Studies on myasthenia gravis in dogs and cats

Thesis submitted to the University of Nottingham for the degree of Veterinary Science Master of Research

Thomas Frederic Nicolas Mignan

Nottingham 2023

Supervisors:

Mike Targett MA VetMB PhD DipECVN SFHEA MRCVS
RCVS & EBVS® European Specialist in Veterinary Neurology
Professor in Veterinary Neurology, University of Nottingham

Mark Lowrie MA VetMB MVM DipECVN MRCVS
RCVS & EBVS® European Specialist in Veterinary Neurology

Winsome West DVM MSc DipACVP AFHEA MRCVS
ABVS® American Specialist in Veterinary Pathology
Associate professor in Veterinary pathology, University of Nottingham

Abstract

Myasthenia is a term used to describe impairment of neuromuscular transmission. Its etymology is derived from the Greek “myasthenia” meaning muscle weakness. Both a congenital and an acquired form exist, each with a different pathophysiology, clinical presentation, list of differential diagnoses, diagnostic approach, treatment, and outcome. Myasthenia is considered one of the most completely understood neuromuscular diseases in dogs and cats, however there are several aspects which warrant further investigations.

The long-term outcome of cats with myasthenia gravis (MG) remains incompletely understood with conflicting results published to date, although it has generally been unfavourable in recent studies. Furthermore, the natural course of MG in cats has not been investigated to date.

Myasthenia gravis is currently classified in dogs and cats according to generalisation, progression, and severity of skeletal muscle weakness and fatigability based on a previous classification system used for humans which has not been updated in over twenty-five years. However, factors such as the presence or absence of a thymoma, or administration of thiourylene medication in cats, can influence treatment, outcome, or both, frequently leading to the division of affected dogs and cats into separate disease groups based on these factors.

Historically, congenital myasthenic syndromes (CMSs) were termed congenital MG which was thought to solely be the result of a postsynaptic deficiency of acetylcholine receptors (AChRs) in the absence of antibodies directed against them. However, it is now clear a congenital post-synaptic acetylcholine receptor (AChR) deficiency is only one of several

clinically heterogeneous congenital myasthenic syndromes affecting the neuromuscular junction (NMJ), resulting in skeletal muscle weakness and fatigability. These syndromes are collectively referred to as CMSs and considered as a separate disease entity from MG. There is currently no established classification system for CMSs in dogs and cats.

A significantly higher incidence of MG associated megaoesophagus (88%) is reported in dogs compared to cats (40%), or even humans in which there are only a few cases reported. This has been attributed to the tunica muscularis of the canine oesophagus being described as solely composed of skeletal muscle as opposed to that of cats or humans in which the distal third and two thirds are respectively reported to contain smooth muscle. Recently, however, the distal third of the tunica muscularis was reported to be composed of smooth muscle in a population of eight Iraqi dogs.

From this, the aims of the present thesis were the following:

- Evaluate the long-term outcome of cats with MG without evidence of a cranial mediastinal mass (CMM).
- Evaluate the natural course of the disease in cats with myasthenia gravis without evidence of a CMM.
- Update the classification system used for MG in dogs and cats based on comparison with published classification systems in human beings.
- Establish a classification system for CMSs in dogs and cats.
- Evaluate the tunica muscularis composition of the oesophagus in dogs.

A set of hypotheses were proposed:

- The long-term outcome of cats with MG without evidence of a CMM is often favourable.
- The natural course of the disease can involve spontaneous remission in cats with MG without evidence of a CMM.
- The tunica muscularis of the canine oesophagus is solely composed of skeletal muscle.

To address these questions, two studies, one retrospective, one prospective, and a literature review, were undertaken.

Study 1 – Long term outcome of cats with myasthenia gravis without evidence of a cranial mediastinal mass.

Eight cats diagnosed with MG without evidence of a CMM. Retrospective case series. The medical records of cats diagnosed with MG between 2005 and 2018 from two veterinary referral hospitals were reviewed for inclusion. Inclusion criteria consisted of a diagnosis of MG, as well as for thoracic imaging, serum biochemistry including measurement of creatine kinase activity, and a haematology profile to have been performed. Exclusion criteria were the presence of an identifiable CMM, or administration of methimazole or carbimazole. All cats had an excellent long-term outcome, achieving immune remission within six months of diagnosis, including four cats that did not receive any treatment and whose natural course of disease involved spontaneous remission. Clinical presentation was heterogeneous, and skeletal muscle weakness and fatigability induced or exacerbated by the wheelbarrow exercise stress test were the most consistent abnormalities associated with MG. Cats

diagnosed with MG without evidence a CMM have a favourable outcome and frequently achieve immune remission. Moreover, the natural history of MG in cats includes spontaneous remission when there is no evidence of a CMM. Attempting to rule out the presence of a CMM therefore refines prognosis, and treatment is not always necessary in this disease population.

Study 2 – Classification of myasthenia gravis and congenital myasthenic syndromes.

