respondents answered each question and therefore the denominator varies in the results.

Baseline population

The baseline population consisted of 22 WB horses in the same region as PSSM2 WB. Horses were 13 ± 6 years of age with 6 mares, 15 geldings, and 1 stallion. Most were used for dressage (14/22, 63%) and hunter/jumper disciplines (11/22, 50%). The highest level of competition was Prix St. George for dressage and 1.1–1.5 meter for jumpers. Lameness was present in 27% (6/22) of WB in the baseline population within in the last year. All of those horses had a lameness exam by a veterinarian and 67% (4/6) had a forelimb and 33% (2/6) a hind limb lameness. Only 1 control horse had nuclear scintigraphy, for which the scan had positive uptake in a forelimb. The frequency of traits and exercise responses in the baseline WBs were; difficulty with canter transitions/leads (4/11; 36%), reluctance to go forward (3/11; 27%), generalized atrophy (2/11; 18%), mild lameness (2/11; 18%), poor topline (3/11; 27%), difficulty backing up (1/11; 9%), prolonged recumbency (1/11; 9%), sensitivity to grooming (1/11; 9%), bucking (1/11; 9%), and resentment to girthing (3/11; 27%).

PSSM2 WB Signalment

At the time of the questionnaire, the 42 PSSM2 WB were 12 ± 6 y of age with 13 mares, 28 geldings, and 1 stallion.

Disciplines

Most PSSM2 WB were used for dressage (33/41, 80%) and hunter/jumper disciplines (6/41, 14.6%). The majority of horses competed at training (n = 5), first (n = 5) or second (n = 8) level dressage from United States Dressage Federation standards. The highest level of competition was Prix St. George for dressage and 1.1–1.5 meter for jumpers.

Time in respondent's possession

Over 90% of the respondents were owners and ≤ 10% were either the trainer, primary rider, or primary caretaker. The majority of respondents had their horses for > 5yrs (64%). Most horses were alive at the time respondents answered the questionnaire (33/41, 80%); however, 20% (8/41) of PSSM2 WB were euthanized at some point after PSSM2 diagnosis.

History

Respondents reported a mean appetite score of 4.4 ± 0.9 and a mean BCS of 4.9 ± 1.3. A few horses had a history of colic (11/38, 29%), laminitis (2/39, 5%), pituitary pars intermedia dysfunction (1/41, 2%), heaves (0/39, 0%), equine metabolic syndrome (1/39, 2%), shivers (2/39, 5%), stringhalt (1/39, 2%), or equine protozoal myeloencephalitis (EPM) (2/39, 5%). Several PSSM2 WB have been diagnosed with gastric ulcers via endoscopy (13/36, 36%). Of the horses that pursued treatment(s), 88% (15/17) used omeprazole, 29% (5/17) used other treatments such as sucralfate or ranitidine, and 35% (6/17) used hindgut buffers. When asked if treatment of gastric ulcers improved any clinical signs that led to a muscle biopsy, 17% (4/23) said yes, 39% (9/23) said no, 35% (8/23) indicated that the GI ulcers did not occur at the same time as clinical signs of PSSM2 and 9% (2/23) were unsure.

Seventeen PSSM2 WB respondents indicated that serum vitamin E concentrations had been measured. Of those, 41% (7/17) had adequate levels (> 2ug/ml), 6% (1/17) had marginal levels (1.5-2ug/ml), 24% (4/17) were deficient (< 1.5ug/ml), and 29% (5/17) were unsure.

Lameness

Of horses affected by a lameness within the last 6 months, 47% (9/19) of PSSM2 WB still had lameness affecting the horse's performance at the time of the questionnaire. Of those that had nuclear scintigraphy, 63% (5/8) had positive uptake and 37% (3/8) had a negative scan. Of the 5 PSSM2 WB with positive scans, 40% (2/5) had uptake in a forelimb, 60% (3/5) had uptake in a hind limb, 40% (2/5) had uptake in the neck, and 80% (4/5) had uptake in the hips or pelvis.

Onset of abnormal exercise responses

PSSM2 WB were first noted to have abnormal exercise responses related to PSSM2 at 6.4 ± 3.5 y of age with 48.8% (20/41) being ≤ 5y. The length of time over which owners perceived these abnormal responses developed was highly variable for PSSM2 WB, ranging from a few days or weeks (28%, 11/39), 1–2 months (31%, 12/39), 3 or more months (28%, 11/39) or unknown (13%, 5/39). Prior to the onset of abnormal exercise responses, 42% (16/38) of PSSM2 WB experienced a change in living situation, such as moving barns, and 37% (14/38) had a change in their diet. A consistent association with a weather pattern and these traits of PSSM2 was not reported.

Abnormal exercise responses

Before diagnosis of PSSM2 and recommendations, over half of the PSSM2 WB displayed abnormal performance related traits to include: decline in performance, reluctance to collect and a reluctance to go forward. General atrophy, difficulty with transitions, mild lameness, muscle fasciculations, poor topline, change in behavior, rhabdomyolysis, difficulty backing and resentment of saddling were only present in 30–48% of PSSM2 horses. The least common traits reported by PSSM2 respondents were bucking, sensitivity to grooming, prolonged recumbency and focal atrophy (Table 2.1).

Table 2.1: Traits, behaviors and exercise responses in a baseline WB population and PSSM2 WB alive at the time of questionnaire. *Significant differences comparing PSSM2 WB before and after recommendations.

Clinical Signs	Baseline WB Population	PSSM2 Warmbloods		
	n=11(%)	Before Recommendations	After Recommendations	p value
		n=33 (%)	n=33 (%)	
Performance				**Significance adjusted p = 0.013**
Reluctance to go forward	3 (27)	25 (76)	15 (45)	0.023
Decline in performance	0	22 (67)	7 (21)*	0.004
Reluctance to collect	0	19 (58)	10 (30)	0.046
Difficult canter transitions/ leads	4 (36)	15 (45)	11 (33)	0.45
Neuromuscular				**Significance adjusted p = 0.006**
Generalized atrophy	2 (18)	16 (48)	5 (15)	0.007
Mild lameness	2 (18)	15 (45)	8 (24)	0.12
Muscle fasciculations	0	15 (45)	8 (24)	0.12
Poor topline muscle	3 (27)	15 (45)	10 (30)	0.31
Rhabdomyolysis	0	12 (36)	2 (6)*	0.005
Difficulty backing up	1 (9)	10 (30)	5 (15)	0.24
Prolonged recumbency	1 (9)	4 (12)	1 (3)	0.355
Focal area of atrophy	0	3 (9)	6 (18)	0.475

Table 2.1 (cont'd)

Clinical Signs	Baseline WB Population	PSSM2 Warmbloods		
	n=11(%)	Before Recommendations	After Recommendations	p value
		n=33 (%)	n=33 (%)	
Behavior				**Significance adjusted p = 0.013**
Overall change in behavior	0	12 (36)	3 (9)	0.017
Resentment of saddling/ girthing	3 (27)	10 (30)	6 (18)	0.389
Bucking	1 (9)	8 (24)	5 (15)	0.537
Sensitivity to grooming	1 (9)	7 (21)	4 (12)	0.511

Performance prior to PSSM2 diagnosis

A decline in performance was reported for 73% of PSSM2 WB, 27% of respondents noted that training had plateaued without advancing as expected and none felt performance was advancing as expected (Table 2.2).

Muscle histopathology

Histopathology scores ≥ 1 were found for: subsarcolemmal glycogen 50% (21/42); cytoplasmic glycogen 43% (18/42); anguloid atrophy 48%, (20/42); central nuclei 21% (9/42); subsarcolemmal vacuoles 12% (5/42); amylase resistant polysaccharide 5% (2/42). Desmin scores > 2, consistent with a potential myofibrillar myopathy, were found in 4/42 PSSM2 WB. Two of these horses were included in a previous study of myofibrillar myopathy (22).

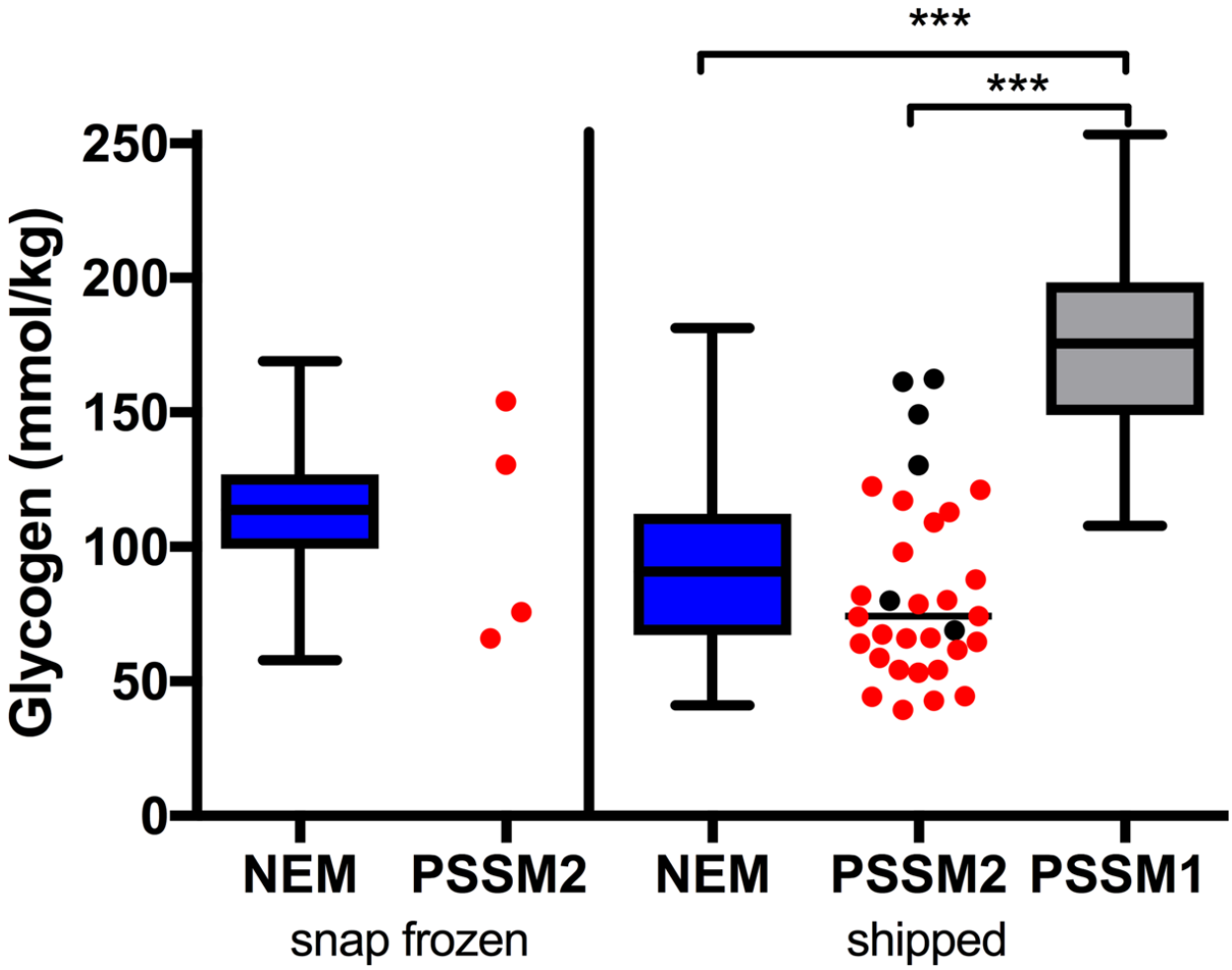
Muscle glycogen concentrations

A wide range in muscle glycogen concentrations was found for PSSM2 WB with some horses overlapping concentrations in PSSM1 horses and many falling below median values in NEM horses (Figure 2.1). Muscle glycogen concentrations in shipped PSSM2 WB samples were not significantly different from shipped NEM horses and were significantly lower ($p < 0.0001$) than PSSM1 horses (Figure 2.1). Four of the PSSM2 WB horses with the highest shipped glycogen concentrations were euthanized because of the severity of traits and one of the horses with the highest snap frozen glycogen was donated to a University due to the severity of abnormal exercise responses.

Table 2.2: Respondent's perception of the horse's performance prior to the diagnosis of PSSM2 (before) and at least 5 months after receiving diet and exercise recommendations.

Performance	Before Recommendations	After Recommendations
	n (%)	n (%)
Total number horses	n = 41	n = 34
Advancing	0 (0)	16 (47)
Not progressing	11 (27)	12 (35)
Declining	30 (73)	6 (18)
% decline		
1-25%	7 (17)	1 (3)
26-50%	6 (15)	1 (3)
51-75%	6 (15)	0 (0)
76-99%	7 (17)	2 (6)
100%	4 (10)	2 (6)

Figure 2.1: Box and whisker plots indicate minimum to maximum values for snap frozen and shipped samples of control horses that had no evidence of a myopathy (NEM), type 1 PSSM (PSSM1), and individual values are shown for PSSM2. Black circles indicate euthanized WB horses. Glycogen concentrations were not different between PSSM2 WB and NM. PSSM1 glycogen concentrations were significantly higher than PSSM2 WB and NM horses ($p < 0.0001$).



Diet change

After the PSSM2 diagnosis, 95% (39/41) of respondents felt they had changed their horse's diets according to the recommendations provided. For those that changed their horse's diet, 85% (33/39) of respondents reported that the diet had produced an overall improvement in exercise and behavioral traits. No improvement was reported for 13% (5/39) PSSM2 WB whereas 3% (1/39) were unsure if diet had made a difference.

In general, 87% (33/38) of PSSM2 WB respondents changed their horse's sources of calories by increasing the amount of dietary fat fed, and/or increasing the amount of low starch/ fat supplemented concentrate (Tables 2.3 and 2.4). A change in protein intake was reported for 63% (24/38), dietary supplements were changed for 76% (29/38), and access to fresh grass was changed for 55% (21/38) (Tables 2.3 and 2.4). It was difficult to discern the precise diet changes that could have been associated with improved exercise and behavioral traits because some owners changed the diet prior to the diagnosis of PSSM2. Most respondents did not have precise measures of the amount of various feeds being consumed and simultaneous changes in exercise regimes may have been adopted.

Table 2.3: Components of the diet of PSSM2 WB after recommendations were provided for a low starch, high fat diet.

Diet Components	After recommendations
	PSSM2 WB n (%)
Hay	**n=41**
Alfalfa	16 (39)
Grass	39 (95)
Fat Source	**n=36**
Low starch + fat concentrate	15 (42)
Solid	11 (31)
Oil	16 (44)
None	7 (19)
Protein	**n=33**
Alfalfa	17 (52)
Amino acid supplement	7 (21)
Ration balancer - 20-30% protein	5 (15)
No additional source	10 (30)
Supplements	**n=27**
Vitamin E (1000-6000 IU/day)	20 (74)
Magnesium	9 (33)
Vitamin & mineral supplement	9 (33)
Acylcarnitine	7 (26)

Table 2.4: Changes owners made to their horse's diet after receiving recommendations for PSSM2.

Diet Changes	PSSM2 WB (n=37) n (%)
↓ grass hay fed	7 (19)
↑ grass hay fed	7 (19)
↓ alfalfa hay fed	8 (22)
↑ alfalfa hay fed	9 (24)
↓ fat supplement	2 (5)
↑ fat supplement	20 (54)
↓ complete feed (Equine Sr. Grain)	13 (35)
↑ complete feed (Equine Sr. Grain)	1 (3)
↓ low starch high fat concentrate	6 (16)
↑ low starch high fat concentrate	19 (51)
↓ low starch ration balancer	2 (5)
↑ low starch ration balancer	8 (22)
↓ time on fresh grass	12 (32)
↑ time on fresh grass	4 (11)

Exercise regime

After diagnosis, PSSM2 WB were ridden for 4 ± 2 days/week for a duration of 42 ± 11 min. Respondents reported that 53% (19/36) of WB were ridden for an average of 45 minutes, 25% (9/36) were ridden for 30 minutes or less, and 22% (8/36) were ridden for 1 hour. Half of the participants warmed their horse up for 11–15 minutes, 30% (12/40) warmed their horse up for 6–10 minutes, and the rest warm their horses up for 16–20 + minutes. The majority of respondents provide their horse with 3 or more breaks during a ride (33/38, 87%). Additionally, 70% of PSSM2 WB get some form of exercise every day and 61% (23/41) get actively worked under saddle 5 or more days a week.

Impact of changes to exercise regime

After diagnosis of PSSM2 and receipt of PSSM recommendations, 76% (31/41) of WB had their exercise regime changed and 24% (10/41) had no change. Of the changes made, 32% (10/31) of respondents increased the amount of time the horse was turned out, 42% (13/31) increased the frequency of exercise throughout the week and 13% (4/31) decreased both riding time and the level of intensity. In addition, 71% (22/31) of respondents increased the duration of their warm-up time, 65% (20/31) started to allow breaks throughout the ride and 67% (20/30) implemented long and low stretching techniques.

Of the PSSM2 WB respondents, 70% (21/30) felt that the change in exercise helped improve exercise responses, while 17% (5/30) saw no improvement, and 13% (4/30) were unsure if an improvement was present. Fifty-five % (11/20) of respondents that used long and low stretching felt it improve exercise responses while 45% (9/20) saw no difference. The time frame for

general improvement was less than a month in 45% (10/22) of horses, 1–2 months in 18% (4/22), 3–4 months in 23% (5/22) and greater than 4 months for 14% (3/22).

Change in exercise responses and behavior

An overall improvement in responses and behavior was reported for 80% (32/40) of PSSM2 WB after receiving recommendations with the PSSM2 diagnosis. When comparing the responses after diagnosis and recommendations to before diagnosis for horses alive at the time of the questionnaire, PSSM2 WB were less likely to experience a decline in performance ($p = 0.004$) and rhabdomyolysis ($p = 0.005$) (Table 2.1). Although the traits of reluctance to collect, reluctance to go forward, overall change in behavior, and generalized muscle atrophy were also reported to improve they did not reach significance after Bonferroni correction (Table 2.1).

Change in performance

With the changes instituted after PSSM2 diagnosis, 47% of PSSM2 WB respondents reported that training was advancing as expected, 35% reported that performance had plateaued and not advance as expected and 18% reported that performance had declined (Table 2.2). Because additional recommendations of adding an amino acid supplement and using long and low warm up techniques were added in 2014, we analyzed the overall response of those with the additional recommendations compared to those without. For those without the additional recommendations, 18% (3/17) of respondents reported a decline in performance, 29% (5/17) indicated no advancement, and 53% (9/17) were reported to be advancing as expected. For those receiving the additional recommendations, 18% (3/17) reported a decline, 41% (7/17) indicated no advancement, and 41% (7/17) were reported to be advancing as expected. There was no

significant difference in the respondent's perception of the horse's overall improvement with respect to the additional recommendations made in 2014.

Five of 31 PSSM2 WB respondents (16%) changed the horse's discipline(s) after diagnosis and recommendations: 2 horses changed from dressage to hunter/jumper, 2 horses from dressage to trail and pleasure riding, and 1 dressage horse was retired. At some time point after PSSM2 diagnosis, 22% (9/41) of PSSM2 WB were euthanized (n = 8) or donated to a University (n = 1). Respondents indicated euthanasia was due to severe traits of PSSM2 (n = 5; 1 with concurrent gastric ulcers and laminitis), epilepsy (n = 1), neurologic disease (n = 1) or prolonged recumbency (n = 1).

Behavior and exercise responses relative to muscle glycogen concentrations

A decline in performance was reported for PSSM2 WB in both above and below median glycogen groups, however, a significantly larger proportion of horses above the median glycogen concentration had a decline in performance before diagnosis compared to those below the median glycogen concentrations ($p = 0.005$) (Table 2.5). All participants that had horses that were deceased at the time of the questionnaire answered questions describing exercise responses and traits before diagnosis (n = 5 above; n = 1 below median glycogen concentration). Only one participant with a deceased horse (above median glycogen) answered questions regarding responses and traits after recommendations. After the recommendations, the proportion of horses with a reported decline in performance significantly decreased in the above and below median glycogen groups ($p = 0.0002$, $p = 0.006$, respectively) (Table 2.5). Sixty-nine versus 30% of owners reported an overall change in behavior before diagnosis in the above vs. below median

glycogen concentration groups, respectively. After recommendations, there was a significant decrease in the number of owners reporting a change in behavior in the above median glycogen group and no horse owners reported a change in behavior in the below median group after the recommendations ($p = 0.009$) (Table 2.5).

Table 2.5: Exercise responses and traits noted in at least 30% of horses before the diagnosis of PSSM2 and after diet and exercise recommendations subdivided into those horses with glycogen concentrations above the median glycogen concentration and those horses below the median glycogen concentration. * indicates significant difference within the above or below median glycogen groups between before and after. † indicates a significant difference between the above and below median glycogen groups for the corresponding clinical signs in the same time frame.

Clinical Signs	**Glycogen above median**		**Glycogen below median**	
	Before Diagnosis	After Recommendations	Before Diagnosis	After Recommendations
	n=16 (%)	n=12 (%)	n=20 (%)	n=16 (%)
Performance				**Significance adjusted p = 0.013**
Decline in performance	16 (100%)†	4 (33%)*	12 (60%)	2 (13%)*
Reluctance to collect	11 (69%)	7 (58%)	11 (55%)	3 (19%)
Reluctance to go forward	15 (94%)	6 (50%)	13 (65%)	7 (44%)
Neuromuscular				**Significance adjusted p = 0.006**
Generalized atrophy	9 (56%)	3 (25%)	7 (35%)	1 (6%)
Rhabdomyolysis	7 (44%)	1 (8%)	9 (45%)	1 (6%)
Muscle fasciculations	6 (38%)	4 (33%)	9 (45%)	3 (19%)
Behavior				**Significance adjusted p = 0.013**
Overall change in behavior	11 (69%)	2 (17%)*	6 (30%)	0 (0%)

DISCUSSION

The results of the present study provide a comprehensive owner reported description of the characteristics of PSSM2 in WB and for the first time provide valuable prognostic information with regard to response of PSSM2 WB to current diet and exercise management as perceived by owners. The onset of abnormal exercise responses and behaviors in PSSM2 WB was insidious with an average age of onset of 6 years, an age when WB horses are expected to be advancing in their training (23). The three most common complaints of respondents owning PSSM2 WB were reluctance to collect under saddle, decline in performance, and reluctance to go forward reported by 58%, 67%, 76% of respondents, respectively. These exercise responses were uncommon in the baseline WB population at 0–27%. Respondents also reported that 6–24% of PSSM2 WB had mild lameness, muscle atrophy, muscle fasciculations and intermittent rhabdomyolysis. No horses in the baseline population had fasciculations or rhabdomyolysis whereas 18% had lameness (largely forelimb) or atrophy. Other traits and responses common to both PSSM2 WB and the baseline group included difficulty with canter transitions/leads, poor topline, sensitivity to grooming, resentment to girthing and bucking with frequencies ranging from 9–36% in baseline and 12 to 33% in PSSM2 WB. In a previous study of PSSM in WB and non-WB breeds, which included 25 WB in the present study, veterinarians reported that 66% of 188 PSSM2 WB horses had a gait abnormality and 26% had rhabdomyolysis (14). To provide a more thorough description of the gait abnormality, participants in the present study were given detailed questions about their horse's gait and lameness. The results revealed that the gait abnormality in PSSM2 WB consisted largely of an undiagnosed lameness that did not resolve with veterinary treatment in 60% of PSSM2 WB. Supplementing the finding of a gait abnormality by

Lewis *et. al*. 2017, our results indicate that reluctance to go forward and to collect are prominent features of PSSM2 in WB which significantly impact the horse's performance.

The lameness and decline in performance in PSSM2 WB described in the present study may overlap with signs associated with orthopedic disorders. In particular, sacroiliac disease is characterized by poor development of epaxial muscles, asymmetric hind limb muscles, stiffness, unwillingness to work on the bit and poor quality canter (24; 25). Scintigraphic evaluation is commonly used to diagnose back and sacroiliac disease and was performed in 8 horses in the present study (26). Of those 8 PSSM2 WB, 4 scans had positive results, but only 1 respondent indicated that their horse had been diagnosed with sacroiliac disease; however, the 3 remaining horses had reported positive uptake in the pelvic area. The overlap in clinical signs and common occurrence of sacroiliac disease in WB certainly suggests that diseases of the back and sacroiliac joint should be ruled out prior to performing a muscle biopsy. Additionally, a wide range of training and saddling issues could also produce many of the performance-related signs seen in PSSM2 horses and it seems prudent to fully explore these issues prior to a muscle biopsy (27).

Fortunately, by following the recommended diet and exercise regimes, 80% of PSSM2 WB were reported to show overall improvement as well as a significant decrease in signs of rhabdomyolysis, muscle atrophy, change in behavior and decline in performance. This is in agreement with a 2007 epidemiologic study of WB diagnosed with PSSM that reported 71% of WB with PSSM (type 1 or type 2 not specified) improved with recommendations for a low starch, fat supplemented diet and regular exercise (12). The reported improvement is also higher than the placebo effect found in a caregiver study in which 40% of owners of dogs with

osteoarthritis reported a beneficial effect when they unknowingly administered a placebo (28). It is important to recognize, however, that although abnormal exercise responses and behaviors improved, one third of respondents felt that their horse's training had plateaued and 18% felt their horse's performance had declined after implementing recommendations. The most persistent traits were reluctance to go forward (45% still present), difficulty with transitions (33% still present), reluctance to collect (30% still present), poor topline muscle (30% still present) and muscle fasciculations (24% still present). Thus, owners of PSSM2 WB should be aware that although the majority of horses improve with the current diet and exercise recommendations, half of the horses are unable to achieve the owner's performance expectations. Sixteen percent of respondents in the survey changed the horse's discipline to one requiring less collection and 22% of PSSM2 WB were eventually euthanized or donated to a University. Our results suggest that even when following the current diet and exercise recommendations, there are residual effects of the muscle disorder underlying PSSM2. Potentially, this study's results may have been biased since owners of more severely affected horses could have been more motivated to participate in the study.

With the present study design, there was no means to determine how stringently recommendations were followed by horse owners or what specific diet changes were responsible for the perceived improvement amongst horse owners. The most consistent changes in diet made by owners of PSSM2 WB were the addition of a fat supplement in 54% of horses, use of a low starch high fat feed in 51% and supplementation with vitamin E in 71% of PSSM2 WB. Generally, the dietary recommendations may have benefited horses because owners sought nutritional advice ensuring that horses received a balanced diet that was relatively low in starch

and sugar, supplemented with fat and contained a quality protein source and vitamin E. Beginning in 2014, recommendations for PSSM2 horses included adding a protein source or amino acid supplement to build topline muscles and providing long and low warm up exercise to relax the back and strengthen topline. Provision of amino acid supplements has been shown to increase muscle mass in exercising horses as assessed by subjective muscle scoring (29). However, there was no significant difference in performance when comparing horses that received recommendations before 2014 to those after–as shown by 50 vs 44% of PSSM2 WB horses advanced in their training, respectively. A clearer understanding of the basis for PSSM2 in WB and design of controlled diet trials and are needed to optimize diet and exercise regimes for these horses.

The diet and exercise regime recommended for PSSM2 horses was originally designed to decrease glycogen synthesis in skeletal muscle and improve oxidative metabolism of glycogen and fat during exercise. In PSSM1 horses, the diet effectively lowers serum insulin concentrations which could thereby decrease glucose uptake and decrease activation of glycogen synthase in skeletal muscle (9; 30). Whereas glycogen concentrations are >1.5 fold normal in PSSM1 WB, a recent study did not find significantly elevated mean glycogen concentrations in the 13 PSSM2 WB studied (14). Similarly, glycogen concentrations in the present study were not significantly different from horses with no evidence of a myopathy in muscle samples and were lower than PSSM1 horses. However, there was a large variation in glycogen content in PSSM2 WB both in snap frozen and shipped samples. Glycogen continues to be metabolized during shipping, and low levels could be a reflection of continued metabolism; however, control samples were also shipped in a similar fashion. It is possible that the horses with elevated muscle

glycogen concentrations represent the extremes within one phenotype embodied by a disorder of glycogen metabolism. Most WB horses diagnosed with PSSM2 had very similar clinical signs, and glycogen storage disorders in humans can have variable muscle glycogen concentrations (31). Alternatively, those PSSM2 WB with glycogen concentrations as high as PSSM1 could represent a group of horses with a separate disease from horses with lower glycogen concentrations. The histopathologic criteria for PSSM2 are nonspecific, which increases the possibility that more than one etiology for muscle disease falls under this histopathologic diagnosis. Four horses were later found to have histochemical characteristic of abnormal aggregates of desmin within muscle biopsies suggestive of myofibrillar myopathy (MFM) (22). This finding again could represent progression of the PSSM2 phenotype or could represent a separate disease entity. Determining whether horses within the PSSM2 diagnosis have one etiopathology, or separate etiologies, will require further biochemical and genetic analyses.

An unexpected finding in the present study was the high prevalence of gastric ulcers (32%) confirmed by endoscopy in the PSSM2 WB horses. This is slightly lower than the prevalence of squamous ulceration (40%) reported in Warmblood show jumping horses (32). It is unlikely that gastric ulcers themselves accounted for clinical signs of PSSM2 in WB horses because many of the horses had been effectively treated for ulcers yet performance problems persisted. The higher prevalence of ulcers in the PSSM2 WB could be related to high exercise frequency and performing at competitions; information that was unfortunately not obtained in our study (33).

The authors acknowledge the limitations of the present study, particularly that the results of the questionnaires are based on the perceptions of owners with varying levels of experience, and not those of a veterinarian.

CONCLUSION

In summary, the results of the present study indicate that PSSM2 in WB has a strong impact on performance affecting a horse's willingness to collect and go forward. By following diet and exercise recommendations, exercise responses appear to improve in 80% of horses; however, 30–45% of horses have residual signs that impacted the horse's ability to advance in training. While a small proportion of PSSM2 WB had elevated muscle glycogen concentrations, the majority of PSSM2 WB had concentrations at or below concentrations in horses without histopathologic evidence of a myopathy.

APPENDIX

Supplement 2.1: This supplemental document provides recommendations and information that was provided to owners of horses with a PSSM diagnosis. They were obtained with the courtesy of Dr. Valberg and the Neuromuscular Diagnostic Lab.

