Immune-Mediated Myositis in Horses:
From phenotype to genotype

A Thesis

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ABSTRACT

Background: Equine immune-mediated myositis (IMM) is a painful and debilitating condition of predominantly Quarter Horse (QH) and related breeds. The epaxial and gluteal muscles are most severely affected and muscle atrophy can be dramatic, with 50% of the affected muscle mass being lost in <72 hours. Diagnosis is based on a muscle biopsy of affected muscles and the identification of lymphocytes invading myofibers and in some cases surrounding blood vessels. The pathophysiology is presumed to be immune-mediated, but further evidence is needed to confirm this. Abnormal expression of major histocompatibility Complex (MHC) has been identified on muscle fibers from most human IMMs and provides the most consistent indication of an immune-mediated mechanism. The restriction of IMM to primarily QH and related breeds, particularly in certain bloodlines suggests that there is a genetic susceptibility underlying IMM.

Hypothesis: Quarter Horses are genetically susceptible to an immune mediated myositis that is characterized by abnormal expression of MHC class I and/or class II on the sarcolemma of myofibers.

Specific Aim 1: To determine if abnormal MHC class I and II expression is present on the sarcolemma of myofibers of horses with active IMM in the presence or absence of myofiber lymphocytic infiltrates.

Specific Aim 2: To characterize the subtypes of lymphocytes in the myofibers of horses with active IMM and correlate this with MHC expression.

Methods: Immunohistochemical staining for MHC I, II, CD4+, CD8+, CD20+ lymphocytes was performed on archived muscle samples of IMM (21 horses) and
controls (3 healthy and 6 disease controls). Scores were given for MHC I and II and for lymphocytic subtypes.

**Results:** A degree of sarcolemmal MHC I and II expression was present in 81% and 71% of IMM horses, respectively. CD4+, CD8+, and CD20+ cells were present in 20/21 IMM horses with a CD4+ predominance in 48% of cases. MHC I score was positively correlated with MHC II (r = 0.89, p = <0.001) and CD8+ (r = 0.64, p = 0.002) and CD20+ (r = 0.66, p = 0.001) lymphocyte and macrophage scores (r = 0.70, p = <0.001). MHC II scores were positively correlated to CD8+ (r = 0.59, p = 0.005), CD20+ (r = 0.61, p = 0.004) lymphocyte and macrophage (r = 0.70, p = <0.001) scores.

**Specific Aim 3:** To determine if equine IMM is significantly associated with a region of the equine genome using a genome-wide association (GWA) study.

**Methods:** DNA was extracted from blood and muscle of 36 IMM horses and 54 healthy controls of QH-related breeds that were housed in similar environments. Sequencing was performed on equine 50K and 70K single nucleotide polymorphism (SNP) arrays. A GWA was performed across 40,811 SNPs that passed quality control. To account for elevated genomic inflation, statistical analysis was performed using GEMMA and GRAMMAR-GC software.

**Results:** A significant association was identified between IMM and a 2 MB region on equine chromosome 11. Five SNPs in 3 haplotype blocks reached genome-wide significance using the 2 different statistical methods to account for population stratification. The significant region contains 6 myosin heavy chain genes expressed in skeletal muscle, including MYH2, which has been associated with a human IMM.
Conclusions: Equine IMM is characterized by MHC I and II expression on the sarcolemma of myofibers during an acute CD4+ and CD8+ lymphocytic inflammatory episode. There is an approximately 2MB region on equine chromosome 11 that is associated with the development of the disease. Sequencing of the MYH genes in IMM cases and unaffected controls is warranted to identify variants that cause IMM in Quarter Horses.
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<tbody>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AIRE</td>
<td>autoimmune regulator protein</td>
</tr>
<tr>
<td>AKT</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>apoptotic protease-activating factor 1</td>
</tr>
<tr>
<td>AST</td>
<td>aspartate transaminase</td>
</tr>
<tr>
<td>CK</td>
<td>creatine kinase</td>
</tr>
<tr>
<td>CMMM</td>
<td>canine masticatory muscle myositis</td>
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<tr>
<td>DAMPs</td>
<td>damage associated molecular pathogens</td>
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<td>DM</td>
<td>dermatomyositis</td>
</tr>
<tr>
<td>ECA</td>
<td>equine chromosome</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>FasL</td>
<td>fas ligand</td>
</tr>
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<td>FoxO</td>
<td>forkhead box group O</td>
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<td>GEMMA</td>
<td>genome-wide efficient mixed model association</td>
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<td>GRAMMAR</td>
<td>genome-wide rapid association using mixed model and regression</td>
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<td>GRAMMAR-GC</td>
<td>GRAMMAR genomic control</td>
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<tr>
<td>GWA</td>
<td>genome-wide association</td>
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<tr>
<td>GYS1</td>
<td>glycogen synthase 1 gene</td>
</tr>
<tr>
<td>HE</td>
<td>hematoxylin eosin</td>
</tr>
<tr>
<td>IBM</td>
<td>sporadic inclusion body myositis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ICAM</td>
<td>intercellular adhesion molecule</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon gamma</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin growth factor</td>
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<td>IHC</td>
<td>immunohistochemistry</td>
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<tr>
<td>Ik-Bα</td>
<td>inhibitor of nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IMM</td>
<td>immune-mediated myositis</td>
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<tr>
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<td>linkage disequilibrium</td>
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<td>MAFbx</td>
<td>muscle atrophy F-box protein</td>
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<td>mitogen activated protein kinase</td>
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<td>MDS</td>
<td>multi-dimensional scaling plot</td>
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<td>major histocompatibility class I</td>
</tr>
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<td>MHC II</td>
<td>major histocompatibility class II</td>
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<tr>
<td>mTORC1</td>
<td>mammalian target of rapamycin complex 1</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MSA</td>
<td>myositis-specific autoantibody</td>
</tr>
<tr>
<td>MuRF1</td>
<td>muscle RING-fiber protein 1 promoter</td>
</tr>
<tr>
<td>MYH</td>
<td>myosin heavy chain</td>
</tr>
<tr>
<td>MYO5A</td>
<td>myosin VA gene</td>
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<tr>
<td>NAM</td>
<td>necrotizing acute myositis</td>
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<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NMDL</td>
<td>University of Minnesota Neuromuscular Laboratory</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>PAS</td>
<td>periodic acid Schiff</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3 kinase</td>
</tr>
<tr>
<td>PM</td>
<td>polymyositis</td>
</tr>
<tr>
<td>PPID</td>
<td>pars-pituitary intermedia dysfunction</td>
</tr>
<tr>
<td>PSSM</td>
<td>polysaccharide storage myopathy</td>
</tr>
<tr>
<td>QH</td>
<td>Quarter Horse</td>
</tr>
<tr>
<td>SINE</td>
<td>short interspaced element</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
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<td>STAT4</td>
<td>signal transducer and activator of transcription 4 gene</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TLR</td>
<td>toll like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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LITERATURE REVIEW

I. INTRODUCTION

Unlike other less athletic species, over 50% of the mass of the horse is comprised of skeletal muscle,\(^1\) which provides power and strength for athletic endeavors. Quarter Horses are one of the most versatile breeds of horse and have many uses, from relatively sedentary work (halter horses) to high levels of speed and agility (cutting horses). Muscle disease is relatively common in horses and diverse arrays of syndromes are recognized.\(^2\) Each muscle disease can have a significant impact on equine performance and in some cases can cause severe morbidity and even mortality. Immune-mediated myositis (IMM) is a severe, debilitating muscle disease, which can lead to atrophy of over 50% of the epaxial and gluteal muscles in less than 72 hours.\(^2\) In horses, the disease appears to predominantly affect Quarter Horses\(^3\) particularly in specific blood lines, and therefore a genetic predisposition is suspected.

a. Basic muscle cell anatomy

Each individual muscle is made up of numerous muscle cells (fibers) that originate and end on tendinous insertions.\(^4\) Each muscle fiber contains numerous strategically aligned myofibrils that are composed of regular arrangements of thick and thin filaments secured in place by an abundance of intermediate filaments and cytoskeletal proteins.\(^5\) The thick filaments are made up of approximately 300 myosin molecules. Each myosin molecule is made up of six polypeptide chains (2 heavy chains and 2 pairs of light chains), with the
heavy chains forming the tail and head of the myosin molecule and the light chains being arranged within the globular head of the myosin molecule. Different forms of myosin heavy chains (neonatal, slow, fast type 2a, fast type 2x or a 2a/2x hybrid) are expressed in different muscle types. The myosin chain types are important in determining the contractile speed. There are multiple myosin isoforms found in most mammalian species. The isoforms are specific to the type of muscle they are expressed in. For example, there are cardiac specific myosin heavy chain (MYH) isoforms that are not expressed in skeletal muscle. There are also isoforms that are only expressed during muscle development in the embryo and neonate. Each MYH is the product of a different gene. In humans, most of the skeletal muscle sarcomeric MYH genes are located on chromosome 17. Several conserved regions of chromosome 17 in humans map strongly to equine chromosome 11. In equine gluteal muscle, three MYHs have been identified. They are consistent with type I, type IIa and type IIx MYH isoforms.

Each thin filament is made up of two F-actin strands twisted into a double helix and possessing a binding site, which is complementary for the globular head of the myosin molecule. The association between actin molecules and the myosin globular heads is controlled by tropomyosin and troponin molecules which inhibit actin and myosin interaction in the absence of calcium. The sarcoplasm surrounding the myofibrils contains large amounts of potassium, magnesium, phosphate, enzymes and mitochondria.
Each muscle is made up of a combination of fast (type II) and slow (type I) twitch fibers. The slow twitch muscle fibers are generally smaller in cross-sectional area and have an extensive capillary network, large numbers of mitochondria and more myoglobin, all of which combine to give a greater oxidative metabolic capacity. Fast twitch muscle fibers are larger in cross-sectional area, have an extensive sarcoplasmic reticulum for rapid release of calcium ions for muscle contraction, have high glycolytic capacity, a less extensive capillary network and fewer mitochondria. These differences enable the type 2 muscle fibers to perform rapid and strong contractions in a relatively anaerobic environment. The proportion of each fiber type in a given muscle is determined by the predominant purpose of that muscle. For example, the epaxial muscles in humans are mostly used as postural muscles and have a predominance of slow twitch (type I fibers), however in horses there is a relatively equal proportion of type I and II fibers in the epaxial muscles, suggesting they play a combined role in both posture and locomotion in the horse. Muscle fiber types also vary depending on the breed and use of the horse. For example, Arabian horses used for endurance tend to have a higher proportion of type I fibers as compared with Quarter Horses that are used for high speed and short distance races. Quarter Horses have a higher proportion of type II fibers in the *gluteus medius* muscle than other breeds. Different contractile fiber types can be differentiated using myosin-adenosine triphosphatase (ATPase) staining and preincubation at different acid and alkaline pH.

Each muscle contains numerous motor units. These motor units are the group of muscle fibers innervated by a single nerve branch at the neuromuscular junction. In large
muscles used predominantly for strength, such as the semitendinosus muscle, there are a large number of muscle fibers in each motor unit. Smaller muscles that require finer control, such as the laryngeal muscles, have much smaller numbers of muscle fibers within each motor unit.

II. REGULATION OF MUSCLE MASS

The muscle mass is constantly undergoing remodeling throughout an animal’s life. Muscle diameter, length, strength, vascular supply and to a small degree muscle fiber type can change depending on the requirements of the individual muscle. The average size of an animal’s muscle prior to training is related to their genetics and to a variety of other factors including the amount of testosterone secretion. The size (or cross-sectional area) of a muscle fiber is maintained through a balance in muscle degradation and protein synthesis.

a. Increase in muscle fiber size

The nuclei of mature myofibers are post mitotic and do not undergo DNA replication under normal circumstances. Therefore any increase in muscle size is mostly based on hypertrophy (the increase in size of individual muscle fibers) rather than hyperplasia (an increase in the number of muscle fibers). This hypertrophy results from an increase in the number of actin and myosin filaments in each muscle fiber leading to enlargement of the individual muscle fibers. Stimulus for a muscle to hypertrophy comes from repetitive loading of a muscle that is contracting. In addition to the increase in muscle fiber size,
there are also significant increases in mitochondrial enzymes and increases in the amounts of stored glycogen and triglycerides depending on the intensity and duration of training.\textsuperscript{6} To maintain the DNA/protein ratio in muscle, significant muscle hypertrophy is accompanied by stimulation of satellite cells to increase the number of myonuclei.\textsuperscript{19}

i. Insulin Growth Factor

Insulin Growth Factors (IGFs) are highly conserved across species. IGF-1 is known to be the primary mediator of growth hormone, while IGF-2 is apparently more important during embryonic development.\textsuperscript{9} Both hormones are produced by a number of tissues and are protein bound in the circulation.\textsuperscript{4} To date, six IGF binding proteins have been recognized.\textsuperscript{19} The release of IGF leads to a strong stimulus for protein synthesis, enhanced uptake of amino acids, differentiation of satellite cells and, in combination with fibroblast growth factors, proliferation of muscle cells.\textsuperscript{21} This process is mediated by individual cell surface receptors.

The IGF-1 receptor is closely related to the insulin receptor and is a multiunit complex. Both IGFs act on this receptor, which is present on myoblasts and myotubes.\textsuperscript{9} The IGF-1 receptor has tyrosine kinase activity and influences the cell cycle through the phosphatidylinositol 3 kinase/protein kinase B (PI3K/AKT) pathway. This pathway plays an extremely important role in increasing muscle mass.\textsuperscript{22} The PI3K/AKT pathway is activated by exercise and shifts the balance of protein synthesis and degradation by activating synthesis and inhibiting degradation.\textsuperscript{19}
IGF-1 mRNA levels increase following exercise. Following a muscle injury, mRNA for IGF-1 is upregulated within 24 hours and remains elevated for approximately 10 days, which corresponds with satellite cell proliferation and muscle regeneration. IGF-1 is also important in the prevention of muscle atrophy by the ubiquitin-proteasome pathway.

ii. Growth Hormone

Growth Hormone (GH) is produced by the anterior hypophysis and under the regulation of hormones from the hypothalamus is secreted in a pulsatile manner. Concentrations of GH in the blood vary greatly throughout the day depending on different factors including diet, exercise, sleep and gender. One of the important effects of GH is the stimulation of IGF-1 synthesis, predominantly by the liver. GH is essential for growth following birth, which is demonstrated by individuals with GH insensitivity who are born at a relatively normal birth weight but fail to grow and develop muscle mass properly. However GH does not seem to play a significant role in muscle mass regulation in the healthy adult.

b. Decrease in muscle fiber size

Muscle atrophy is a decrease in muscle fiber size. In the healthy individual, muscle protein synthesis and catabolism is balanced and therefore muscle mass remains relatively constant without the input of additional factors. There are many stimuli for a decrease in muscle fiber size, including disuse, old age, cachexia and denervation. Whatever the cause of the atrophy, the rate of degradation of the muscle proteins exceeds the rate of replacement leading to decreased protein concentrations in the muscle and
decreased fiber size. The main feature of muscle atrophy is a decrease in the cross-sectional area of the muscle fibers due to the loss of contractile proteins.

i. Imbalance in anabolic and catabolic factors

Anabolic factors including IGF-1 are essential for maintaining growth and repair of muscle fibers. IGF-1 is reported to be decreased in numerous disorders associated with muscle atrophy. Patients with diabetes that have insulin resistance develop muscle atrophy and this is believed to be due to failure of activation of the PI3K/Akt pathway, which is the same pathway that is activated by IGF-1. Upregulation of inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and transforming growth factor beta (TGF-β) compounds (including myostatin), and increased production of glucocorticoids all have catabolic effects.

ii. Ubiquitin-proteasome pathway

Ubiquitin is present in all cell types including muscle and has many roles including forming covalent bonds with other proteins. Ubiquitination is a complex process with three main steps: ubiquitin is activated, binds to the specific substrate and is then labeled for a specific downstream event such as degradation by proteasomes. If a protein is monoubiquitinated, then this is usually targeted for degradation by the lysosome pathway. If a protein is polyubiquitinated, this protein is then targeted for destruction by the proteasome.
To date there are three main muscle-specific ubiquitin ligases (Muscle atrophy F-box protein (MAFbx)/Atrogin-1, muscle RING-finger protein 1 promoter (MuRF1) and TRIM32) that play different roles in the ubiquitination process.\textsuperscript{43-45} MAFbx and MuRF1 are the main atrophy genes which are activated by forkhead (FoxO) transcription factors 1 and 3.\textsuperscript{46} MuRF1 binds to the MYH\textsuperscript{47} leading to degradation. IGF-1 plays an important role in preventing muscle atrophy by these pathways.\textsuperscript{25} IGF-1 activates the pathway that leads to AKT1 production, which phosphorylates FoxO transcription factors, preventing the entry of FoxO3 into the nucleus and thus preventing transcription of specific ubiquitin ligases and autophagy-related genes, which are involved in the ubiquitin-proteasome and autophagy/lysosomal pathways.\textsuperscript{48}