We review the published literature on MG and CMSs in dogs and cats, and by comparison with published classification used in humans, propose a classification system for MG and CMSs in dogs and cats. Myasthenia gravis is first classified based on focal, generalised, or acute fulminating presentation. It then is subclassified according to the autoimmune disease mechanism or seronegativity. Autoimmune disease mechanism relates to the presence or absence of a thymoma, or administration of thiourylene medication in cats. Congenital myasthenic syndromes are classified according to the affected NMJ component, the mechanism of the defect of neuromuscular transmission, the affected protein, and ultimately the mutated gene responsible. In proposing this categorisation of MG and CMSs, we hope to aid recognition of the disease groups for both conditions, as well as guide treatment, refine prognosis, and provide a framework for additional studies of these conditions.

Study 3 – Composition of the tunica muscularis of the oesophagus in dogs.

A population of thirty dogs comprising ten small, ten medium, and ten large breed dogs without any history or pathological evidence of oesophageal disease were prospectively recruited. Samples of the cervical, thoracic, and abdominal region of the oesophagus were collected from all dogs. Phosphotungstic acid haematoxylin staining (PTAH) was consistent

across all thirty dogs, and throughout the cervical, thoracic, and abdominal regions of the oesophagus, revealing strong positive staining of the entire tunica muscularis layer, revealing cross-striations consistent with skeletal muscle. Smooth muscle actin (SMA) staining was consistent across all thirty dogs and throughout the cervical, thoracic, and abdominal regions of the oesophagus, revealing strong positive staining in the mucosal layer consistent with the smooth muscle layer that is the muscularis mucosa but no staining of the tunica muscularis. These findings support the theory that MG associated megaoesophagus incidence is negatively correlated with the amount of smooth muscle in the tunica muscularis layer of the oesophagus. Given the lack of smooth muscle in the tunica muscularis, medications targeting the skeletal muscle should be prioritised in management of MG associated megaoesophagus.

In conclusion, the results of this thesis strengthen the understanding of treatment and outcome of MG and CMSs, and highlight future research avenues as well as provide a framework for such studies.

Acknowledgements

I would like to express a heartfelt thank you to my supervisors Mike, Mark, and Winsome for continuously providing me with encouragement and willingly and enthusiastically assisting me throughout the research projects that make up this thesis. Their expertise and mentoring skills proved invaluable, and I hope to one day emulate this. A special thank you also goes to my partner Corinne and to my family who have always provided me with unwavering support.

Abbreviations

ACh – Acetylcholine

AChE-I – Acetylcholinesterase inhibitor

AChE-Is – Acetylcholinesterase inhibitors

AcCoA – Acetyl coenzyme A

AChR – Acetylcholine receptor

AChRs – Acetylcholine receptors

CMS – Congenital myasthenic syndrome

CMSs – Congenital myasthenia syndromes

CMM – Cranial mediastinal mass

CHAT – Choline acetyltransferase gene

ChAT – Choline acetyltransferase

COLQ – Collagen-like tail subunit of asymmetric acetylcholinesterase gene

CHRNE – Cholinergic receptor nicotinic ε subunit gene

FIV – Feline immunodeficiency virus

FeLV – Feline leukemia virus

LRP4 – Low-density lipoprotein receptor related protein 4

MG – Myasthenia gravis

NMJ – Neuromuscular junction

PTAH – Phosphotungstic acid haematoxylin

SMA – Smooth muscle actin

MUSK – Muscle specific kinase

VAChT – Vesicular acetylcholine transporter

WEST – Wheelbarrow exercise stress test

3,4-DAP – 3,4-diaminopyridine

Table of content

List of tables

List of figures

Publications

Mignan, T, Targett, M, Lowrie, M. Classification of myasthenia gravis and congenital myasthenic syndromes in dogs and cats. *Journal of Veterinary Internal Medicine*. 2020;34: 1707–1717. https://doi.org/10.1111/jvim.15855

Mignan, T, Garosi, L, Targett, M, Lowrie, M. Long-term outcome of cats with acquired myasthenia gravis without evidence of a cranial mediastinal mass. *Journal of Veterinary Internal Medicine*. 2020;34:247–252. https://doi.org/10.1111/jvim.15655

Chapter I: Review of myasthenia gravis and congenital myasthenic syndromes in dogs and cats

1. Introduction

The neuromuscular junction (NMJ) is a highly specialised synapse between a lower motor neuron axon terminal and the muscle fibres it innervates (Sanes 2001). Neuromuscular transmission involves a series of complex and tightly regulated sequential events during which electrical activity in the form of an action potential in the motor neuron is transmitted to the muscle fibres it innervates where it is subsequently converted into mechanical activity in the form of muscle contraction (Hong and Etherington 2011, Rodríguez Cruz et al. 2020). Myasthenia is the term used to describe impairment of neuromuscular transmission (Shelton 2002). Its etymology is derived from the Greek "myasthenia" meaning muscle weakness (Hughes 2015). Both a congenital and an acquired form exist, each with a different pathophysiology, clinical presentation, list of differential diagnoses, diagnostic approach, treatment, and outcome (Shelton 2002).