Neuromuscular Diagnostic Laboratory
Polysaccharide Storage Myopathy

What Causes PSSM?
Horses with polysaccharide storage myopathy (PSSM) accumulate excessive amounts of the normal form of sugar in their muscles (glycogen) as well as an abnormal form of sugar (polysaccharide) in muscle tissue. Clinical signs of are usually those of tying-up where horses develop muscle stiffness, soreness and reluctance to work with exercise. However, signs found in Draft, Draft crossbreeds, include muscle atrophy, weakness and gait abnormalities. Some horses with PSSM that are managed well show no clinical signs. Type 1 PSSM is caused by an inherited defect in a gene called glycogen synthase that results in abnormal regulation of glycogen in skeletal muscle. This appears to disrupt energy metabolism. We can now perform a genetic test for PSSM if you did not do so when the biopsy was submitted. You can request this test from hair or blood samples. Forms are on our website http://www.vdl.umn.edu/vdl/ourservices/neuromuscular.html We do not know yet what the defect is that causes type 2 PSSM.

More information regarding PSSM can be found at the Neuromuscular Disease Laboratory website http://www.cvm.umn.edu/umec/lab/home.html. We are unable to discuss individual cases with horse owners by telephone. We are pleased to discuss cases with your veterinarian. If owners of horses we have evaluated have questions please email or mail them to us. Email address is NMDL@umn.edu

Management of PSSM
Signs of muscle pain, atrophy and stiffness in horses with both types of PSSM can be managed through specific diet and training regimes. Both diet and training must be changed to see a beneficial effect. The diet is altered to minimize starch and sugar content. This means eliminating sweet feed, corn, wheat, oats, barley, and molasses. Alternative calories are supplied in the form of fat. An equally important part of PSSM horse management is daily exercise. Consistent exercise enhances glycogen utilization, increases enzymes needed to burn fat and improves energy metabolism in skeletal muscle. To burn fat efficiently, horses need a gradual training program in addition to more fat in their diet. Approximately 90% of horses experienced fewer or no episodes of tying-up if the recommendations provided below are followed. At present, the best we can do is to use management techniques in order to reduce recurrence. Many horses with this muscle disorder have recurrent episodes of tying- up.

1) **Exercise recommendations**
   Provide daily turnout: Provide daily turn out for as long as possible with other horses to keep the horse active. Decrease the amount of time the horse is left in the stall as much as possible.

Starting exercise: If the horse has suffered from stiffness within the last two weeks, or has just been diagnosed as having PSSM, start by turning the horse out for two weeks while implementing the recommended dietary changes. Once the horse has been on the diet for two to four weeks, start round pen or light lunge line work once a day for three minutes at a walk and trot. This initial work should be very mild and very short in duration. Gradually increase the time in work by two minutes each day, with a two minute walk break before the increasing the exercise duration. If the horse seems stiff at any time, slow to a halt and rest for a minute if the stiffness persists, stop there; if not, continue after a two-minute walk. If the horse seems tired, do not continue to increase the work load, but let the horse become acclimated to the workload in an even more gradual manner.

When the horse can exercise for 15 minutes, provide a five-minute break at a walk, and then gradually increase walking and trotting for longer periods. At least three weeks of groundwork will precede any mounted exercise. When the horse can comfortably walk and trot for 30 minutes on a lunge line, you may start riding the horse at the same exercise intensity, starting with 20 minutes of combined walk/trot. Gradually increase the time the horse is ridden using intervals of walk and trot and then work into walk, trot, and canter. Be sure to monitor the horse's level of exertion. Your patience will be rewarded.

Maintaining exercise: Try to exercise the horse on a daily basis even if only for 15 minutes on a lunge-line. Research has consistently shown that even 10-15 min a day will improve the function of muscle in PSSM horses. Once your horse is fit it may well manage with turn out rather than daily exercise from time to time. The number of days off a horse can manage is highly individual. If more than 3 or 4 days have gone buy begin with a small amount of exercise.

2) Dietary Changes for PSSM Horses

Scientific evidence proves that <u>it is not necessary to feed 1 lb of fat a day</u> as you may see recommended on the internet. Excessive dietary fat will lead to obesity and metabolic syndrome. Judge the number of calories your horse needs first and adjust the diet accordingly.

Forage

A high-quality grass or oat hay should form the basis of the diet. If alfalfa hay must be fed, combine it with another low sugar hay like Bermuda grass hay or timothy. Vitamin and mineral supplements containing Vitamin E and selenium are beneficial; however, some feeds (including some recommended below) contain enough selenium and other vitamins and minerals and do not require additional supplements. Check with the feed company if there are any questions.

Electrolytes

Ensure that salt is always available. If horses will not use a salt block, add 1-3 tablespoon of loose iodized table salt in the feed, particularly in hot weather. If the horse

is sweating a great deal, an additional tablespoon of lite salt (containing potassium chloride) can be added.

Complete Feeds
In my experience it is easiest to provide a balanced ration if a complete feed is fed. These feeds do not require additional protein/vitamin/mineral supplements and are to be fed along with hay. No additional grain or mineral/vitamin mix should be added.

Re-Leve®** by Hallway Feeds (www.Re-leve.com) Phone 1-800 753-4255
In Minnesota, Re-Leve is carried by Assurance Feeds Phone (651) 463-8041.

Developed with University of Minnesota researchers, Re-Leve is the only feed proven to be effective for PSSM and is good for finicky eaters. Starch content is low (9.0% by weight) and fat content high (12.5% by weight). Additional selenium should not be fed.

- Two forms of Re-Leve exist:
  - **Re-Leve original is for hard keepers needing more concentrate to maintain body weight.**
    - Feed 8-10 lbs for thin horses or horses in heavy work
  - **Re-Leve Concentrate is best for many PSSM horses that are easy keepers**
    - 3-5 lbs fed for light to moderate work

For overweight horses, work with your veterinarian and avoid excessive fat supplementation. One approach would be to reduce hay to 1% of body weight and feed 1-3 lbs of Re-Leve Concentrate.

For growing horses:
Weanlings: 6.5 lbs Re-Leve® and a mixed grass/alfalfa hay (8lbs per day) Yearlings: 8 lbs of Re-Leve® and a 50-50 alfalfa/grass hay (9 lbs per day).
(**a portion of the proceeds from the sale of Re-Leve® are directed to Dr. Valberg)

Ultium® by Purina in the USA: www.purinamills.com. Or Phone 800-227-8941

- 6-8lbs per day

Safe Choice® by Nuterna. www.nutrenaworld.com.

- 6 lbs per day combined with Empower® at 2 lbs/day

UnTi available in Canada is a low starch and high fat feed

There are now several other low starch and high fat feeds available. Please speak to a nutritionist regarding these feeds. The quality of ingredients may vary so make sure you are working with a reputable company. At a minimum they should meet the nutritional requirements provided in Table S1. In general, the starch content of the feed should not be greater than 15-20% by weight and the fat should be greater than 10% by weight.

Blending of individual feeds
Fat supplements combined with additional protein/ vitamin /mineral mixes and a fiber base can be custom blended. You may find in the end that this is not a cost saver. Consult with the manufacturer's nutritionists to formulate the correct blend for a PSSM horse which is specific to breed and level of use using the values in Table S1.
Stabilized rice bran fat supplements:
EquiJewel® Kentucky Equine Research, Phone 859 873 1988, Fax 859 873 3781, Email info@ker.com. ker.com/supplements/Equijewel.html

Natural Glo® (rice bran) Alliance Nutrition. Phone 1-800 680-8254 email AM_EquineHelp@admworld.com, www.admani.com/Alliance Equine/

Ultimate Finish® by Buckeye Nutrition, PO Box 505, 330 E. Schultz Ave. Dalton, OH 44618. Phone: 1-800-898- 9467 Fax: (330) 828-2309, www.buckeyenutrition.com/equine

Vegetable Oils: Soy oil or Corn oil gradually can be added at 1-2 cups per day to a fiber base such as hay cubes or alfalfa pellets. Add 600 U of vitamin E/cup of oil per day.

Remember to weigh the feeds using a scale and not use volume to determine actual weight.

Table S1: Nutritional requirements for an average sized horse (500 kg /1100 lbs) with PSSM at varying levels of exertion*.

	Maintenance	Light Exercise	Moderate Exercise	Intense Exercise
Digestible Energy (DE) (Mcal/day)	16.4	20.5	24.6	32.8
% DE as NSC	<20%	<20%	<20%	<20%
% DE as fat	15%	15%	15%-20%	20-25%
Forage % bodyweight	1.5- 2 %	1.5- 2 %	1.5- 2 %	1.5- 2 %
Protein (g/day)	697	767	836	906
Calcium (g/day)	30	33	36	39
Phosphorus (g/day)	20	22	24	26
Sodium (g/day)	22.5	33.5	33.8	41.3
Chloride (g/day)	33.8	50.3	50.6	62
Potassium (g/day)	52.5	78.3	78.8	96.4
Selenium (mg/day)	1.88	2.2	2.81	3.13
Vitamin E (IU/day)	375	700	900	1000

***** Disclosure of financial interest: Drs. McCue, Mickelson and Valberg are the patent owners for the genetic testing for GYS1. A portion of the proceeds from this test will go towards their continued research as well as patent royalties.

Although the type 1 PSSM (GYS1 mutation) is the most common genetic cause of PSSM and tying up in Draft and Quarter Horse related breeds there are other causes of tying-up. If horses affected with chronic muscle diseases are negative for type 1 PSSM we recommend follow up with a muscle biopsy to investigate other possible causes, see

http://www.cvm.umn.edu/umec/lab/home.html, for information on obtaining and submitting a muscle biopsy to the Neuromuscular Diagnostic Laboratory.

Supplement 2.2: This supplemental document provides recommendations and information that was provided to owners of horses with a PSSM2 diagnosis. They were obtained with the courtesy of Dr. Valberg and the Neuromuscular Diagnostic Lab.

Neuromuscular Diagnostic Laboratory Michigan State University, College of Veterinary Medicine
Dr. Stephanie J. Valberg

10/5/2015

Type 2 Polysaccharide Storage Myopathy

Terminology: PSSM, EPSM and EPSSM are terms first used to describe horses that have abnormal appearing glycogen in muscle biopsies. Considerable controversy existed as to whether these acronyms encompassed one muscle condition or several muscle conditions. In 2008, a mutation in the glycogen synthase 1 gene was found to be highly associated with one form of excessive storage of polysaccharide in muscle. Genetic testing of hundreds of horses previously diagnosed with PSSM showed that not all horses diagnosed with PSSM have this genetic mutation. This suggested that there are at least two forms of PSSM. For clarity, the form of PSSM caused by a glycogen synthase 1 (*GYS1)* gene mutation is now termed **type 1 (PSSM1)** whereas the form of PSSM that is not caused by the *GYS1* mutation and whose origin is yet unknown is now termed **type 2 (PSSM2)**. PSSM1 is likely the same disorder described as "Azoturia " or "Monday Morning Disease" in work horses in the 19th and 20th centuries. We believe that there are probably several causes of type 2 PSSM.

Type 2 PSSM

There is much less known about type 2 PSSM, because as it turns out, previous research on PSSM has largely involved horses with type 1 PSSM. Current knowledge of type 2 PSSM is based on retrospective evaluation of cases diagnosed with PSSM by muscle biopsy that are now known be free of the *GYS1* mutation and a few years of prospective clinical cases.

Prevalence: Approximately 28% of cases of PSSM diagnosed by muscle biopsy in Quarter Horses do not have the *GYS1* mutation. Type 2 PSSM seems to be more common in higher performance horses such as barrel racing, reining and cutting horses compared to the high prevalence of type 1 PSSM in halter horses. About 80% of cases of PSSM diagnosed by biopsy in Warmbloods have type 2 PSSM. Breeds affected include Dutch Warmbloods, Swedish Warmbloods, Hanoverians, Friesians, Selle Francais, Westfalian, Canadian Warmblood, Irish Sport Horse, Gerdlander, Husien, and Icelandic horses. Many other light breeds have also been diagnosed with type 2 PSSM including Morgans, Arabians, Standardbreds and Thoroughbreds.

Pathophysiology: The cause of type 2 PSSM is currently unknown. It may well be that there are a group of conditions that have separate causes but share common findings of

glycogen accumulation and poor performance. A heritable predisposition is suspected in Quarter Horses but yet to be proven. Recent research does not support higher than normal muscle glycogen concentrations in muscle from horses with type 2 PSSM. The glycogen aggregates within the cells but the total amount is not increased as in type 1 PSSM.

Acute Clinical signs: Horses with type 2 PSSM do not necessarily have the same calm temperament as horses with type 1 PSSM. In adults, acute clinical signs of rhabdomyolysis are similar between type 1 and type 2 PSSM. Muscle atrophy after rhabdomyolysis is a common complaint in Quarter Horses with type 2 PSSM and this may not be preceded by exercise. There are more Quarter Horses less than one year of age reported with type 2 PSSM than type 1 PSSM and these foals may present with an inability to rise or a stiff hind limb gait.

Chronic clinical signs: Chronic signs of type 2 PSSM are often most closely related to poor performance rather than recurrent ER and elevations in serum CK activity. An undiagnosed gait abnormality, sore muscles and drop in energy level and unwillingness to perform after 5 -10 min of exercise are common complaints with type 2 PSSM. Warmbloods with type 2 PSSM have painful firm back and hindquarter muscles, reluctance to collect and engage the hindquarters, poor rounding over fences, gait abnormalities, and slow onset of atrophy especially when out of work. The mean age of onset of clinical signs in Warmbloods is between 8 and 11 years of age with the median CK and AST activity being 323 and 331U/L, respectively.

Diagnosis: Type 2 PSSM must be diagnosed by muscle biopsy where increased or abnormal PAS positive material that is usually amylase-sensitive is apparent particularly in subsarcolemmal locations. False positive diagnosis is possible for type 2 PSSM in highly trained horses that normally have higher muscle glycogen concentrations or in formalin fixed sections which show a greater deposition of subsarcolemmal glycogen even in healthy horses. Our laboratory grades polysaccharide accumulation as mild, moderate, and severe where mild accumulation represents a category which has a higher chance of being a false positive diagnosis. Mild PSSM cases in particular should receive a full physical examination to ensure that there are not other underlying causes for performance problems.

Management of PSSM

Signs of muscle pain, atrophy and stiffness in horses with both types of PSSM can be managed through specific diet and training regimes. Both diet and training must be changed to see a beneficial effect. The diet is altered to provide a moderate starch and sugar content, a slightly higher protein content with high quality amino acids and if needed for energy fat supplementation. This means reducing or replacing sweet feed, corn, wheat, oats, barley, and molasses with a ration balancer that contains vitamins, minerals and at least 20% protein. In addition, alfalfa hay, meal or cubes can be added to the diet. If the horse needs more energy to maintain weight, calories can be supplied in the form of fat. An equally important part of PSSM horse management is daily exercise. Consistent exercise enhances glycogen utilization, increases turnover of structural proteins in the muscle and builds enzymes needed to burn energy as fuel. At present, the best we can do is to use management techniques in order to reduce recurrence. Many horses with this muscle disorder have recurrent episodes of tying-up. An addition protein supplement such as Progressive's Topline extreme may be of value in horses with muscle atrophy and feeding after exercise

may enhance its incorporation into muscle proteins. Ensure horses have normal serum vitamin E and supplement if necessary.

Avoid Rest: For chronic cases, prolonged rest after an episode appears to be counterproductive and predisposes PSSM horses to further episodes of muscle pain. With PSSM it is NOT advisable to only resume exercise when serum CK activity is normal. Rather, horses should begin small paddock turn out as soon as reluctance to move has abated. Providing daily turn out with compatible companions can be very beneficial as it enhances energy metabolism in PSSM horses. Grazing muzzles may be of benefit to PSSM horses turned out on pastures for periods when grass is particularly lush. Most PSSM horses are calm and not easily stressed, however, if stress is a precipitating fact, stressful environmental elements should be minimized.

Reintroducing exercise: Re-introduction of exercise after an acute episode of ER in PSSM horses needs to be gradual. Important principles include 1) providing adequate time for adaptation to a new diet before commencing exercise (2 weeks), 2) recognizing that the duration of exercise is more important to restrict than the intensity of exercise (no more than 5 min walk/trot to start) 3) ensuring that exercise is gradually introduced and consistently performed and 4) minimizing any days without some form of exercise. Exercise should begin with light slow uncollected work on a longe-line or under saddle beginning with once a day for 3-5 minutes at a walk and trot. This initial work should be very mild and very short in duration. Work at a walk and trot can be gradually increased by two minutes each day. When the horse can exercise for 15 minutes, a five-minute break at a walk can be provided, and then a few intervals of walk and trot can gradually be increased. At least three weeks of walk and trot should precede work at a canter.

Exercise: Regular daily exercise is extremely important for managing horses with PSSM. Even 10 min of exercise has been shown to be extremely beneficial in reducing muscle damage with exercise. Once conditioned, some PSSM horses thrive with 4 days of exercise as long as they receive daily turn out. For riding horses with type 2 PSSM, a prolonged warm-up with adequate stretching is recommended. Rest periods that allow horses to relax and stretch their muscles between 2 – 5 min periods of collection under saddle may be of benefit. Horses should be worked in a long and low frame for at least 4 weeks initially with a very gradual reintroduction of collected work. The collected work should be performed in intervals lasting no more than 5 min with a period of stretching provided between intervals. The time of active collection can be gradually increased as the horse works more underneath himself and in balance.

Try to exercise the horse on a daily basis even if only for 15 minutes on a lunge-line. Research has consistently shown that even 10-15 min a day will improve the function of muscle in PSSM horses. Once your horse is fit it may well manage with turn out rather than daily exercise from time to time. The number of days off a horse can manage is highly individual. If more than 3 or 4 days have gone by begin with a small amount of exercise.

A potential scenario for topline strength building exercises include: Relaxed work on a lunge-line before riding or just by itself 5 days a week. Aids that help create a long low frame may be useful such as Vienna reins. Begin with a couple of minutes of walk and then ask for a very relaxed trot to get the back swinging long and low without the need for

hindlimb impulsion. Watch to see the base of his neck muscles release, the back stretch and rump muscles contract. To begin with, the length of time at a trot should be no more than 5 minutes in each direction. After that allow the horse to walk and stretch. After a few weeks, add canter after the trot is relaxed and continue foras long as the horse can carry himself with a relaxed base of his neck, impulsion and rounded back. Start with just a minute or less. When the horse releases at the base of its neck at the canter I would do a few more strides then come back to trot and do some trot with the horse moving well forward underneath him/herself and try to get him/her to stretch down at the same time. It takes several weeks in my experience to get them to round at a canter through their back. They often cannot hold the canter at first for more than a few strides in the correct position. Once this is working well add more transitions. After about 4 weeks If doing this for 20 minutes before you ride you should start see more energy and better carriage and with the diet change more muscle mass.

Dantrolene: For horses with highly recurrent forms of type 2 PSSM (Barrel horses for example), the use of dantrolene may be beneficial for a period of time in bringing horses back into training. A dose between 2 and 4 mg/kg should be given 1 hour before exercise and its peak effect is 90 min after administration. Dantrolene may cause a positive drug test in race horses or show horses. It should be gradually withdrawn once horses have settled into a training regime.

Dietary recommendations for PSSM

We strongly encourage owners to utilize an equine nutritionist to provide a balanced diet. Most feed companies have a nutritionist that will provide support for veterinarians. The Neuromuscular Diagnostic Laboratory does not have a nutritionist on staff but can work with a feed company to meet your horse's needs if you let us know what company you want to utilize.

Caloric balance: The first step in designing a diet for PSSM horses is to decide what the horse's caloric requirements are and what the horse's ideal body weight is or should be. Many horses with PSSM are easy keepers and may be overweight at the time of diagnosis. Adding excessive calories in the form of fat to an obese horse may produce metabolic syndrome and is contraindicated. If necessary, caloric intake can be reduced by using a grazing muzzle during turn-out, feeding hay with a moderate nonstructural carbohydrate content (less than 12 % NSC) at 1 to 1.5% of body weight, providing a low calorie, protein supplemented ration balancer and gradually introducing daily exercise. Rather than provide dietary fat to an overweight horse, fasting for 6 h prior to exercise can be used to elevate plasma free fatty acids prior to exercise and alleviate any restrictions in energy metabolism in muscle.

Selection of forage: We recommend a high quality hay with an NSC content of about 12% NSC While it is important to feed a low NSC hay to horses with type 1 PSSM, this is likely not as important for type 2 PSSM. Our current recommendation for type 2 PSSM is to increase the quality of dietary protein by feeding half of the forage in the form of alfalfa hay or alfalfa cubes/meal/ and about half as grass hay. Of course this balance varies depending on the type of hay available in your area.

Low starch, fat enriched concentrates: A number of low starch, fat enriched concentrates are currently on the market and they have been tested on horses with type 1 PSSM. They may work very well for your horse with type 2 PSSM, however, the important principle to be met by such feeds is that they are fed in the amount recommended by the manufacturer on the feed bag or web site in order to provide the right balance of protein, vitamins and minerals. If that amount of feed produces excessive weight gain then select another product such as a ration balancer pellet with added protein (20 – 30%). Don't feed a lesser amount of the feed than recommended by the manufacturer, select a product better suited for your individual horse. Many times owners of horses with type 2 PSSM are so focused on low starch and high fat in the diet that they provide a diet that is deficient in the amino acids needed by the muscle and this exacerbated the horses condition.

Selection of fat source: If additional calories are needed to maintain weight, fat can be added to the diet. It may not be necessary for horses with type 2 PSSM to consume a high fat diet. We recommend providing some fat in the form of fish oil or flax seed, which has a high Omega 3 content. If more fat is needed to maintain weight and energy, oils or solid fats can be added. Suitable forms of vegetable oils include soybean, corn, safflower, canola, flaxseed, linseed, peanut and coconut or solid fat products may also be used. The amount of oil added to the diet varies for each horse and should not be provided in amounts that produce excessive weight gain or a cresty neck. Two cups of oil is often the maximum amount recommended. Due to the potential additional oxidant stress of fats, vitamin E (1000 – 6000 U/day) should be fed to horses receiving high oil diets.

Protein supplements: Addition of a protein supplement is recommended for type 2 PSSM horses that do not have an appropriate muscle mass, or horses that do not have the endurance/strength they need for higher level performance. Whey based formulations are recommended at the manufacturers recommended amount. Progressive's topline Xtreme or Purina's Supersport are two such supplements. Feeding within 45 min of exercise may enhance building muscle mass. If horses have evidence of a cresty neck, excessive fat pads or a history of metabolic syndrome or laminitis, sore feet consult your veterinarian before feeding higher protein feeds.

Expectations: It is important to note that a horse diagnosed with PSSM will always have an underlying predilection for muscle soreness and the best that can be done is to manage horses to minimize clinical signs. With adherence to both the diet and exercise recommendations about 70% of Warmblood horses show notable improvement in clinical signs and many return to acceptable levels of performance. There is, however, a wide range in the severity of clinical signs shown by horses with PSSM; those horses with severe or recurrent clinical signs will require more stringent adherence to diet and exercise recommendations in order to regain muscle function.

Prognosis for PSSM

The best indicator as to whether horses with PSSM will be productive athletes is their past performance combined with response to changes in exercise regimes and diet. Genotype or biopsy findings cannot predict future athletic potential. Horses with PSSM will always have a predisposition to muscle soreness and will require long-term management of their condition.

Conflict of Interest Statement: Drs. Valberg, Mickelson and McCue own the license for PSSM testing and receive sales income from its use. Their financial and business interests have been reviewed and managed by the University in accordance with its conflict of interest policies. Dr. Valberg receives a portion of the profits of the sale of Re-Leve.

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CHAPTER 3

CANDIDATE GENE EXPRESSION, CODING SEQUENCE VARIANTS AND SKELETAL MUSCLE CONTRACTILE FORCE IN WARMBLOOD HORSES WITH MYOFIBRILLAR MYOPATHY

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ABSTRACT

Myofibrillar myopathy (MFM) characterized by myofibrillar disarray has recently been identified in Warmblood (WB) horses. In humans, MFM is known to be associated with mutations in 16 genes, whereas the etiology of equine MFM is unknown. Our objective was to compare differential expression (DE) and allele frequencies (AF) of coding missense variants in 16 candidate MFM genes among MFM WB, non-MFM WB and publicly available data of other breeds. This case-control study used 8 MFM WB, 8 non-MFM WB, and public databases for 33 additional WB, 32 Thoroughbreds, 80 Quarter Horses and 77 other breeds. Using gluteal muscle mRNA sequences aligned to EquCab3.0, candidate gene DE was determined between MFM and non-MFM WB by fitting a negative binomial generalized log-linear model per gene (FDR < 0.05). Variants were called within transcripts of 16 candidate genes and AF of identified coding variants compared by Fisher's exact test among MFM WB, non-MFM WB and public sequences across breeds. DE of candidate genes was not detected between MFM and non-MFM WB. Eleven candidate genes contained 26 coding missense variants with similar AF among MFM WB, non-MFM WB and other WBs. Some variants had breed specific differences in AF. In conclusion, coding sequence variants are a common occurrence in the equine genome. The variants identified in candidate genes for human MFM were not associated with WB MFM phenotype and had no effect on total gene expression.

INTRODUCTION

In humans, myofibrillar myopathies (MFM) are usually familial disorders that can cause progressive muscle weakness and atrophy with the potential for respiratory compromise, cardiomyopathy, and death (1–4). Histologically, MFM is characterized by disorganized myofibrils and ectopic accumulation of cytoskeletal or Z-disc proteins in skeletal, and potentially cardiac muscle (5–7). MFM in adult humans is often inherited as a late onset autosomal dominant disorder with clinical signs becoming apparent after the 4th decade of life (2,8–12). However, dominant or recessive forms of MFM can manifest in early childhood (1,2,13–15). Numerous mutations have been associated with the MFM phenotype including approximately 70 mutations in desmin *(DES)* (16,17) that can have incomplete penetrance and variable disease severity (18). Although numerous mutations cause MFM in humans, they all have a classic histopathologic appearance of protein aggregation and myofibrillar disruption (19).

MFM has been diagnosed in Arabian and Warmblood horses (WB) based on the light microscopic appearance of desmin aggregates and electron microscopic appearance of myofibrillar disarray in skeletal muscle biopsies (20,21). The clinical presentation appears to be late onset, with horses diagnosed with MFM at the age of 11-15 years for Arabians and on average 11 years of age for WB horses (20,21). The principle clinical signs associated with MFM in WB are exercise intolerance, a reluctance to move forward under saddle and a mild lameness not attributable to an underlying orthopedic cause (21–23). The finding of desmin aggregates in a 3-generation family of WB horses suggested a potential familial basis for WB MFM (21). The clinical and histopathologic features as well as the potential familial basis for MFM in horses draws several parallels to human MFM. Some differences, exist, however,

including less extensive ectopic protein aggregation in MFM horses compared to that reported in the human literature and lack of any associated genetic mutations for equine MFM (21,24,25).

The advent of high-throughput next-generation sequencing has provided a successful and cost-effective strategy for identification of novel causative genes in human MFM (19,26). Such studies have determined that roughly half of human MFM cases are associated with mutations in Z-disc as well as extrasarcomeric genes. These include desmin (*DES*) (5,6,27), αβ-crystallin (*CRYAB*) (10,28,29), myotilin (*MYOT*) (11,30,31), Z band alternatively spliced PDZ-containing protein (*LDB3/ZASP*) (9,32), filamin C (*FLNC)* (8,33), bcl-2-associated athanogene-3 (*BAG3*)(14,34), kyphoscoliosis peptidase (*KY*) (35,36), and pyridine nucleotide-disulfide oxidoreductase domain-containing protein 1 (*PYROXD1)* (15,19). Additionally, other genes have been recently associated with desmin aggregates and MFM-like phenotypes: four-and-half LIM domain 1 (*FHL1*) (12,37–39), DNAJ/HSP40 homolog subfamily B, member 6 (*DNAJB6)* (19,40,41), plectin (*PLEC*) (41–43), lamin A/C (*LMNA*) (44,45), alpha-actin (*ACTA1*) (19,46), heat-shock 22-kd protein 8 (*HSPB8*) (19,47), and digenic mutations in sequestosome 1 and cytotoxic granule-associated RNA-binding protein (*SQSTM1* and *TIA1*) (48). Mutations in these genes cause scapuloperoneal myopathy, limb-girdle muscular dystrophy, Emery-Dreifuss muscular dystrophy, nemaline myopathy, Charcot-Marie-Tooth, Welander distal myopathy, and MFM-like inclusion body myopathy (12,19,37–48). Exome sequencing has been a successful approach in human medicine to identify mutations in genes that are causative or associated with the MFM phenotype.

In the present study, we hypothesized that potential pathogenic coding sequence variants and differential gene expression of orthologous human MFM candidate genes could be identified from RNA-seq analyses of skeletal muscle from severely affected MFM WB. Further, we hypothesized that MFM would be associated with altered contractile force.

The first objective of the present study was to determine whether differential expression (DE) of candidate genes was present in WB MFM compared to healthy WB (non-MFM). The second objective was to identify candidate gene variants and compare allele frequencies among MFM WB, non-MFM WB and a variety of other breeds. The final objective was to determine whether the contractile force of isolated fibers from gluteal muscle differed between MFM and non-MFM WB.

METHODS

Warmblood Selection

The database of the Neuromuscular Diagnostic Laboratory was searched to identify WB horses with both a history of poor performance and exercise intolerance that had been diagnosed with MFM (MFM WB) based on the presence of cytoplasmic aggregates of desmin in myofibers and that also had snap frozen samples of gluteus medius muscle available for further study (21,22). Healthy control WB (non-MFM WB) horses with a history of satisfactory performance were selected for study if snap frozen samples of gluteus medius muscle were available and there was no evidence of myopathic changes or desmin aggregation in muscle biopsies. MFM WB were 13.6 ± 4 years-of-age and included 4 castrated males, 1 intact male and 3 females. Non-MFM WB were 13.9 ± 3 years-of-age including 4 castrated males, 1 intact male and 3 females.