\textbf{iii. Autophagy-lysosome pathway}

This pathway is one of the most important in maintaining normal muscle homeostasis by removing components of muscle that develop an abnormality such as abnormal mitochondria.\textsuperscript{31} However, following oxidative stress or conditions such as denervation, it can be excessively stimulated leading to muscle fiber death and atrophy.\textsuperscript{49,50} Although there are different autophagy-lysosomal pathways, all of them involve exposure of cytoplasmic contents to the lysosome.\textsuperscript{31} FoxO3 also plays an important role in activating the autophagy pathway.\textsuperscript{51}

\textbf{iv. Caspase pathway}

The caspases are a subset of proteolytic enzymes that are extremely important for apoptosis. They can be activated intrinsically within the cell, due to a response to stress
such as that created by DNA damage.\textsuperscript{52} The stressed cell alters the permeability of the mitochondrial outer membrane leading to release of cytochrome C, which combines with apoptotic protease-activating factor 1 (Apaf-1).\textsuperscript{31} This complex recruits and activates procaspase-9 leading to downstream activation of the caspase pathway that leads to degradation of the intracellular substrates. Caspases can also be activated extrinsically by ligands such as fas ligand (FasL) and TNF-\(\alpha\) leading to binding of specific death receptors on the cell surface.\textsuperscript{31} During the process of aging, the amount of apoptosis increases in normal individuals,\textsuperscript{53} however apoptosis also plays an important role in numerous disorders that lead to muscle atrophy, including cancer cachexia\textsuperscript{54} and disuse atrophy.\textsuperscript{55}

v. NF-\(\kappa\)B pathway

The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-\(\kappa\)B) pathway is a pro-inflammatory pathway that is frequently activated by cytokines (IL-1, IL-6 and TNF-\(\alpha\)) which promote degradation of the NF-\(\kappa\)B inhibitor (I\(\kappa\)-B\(\alpha\)) particularly in patients with cachexia.\textsuperscript{56} NF-\(\kappa\)B can directly trigger proteasome-dependent muscle atrophy by binding and activating the MuRF1 promoter.\textsuperscript{57} NF-\(\kappa\)B can also activate expression of inducible nitric oxide synthase leading to increased nitric oxide concentrations, which have been implicated in age related muscle atrophy via apoptosis.\textsuperscript{58} The nitric oxide species also contribute to disuse atrophy via increased production of FoxO transcription factors.\textsuperscript{59}
vi. Myostatin

Transforming growth factor beta is a negative growth factor that plays a role in decreasing satellite cell proliferation\textsuperscript{9} and myostatin is a member of this family. Myostatin plays a role in inhibition of the cell cycle\textsuperscript{60} and has been identified in muscle satellite cells that have regenerated.\textsuperscript{61} Myostatin also inhibits the differentiation of myoblasts into myotubes.\textsuperscript{62} In the serum of a healthy animal, a majority of myostatin is bound to an inhibitory propeptide.\textsuperscript{63} Multiple proteins have been identified that regulate myostatin activity both through the interaction with the propeptide and at a receptor level.\textsuperscript{61}

Following strength training in men and women, myostatin expression is decreased.\textsuperscript{64} Selective breeding for increased muscle production in cattle has led to double muscling in certain breeds e.g. the British Blue cattle. The double muscling is due to mutations in the myostatin gene.\textsuperscript{65} A short interspaced element (SINE) insertion and single nucleotide polymorphisms (SNP) have been identified in the promotor region of the equine myostatin gene that are associated with an increase in type 2x fibers and enhanced ability for racing over shorter distances.\textsuperscript{66} Myostatin expression has also been found to be upregulated in a number of disease states.\textsuperscript{67,68,69}

III. MUSCLE ATROPHY IN THE HORSE

Due to the athletic requirements of a horse, a small decrease in the amount of muscle mass can significantly impact the performance potential of that animal. Loss of muscle
mass, or more appropriately gross muscle atrophy, can occur either across all muscle groups (generalized) or within a specific muscle (focal). Muscle atrophy can be secondary to dysfunction or damage of the nerve supplying the muscle leading to neurogenic atrophy. Myogenic atrophy can be due to systemic diseases or due to a primary disorder involving the muscles themselves.

a. Causes of diminished fiber sizes

i. Neurogenic Atrophy

Innervation is essential for maintaining normal muscle mass. Regardless of the cause of nerve injury, decreased stimulation of the muscle leads to muscle atrophy. In cases of complete removal of nerve stimulation, a 70% reduction in muscle cross-sectional area is seen within two months of the injury. Without re-innervation, the affected muscle is typically replaced with fibrous and fatty tissue.

1. Focal neurogenic atrophy

Focal atrophy is typically caused by an injury to an individual neuron or a group of neurons. The most common cause is trauma to a relatively exposed section of the nerve. For example, trauma to the suprascapular nerve in the horse leads to a syndrome called Sweeney, which manifests as rapid and dramatic atrophy of the suprascapular muscle. Equine protozoal myeloencephalitis is another cause of focal atrophy which is secondary to disruption of the cell body of the motor nerve within the ventral horn of the spinal cord. Motor nerve damage affects nerve fibers supplying both type 1 or type 2 muscle
fibers which leads to the classic finding in muscle biopsies of angular atrophy of the slow
twitch type 1 and fast twitch type 2 muscle fibers.70

2. Generalized neurogenic atrophy

Generalized neurogenic atrophy manifests as muscle atrophy of multiple muscle
groups.72 The underlying pathogenesis varies. Equine Motor Neuron Disease is a chronic
progressive disorder that is associated with low serum vitamin E levels75 and can be
induced by chronic low Vitamin E diets.76 Vitamin E is involved in the protection of
neurons from free radicals and, when it is chronically deficient, this can lead to oxidative
damage to neurons.77 It is hypothesized that the vitamin E deficiency leads to
deterioration of parent motor neurons, resulting in the death of the peripheral motor
neurons and secondary neurogenic atrophy.78 Affected horses develop dramatic
generalized muscle atrophy, weakness; narrow based stance, and an elevated tail head.79
The clinical diagnosis is based on a muscle biopsy of the sacrocaudalis dorsalis medialis
muscle, which contains angular atrophy of predominantly type 1 muscle fibers.80
Definitive diagnosis requires examination of the ventral horn of the spinal cord and
identification of degeneration and loss of motor neuron cell bodies.78

ii. Myogenic atrophy

Myogenic atrophy can arise from disuse, endocrinopathies, a systemic negative energy
balance or a primary muscle disease.81 The progression of the atrophy is typically slower
than with neurogenic atrophy and it is characterized by a generalized decrease in the size
of type 2; often type 2x muscle fibers.2 The shape of the atrophied fiber is often
characterized as anguloid, indicating one or two sides are concave but usually not to the extreme of causing triangular shaped angular atrophied fibers.9

1. Disuse
Disuse atrophy occurs with decreased exercise or when a limb is immobilized, such as with casting. Atrophy can be rapid82 but is usually slower than with neurogenic atrophy and is characterized by a general reduction in muscle fiber sizes due to decreased protein synthesis.83 In many animals, there is also evidence of an increase in muscle protein catabolism.84 With complete disuse, such as in hospitalized non-ambulatory humans, significant atrophy and loss of muscle strength can be seen within 10 days.83 This is most often seen in horses following an injury leading to casting or severe lameness and disuse of the limb.85 Prolonged muscle disuse increases reactive oxygen species production leading to activation of the cytosolic calcium-dependent calpains, the mitogen activated protein kinase (MAPK) system (including Jnk), and the NF-κB pathway.83

2. Endocrinopathy
Pars-pituitary intermedia dysfunction (PPID) is the most common primary endocrinopathy that causes muscle atrophy in horses and ponies over the age of 15 years.86 Clinical signs include hirsutism, polyuria and polydipsia, marked muscle atrophy and fat redistribution.87 The disease is caused by hypertrophy, hyperplasia, or the development of a microadenoma in the pars intermedia leading to an increased production in proopiomelanocortin peptides including α-melanocyte-stimulating hormone, β-endorphin related peptides, and adrenocorticotropic hormone (ACTH) due to
decreased sensitivity to inhibition of the Pars Pituitary Intermedia by dopamine. Elevated ACTH leads to high corticosteroid levels, which impairs protein synthesis resulting in a decrease in the size of type 2A and 2x muscle fibers loss of type 2x fibers and gross muscle atrophy. Ubiquitin expression and proteasome activation are also increased following glucocorticoid administration.

3. Sarcopenia
Muscle atrophy commonly develops as the body ages. One of the main causes appears to be a decreased anabolic response. Other potential causes of sarcopenia include insulin resistance and decreased circulating IGF-1. Over time, inflammation also appears to play a role in sarcopenia with high levels of TNF-α being negatively correlated with MYH protein synthesis.

4. Starvation
Starvation leads to a negative energy balance that causes gross muscle atrophy due to enhanced catabolism of muscle proteins and release of free amino acids to provide the body with an energy supply. When nitrogen becomes insufficient for protein anabolism, protein catabolism occurs from skeletal muscle within 48-72 hours. Malnutrition leads to inhibition of IGF-1 production and release, which leads to decreased protein synthesis. Muscle atrophy in patients with starvation is usually a function of both increased proteolysis and decreased protein synthesis. Proteasome activation and ubiquitin expression are increased in individuals suffering from starvation.
5. Sepsis

Sepsis is characterized by a severe inflammatory response secondary to overwhelming infection. Inflammation, and in particular high levels of TNF-α, lead to direct inhibition of muscle protein synthesis.\(^9\) Increased protein degradation may play a role in the severity of muscle atrophy, however, there are conflicting results in studies using muscle from human patients with sepsis.\(^9\)

6. Cachexia

Cachexia is characterized by weakness and muscle atrophy secondary to a severe and usually chronic illness.\(^9\) Potential causes include neoplasia,\(^9\) chronic cardiac failure, chronic obstructive pulmonary disease, and chronic renal failure.\(^9\) The cause of the muscle atrophy is multifactorial. Protein synthesis rates are decreased\(^9\) and muscle becomes resistant to the normal anabolic pathways even after provision of excess nutrition.\(^9\) Mammalian target of rapamycin complex 1 (mTORC1) signaling decreases, which is another likely cause of the decreased protein synthesis rate.\(^9\) Decreased mTORC1 signaling appears to be secondary to increased levels of pro-inflammatory cytokines, particularly IL-6.\(^9\) In more advanced stages of cachexia, muscle atrophy is also impacted by mitochondrial dysfunction and activation of AMPK leading to activation of genes (including FOXO and MuRF1) involved in autophagy.\(^9\) Other factors related to atrophy include reduced expression of IGF-1.\(^9,9\)
7. Rhabdomyolysis

Severe rhabdomyolysis can cause gross atrophy of muscle as a result of phagocytosis of degenerating sarcoplasmic material in a large number of myofibers. The syndrome is common in horses and there are many potential causes including infectious, toxic, nutritional, ischemic and idiopathic factors. In horses the underlying etiology is usually split into exertional and nonexertional causes. Whatever the underlying cause, the end pathway is the same with either a failure of the energy supply to maintain calcium homeostasis or damage to the sarcolemma. This dysfunction leads to excessive accumulation of calcium in the sarcoplasm, which overwhelms the normal mitochondrial pathways leading to cessation of oxidative metabolism. Disruption of mitochondrial pathways can lead to release of components of the mitochondria including cytochrome C being released from the cell which can lead to increased apoptosis and muscle atrophy. Macrophage infiltration occurs within 24-48 hours of rhabdomyolysis and begins the process of clearing proteinaceous debris from the damaged segments of the myofibers or, in some cases, leads to apoptosis. Regeneration of the damaged segment subsequently occurs as satellite cells migrate along the basement membrane within 3-4 days of the rhabdomyolysis. Myoblasts formed from satellite cells fuse to form myotubes, which have large centrally located nuclei. Synthesis of new myosin and actin filaments occurs and nuclei become more peripheral occupying a subsarcolemmal position in normal sized fibers within approximately one month after the episode. This review will focus on inflammatory causes of rhabdomyolysis in horses.
IV. INFLAMMATORY MYOPATHIES IN HORSES

Inflammatory myopathies have been identified in many species including humans, dogs and horses. They are characterized by infiltration of the muscle by inflammatory cells including lymphocytes and macrophages. Some inflammatory myopathies arise from a primary infection and others have an immune-mediated basis. Muscle atrophy is associated primarily with immune-mediated rather than inflammatory myopathies.

a. Clostridial myonecrosis

Clostridial myonecrosis can be caused by many different Clostridia species including; Clostridium perfringens, chauvoei, septicum, sordelli, novyi type B, perfringens A, and carnis. The most common cause in horses is C. perfringens. The disease most frequently occurs following contamination of an intramuscular injection with the actual bacteria, however cases have also been reported following lacerations. The most commonly affected muscle group is the musculature in the cervical region, which is the muscle most commonly used for intramuscular injections. Affected animals demonstrate marked depression and the affected muscles are usually swollen and hot, with areas of emphysema palpable under the skin. The pathogenesis is due to release of toxins by the bacteria, particularly lecithinase and hemolysin. The history and clinical findings are often diagnostic and muscle biopsies are rarely performed.
b. *Streptococcus equi ss equi* rhabdomyolysis

*Streptococcus equi ss equi* infection (Strangles) is relatively common in horses, and generally causes a mild respiratory disease. However, in some cases, severe secondary complications can develop including acute rhabdomyolysis or infarctive purpura hemorrhagica. In young (less than 7 years old) Quarter Horses (QHs) severe acute, rhabdomyolysis has been reported. These horses develop typical signs of Strangles infection (including submandibular lymphadenopathy, pyrexia and sometimes guttural pouch empyema) and due to the acute nature of the infection, affected horses usually have low levels of antibody to the *Streptococcus equi ss equi* M protein. Horses with *S. equi equi* rhabdomyolysis develop firm, swollen and painful gluteal and epaxial muscles, a stiff gait and rapidly progress to recumbency and frequently death within one week of infection. At necropsy, the necrotic muscles have severe, acute myonecrosis with varying degrees of macrophage infiltration. The cause of this condition is currently unknown, however there are two current theories. The first is that release of streptococcal superantigens stimulates T cells to produce large quantities of pro-inflammatory cytokines leading to a toxic-shock like reaction. The second suggests that systemic spread of the bacteria leads to production of exotoxins and or proteases in skeletal muscle leading to severe myonecrosis.

c. Infarctive hemorrhagic purpura

Horses with Infarctive Purpura Hemorrhagica generally have a history of exposure to *Streptococcus equi ss equi* or prior vaccination resulting in high titers to M protein and then a more recent episode of Strangles or recent vaccination. Clinical signs include
lameness, muscle stiffness, edema and colic.\textsuperscript{2} There are often multiple petechiae on the mucous membranes and focal areas of muscles along the horses pectoral and stifle regions are usually hot, swollen and firm.\textsuperscript{110} Biopsies of the firm regions of infarction show acute coagulative necrosis with neutrophilic infiltration.\textsuperscript{110} The pathophysiology is believed to be deposition of immune complexes in vessel walls leading to vascular occlusion and regions of muscle infarction.\textsuperscript{111}

d. Suspected Immune-Mediated Myositis

Suspected equine immune-mediated myositis (IMM) has only been briefly described in one case series\textsuperscript{3} and one case report.\textsuperscript{112} There is no sex predilection, but the disease is more prevalent in QHs and related breeds (Paints, Appendix horses and Appaloosas),\textsuperscript{28} although it has been reported in other breeds.\textsuperscript{112} Affected horses are usually eight years and younger, or sixteen years and older.\textsuperscript{3} Thirty-nine percent have a history of being exposed to a ‘triggering’ factor such as \textit{S. equi} infection, a respiratory virus, or vaccination.\textsuperscript{3}

The clinical signs of IMM include stiffness, malaise and a rapid onset of muscle atrophy. Some affected horses can lose over 50\% of the affected muscle mass in 48 hours and usually the gluteal and epaxial muscles are most severely affected.\textsuperscript{3} In one pony with IMM, focal and symmetrical atrophy of the cervical muscles was observed.\textsuperscript{112} There is also a report of a horse with multifocal atrophy that had lymphocytic infiltrates in a muscle biopsy consistent with IMM.\textsuperscript{113} Hematologic parameters are usually normal, and mild to severe elevations in serum creatine kinase (CK) and aspartate transaminase (AST)
may be noted on a chemistry profile but they can remain within normal limits. This is likely dependent upon time of sampling relevant to disease onset.