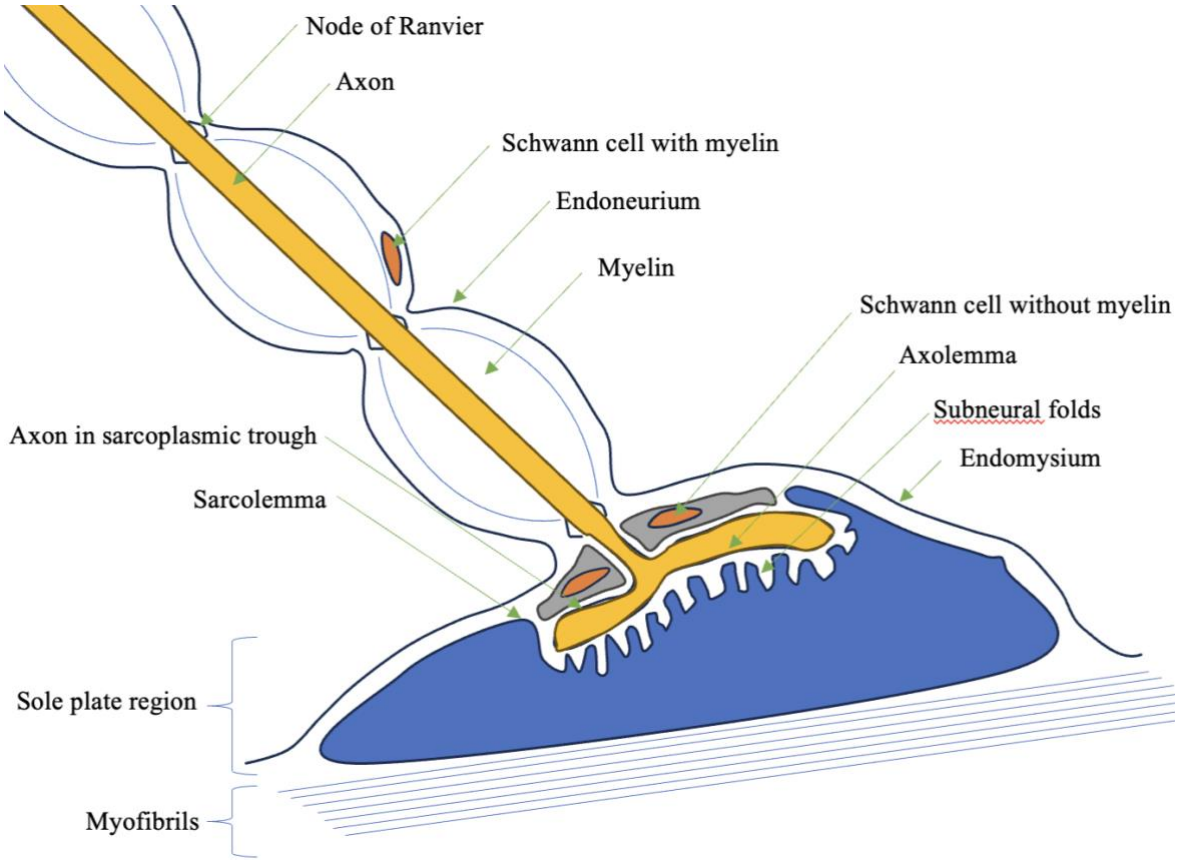
1.1. Anatomy and physiology of the neuromuscular junction

1.1.1. Anatomy of the neuromuscular junction

The presynaptic terminal consists of a distended unmyelinated lower motor neuron axon terminal covered by a Schwann cell until the endoneurium becomes continuous with the endomysium (Desaki and Uehara 1981, Feng and Ko 2008). The distended axon terminal contains numerous mitochondria and acetylcholine (ACh) containing synaptic vesicles and is juxtaposed with the sarcolemma (Desaki and Uehara 1981, Hong and Etherington 2011). It is estimated that each synaptic vesicle contains approximately 5000-10000 ACh molecules (Kuffler and Yoshikami 1975, Martin 1965). The synaptic cleft is the space between the juxtaposed presynaptic axon terminal and the postsynaptic muscle membrane, and it is filled

with a specialised form of extracellular matrix called synaptic basal lamina (Hong and Etherington 2011, Sanes 2003). The surface area of the post synaptic membrane is increased by invaginations forming postsynaptic membrane folds, also called subneural clefts (De Harven and Coers 1959, Hall and Hall 2016). Nicotinic cholinergic acetylcholine receptors (AChRs) are located at the mouth end of the folds, and voltage gated sodium channels are located at the bottom of these folds (Flutcher and Daniels 1989, Wood and Slater 1998). The nicotinic cholinergic acetylcholine receptor (AChR) complex is a transmembrane glycoprotein arranged in a circular shape that forms a direct ionotropic ACh ligand gated sodium channel (Miyazawa et al. 2003). The foetal AChR complex is composed of five subunit proteins, two alpha proteins and one each of beta, delta, and gamma proteins (Cetin et al. 2020). In adulthood, the gamma subunit switches to the epsilon subunit in this receptor complex (Cetin et al. 2020).