Of the horses selected above, four MFM WB (3 castrated males and 1 female) and four non-MFM WB (1 male, 1 castrated male, and 2 females) were also used for contractile force testing.

RNA isolation

Total RNA was isolated from flash frozen gluteus medius samples using TRIzol/Chloroform extraction after homogenization with a biopulverizer (BioSpec Products, Inc, Bartlesville, OK, USA) as previously described (49). Samples underwent DNase treatments on columns (Direct-zolTM RNA MiniPrep Plus, Zymo, Irvine, CA) with RNase-free DNase I (New England BioLabs, Inc.) according to manufacturer's instructions. Samples then underwent quantification with a Qubit Fluorometer and RNA HS Assay Kit (Thermo Fischer Scientific) and RIN scores

were measured with an Agilent 2100 Bioanalyzer and Agilent RNA 6000 Pico Kit (Agilent Technologies, Santa Clara, CA) and samples with RIN scores > 7.0 were used.

RNA Library preparation

All libraries were constructed by the Michigan State University Genomics Core using the Illumina TruSeq Stranded mRNA Library Preparation Kit per manufacturer's instructions (Illumina, San Diego, CA). All libraries underwent quality control and were quantified using Qubit dsDNA High Specificity and Caliper LabchipGX High Specificity DNA assays. Libraries were then pooled in equimolar concentration for multiplex sequencing.

Illumina Hiseq 4000

Kapa Biosystems Illumina Library Quantification qPCR kit was used to quantify the pooled libraries. Samples were then loaded onto 2 lanes of an Illumina HiSeq 4000 flow cell. Sequencing was performed in a 150bp paired-end format using HiSeq 4000 SBS reagents for a target of 35-40 million reads for each sample. Base calling was done by Illumina Real Time Analysis v2.7.7 and output of RTA was sorted and converted to FastQ format with Illumina Bcl2fastq v2.19.1 for analysis.

Candidate Genes

Candidate genes were identified by reviewing the current human literature for any genes with mutations known to cause MFM, desmin aggregate myopathies, or protein aggregate myopathies with myofibrillar disorganization (search date August 2019).

Assembly, Mapping, and Variant Calling

FastQC (50) and MultiQC (51) were used to evaluate the quality of all 150-nucleotide paired end RNA-seq reads. Trimmomatic (52) was used to filter adapter sequences and ConDeTri (53) was used to filter low quality reads. A quality threshold of 30 (Q ≥30) was used to filter samples. Next, Bowtie2 (54)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 (55) was used to map all the reads. SAMTools (56) was utilized to retain all reads that were uniquely aligned (76%). Cufflinks (57) was used to assemble the transcriptome. HTSeq (58) was used to count the number of normalized 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 (59). To maintain statistical power, called variants were retained for downstream analysis if they were present in at least 7 (≥ 43%) horses and had a Phred score of 30 and ≥ 10 reads.

Variant Predictions

Ensembl's Variant Effect Predictor version 98 (60)(https://useast.ensembl.org/Tools/VEP) was used referencing EquCab 3.0 to specify variant location, predict the effect defined as synonymous or missense based on the nucleotide and associated amino acid substitution, and estimate the impact score defined as low, moderate, or severe.

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

Neuromuscular Diagnostic Lab, collaborators, and data (accession numbers PRJEB30116, PRJEB28306) from the European Variation Archive (https://www.ebi.ac.uk/eva/).

The NCBI remap tool (https://www.ncbi.nlm.nih.gov/genome/tools/remap) was used to remap variants found in EquCab 3.0 to Equcab 2.0 for sequencing data that was mapped to second version of the equine genome. Variants from the 16 genes were called from the VCF files using SAMTools bcftools mpileup (58).

Gene expression and allele frequency statistics

For the MFM WB and non-MFM WB, raw read counts per gene were normalized using the trimmed mean of M-values (TMM) (61). Differential gene expression (DE) was determined by fitting a negative binomial generalized log-linear model per gene with diagnosis of MFM as coefficient of interest using EdgeR (62) and corrected with the Benjamini-Hochberg method with a false discovery rate ≤ 0.05.

Allele frequencies from all identified variants within candidate genes were compared between MFM WB and non-MFM WB using a Fisher's exact test and corrected with a Benjamini-Hochberg method (FDR≤ 0.05). Allele frequencies of missense variants were further compared among MFM WB (n=8), non-MFM WB (n=8) and public databases containing non-phenotyped WB (n=33), TB (n=32), QH (n=80) and OB (n=77) using a Fisher's exact test and Benjamini-Hochberg method for multiple testing (FDR≤ 0.05). All significant results were followed by post-hoc testing consisting of pairwise comparison and corrected for multiple testing again with the Benjamini-Hochberg method (FDR≤ 0.05).

Single myofiber force production

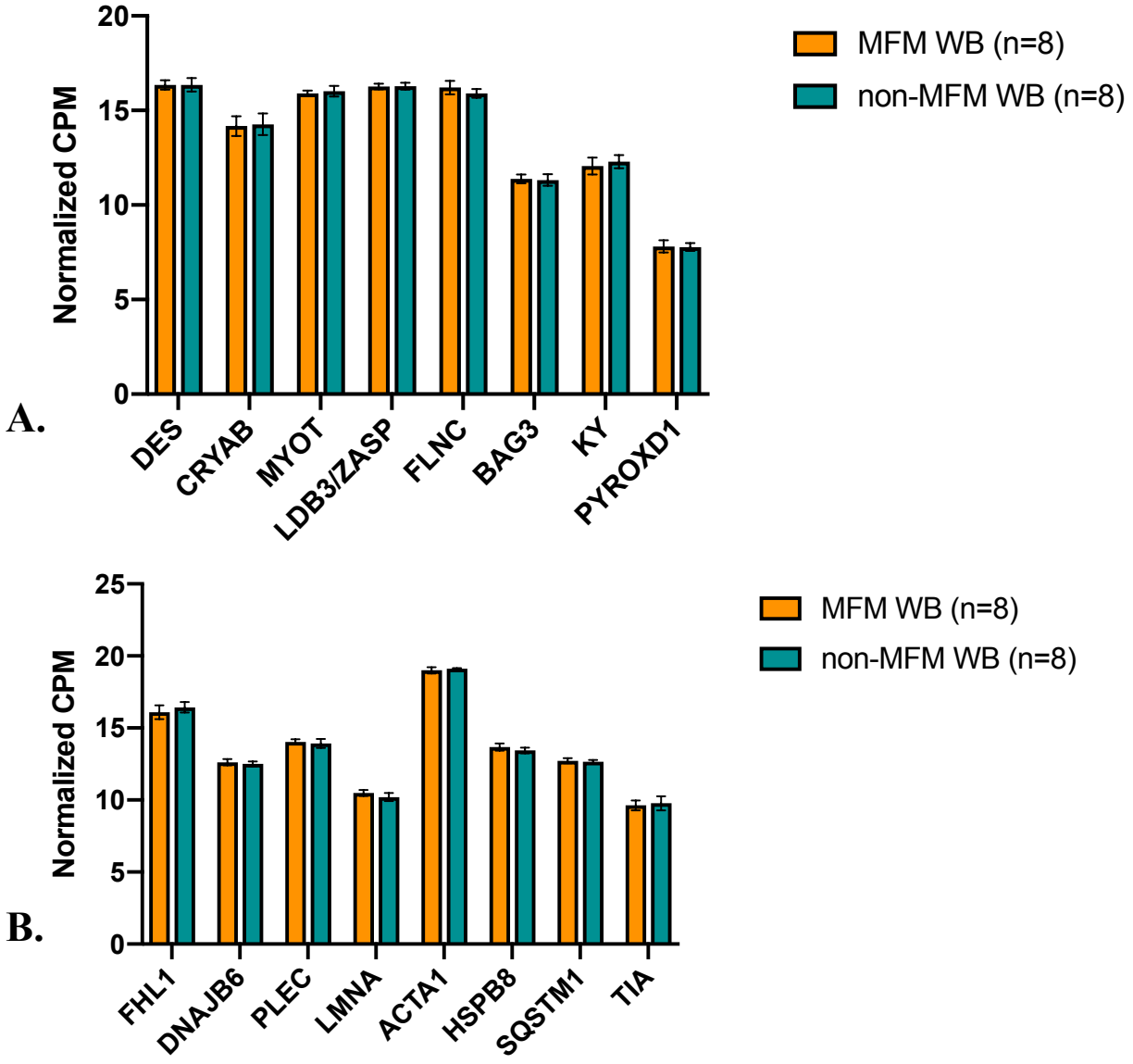
On the day of experiment, 6-7 single myofibers per horse were dissected from muscle biopsy specimens in a relaxing solution. Relaxing and activating solutions contained 4 mM Mg-ATP, 1 mM free $Mg^{2+,}$ 20 mM imidazole, 7 mM EGTA, 14.5 mM creatine phosphate, and KCl to adjust the ionic strength to 180 mM and pH to 7.0. The concentrations of free Ca^{2+} were pCa ($-\log_{10}[Ca^{2+}]$) 9.0 M (relaxing solution) and pCa 4.5 M (activating solution). Myofibers 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. Fiber cross-sectional area (CSA) was estimated in μm^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 myofiber while in the relaxing solution (pCa 9.0). Specific force was defined as absolute force divided by CSA (KPa). The specific force per myofiber was recorded and means were compared in MFM (n = 27 fibers) versus non-MFM WB (n = 27 fibers) using an unpaired t-test after normality was determined with the Shapiro-Wilk method.

RESULTS

Differential gene expression

All candidate genes passed quality control and filtering criteria. The normalized coverage for all of the candidate genes averaged 13.64 counts per million ± 0.28 for the non-MFM WB and 13.65 CPM ± 0.28 for MFM WB (Figure 3.1,Table A.3.1). There was no significant differential expression in 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 0.31 - 0.86 (Figure 3.1, Table A.3.1).

Figure 3.1: A.) Expression of the 8 genes known to cause MFM types 1-8 reported by normalized counts per million reads (CPM). B.)Expression of the 8 additional genes known to have MFM-like features reported by normalized CPM. There were no significant differences in gene expression between MFM WB and non- MFM WB.



Variant identification and prediction

After filtering, a total of 426 variants in MFM WB and non-MFM WB were found in the 16 candidate MFM genes (DES, CRYAB, MYOT, LDB3/ZASP, FLNC, BAG3, KY, PYROXD1, FHL1, DNAJB6, PLEC, LMNA, ACTA1, HSPB8, SQSTM1, and TIA). The identified variants had 3,857 different predicted annotations depending on gene isoform expression. Of these variant annotations, 454 were missense (11.7%), 1,161 were synonymous (30.0%), and the rest were categorized as splice site, 5' untranslated region (UTR), 3' UTR, non-coding, intronic, upstream or downstream.

LDB3/ZASP had the most identified variants (n = 126) and *CRYAB* only had 1 identified variant (Figure 3.2). Ninety of the variants were annotated in exonic regions in *ACTA1, BAG3, DES, DNAJB6, FLNC, HSPB8, KY, LDB3/ZASP, LMNA, MYOT, PLEC, PYROXD1, SQSTM1*. No coding variants were found in *CRYAB, FHL1* and *TIA1*. Of the 90 coding variants, 62 were synonymous with low impact scores, 26 were missense with moderate impact scores and 2 were splice site variants with low impact scores. The 26 missense variants were identified in 11 genes: *LDB3/ZASP, SQSTM, PYROXD1, PLEC, MYOT, LMNA, KY, FLNC, DES, BAG3, DNAJB6* (Figure 3.2).

Variant allele frequencies

None of the identified variants, regardless of location within the candidate gene or predicted consequence, were significantly associated with the MFM WB phenotype when compared to the non-MFM WB. The allele frequencies of the 26 missense coding variants were not significantly

different among the MFM WB, non-MFM WB (n=8) and the non-phenotyped WB from publicly available data (Figure 3.3).

When evaluating publicly available data from other breeds, 8 variants had significantly different AF after multiple test correction when comparing non-phenotyped *WB, TB, QH* and OB in a pairwise fashion. *LDB3* (1 variant), *PYROXD1*(2 variants), *PLEC* (3 variants), *MYOT* (1 variant) and *KY* (1 variant) had significant differences in AF between the breeds after pairwise comparisons (Table 3.1).

There was an insufficient number of quality reads for *DES* chr6:8696183 in the non-phenotyped WB (n=33), QH (n=80), and OB (n=77) for evaluation. Additionally, some variants did not have sufficient data that met read count requirements and quality thresholds for certain individuals within a breed population. *PLEC* chr9:84703610 and *KY* chr16:71665337 only had data in one horse in the non-phenotyped WB (n=33).

Figure 3.2*:* The total number of identified variants and missense coding variants found in the 8 MFM genes and 8 MFM-like candidate genes from RNA sequencing data of 8 MFM WB and 8 non-MFM WB. *LDB3/ZASP* had the most total variants and *CRYAB* had the least total variants identified. The most missense coding variants were identified in *PLEC*, while *CRYAB, FHL1, ACTA, HSPB8*, and *TIA1* had no missense variants identified.

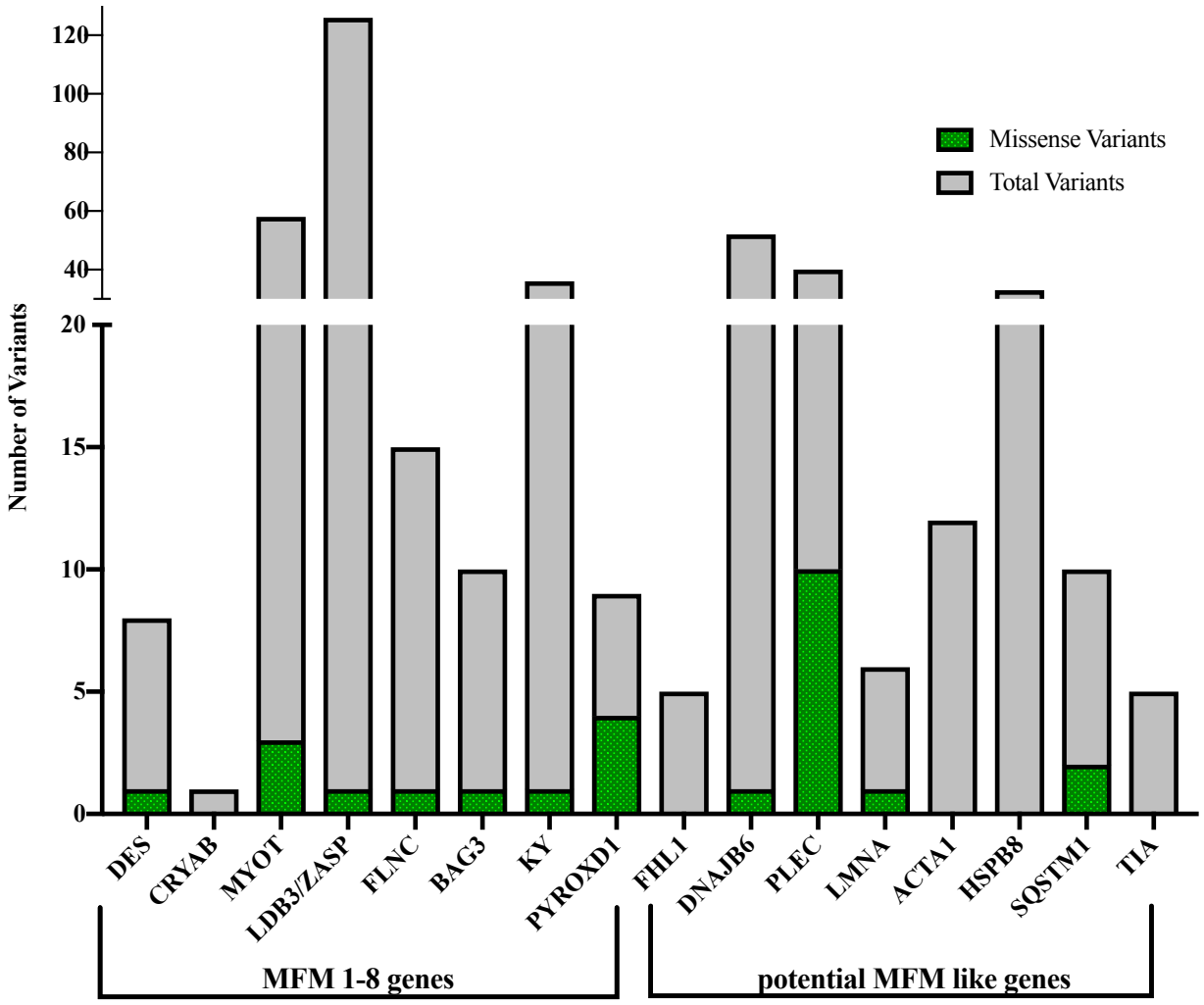
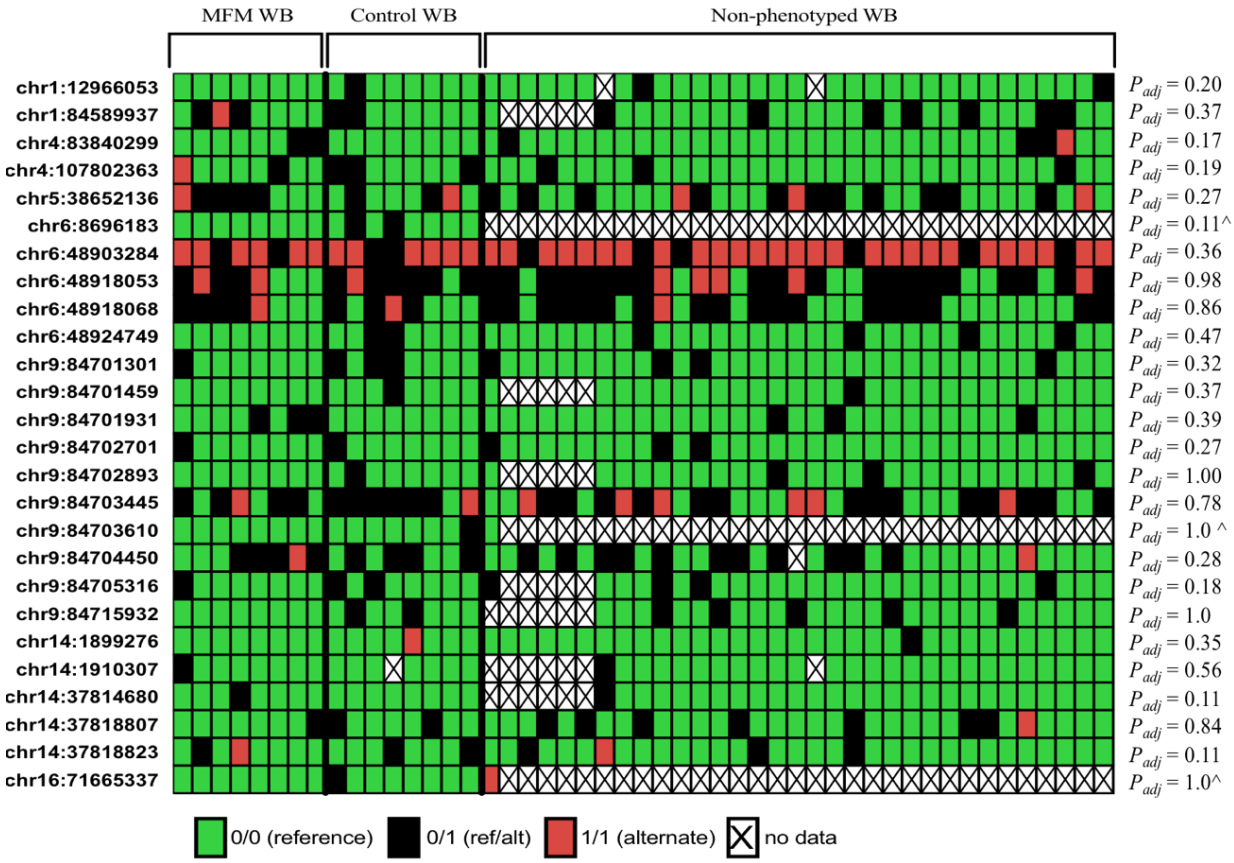


Figure 3.3: A heat map of the 26 detected missense coding variants among MFM, non-MFM and non-phenotyped WB. Each identified missense coding variant is noted by its chromosomal location per row and each column references a horse grouped with its phenotype. There were no significant differences among WB groups. ^ indicates that there was minimal data for comparison in the non-phenotyped WB group.



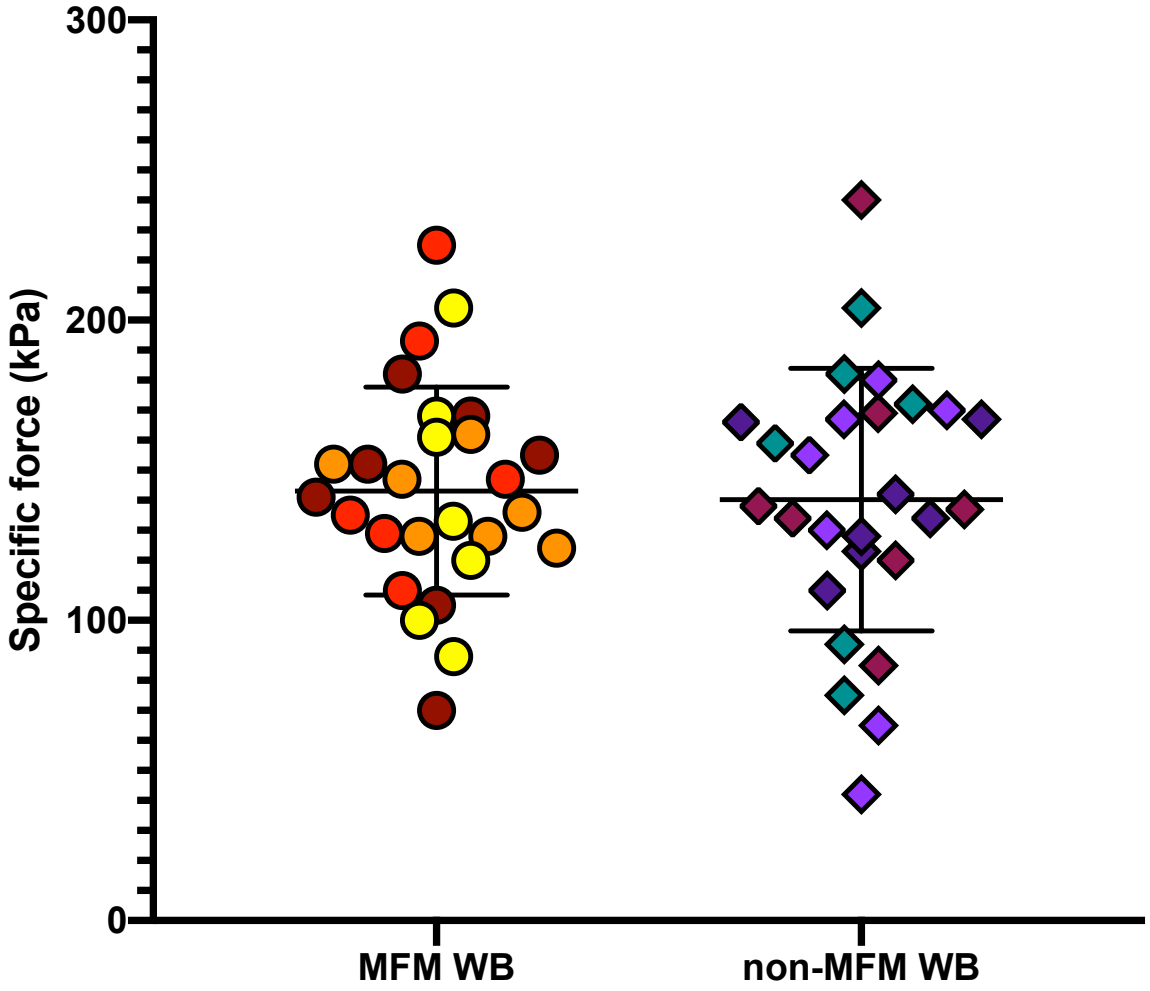
Contractile force

To investigate whether the force generated by myofibrillar proteins was impaired in MFM WB compared to non-MFM WB, the specific force of isolated membrane-permeabilized muscle fibers was evaluated. The mean cross-sectional area of muscle fibers was not significantly different between MFM WB (3365.4 ± 992.4 μm^2) and non-MFM WB (3570.1 ± 1099.0 μm^2). The force measurements were normally distributed in the MFM and non-MFM WB. There was no significant difference in specific myofiber force generated between MFM WB (143.1 ± 34.7 kPa) and non-MFM WB (140.2 ± 43.7 kPa) ($P = 0.79$)(Figure 3.4). Variation in contractile force occurred between myofibers of different horses in a similar fashion between MFM and non-MFM WB (Figure 3.4) . For contractile force, MFM WB had a coefficient of variation ranging from 10.3% to 29.5% for individual horses and non-MFM WB had a coefficient of variation ranging from 15.5% to 42.3% for individual horses. The coefficient of variation for fiber cross sectional area ranged from 24.1% to 29.0% for MFM WB and from 27.3% to 40.5% for non-MFM WB.

Table 3.1: Missense variant allele frequencies that were significantly different between breeds. There were no differences among MFM WB, non-MFM WB or publicly available sequences of non phenotyped WB but there were significant differences when comparing WB groups to 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)	0.01
		non phenotyped WB (n=28)	TB (n=29)	0.02
		OB (n=71)	non phenotyped WB (n=28)	0.03
		QH (n= 66)	TB (n=29)	0.01
		OB (n=71)	TB (n=29)	< 0.0001
		OB (n=71)	QH (n=66)	0.01
PYROXD1	chr6:48918068 G /A	non phenotyped WB (n=33)	QH (n=79)	0.01
		QH (n= 79)	OB (n=77)	< 0.0001
PLEC	chr9:84702893 A /G	OB (n=70)	TB (n=29)	0.03
		OB (n=70)	QH (n=66)	0.03
	chr9:84704450 T /C	MFM WB (n=8)	TB (n=32)	0.03
		MFM WB (n=8)	QH (n=41)	0.03
		non phenotyped WB (n=32)	QH (n=41)	0.04
		OB (n=75)	QH (n=41)	0.03
	chr9:84715932 C /A	non phenotyped WB (n=27)	OB (n=52)	0.06
SQSTM1	chr14:1899276 G /A	non-MFM WB (n=8)	OB (n=76)	0.06
		QH (n= 80)	OB (n=76)	0.06
MYOT	chr14:37818807 A /G	QH (n= 41)	TB (n= 32)	0.01
		OB (n=77)	TB (n= 32)	0.01
KY	chr16:71665337 C /T	QH (n= 19)	MFM WB (n=8)	< 0.0001
		OB (n=9)	MFM WB (n=8)	< 0.0001
		QH (n=19)	non-MFM WB (n=8)	< 0.0001
		OB (n=9)	non-MFM WB (n=8)	< 0.0001
		QH (n=19)	TB (n=28)	< 0.0001
		OB (n=9)	TB (n=28)	< 0.0001

Figure 3.4: Specific force for individual muscle fibers from MFM and non-MFM WB. The specific force (kPa), was not different between MFM and non-MFM WB *(P = 0.8)*. Each point corresponds to single permeabilized fiber. Fibers from the same horse are indicated by the same color.



DISCUSSION

A diagnosis of MFM is initially based on myopathic clinical signs combined with specific muscle histopathology (2). Both late-onset forms of human MFM and WB MFM share clinical features of progressive weakness, exercise intolerance and muscle atrophy (2,24,63). Additionally, both human and WB MFM share the histopathologic features of desmin aggregation, myofibrillar disruption, and Z-disc streaming (15,21,33,42,64–66). Further diagnostic steps for MFM diagnosis in human medicine include exome-sequencing of candidate genes to identify a specific mutation that underlies a familial basis for the disease (67,68). In the absence of an exome sequencing panel for equine myopathies, we utilized RNA sequencing data from skeletal muscle to investigate potential coding sequence variants associated with WB MFM. RNA-seq previously has been utilized in humans to examine coding variants in expressed transcripts in a variety of diseases ranging from muscular dystrophy to acute myeloid leukemia (69–71).

According to the database Online Mendelian Inheritance in Man (www.omim.org), mutations known to cause human MFM type 1- 8 are found in *DES, CRYAB, MYOT, LDB3/ZASP, FLNC, BAG3, KY,* and *PYROXD1* genes (5,6,9–11,14,15,19,27–32,34–36). Numerous different mutations have been identified within these genes in human MFM that have either autosomal dominant or recessive inheritance or are de novo mutations (19). We chose to evaluate variants in the skeletal muscle transcriptome for the MFM type 1- 8 genes as well as transcripts of *FHL1, DNAJB6, PLEC, LMNA, ACTA1, HSPB8, SQSTM and TIA1* because mutations in these genes create overlapping features of desmin aggregation, myofibrillar disarray, progressive muscle weakness and atrophy (19).

There were 426 variants in the candidate genes identified in MFM WB and non-MFM WB. Of those, 26 variants were missense variants with "moderate" impact scores predicted by the Ensembl Variant Effect Predictor. Notably, none of these variants were associated with the MFM disease phenotype. The abundance of variants, including missense variants, detected in the present study is in agreement with a recent report that found 23.5 million single nucleotide variants in whole-genome sequencing data from 88 horses of different breeds (72). We also found differences in variant AF between breeds. Our results do not support a coding sequence variant in the candidate genes as the cause for MFM. Because missense variants are common in horses, AF need to be evaluated in well-phenotyped affected and control before assuming a discovered coding sequence variant causes a disease. Further our results also strongly suggest that studies claiming variant pathogenicity need to account for breed differences in AF by using appropriate breeds in control populations.