As with other myopathies, an accurate and early diagnosis is important so that treatment with appropriate immunosuppressive therapy can be instituted early. Diagnostic findings include varying degrees of lymphocytic infiltration of the myofibers, usually with a high CD4+/CD8+ ratio and mononuclear cuffing of blood vessels. Biopsies from the unaffected muscles such as semimembranosus/tendinosus or the cutaneous colic muscle are often normal. Immunoglobulin G staining has not been found on myofibers of IMM horses. Treatment is based on anti-inflammatory doses of corticosteroids for approximately one month. Although muscle regeneration usually occurs with or without treatment with corticosteroids, the disease can recur.

Limited information is known about IMM in horses, and although the etiology is suspected to be immune-mediated, direct proof is lacking. At present, there is no accurate method to diagnose horses with IMM after the acute phase of atrophy has passed. The previous equine IMM study defined the clinical signs of suspected IMM and the predominant inflammatory cell type in affected muscles. However, the presence of inflammatory cells alone is insufficient to diagnose an immune-mediated disorder, as demonstrated by the studies identifying lymphocytes in cases of non-immune-mediated disease. Without definitive evidence that equine IMM is immune-mediated, it is difficult to recommend treating with corticosteroids, which would be contraindicated in a disease without an immune-mediated basis. Thus, one of the primary objectives of this
thesis was to determine if there were further characteristics of skeletal muscle biopsies of IMM horses that would further support an immune-mediated basis for this disease.

V. IMMUNE-MEDIATED MYOPATHIES IN HUMANS AND DOGS

Immune-mediated myopathies have been identified in humans, dogs and several other species. The clinical signs can be acute, sub-acute or chronic. Many patients develop moderate to severe muscle weakness. In humans Dermatomyositis (DM), Polymyositis (PM), Sporadic Inclusion Body Myositis (IBM) and Necrotizing Acute Myositis (NAM) are the most common categories of IMMs. In dogs; the most common IMMs are DM, PM and Canine Masticatory Muscle Myositis (CMMM). By definition, evidence of inflammatory cell infiltration in a muscle biopsy are a requirement for diagnosis. The diagnosis of IMMs can be challenging, however, because depending on the phase of disease inflammatory infiltrates are not always present and overlap also exists with some non-IMM diseases that have inflammatory cell infiltrates.

a. Mechanisms of autoimmunity

The innate immune response in combination with the body’s external barriers including the mucous membranes and skin is the first line of defense against invading pathogens. A vast majority of pathogens are neutralized by the innate response, however the innate response is not specific to a particular pathogen and no memory develops following the resolution of an invading pathogen. To mount an effective immune response against more virulent pathogens and to develop memory to that pathogen, a response by the
adaptive immune system is required. Both the innate and adaptive immune systems have numerous mechanisms in place to prevent autoimmunity. As the immune system evolves, there are several steps where immune cells with receptors specific for ‘self’ proteins are deleted or converted into regulatory T cells to prevent autoimmune reactions. However the process is not 100% effective and some ‘self’ reactive cells are not deleted by mistake while others that are only weakly specific for ‘self’ peptides are intentionally not deleted because there are many pathogens with peptides similar to ‘self’ peptides and deletion of all weakly ‘self’ reactive cells would likely lead to immunosuppression. Despite the presence of these potentially ‘self’ reactive cells, autoimmune disease is still rare. This is because there are many steps required to activate the immune system. Even if an immune cell mistakenly does not recognize a ‘self’ peptide as ‘self’, two further steps; ligation of the T cell receptor to the specific antigen and major histocompatibility complex of that receptor, and the presence of costimulatory molecules (e.g. CD80+ and CD86+) and inflammatory cytokines, are required before the next step in the immune response can occur. There is evidence that both the innate and adaptive immune systems play a role in the failure of recognition of a ‘self’-peptide as ‘self’.

i. Role of Major Histocompatibility Complex

Research into IMMs and the pathophysiology of the autoimmune response has focused on the upregulation of major histocompatibility complex (MHC) class I and II. MHC is critical to the adaptive immune response and is required for activation and clonal expansion of CD8+ and/or CD4+ T cells. The most important role of MHC is to bind
foreign peptides and present them to the immune system, particularly T-cells. There are two main classes of MHC. MHC class I is expressed by virtually every nucleated cell in the body with few exceptions and one of its roles in the normal cell is to present ‘self’ proteins to the immune system. The immune system typically recognizes the MHC class I and the self-antigen complex as being self, which is important for survival of circulating T cells. In some individuals with autoimmune disease, the T cell fails to recognize the MHC and self-antigen complex as self and when the appropriate environment develops, the immune system is activated against that target cell. If the MHC class I molecules are lost from a cell, such as with certain types of cancer, the cells can be recognized by Natural Killer (NK) cells, which trigger programmed cell death. MHC class II is more specific to antigen presenting cells of the immune system, such as dendritic cells, B cells, and macrophages and plays a primary role in the presentation of foreign material that has been phagocytized by those cells. Foreign antigen, such as from a virus or bacteria, binds to a T cell with a specific receptor for that MHC antigen complex and, in the presence of the correct cofactors, is activated leading to upregulation of the adaptive immune response.

MHC class I molecules have beta2 microglobulin subunits and are only recognized by CD8+ T cells. For CD8+ T cell mediated cytotoxicity to occur (leading to cell death), MHC Class I expression must be present on target cells. MHC class II molecules do not have beta2 subunits and are only recognized by CD4+ T cells. Following translation of the MHC class I molecule, during processing in the endoplasmic reticulum (ER), they bind to peptides that have been produced by the proteasome system in the
cytosol of the cell before migrating to the cell membrane.\textsuperscript{126} Whereas during MHC class II processing by the ER, the MHC II molecules bind to peptides produced in the ER, golgi apparatus, endosomes, lysosomes, and any other intracellular vesicles prior to the MHC II complex migrating to the cell membrane.\textsuperscript{122} Cathepsins play important roles in the development of MHC class II and binding of peptides prior to presentation on the surface of a muscle cell.\textsuperscript{130} Upregulation of MHC and the presentation of peptides is not enough to activate the immune system.\textsuperscript{122} Antigen presenting cells must provide adequate co-factors, in particular intercellular adhesion molecule-1 (ICAM-1), inflammatory cytokines, and co-stimulatory molecules, especially CD80\textsuperscript{+} and CD86\textsuperscript{+}, to stimulate full activation of the adaptive immune system.\textsuperscript{131} Some pathogens do not invade typical antigen presenting cells, for example the HIV virus. In this case it is possible for antigen presenting cells (particularly dendritic cells) to cross-present with the infected cell, and can provide the appropriate cofactors required for activation of the adaptive immune response.\textsuperscript{122}

During muscle development both myoblasts and myotubes express MHC class I but not MHC class II antigens.\textsuperscript{132} They can be induced to express MHC class II in the presence of interferon-gamma (IFN-\(\gamma\))\textsuperscript{132} and appear to share some functions and molecules with dendritic cells and therefore function as antigen processing cells.\textsuperscript{133} This would suggest that myoblasts and myotubes have the potential to function as antigen presenting cells, which could lead to the proliferation of T cells specific for the particular antigen being presented if an appropriate environment is present.\textsuperscript{133} However these functions are lost as myotubes develop into myofibers. MHC class I and class II are not normally expressed at
detectable levels in normal muscle. The MHC class I expression has been observed on the sarcolemma of apparently normal and regenerating fibers, myofibers with infiltrating mononuclear cells and on endothelial cells in patients with DM, PM, IBM, and CMMM. The MHC class II DR antigen was identified on the sarcolemma of affected muscle in 33% of patients with DM, 29% of patients with PM, and 100% of patients with IBM. The exact cause of MHC upregulation in adult myofibers is not known in patients with IMMs, however it is believed to be secondary to increased pro-inflammatory cytokines, as almost all studies looking at cytokine expression in IMMs show upregulation of various pro-inflammatory cytokines. IFN-γ, TNF-α, IL1-α and 1-β, and macrophage inflammatory protein-1-α have been shown to induce or increase MHC class I expression. In mouse models of PM, IFN-γ is important for the development of immune-mediated disease with significant infiltration of CD8+ T lymphocytes. TGF-β has been shown to reduce the expression of MHC class I. Matrix metalloproteinases (MMP-9 and MMP-2) have been identified in patients with PM and IBM, and are suspected to contribute to muscle damage by facilitating lymphocyte adhesion. There are two main mechanisms that may lead to muscle damage in patients with IMMs. The first is related to CD8+ cytotoxicity where the myofiber is damaged by the secretion of perforin (a pore-forming toxin) by cytotoxic T cells (CD8+). The second is related to T cell activation, leading to upregulation of Fas ligand, which interacts with the Fas receptor on the myofiber. Both lead to myofiber damage and ultimately muscle atrophy. Abnormal expression of MHC could allow the myofiber to become an antigen presenting cell, potentially presenting viral, bacterial or autoantigens to cells of the immune system.
An individual has a diverse array of MHC because there are multiple genes related to MHC expression leading to a highly polymorphic repertoire that allows recognition of numerous antigens.\textsuperscript{122} There are several specific MHC genotypes that have been linked to increased risk of autoimmunity.\textsuperscript{142} The major MHC loci are found on chromosome 6 in humans\textsuperscript{143} and chromosome 20 in horses.\textsuperscript{144}

ii. Autoreactive lymphocytes

There are many stages in the development of the immune system that are crucial to prevent the production of autoreactive immune cells.\textsuperscript{122} However occasionally mistakes are made, or there is a defect in a particular enzyme required for the development of tolerance, such as a deficiency in autoimmune regulator protein (AIRE) leading to autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy.\textsuperscript{145} Due to the marked polymorphism of T-cell receptors, many lymphocytes have a weak affinity for some self-antigens.\textsuperscript{146} In a majority of individuals, these lymphocytes with a weak self-antigen affinity are never activated. However, in some circumstances, the T-cell receptors with weak self-affinity can be activated leading to autoimmune disease.\textsuperscript{147}

A self-antigen may also be a ligand for another receptor in the immune system, such as a toll-like receptor (TLR),\textsuperscript{122} which can occur following excessive cell apoptosis. B cells can phagocytose segments of unmethylated DNA and if specific CpG sequences interact with the intracellular TLR-9 this can lead to activation of the B cell leading to the
production of anti-chromatin antibodies.\textsuperscript{148} This process is believed to be one of the mechanisms causing systemic lupus erythematos.

Many self-antigens are not normally exposed to the immune system because they are normally intracellular.\textsuperscript{122} In certain cases of massive tissue destruction or marked inflammation, intracellular components may be released and exposed to the immune system. This process can occur following a myocardial infarction, with ongoing myocardial destruction due activation of autoimmune lymphocytes.\textsuperscript{149}

Immune privileged tissues, such as the brain, have additional barriers in place to prevent autoimmunity such as the brain and the blood-brain barrier\textsuperscript{122} that prevent the passage of naïve lymphocytes.\textsuperscript{150} Cytokines, such as TGF-\(\beta\) are often produced in the immune privileged areas and encourage any immune response against particular antigens to be regulatory T cell responses rather than pro-inflammatory T cell responses\textsuperscript{151} that could have devastating consequences. FasL is also expressed in these sites, and plays a crucial role in inducing apoptosis of any activated lymphocytes entering the area.\textsuperscript{151} However, abnormal exposure of the immune system to tissues from the immunologically privileged sites can lead to autoimmunity.\textsuperscript{150} Multiple sclerosis, where antibodies are produced against myelin basic protein, is an example of this.\textsuperscript{122}

Autoimmune disease usually occurs sporadically and the events that lead to the initiation of the disease process are not fully understood.\textsuperscript{122} Autoimmune diseases often involve multiple components of the immune system and B cells and T cells are usually both
involved to some extent. Diseases predominantly involving T cells include PM, IBM, Type I Diabetes, psoriasis, rheumatoid arthritis and multiple sclerosis. These diseases manifest due to presence of autoreactive T cells that become activated, leading to either direct T-cell damage to the affected tissue or by recruitment of other immune cells leading to inflammation of the affected tissue. Some patients develop autoimmune disease due to the presence of autoantibodies. This includes patients with Grave’s disease, immune-mediated hemolytic anemia and myasthenia gravis. Many diseases involve both autoantibodies and autoreactive T cells, including systemic lupus erythematosus, CMMM, and Sjogren’s syndrome.

Antibody mediated autoimmune disease manifests as antibodies binding to their specific antigen on the surface of a cell. For example, with immune-mediated hemolytic anemia, IgG or IgM antibodies bind the surface of red blood cells. This typically leads to the removal of affected red blood cells due to recognition of the Fc receptor by the mononuclear phagocytic system of the spleen. Or, in some cases, activation of the complement system leads to formation of a membrane attack complex in the cell leading to red cell lysis. In nucleated cells, lysis is rare but accumulation of complement provides a strong signal for inflammation including cytokine release, generation of reactive oxygen species, and activation of the pathways that lead to the production of prostaglandins and leukotrienes. The release of certain complement molecules including C5a, leads to chemotaxis of other immune cells including lymphocytes, macrophages, and NK cells producing more inflammation. In some cases, it is not the cell that is destroyed; instead the antibody is specific for a particular cell receptor. In
cases of myasthenia gravis, the antibody binds to the acetylcholine receptor and prevents binding and internalization of acetylcholine, which leads to the clinical signs of progressive weakness.\textsuperscript{156} One of the key components of defining an autoimmune antibody mediated disease is that the autoantibodies can be transferred into an appropriate recipient, which will also develop the disease.\textsuperscript{122}

T cell specific autoimmune disease leads not only to direct tissue injury but in many cases to the development of an autoantibody response as well.\textsuperscript{157} T cell mediated autoimmune disease is different from autoantibody mediated autoimmune disease because autoreactive T cells transferred into a recipient would not cause the disease in the recipient.\textsuperscript{122} This is because the T cells must not only recognize the self-antigen, but also the MHC that the self-antigen is bound to. Multiple sclerosis is one of the most well recognized T cell mediated autoimmune diseases. T cells specific for several self-antigens, but in particular myelin basic protein, attack these proteins leading to damage to the myelin sheaths that normally surround axons leading to decreased efficiency of neuronal transport.\textsuperscript{158}

Autoimmune disease typically becomes chronic because there is the constant presence of self-antigen.\textsuperscript{122} Due to epitope spreading, the disease can become more extensive as the disease becomes chronic.\textsuperscript{159} As the auto reactive cells or antibodies break down the affected tissue, these tissues are broken down and processed releasing more peptides than can be presented to T cells.\textsuperscript{160} Due to the ongoing inflammation, these T cells are more likely to become activated leading to further auto reactivity and increased severity of the
disease, this is often the case with pemphigus vulgaris.\textsuperscript{159} In some cases, the autoimmune disease can resolve. This occurs when the self-antigen that the autoreactive cells are specific for are removed, and occurs in patients with Type I Diabetes, as the beta cells are completely destroyed.\textsuperscript{122}

iii. Autoantibodies in the immune-mediated myopathies

The recognition of autoantibodies in the serum of patients with IMMs has become important for the diagnosis of different subtypes of IMMs in humans and dogs. To date, autoantibodies have been found in patients with PM, DM, NAM\textsuperscript{161} and CMMM.\textsuperscript{152} Autoantibodies appear to be less prevalent in patients with IBM, which may be useful in differentiation between patients with PM and IBM.\textsuperscript{161} However the autoantibodies are not always specific to patients with myositis\textsuperscript{152} and may be found in patients with other autoimmune diseases.\textsuperscript{161} Autoantibodies that are specific for myositis (myositis-specific autoantibodies) or those that are associated with myositis (myositis-associated autoantibodies) have been found in approximately 60\% of humans with IMMs.\textsuperscript{162,161} Although these autoantibodies appear to be useful diagnostically, the exact role in the pathophysiology of IMMs has not been identified, although there is evidence that they appear to be disease specific rather than secondary to damage to myofibers.\textsuperscript{152}