Figure 1: Anatomy of the neuromuscular junction as adapted from De Lahunta et al. (2020)



1.1.2. Physiology of neuromuscular transmission

The enzyme choline acetyltransferase (ChAT) synthesizes ACh from acetyl coenzyme A (AcCoA) and choline in the cytoplasm of the axon terminal (Nachmansohn and Machado 1943). Subsequently, vesicular acetylcholine transporter (VAChT) packs ACh into synaptic vesicles (Roghani et al. 1994). Synaptic vesicles accumulate in the presynaptic terminal at release sites referred to as active zones (Sud-hof et al. 2012). Upon arrival of an action potential, voltage-gated calcium channels open, and calcium ions flow into the cytoplasm of the axon terminal (Flucher and Daniels 1989, Martin 1994, Wood and Slater 1997). The inflow of calcium ions activates fusion and release termed exocytosis of ACh from the active

zones into the synaptic cleft through soluble N-ethylmaleimide-sensitive factor attachment protein receptor complex (Baker and Hughson 2016). Acetylcholine molecules then bind to the post-synaptic nicotinic cholinergic AChR, and when bound to both binding sites located at the border between the alpha subunits and the adjacent gamma/epsilon or delta subunit of a given AChR, then a conformational change occurs causing opening of the AChR and an influx of sodium ions inside the myofiber (Cetin et al. 2020, Miyazawa et al. 2003). This influx of sodium ions causes depolarisation of the motor endplate, called endplate potential (Wood and Slater 1995). If the endplate potential reaches the threshold potential, defined as the membrane potential value that must be exceeded in order to activate a sufficient number of voltage gated sodium channels, then an action potential is generated which spreads across the myofiber (Wood and Slater 1995). Under normal circumstances there is both an abundance of ACh molecules released into the synaptic cleft, and an abundance of post-synaptic nicotinic cholinergic AChRs, resulting in an endplate potential that is several times larger than that required to generate an action potential (Wood and Slater 1997). This is called the neuromuscular safety factor (Wood and Slater 1997). The abundance of AChRs is important because with sustained muscle contraction, a phenomenon termed “run down” occurs whereby progressively less ACh is released into the synaptic cleft (Kamenskaya et al. 1975, Wood and Slater 2001). Hence the excess AChRs compensates for the decreased amount of ACh being released into the synaptic cleft during sustained contraction, permitting an action potential to still be generated (Wood and Slater 2001). Following the spread of the action potential across the myofiber, a process called excitation-contraction coupling ensues, and describes the series of events by which the myofiber action potential elicits contraction of the myofiber (Calderon 2014, Sandow 1952). The generated action potential spreads across the sarcolemma and penetrates the myofiber through the transverse tubules which are small,

run transversally, and are internal extensions of the cell membrane (Adrian 1969, Franzini-Armstrong and Porter 1964, Huxley 1964). The spread of the action potential through the transverse tubules causes current flow into the sarcoplasmic reticular cisternae which lie adjacent to the transverse tubules (Fill and Coronado 1988). As the action potential reaches the transverse tubule, the voltage change is sensed by dihydropyridine receptors that are linked to calcium release channels, also called ryanodine receptor channels, in the adjacent sarcoplasmic reticular cisternae (Tanabe et al. 1990). Activation of dihydropyridine receptors triggers the opening of the calcium release channels in the cisternae (Tanabe et al. 1990). Influx of calcium ions into the sarcoplasm surrounding the myofibrils is thought to cause a conformational change in the Troponin-Tropomyosin complex, "uncovering" the active site of the actin filament, thus permitting attachment to the myosin cross-bridge heads and muscle contraction trough a "walk along" or "ratchet" process (Hall and Hall 2016, Huxley 1969, Ebashi 1974). Following release into the synaptic cleft, ACh is rapidly removed both by degradation hydrolysis by acetylcholinesterase located in the basal lamina, and by diffusion away from the synaptic cleft (Nachmansohn and Wilson 1951). Hydrolysis of ACh produces choline and acetate (Nachmansohn and Wilson 1951). Choline is recycled and up taken by the presynaptic axon terminal and combined with AcCoA to create ACh through the enzyme ChAT (Nachmansohn and Machado 1943, Nachmansohn and Wilson 1951). A continuous calcium pump located in the wall of the sarcoplasmic reticulum pumps calcium ions from the sarcoplasm into the sarcoplasmic reticulum following muscle contraction (Hasselbach 1964).

2. Congenital myasthenic syndromes

2.1. Pathophysiology

Congenital myasthenic syndromes (CMSs) are a group of heterogenous inherited disorders caused by mutations in genes encoding proteins whose function is essential for the integrity of neuromuscular transmission (Vanhaesebrouck and Beeson 2019). The mechanism by which a given congenital myasthenic syndrome (CMS) interferes with neuromuscular transmission is specific to the role of the affected protein (Vanhaesebrouck and Beeson 2019). There are no circulating antibodies directed against the AChR.