Two coding variants detected in *FLNC* (chr4: 83738769 EquCab 2.0; chr4:83840299 EquCab 3.0) and *MYOT* (chr14:38519183 EquCab 2.0; chr14: 37818823 EquCab 3.0) are commercially offered as a genetic test for equine MFM and type 2 polysaccharide myopathy (PSSM2) (patent # WO2017165733A1).We were unable to locate a peer-reviewed manuscript to support an association between these two test variants and a diagnosis of MFM. The V238A *MYOT* variant is predicted to be "tolerated" by SIFT classification– a predictive algorithm used to predict a phenotypic outcome from a genomic mutation (73,74). The same position in human *MYOT* encodes one of three different amino acids (N238, T238, Y238) depending on isoform expression. The variability in amino acids in this position suggest that this region is either not

conserved, or that there are other isoforms that are not yet annotated in the current version of the equine genome. The two variants in *FLNC* and *MYOT* offered as diagnostic tests for equine MFM/PSSM2 were present in equal frequency in MFM WB, non-MFM WB and 33 publicly available sequences of other WB horses. Thus, the results of the present study do not support utilizing this commercial test to diagnose MFM in WB. Skeletal muscle biopsies therefore remain the gold standard to identify MFM in horses at the present time.

The expression of the 16 MFM candidate genes in skeletal muscle did not differ between MFM WB and non-MFM WB. In human MFM, the effect of the mutation on gene expression varies with the specific gene and the mutation. A recent research abstract found that mutations in *DES, FLNC*, and *LDB3/ZASP* in human patients increase gene expression when compared with healthy controls (75,76). In contrast, coding sequence variants in *MYOT* in humans and W2710X *FLNC* in mice are not DE compared to healthy/wild type controls (75,76). A similar variable effect of mutations on gene expression is reported for other equine genetic myopathies. In glycogen branching enzyme deficiency, a Y34X *GBE1* premature stop codon produces higher *GBE1* transcript expression in affected versus control skeletal muscle with low GBE protein expression (77). In contrast, no difference in *GYS1* skeletal muscle expression occurs between affected and control horses with the R309H *GYS1* mutation that causes type 1 polysaccharide storage myopathy (78). Thus, our gene expression results did not rule out any of the candidate genes 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 of utilizing RNA-seq data to identify potential disease-causing mutations, include variability in the depth of coverage, reduced ability to quantify lowly expressed genes and short-read length that are challenging to map for repetitive regions. Furthermore, the present study presumed that the cause of MFM in WB primarily originates in skeletal muscle. With samples frozen in liquid nitrogen that met our phenotypic criteria, we could only evaluate mRNA transcripts that are expressed in skeletal muscle at the time of sample acquisition and are annotated on the current version of the equine genome.

Mouse models of muscular dystrophies, nemaline myopathies and centronuclear myopathies have all shown decreases in absolute isometric, specific force, and absolute force, respectively (79–81). Similarly, mouse models of MFM reveal decreases in absolute force and maximum specific isometric force (82,83). We hypothesized that myofiber specific force in MFM WB would be less than non-MFM WB. Interestingly, that was not the case. Force was measured in 6-7 fibers per individual because previous studies have found that this number of fibers is adequate to show a 20% difference in force while maintaining a power of 80% (84). The contractile force measured in the present study did not specifically identify fiber types based on previous studies that did not find a predilection for desmin aggregates in specific fiber type (21). Our results could indicate a small or total lack of effect of MFM on isolated fiber force in equine MFM. Alternatively, our results could reflect an effect of MFM on specific fiber types or solely on fibers with desmin aggregates. A recent histochemical study found desmin aggregates occur in type 2A and a few type 2X fibers of MFM horses (21). An ideal study would evaluate contractile force in fibers of known type containing desmin aggregates. The number of fibers containing desmin aggregates, however, is very small within a biopsy sample (21).

A genome wide association study (GWAS) would ideally be utilized to identify a genomic locus for WB MFM prior to candidate gene sequencing. However, many more horses than were available for the present study would be needed to achieve the statistical power needed for a GWAS (85), particularly with a proposed dominant mode of inheritance seen in select human MFM. Large numbers of horses were difficult to achieve because of the unwillingness of owners of healthy WB to have a muscle biopsy performed to provide an accurate phenotype. Genetic studies in WB are also complicated by the fact that WB breed registries do not have a closed stud book and horses of a variety of breeds may be registered as WB. At this time, a heritable basis for WB MFM has yet to be definitively established.

CONCLUSION

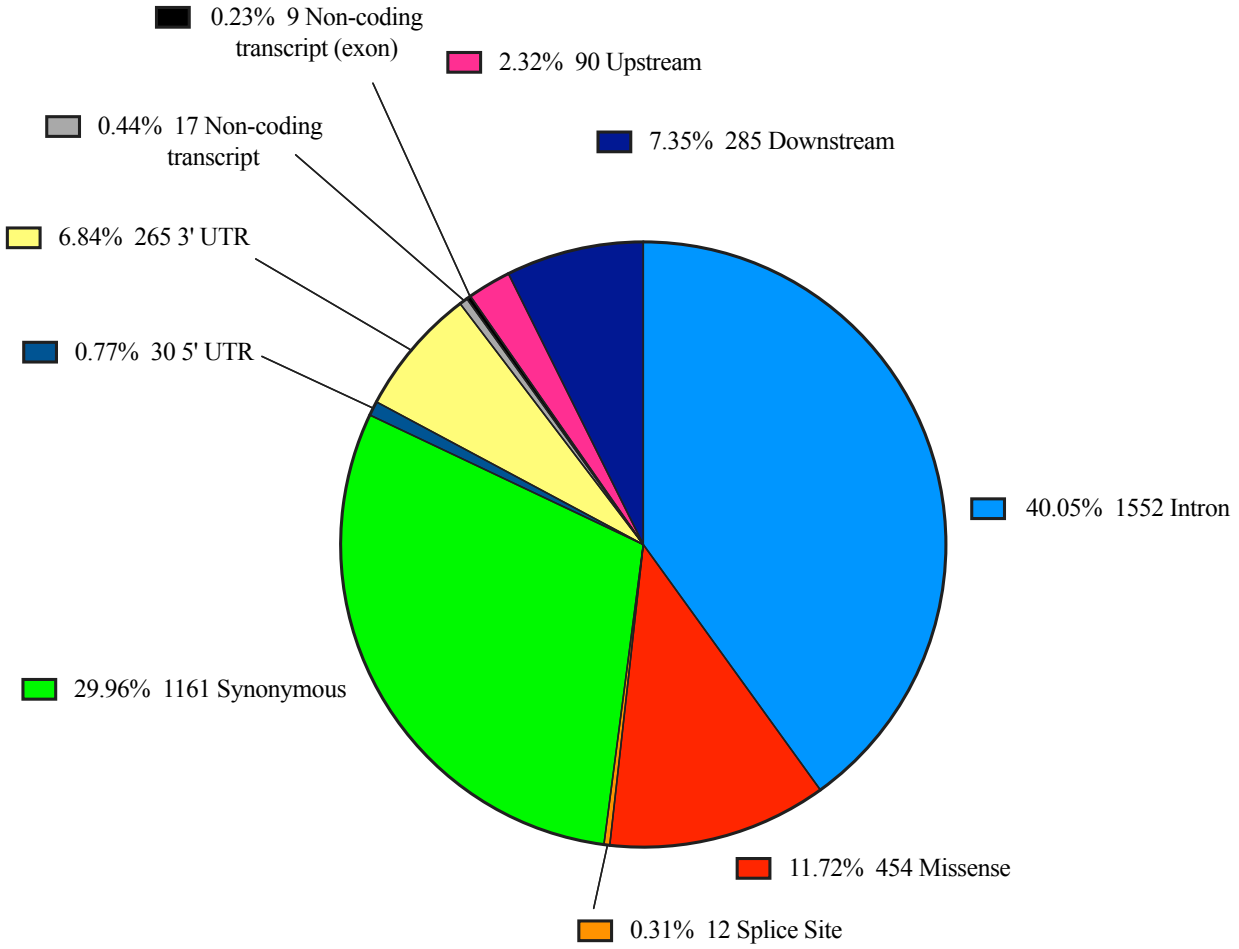
In conclusion, no differences were found in isolated skeletal muscle fiber contractile force, gene expression or AF of missense variants identified in 16 MFM candidate genes between MFM WB and non-MFM WB. The detection of numerous variants, including coding sequence variants, within the equine genome underscores the importance of comparing variant frequencies between well-phenotyped affected and control populations to validate commercially offered genetic tests and is in line with the recommendations of the American College of Medical Genetic and Genomics (86).

APPENDIX

Table A.3.1: Expression of candidate gene in MFM and non-MFM WB using RNA sequencing shows no significant differences.

Gene name	MFM WB		non- MFM WB		Gene Coverage (genomic)	strand	Log_2 Fold Change	Log Counts Per Million	*P*	P_{adj}
	Mean reads	Stdev	Mean reads	Stdev						
FLNC	211844	46836	142594	57157	chr4:83825176-83853232	+	0.33	16.1	0.03	0.31
LMNA	3922	867	2761	1107	chr5:38649856-38684406	-	0.27	10.37	0.04	0.31
HSPB8	35997	7959	25807	10344	chr8:17039457-17053877	-	0.24	13.58	0.06	0.31
FHL1	198201	43820	209740	84072	chrX:112289307-112348135	+	-0.32	16.33	0.10	0.39
KY	12113	2678	11800	4730	chr16:71621957-71666143	-	-0.20	12.23	0.28	0.70
MYOT	166752	36867	155045	62148	chr14:37813838-37829957	-	-0.13	15.98	0.28	0.70
DNAJB6	17269	3818	13494	5409	chr4:107733722-107823595	+	0.12	12.58	0.31	0.70
TIA1	2200	486	2122	850	chr15:33263651-33301177	+	-0.19	9.76	0.35	0.70
ACTA1	1443463	319132	1297661	520154	chr1:68958999-68961967	+	-0.09	19.06	0.41	0.70
PLEC	45922	10153	36378	14582	chr9:84622174-84754010	-	0.10	14.00	0.47	0.70
SQSTM1	18448	4079	14849	5952	chr14:1897388-1910384	-	0.07	12.69	0.48	0.70
CRYAB	53464	11820	48585	19475	chr7:20964634-20968038	-	-0.10	14.32	0.69	0.83
PYROXD1	621	137	507	203	chr6:48901028-48925072	+	0.06	7.82	0.70	0.83
BAG3	7337	1622	6009	2408	chr1:12964989-12989510	-	0.05	11.38	0.73	0.83
LDB3	214951	47523	184794	74073	chr1:84575090-84576077	+	-0.02	16.28	0.84	0.86
DES	229437	50726	198109	79410	chr6:8695940-8705770	+	-0.03	16.38	0.86	0.86

Figure A.3.1: The Ensembl variant effect predictor (version 98) classification of the 3,875 annotations belonging to the 426 identified variants in the 16 candidate genes. The majority of variants were intronic and the second largest variant class was coding synonymous variants followed by missense variants.



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CHAPTER 4

PROTEOMIC AND TRANSCRIPTOMIC PROFILING IDENTIFIES Z-DISC, MITOCHONDRIAL COMPLEX I, AND ENRICHED TRANSCRIPTION FACTORS AS KEY PLAYERS IN THE ETIOPATHOLOGY OF MYOFIBRILLAR MYOPATHY IN WARMBLOOD HORSES

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ABSTRACT

Myofibrillar myopathy (MFM) in humans is a progressive late onset disease characterized by myofibrillary disarray and protein aggregation, resulting in progressive weakness. Numerous mutations are known to cause MFM in humans, but the cause in Warmblood horses (WB) is unknown. The objective of this study was to elucidate the cellular pathways and molecular drivers of WB MFM by integrating transcriptomic profiling (8 MFM WB vs. 8 non-MFM WB), proteomic profiling (5 MFM WB vs. 4 non-MFM WB), and a transcription factor motif analysis (TFMA) of DE genes and proteins. Gluteal muscle mRNA was analyzed by Illumina HiSeq 4000 150 nucleotide paired end sequencing. Gene transcripts were mapped to EquCab3.0, normalized and quantified. Extracted gluteal muscle protein was analyzed by tandem mass tag 10-plex MS/MS quantitative analysis. Differentially expressed (DE) proteins (*Equus caballus* 3.0 complement of UniProtKB) were determined with a permutation test and FDR <0.05. All significantly DE genes and DE proteins underwent gene ontology (GO) analysis, reactome pathway enrichment and TFMA. There were 47 DE genes out of 27,690, 93 DE proteins out of 1533, and 3 enriched transcription factors (TF) in MFM WB vs. non-MFM WB. Amalgamated transcriptomic and proteomic datasets revealed 3 distinct enriched GO cellular locations: sarcomere structure (Z-disc & I band), complex I of mitochondria, and extracellular matrix. Enriched GO terms of TF DE target genes included; Y-Box binding protein 1 (YBX1) targets - *response to oxygen/reactive oxygen species* (4/11 GO terms); Signal transducer & activator of transcription 3 (STAT3) targets – *response to oxygen/reactive oxygen species* (4/19 GO terms); and Activating Transcription Factor 3 (*ATF3)* targets - *response to amino acid starvation/protein phosphorylation* (7/14 GO terms). The greatest log fold change (FC) for DE genes in MFM WB occurred for oxidoreductase genes glutathione specific gamma-glutamylcyclotransferase1

(*CHAC1*, $\log_2$ FC 4.8) and hemoglobin subunit beta (*HBB,* $\log_2$ FC -6.7). The greatest $\log_2$ FC for DE proteins were for the Z-disc protein cysteine and glycine-rich protein 3 (CSRP3, $\log_2$ FC 0.74) and D-dopachrome decarboxylase (DDT*,* $\log_2$ FC -0.61). Our integrated molecular approach identified the Z-disc, complex I of the ETC, and signaling pathways for oxidative stress/ amino acid metabolism as key drivers of WB MFM and revealed exciting new candidate genes for future analysis.

INTRODUCTION

Myofibrillar myopathy (MFM) in humans is classically known as a late onset protein aggregate myopathy that can affect skeletal and cardiac muscle– leading to muscle atrophy, immobility, need for assisted ventilation, and cardiomyopathy (1–4). At least 8 genes, some containing more than 70 different mutations (5,6), cause MFM types 1-8 and an additional 8 genes are associated with MFM-like protein aggregate myopathies. The variety of genes causing desmin aggregate myopathies and heterogeneous clinical signs suggest that the underlying basis for MFM is complex, influenced by both genetic and environmental factors (7–9).

The defining histologic features of MFM include ectopic protein aggregation and myofibrillar disarray (10). Proteomic profiling of MFM protein aggregates has shown that different MFM etiologies result in different protein expression profiles (11–13). Some of the unexpected proteins found in aggregates appear to be explained by an unexpected role of transcription factors (TF) (9).

TF are proteins that translocate from the cytoplasm to the nucleus, bind to silencer or enhancer regions of DNA and regulate the rate of gene transcription (14). They orchestrate cellular differentiation and response to disease or metabolic stress (15), and thus, TF analysis can provide invaluable insight into disease drivers and potential therapeutic targets (16). Mutations in TF can cause neurodevelopment disorders (17,18), certain types of diabetes (19–21), autoimmune disorders (22), and cancer (23,24). Studying the role TF have in cellular regulation and dysregulation in skeletal and cardiac muscle has shed new light on their underlying role in myopathies (25-28).

MFM has recently been described in both Arabian and WB horses (29,30). Paralleling human MFM, WB horses with MFM have myofilament disarray, Z-disc disruption, desmin aggregation, focal accumulation of granulofilamentous material, and clusters of degenerate mitochondria in skeletal muscle (29,30). Candidate genes causing human MFM have been investigated in MFM WB, but a specific mutation associated with MFM has not yet been identified (Chapter 3). Transcriptomic and proteomic approaches in Arabian horses with MFM found altered cysteine metabolic pathways– required for synthesis of thiol-based antioxidants– and proposed oxidative stress as a key driver of the disease (31). Whereas Arabian horses with MFM develop muscle stiffness and pain after many hours of endurance exercise, WB MFM exhibit exercise intolerance and a mild lameness soon after exercise commences (30,32,33). Due to the different clinical presentation, genetics, and environmental backgrounds, it remains unclear if WB with MFM have the same underlying etiopathology as Arabians.

We hypothesized that a unique pathologic process causes clinical signs of exercise intolerance and abnormal Z-disc morphology in MFM WB and that the etiopathology could be identified by integrating transcriptomic and proteomic profiling as well as TFMA. Our objectives were to 1) perform mRNA sequencing and transcriptomic profiling of MFM and non-MFM WB to determine DE of genes, 2) conduct proteomic profiling to identify DE of proteins, and 3) amalgamate the DE data to identify enriched pathways and TFs reflective of the MFM WB etiopathology. The overall objective was to thereby identify new candidate genes and proteins for further hypothesis driven research.

METHODS

Case selection

The Neuromuscular Diagnostic Laboratory database was queried to identify WB horses with snap frozen gluteus medius biopsies available for analysis MFM and cases were defined as horses with a chronic history of poor performance and exercise intolerance as well as accumulation of desmin aggregates in > 4 myofibers in immunohistochemical stains (30). Non-MFM control horses were defined as horses with no history of exercise intolerance and no evidence of desmin accumulation or other myopathies in muscle biopsies.

For transcriptomic analysis, muscle from eight MFM WB (3 castrated males, 1 male and 3 females) with a mean age of 13.6 ± 4yrs and eight healthy WB (3 castrated males, 1 male, and 3 females) with a mean age of 13.9 ± 3yrs were available for analysis. For proteomics, muscle samples from 10 of these horses were analyzed based on a 10-plex assay size and tissue availability. This included 5 MFM WB (2 castrated males, 1 male and 2 females) with a mean age of 14.4 ± 3yrs and 4 non-MFM WB (2 castrated males and 2 females) with a mean age of 13.8 ±4.6 yrs. Muscle biopsies were taken with owner informed consent under Michigan State University Institutional Animal Care and Use Committee animal use approval # 04/16-045-00.

Electron microscopy

Gluteal muscle from three MFM WB (2 castrated males and 1 female) with a history of muscle atrophy, exercise tolerance leading to poor performance and myofiber desmin aggregation present on muscle biopsy was placed in 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer. Samples were dissected to approximately 5 mm^2 oriented longitudinally and processed as previously described (34).

RNA isolation

Total RNA was isolated from flash frozen *gluteus medius* samples using TRIzol/Chloroform extraction after homogenization with a biopulverizer (BioSpec Products, Inc, Bartlesville, OK, USA) as previously described (35). Samples underwent DNase treatments on columns (Direct-zol™ RNA MiniPrep Plus, Zymo, Irvine, CA) with RNase-free DNase I (New England BioLabs, M0303S) according to manufacturer's instructions. Samples then underwent quantification with a Qubit Fluorometer and RNA HS Assay Kit (Thermo Fischer Scientific) and RNA integrity (RIN) scores were measured with an Agilent 2100 Bioanalyzer and Agilent RNA 6000 Pico Kit (Agilent Technologies, Santa Clara, CA) and samples with RIN scores > 7.0 were used.

Library preparation

All libraries were constructed with the Illumina TruSeq Stranded mRNA Library Preparation Kit per manufacturer's instructions (Illumina, San Diego, CA) by the Michigan State University Genomics core. All libraries underwent quality control and were quantified using Qubit dsDNA High Specificity and Caliper LabchipGX High Specificity DNA assays. Next, libraries were pooled in equimolar concentration for multiplex sequencing.

Illumina Hiseq 4000

Kapa Biosystems Illumina Library Quantification qPCR kit was used to quantify libraries. Samples were then loaded onto 2 lanes of an Illumina HiSeq 4000 flow cell. Sequencing was performed in a 2x150bp paired end format using HiSeq 4000 SBS reagents for a target of 35-40 million reads for each sample. Base calling was done by Illumina Real Time Analysis (RTA) v2.7.7 and output of RTA was sorted and converted to FastQ format with Illumina Bcl2fastq v2.19.1 for analysis.

Assembly and Mapping

The quality of all 150-nucleotide paired end RNA-seq reads were evaluated with FastQC (36) and MultiQC (37). Adapter sequences were filtered usising Trimmomatic (38) and ConDeTri (39) was used to filter low quality reads with a quality threshold score of 30 (Q ≥30) or greater. Next, Bowtie2 (40) 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 (41) was used to map the reads. SAMTools (42) was utilized to retain all reads that were uniquely aligned (76%). Cufflinks (43) was used to assemble the transcriptome. HTSeq (44) was used to count the number of normalized gene reads aligning to the genes of interest with expression abundance observed across all horses in this study.

Differential Expression and Statistics

For the MFM WB and non-MFM WB, raw read counts per gene were normalized using the trimmed mean of M-values (TMM) (45). DE was determined by fitting a negative binomial generalized log-linear model per gene with diagnosis of MFM as coefficient of interest using

EdgeR (46) and corrected for multiple testing with the Benjamini-Hochberg method with a false discovery rate ≤ 0.05.

Validation by quantitative real time polymerase chain reaction

Differential gene expression was validated using quantitative real time polymerase chain reaction. Primers were designed for four genes with the most abundant significant differential expression (DE)– two genes with increased DE and two with decreased DE. The primers were designed to bridge exon-exon junctions using NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) referencing NCBI EquCab 3.0 (Table A.4.1). Glyceraldehyde phosphate dehydrogenase (GAPDH) and β-actin (ACTB) were used as housekeeping genes for an endogenous control due to the minimal variability of expression between samples.

Thermocycling for quantitative real-time polymerase chain reaction (qRT-PCR) was conducted using EvaGreen dye (Biotium, Inc, Fremont, California), ROX Reference Dye (Invitrogen, Life Technologies Carlsbad, CA), and Hot Start taq DNA Polymerase (New England BioLabs, Inc Ipswich, MA), using the QuantStudio 3 Real-Time PCR System (ThermoFisher Scientific Waltham, MA). The PCR reactions were run in triplicate (20 μL volume reactions). Each reaction contained 2 μL of sample cDNA made from 1200 ng of total RNA, 2 μL of 2.5 mM dNTPs, 2 μL of 10× PCR buffer (New England Biolabs, B9014S), 1 μL of EvaGreen dye (Biotium, 31000), 1.5 μL of 1:10 ROX reference dye dilution (Biotium, 29052), 0.125 μL of Hot Start taq DNA Polymerase (5,000 U/mL, New England Biolabs, M0495L), 2 μL of 800 nM forward primer, 2 μL of 800 nM reverse primer, and 7.4 μL of sterile nuclease-free distilled water. Reactions were run for 40 cycles under the following conditions: denaturation at 95 ºC for

10 minutes, annealing at 60 ºC for 1 minute; melt curve stages at 95 ºC for 15 seconds, 60 ºC for 1 minute, and 95 ºC for 15 seconds. Cycle thresholds (CT) were automatically calculated by the QuantStudio 3 Real-Time PCR System. For each gene of interest, > 90% geometric efficiency was established. Non template controls run for each gene showed no consistent amplification across triplicates. Normalized results were compared between MFM and non-MFM WB using a t-test and expressed as $2^{\Delta\Delta CT}$ in GraphPad Prism version 8.0 for Mac (GraphPad Software, La Jolla California USA, www.graphpad.com).

Protein Extraction and Proteolytic digestion

Protein was extracted from snap frozen gluteal muscle biopsies with a radioimmunoprecipitation lysis buffer (G Biosciences, 786-489) and protease inhibitor. Protein was then quantified with a bicinchoninic acid assay and pelleted for submission to the Michigan State University proteomics core.

From each sample, 500 μg of protein was digested in trypsin with a Filter-Aided Sample Preparation protocol and spin ultrafiltration unit cutoff of 30,000 Da (47). Reverse phase C18 SepPaks were used to de-salt the resulting peptides (Waters Corporation, Milford, MA) which were then dried by vacuum centrifugation. Peptide quantification was verified by colorimetric peptide concentration using 5μL from each sample digest.

Isobaric Labeling

Samples containing 100 μg of peptide were re-suspended in 100 μL of 100 mM of tetraethylammonium bicarbonate (TEAB) and labeled with tandem mass tag (TMT)10 reagents

(Thermo Scientific) per manufacturer's protocol. Labeling efficiency was tested by mass spectrometry with 5 μL from each sample. Equal sample proportions were mixed, de-salted, dried to a resulting volume of 2 μL and stored at -20 ºC.

Gel Fractionation

Dried peptides were suspended in Agilent Offgel buffer to a volume of 1.5 mL and an Agilent 3100 OFFGEL Fractionator (Agilent, Santa Clara, CA) was used to fractionate the samples into 12 portions over a non-linear 3-10 pH gradient per manufacturer's instructions. Following electrophoresis, fractions were de-salted with C18 stageTips (48). Samples were then dried to 2 μL via vacuum centrifugation and frozen at -20 ºC.

LC/MS/MS Analysis

Samples were suspended in 2%ACN/0.1% Formic Acid to 20uL and an injection of 5uL was made automatically using a Thermo EASYnLC 1200 (Thermo Scientific) onto a Thermo Acclaim PepMap RSLC 0.1mm x 20mm C18 trapping column and washed for ~5min with buffer A. Bound peptides were then eluted onto a Thermo Acclaim PepMap RSLC 0.075mm x 250mm C18 resolving column over 95min with a gradient of 2% B to 32% B in 84 min, ramping to 100% B at 85min and held at 100%B for the duration of the run (Buffer A = 99.9% Water/0.1% Formic Acid, Buffer B = 80% Acetonitrile/0.1% Formic Acid/19.9%H2O) at a constant flow rate of 300nl/min. Column temperature was maintained at a constant temperature of 50ºC using an integrated column oven (PRSO-V1, Sonation GmbH, Biberach, Germany).

Eluted peptides were sprayed into a ThermoScientific Q-Exactive HF-X mass spectrometer (Thermo Scientific) using a FlexSpray spray ion source. Survey scans were taken in the Orbi trap (120,000 resolution, determined at m/z 200), and the top twenty ions in each survey scan were then subjected to automatic higher energy collision induced dissociation with fragment spectra acquired at resolving power of 45,000. The resulting MS/MS spectra were converted to peak lists using Proteome Discoverer, v2.2 (Thermo Scientific) and searched against UniprotKB for all *Equus caballus* sequences. Results were appended with common laboratory contaminants using the Sequest HT search algorithm. The search output was then analyzed using Scaffold, v4.8.4 (Proteome Software, Inc, Portland, Oregon), to probabilistically validate protein identifications (FDR < 0.01). Quantification of reporter ion intensities is done using the Q+S module within Scaffold.

Quantitative data analysis

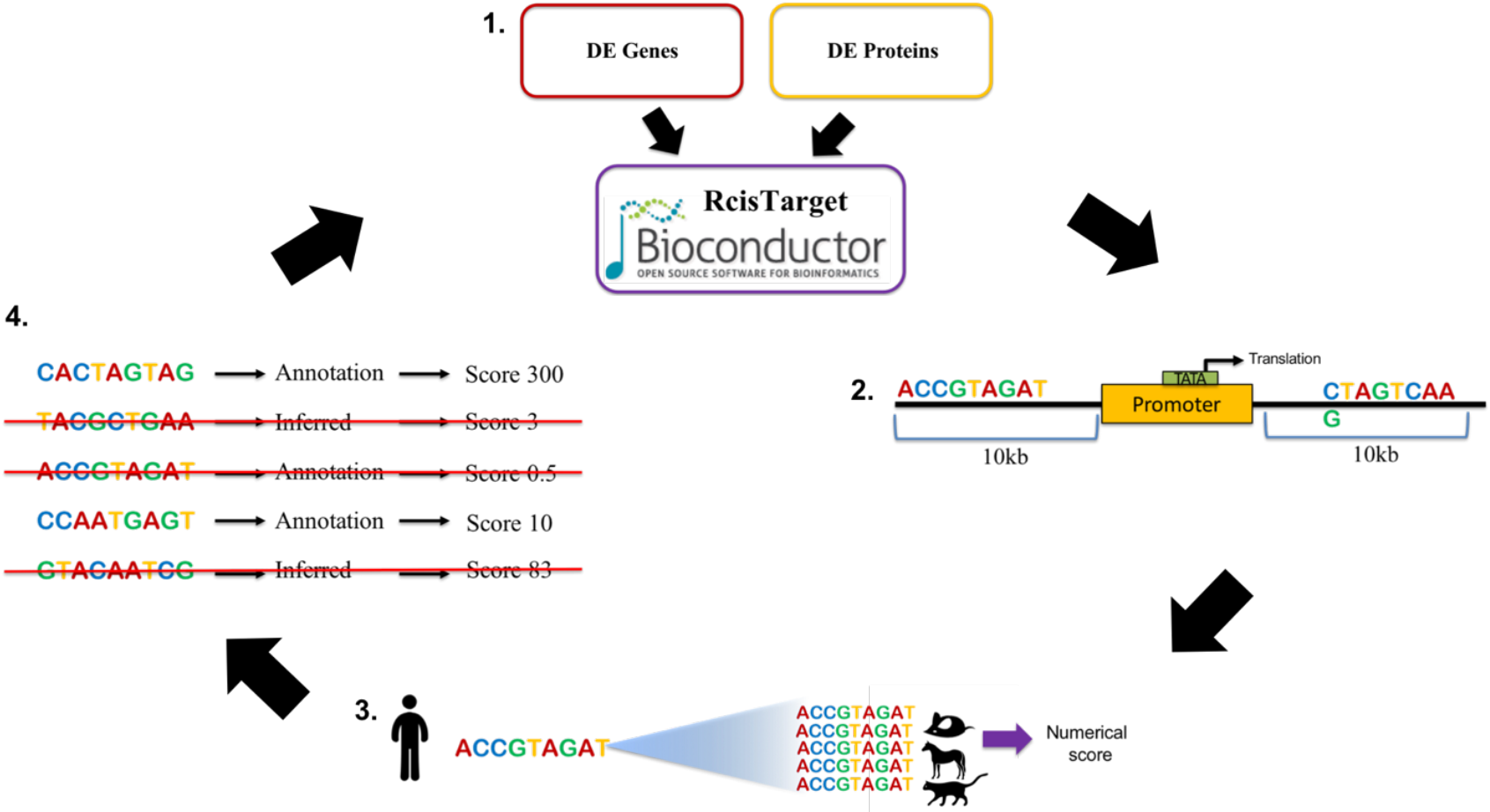
Scaffold Q+ (version Scaffold 4.9.0, Proteome Software Inc., Portland, OR) was used to quantitate TMT Label Based Quantitation peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99.9% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (49). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. Channels were corrected by the matrix in all samples according to the algorithm described in i-Tracker (50). Normalization was performed iteratively (across samples and

spectra) on intensities, as described in Statistical Analysis of Relative Labeled Mass Spectrometry Data from Complex Samples Using ANOVA (51). Medians were used for averaging. Spectra data were log-transformed, pruned of those matched to multiple proteins, and weighted by an adaptive intensity weighting algorithm. Of 108665 spectra in the experiment at the given thresholds, 79921 (74%) were included in quantitation. Differentially expressed proteins were determined by applying Permutation Test with unadjusted significance level $P <$ 0.003 corrected by Benjamini-Hochberg.