The most well recognized subtypes of Myositis-Specific Autoantibodies (MSAs) are components involved in translation, such as aminoacyl-tRNA tRNA synthetases,\textsuperscript{163} components that form part of the nucleosome remodeling complex, such as MI-1,\textsuperscript{164} and components that form part of the signal recognition particle complex.\textsuperscript{162} However there
are numerous other autoantibodies that have been reported that appear to be related to separate subtypes of the IMM diseases, particularly DM; for example anti-MDA5. The usefulness of recognizing different autoantibodies in different subsets of patients with a particular IMM is mostly related to the prognosis and the likelihood of response to treatment. It may also be possible to screen patients for autoantibodies as there is evidence that the MSA can be identified before the onset of clinical signs of myositis.

b. Susceptibility to autoimmune disease

Almost every individual has lymphocytes with a low affinity for self-antigens in their bloodstream, however only approximately 3% of the human population in the U.S. develop any form of autoimmune disease. The immune system is highly evolved to prevent autoimmune disease from occurring, and therefore for an autoimmune event to occur there has to be a breakdown in the normal tolerance mechanisms. One of the most important factors required for the development of autoimmune disease seems to be the presence of genetic susceptibility. However, other factors are usually required before an autoimmune disease will develop.

i. Genetic susceptibility

Since the completion of the human genome sequence in 2003, numerous genetic variants have been discovered in an effort to identify the exact mutations involved in human autoimmune disorders. In some individuals, a mutation in a single gene is sufficient to cause autoimmune disease. Most of these genes are genes involved in the early steps of the development of immune tolerance. A mutation in the gene that mediates apoptosis
of autoreactive cells by the Fas pathway leads to an autoimmune lymphoproliferative syndrome with variable penetrance.\textsuperscript{169,146}

Most autoimmune diseases involve multiple genes and are considered complex diseases. Polymorphisms in these genes are present in many individuals, but only when multiple polymorphisms exist that increase susceptibility to autoimmune disease does the development of autoimmunity occur.\textsuperscript{170} Some of the most important polymorphisms involve the MHC loci.\textsuperscript{146} Autoimmune disease in a majority of individuals can be linked to the MHC class I or II loci,\textsuperscript{143} although other genetic polymorphisms may be required for autoimmune disease to be expressed. Rheumatoid arthritis is a disease where MHC alleles appear to be particularly indicative of disease susceptibility.\textsuperscript{171} Particular MHC alleles also appear to play protective roles in preventing autoimmune disease, even in the presence of another MHC allele that is linked to autoimmune disease.\textsuperscript{143} Interestingly although two types of MHC class II alleles (HLA-DRB1*0401 and DRB1*0404) are highly associated with the development of rheumatoid arthritis in people with North European roots,\textsuperscript{171} the same alleles are not associated with rheumatoid arthritis in people with African-American roots.\textsuperscript{172} Mouse models have played an important role in investigating autoimmune disease and they have led to the understanding of two key concepts: 1) regardless of the gene polymorphism, the whole host genetic makeup is ultimately important for the disease phenotype and 2) some genetic polymorphisms leave an individual at increased risk of multiple autoimmune diseases.\textsuperscript{146,167}
ii. Environment

Autoimmune disease can occur spontaneously\textsuperscript{122} but often an external trigger leads to the development of clinical signs, even in a genetically susceptible individual.\textsuperscript{146} The precise environmental trigger is often not known, but there is evidence that environmental factors can lead to autoimmune disease. In monozygotic twins with identical genetic susceptibility to an autoimmune disease, there can be lower than expected rates of disease in one of the twins if they live in different environments.\textsuperscript{173} There is also evidence that patients in Brazil with a genetic susceptibility to pemphigus foliaceus have a lower likelihood of developing the autoimmune disease the further they are away from areas of endemic disease.\textsuperscript{174}

iii. Infection

There are three main mechanisms by which pathogens can cause increased risk to autoimmune disease.\textsuperscript{146} The first is molecular mimicry, where the peptide sequence of the pathogen is similar to a particular human peptide and when exposed to the pathogen the host immune system is activated against that pathogen leading to clonal expansion of lymphocytes specific to that antigen.\textsuperscript{122} This is the case in patients with rheumatic fever where infection by \textit{Streptococcus} bacteria leads to autoimmune destruction of cardiac myosin.\textsuperscript{175} The second pathway is polyclonal activation, where there is clonal expansion of lymphocytes against a particular pathogen peptide.\textsuperscript{122} In some cases, the pathogen may trigger clonal expansion of autoreactive lymphocytes leading to autoimmunity.\textsuperscript{146} The third pathway is the release of sequestered antigens secondary to massive tissue destruction.\textsuperscript{146} The second and third pathways often occur simultaneously, for example in
patients with Type 1 Diabetes, there is evidence that infection with the Coxsackie virus leads to infection of pancreatic islet cells, leading to local inflammation and release of self-antigens that are then exposed to activated T cells which results in autoimmune destruction of pancreatic islet cells.\textsuperscript{176}

iv. Non-infectious causes

Autoimmune diseases are often more common in female individuals than males.\textsuperscript{122} This may be hormone related, and there is evidence in mouse models of systemic lupus erythematosus that estrogen exacerbates the phenotype by affecting the development of tolerance in B cells.\textsuperscript{177} Certain medications, for example cephalosporins, have been shown to increase risk of autoimmune hemolytic anemia.\textsuperscript{178} Gluten is becoming a more common cause of autoimmune disease, and the presence of gliadin (one of the components of wheat gluten) bound to tissue transglutaminase can induce antibody mediated destruction of both components of the gliadin-transglutaminase complex and damage to the intestinal epithelium.\textsuperscript{179} Although the mechanism is not understood, certain cytokines such as TNF-\textgreek{a} appear to have beneficial effects in decreasing the disease severity of autoimmune disease by inhibiting activated T cells when administered chronically.\textsuperscript{180} Regulatory T cells are crucial for decreasing and controlling autoreactivity and are often decreased in patients with autoimmune disease, although the precise cause of the downregulation is not known.\textsuperscript{146}
c) Human immune-mediated myopathies

i. Dermatomyositis

Dermatomyositis is relatively easy to diagnose based on the combination of muscle atrophy, weakness and skin abnormalities.\textsuperscript{181} DM affects both children and adults, and is more common in woman than men.\textsuperscript{120} This disease is different to the other immune-mediated myopathies in that patients develop a rash either at the same time as the muscle weakness begins, or before it starts.\textsuperscript{104} There is an increased prevalence of malignancy in patients that develop DM over the age of 50 years.\textsuperscript{104} The onset of clinical signs is considered subacute and occurs over weeks. On routine blood work, the CK can be normal in cases of DM.\textsuperscript{104}

Muscle biopsy of a moderately affected muscle is important for a definitive diagnosis.\textsuperscript{117} Histopathology typically identifies perifascicular atrophy and inflammation around myofibers and perivascularly (predominantly B cells and CD4+ cells).\textsuperscript{120} The main antigen for DM is the vascular endothelium of the endomysial capillaries. It is postulated that initially antibodies bind to the endothelial cells, leading to the activation of the complement cascade through the classical pathway, resulting in the development of the membrane attack complex that punches holes in the endothelial cells.\textsuperscript{182} The destruction of the endothelial cells and activation of complement leads to the release of pro-inflammatory cytokines including IFN-\(\gamma\) and TNF-\(\alpha\).\textsuperscript{183} The perifascicular atrophy and inflammation is thought to be as a result of decreased perfusion secondary to decreased capillary function.\textsuperscript{120} The inflammatory infiltrate of predominantly CD4+ T cells, B cells,
and macrophages can be so severe that centers similar to germinal centers appear.\textsuperscript{184} MHC class I was present in 100\% of DM cases in one study\textsuperscript{185}, with 60\% having marked detection of MHC I, 30\% moderate and 10\% mild. However the scoring system, as with many other studies, was not well described. Overall, across multiple studies, the detection of MHC class I on the muscle sarcolemma appears to be present in approximately 50\% of patients with DM. MHC class II has also been shown to be present on the sarcolemma in patients with DM\textsuperscript{186} but appears to be more variable in both severity of scoring and the percentage of patients with detectable MHC class II staining.\textsuperscript{185}

ii. Polymyositis

Polymyositis is rare in childhood, and usually presents in people over the age of 18.\textsuperscript{118} The onset of clinical signs is considered subacute and occurs over weeks. Proximal weakness can progress over months to years leading to the necessity of using a wheelchair.\textsuperscript{104} The underlying cause of the development of PM is yet to be identified.\textsuperscript{118} There appears to be an association between the development of PM and the patient being affected with a retrovirus including HIV or HTLV-1.\textsuperscript{187}

Muscle biopsies of affected muscles identify multifocal inflammation, with a predominance of CD8+ T cells, and MHC class I is detected on the sarcolemma of apparently healthy and affected muscle fibers.\textsuperscript{117} There are many similarities in the immunopathology between PM and IBM. When evaluating the invading CD8+ cells, the expression of markers on the cell surface, is consistent with activation and therefore pathogenicity. The presence of MHC class I upregulation on non-necrotic muscle fibers
in combination with the presence of invading CD8+ T cells that are expressing activation markers provides evidence for the mechanism of pathogenicity.\textsuperscript{117} Abnormal MHC expression on apparently healthy muscle fibers is significant, as it is not present in most inflammatory dystrophies or non-immune-mediated myopathies.\textsuperscript{188,189} MHC class I expression in myofibers of PM patients is likely related to the increased expression of pro-inflammatory cytokines because MHC class I can be upregulated in the presence of inflammatory cytokines.\textsuperscript{190} When evaluating the CD8+ T cells, only certain T cell families with particular T cell receptors are present in the affected muscle, which is consistent with them being specific for the self-peptide:MHC-I complexes and the creation of a specific adaptive immune response.\textsuperscript{191} There is also evidence for the presence of co-stimulatory molecules (including CD80+) giving further evidence of immune system upregulation and the secondary signal required for full activation of the cytotoxic response.\textsuperscript{192} The increased inflammatory cytokines in the muscle of PM patients is consistent with the third signal required for activation and expansion of the specific CD8+ cell population.\textsuperscript{190} There are also increased numbers of plasma cells and clonally expanded B cells\textsuperscript{193} in patients with PM.

iii. Sporadic Inclusion Body Myositis
IBM has many similarities to PM.\textsuperscript{118} It is generally observed in people over the age of 50 and is more common in men than women as compared to DM.\textsuperscript{120} Serum CK activity can be normal in patients with IBM. There appears to be an association between the development of IBM and the patient being affected with a retrovirus including HIV or HTLV-1.\textsuperscript{187} Patients positive for HIV who develop IBM have invasion of MHC class I
positive myofibers by cytotoxic CD8+ T cells that are specific for the retrovirus.\textsuperscript{194}

Similar muscle biopsy findings to PM are observed, with the invasion of predominantly CD8+ T cells and upregulation of MHC class I ubiquitously in the biopsy sample (on both normal and necrotic fibers).\textsuperscript{117} Patients with IBM have additional findings of vacuoles containing amyloid, and amyloid-related substances such as apolipoprotein E as well as oxidative or stress related proteins near the vacuoles.\textsuperscript{195,196}

iv. NAM

Clinical signs of NAM develop acutely over a few days and serum CK activity is usually extremely high.\textsuperscript{118} NAM muscle biopsies are characterized by necrotic fibers that have been invaded by macrophages. The disease was traditionally thought to be immune-mediated,\textsuperscript{197} but T cells are not usually present and MHC class I is only upregulated in necrotic fibers.\textsuperscript{117} However, there is evidence that the disease could be antibody mediated\textsuperscript{198} and there is evidence of complement deposition on thickened vessels in some patients.\textsuperscript{199} The underlying cause of NAM varies and includes infection with HIV, cancer or treatment with statins.\textsuperscript{197}

v. Therapy of IMMs

Therapy of IMMs is a challenge as the specific antigens targeted by DM, PM, IBM, and NAM are not fully understood. Therefore nonspecific immunosuppressive therapy has been used most commonly. Most patients with PM and DM improve with corticosteroids at an immunosuppressive dose.\textsuperscript{200} Intravenous immunoglobulin has been trialed with some success in patients with DM, PM and NAM.\textsuperscript{201} New therapeutic agents are
constantly being trialed including Rituximab (a B-cell depleting agent) that has been successful in some cases of DM and NAM. IBM carries a poor prognosis and no treatments are effective long term, although they may be partially effective in the short term.

c) Canine immune-mediated myopathies

i. Polymyositis

Polymyositis is one of the most common immune-mediated myopathies in dogs. The disease is characterized by signs of muscle weakness, muscle atrophy and a stilted gait. Similar to humans, autoantibodies to particular muscle sarcolemmal antigens have also been identified in canine patients with PM. Serum CK activity is usually elevated in patients with the disease. In dogs, there are three main subgroups of PM depending on the muscle groups affected; diffuse, extraocular and laryngeal. PM has been reported in numerous breeds and any age of dog, but appears to be more common in large breed mature dogs. A muscle biopsy of the affected muscle is usually considered the gold standard diagnostic test. Canine PM is characterized by T cell mediated destruction of muscle fibers and has many similarities to the human form of PM, including evidence of T cell receptor activity, primarily of α/β T cells with only occasional γ/δ T cells being present. The predominant inflammatory cellular infiltrate is lymphocytes, with more CD8+ T cells than CD4+ T cells. B cells are generally not present in PM cases. A large proportion of the cases have abnormal expression of MHC class I and MHC class II on the myofibers with and without lymphocytic infiltration.
ii. Canine Masticatory Muscle Myositis

Canine Masticatory Muscle Myositis is a condition seen in all breeds of dog, although larger breed dogs may be more commonly affected and it does not have an age or sex predilection.\textsuperscript{153} This disease is often recurrent and leads to progressive atrophy and fibrosis of the masticatory (masseter, temporalis, and pterygoid) muscles.\textsuperscript{208}

Masticatory muscles contain a unique isoform of myosin that differs both histochemically\textsuperscript{209} and biochemically\textsuperscript{210} from myosin isoforms found in the limbs. Autoantibodies against the specific masticatory muscle myosin isoform found in canine type 2M fibers are present in 85-90\% of patients with CMMM and these are suspected to play an important role in the pathogenesis.\textsuperscript{153} To date, three muscle specific autoantibodies have been identified in patients with CMMM; directed against myosin binding protein-C,\textsuperscript{152} and masticatory myosin heavy and light chains.\textsuperscript{153} These autoantibodies are not found in patients with inflammatory myopathies caused by infectious agents or PM and therefore appear to be specific to the CMMM disease.\textsuperscript{152} Diagnosis of CMMM can be based on identification of serum autoantibodies against type 2M muscle fibers.\textsuperscript{211}