2.2. Clinical presentation

2.2.1. Signalment

Congenital myasthenic syndromes have been reported in dogs in the Jack Russell Terrier, Springer Spaniel, Smooth-Haired Fox Terrier, Miniature Short-Haired Dachshund, and Old Danish Pointing dog, Labrador Retriever, Golden Retriever, Heideterrier, as well as in two mixed breed dogs, and in cats in the Sphynx, Devon Rex, Siamese, and Domestic Shorthair breeds with onset of clinical signs from a few weeks of age, and without any reported sex predisposition (Dickinson and LeCouteur 2004, Dickinson et al. 2005, Indieri et al. 1983, Johnson et al. 1975, Jenkins et al. 1976, Joseph et al. 1988, Palmer and Barker 1974, Palmer and Goodyear 1978, Palmer et al. 1980, Miller et al. 1983, Miller et al. 1984, Shelton 2002, Van Ham 1992, Wallace and Palmer 1984, Rinz et al 2014, Rinz et al. 2015, Abitbol et al. 2015, Gandolfi et al. 2015, Blakey et al. 2017, Herder et al. 2017, Tsai et al. 2019) (Table 1).

Table 1: Reported cases of congenital myasthenic syndrome in dogs and cats

Study	Species	Breed	Age	Sex
Palmer and Barker 1974	Dog	Jack Russel Terrier	6 weeks	Male
Johnson et al. 1975	Dog	Springer Spaniel	6 weeks	Litter of males and females
Jenkins et al. 1976	Dog	Fox Terrier	5 weeks	Litter of males and females
Palmer and Goodyear 1978	Dog	Jack Russel Terrier	6-8 weeks	Litter of males and females
Palmer et al. 1980	Dog	Jack Russel Terrier	8 weeks	Male
Indieri et al. 1983	Cat	Siamese	16 weeks	Female
Miller et al. 1983	Dog	Smooth Fox Terrier	6-12 weeks	Litter of both males and females
Miller et al. 1984	Dog	Smooth Fox Terrier	6-12 weeks	Litter of both males and females
Wallace and Palmer 1984	Dog	Jack Russel Terrier	6-8 weeks	Litter of males and females
Joseph et al. 1988	Cat	Domestic Shorthair	20 weeks	Male
Flagstad et al. 1989	Dog	Old Danish Pointing Dog	12-16 weeks	Litter of both males and females
Van Ham 1992	Dog	Mixed breed	5-9 weeks	Litter of both males and females
Dickinson et al. 2005	Dog	Smooth-Haired Miniature Dachshund	6 weeks	Litter of both males and females
Rinz et al. 2014	Dog	Labrador Retriever	2-3 weeks	Female
Abitbol et al. 2015	Cat	Devon Rex and Sphynx	3-23 weeks	Litter of both males and females
Herder et al. 2017	Dog	Heideterrier	1 week	Unknown

Blakey et al. 2017	Dog	Mixed breed	12 weeks	Male
Tsai et al. 2019	Dog	Golden Retriever	6-8 weeks	Litter of both males and females

2.2.2. Clinical signs

Congenital myasthenic syndromes present as progressive generalised skeletal muscle weakness and fatigability, ultimately rendering the affected dog or cat unable to walk (Dickinson and LeCouteur 2004, Dickinson et al. 2005, Indieri et al. 1983, Johnson et al. 1975, Jenkins et al. 1976, Joseph et al. 1988, Miller et al. 1983, Palmer and Barker 1974, Palmer and Goodyear 1978, Palmer et al. 1980, Shelton 2002, Van Ham 1992). There are no specific clinical forms reported, and megaoesophagus is uncommon except in the Smooth-Haired Fox Terrier (Bailey 2012, Penderis and Martin-Vaquero 2016, Shelton 2002).

2.2.3. Neurological examination findings

The neurological examination reveals persistent and progressive weakness that is exacerbated by exercise (Blakey et al. 2017, Shelton 2016, Tsai et al. 2019). Cervical ventroflexion is commonly seen cats, due to the absence of a nuchal ligament (Abitbol et al. 2015). Proprioception remains unaffected (Blakey et al. 2017, Tsai et al. 2019). The withdrawal reflex can be decreased to absent (Blakey et al. 2017). Tendon reflexes such as the patellar reflex, and facial, oesophageal, laryngeal, and pharyngeal reflexes can remain normal (Blakey et al. 2017, Tsai et al. 2019).

2.3. Diagnosis

2.3.1. Differential diagnoses

Differential diagnoses include any condition capable of causing diffuse lower motor neuron tetraparesis including junctionopathies such as MG, botulism, tick paralysis, and numerous drugs or toxins including organophosphates and snake envenomation; myopathies including centronuclear myopathy, myotubular myopathy, muscular dystrophy, immune mediated polymyositis, glycogen storage myopathy, lipid/mitochondrial myopathies, hyperadrenocorticoid myopathy, hypoadrenocorticoid myopathy, hypothyroid myopathy, hyperthyroid myopathy, hypokalemic myopathy, hyperkalemic myopathy, hypoglycemic myopathy; motor or mixed polyneuropathies including acute idiopathic polyradiculoneuritis, degenerative polyneuropathies, diabetic polyneuropathy, hyperadrenocorticoid polyneuropathy, hyperchylomicronemia, hyperoxaluria, hypothyroid neuropathy, paraneoplastic neuropathies, chronic inflammatory demyelinating/relapsing polyneuropathy, protozoal polyradiculoneuritis, toxic polyneuropathies (Bailey 2012, Khorzad et al. 2011, Penderis and Martin-Vaquero 2016, Platt and Shelton 2013). However, given the very young age of onset of CMSs, consideration of conditions with a similar age of onset must be prioritised (Bailey 2012, Penderis and Martin-Vaquero 2016, Platt and Shelton 2013).