Transcription factor motif analysis- RcisTarget

Gene identification (ID) lists were generated from significantly differentially expressed (DE) gene transcripts (n = 47) and proteins (n = 93). These lists were then input into RcisTarget for identification of enriched transcription factor binding motifs that were within 10kb centered around the annotated transcription start site for that gene ID (52). Next, a recovery-based method was used referencing the human species to query genome wide cross-species rankings for identified motifs that were conserved in at least 10 species. Identified motifs were retained and annotated to TFs if they had a normalized enrichment score > 3.0 (Figure 4.1). The resulting list of enriched TFs was further filtered based on expression of those TFs within the transcriptomic or proteomic datasets. The target gene lists of the resulting TFs were generated for those that were DE in the proteomic or transcriptomic data sets. Circos plots were generated using BioCircos.js (53) to depict the enriched TFs and the chromosomal location and direction of DE of their target genes. Motifs sequence logos were searched and obtained from https://motifcollections.aertslab.org/v7/logos/?C=M;O=A.

Figure 4.1: The RcisTarget methods. 1) the gene ID lists from DE genes and DE proteins are imported into RcisTarget, available R package from Bioconductor. 2) Transcription factor binding motifs are identified within 10 kb centered around the transcription start site for the DE gene IDs. 3) A recovery based method is used on a database of genome-wide cross species ranking of each motif. The reference is set for the human species with the motif retained if it is conserved across at least 10 other species. 4) Motifs are ranked based on their cross species comparisons to yield a normalized enrichment score. Those scores greater than 3.0 and directly annotated to TF are retained and target DE gene ID lists generated.



Gene ontology and pathway enrichment

Gene Ontology (GO) enrichment analyses was performed using significant results from the transcriptomic dataset, the proteomic dataset, amalgamated dataset, and DE target genes of enriched TF. Significant DE gene symbols were translated to ENTREZ gene IDs in the R package org.Hs.eg.db using the human annotation (54) and were then analyzed and depicted with the default settings in clusterProfiler R package (55). This resulted in pathway enrichment analysis for biological processes, cellular location, and molecular function.

Reactome pathway analysis

A reactome pathway analysis was performed for amalgamated DE genes and DE proteins. The converted ENTREZ gene IDs annotated to human were analyzed with the R package ReactomePA (56) and depicted with the default settings in clusterProfiler R package (55).

RESULTS

Electron microscopy

The majority of fibers had well aligned myofibrils (Figure 4.2-A); however, several regions within each sample showed, streaming of Z-discs, and disarray of sarcomeres and myofilaments (Figure 4.2-B). In a few regions, areas of severe myofibrillar disruption were apparent with marked streaming and aberrant accumulation of Z-disc material (Figure 4.2-C). Some areas had abnormal variation in mitochondrial shape and others had both normal and degenerate mitochondria with less compact cristae (Figure 4.3-A, B).

mRNA reads and mapping

A sequencing depth of approximately 75.6 X per horse was achieved. An average of 56 ± 13.9 million reads per horse was filtered resulting in 76.4% of the filtered reads mapping to the equine genome, EquCab 3.0. Of those reads, 97.2% were unique and retained for downstream analysis. After filtering out genes with low read counts, 14,366 total genes (51.9% of the total raw reads) were used for DE analysis between MFM and non-MFM WB (Figure 4.4).

Transcriptomics- differential expression

A total of 27,690 genes were identified. Of those, 47 were significantly DE in MFM WB when compared to control WB (Figure 4.5-A). The $\log_2$ FC of the DE genes ranged from -6.7 to 4.8. Thirty-four gene transcripts had increased DE and 13 had decreased DE. Eight of the 47 genes were not annotated in the current equine reference genome and 2 of the genes with locus identification were uncharacterized. Twenty-four DE genes had a $\log_2$ FC > 1.5; many of which are transcription factors, have cell surface and extracellular matrix functions, code for

metalloproteins, or have roles in glutathione, protein, glucose and nucleotide metabolism (Table 4.1). The greatest log FC occurred for glutathione specific gamma-glutamylcyclotransferase 1 (*CHAC1*, $\log_2$ FC 4.8) and hemoglobin subunit feta (*HBB,* $\log_2$ FC -6.7).

Figure 4.2: Electron micrographs of MFM WB gluteal muscle. A.) Areas of well aligned myofibrils and organized sarcomeres. 14k x B.) Areas were hypercontracted sarcomeres, mild Z-disc streaming (arrows) and pooling of granular material containing glycogen. 10 k x C.) Severe myofibrillar disarray with complete loss of sarcomeres, dense streaming of Z-disc material (arrows). 40k x.

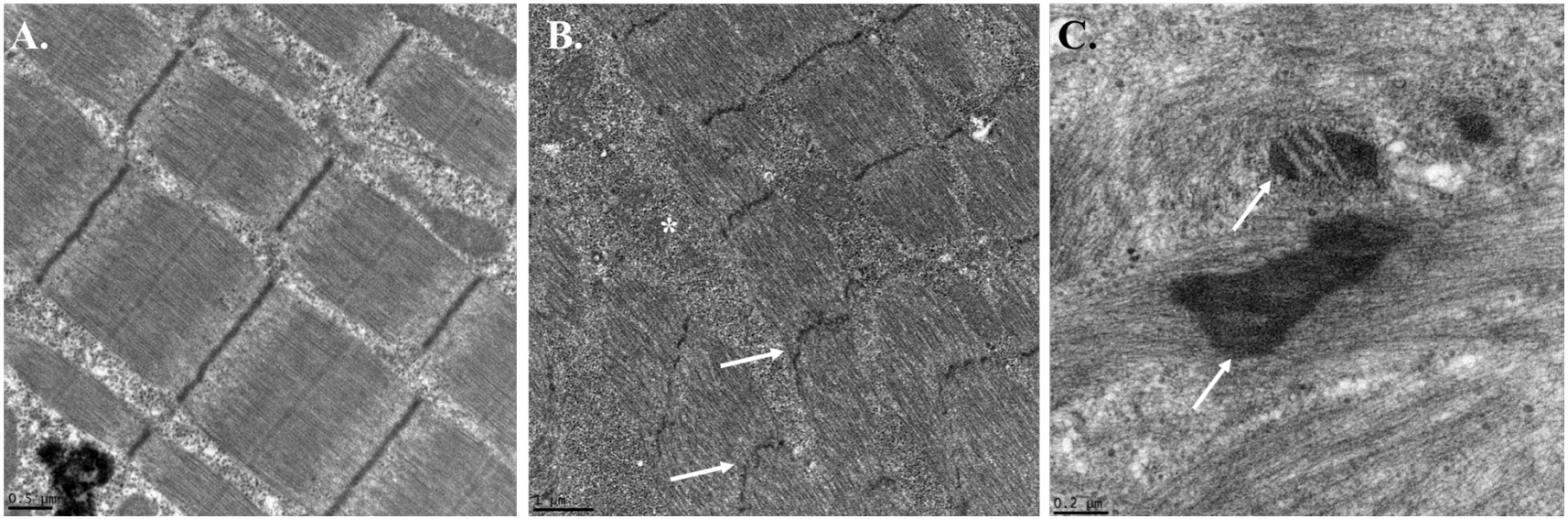


Figure 4.3: Electron micrograph of MFM WB gluteal muscle A.) Subsarcolemmal mitochondria with increased variability in shape and size (arrow) 14k x B.) Budding mitochondria (arrow), and degenerate mitochondria with enlarged cristae (asterisk).

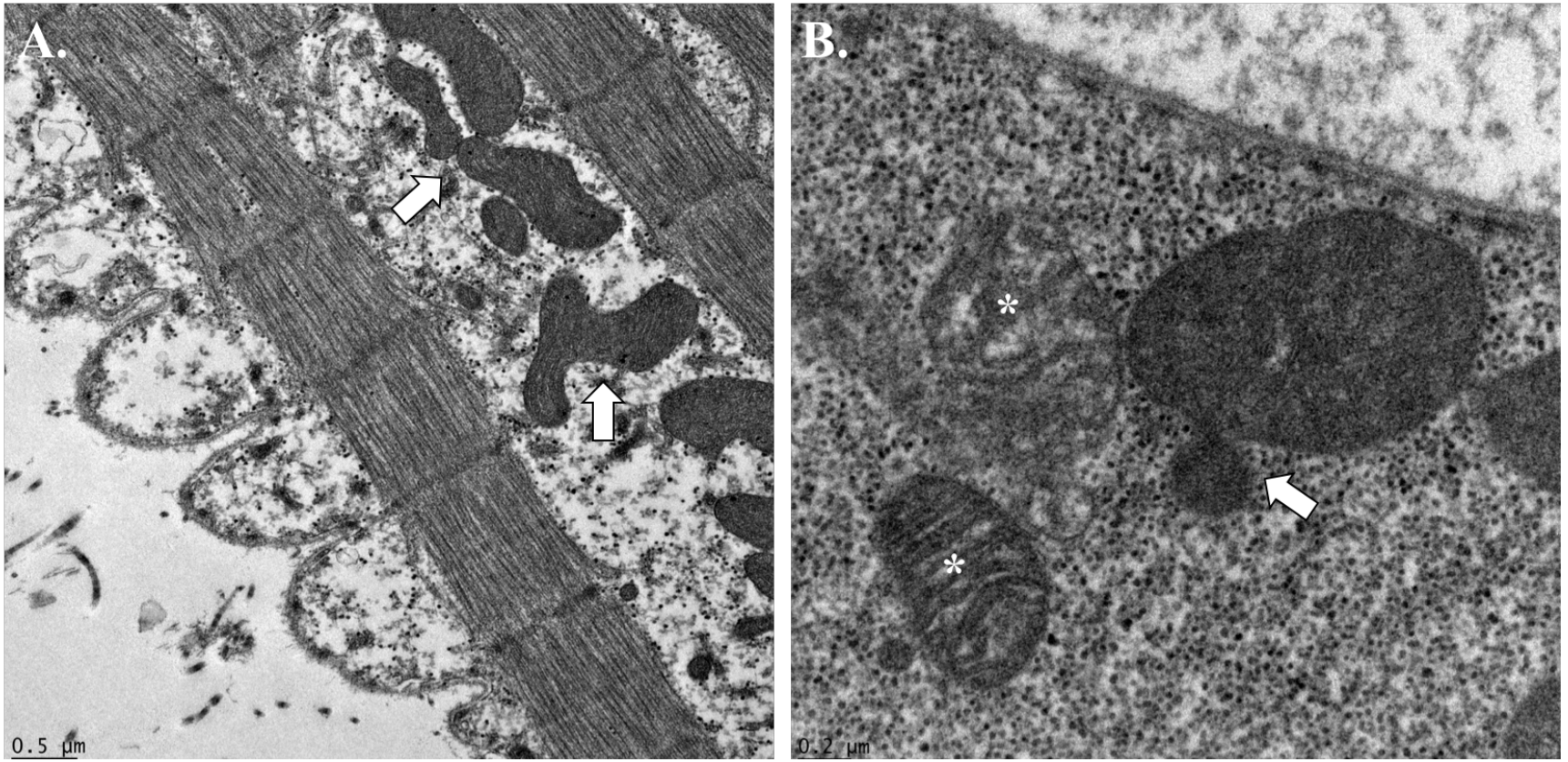


Figure 4.4: The RNA-seq bioinformatic pipeline used including the filtering steps used, the resulting reads per million and the expressed genes used for differential expression (DE) analysis. After stringent filtering, 55.6% of reads and 51.9% of expressed genes were used for DE analysis.

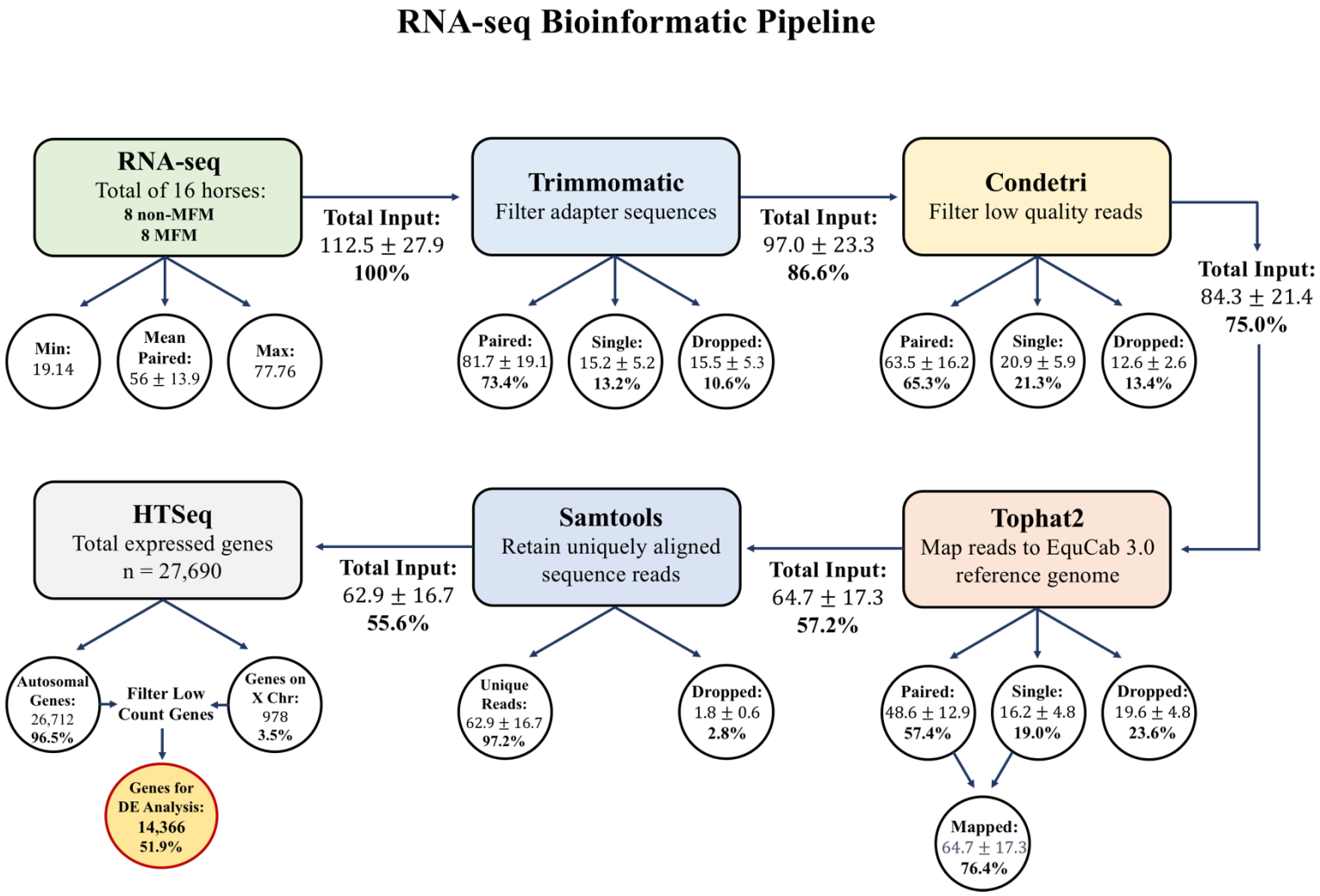


Figure 4.5: A.) A volcano plot depicting gene expression according to the adjusted *P* value and the $\log_2$ fold change for 14,366 genes. Forty-seven of the DE genes were significantly DE ($P \leq 0.0001$) between MFM and non-MFM WB. B.) Protein expression according to the adjusted *P* value and the $\log_2$ fold change for 1,533 proteins. Ninety-three of the proteins were significantly DE ($P \leq 0.0027$) between MFM and non-MFM WB .

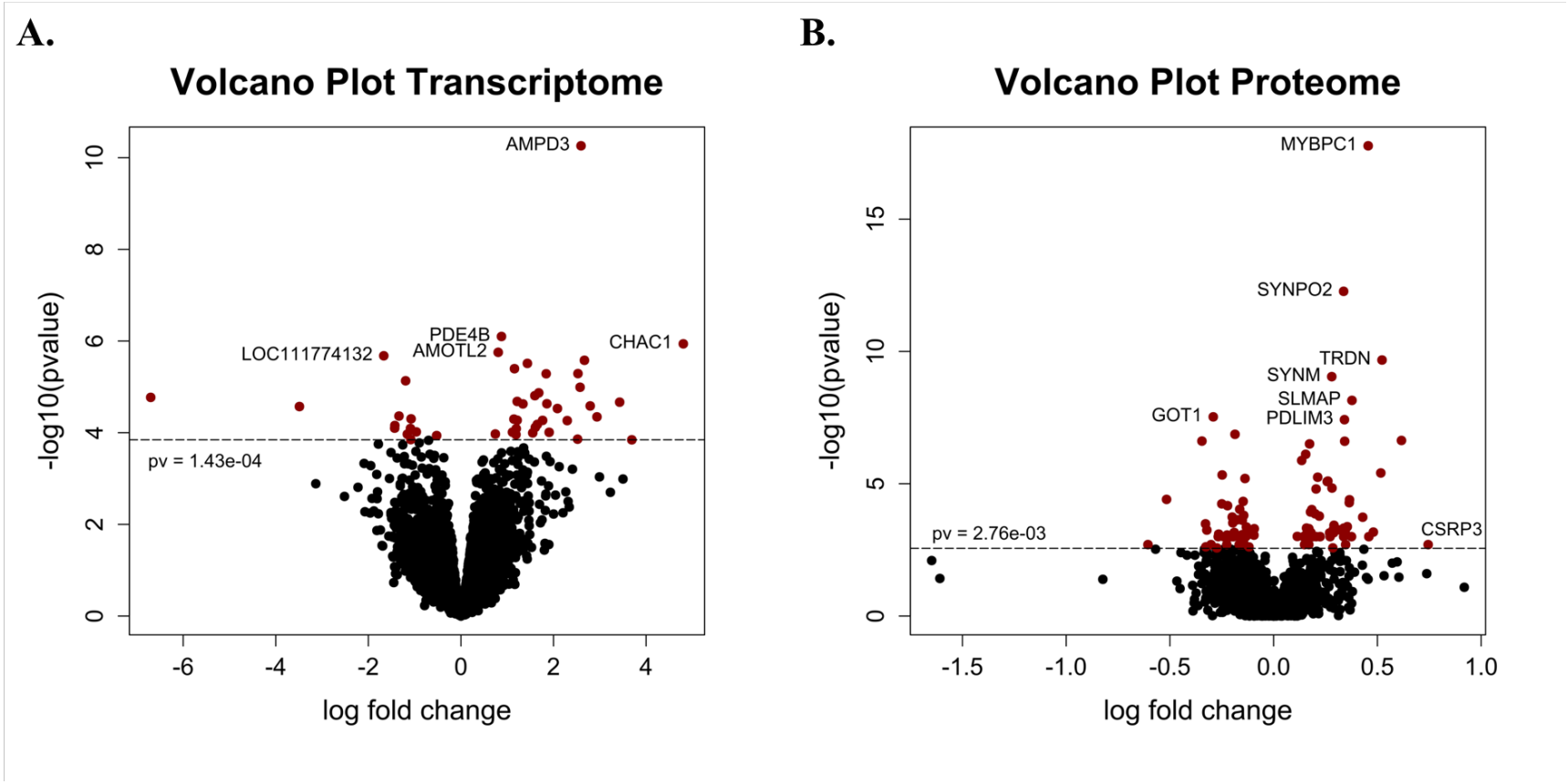


Table 4.1: Significantly DE genes with a $\log_2$ fold change of ≥ 1.5 in MFM WB compared to non-MFM WB.

Category	ID	Gene name	Function	$\log_2$ FC >1.5	*P adjusted*	detected in proteomics?
Cell surface/ extracellular matrix	CCR7	C-C Motif Chemokine Receptor 7	G protein-coupled receptor	3.43	0.0203	no
	THBS1	Thrombospondin 1	adhesive glycoprotein, cell to matrix interactions, and integrin binding	2.67	0.0072	no (THBS4 found)
	ELN	Elastin	elastic extracellular matrix constituent	1.91	0.0412	no
	NCAM1	Neural cell adhesion molecule 1	cell-cell adhesion (neural)	1.84	0.0085	no
	TNC	Tenascin C	extracellular matrix, synaptic plasticity, neuronal generation	1.61	0.0382	no
Transcription factor	NR4A2	Nuclear Receptor Subfamily 4 Group A Member 2	transcription factor	2.94	0.0307	no
	GADD45G	Growth Arrest And DNA Damage Inducible Gamma	transcription factor - growth and apoptosis	2.57	0.0139	no
	CEBPD	CCAAT Enhancer Binding Protein Delta	transcription factor - enhances IL 6	2.53	0.0085	no
	ATF3	Activating Transcription Factor 3	transcription factor	2.52	0.0498	no
	RTL3	Retrotransposon Gag Like 3	transcription factor- post natal myogenesis	1.68	0.0170	no
	PER1	Period Circadian Regulator 1	transcription factor	1.60	0.0182	no
	DDIT4	DNA Damage Inducible Transcript 4	transcription factor- regulates mTORC1 and responses to cellular energy levels and cellular stress	1.55	0.0413	no
Metalloprotein	LOC100630543 (MT1B)	Metallothionein 1B	metal binding	1.76	0.0307	no
	ADAMDEC1	ADAM Like Decysin 1	disintegrin metalloproteinase	-3.49	0.0209	no
	HBB	Hemoglobin Subunit Beta	iron and oxygen binding	-6.70	0.0185	yes

Table 4.1 (cont'd)

Category	ID	Gene name	Function	log_2 FC >1.5	*P adjusted*	detected in proteomics?
Glutathione degradation	CHAC1	ChaC Glutathione Specific Gamma-Glutamylcyclotransferase 1	oxidative imbalance, degrades glutathione	4.80	0.0063	no
Protein modification	OTUD1	OTU Deubiquitinase 1	cleaves ubiquitin	2.79	0.0209	no
Nucleotide metabolism	AMPD3	Adenosine Monophosphate Deaminase 3	deamination of adenosine monophosphate to inosine monophosphate	2.59	< 0.0001	no (AMPD1 found)
Glucose metabolism	PFKFB3	6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3	glucose metabolism, anti-apoptosis	1.65	0.0360	no (PFKFB1 found)
Unknown	NA	chr21:44470419-44471212	unknown	3.69	0.0498	no
	NA	chr10:42480066-42482237	unknown	2.30	0.0307	no
	LOC111773016	Uncharacterized	unknown	2.08	0.0221	no
	LOC111769187	Uncharacterized	unknown	1.86	0.0203	no
	LOC111774132	Uncharacterized	unknown	-1.67	0.0069	no

qRT-PCR

The geometric mean calculated from *GAPDH* and *ACTB* to act as an endogenous control for each sample was on average for all samples 23 cycle thresholds (CT) ± 1.5. The direction of DE concurred with the transcriptomic analysis (Figure A.4.1). There were no significant differences in qRT-PCR detected gene transcripts when comparing MFM WB versus non-MFM WB.

Proteomics- differential expression

Proteomic analysis identified a total of 1,533 equine proteins. Of those, 93 proteins were significantly DE in MFM *vs.* controls WB ($P < 0.0030$) (Figure 4.5-B). Forty-nine proteins had increased DE and 44 had decreased DE, with the log FC ranging from -0.47 to 0.61. Fifty-six DE proteins had a log FC ≥ 0.2 which generally had cytoskeletal functions, mitochondrial functions, and functions in protein metabolism or modification (Table 4.2). The proteins with the greatest positive or negative $\log_2$ FC were a Z-disc protein cysteine and glycine-rich protein 3 (CSRP3, FC 0.74) and D-dopachrome decarboxylase (DDT, $\log_2$ FC -0.61).

Table 4.2: Significantly DE proteins with a log fold change of ≥ 0.20 in MFM WB compared to non-MFM WB.

Category	ID	Protein name	Function	Fold change > 0.2	*P adjusted*	Detected in transcript-omics
Mitochondrial function	VDAC3	Voltage Dependent Anion Channel 3	mitochondrial anion porin	0.20	0.0001	yes
	TST	Thiosulfate Sulfurtransferase	mitochondrial sulfur amino acid metabolism	-0.20	0.0002	yes
	PDHA1	Pyruvate Dehydrogenase E1 Alpha 1 Subunit	converts pyruvate to acetyl-CoA and CO_2	-0.22	0.0009	yes
	HCCS	Holocytochrome C Synthase	links a heme group to the apoprotein of cytochrome c	-0.24	0.0010	yes
	APOO	Apolipoprotein O	transports and metabolizes lipids, role cristae formation and function	-0.33	0.0024	yes
	NDUFV3	NADH:Ubiquinone Oxidoreductase Subunit V3	complex I accessory subunit-NADH dehydrogenase module	0.48	0.0007	yes
	NDUFA13	NADH:Ubiquinone Oxidoreductase Subunit A13	necessary for complex I assembly and electron transfer, prevents the transactivation of STAT3	-0.21	0.0010	no
	NDUFB5	NADH:Ubiquinone Oxidoreductase Subunit B5	complex I accessory subunit	-0.27	0.0010	yes
	MTND4	Mitochondrially Encoded NADH:Ubiquinone Oxidoreductase Core Subunit 4	required for complex 1 assembly and catalysis	-0.52	< 0.0001	no
Cytoskeletal organization/ contraction	SMTNL1	Smoothelin Like 1	regulates contractile properties	0.62	< 0.0001	yes
	MYBPC1	Myosin Binding Protein C, Slow Type	regulates contractile properties, recruits muscle-type creatine kinase to myosin filaments	0.46	< 0.0001	yes
	EML1	Echinoderm Microtubule-Associated Protein-Like 1	microtubule cytoskeleton	0.35	0.0020	yes

Table 4.2 (cont'd)

Category	ID	Protein name	Function	Fold change > 0.2	*P adjusted*	Detected in transcript-omics
Cytoskeletal organization/ contraction	PFN2	Profilin 2	responds to extracellular signals by regulating actin polymerization	0.29	0.0027	yes
	SMTNL2	Smoothelin Like 2	possible cytoskeletal/membrane regulation	0.21	0.0010	yes
	NEB	Nebulin	cytoskeletal organization	-0.31	0.0005	yes
	TNNT1	Troponin T1, Slow Skeletal Type	contractility	-0.33	0.0003	yes
	CSRP3	Cysteine And Glycine Rich Protein 3	transcription factor involved in myogenesis and cytoskeletal Z-disc assembly	0.74	0.0020	no
	SYNPO2	Synaptopodin 2	cytoskeletal organization and maintenance at Z-disc	0.34	< 0.0001	yes
	PDLIM3	PDZ And LIM Domain 3	cytoskeletal organization, colocalizes with alpha - actinin at Z-disc	0.34	< 0.0001	yes
	SYNM	Synemin	intermediate filament that binds desmin and the extracellular matrix	0.28	< 0.0001	yes
Sarcoplasmic/ endoplasmic reticulum	TRDN	Triadin	regulates of lumenal calcium release by RYR1 and RYR2 channels	0.52	< 0.0001	yes
	BCAP31	B Cell Receptor Associated Protein 31	endoplasmic reticulum chaperone	0.34	0.0005	yes
	JPH1	Junctophilin 1	links T-tubule and sarcoplasmic reticulum, has role in calcium sensing	0.26	< 0.0001	yes
Cell membrane	SLMAP	Sarcolemma Associated Protein	possible role in myoblast fusion	0.38	< 0.0001	yes
	CDH13	Cadherin 13	cell-cell adhesion	0.35	0.0004	yes
Transcription factor	KLHL41	Kelch Like Family Member 41	transcription factor involved in myoblast differentiation and myofibril assembly	0.22	0.0002	yes
	ZNF592	Zinc Finger Protein 592	transcription factor	0.26	< 0.0001	yes
Nuclear proliferation/location	LMNA	Lamin A/C	involved in skeletal muscle and satellite cell proliferation	0.20	< 0.0001	yes
	TACC2	Transforming Acidic Coiled-Coil Containing Protein 2	microtubule-dependent coupling of the nucleus and the centrosome	0.29	0.0004	yes

Table 4.2 (cont'd)

Category	ID	Protein name	Function	Fold change > 0.2	*P adjusted*	Detected in transcript-omics
mRNA processing	HNRNPA1	Heterogeneous Nuclear Ribonucleoprotein A1	mRNA binding and pre-mRNA processing	0.43	0.0002	yes
	SFPQ	Splicing Factor Proline And Glutamine Rich	pre-mRNA splicing factor, regulates circadian clock genes	0.27	0.0010	yes
mRNA processing Translation/ protein synthesis	EIF4B	Eukaryotic Translation Initiation Factor 4B	binding of mRNA to ribosomes	0.29	0.0005	yes
	EEF2K	Eukaryotic Elongation Factor 2 Kinase	protein synthesis and elongation	0.37	0.0010	yes
	EIF3C	Eukaryotic Translation Initiation Factor 3 Subunit C	component of translation initiation complex	0.32	0.0006	no
Translation/ protein synthesis Protein metabolism or modification	RPL21	Ribosomal Protein L21	60S ribosomal subunit	0.29	0.0007	yes
	RPS4X	Ribosomal Protein S4 X-Linked	40S ribosomal subunit	-0.20	0.0010	yes
	RPS9	Ribosomal Protein S9	40S ribosomal subunit	-0.25	< 0.0001	yes
	UCHL1	Ubiquitin C-Terminal Hydrolase L1	thiol protease	0.34	0.0010	yes
	NTAN1	N-Terminal Asparagine Amidase	ubiquitin-dependent protein degradation	0.27	0.0007	yes
Protein metabolism or modification Amino acid metabolism	PPP1R2	Protein Phosphatase 1 Regulatory Inhibitor Subunit 2	protein phosphatase	0.22	0.0010	yes
	ASNA1	Guided Entry Of Tail-Anchored Proteins Factor 3, ATPase (GET3)	post-translational endoplasmic reticulum protein transport, involved in unfolded protein response	-0.32	0.0006	yes

Table 4.2 (cont'd)						
Category	**ID**	**Protein name**	**Function**	**Fold change > 0.2**	***P adjusted***	**Detected in transcript-omics**
Protein metabolism or modification Amino acid metabolism	UBA3	Ubiquitin Like Modifier Activating Enzyme 3	down-regulates steroid receptor activity	-0.20	0.0003	yes
	S100A1	S100 Calcium Binding Protein A1	calcium signal transducer, inhibits protein phosphorylation	-0.23	0.0020	yes
	FAH	Fumarylacetoacetate Hydrolase	tyrosine catabolism	-0.27	0.0027	yes
	GOT1	Glutamic-Oxaloacetic Transaminase 1	biosynthesis of L-glutamate	-0.29	< 0.0001	yes
Amino acid metabolism Nucleoside/nucleotide metabolism	DDT	D-dopachrome tautomerase	converts D-dopachrome into 5,6-dihydroxyindole	-0.61	0.0020	no
	NIT2	Nitrilase Family Member 2	converts alpha-ketoglutaramate to alpha-ketoglutarate and alpha-ketosuccinamate to oxaloacetate	-0.22	< 0.0001	yes
	NT5C1A	5'-Nucleotidase, Cytosolic IA	dephosphorylates nucleoside monophosphate	-0.25	< 0.0001	yes
	IMPDH2	Inosine Monophosphate Dehydrogenase 2	converts IMP to XMP	0.22	0.0010	yes
Lipid metabolism	APOA1	Apolipoprotein A1	may play a role in lipid metabolism in skeletal muscle (57)	-0.35	< 0.0001	no
	FASN	Fatty Acid Synthase	synthesis of long-chain fatty acids from acetyl-CoA, malonyl-CoA and NADPH	-0.26	0.0008	yes
Antioxidant	CAT	Catalase	antioxidant enzyme	-0.30	0.0020	yes
	HP	Haptoglobin	antioxidant needed to prevent muscle atrophy (58)	0.52	< 0.0001	no
Blood glycoprotein	FGB	Fibrinogen Beta Chain	muscle mitochondrial dysfunction (59) and fibrosis (60)	0.46	0.0010	yes
	FGG	Fibrinogen Gamma Chain	muscle mitochondrial dysfunction (59) and fibrosis (60)	0.36	< 0.0001	no

Table 4.2 (cont'd)						
Category	**ID**	**Protein name**	**Function**	**Fold change > 0.2**	***P adjusted***	**Detected in transcript-omics**
Blood glycoprotein	FGA	Fibrinogen Alpha Chain	muscle mitochondrial dysfunction (59) and fibrosis (60)	0.34	0.0006	no

Amalgamated data

There were 1,229 analyzed gene IDs that were common to both the transcriptomic and proteomic data. There was, however, no overlap in the protein gene IDs or gene transcript IDs that were significantly DE. Only *HBB* from the transcriptomic data ≥ 1.5 $\log_2$ FC was also identified in the proteomic analysis, however it was not DE as a protein (Table 4.1). In contrast, 46 DE proteins with ≥ 0.2 $\log_2$ fold change were also expressed in the transcriptomic data, but none had DE gene transcripts (Table 4.2).