Muscle biopsies of patients with CMMM often contain both CD4\(^+\) and CD8\(^+\) T cells infiltrating the myofibers to a varying extent.\textsuperscript{114} However, in most cases of CMMM, CD4\(^+\) cells are present in greater numbers than CD8\(^+\).\textsuperscript{206} Variable amounts of MHC class I expression occurs on the surface of muscle fibers, with expression documented in 68\% of CMMM cases that had lymphocyte infiltrates.\textsuperscript{114} MHC class II is upregulated to
varying degrees in a majority of the affected muscle from dogs with acute or chronic CMMM.\textsuperscript{207} B cells are present in most cases of CMMM in varying numbers.\textsuperscript{206} CMMM has some features that are similar to human DM.\textsuperscript{205} Interestingly, patients with more chronic CMMM have less MHC class I and class II expression than patients with acute disease.\textsuperscript{207}

iii. Dermatomyositis

Canine DM is limited to select families of specific breeds of dogs, particularly Collies\textsuperscript{212} and Shetland Sheepdogs.\textsuperscript{213} Affected dogs often show signs early in life, with a majority showing signs before they are 6 months old.\textsuperscript{214} Clinical signs include skin lesions, particularly of the face and ears\textsuperscript{202} and muscle weakness and atrophy.\textsuperscript{214} Signs are typically only generalized in severely affected dogs and the lesions are usually identified in the temporalis and distal limb muscles.\textsuperscript{215} Serum CK activity is usually within normal limits.\textsuperscript{202} Biopsies of the skin lesions identify inflammation, follicular atrophy, ulceration and in some cases the development of vesicles.\textsuperscript{202} Muscle biopsies identify inflammation of the perivascular areas and infiltration by lymphocytes, macrophages, neutrophils, eosinophils and plasma cells, and in severe cases myofiber necrosis.\textsuperscript{215} As the disease progresses, myofibers are replaced with collagen.\textsuperscript{202}

d. Non-Immune-Mediated Myopathies with detectable MHC Expression

i. Dysferlinopathies

Dysferlin is a protein important for myofiber membrane repair and movement of vesicles.\textsuperscript{216} Deficiencies in dysferlin lead to failure of sarcolemmal repair following
injury, and therefore leakage of intracellular contents into the extracellular environment of the muscle.\textsuperscript{216} Individuals with an autosomal recessive mutation in the dysferlin gene develop distinct myopathies, including Miyoshi myopathy and limb girdle muscular dystrophy type 2B.\textsuperscript{217} Patients with limb girdle muscular dystrophy often show clinical signs in their later teens, which are characterized by progressive proximal limb muscle atrophy and weakness.\textsuperscript{216,218} Miyoshi myopathy has similar signs that are restricted to the distal muscles of the limb.\textsuperscript{216,217} Typically, patients undergo slow progression and have elevations in serum CK activity.\textsuperscript{188,216,218} Muscle biopsies from patients with dysferlinopathies are characterized by perivascular and endomysial inflammatory cell infiltration and detection of MHC class I on muscle fibers.\textsuperscript{188} Compared with immune-mediated myopathies, there are typically more macrophages than T lymphocytes.\textsuperscript{188,216} and T lymphocytes are rarely seen invading myofibers in patients with a dysferlinopathy.\textsuperscript{188} B cells were not seen in the muscle of patients with a dysferlinopathy.\textsuperscript{188} However in another study, patients with dysferlinopathy appeared to more closely resemble the histopathologic findings of patients with DM, with a high CD4+/CD8+ ratio, and large percentage of B cells.\textsuperscript{219}

70-71\% of patients with dysferlinopathy have evidence of MHC I staining on apparently normal myofibers.\textsuperscript{188,216} In one study, the staining was on myofibers that were close to areas of CD8+ cell infiltration, with most showing <25\% sarcolemmal positivity for MHC class I staining and only one individual having 50-75\% sarcolemmal positivity for MHC class I.\textsuperscript{188} However, another study of patients with dysferlinopathy reported only very mild MHC class I staining.\textsuperscript{219} Only very mild MHC class II staining was found in
2/10 dysferlinopathy patients. Interestingly, in mouse models of dysferlinopathy, MHC class I does not seem to be required for the development of weakness and atrophy as it is not present in all mice, despite all the mice with the dysferlin mutation developing clinical signs. Despite the upregulation of MHC class I and the presence of inflammatory cell infiltration into the muscle, most patients with dysferlinopathies do not respond to treatment with steroids.

ii. Infectious myopathies

Most patients with clinical signs associated with infectious muscle disease, such as Sarcocystis, are presumed to have an underlying immunodeficiency. Clinical signs are similar to many of the inflammatory myopathies and are characterized particularly by malaise and weakness. However, affected animals and humans often have evidence of an inflammatory leukogram. Muscle biopsy findings in dogs are consistent with a marked inflammatory myopathy with severe necrosis. Inflammatory cells include macrophages, CD8+ lymphocytes, and eosinophils. Although MHC class I expression was identified on the surface of sarcocysts, no MHC class I or II sarcolemmal staining was seen on the sarcolemma of myofibers. In contrast, in human patients with toxoplasma myopathy, MHC class I staining of the sarcolemma of both normal and degenerate myofibers was identified in several patients.
There are three main approaches when investigating the heritability of a particular disorder. The first is the candidate gene approach. It requires that a specific gene is thought to be the likely cause of the condition and compares the sequence of one specific gene between cases and controls. However, this approach is limited only to the gene of interest, and other genes that may be associated with the disease cannot be identified. The second approach is linkage analysis, which is useful for diseases with multiple generations of a family available to study, as well as high disease penetrance and a Mendelian pattern of inheritance. This is not often useful in autoimmune diseases because inheritance is usually complex and disease penetrance variable. Linkage analysis, however, has been successful in the investigation of some autoimmune diseases including Crohn’s disease. The third approach to investigating genetic diseases is the genome-wide association (GWA) study, which has become the most important tool for genetic investigation of autoimmune disease. A GWA study compares the frequency of alleles across the genome of affected individuals and controls, identifying variations that could be linked with the disease. The goal of a GWA study is to identify SNPs in one region of the genome that are statistically significantly associated with the disease or trait being studied. Once a region of association is identified, the reference genome can be evaluated to determine if there are genes in this region that could be associated with the disease based on an understanding of the disease pathogenesis and tissue gene expression patterns. These putative candidate genes can then be sequenced to determine if a
polymorphism can be identified in the coding or regulatory region that is consistently present in affected individuals and not in controls.224

Genetic testing has played a significant and increasing role in the medical field over the last few decades.226 Understanding genetic variants behind various diseases is important as it can establish the risk that an individual has for a particular disease, but can also help to improve the understanding of the pathophysiology behind the disease.225 For example, the identification of a MHC locus as associated with a particular disease suggests that the disease is more likely to have an immune-mediated etiology.227 In the horse, identification of genetic variants and the development of genetic tests for particular disease phenotypes is important for the diagnosis of several diseases.228 Screening for some diseases with genetic tests is now required and individuals with certain genotypes are not allowed to be used as show horses.229 The goal of this application of genetic testing is to reduce the incidence of the disease in the population.

a. Genome-wide association studies in horses

Equine GWA studies have become increasingly popular since the publication of the equine genome in 2009.230 A variety of SNP arrays are available with different numbers of SNPs for analysis, including 50,000 (50K) and 70,000 (70K) SNP arrays. To date, equine GWA studies have been successful in identifying both dominant and recessive alleles associated with a variety of health related229 and performance traits.66 One of the first successful GWA studies in the horse was performed on Type 1 Polysaccharide Storage Myopathy (PSSM) a disease known to cause repetitive episodes of
rhabdomyolysis. The GWA study of 100 QHs affected by PSSM and 92 unrelated QHs without PSSM across 105 microsatellite markers identified a region on equine chromosome 10 that was associated with the disease. The glycogen synthase 1 gene (GYS1) was within the region of interest, and sequencing of this gene identified a dominant non-synonymous substitution of a G-to-A base, leading to a change at codon 309 of arginine to histidine was the cause of Type 1 PSSM. Since that study was performed, the mutation has been identified as the cause of Type 1 PSSM in other breeds. Another successful GWA study in the horse was in ‘lavender foal syndrome’, a lethal neurologic disease of Arabian horses. This study used SNP array data from 6 affected foals and 30 healthy related horses and identified a significant region of interest on equine chromosome 1. Further investigation and sequencing of one of the candidate genes led to the identification of a recessive deletion leading to a frame shift and a premature stop codon in exon 30 of the myosin VA gene (MYO5A). Genetic tests are now available for both of these diseases.

The genetic factors involved in diseases with suspected immune-mediated etiologies in the horse have not been particularly well studied. However, two diseases with suspected immune-mediated components have been investigated; equine recurrent uveitis (ERU) and insect bite hypersensitivity. A GWA study of 79 horses with ERU and 65 unaffected horses and an additional 286 horses in an extended control group, across 37,040 SNPs identified a significant region equine chromosome 20. A significantly associated SNP was intergenic, but in proximity to interleukin 17, which could be associated with the disease pathogenesis. The GWA study of insect bite
hypersensitivity explored two different breeds; Shetland ponies (103 affected and 97 unaffected) across 46,888 SNPs and Icelandic horses (73 affected and 73 unaffected) across 51,453 SNPs. A region on equine chromosome 20 explained the greatest amount of the phenotype variance (13%) that could be explained by the individual genotypes in the Shetland pony. In the Icelandic horses, a region on chromosome X explained the greatest amount of the phenotype variance (28%) that could be explained by the individual genotypes.\textsuperscript{235} As with many GWA studies, a specific genetic mutation has yet to be identified that is associated with these diseases. This is likely due to the fact that both studies are likely underpowered as case sizes were smaller than typically recommended\textsuperscript{224} and both diseases are likely multifactorial (where both environment and genetic factors play important roles in the phenotype).\textsuperscript{225}

b. Genetic associations in human immune-mediated myopathies

As with many other autoimmune diseases, the IMMs are considered to be complex genetic diseases, with disease occurring in genetically susceptible individuals\textsuperscript{236} when specific environmental conditions occur.\textsuperscript{237} The most important genetic association in human patients with IMMs to date is on the MHC locus\textsuperscript{238} and recent studies have shown overlap with genetic susceptibility to other autoimmune diseases.\textsuperscript{142} The most basic genetic susceptibilities were identified when it was first noted that there were several families with multiple individuals affected by a particular IMM.\textsuperscript{236} Further evidence was identified when looking at immediate relatives of patients with IMM and noting that there was an increased prevalence of autoimmune disease (21.9\%) in the relatives or affected patients when compared to the age-, gender-, and race- matched controls (4.9\%).\textsuperscript{239} As
with other autoimmune diseases, the prevalence of the familial IMMs was higher in women than men.\textsuperscript{236} Since then, more detailed analysis including candidate gene studies and GWA studies have been performed.

i. MHC associated alleles

One of the first IMM alleles identified as a cause for increased genetic susceptibility in patients with juvenile DM was the HLA B8 MHC Class I allele.\textsuperscript{240} However, ongoing research suggested that this finding may be secondary to linkage disequilibrium (LD) with several HLA class II genes.\textsuperscript{241} Subsequently, HLA DRB1*0301 and HLA DQA1*0501 were identified as increasing the genetic susceptibility to IMMs.\textsuperscript{241} Other potential MHC class II alleles that increase the risk of the development of juvenile DM include HLA DMA*0103 and HLA DMB*0102.\textsuperscript{242} Interestingly, these associations with particular MHC alleles are specific to Caucasian populations.\textsuperscript{236}

In the Japanese population, HLA-DRB1 (an MHC Class II isotype) has been identified as a risk locus for a particular type of DM (anti-melanoma differentiation-associated gene 5-positive).\textsuperscript{243} In other ethnic minorities, the HLA DRB1*0301 does not seem to play a role in the development of IMM but HLA DQA1*0501 does appear to be important for the increased risk of development of juvenile DM.\textsuperscript{244} In Korean patients, no HLA alleles have been identified that increase the risk of IMMs\textsuperscript{236} but there is evidence that HLA DRB1*14 plays a protective role against the development of IMMs.\textsuperscript{245}
One particular environmental risk for IMM is taking a statin drug for high cholesterol. In genetically susceptible individuals (with the MHC Class II allele DRB1*11:01), statins can lead to NAM.\textsuperscript{246} Other HLA alleles have been identified that appear to predispose patients to myositis secondary to other environmental factors such as exposure to D-penicillamine.\textsuperscript{247}

ii. Non MHC associated alleles

In the Japanese population, candidate gene studies have suggested that the signal transducer and activator transcription 4 gene (\textit{STAT4}) is a risk locus for PM/DM.\textsuperscript{248} There are other studies that have identified genes for cytokines (such as TNF-\textit{\alpha}),\textsuperscript{249} cytokine receptors (such as the IL-1 receptor antagonist),\textsuperscript{250} and immunoglobulins that could play an important role in susceptibility to IMMs.\textsuperscript{245,251}

c. Genetic associations in canine immune-mediated myopathies

Similar genetic associations have been found in dogs, although much less research has been performed. In Hungarian Vizsla dogs with PM, there was a significant association with a particular MHC Class II haplotype in the affected dogs.\textsuperscript{252} There is strong evidence that DM is an inherited disease, although the precise genetic anomaly has not yet been identified.\textsuperscript{253} There is one report of a possible autosomal dominant component to the inheritance pattern in Collies.\textsuperscript{254} In Shetland Sheepdogs with DM, there was an association with affected dogs and the region FH3570 on chromosome 35 although the function of the gene is not yet known.\textsuperscript{255} The prevalence of autoantibodies in certain
breeds of dogs with PM (Boxers and Newfoundlands) may be consistent with a genetic susceptibility.256

d. Genetic Associations in equine immune-mediated myositis

Very little is known about the genetic susceptibility of horses with suspected IMM. There is evidence that there may be a genetic susceptibility, in that 91% of the reported cases of IMM were QH or related breeds, which was different to the control population of horses with other neuromuscular disorders.3 The study also reported that there were clusters of horses that shared relationships within six generations.3 The increased prevalence within one breed and particularly within certain families along with the presence of lymphocyte infiltration is similar to many of the human and canine IMMs that have evidence of genetic susceptibility reported.236,252

It is likely that equine IMM is a complex trait. Many immune-mediated disorders are complex, as demonstrated in twin studies where different environments lead to different disease phenotypes despite identical genetic backgrounds.173 There is evidence that environmental factors, such as exposure to respiratory disease, play a role in the development of disease in some Quarter Horses with IMM.3 Despite this, a GWA study is an important first step to identify genetic variants that may be associated with the disease. Typically, large numbers of individuals are recommended to provide sufficient power to detect genetic variants associated with disease.224 However, studies into immune-mediated myopathies have successfully identified plausible genetic variants associated with disease phenotype using smaller numbers of cases and controls.252 During the GWA
analysis, each SNP is tested for association with the phenotype, therefore controlling for multiple testing is important to decrease the risk of a false positive SNP-disease association.\textsuperscript{225} The bonferroni significance threshold (set at the total number of SNPs) is the most stringent p-value threshold. However, due to the conservativeness of the bonferroni p-value, there is a risk of overcorrection leading to a type II error. Therefore it is recommended to utilize a suggestive p-value threshold in addition to the bonferroni threshold.\textsuperscript{283}

When designing a GWA study it is extremely important to define the disease phenotype accurately and select appropriate controls.\textsuperscript{222} The ability to define horses with IMM based on signalment, history, clinical signs, and muscle biopsy findings should provide the most accurate means of selecting one specific form of IMM in horses to study. When selecting controls for a study of IMM, it is important to select a representative sample of the same breed ideally housed under the same environmental influences and matched as closely as possible for age and gender.\textsuperscript{257}

If a region of interest were identified in a GWA, the next steps would include evaluating the reference genome for potential candidate genes and sequencing candidate genes to determine if a mutation exists in IMM affected horses that is not present in controls.

From this literature review, it is clear that there is preliminary evidence for an immune-mediated etiology for suspected IMM in horses, however, further evidence for an
immune-mediated basis is needed. Confirming that a disease is immune-mediated can be challenging. Identification of ubiquitous MHC expression in the affected muscles, on myofibers surrounded by cellular infiltration and myofibers that appear normal and have no cellular infiltrate around them appears to be consistent with an immune-mediated mechanism of disease.\textsuperscript{181} The localization of equine IMM almost exclusively to the gluteal and epaxial muscles\textsuperscript{3} is interesting and has some similarities to the specific localization of CMMM to the muscles of mastication.\textsuperscript{208} The identification of the unique type 2M myosin of masticatory muscle fibers led to greater understanding as to why CMMM does not become generalized.\textsuperscript{210} However, to date, no unique isoforms of myosin have been identified in horses. The evidence to support an immune-mediated etiology for equine IMM could be provided by determining if MHC I or II is expressed on the sarcolemma of myofibers of IMM horses and by excluding genetic disorders that have a similar histologic appearance to IMM, such as dysferlinopathy. Finally, with the evidence in other species for certain IMMs being linked to genomic regions encoding MHC and the strong breed association of IMM, further support for an immune-mediated etiology could be provided by linkage of equine IMM to the region of MHC on equine chromosome 20 through a GWA study.

**Hypothesis:**

Quarter Horses are genetically susceptible to an immune-mediated myositis that is characterized by abnormal expression of MHC class I and/or class II on the sarcolemma of myofibers.
This hypothesis will be tested by the following specific aims:

**Specific Aims:**

1. To determine if abnormal MHC class I and II expression is present on the sarcolemma of myofibers of horses with active IMM in the presence or absence of myofiber lymphocytic infiltrates.

2. To characterize the subtypes of lymphocytes in the myofibers of horses with active IMM and correlate this with MHC expression.