2.3.2. Pharmacological testing

Pharmacological testing involves inducing exercise induced weakness followed by administration of a short acting acetylcholinesterase inhibitor (AchE-I) (Dickinson et al. 2005). A dramatic improvement in skeletal muscle weakness and fatigability following pharmacological testing is supportive of the diagnosis of post-synaptic CMS (Shelton 2002).

Short acting acetylcholinesterase inhibitors (AchE-Is) transiently prevent the acetylcholinesterase enzyme from hydrolysing ACh thereby lengthening its residence time in the neuromuscular cleft, hence enhancing binding of ACh to the functional AChRs, resulting in an improvement in neuromuscular transmission and ultimately muscle strength (Pascuzzi 2003, Webb 1971). However pharmacological testing lacks both specificity and sensitivity and does not provide a definitive diagnosis (Dickinson et al. 2005, Flagstad et al. 1989). Indeed, MG and other neuromuscular disorders such as polymyositis can yield a positive response, while AchE-Is have no effect or even cause exacerbation of some CMSs as is discussed in Chapter III (Cridge et al. 2020, Flagstad et al. 1989, Rinz et al. 2015, Tsai et al. 2019). Furthermore, this test is subjective and there is no clear definition of what constitutes a marked improvement (Bailey 2012, Khorzad et al. 2011). Nonetheless, the value of this test lies in its ability to offer a rapid presumptive diagnosis so that treatment can be started promptly while serological measurement of AChR antibody concentration is pending (Khorzad et al. 2011). Both edrophonium chloride and neostigmine methylsulphate can be used, however edrophonium production by the sole producer was discontinued in December 2017 (Dickinson et al. 2005, Cridge et al. 2020). The edrophonium chloride dosage is 0.1-0.2 mg/kg intravenously in dogs, and 0.25-0.5 mg/kg intravenously in cats (Shelton 2002). The neostigmine methylsulphate dosage is 0.01-0.05 mg/kg intravenously (Cridge et al 2020). This test is not without risks as inhibition of acetylcholinesterase can result in overstimulation of the nicotinic and muscarinic cholinergic receptors which constitutes a “cholinergic crisis” (Newark 2007, Rusyniak et al. 2004). The clinical signs associated with overstimulation of the muscarinic receptors include salivation, vomiting, defecation, urination, bronchoconstriction, and bradycardia (Newark 2007, Rusyniak et al. 2004). Whereas overstimulation of the nicotinic cholinergic receptors results in a depolarisation block causing

worsening of skeletal muscle weakness and in severe cases, respiratory paralysis and death (Newark 2007, Rusyniak et al. 2004). Given this, pre-emptive placement of an intravenous cannula, and pre-emptive premedication with an antimuscarinic agent such as atropine at a dosage of 0.02-0.04 mg/kg subcutaneous, intramuscular, or intravenous or glycopyrrolate at a dosage of 0.01-0.03 subcutaneous, intramuscular, or intravenous is recommended (Penderis and Martin-Vaquero 2016).

2.3.3. Electrophysiology

Electromyography which involves recording the electrical activity in skeletal muscle, and motor nerve conduction studies which evaluates both the conduction velocity and the functional integrity of the axons are usually normal in CMSs (Cuddon 2002). Supramaximal repetitive nerve stimulation involves repeatedly stimulating a motor nerve using a stimulus that will activate all of the motor axons, and recording the resulting compound muscle action potential (Cuddon 2002). A stimulation frequency of 2-3 hertz is used because it is fast enough to deplete the immediately available ACh stores yet slow enough to prevent the neurosecretory mechanisms that enhance neuromuscular transmission with post-synaptic CMSs (Cuddon 2002). A train of 10 stimulations is usually used and in CMSs a 10% or more decrease in the amplitude or area of the compound muscle action potential is usually observed with post-synaptic CMSs (Cuddon 2002). Repetitive nerve stimulation can elicit different responses with pre-synaptic or synaptic CMSs and these are discussed in more detail in chapter III. Electrodiagnostic findings of a decremental response are non-specific given that they can also occur with MG and other neuromuscular diseases (Cuddon 2002, Shelton et al. 2002). Although electrophysiology does not permit a definitive diagnosis to be made, similar to pharmacological testing, it allows for a rapid presumptive diagnosis to be

established, so that treatment can be started promptly while serological measurement of AChR antibody concentration is pending (Khorzad et al. 2011). Electrophysiology is usually performed under general anaesthesia in dogs and cats, however general anaesthesia can present a significant risk in animals with a CMS (Khorzad et al. 2011).