Transcription factor motif analysis

A total of 186 enriched TF were obtained from the DE gene ID list. After filtering for those that were either differentially expressed themselves or expressed in both our transcriptomic and proteomic datasets, only 3 enriched TF remained (Figure A.4.2). Those included Signal transducer & activator of transcription 3 (STAT3) and Y-Box binding protein 1 (YBX1), which was expressed in both the transcriptomic and proteomic datasets, and Activating transcription factor 3 (ATF3), which was DE (↑ 2.5 $\log_2$ FC) in the transcriptomic data, but not found in the proteomic data. This was because the previously used equine protein annotation did not contain ATF3.

Target DE gene IDs

Forty-three DE targets were identified for the 3 enriched TF. STAT3 shared 2 DE targets with ATF3 and 3 DE targets with YBX1. ATF3 shared 4 DE targets with YBX1 (Table 4.3). For STAT3, 18 target gene IDs were identified that were also DE (15↑, 3↓) (Figure A.4.3-A). YBX1 also had 18 DE target gene IDs (12↑, 6↓) (Figure A.4.3-B). Including itself, ATF3 only had 7 DE target gene transcripts which all had increased expression (↑7) (Figure 4.6).

Transcriptomics- GO analysis

GO analysis for significant transcripts after background correction revealed 15 significantly enriched GO biological process terms. The GO term with the lowest adjusted *P* value was response to ketone (GO:1901654, q = < 0.0001, 8 DE gene transcripts (Figure 4.7). Seven of these 8 DE genes were also defined as response to steroid hormone. Many DE genes were found within multiple GO terms (Figure 4.7). One of those 4 DE gene transcripts that fell within those 3 GO terms was *HBB*, which had the lowest DE in MFM WB. There were no significantly enriched GO terms for GO molecular function or GO Cellular location terms.

Table 4.3: The 3 enriched transcription factors STAT3, YBX1, and ATF3 and their DE target genes with direction of DE.

STAT3 DE Targets		YBX1 DE Targets		ATF3 DE Targets	
Gene ID	Direction of DE	Gene ID	Direction of DE	Gene ID	Direction of DE
DDIT4	↑	CHAC1	↑	CHAC1	↑
MAP1A	↑	PNRC1	↑	PNRC1	↑
THBS1	↑	PER1	↑	PER1	↑
AMOTL2	↑	NR4A2	↑	AMOTL2	↑
CDKN1A	↑	CDKN1A	↑	CDKN1A	↑
KLF9	↑	KLF9	↑	ITPRIP	↑
NR4A2	↑	TXNIP	↑	ATF3	↑
TNC	↑	HEY1	↑		
PDE4B	↑	TRDN	↑		
LMNA	↑	EEF2K	↑		
SYNPO	↑	P4HB	↑		
CLIP1	↑	HNRNPA1	↑		
IMPDH2	↑	NEB	↓		
MYBPC2	↑	ASNA1	↓		
PRDX1	↑	CAND1	↓		
HCCS	↓	CAT	↓		
RPS3	↓	GOT1	↓		
TNNT1	↓	ALDH6A1	↓		

Figure 4.6: Circos plot depicting the chromosomal location and direction of expression of DE genes and proteins identified in the transcriptomic and proteomic analysis. The outer most ring shows the equine chromosomal locations, the yellow middle ring shows DE proteins and the inner ring shows DE transcriptomic data. The direction of expression is marked as a blue line for increased expression and a red line for decreased expression. The target genes for ATF3 are shown by the connecting lines in the inner most circle.

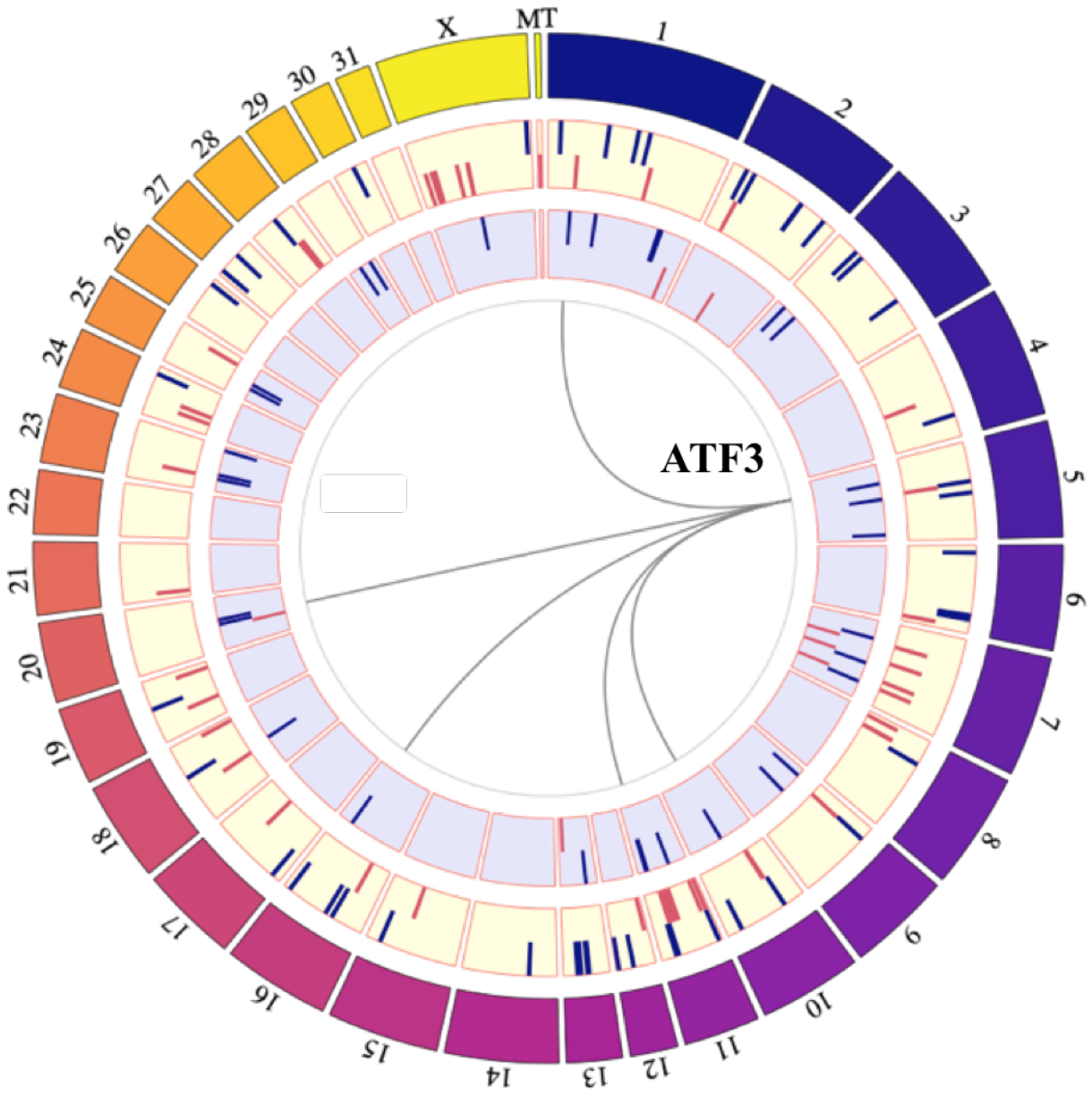
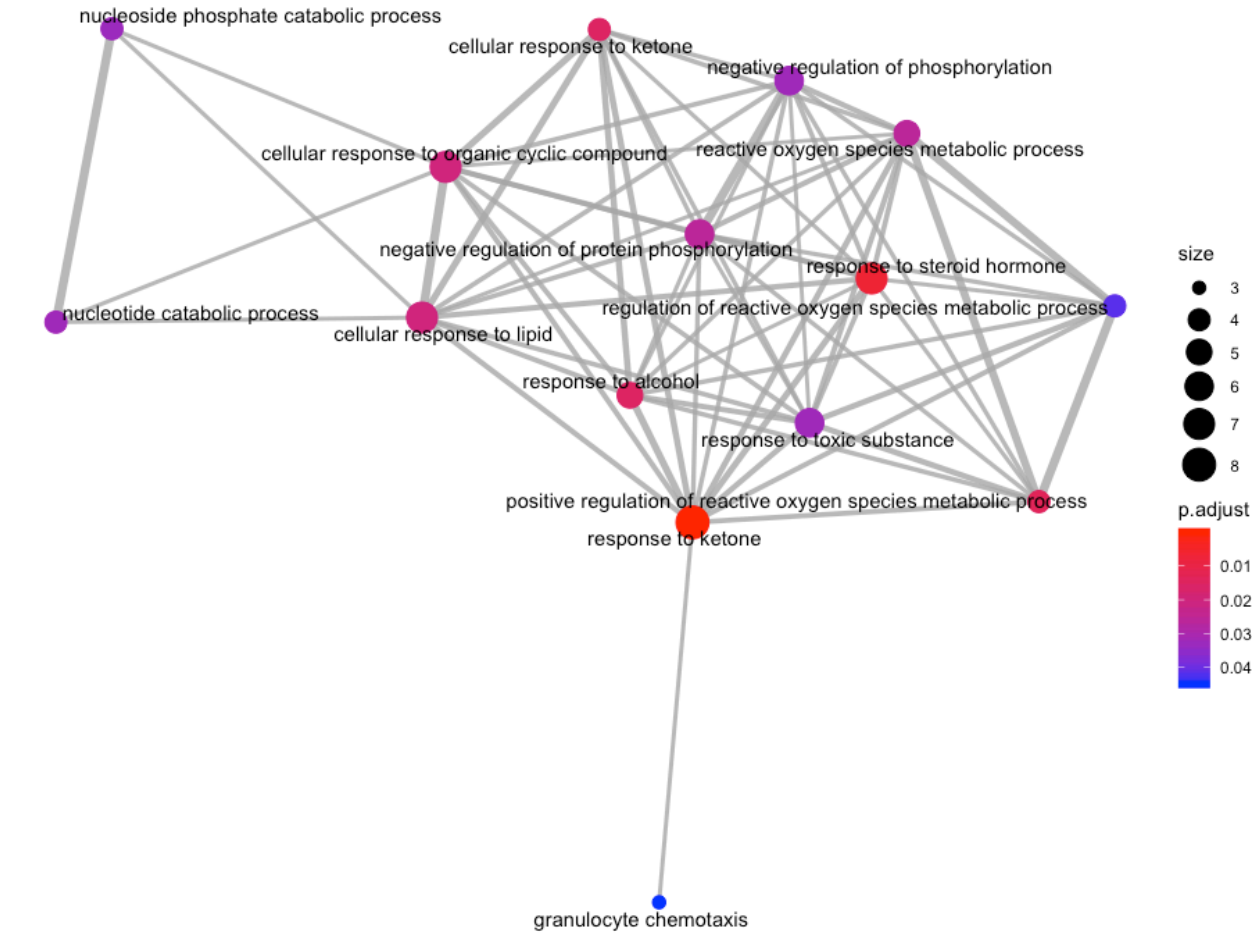


Figure 4.7: Enriched GO biological function terms for DE gene transcripts in MFM WB. The size of the vertex indicates the number of DE target genes in that term. The color of the vertex indicates the adjusted *P* value and the edges (lines) connecting the vertices represents DE target genes that were common between the GO terms.



Proteomics-GO analysis

GO biological process yielded one significant enrichment term, cytoskeletal organization (GO:0007010) containing 26 DE proteins. After background correction, there was no significant enrichment in either GO molecular function or GO cellular location.

Amalgamated data- GO analysis

After merging both the DE protein gene IDs and the DE gene transcript ID with a merged background correction, there was significant GO enrichment in biological process, molecular function, and cellular location terms. In total, there were 126 GO terms for biological processes, 45 for cellular locations and 12 for molecular functions. Many DE gene transcripts and proteins appeared in multiple terms within their respective GO category (Figures 4.8, 4.9, 4.10). Interestingly, the GO cellular location terms had 3 distinct clusters that fell within 1) Z-disc and sarcomere structure, 2) complex I and the respiratory chain of mitochondria, and 3) extracellular matrix and vesicles (Figure 4.9).

Figure 4.8: Eight GO biological function terms with the lowest *P* values for DE gene transcripts merged with DE proteins in MFM WB. The size of the bars indicate the number of DE genes/DE proteins in each GO term and the color of the bar reflects the adjusted *P* value.

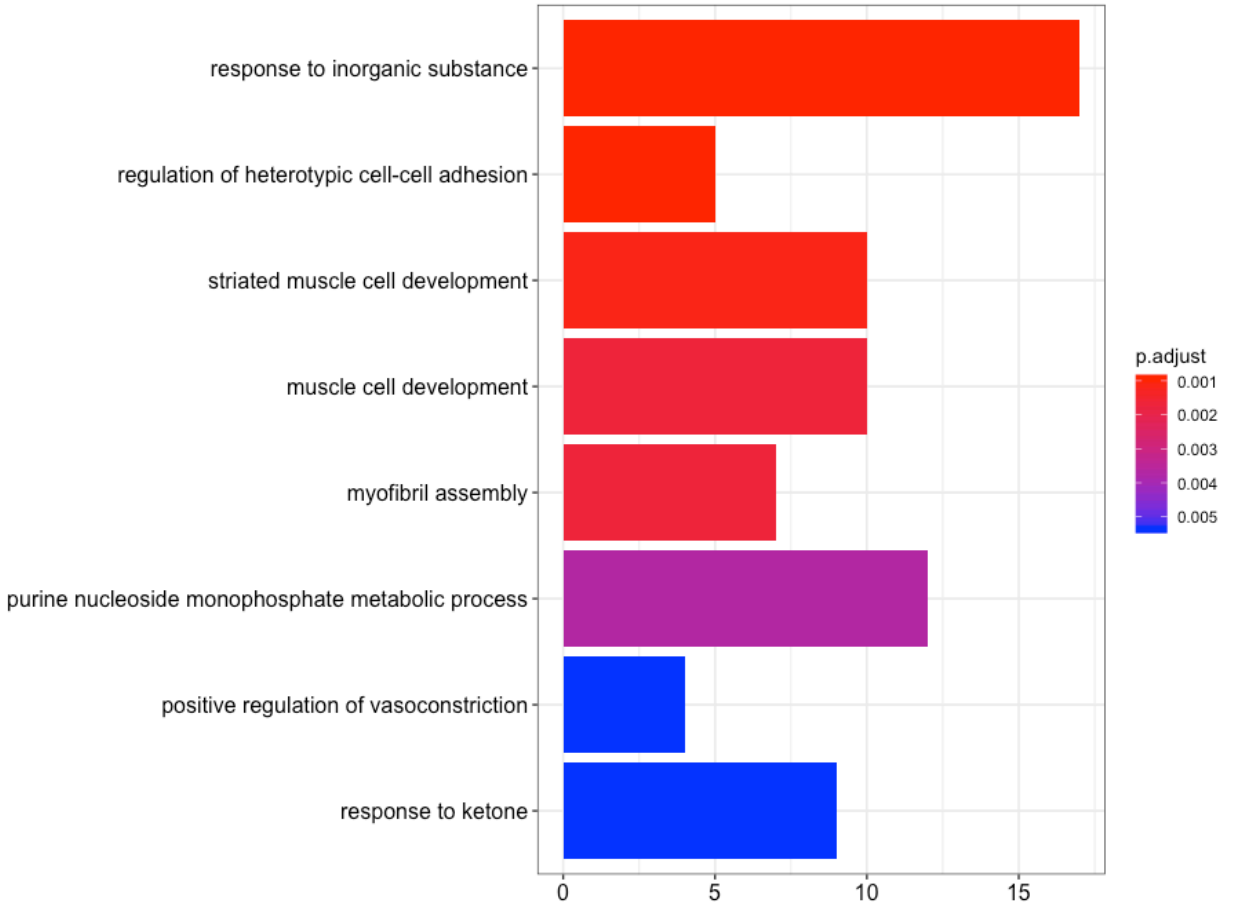


Figure 4.9: Enriched GO cellular location terms for DE gene transcripts merged with DE proteins in MFM WB. The size of the vertex indicates the number of DE target genes in that term. The color of the vertex indicates the adjusted *P* value and the edges (lines) connecting the vertices reflect DE target genes that were common between the GO terms.

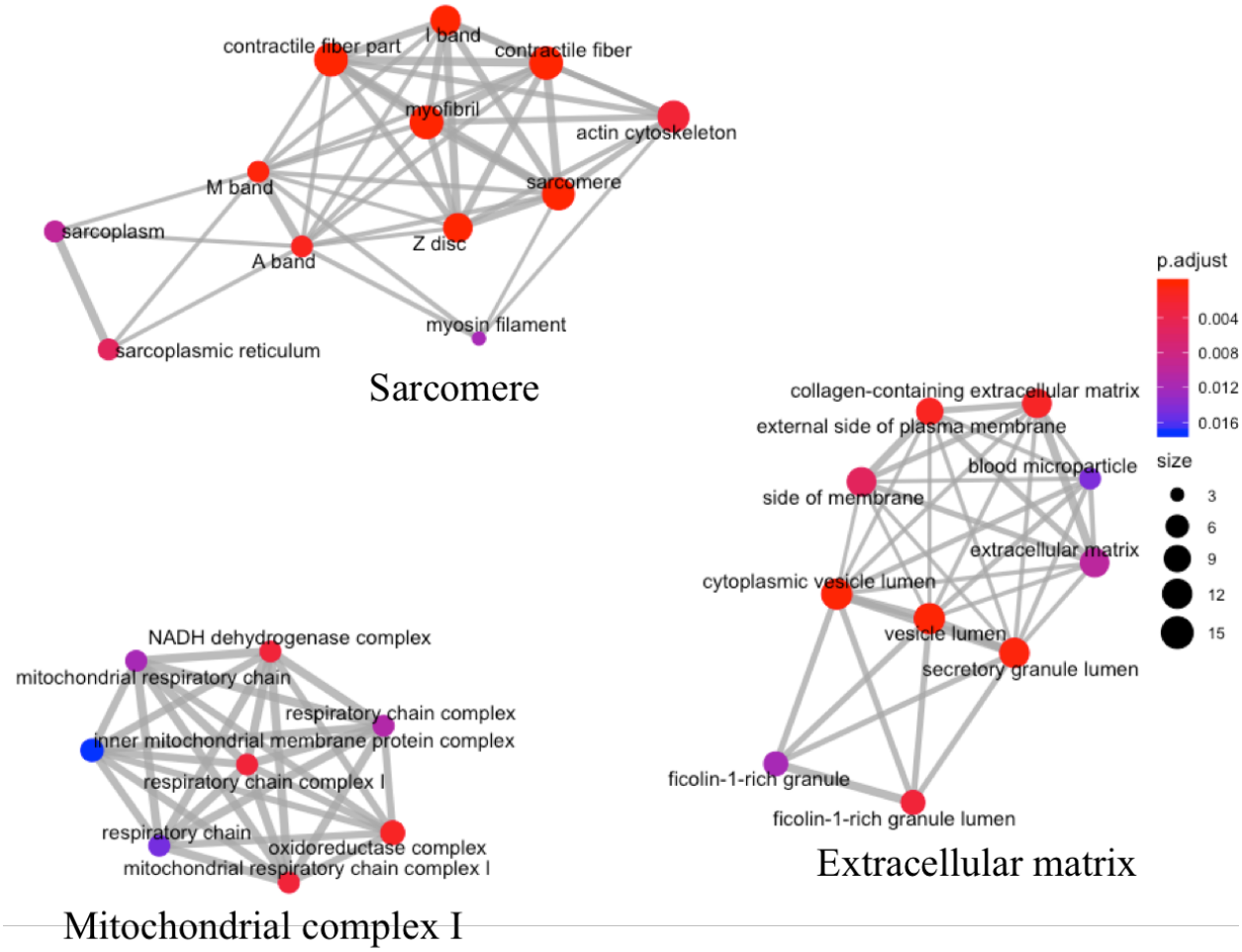
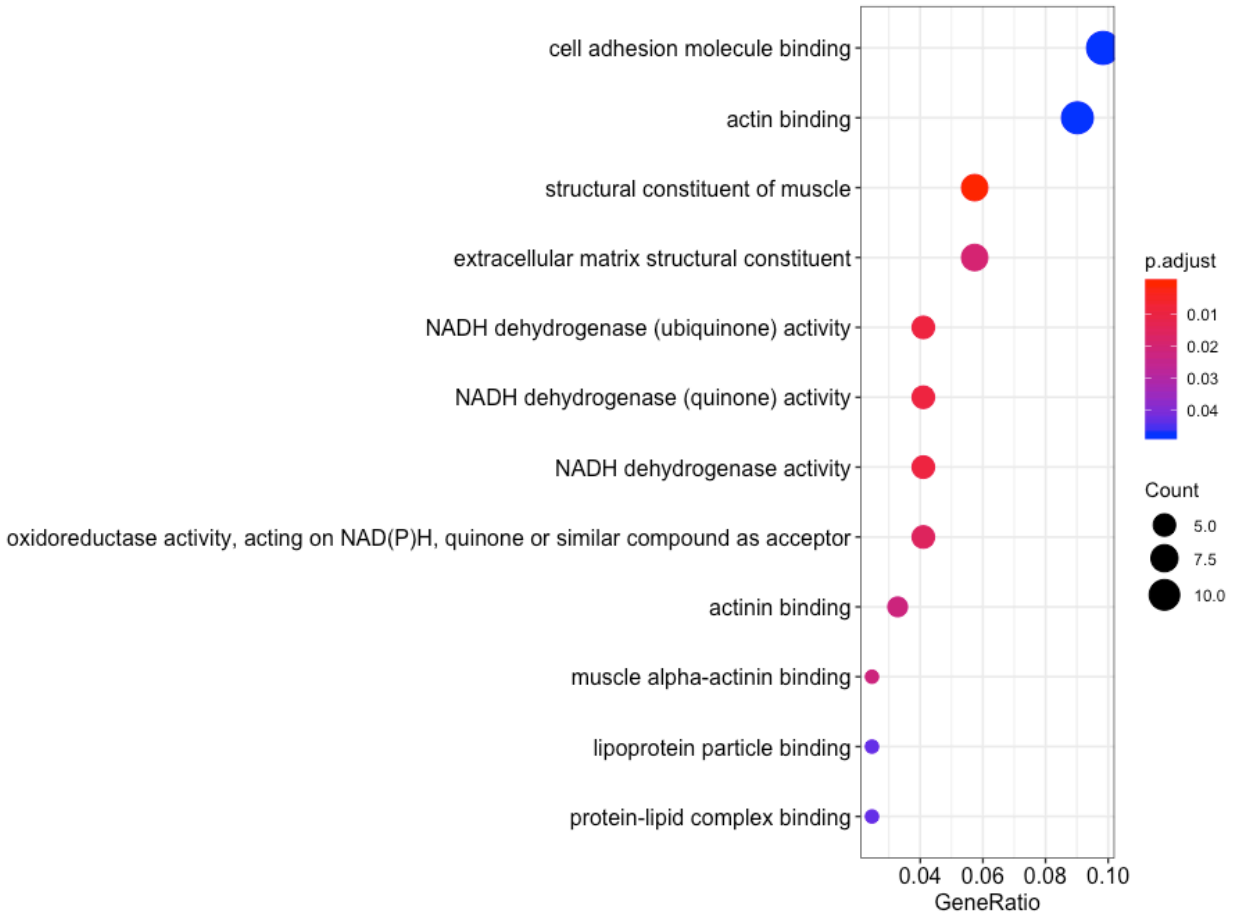


Figure 4.10: The GO molecular function terms for the merged DE gene transcripts and DE proteins. The color of the dots reflects the adjusted *P* value, the size of the dot reflects how many DE genes/proteins were included in that term, and the gene ratio indicates the number of DE genes/proteins in that term divided by the total significantly DE merged data count.



Transcription factor motif analysis- GO analysis

For STAT3 targets, there were 19 enriched GO biological function terms, 9 enriched GO cellular location terms, and 2 enriched GO molecular function terms. The 4 GO biological function terms with the lowest adjusted P values were response to ketone (GO:1901654, $P_{adj} = 0.002$), response to oxygen levels (GO:0070482, $P_{adj} = 0.02$), apoptotic signaling pathway (GO:0097190, $P_{adj} = 0.02$) and response to steroid hormone (GO:0048545, $P_{adj} = 0.02$) (Figure 4.11-A). The GO cellular locations terms included sarcomere (GO:0030017, $P_{adj} = 0.006$) and contractile fiber part (GO:0044449, $P_{adj} = 0.006$). Interestingly, there was specific enrichment of the Z disc (GO:0030018, $P_{adj} = 0.01$) and I band (GO:0031674, $P_{adj} = 0.01$) within the sarcomere (Figure 4.11-B). The 2 GO molecular function terms were tubulin binding (GO:0015631, $P_{adj} = 0.03$) and proteoglycan binding (GO:0043394, $P_{adj} = 0.03$).

For YBX1 targets, there were 11 enriched GO biological process terms. Similar to STAT3, there was also enrichment in response to steroid hormone (GO:0048545, $P_{adj} = 0.003$) and response to oxygen levels (GO:0070482, $P_{adj} = 0.01$) (Figure 4.12).

For ATF3 targets, there were 14 enriched biological process terms. Five of these terms were associated with amino acid or nutrient starvation– cellular response to amino acid starvation (GO:0034198, $P_{adj} = 0.006$), response to amino acid starvation (GO:1990928, $P_{adj} = 0.006$), cellular response to starvation (GO:0009267, $P_{adj} = 0.01$), response to starvation (GO:0042594, $P_{adj} = 0.01$), and cellular response to nutrient levels (GO:0031669, $P_{adj} = 0.02$) (Figure 4.13).

Figure 4.11: A.) Enriched GO biological terms for STAT3 DE target genes. The size of the vertex indicates the number of DE target genes in that term. The color of the vertex indicates the adjusted *P* value and the edges connecting the vertices represents DE target genes that were common between the GO terms. B.) Enriched GO cellular location terms for STAT3 DE target genes.