3. To determine if equine IMM is significantly associated with a region of the equine genome using a GWA study.
CHAPTER 1

Major histocompatibility complex I and II expression and lymphocytic subtypes in muscle of horses with immune-mediated myositis

Introduction

Suspected immune-mediated myositis was first described in horses in 2007. Affected horses were predominantly QHs or related breeds and presented with severe atrophy of gluteal and epaxial muscles and high serum CK and AST activities. An immune-mediated basis for muscle degeneration was suspected because myofibers within muscle biopsies of IMM horses contained lymphocytic infiltrates, with on average more CD4+ than CD8+ lymphocytes. Lymphocytic infiltrates are typically found in muscle of humans and dogs affected with various types of inflammatory and immune-mediated myopathies with specific lymphocytic subtypes predominating in some forms and not others. For example, a predominant CD4+ infiltrate is described in CMMM and DM whereas a predominant CD8+ infiltrate is found in PM.

Lymphocytic infiltrates, however, are not restricted to immune-mediated myopathies and are also a feature of inflammatory myopathies caused by infectious agents such as Toxoplasma gondii and Neospora caninum. Inflammatory myopathies secondary to
infectious causes, however, often have eosinophilic infiltrates in addition to lymphocytes. Eosinophils have also been found in immune-mediated myopathies such as CMMM in dogs and eosinophilic polymyositis in humans. Muscle inflammation may also be a predominant pathological finding in dysferlin deficient muscular dystrophy in humans.

Expression of MHC class I and in some cases MHC class II on the sarcolemma and occasionally within muscle fibers is another diagnostic tool for assessing many forms of immune-mediated myositis in humans and dogs. Mature muscle fibers in healthy individuals are quite unique in that MHC antigens are not normally detectable on the sarcolemma and staining is limited to the capillary network and endothelial cells of blood vessels. MHC I, however, is detectable on the sarcolemmal surface of developing myoblasts and regenerating myofibers. In addition to sarcolemmal expression of MHC I and II in immune-mediated myopathies, MHC class I expression also occurs in some forms of muscular dystrophy such as limb girdle muscular dystrophy caused by a mutation in the dysferlin gene.

The purpose of the present study was to further explore the pathogenesis of IMM in horses by determining 1) if MHC I and or II expression is detectable on the sarcolemma of myofibers of horses with active IMM, 2) whether there is a relationship between MHC expression and the predominate subtype of lymphocytes within muscle infiltrates and 3) whether dysferlin is expressed on the myofibers of horses with IMM.
MATERIALS AND METHODS

Horses

**IMM:** Records of muscle biopsies submitted to the University of Minnesota Neuromuscular Laboratory (NMDL) between 1995 and 2013 were reviewed to identify the breed, age, gender, and clinicopathologic abnormalities of horses with a primary diagnosis of IMM. All muscle samples had been shipped overnight on frozen gel packs to the NMDL where they were frozen in isopentane pre-cooled in liquid nitrogen and stored at -80°C until further processed. The diagnosis of IMM was based on identification of lymphocytic infiltration having an endomysial or perimysial distribution with invasion of non-necrotic fibers or surrounding blood vessels in hematoxylin eosin (HE) stained muscle sections.

Twenty one muscle biopsies from 18 Quarter Horses and 3 Paint horses that had a history of muscle atrophy were selected for inclusion in the study based on; large numbers of lymphocytes invading myofibers; well preserved frozen muscle tissue with minimal freeze artifact; and the absence of evidence of polysaccharide storage myopathy (PSSM) in periodic acid Schiff’s stains. The mean age was 5.0 years (range: 0.5-19 years). There were 10 mares, 5 stallions and 6 geldings. Muscle biopsies were from the gluteal (n=7), epaxial (n=2) and semimembranosus (n=12) muscles. Median reported serum CK activity was 3,165 U/L; range 123 - 130,000 U/L and median serum AST activity was 3,356 U/L; range 460 – 79,700 U/L. Eighty nine percent (18/21 horses) with reported CK or AST results had elevated enzyme activities.
**Healthy and Disease Controls:** Muscle samples from the gluteus medius muscle of 3 Quarter Horses, 2 mares and 1 gelding, with a mean age of 4.7 years (range: 4-6 years) were selected as healthy controls. The disease controls consisted of 3 horses with acute seasonal pasture myopathy and 3 horses with amylase-resistant polysaccharide storage myopathy (PSSM). The 3 horses with pasture myopathy consisted of 1 Quarter Horse, 1 Paint horse and 1 Appaloosa, 2 geldings and 1 stallion, with a mean age of 5.5 years (range: 1-13 years). Samples were from the gluteus medius (n=2) and semimembranosus (n=1) muscles. The three geldings with PSSM consisted of 1 Quarter Horse and 2 Appaloosas with a mean age of 5 years (range: 3-7 years). Samples were all from the semimembranosus muscle. Review of archived stains determined that muscle samples from horses with pasture myopathy had excessive myofiber lipid in oil Red O stains and evidence of acute myodegeneration without regeneration in HE stains. PSSM muscle samples did not contain regenerating myofibers based on the absence of small basophilic myofibers with prominent central nuclei in HE stains.

**Muscle Analysis**

**Immunohistochemistry:** Immunohistochemical (IHC) staining was performed on 10 μm thick frozen sections that were fixed in acetone (75%) and ethanol (25%) at 4°C for 10 min and had nonspecific binding blocked with normal goat serum (1:10). Muscle sections were incubated with monoclonal antibodies for MHC class I (mouse anti-horse CVS22), MHC class II (mouse anti-horse CVS20), CD4+ (mouse anti-horse HB61A), CD8+ (mouse anti-horse CVS8) and CD20+ (rabbit anti-horse). Secondary antibodies against MHC class I, MHC class II, CD4+, CD8+ (horseradish peroxidase goat anti-mouse IgG)
and CD20+ (HRP goat anti-rabbit IgG) were from EnVision. Immunoreactivity was detected with 3-Amino-9-Ethylcarbazole. Slides were counterstained with Mayer’s hematoxylin. Positive control tissue consisted of normal adult horse tonsil. Negative controls used dilute negative control mouse (or rabbit for CD20+) fraction in place of the primary antibody. As staining for the macrophage marker MAC387 cross reacts with other inflammatory cells, histochemical staining for macrophages was performed with the acid phosphatase reaction. 

Immunofluorescent staining for sarcolemmal localization of dysferlin, alpha sarcoglycan and dystrophin was performed on cryopreserved muscle samples from 5 Quarter Horses with IMM and 1 control horse using acetone/methanol fixation. Antibodies include NCL-Hamlet and NCL Hamlet 2 monoclonal anti-dysferlin antibodies, a polyclonal antibody against alpha sarcoglycan and a monoclonal antibody against the rod domain of dystrophin (DYS1) by methods previously described. Slides were assessed for the presence or absence of dysferlin, dystrophin and alpha sarcoglycan on the sarcolemma compared to control muscle.

**Assessment of MHC I and II:** A scoring system was devised to assess both the percentage of fibers that had some degree of sarcolemmal staining for MHC as well as to assess the extent of MHC staining around the periphery of a myofiber. The whole muscle section was examined at 20x magnification and three separate areas with the most extensive MHC staining and least cellular infiltrate were selected. These selection criteria were used to avoid confusion of MHC staining of lymphocytes with MHC staining of the
sarcolemma. Each selected area was then examined at 40 x magnification to estimate the number of myofibers with MHC staining on some or all of the sarcolemma and scored using; score 0 (none positive), score 1 (<25% myofibers positive), score 2 (25-50% positive), score 3 (51-75% positive) and score 4 (>75% positive). In addition, the average extent to which sarcolemmal staining for MHC extended around the sarcolemma was estimated for all fibers in the region and scored according to 0 (no positive sarcolemma staining), score 1 (<25% of sarcolemma staining positive), score 2 (25-50% of sarcolemma staining positive), score 3 (51-75% staining positive) and score 4 (>75% staining positive). The myofiber and sarcolemma scores for each objective field were multiplied, and then summed to produce an overall MHC I and MHC II score (maximum score of 48).

Assessment of Inflammatory cells

Vascular cuffing: Three areas with the most extensive cellular infiltration surrounding vessels were selected at 20x magnification. Each selected area was then examined at 40x magnification and the percentage of the specific lymphocyte subtype (or macrophages) that stained positively out of the total number of mononuclear cell infiltrates in the area was estimated.

Myofiber infiltrates: To compare the predominance of lymphocyte subtypes and macrophages with respect to the total mononuclear infiltrates the whole muscle section was examined at 20x magnification and the 3 areas with the most extensive myofiber cellular infiltration were selected. Each selected area was then examined at 40 x
magnification and the percentage of the specific lymphocyte subtype (or macrophages) that stained positively out of the total number of mononuclear cell infiltrates in the area was estimated according to; score 0 (none positive), score 1 (<25% of cells positive), score 2 (25-50% positive), score 3 (51-75% positive) and score 4 (>75% positive). In order to also assess the amount of inflammation in the areas examined, a score was also given for the total number of inflammatory cells in the field; score 0 (no inflammatory cells), score 1 (<25% of the field contained inflammatory cells), score 2 (25-50% of the field contained inflammatory cells), score 3 (51-75%) and score 4 (>75%). For each field, the scores for the percentage of cells staining positively and the percentage of the field containing inflammatory cells were multiplied and the scores for the fields in each horse were then summed to produce an overall myofiber infiltrate score (maximum total score of 48).

Archived PAS stains were examined from horses with PSSM to compare the distribution of myofibers with PAS positive inclusions with the distribution of MHC I and II positive fibers.

**Statistical Analysis**

Based on Shapiro-Wilk testing data were not normally distributed. A Mann-Whitney U test was used to compare scores for lymphocytic subtypes within muscle of IMM cases and scores for MHC, lymphocytes and macrophages between IMM cases and disease controls. A Spearman Correlation was used to assess the relationship between the cell
RESULTS

MHC Immunohistochemistry

**Healthy control horses:** Vascular endothelium stained positively for MHC I and MHC II whereas MHC I or II staining was not observed on the sarcolemma of myofibers of healthy control horses (Figure 1A).

**IMM horses:** Vascular endothelium and mononuclear cells were darkly stained for MHC I and MHC II in IMM horses (Figure 2A,B). There was a wide range of MHC I and II staining of the sarcolemma within the same muscle biopsy and among different horses with IMM (Figure 1C-F 3A,B). Both nondegenerate and degenerate myofibers had MHC I or II sarcolemmal staining in IMM horses. Fibers with MHC I or II sarcolemmal staining were scattered throughout the biopsy in areas without mononuclear infiltrates (Figure 1C-F) and concentrated in areas with infiltrates (Figure 3A,B). Fibers with MHC I sarcolemmal staining did not consistently have MHC II staining (Figure 1D,E). In regions with minimal lymphocytic infiltrates, some degree of MHC I sarcolemmal staining was present in 81% (17/21) of IMM horses and MHC II staining present in 71% (15/21) of IMM horses. MHC I scores were higher than MHC II scores in 82% (14/17) of IMM horses that had myofibers staining for MHC (Table 1).
**Disease control horses:** MHC I staining was not present in muscle samples from horses with seasonal pasture myopathy (Figure 1B) but MHC II staining was observed in a few degenerating myofibers of two horses with seasonal pasture myopathy (Table 1). Sarcolemmal and aggregates of cytoplasmic MHC I and II staining were observed in the same myofibers of all three PSSM horses (Figure 4B,C) (Table 1). Unlike IMM cases, myofibers with MHC staining were often clustered along the edge of muscle fascicles, in regions containing fibers with abnormal polysaccharide (Figure 4A), the cytoplasm had stippled MHC staining and the fibers with MHC staining did not appear to be regenerating based on review of HE sections.

**Mononuclear cell histochemistry/immunohistochemistry**

**Healthy control horses**

Macrophages, CD4+ and CD8+ lymphocytes were not observed near blood vessels or among myofibers in healthy control horses (Table 1). A muscle sample from one control horse had a few CD20+ lymphocytes between myofibers.

**IMM horses**

**Vascular Cuffing:** Mononuclear cells, predominantly lymphocytes, were present around at least one blood vessel in the muscle biopsies of all IMM horses. Only a few macrophages were present around blood vessels in IMM horses (Figure 2F). CD4+ cells were present around blood vessels in 76 % (16/21) of IMM horses, CD8+ cells in 71% (15/21) and CD20+ cells in 62 % (13/21) of IMM horses (Figure 2C,D,E).
Myofiber Infiltrates: Mononuclear cell infiltrates were present around or in myofibers in 95% (20/21) of muscle samples from IMM horses. One IMM case only had vascular cuffing. Five horses had >50% of the biopsy consisting of inflammatory infiltrates consisting of CD4+, CD8+, CD20+ and macrophages (Figure 3C,D,E,F). In 48% (10/21) of IMM horses CD4+ lymphocytes predominated over CD8+ and CD20+ cells and in 28% (6/21) of IMM horses CD8+ lymphocytes predominated (assessed by comparison of scores within individuals). The median score for CD4+ lymphocytes in IMM horses was not significantly different from CD8+ scores (p=0.35), CD20+ lymphocytes (p=0.07), or macrophages (p=0.90) in IMM horses (Table 1). Scores for CD8+ and CD20+ lymphocytes (p=0.22), CD8+ lymphocytes and macrophages (p=0.36) and CD20+ lymphocytes and macrophages (p= 0.07) in IMM horses were similar (Table 1). The CD4+ lymphocyte score was positively correlated with CD8+ lymphocyte score (r = 0.53, p = 0.01) (Figure 6) and macrophage score (r = 0.46, p = 0.037). No significant correlation between CD4+ and CD20+ score was identified (r = 0.38, p = 0.09). The CD8+ lymphocyte score was positively correlated with CD20+ lymphocyte score (r = 0.70, p = <0.001) and macrophage score (r = 0.64, p = 0.002).

MHC I scores were positively correlated to MHC II scores (r = 0.89, p = <0.001) (Figure 6) and CD8+ (r= 0.64, p = 0.002) and CD20+ (r = 0.66, p = 0.001) lymphocyte and macrophage scores (r = 0.70, p = <0.001). MHC I scores were not significantly correlated with CD4+ (r = 0.22, p = 0.34) lymphocyte scores. MHC II scores were positively correlated to CD8+ (r = 0.59, p = 0.005), CD20+ (r = 0.61, p = 0.004) lymphocyte and
macrophage \( (r = 0.70, p = <0.001) \) scores. MHC II scores were not significantly correlated with CD4+ \( (r = 0.11, p = 0.63) \) lymphocyte scores.

**Disease control horses**

Seasonal pasture myopathy: CD4+ lymphocytes were not observed in any horses (Table 1). CD8+ and CD20+ lymphocytes were not observed in muscle of 2/3 horses, whereas 1/3 horses had a few CD8+ and CD20+ lymphocytes in the muscle sample (Table 1). A few macrophages were identified in necrotic myofibers in all horses.

PSSM: A few CD4+ and CD8+ lymphocytes were present in muscle samples of one of the three PSSM horses (Table 1). Two of three PSSM horses had a few CD20+ lymphocytes. All 3 PSSM horses had a few scattered macrophages in myofibers.

**Dysferlin, Dystrophin and alpha Sarcoglycan**

Compared to control muscle, muscle samples from all IMM horses tested showed a normal pattern of uniform sarcolemmal staining for dysferlin, dystrophin and alpha sarcoglycan (Figure 5).

**DISCUSSION**

The results of the present study expand upon the previous equine study of suspected IMM in horses\(^3\) and with the presence of lymphocytic infiltration, without evidence of a dysferlinopathy, support an immune-mediated basis for this disorder in Quarter Horses.
Similar to cases of immune-mediated myopathies in humans and dogs infiltrates of CD8+ and CD4+ T cells are observed in myofibers of IMM horses and, when inflammatory infiltrates are present, the majority of Quarter Horses with IMM express detectable levels of MHC I or II on the sarcolemma of some myofibers. This is significant because MHC expression is normally not detectable on mature skeletal muscle fibers but is detectable on myofibers of humans and dogs with immune-mediated myopathies. Investigations into canine and human immune-mediated myopathies have found antigen processing and presentation by myofibers expressing MHC. Thus, MHC expression in myofibers of horses with IMM suggests that these fibers have become immunologically activated and could serve as antigen presenting cells, presenting autoantigens to inflammatory cells.