2.3.4. Serum acetylcholine receptor antibody concentration measurement

Serology for circulating nicotinic cholinergic acetylcholine receptor antibodies is negative (Shelton 2002).

2.3.5. Ultrastructural quantification of post-synaptic acetylcholine receptor antibody concentration

Ultrastructural demonstration of a reduced concentration of nicotinic post-synaptic AChRs in the motor endplate of a fresh-frozen biopsy specimen from the external intercostal muscles is consistent with a post-synaptic CMS (Dickinson et al 2005).

2.3.6. Genetic analysis

Definitive diagnosis of a CMS requires demonstration of a genetic mutation affecting a protein involved in neuromuscular transmission (Rinz et al. 2014, Tsai et al. 2019). Several CMSs have been genetically confirmed in dogs and cats and are further discussed in chapter III.

2.4. Treatment

Therapy for CMSs is based on the affected protein and whether the CMS is pre-synaptic, synaptic, or post-synaptic. Treatment of CMSs is further discussed in chapter III.

2.5. Prognosis

Long-term term prognosis is reported to be unfavourable as the clinical signs are often progressive, ultimately leading to death (Shelton 2002). However, some affected Jack Russell Terriers and cats have been reported to survive many years (Joseph 1988, Shelton 2002). Furthermore, in Smooth-Haired miniature Dachshunds, the condition appears to resolve spontaneously around 6 months of age (Dickinson et al. 2005). This is suspected to be due to the replacement of the gamma subunit of the AChR by the epsilon subunit (Dickinson et al. 2005).

3. Myasthenia gravis

3.1. Pathophysiology

Myasthenia gravis is an autoimmune disease that involves the production of antibodies, predominantly of the immunoglobulin G class, against post-synaptic nicotinic cholinergic AChRs in skeletal muscle (Shelton et al. 1988). The majority of these antibodies are directed towards a specific region of the alpha subunit of the AChR complex, termed main immunogenic region (Shelton et al. 1988). The main immunogenic region is separate and distinct from the AChR binding site though it is adjacent to it on the alpha subunit (Shelton et al. 1988). The main immunogenic region is located on the extracellular surface of the AChR complex and is hence accessible to circulating antibodies (Shelton et al. 1988). Antibodies are thought to interfere with the neuromuscular junction through three mechanisms (Drachman et al. 1978, Drachman et al. 1982, Engel 1984, Engel and Arahata 1987). Firstly, through complement-dependent lysis of the postsynaptic membrane caused by antibodies bound to AChRs and simplification of the post-synaptic membrane (Engel 1984, Engel and

Arahata 1987). Secondly, by cross-linking AChRs on the surface of the membrane, leading to increased internalisation of AChRs (Drachman et al. 1978). And lastly, by directly interfering with AChR function (Drachman et al. 1982). This results in a decreased number of functional post-synaptic nicotinic cholinergic AChRs in skeletal muscle which therefore cannot compensate for the decreased amount of acetylcholine released into the synaptic cleft during "run down", thereby impairing neuromuscular transmission (Wood and Slater 2001).

3.2. Clinical presentation

3.2.1. Signalment

Dog breeds predisposed to MG are the Akita, Scottish Terrier, German Shorthaired Pointer, Chihuahua, and the Terrier group (Shelton et al. 1997). Cat breeds predisposed to MG are the Abyssinian and Somali breeds (Shelton et al. 2000). There is a bimodal age of onset in both dogs and cats (Shelton et al. 1997, Shelton et al. 2000). Onset is most common in dogs between 4 months and 4 years of age and between 9-13 years of age (Shelton et al. 1997). In cats, onset is most common between 2-3 years of age and between 9-10 years of age (Shelton et al. 2000). Intact dogs appear slightly less likely to develop the disease compared to neutered dogs (Penderis and Martin-Vaquero 2016). Additionally, MG has been diagnosed in a group of young adult Newfoundland dogs from two distinct lineages, and in three young Great Dane littermates suggesting a familial or genetic predisposition in these breeds (Kent et al. 2008, Lipsitz et al. 1999).

3.2.2. Clinical signs and clinical forms

MG presents as exercise induced skeletal muscle weakness and there are 3 forms; focal, generalised, and fulminant MG (Dewey et al. 1997, King and Vite 1998, Shelton 1990,