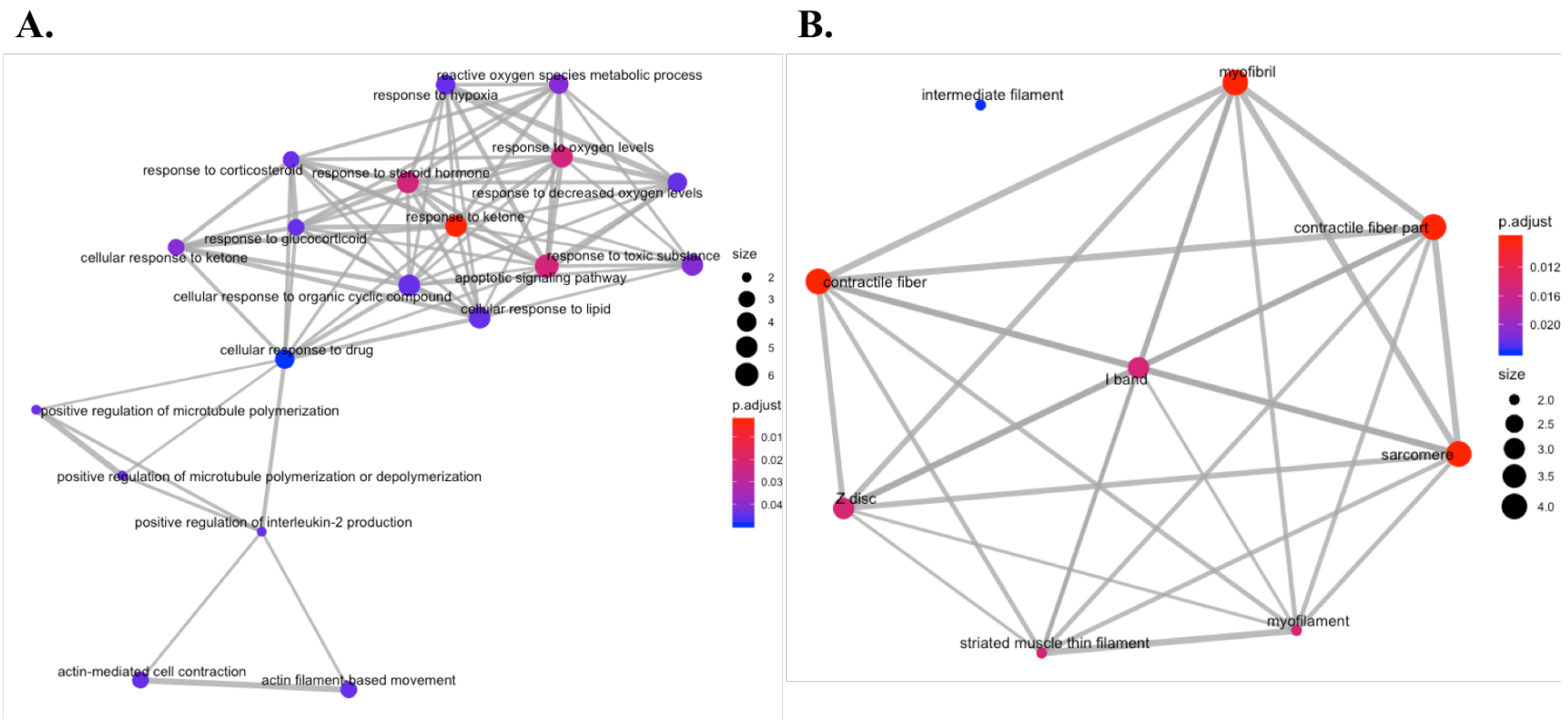


Figure 4.12: Enriched GO biological terms for YBX1 DE target genes. The size of the vertex indicates the number of DE target genes in that term. The color of the vertex indicates the adjusted *P* value and the edges connecting the vertices represents DE target genes that were common between the GO terms.

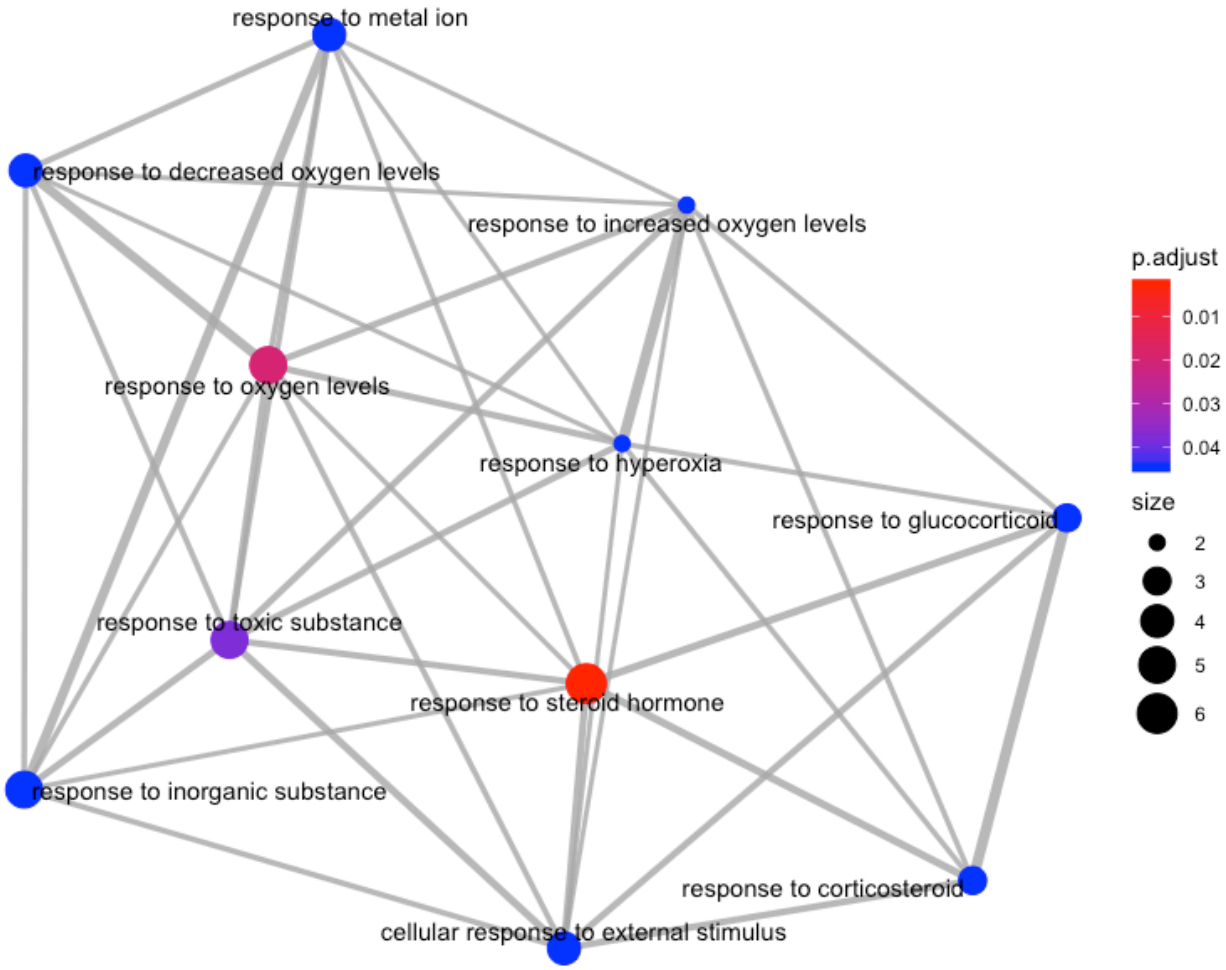
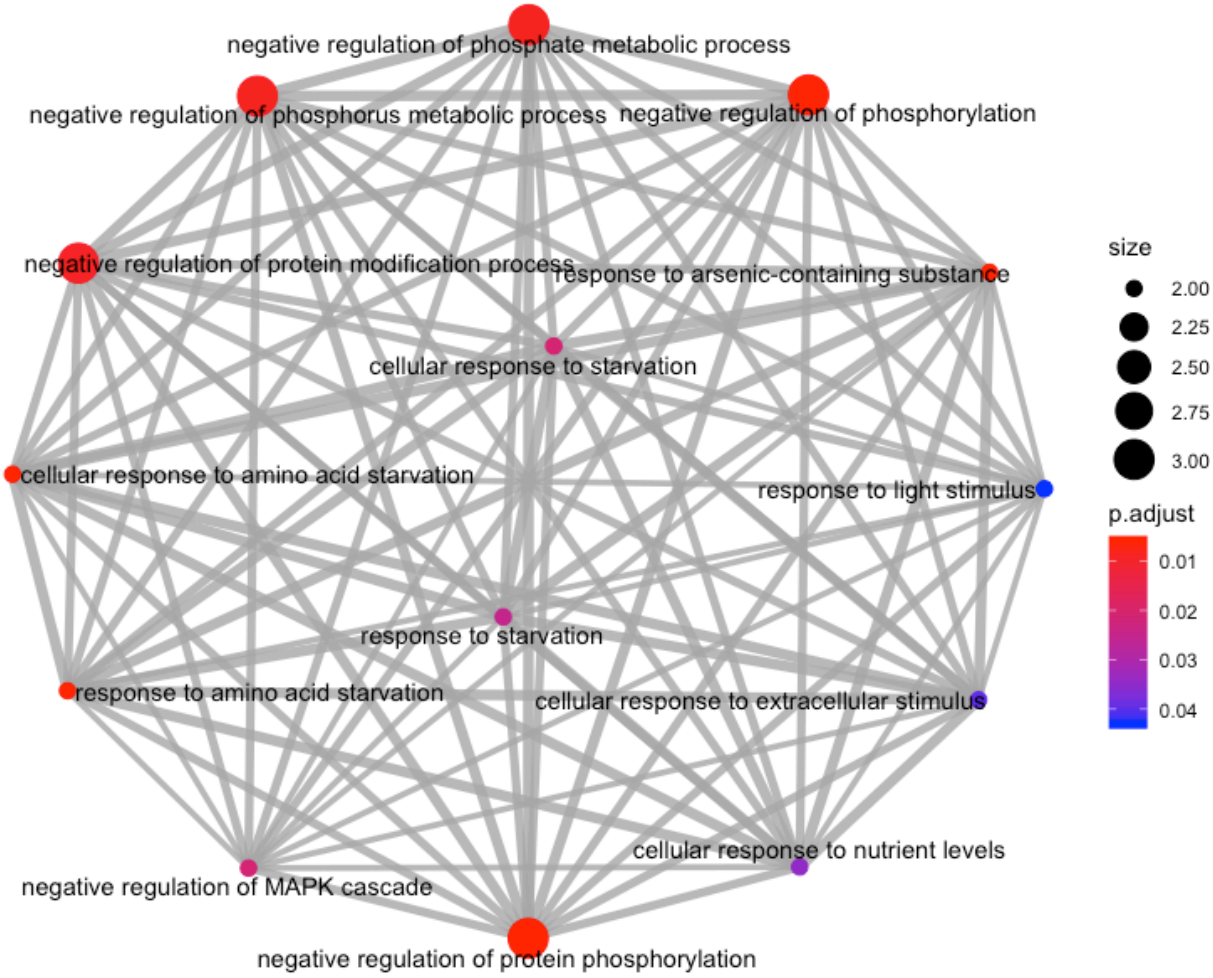


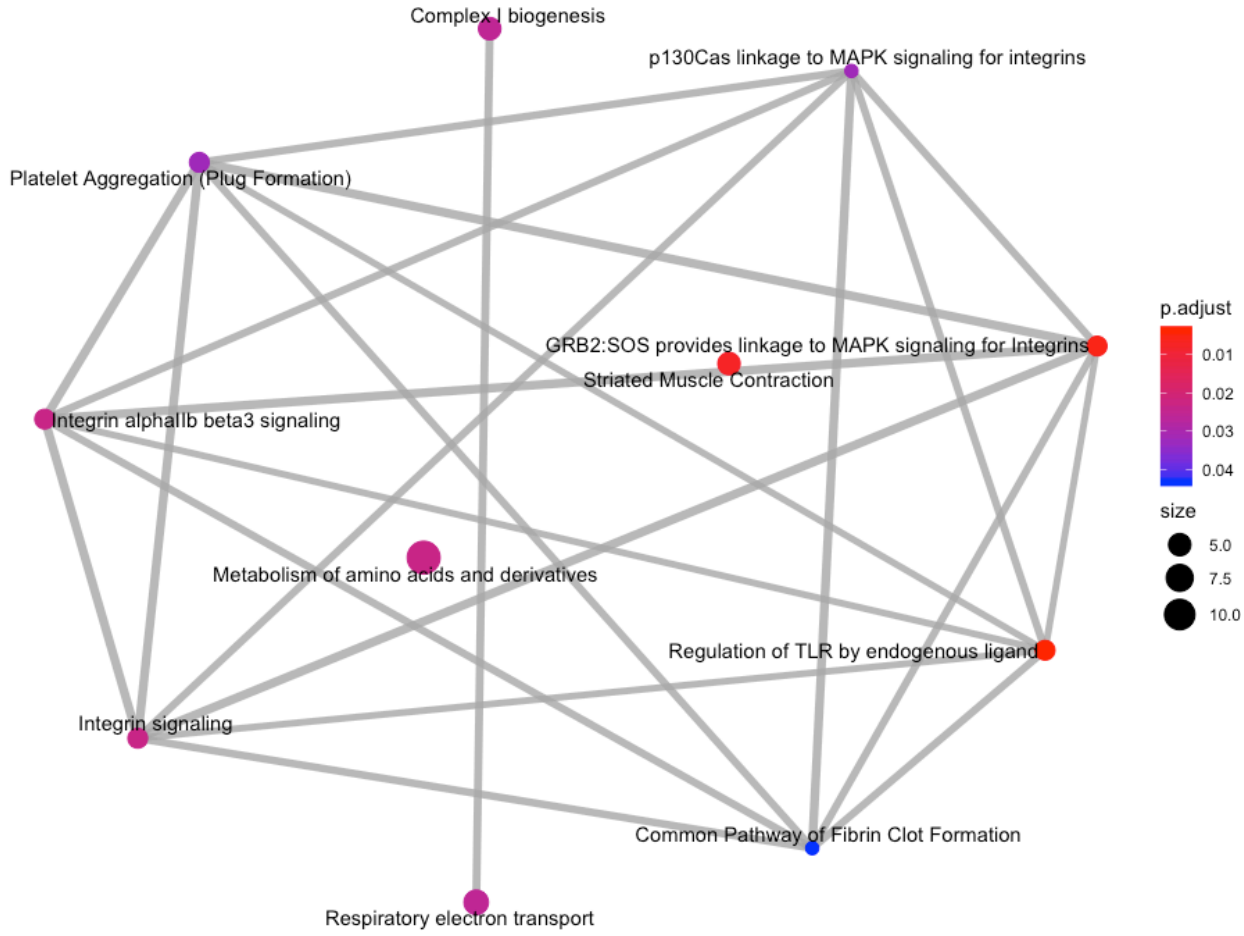
Figure 4.13: Enriched GO biological terms for ATF3 DE target genes. The size of the vertex indicates the number of DE target genes in that term. The color of the vertex indicates the adjusted *P* value and the edges connecting the vertices represents DE target genes that were common between the GO terms.



Reactome pathway analysis

Reactome pathway analysis of merged data revealed 11 significantly enriched pathways. The pathway with the most DE proteins and DE gene transcripts was metabolism of amino acids and derivatives (R-HSA-71291, q = 0.02). Similar to the GO analysis, there was overlap between pathways and DE gene IDs (Figure 4.14), but metabolism of amino acids and derivatives (R-HSA-71291) and striated muscle contraction (R-HSA-390522, q = 0.07) were pathways that had no overlap. The remaining pathways were integrin signaling (R-HSA-9006921) with the largest amount of overlap in related pathways, and complex I biogenesis (R-HSA-6799198) which shared DE genes with respiratory chain electron transport (R-HSA-611105).

Figure 4.14: The enriched reactome pathways of the merged DE gene transcripts and proteins. The size of the vertex indicates the number of DE target genes in that term. The color of the vertex indicates the adjusted *P* value and the edges connecting the vertices represents DE target genes that were common between the GO terms.



DISCUSSION

The present study used an integrated multi-omic approach to identify differentially expressed genes and proteins and enriched pathways that provide insights into the etiopathology of MFM in WB horses. Three distinct enriched cellular locations were identified by our amalgamated analysis. DE genes and DE proteins in MFM WB were located in the sarcomere and Z-disc, the mitochondrial electron transport chain, and the extracellular matrix. A transcription factor motif analysis further identified 3 enriched transcription factors – STAT3, ATF3, YBX1 – that had DE targets in the transcriptomic or proteomic analyses. The DE genes and DE proteins regulated by the three enriched TF were linked to the MFM phenotype through impacts on muscle mass (amino acid metabolism and response to steroid hormones) and oxidative stress (oxygen/oxidative responses).

The Z-disc and the I band were identified as enriched cellular locations with many of the DE genes and DE proteins residing in these sarcomere regions. The Z-disc, located on either end of the sarcomere, is comprised of > 200 proteins that provide mechanical stability to the sarcomere (61,62). The Z-disc is the structure in the sarcomere that is most vulnerable to exercise-induced muscle damage (63). Z-disc streaming similar to that seen in eccentric exercise models was clearly apparent in the electron micrographs of the MFM WB in our study prior to performing any strenuous exercise. Thus, our ultrastructural results suggest that weakness in the Z-disc structure is one component of the etiolopathology of WB MFM. Breaks in the Z-disc and streaming of Z-disc-material are hallmark histologic features of MFM in both humans and horses (30,34,64–66)

The Z-disc also plays an essential role in mechanosensing and mechanotransduction in which Z-disc signaling proteins translocate to the nucleus when activated resulting in altered gene expression (62,67,68). MFM WB had increased expression of 4 proteins that bind to the alpha-actinin back bone of the Z-disc and act as mechanotransducers; alpha-actinin: CSRP3 (also known as MLP) (log_2 FC 0.74), PDLIM3 (also known as ALP) (log_2 FC 0.34), SYNM (log_2 FC 0.28), and SYNOP2 (also known as myopodin) (log_2 FC 0.34) (4,69–75). Additionally, the enriched TF YBX1 complexes with a mechanosensor, Ankrd2, at the Z- I band interface (76–78) and translocates to the nucleus to influence gene transcription (62,67,68). In regulating nucleo-cytoplasmic functions, these Z-disc associated proteins may serve to signal or act as transcription factors to achieve appropriate gene regulation. Thus, genes encoding these 4 proteins represent exciting new candidate genes for MFM in WB. Notably, CSRP3 had the greatest log_2 FC in the proteome of MFM WB and mutations in CSRP3 have been associated with myofibrillar disarray and hypertrophic cardiomyopathy in humans making this a top candidate gene (79–83).

Mechanosignaling normally results in mechanical and physiological exercise adaptations that protect the sarcomere from damage in subsequent bouts of exercise (84). In the presence of pathogenic genetic variants in CSRP3, perturbations of signaling functions have been suggested to contribute to cardiomyopathies (85). It is possible therefore, that the Z-disc alterations, exercise-associated stiffness and reluctance to exercise exhibited by MFM horses arises from maladapted Z-disc-signaling events that results in Z-discs that are unable to withstand the forces that arise with normal exercise. Z-disc streaming, a decrease in force generation, and delayed onset of muscle soreness result from eccentric exercise in humans and could be experienced by MFM horses during regular exercise sessions.

Interestingly, as part of its mechanosensing role, CSRP3 interacts with the signal transducer calcineurin (69) and calcineurin is known to mediate mitochondrial fission (86,87). Mice with calcineurin knocked out in skeletal muscle exhibit elongated mitochondria, increased respiratory chain activity and diminished exercise performance (87). Thus, it is possible that altered CSRP3 and Z-disc signal transduction could result in the altered mitochondrial membrane morphology seen in the electron micrographs of MFM WB. Interestingly, another key cellular location that was identified in the amalgamated omic analysis of MFM WB was complex I (NADH: Ubiquinone oxidoreductase) of the mitochondrial electron transport chain. WB horses with MFM exhibited altered expression of 17 mitochondrial proteins and decreased expression of 4 of the 42 complex I subunits detected compared to non-MFM WB. Arabian horses with MFM and human cases of MFM also have decreased expression of complex I subunits (31,88,89). Complex I dysfunction is often observed in myopathies (90,91) and other diseases involving post-mitotic tissue such as Parkinson's and Alzheimers, involving post mitotic tissue (92,93).

Mitochondrial complex I generates most of the cell's reactive oxygen species (ROS) in the form of superoxide anions, particularly during exercise (94,95). In MFM WB, we found reactome enrichment and enriched GO cellular terms involving complex I biogenesis (R-HSA-6799198), oxidoreductase activity (GO:0016655) and response to reactive oxygen species (GO:0006979, GO:0042542, GO:2000379, GO:0072593). Low levels of oxidative stress promote cell signaling, training adaptations and protein turnover within the cell (96,97), but pathologic amounts of ROS result in protein alterations, aggregation, and impaired function which could further exacerbate sarcomere disarray in MFM (98,99). Enhanced generation of ROS via complex I and deficiencies in cysteine-based antioxidants have been suggested to be important in the etiology

of MFM in Arabian horses (31). We found low expression of the complex I subunit NDUFA13 in MFM WB and this is known to activate the enriched TF STAT3 which partially blocks complex I to decrease ROS production (100,101). The TFs enriched in WB MFM, STAT3, YBX1, and ATF3, are also activated by ischemia and oxidative stress which would promote expression of antioxidant and anti-apoptotic genes (102–113). Provision of antioxidants such as N-acetyl cysteine have been shown to prevent cellular oxidative stress-mediated apoptosis and prevent desmin aggregation in mouse myoblast cell culture models of desminopathies (96,98,114,115). Thus, similar to other studies of MFM, the results of the present study support a role for complex I and oxidative stress in the pathogenesis of MFM in WB horses and suggest potential antioxidant therapeutic targets (98,114,115).

Several DE genes and DE proteins could have contributed to oxidative stress in MFM WB through an imbalance between ROS generated and counteracting muscle antioxidants. The antioxidant enzyme CAT (catalase) had decreased expression in MFM WB, and CHAC1 (gamma-glutamylcyclotransferase) had markedly increased expression in MFM WB (↑4.8 log2FC). CHAC1 acts to degrade the ubiquitous antioxidant glutathione as a proapoptotic participant (116). Low levels of glutathione could readily have contributed to oxidative stress, and CHAC1 therefore represents a further candidate gene for follow up in MFM WB.

Excessive amounts of oxidative stress trigger apoptotic signaling cascades (96,97,117,118). Apoptosis is necessary for the survival of most mitotic tissues and is a regulatory mechanism to eliminate pathogens or neoplastic cells (118). Post-mitotic cells such as muscle and neural tissue, however, cannot divide and have limited mechanisms to repair (93,119). The repair process in

skeletal muscle involves autophagy and satellite cell mediated repair (119). As multi-nucleated cells, muscle can selectively degrade nuclei and replace them with a satellite cell nucleus, rather than apoptose the entire fiber (119). Unfortunately, degenerative myopathies deplete their satellite cell reserve (120), resulting in an apoptotic turning point that leads to cell death, fibrosis and fatty infiltration (121–123). Since increased CHAC1 expression promotes apoptosis (124), and it was upregulated in MFM WB, it is possible that the long-term structural and oxidative damage in MFM WB myofibers initiates a proapoptotic cascade leading to fiber atrophy and cell death.

The extracellular matrix was the third cellular location that was enriched in the amalgamated analysis of MFM WB compared to non-MFM WB. The skeletal muscle extracellular matrix has a crucial role in mechanical force transduction, recruitment of satellite cells for repair, and muscle maintenance (125). The extracellular matrix is connected to the sarcolemma through dystroglycan complexes and integrin mediated adhesion, and these structures are in turn connected to the Z-disc by intermediate filaments and the cytoskeleton (126). Several of the DE genes and DE proteins localized to the extracellular matrix (GO:0009897) in MFM WB, including increased expression of three fibrinogen subunits, thrombospondin and neural cell adhesion protein. Fibrinogen is a driver of dystrophic skeletal muscle fibrosis, which is a key feature of limb-girdle muscular dystrophies that have clinical features incommon with MFM (60). Other extracellular matrix DE genes and DE proteins in MFM WB were involved in integrin signaling pathways (R-HSA-9006921) (127,128). Upregulation of α7-integrin protein is believed to be a mechanism to reinforce muscle load-bearing structures and resist injury with repeated bouts of exercise (126). Thus, upregulation of the extracellular matrix and proteins

related to integrin signaling pathways could reflect a stabilizing adaptation in MFM WB muscle to prevent further sarcomere instability and damage.

Integrin signaling can be initiated by transduction of mechanical forces at the Z-disc to the sarcolemma through intermediate filaments such as desmin and synemin (61,62,129–131). Intermediate filaments act to stabilize sarcomeres and complex with the sarcolemma, mitochondria, and other organelles (132,133). Mutations in desmin are a well-recognized cause of MFM in humans (7,8,134), and desmin is found in almost all MFM aggregates (1,2,135–137). Despite using desmin aggregation as a key diagnostic feature for MFM WB, we did not find the protein or gene to be DE. Human MFM aggregates aren't reported to be fiber type specific, but MFM WB only appear to have desmin aggregation in a small number of type 2A and some type 2X fibers (30). Coding sequence variants in desmin were not identified in WB or Arabian MFM horses using RNA sequencing (18, Chapter 3). Desmin aggregation in WB MFM could be a secondary response to stabilize the Z-disc because of instability created by another primary protein aberration. Proposed mechanisms for protein aggregation in MFM include dysfunctional ubiquitin-proteasome systems, aggresome formation, mutated ubiquitin, abnormal p62 expression, chaperone inhibited aggresome formation and oxidative stress (98,138–143).

Interestingly, MFM WB did have increased expression of another intermediate filament called synemin (SYNM) ($\log_2$ FC = 0.28). SYNM binds to both alpha-actinin and desmin at the Z-disc, mechanically stabilizing the sarcomere and distributing myofibrillar forces to the extracellular matrix (129,130). SYNM has been found to abnormally accumulate in MFM and other

myopathies (144) and is a candidate intermediate filament for further diagnostic and genetic investigation in WB MFM.

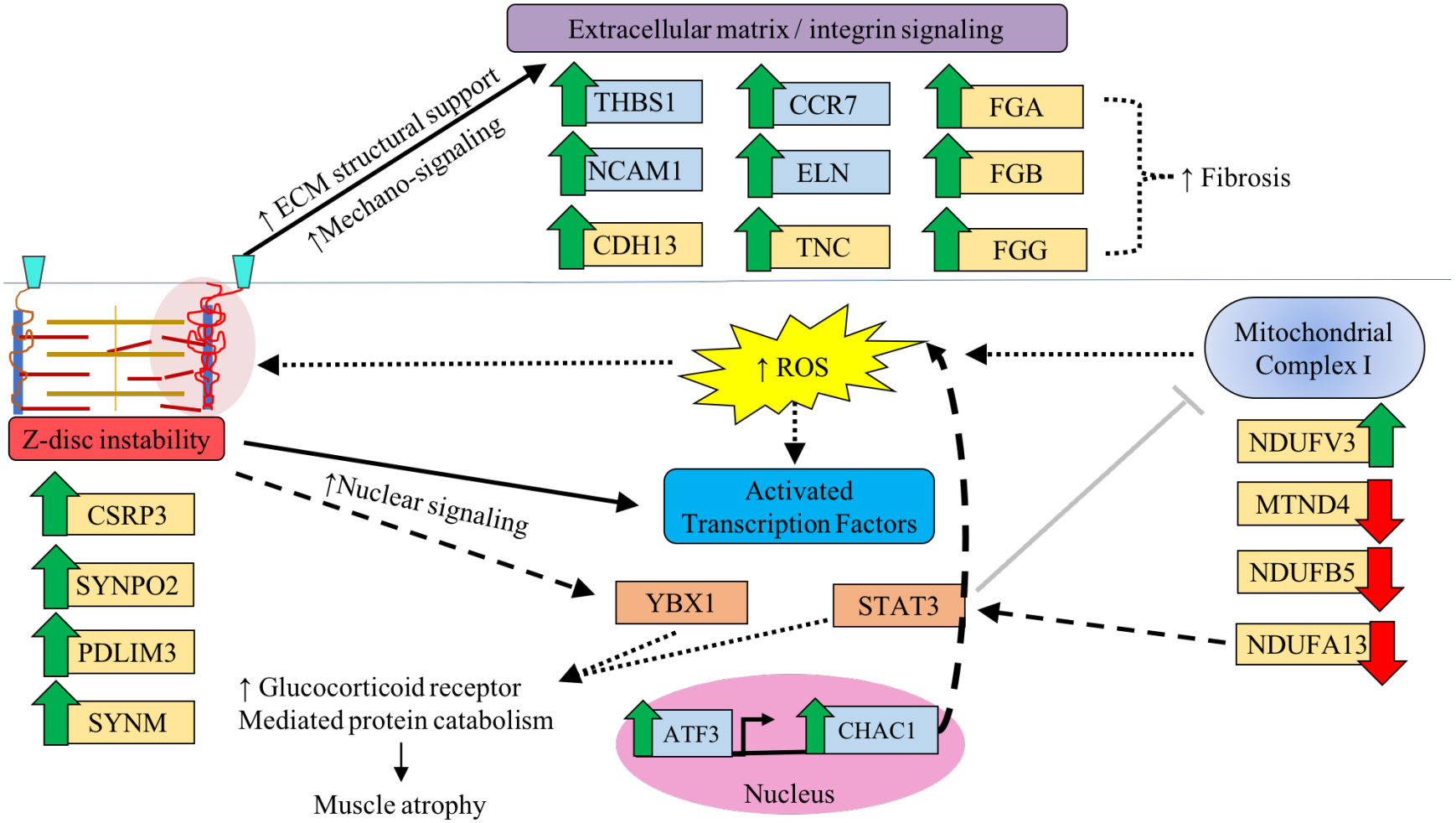
Lastly, muscle mass is often reduced in WB horses with MFM. The enriched GO terms in MFM WB that related to muscle atrophy were response to glucocorticoid, response to steroid hormone and cellular response to amino acid starvation. These coincided with the enriched TFs STAT3, a glucocorticoid receptor coactivator (145,146) and YBX1 a functional component of glucocorticoid receptor mediated mRNA decay (147). Transient increases in STAT3 activation promote exercise adaptations and regulate muscle mass and repair (148,149). However, increased activation, found in Duchenne muscular dystrophy (150,151) and congenital myotonic dystrophy (152), is linked to muscle wasting and atrophy(153,154). Thus, our results suggest that muscle atrophy in MFM WB is likely related to activation of specific TFs resulting in glucocorticoid induced protein degradation.

CONCLUSION

From the present results, it is clear that MFM WB have cellular enrichment of pathways that implicate the Z-disc, complex I and the extracellular matrix in the etiopathology of MFM. Furthermore, the DE of genes and proteins in these locations suggest that MFM WB have increased expression of Z-disc mechanosensing and extracellular matrix proteins potentially as a response to sarcomere instability. The decreased expression of complex I proteins in the electric transport chain could be a protective mechanism to decrease ROS production arising from STAT3 activation. Increased CHAC1 expression as a potential proapoptotic response in MFM WB suggests that the redox homeostasis may be compromised through excessive degradation of glutathione, thereby promoting increased oxidative stress.