Over-expression of MHC I is not specific to inflammatory myopathies, however, and may also occur in dystrophin deficient muscular dystrophy, some human limb-girdle muscular dystrophies and limb-girdle muscular dystrophy caused by a dysferlin deficiency. The histopathology of dysferlinopathy bears a resemblance to equine IMM in that infiltrates of CD4+ lymphocytes can be present together with MHC I and rarely MHC II expression on myofibers. A lack of dysferlin on the sarcolemma, a characteristic of dysferlinopathies, was not identified in immunofluorescent stains of IMM horse muscle. Neither do our results support dystrophin or sarcoglycan deficient muscular dystrophy as a cause of IMM since dystrophin and alpha sarcoglycan were clearly evident on the sarcolemma of IMM affected horses.
MHC II antigens are typically restricted to antigen presenting cells of the immune system and interact with CD4+ lymphocytes. MHC II staining of the sarcolemma was apparent in nondegenerate myofibers of some IMM horses as well as in degenerate myofibers of IMM and some disease control horses. In other species, MHC II sarcolemmal staining does not appear to be as consistent a feature of inflammatory myopathies as MHC I sarcolemmal staining, however, when present, MHC II is specific for the idiopathic inflammatory myopathies.\textsuperscript{185} Although scores for MHC II expression correlated with scores for MHC I expression, MHC I and II expression did not necessarily co-localize to the same myofibers in IMM horses.

MHC expression on the sarcolemma of myofibers of IMM horses was not uniform and often only involved scattered myofibers or a portion of the sarcolemma. Variability in MHC expression with immune-mediated myopathies may relate to the degree of cytokine expression in myofibers, type of fibers affected, severity of inflammation or stage of disease.\textsuperscript{185} Cytokines have been demonstrated to up-regulate MHC I and may play a role in muscle expression of MHC I.\textsuperscript{131} In dogs with acute, severe CMMM, MHC I and II staining commonly occurs in over 70% of myofibers whereas in dogs with more chronic disease, MHC I and II staining is much less extensive.\textsuperscript{207} Unfortunately, the onset of clinical signs relative to the time of muscle biopsy was not recorded for many of the cases in the present study and it was not possible to determine if there was a temporal relationship between time of biopsy, the amount of MHC staining on the sarcolemma and time course of infiltration of myofibers with lymphocytes. Since the presence of lymphocytic infiltrates was an inclusion criteria for the study, it is not possible to know
whether MHC I or II expression occurred without active inflammation, but subjectively it appeared that those biopsies with the most severe lymphocyte scores usually had the most extensive MHC expression and those with the least infiltrates usually had the least MHC expression. In addition, sarcolemmal membrane integrity is important for reliable MHC antibody binding and it is possible that variable MHC staining was related to degradation of some of the muscle samples prior to being frozen or during shipping. To manage this possibility, IMM samples selected for inclusion in the present study were selected so that they had minimal shipping artifacts, but delay in freezing could have impacted the results of some biopsies.

Most horses with IMM have elevations in muscle enzymes and it was important to rule out rhabdomyolysis in itself as a cause for upregulation of MHC on myofibers. Horses with PSSM and pasture myopathy have rhabdomyolysis without marked myofiber infiltration by lymphocytes. Neither MHC class I or II were detected in normal appearing muscle fibers of horses with PSSM or pasture myopathy. Interestingly, clusters of mature myofibers from horses with PSSM expressed MHC class I and II in the absence of any lymphocytic infiltrates and in the absence of regenerative features such as prominent centrally displaced nuclei or basophilic cytoplasm in HE stains. The distribution of MHC positive myofibers in PSSM horses was very similar to the distribution of fibers with aggregates of abnormal polysaccharide. A number of protein aggregates have been found in myofibers of horses with type 1 PSSM including myoglobin and desmin suggesting that the cytoplasmic aggregates of MHC protein could be a nonspecific finding. Another possibility is that sarcolemmal MHC expression in fibers with abnormal
polysaccharide results from upregulation due to the presence of the abnormal polysaccharide within the myofiber and recognition of the polysaccharide as a foreign material. The lack of cellular inflammatory response in the face of MHC I or II expression, however, suggests that the sarcolemmal MHC:peptide complexes are recognized as ‘self’ peptides in PSSM horses and therefore no additional activation of the immune response occurs. A further explanation for a lack of inflammatory response in the face of MHC expression could be a lack of upregulation of those pro-inflammatory cytokines that directly incite an immune response. MHC expression has been found to occur in other myopathies that do not have an immune-mediated etiology and the reason for this is not fully understood.

Scores for MHC I expression were significantly correlated with scores for predominance of CD8+ lymphocytes, which considering that CD8+ lymphocytes require MHC I expression for activation is not surprising. However scores for MHC II expression were not correlated with scores for predominance of CD4+ lymphocytes in IMM muscle biopsies even though CD4+ lymphocytes require MHC II expression for activation. This could be due to the fact that both CD8+ and CD4+ lymphocytes were present in significant numbers in IMM muscle with 48% of IMM horses having a predominance of CD4+ cells compared to CD8+ and CD20+ lymphocytes. The mixed population of CD4+ and CD8+ lymphocytes in horses with IMM would suggest that multiple immune-mediated events lead to myofiber destruction. The CD8+ lymphocytes lead to direct cell mediated cytotoxicity and the CD4+ cells typically mediate disease through interaction with B cells and the antibody response. In many of the human and canine immune-
mediated myopathies there is a characteristic predominance of either CD4+ or CD8+ lymphocytes.\textsuperscript{268} For example, PM is characterized by a predominantly CD8+ infiltrate\textsuperscript{117} and DM and CMMM are characterized by predominantly CD4+ lymphocytic infiltrates.\textsuperscript{116,269} Although CD4+ cells predominate in CMMM, affected masticatory muscle are reported to have higher MHC class I than II scores suggesting that there may not be a strong correlation between MHC I and II expression and scores for CD8+ and CD4+ lymphocytes in muscle biopsies.\textsuperscript{207} DM is relatively unique in that it typically presents with characteristic skin lesions in addition to muscle weakness.\textsuperscript{256}

In conclusion, MHC I and II expression occur in mature nondegenerate myofibers of horses with IMM in conjunction with a phase of active lymphocytic infiltration; findings which together support an immune-mediated etiology for the disease. Unlike IMM, MHC expression in select myofibers of PSSM horses occurs in the absence of lymphocytic infiltrates. Because the degree of MHC sarcolemmal expression varies in IMM affected horses and is also present in horses with PSSM, MHC staining alone is not a diagnostic test for IMM in horses. Muscular dystrophies associated with dysferlin, dystrophin and sarcoglycan deficiencies were ruled out based on normal IF staining of IMM muscle samples.

**FOOTNOTES**

a. EnVision Systems, Dako North America, Inc. 6392 Via Real, Carpinteria, CA 93013

b. Novocastra, Leica Biosystems, Buffalo Grove, Illinois, USA

c. Gift from Eva Engvall
Table 1. IMM horses muscle scores: MHC I and II, lymphocyte and macrophage scores of myofiber infiltrates in IMM horses. The higher the MHC scores the more fibers with MHC sarcolemmal staining. The higher the lymphocyte or macrophage score, the higher the proportion of mononuclear cells of that specific subtype (CD4+, CD8+, CD20+ or macrophages) was relative to the total number of inflammatory cells. There was no significant difference between scores for MHC I vs II staining or among lymphocyte subpopulations in IMM muscle indicating most IMM muscle had a mixture of inflammatory cell infiltrates.

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**Figure 1. Control and IMM horse muscle:** A. MHC I staining of a healthy control muscle showing endothelial staining of capillaries but no staining of the sarcolemma. B. MHC I staining of a pasture myopathy disease control muscle showing endothelial staining of capillaries but no staining of the sarcolemma of degenerate myofibers. C. Focal sarcolemmal staining of an individual myofiber in a horse with IMM (IMM horse 1). D. Generalized MHC I staining of the sarcolemma in a horse with IMM (IMM horse 2). E. A lack of sarcolemma MHC II staining in a serial section of muscle from the IMM horse 2. F. MHC II staining of an individual degenerating myofiber containing mononuclear infiltrates (IMM horse 3).
Figure 2. IMM horse 3 muscle. A. MHC I staining of the endothelium and the mononuclear infiltrates that surround two arterioles. B. MHC II staining of the endothelium and the mononuclear infiltrates that surround two arterioles. C. IHC staining for many CD4+ lymphocytes that are surrounding the arterioles. D. IHC staining of a small number of CD8+ lymphocytes surrounding the arterioles. E. IHC staining of a small number of CD20+ lymphocytes found scattered around arterioles. F. Acid phosphatase staining of macrophages (aggregates of deep red stain) around an arteriole.
Figure 3. IMM horse 2 muscle. A. MHC I staining of mononuclear infiltrates that is so dense it obscures potential sarcolemmal MHC I staining. B. MHC II staining of mononuclear infiltrates without evident MHC II sarcolemmal staining. C. IHC staining of numerous CD4+ lymphocytes in the endomysium and within myofibers. D. IHC staining of CD8+ lymphocytes in the endomysium. E. IHC staining of a few CD20+ lymphocytes scattered in the endomysium. F. Acid phosphatase staining of numerous macrophages (red aggregates) infiltrating myofibers.
Figure 4. PSSM horse muscle. A. Abnormal Periodic acid Schiff (PAS) positive inclusions in myofibers (arrow). B. MHC I staining of the sarcolemma and granular material within myofibers. C. MHC II staining of the sarcolemma and granular material in the same fibers.

Figure 5. Immunofluorescent staining of IMM (horse 2) and healthy control muscle for localization of dysferlin, dystrophin and alpha-sarcoglycan. Cryosections stained with antibodies against dysferlin, dystrophin and alpha-sarcoglycan show comparable staining of the sarcolemma in IMM and control horse muscle.
Figure 6. Correlation between CD4+ and CD8+ lymphocyte and MHC class I and II scores. MHC I scores were positively correlated to MHC II scores \( (r = 0.89, \ p < 0.001) \). CD4+ scores were positively correlated to CD8+ scores \( (r = 0.53, \ p = 0.01) \).
CHAPTER 2:

Identification of a Genetic Locus for Immune-Mediated Myositis in Quarter Horses

Introduction

Immune-mediated myopathies are an important cause of morbidity and, in some cases, mortality in several species including humans, dogs, and horses. Common features include muscle atrophy, weakness and infiltration of the muscles with inflammatory cells, particularly lymphocytes and abnormal expression of MHC on the myofiber sarcolemma. There are several different IMM subtypes, including IBM in humans, PM and DM, which are reported in dogs and humans, and CMMM.

The cause or causes of autoimmune diseases such as IMM are not well understood, but typically a genetic predilection combined with environmental stimuli appear to be involved. This is exemplified by the fact that autoimmune disease has occurred in one monozygous twin but not the other when they live in different environments. Potential environmental triggers include viral or bacterial infections, vaccination, and therapeutics such as statins. Although the precise environmental trigger for equine IMM is not clear, 39% of horses with IMM have a recent history of respiratory disease.

Genetic associations with IMM have been found at various MHC loci in humans and dogs. Since IMM affects predominantly QH and QH-related breeds and certain stallions appear to be overrepresented in the genetic lineage of QHs with IMM, we
hypothesize that there is an underlying genetic variant that causes susceptibility to IMM. The purpose of this present study was to identify genetic variants associated with increased risk for developing IMM by performing a genome-wide association (GWA) study using horses of QH and QH-related breeds with and without IMM that were housed in the same environments.

**MATERIALS AND METHODS**

**Case selection**

DNA was extracted from blood or muscle of 32 QHs and 4 Paint horses with IMM (median age 2 years; range 0.1-19, 15 females and 21 males) and 54 unaffected QHs (median age 2 years; range 1-27; 33 females and 21 males). The IMM cases were selected based on a history of muscle atrophy (particularly of the epaxial or gluteal muscles) and the presence of lymphocytes invading myofibers in a muscle biopsy. Horses with Type 1 Polysaccharide Storage Myopathy based on amylase-resistant polysaccharide in myofibers or the presence of the H309A GYS1 mutation were excluded. Due to the importance of environmental triggers, control QHs were selected from two herds that had active IMM cases. Control horses had no history of muscle atrophy or stiffness consistent with IMM. Horses were selected such that they were not related at least within one generation. Of the horses used for the GWA, 1/36 affected and 41/54 control horses were used in a previous genetic study of neuroaxonal dystrophy.273
Genome-wide association

35 horses with IMM and 13 control horses were genotyped across 74,500 SNP markers with the Equine SNP 70K BeadChip and 1 horse with IMM and 41 control horses were genotyped across 54,602 SNPs with the Equine SNP 50K BeadChip. Datasets were merged and only SNPs that passed quality control settings (minor allele frequency >1%, genotyping across individuals >90% and hardy-weinberg p>0.001) were selected. A case/control standard allelic GWA was performed using plink, genome-wide efficient mixed model association (GEMMA), and GenABEL. Population stratification was estimated by assessing the genomic control inflation factor (λ). If λ=1, there is no population stratification present and therefore the results are not likely to be influenced by the population structure.

As population stratification was evident in this population of horses (Figure 7), two different linear mixed models were used to account for population substructure and relatedness. First, the linear model was performed using an exact method using a genome-wide efficient mixed model association (GEMMA) to avoid repeatedly estimating the variance components when each test was performed. The linear mixed models utilize a kinship matrix estimated from identical by descent distances to produce clustering for relatedness and population substructure. Second, using the R package GenABEL, a genome-wide rapid association using mixed model and regression (GRAMMAR) was performed. This estimates the linear mixed model residues under the null model and treats them as phenotypes for genome-wide analysis by the standard linear model.
The GRAMMAR method used two main steps; the first calculated the individual environmental residuals utilizing an additive polygenic model. 279 The second involved performing an association using the residuals. 276 Although the test has a tendency to lose power in highly heritable traits and in populations with a large number of full siblings, 279 the method was used in this study because full siblings were excluded from the analysis where possible. A minor modification of the GRAMMAR method was utilized due to the lack of complete pedigree information. The GRAMMAR genomic control (GRAMMAR-GC) method had three main steps. The first estimated the heritability of the trait using kinship coefficients that were estimated from the available genomic data and environmental residuals were then calculated. 279 Next a simple linear regression test was performed on the markers using the environmental residuals to examine any association that was present. Finally the genomic control step was applied to the test statistic from the second step of the analysis. 279

**Significance thresholds**

A Bonferroni correction for 40,811 tests (the number of useable SNPs) from the GEMMA analysis and 40,467 tests from the GRAMMAR-GC analysis, based on a P_{genome-wide} of 0.05 was determined as 1.2 x 10^{-6}. The Bonferroni correction was utilized to control type 1 error.
Haplotype analysis

For chromosome 11, which demonstrated the only genome-wide significant associations, haplotypes were reconstructed on the individual chromosome using Haploview and the R package haplo.stats. Association testing of both the single markers and haplotypes was performed using 1108 permutations. The adjusted haplotype-wide significance threshold was $P_{\text{corrected}} = 0.05$.

RESULTS

Genome-wide association study

Following quality control, 40,811 SNPs remained (6146 excluded for minor allele frequency <1%, 31215 excluded for genotyping <90% and 218 excluded for failing hardy Weinberg equilibrium (p<0.001)). Genomic inflation ($\lambda$) was estimated at 1.99, indicative of population stratification. Analysis of multi-dimensional scaling plots revealed clustering (Figure 7). Due to the elevated genomic inflation, two mixed model analyses were performed utilizing GEMMA and GenABEL. Using the GEMMA relationship matrix, with sex as a covariate, testing was performed across 40,811 SNPs in 90 horses (36 cases and 54 controls) with $\lambda = 1.02$. Seven SNPs on chromosome 11 reached genome-wide significance (Table 2 and Figure 8). As $\lambda$ remained slightly elevated, the GRAMMAR model was utilized to account for population stratification. Further quality control prior to performing analysis with GRAMMAR-GC revealed 3 individuals with low SNP call rates and these horses were excluded from ongoing analysis. Using GRAMMAR-GC, the association testing was performed across 40,467 SNPs from 87
horses (35 IMM cases and 52 unaffected horses), with $\lambda = 0.98$ (Figure 9). The 5 most significant SNPs from the GEMMA analysis remained significant with the GRAMMAR-GC analysis (Table 3).

**Haplotype analysis**

Haplotype analysis of equine chromosome (ECA) 11 identified 5 haplotype blocks that were significant ($P_{\text{unadjusted}} < 4.5 \times 10^{-5}$). The 5 SNPs that reached genome-wide significance with the GRAMMAR-GC analysis were contained within 3 of the significant blocks. One of the additional SNPs identified with only GEMMA analysis was approximately 10,000 base pairs 5’ of one of these 3 significant haplotype blocks. The other SNP identified by GEMMA analysis alone, was within 10,000 base pairs of another haplotype block that reached genome-wide significance. Haplotype analysis was also performed using haplo.stats, a 2 MB region was selected spanning either side of the most significant SNP (11_53982070) because whole chromosome analysis created a memory error in R. This identified 3 significant haplotype blocks present in >5 cases that were associated with disease. Of the 5 SNPs contained within the 2 MB region, all 5 were in a significant haplotype region.

**Evaluation of Candidate Region**

Using a window of 1MB either side of the SNP locus, 4 of the 5 SNPs significant in both analyses were within 1MB of several myosin heavy chain (MYH) genes expressed in skeletal muscle, including *MYH1, MYH2, MYH3, MYH4, MYH8* and *MYH13*. Several transmembrane protein-encoding genes were also present within this region. A family of chromodomain helicase DNA binding protein (CHD) encoding genes were also within
this region around 11_51677777, one of the 2 SNPs that reached significance with GEMMA analysis, but was not significant with GRAMMAR-GC analysis.

DISCUSSION

The present study is the first to identify a significant association of an approximately 2MB region on ECA11 with equine IMM. It highlights the ability to utilize GWA in a small population of horses using well-phenotyped cases and the 50K to 70K SNP platforms. A strong association of 7 SNPs on equine chromosome 11 with equine IMM was identified, 5 of which are contained within 3 haplotype blocks. The strength of the findings was enhanced by using two different statistical models to control for population stratification (GEMMA and GRAMMAR-GC). The cut-off for statistical significance has been controversial in GWA studies because using the Bonferroni significance threshold has a tendency to reduce the power of the study but decreases the risk of type I error. However, the results of both analyses identified the same 5 SNPs on ECA11 as being statistically significantly associated with disease, even with the conservative Bonferroni correction.

Of great interest was the fact that several muscle specific genes are located within 2MB of the genome-wide significant SNPs, including several myosin heavy chain genes. Muscle fibers expressing both MYH1 (MYH-2x) and MYH2 (MYH-2a) are found in equine gluteus medius muscle with over 75% of muscle fibers in the equine gluteus medius muscle comprised of muscle fibers containing either MYH-2a and MYH-2x. The myosin heavy chains appear to be highly conserved across species through evolution.
and relatively few pathogenic variants have been described. A missense mutation in \textit{MYH2} has been identified in Japanese patients with the human IMM: IBM. \textsuperscript{286} Histopathologic features of muscle biopsies from these patients consisted of lymphocytic infiltration and vacuolation of myofibers. Abnormal sarcolemmal MHC type I expression in 100\% of patients tested in the study, included the 3 patients in which the c.2542T>C mutation was identified in the \textit{MYH2A} gene. \textsuperscript{286} The mutation led to an amino acid change from valine to alanine at codon 805 which is in the conserved domain of the gene. All 3 patients appeared to have selective loss of type II fibers, 2 patients had immunohistochemical changes consistent with atrophy only of myofibers expressing myosin heavy chain IIa or IIx. \textsuperscript{286} Of the reported variants in \textit{MYH2} there is evidence of variable gene expression depending on the severity of the mutation. \textsuperscript{285}

Similar to patients with IBM and the c.2542T>C identified in the \textit{MYH2A} gene, horses with IMM have abnormal expression of MHC on the sarcolemma of myofibers in atrophied muscles. \textsuperscript{289} In the present study, \textit{MYH2} was within 2MB of 4 out of the 5 SNPs that reached genome-wide significance. It is possible that, due to a variant in the \textit{MYH2} gene, IMM horses produce an abnormal form of myosin. Within the myofiber, the myosin heavy chains are protected from the immune system, but we hypothesize that damage to the myofiber, such as during an episode of rhabdomyolysis could lead to exposure of the abnormal protein, if present, to immune cells and activation of the immune response to the abnormal protein. As with most autoimmune diseases, the environment appears to play a role in the development of equine IMM, with 39\% of cases having a history of respiratory disease prior to the development of the dramatic atrophy.
characteristic of IMM. Activation of the immune system during the episode of respiratory disease could then lead to the episode of IMM.

Although MYH2 is the only MYH gene reported to be associated with a particular IMM in humans, it is possible that other MYH genes play a role in the pathophysiology of equine IMM. Dogs with CMMM have autoantibodies specific to a unique MyHC isoform in the masticatory muscles (type 2M), and the inflammation is mainly limited to type 2M fibers. To date, autoantibodies have not been evaluated in horses with IMM. A unique MYH isoform has not been identified in the horse. Equine IMM affects primarily the epaxial and gluteal muscles although it can be a generalized process. Interestingly, over 75% of muscle fibers in equine gluteus medius muscle are comprised of MYH-2a and MYH-2x fibers and the MYH2 gene encodes the MYH-2a protein. It is therefore possible that a genetic variant in this gene could lead to the production of an abnormal MYH-2a and the development of an immune response to this fiber type. Many other equine muscles, however, contain type 2a muscle fibers including digital flexor and respiratory muscles, which makes it difficult to explain why equine IMM is so localized to specific muscle groups. It could be possible that affected horses have a unique MyHC isoform in affected muscles that contributes to disease susceptibility.

One of the surprising findings in the present study was the lack of association of IMM with the MHC loci on ECA20. Although the underlying pathophysiology of immune-mediated diseases in general are poorly understood, many, including human IMMs and canine polymyositis have genetic variants in the MHC locus that are associated
with disease. It is possible that the MHC locus is not important to the pathophysiology of IMM in horses, however it is also possible that the study had insufficient power to identify an association with the MHC locus due to the relatively small population size and the high genetic variability normally present at the MHC locus.\textsuperscript{122}

Equine IMM is currently diagnosed based on the history, clinical signs and identification of lymphocytes in the affected muscles.\textsuperscript{287} Lymphocytes only appear to be present in the acute phases of disease in many horses and abnormal MHC expression varies markedly across individuals.\textsuperscript{289} Therefore equine IMM can be difficult to definitively diagnose in the more chronic phase. Identification of genetic variants that increase risk to a particular disease will increase understanding of the pathogenesis of the disease lead to improved diagnostic and treatment options.\textsuperscript{168}

In conclusion, the results of this study identified an approximately 2MB region on ECA11 that is significantly associated with the equine IMM phenotype. The significant region contains numerous myosin heavy chain genes, including one that has been associated with the IMM inclusion body myositis in humans. At least one of the SNPs identified are likely to be in linkage disequilibrium with a variant that plays a functional role in the etiology of IMM. Although large populations are typically required to identify genetic associations in complex traits, the results of this study demonstrate that smaller numbers of well-phenotyped individuals from similar environments can be used to identify genetic variants associated with important equine diseases.
FOOTNOTES

a. Illumina, San Diego, CA, 92122, U
Table 2: Significant SNPs following GEMMA analysis, $\lambda = 1.02$. Bonferroni significance threshold = $p < 1.2E-6$

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Table 3: Significant SNPs following GRAMMAR-GC analysis, $\lambda = 0.98$. Bonferroni significance threshold = $p < 1.2E-6$

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Figure 7: An MDS plot showing clustering of IMM cases and controls. A multi-dimension scaling plot demonstrating clustering of IMM horses (black circles) and unaffected controls (open diamonds) consistent with population stratification.
Figure 8: Manhattan plot of genome-wide association analysis with GEMMA. Gender was included as a covariate. Individual chromosomes are represented in different colors along the x-axis. SNPs are represented as individual dots. The y-axis indicates the statistical significance of each SNP (-log10 of the p-value). The red line represents the Bonferroni-corrected p-value cutoff for significant (p<0.05) association.
Figure 9: Manhattan plot of genome-wide association analysis with GRAMMAR-GC. Individual chromosomes are represented in different colors along the x-axis. SNPs are represented as individual dots. The y-axis indicates the statistical significance of each SNP (-log10 of the p-value). The red line represents the Bonferroni-corrected p-value cutoff for significant (p<0.05) association.
CONCLUSIONS

The results of the immunohistochemical study of skeletal muscle from IMM horses support an immune-mediated basis for this disease. The presence of lymphocytes invading myofibers is suggestive of an immune-mediated etiology, particularly in the absence of eosinophils or other evidence of an infectious cause such as sarcocysts.\(^{116}\) The presence of MHC staining in both apparently normal myofibers and myofibers surrounded by lymphocytes is highly suggestive of an immune-mediated mechanism.\(^{181}\) Although there was more extensive MHC I staining than MHC II, the presence of MHC II staining around normal myofibers is highly specific for immune-mediated disease.\(^{185}\) Minimal evidence is available in the literature about the correlation between MHC and lymphocyte subtype but the correlation between MHC I and CD8+ T cells identified is important because MHC I is an essential part of CD8+ cell activation. Although CD4+ and MHC II scores were not correlated, many studies have shown that MHC II severity is less consistent than MHC I and so a correlation would not necessarily be expected.

The results of the GWA study of IMM identified a 2MB region on ECA11 that was associated with IMM, thereby strongly supporting a genetic basis for this disease. Despite using a strict Bonferroni significance threshold as the cut off for genome-wide significance, 5 SNPs remained significantly associated with the disease, when using two different methods of analysis. The SNPs remain significant when adding in haplotypes to the analysis and fall into 3 haplotype blocks suggesting that they are inherited together. ECA11 is particularly interesting because the region of interest contains many of the
myosin heavy chain genes that are known to be expressed in skeletal muscle in the horse, including one (MYH2) that has been associated with an immune-mediated myositis in humans. Two of the genes within the region have been demonstrated to be expressed in the gluteus medius muscle of the horse. Despite the wide usage of GWA studies in the horse, very few have successfully been able to associate regions of the genome that contain likely candidate genes to the disease being studied. PSSM type I and Lavender foal syndrome are the most successful GWA studies performed in the horse that have clearly shown evidence of a genome-wide significant region consistent with the pathophysiology of the disease prior to this study. Both studies identified genome-wide associations with the disease and then subsequently identified mutations by sequencing of genes within the region of interest in cases and controls. In the genetics study, the use of well-phenotyped cases and carefully selected control horses that have been exposed to a similar environment allowed for the identification of a strong genetic association with equine IMM, which adds further support to the disease having an immune-mediated etiology.

Overall, this thesis project has provided evidence to support both parts of our hypothesis that Quarter Horses are genetically susceptible to an immune-mediated myositis that is characterized by abnormal expression of MHC class I and/or class II on the sarcolemma of myofibers. Abnormal MHC staining in the muscles of affected horses, in combination with infiltration of myocytes and blood vessels with predominantly T lymphocytes, supports the conclusion that the disease is immune-mediated. The genome-wide association of several SNPs in one region of ECA11 with the IMM phenotype strongly
supports a genetic component for the etiology of IMM. Although the precise underlying pathophysiology of equine IMM is not fully understood, we hypothesize that a combination of environmental factors and genetic variants lead to the activation of the immune response against the specific muscle groups seen in horses with equine IMM (figure 10).
Figure 10: Current hypothesis of the pathophysiology of equine IMM.

- Damage to myofiber
  Possibly due to rhabdomyolysis
- Release of DAMPs
- Exposure of immune system to DAMPs
  (innate response and subsequently adaptive response leading to memory)
- Trigger factor leading to activation of the immune system
  (infection or vaccination)
- TIME
- Repair of myofibers
- Innate response = cytokine release
- Influx of immune cells, specifically T and B cells
- Upregulation of MHC class I and II
- Equine IMM phenotype

CYTOKINE RELEASE
LIMITATIONS

Relatively small numbers of horses were used in both the immunohistochemistry and GWA part of this study. This likely led to a reduced power of the study to detect a statistical difference between cases and controls. However, both studies demonstrated statistically significant differences between normal controls and cases of IMM, suggesting that there was sufficient power to detect a difference. It is possible that other genetic variants are also associated with equine IMM but did not reach genome-wide significance due to both the stringent Bonferroni value and the small numbers of individuals included in the GWA.

One of the challenges of equine IMM is the ability to diagnose the disease after the acute period. Unfortunately, the MHC staining was variable in many of the individuals included in this study. Due to the retrospective nature of the case collection, it was not possible to determine how long the clinical signs had been present in most of the horses included in the IHC study. This is likely to contribute to the variability of MHC staining. Although CD4+ cells did not correlate with either MHC I or II, CD8+ and CD20+ lymphocyte scores correlated with both MHC I and II, therefore suggesting that horses with active inflammation and marked lymphocytic infiltration are more likely to have higher MHC I and II scores. With this in mind, it is unlikely that MHC staining will be useful for identifying horses with IMM in the chronic stage of infection when the degree of lymphocyte infiltration has decreased. We have insufficient evidence to determine if
abnormal MHC staining is present in the peracute stage, i.e. before lymphocyte infiltration occurs.

The identification of multiple significant SNPs within haplotypes associated with the equine IMM phenotype is not sufficient to determine if the variants are causal in the disease process. The SNPs significantly associated with IMM are likely in linkage disequilibrium with the causal variant, which was not included in the 2 SNP arrays used in this study. However the goal of a GWA study is not to detect the specific variant that causes the disease, it is used to identify a region of interest that can then be explored in greater detail using next generation sequencing.
FURTHER WORK

The evidence from this study that supports an immune-mediated etiology opens several avenues for exploration. One of the challenges of equine IMM is the lack of a suitable diagnostic test to identify at risk individuals, or individuals in the chronic stage of the disease. One possible direction would be to evaluate the serum of affected horses for autoantibodies. MSAs have been reported in most IMMs including DM, PM\textsuperscript{161} and CMMM.\textsuperscript{152,153} Despite the similarities between clinical presentation and immunohistochemical findings of PM and IBM, individuals with IBM are less likely to have MSAs than individuals with other IMMs.\textsuperscript{161} The identification of MSAs in horses with IMM compared with normal horses could provide a gold standard diagnostic test for detecting at risk individuals as there is evidence that MSAs are present before the onset of clinical disease.\textsuperscript{166} The presence of MSAs to a particular myosin isoform identified only in horses with IMM may also support the presence of a unique myosin isoform in horses with IMM.

Equine IMM has many similarities with CMMM including the localization of the clinical signs predominantly to a specific muscle group. CMMM is characterized by autoantibodies to and destruction of a unique myosin heavy chain isoform found only in the masticatory muscles. To date, a unique myosin isoform has not been identified in horses. Another future direction could be to explore RNA sequencing analysis of affected muscle from horses with acute IMM, those with a history of IMM but without active clinical signs and normal horses that have never had a history of IMM. It is possible that myosin heavy chain gene expression is altered in horses with IMM and this would be a
useful exploration to add information to the underlying pathophysiology of equine IMM. Myosin heavy chain protein expression could also be analyzed using immunoelectrophoresis or western blot to detect different protein antigens within the gluteal/epaxial muscle biopsy.

To further explore the genetic risk of IMM, sequencing the region of interest on ECA11 in affected and unaffected horses with IMM will help identify specific variants and whether they lead to coding changes in amino acid sequence or potentially alter regulatory regions of the gene. As many of the variants identified in this study are in intergenic regions of the equine genome, it is likely that the SNPs identified by this study are not truly causative but are in LD with the causal variant, which can be identified by sequencing. Following sequencing, the degree of penetrance that the variant has can be calculated. A fully penetrant variant would be in all IMM horses, but none of the control horses. However, IMM does not appear to be a simple inherited trait and environmental factors appear to play a role in the development of the disease in some individuals. Therefore it is unlikely that the variant will completely segregate with the disease phenotype. This would mean that not individuals with the disease-associated variant develop IMM, which would be classified as incomplete penetrance. Potentially, if the variant has a high penetrance, it could be used to develop a genetic test to identify horses at risk of IMM. Although it would likely be impossible to prevent the disease, this could lead to improved management of these horses and an increased suspicion of IMM in horses developing early clinical signs which will enable the attending veterinarian to treat
the horse earlier on following disease onset, hopefully leading to milder clinical signs and a shorter duration of disease.

If sequencing of the region of interest on ECA11 is not helpful for identifying causative variants, then a new GWA could be performed. It could be beneficial to concurrently recruit additional well-phenotyped horses with IMM and controls ideally in the same environment and submit DNA for SNP genotyping on a larger array platform such as the 670K SNP array that is now commercially available. Increasing both the number of horses and SNPs included in the GWA study should increase the power of the analysis to detect variants associated with the IMM phenotype.
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