Considering the present results, we further hypothesize that instability in the Z-disc of MFM WB is caused by maladaptive mechanosignaling and that the increased expression of extracellular matrix proteins and genes is a compensatory mechanism to provide myofiber stability. We further hypothesize that perturbed redox homeostasis is leads to aggregation of Z-disc proteins, promoting instability and impairing mechanosignaling (Figure 4.15). While functional studies are needed to validate the newly proposed hypothesis, the multi-omic approach to this study has successfully provided previously unknown information about MFM in WB and identified new candidate genes and potential biomarkers for this disease.

Figure 4.15: Diagram of the proposed etiopathology of WB MFM includes perturbed redox homeostasis leading to protein aggregation of Z-disc material, further fueling instability and impaired mechanosignaling.



APPENDIX

Table A.4.1: This table includes the 4 genes (*CHAC1, NA, ADAMDEC1, HBB*) used for transcriptomic validation through RT-qPCR and their forward and reverse primers and primer characteristics.

Gene	Forward	Reverse	Product size
CHAC1	AGCCCTGTGGATTTTCGGAT	CAAGTGCAGCCCTCATGATCT	186
NA: chr21:44470419	CCGTAGTTAACGTCTCGGCA	TTGACCTACCAGCTCCCTCA	172
ADAMDEC1	GGGTTGAGGGGCTACTTCAC	TTGCTGTGGGCTATTGAGGG	198
HBB	CCAATCCTGGTGCTGTGATG	CCCAGGAGCCTGAAGTTCTCA	175

Figure A.4.1: The $2^{-\Delta\Delta CT}$ values from RT-qPCR validation for *CHAC1, NA* (chr21:44470419-44471212), *ADAMDEC1, HBB* for MFM WB vs. non-MFM WB.

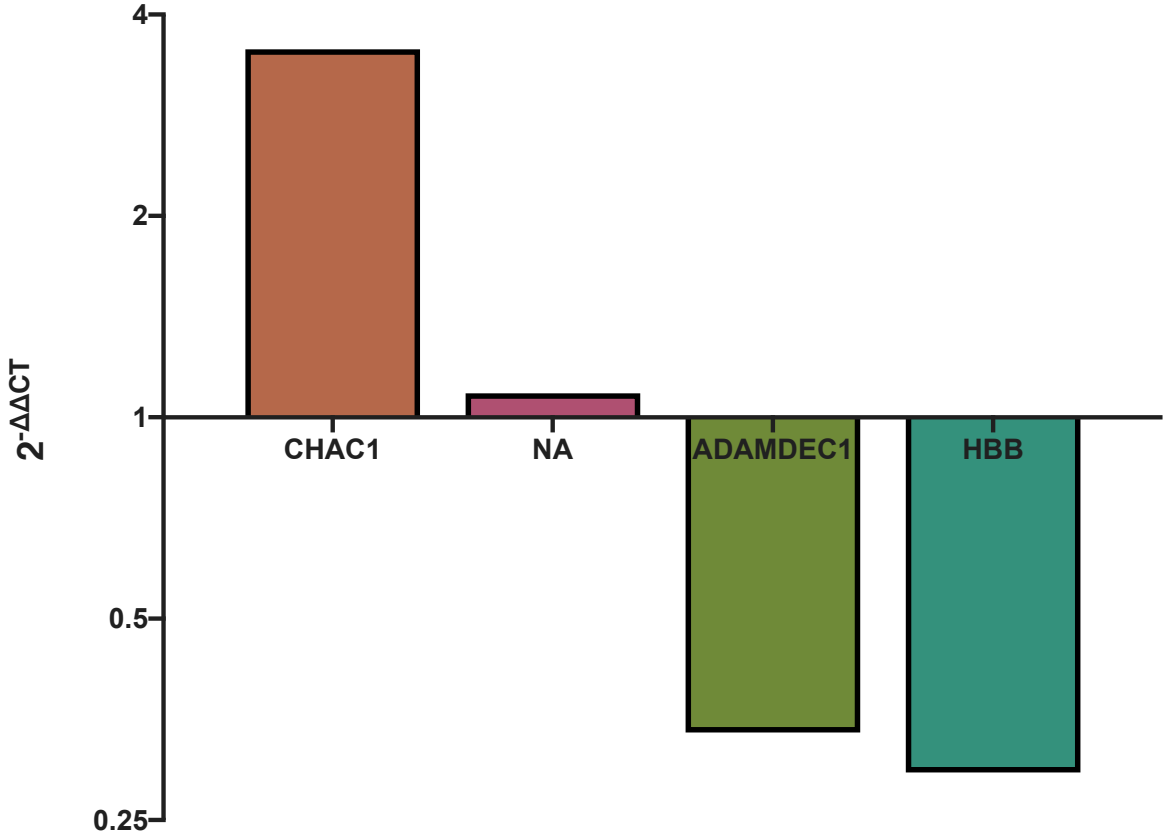


Figure A.4.2: A) This sequence logo pertains to the transcription factor binding motif that annotates to STAT3. The base pair location is mapped on the x-axis and the conservation between species is mapped with a bit score, taking into account expression frequency at that nucleotide position, on the y-axis. The size of the nucleotide represents its conservation. STAT3 has 6 of the 15 nucleotides that have high bit scores B) The sequence logo for YBX1 shows 5 of 11 nucleotides with high bit scores. C) The sequence logo for ATF3has about 8 of 14 nucleotides with a bit score of 1 or above.

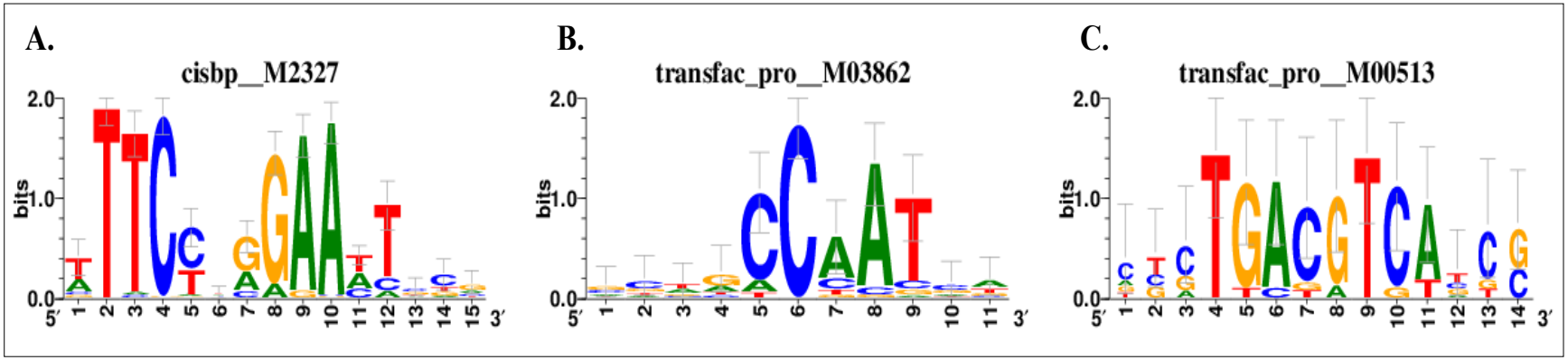
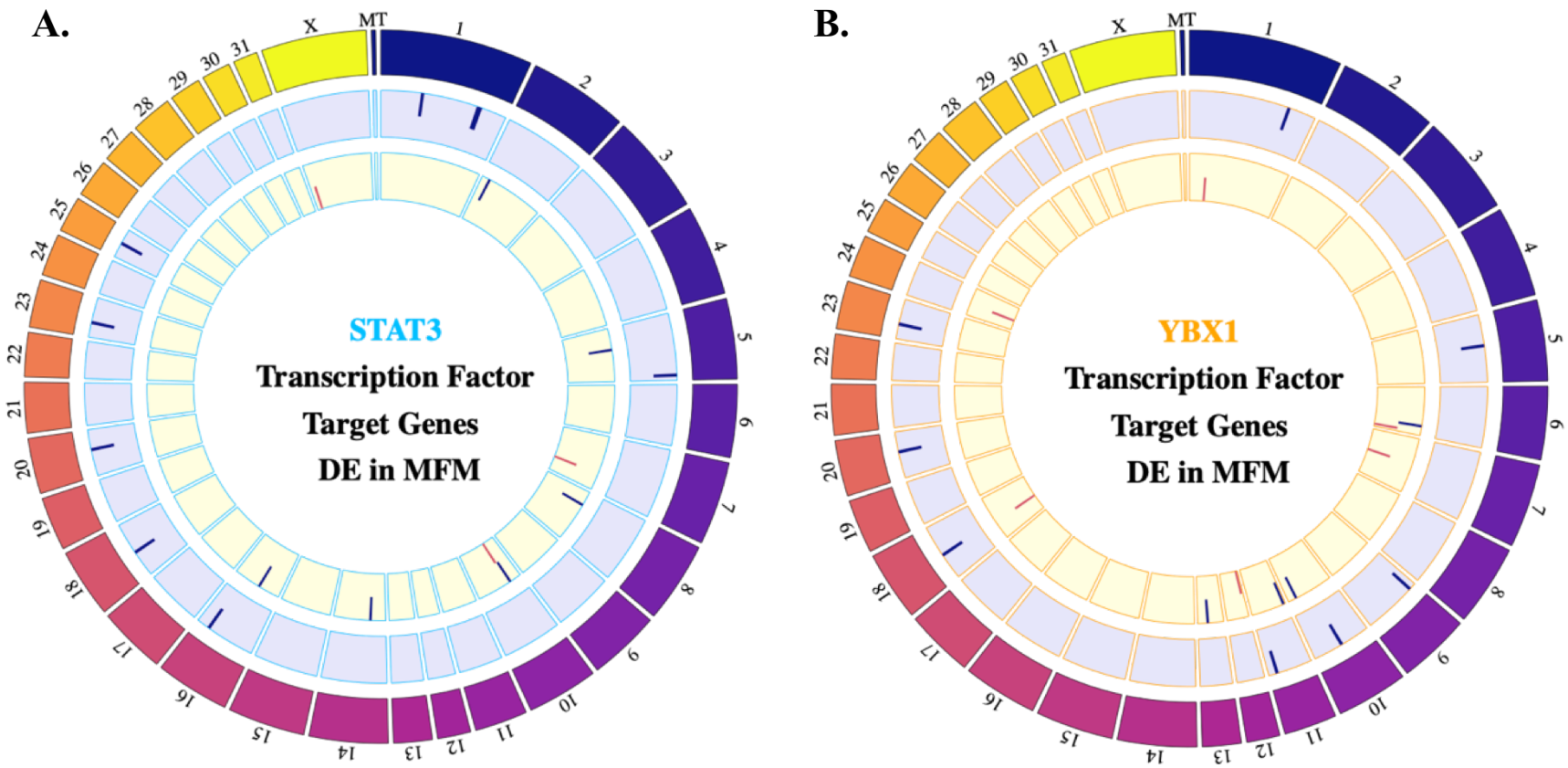


Figure A.4.3: These circo plots depict the chromosomal location and direction of expression of DE target genes for STAT3 (A.) and YBX1(B.). The outer most ring shows the equine chromosomal locations, the middle ring shows target gene that were DE in the transcriptomic data the middle ring shows target genes that were DE as proteins. The direction of expression is marked as a blue line for increased expression and a red line for decreased expression. STAT3 and YBX1 have DE target genes on different chromosomal locations.



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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Background

Metabolic myopathies, such as polysaccharide storage myopathy (PSSM1), respond well to specific diet and exercise changes in both humans and horses (1–6). Studies found that horses with polysaccharide storage myopathy improved greatly with a low starch and fat supplemented diet. However, in 2008 a non-synonymous point mutation was identified to cause a gain of function in the enzyme glycogen synthase. Further testing showed that not all PSSM horses had the mutation. This led to the creation of two categories: horses with type 1 (PSSM1) have the mutation and horse with type 2 (PSSM2) do not. Recently, one study found that Warmblood horses (WB) with PSSM2 do not actually have elevations in skeletal muscle glycogen (7). Those findings suggest that PSSM2 in WB may need alternate management regimes, rather than the one designed for excessive muscle glycogen concentrations.

It was later discovered that some of the PSSM2 WB actually had aggregation of a cytoskeletal protein called desmin. This finding was consistent with a disease in humans called Myofibrillar myopathy (MFM). MFM in WB is a late onset chronic myopathy, previously thought to be PSSM2 (8,9). Only 18% of WB diagnosed with myopathies have PSSM1 (10). Therefore, MFM and PSSM2 are the most commonly diagnosed myopathies in WB horses, yet their etiologies remain unknown. This dissertation evaluated the responses of PSSM2/MFM WB to the recommended PSSM diet and identified candidate genes, proteins, and pathways that appear to have key roles in the aberrant cellular responses and etiopathology of MFM in WB.

PSSM2 & MFM WB: symptoms, responses to PSSM diet, and muscle glycogens

The first study of this dissertation hypothesized that PSSM2 WB have lower skeletal muscle glycogen concentrations compared to PSSM1 WB and, therefore, a significant improvement in clinical signs of PSSM2 WB will not occur with the same low starch and fat supplemented diet shown to benefit PSSM1 horses. Our objectives were to define owner reported symptoms of PSSM2 in WB, determine the responses of PSSM2 WB to the PSSM diet and exercise regime, and determine if PSSM2 WB had changes in skeletal muscle glycogen concentrations.

The first and second objectives were accomplished through a retrospective questionnaire answered by 42 PSSM2 WB horse owners or trainers. We found the most reported PSSM2 symptoms were a reluctance to go forward, decline in athletic performance, and a reluctance to collect –or engage hind end and core muscles. PSSM1 usually presents with exertional rhabdomyolysis and is worse with exercise following prolonged times of rest (11,12). However, the results of PSSM2 and MFM WB suggest that, unlike PSSM1, these horses may be experiencing a chronic myopathy affected by muscle weakness or myalgia that impacts the horse's athletic performance.

After implementing a high fat and low starch diet, 100% of PSSM1 Quarter Horse breeds had a decrease in the frequency of ER with 71% having no further episodes of rhabdomyolysis (4). Interestingly, PSSM2 WB had improvements in performance and episodes of exertional rhabdomyolysis after implementing the recommended PSSM diet. Unfortunately, 53% of the horses were still not advancing in their training and 45% of the horses didn't meet their owner's expectations for athletic potential. These findings were intriguing because only a few horses had

episodes of rhabdomyolysis and responded in a similar fashion to the diet as PSSM1 horses – despite testing negative for the PSSM1 mutation. Although WB has a lower reported "decline in performance" after the diet, it isn't clear if this due to the owner seeking a nutritionally balanced diet opposed to what was previously fed, or if this is a direct result of decreasing non-structural carbohydrates and supplementing with a fat source for energy. Specific caloric and nutritional information about the diets was not obtained.

Of particular note, eight (20%) of the horses were euthanized with five horses suffering from what the owner's reported as extreme and unmanageable PSSM2 symptoms. One additional horse was donated to our Neuromuscular Laboratory because his owner felt the contribution to research was better than electing euthanasia. These results clearly indicated a need to investigate PSSM2 and MFM further in WB in pursuit of providing better diagnostics, treatment, and prognostic information for referring veterinarians and horse owners.

The last objective of this study was the biochemical measurement of muscle glycogen concentrations in samples from horses evaluated in the retrospective questionnaire. The results confirmed that, on average, PSSM2 WB do not have elevated glycogen concentrations in their skeletal muscle like their PSSM1 counterparts. However, we did observe a wide variation in muscle glycogen concentrations and found that horses above the median glycogen concentration in PSSM2 WB, had a higher reported incidence of "decline in performance". The significance of this finding is unknown and questions the role glycogen accumulation may play in the clinical presentation of PSSM2/MFM WB. After retrospective staining for desmin, we found four of the PSSM2 had desmin aggregation in selective fibers – the hallmark feature of MFM (9,13–16).

The ectopic aggregation of this Z-disc protein led to further examination of WB MFM skeletal muscle with electron microscopy. The micrographs suggest that the appearance of amylase sensitive Periodic Acid Schiff's stain (PAS) positive aggregates under light microscopy is likely due to the pooling of glycogen between broken myofibrils secondary to sarcomere disruption and myofibrillar disarray (9). The subset of MFM WB that were diagnosed with PSSM2 suggest that the former diagnosis of PSSM2 may encompass multiple disease phenotypes and/or contain a disease with varying clinical severity. It is possible that desmin aggregation is only seen in later stages of the disease and that there could be better diagnostic markers to detect MFM. The aggregation of desmin leading to the MFM diagnosis launched our investigation to elucidate the etiopathology of this myopathy in WB horses.

MFM candidate genes

The second study of this dissertation hypothesized that genes associated with human MFM and MFM-like protein aggregate myopathies are candidates for WB MFM; therefore, MFM WB will have differential gene expression and/or an associated coding variant in one or more candidate MFM genes. To accomplish this, we employed a candidate gene approach using RNA sequencing data from the skeletal muscle of eight MFM and eight non-MFM WB. There are currently 8 genes associated with MFM types 1-8 and there are an additional 8 genes that cause protein aggregate myopathies with MFM-like features (17–19). Reflecting the heterogeneous presentations and causes of MFM in humans, the mode of inheritance also varies ranges from autosomal dominant, to autosomal recessive, to X-linked, to de novo mutations (17,20–25). With that being said, there are still approximately half of MFM cases in people which do not yet have a defined basis. Considering what is known from the literature about human MFM, we evaluated the 8 human MFM and 8 MFM-like genes as candidates for causing MFM in WB. The objectives of this dissertation were to examine the candidate genes for differential expression (DE), compare identified variant allele frequencies in MFM and non-MFM WB, and compare identified variant allele frequencies in other breeds using publicly available databases.

We accomplished the first two objectives by using mRNA sequencing from eight MFM WB and eight non-MFM WB. When comparing DE between MFM and non-MFM WB, we found no difference. Had we identified DE of a candidate gene, it may have indicated the presence of transcriptional regulation, upstream variants affecting promotor or enhancer regions, or nonsense mediated decay of transcripts. After calling variants in the 16 candidate genes, we identified a substantial number of variants that annotated as coding and intronic variants in the equine

genome. The presence of intronic variants was surprising because mRNA usually contains only coding, or exon, sequences. On gross evaluation of the genes in the Integrative Genomics Viewer (IGV), we did not detect any retained intronic regions in either the MFM or non-MFM WB. Therefore, we attribute the presence of intronic variants to the annotation of the current equine genome (EquCab 3.0). After statistically comparing the allele frequency (AF) of all the variants identified in MFM and non-MFM WB, we did not find any significant association with the MFM phenotype.

To complete the third objective of this study, we utilized publicly available data from both RNA sequencing and whole genome sequencing to compare the frequency of the variants we identified that resulted in non-synonymous amino acids and had a "moderate" or "severe" predicted SIFT impact score. We compared the missense variant AF between MFM WB, non-MFM WB, 33 non-phenotyped WB, 32 Thoroughbreds (TB), 80 Quarter Horses (QH), and 77 other breeds (OB). There were no differences in AF between MFM, non-MFM and non-phenotyped WB. However, there were significant differences comparing AF in different breeds. Interestingly, TB had the lowest variant AF when compared to other groups. This finding is important because the equine genome is primarily annotated to an inbred TB. This suggests that these variants are likely not associated with WB MFM, but instead the frequency of their expression is breed specific. Use of a breed specific reference, or well phenotyped controls, is needed when investigating potentially pathogenic variants.

From this study, it appears that the 16 candidate genes do not have any MFM WB associated variants that we could identify within the limitations of RNA sequencing and subject availability.

While our sequencing modality was adequate for detecting gene expression, it limited our ability to detect upstream variants due to the lack of 5' coverage in some genes. Additionally, RNA sequencing would not be able to identify whether allele specific expression or copy number variants were present. Our sequencing data was compiled with 150 nucleotide paired-end reads, which can make the assembly of a continuous genome sequence difficult and dependent on mapping parameters. Ideally whole genome sequencing or candidate gene sequencing with longer read fragments may strengthen our ability to detect variants. If we ascertain enough subjects to power a genome-wide association study, we may be able to detect genomic loci of interest.

In conclusion, this study did not identify any mRNA variants associated with MFM WB and found no significant DE in the 16 genes evaluated. Coupling our RNA sequencing with whole genome sequencing would be optimal to asses other genomic variations. The absence of candidate gene DE or association of variant AF with the MFM WB phenotype lead us to an alternate hypothesis: WB MFM does not have an etiology caused by coding variants in the 16 candidate MFM and MFM-like genes. This is not an unlikely notion since approximately 50% of all human MFM cases still don't have an identified cause.

Using Omics to investigate MFM in WB

In the third study of this dissertation, we hypothesized that MFM WB have specific proteins, genes, and transcription factors that drive the aberrant cellular response and have key roles in the etiopathology of MFM WB. To test this, we performed electron microscopy of three MFM WB to document ultrastructural abnormalities. We then utilized a multi-omic approach that integrated transcriptomic and proteomic analyses to elucidate the cellular impact of MFM by creating a unique expression profile that highlighted genes, proteins and enriched pathways (26,27)

The first objective was accomplished by obtaining electron micrographs of WB MFM gluteal muscle biopsies. Most regions were unremarkable, but affected areas revealed myofibrillar disarray, Z-disc degeneration, aggregation of Z-disc material, pooling of granulofilamentous material and abnormal mitochondria. These findings are similar to those reported in human MFM (28,29). These results suggest that the Z-disc is a key site of pathology in MFM WB and potentially linked to abnormal mitochondrial pathology.

The second objective of this study identified DE genes and DE proteins utilizing transcriptomic and proteomic analyses. Surprisingly, there were no DE genes that also had DE protein products in MFM WB. Factors such as mRNA decay, variation in protein half-life, changes in post-transcriptional machinery, and post-translational modifications all contribute to variation in mRNA and protein expression, especially in disease states (30,31). Additionally, non-model organisms, such as the horse, may have discrepancies in gene annotation and mapped protein libraries when compared to other species. To account for differences in gene and protein

expression, we used an amalgamated approach to combine DE gene and DE protein datasets for further pathway analyses similar to other studies (32–34).

Objectives three and four were accomplished by performing gene ontology and reactome pathway analyses on the transcriptomic, proteomic and merged DE genes and DE proteins. A significant finding in MFM WB was the enrichment of three distinct locations where the DE genes and proteins were overrepresented: 1) the Z-disc and I band of the sarcomere, 2) extracellular matrix, and 3) complex I of the mitochondrial respiratory chain. This finding is particularly intriguing because it corroborates the pathology seen in MFM WB electron micrographs: Z-disc streaming and aggregation and abnormally shaped and degenerate mitochondria.

Z-disc damage and aggregation of Z disc-material are hallmark features of MFM (9,13,15,25,35). The primary role of the Z-disc is to provide stability to myofibrils and maintain sarcomere organization. These roles are achieved by signaling proteins that translocate to the nucleus to alter gene expression when activated by Z-disc forces. Mechanosignaling usually results in exercise and training adaptations (36). MFM WB had increased DE of CSRP3, SYNPO2, PDLIM3, which are well-known mechanosignaling Z-disc proteins (37–44). Furthermore, CSRP3 mutations have been associated with myofibrillar disarray in cardiomyopathies (45–49). These findings suggest that MFM WB could have increased mechanosensing protein expression in an effort to stabilize compromised Z-discs. Alternatively, maladaptive mechanosensing may result in insufficient training responses and, therefore, result

in sarcomere disorganization and Z-disc compromise. The DE proteins and enrichment of the Z-disc and I band, make these genes candidates for future genetic and diagnostic investigation.

The abnormal mitochondrial morphology seen in WB MFM electron micrographs may also be linked to Z-disc mechanosignaling. CSRP3 complexes with calcineurin, which mediates mitochondrial fission (38)(50,51). Furthermore, mitochondrial complex I was an enriched cellular location in MFM WB. Many of the DE genes and DE proteins that reside there were enriched in pathways pertaining to complex I biogenesis, oxidoreductase activity, and response to reactive oxygen species. Complex I generates most of the reactive oxygen species in the cell (52,53). Pathologic amounts of reactive oxygen species have been linked to protein aggregation and sarcomere disarray in MFM (54,55). Increases in reactive oxygen species may be from increased production or a deficiency in cysteine-based antioxidants. Regardless, altered redox homeostasis has been implicated in both human MFM and MFM in Arabian horses (56–59) (54,57,60). The present data also supports that oxidative stress plays a role in the pathogenesis of WB MFM.

The possible alteration in redox homeostasis may be explained by decreased expression of 4 of the 42 complex I subunits detected in MFM WB. One of these complex I subunits was NDUFA13 which is required for complex 1 biogenesis (61,62). Furthermore decreased expression of NDUFA13 activates the enriched TF STAT3, which was identified by the transcription factor motif analysis in our fifth objective (63). When activated, STAT3 acts to partially block complex I and thereby decreases ROS production (63,64). MFM WB also had decreased expression of the antioxidant enzyme CAT (catalase) in MFM WB and an increase in *CHAC1* (gamma-glutamylcyclotransferase). CHAC1 acts to degrade the ubiquitous antioxidant

glutathione as a proapoptotic participant (65). If the myofiber cannot efficiently attenuate oxidative stress, it is possible that a proapoptotic cascade is initiated.

To ameliorate the oxidative stress associated with MFM, antioxidants such as N-acetyl cysteine have been shown to prevent protein aggregation (54,57,60) and prevent cell apoptosis (66). Targeted treatments to mitigate myofiber pathology and prevent apoptosis are needed because post mitotic cells, such as skeletal muscle, do not divide and have limited ability to repair (67,68). Apoptosis of myofibers leads to the fibrosis and fatty infiltration seen with other degenerative myopathies (69–71).

A key driver of muscle fibrosis is fibrinogen (72). MFM WB had increased expression of three fibrinogen subunits in their muscle. It is possible that there was blood contamination during the biopsy process. However, endomysial fibrosis is associated with limb-girdle muscular dystrophies and occurs in the extracellular matrix (73,74). Surprisingly, MFM WB had enrichment of the extracellular matrix and pathways pertaining to integrin signaling. The extracellular matrix has a necessary role in mechanical force transduction and satellite cell recruitment for repair in skeletal muscle (75). Extracellular matrix signaling is conducted by α7β1 integrins and dystroglycan complexes (76). Increased expression of these proteins may indicate a response to stabilize the myofiber, or may be suggestive of dystrophic and fibrotic changes in MFM WB.

Endogenous integrin signaling can be caused by transduction of mechanical forces at the Z-disc through intermediate filaments such as desmin and synemin (77–81). These filaments not only

stabilize sarcomeres, but also complex with the sarcolemma, mitochondria, and other organelles (82,83). Desmin is found in almost all MFM aggregates, regardless of the cause (14,20,84–86). Despite using desmin aggregation as a key diagnostic feature for MFM WB, we did not find the protein or gene to be DE. We did however, find increased protein expression of synemin in MFM WB. Synemin localizes to the Z-disc by binding to alpha-actinin and desmin and acts to stabilize the sarcomere by dissipating myofibrillar forces to the extracellular matrix (79,80). Aggregation of synemin has been found in MFM and other myopathies (87) and is a new candidate intermediate filament for further diagnostic and genetic investigation in WB MFM.

Conclusion

This dissertation defines the prominent clinical features of PSSM2 and MFM WB as decline in athletic performance, reluctance to go forward, and reluctance collect during work. Of the subset of MFM WB examined, we could not find any association of identified variants or differential expression in 16 candidate MFM genes using RNAseq data. The integration of proteomic and transcriptomic analyses of gluteal muscle from MFM and non-MFM WB showed a DE genes and DE proteins profile unique to MFM WB. This DE and subsequent GO analyses point to Z-disc instability and mechanosignaling, compromised redox potential, and the extracellular matrix as an integral parts of the equine MFM pathogenesis. Lastly, CHAC1, CSRP3, PDLIM3, SYNPO2, and STAT3 are among the newly identified candidate proteins and genes that warrant further genomic and diagnostic evaluation in MFM WB.

Suggestions for continued work

As with most scientific endeavors, this dissertation has unearthed a plethora of additional questions needing further hypothesis driven research:

From the first chapter, it remains unclear if PSSM2 WB without desmin staining have a clinically less severe MFM. To evaluate the ultrastructural features of myofibrillar disarray, a study conducting EM on PSSM2 would be useful to determine if these horses have early MFM. Additionally, staining for other cytoskeletal protein aggregates, such as CSRP3 or synemin, may also prove revealing.

Whole genome sequencing would be a useful tool to add another omics analysis to our investigation of MFM WB. This particular modality would be able to identify genomic variants, where our RNA-seq approach was limited strictly to expressed gene transcripts. A GWAS would be more cost effective than WGS and would help identify a chromosomal locus linked to the MFM phenotype. However, to do such a study would require more horses to obtain statistical power.

Another modality that would be useful in identifying better diagnostic markers for WB MFM is metabolomics. Similar to proteomics, metabolomics looks for differential metabolic intermediate expression. Identifying excess or deficient metabolic intermediates would pinpoint specific metabolic pathways and enzymes for functional deficiencies, lack of substrates, or potential allosteric inhibitors. Furthermore the accumulation of metabolic intermediates may prove a useful diagnostic tool.

The implication that oxidative stress and antioxidant deficiencies are involved in MFM needs to be validated. The quantification of lipid peroxidation coupled with carboxylation, glycosylation and nitrosylation of proteins could show functional changes incited by increased oxidation in the cell. Techniques to accomplish this include western blots, and immunohistochemistry.

Since a decreased antioxidant potential has been implicated as a cause of equine MFM, a *controlled* antioxidant supplement trial would be useful to determine if changes in clinical signs, histopathology, and cellular indicators of oxidative stress, such as lipid peroxidation or carbonylation change with treatment.

We identified three candidate transcription factors in WB MFM. Further investigation utilizing CHIPseq technology would allow for the identification of TF target genes and binding sight annotations.

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