Shelton 1997, Shelton 2002). Focal MG is defined as weakness in ≥1 focal skeletal muscle group that does not involve the appendicular skeletal muscles (Dewey et al. 1997, Ducoté et al. 1999, King and Vite 1998, Shelton 1990, Shelton 1997, Shelton 2002). These focal skeletal muscle groups are the facial, pharyngeal, laryngeal, and oesophageal skeletal muscles, which can respectively cause reduced ability to blink, dysphagia, decreased gag reflex, laryngeal paralysis, dysphonia, and megaoesophagus (Dewey et al. 1997, Ducoté et al. 1999, King and Vite 1998, Shelton 1990, Shelton 1997, Shelton 2002). The latter of which is respectively reported in 88% and 40% of all dogs and cats with MG, and it is thought that this prevalence difference is due to differences in the muscular composition of the tunica muscularis of the oesophagus (Ducoté et al. 1999, Dewey et al. 1997, Shelton et al. 1997). Focal MG accounts respectively for 36-43% and 15% of all dogs and cats with MG (Ducoté et al. 1999, Dewey et al. 1997, Shelton et al. 1997). Generalised MG is defined as appendicular skeletal muscle weakness, which can range from mild to severe, with or without facial, oesophageal, pharyngeal, or laryngeal skeletal muscle involvement (Dewey et al. 1997, Dickinson and LeCouteur 2004, Ducoté et al. 1997, Shelton 2002, Khorzad et al. 2011). Generalised MG accounts respectively for 57-64% and 70% of all dogs and cats with MG (Ducoté et al. 1999, Dewey et al. 1997, Shelton et al. 1997). Acute fulminant MG is defined as an acute, rapidly progressive, and very severe form of generalised MG frequently but not necessarily causing respiratory failure and death (Dewey et al. 1997, Dickinson and LeCouteur 2004, Ducoté et al. 1999, King 1998, Khorzad et al. 2011, Richardson 2011, Shelton 2002). Fulminant MG accounts respectively for <5% and 15% of all dogs and cats with MG (Dewey et al. 1997, Ducoté et al. 1999, Shelton et al. 2000). Additionally, a drug induced form of MG has been reported in cats with hyperthyroidism treated with methimazole (Shelton et al. 1997, Shelton et al. 2000). In dogs, third-degree atrioventricular

block can occur alongside MG and is thought to be due to antibodies directed against the conducting tissue of the heart or because of secondary focal myocarditis (Hackett et al. 1995). In humans with MG, cardiac manifestations including arrhythmias are thought to be associated with the presence of striational Kv 1.4 antibodies (Shivamurthy and Parker 2014).

3.2.3. Neurological examination findings

The neurological examination is often normal at rest (Shelton 1997, Shelton 2000). However, exercise will induce symmetric progressive skeletal muscle weakness (Shelton 1997, Shelton 2000). Exercise induced skeletal muscle weakness can initially be restricted to the pelvic limbs before progressing to the thoracic limbs, and can thereafter remain more severe in the pelvic limbs than in the thoracic limbs in some dogs (Shelton 1997, Shelton 2000). Cervical ventroflexion is commonly seen in cats, due to the absence of a nuchal ligament (Shelton et al. 2000). As a rule, proprioception remains unaffected (Khorzad et al. 2011). The withdrawal reflex can initially be intact but can become decreased to absent following exercise (Shelton 1997, Shelton 2000). Tendon reflexes such as the patellar reflex are often preserved at rest but can become decreased following repeated stimulation due to fatigue of the quadriceps muscles (Shelton 1997, Shelton 2000). While the cranial nerves are unaffected, the muscles they innervate can demonstrate weakness. The menace response and palpebral reflex can be intact at rest but can become decreased upon repeated stimulation due to fatigue of the orbicularis oculi muscles (Shelton 1997, Shelton 2000). Jaw tone can be decreased due to weakness of the masticatory muscles (Shelton 1997, Shelton 2000). Stridor can be detected if there is laryngeal muscles weakness (Shelton 1997, Shelton 2000). The gag reflex can be decreased due to weakness of the pharyngeal muscles (Shelton 1997, Shelton 2000). Finally, regurgitation as a result of megaoesophagus due to weakness of the oesophageal muscles

occurs in 88% of dogs and 40% of cats (Ducoté et al. 1999, Dewey et al. 1997, Shelton et al. 1997).

Figure 2: Cat with myasthenia gravis exhibiting cervical ventroflexion

3.2.4. Co-morbidities

Several co-morbidities have been reported to occur alongside MG. Most commonly is the presence of a cranial mediastinal mass (CMM), almost invariably diagnosed as a thymoma, which is respectively identified in 3.4-11% and 25.6-52% of all dogs and cats with MG (Forgash et al. 2021, Hague et al. 2015, Shelton et al. 1997, Shelton et al. 2000). Other conditions reported alongside MG in dogs include hypothyroidism, meningomyelitis of unknown origin, nonepitheliotropic cutaneous lymphoma, cholangiocellular carcinoma, anal sac adenocarcinoma, osteosarcoma, oral sarcoma, masticatory muscle myositis, and dysautonomia (Dewey et al. 1995, Levine et al. 2005, Krotje et al. 1990, Moore et al. 1990, Penderis and Martin-Vaquero 2016, Stepaniuk et al. 2011, Ridyard et al. 2000, Shelton et al. 1998, Stenner et al. 2003). In cats with MG, an additional co-morbidity is immune mediated polymyositis (Mayousse et al. 2017).

3.3. Diagnosis

3.3.1. Differential diagnoses

Although the clinical signs of MG are characteristic, there are several other conditions that can mimic the presentation. These differential diagnoses include any condition capable of causing diffuse lower motor neuron tetraparesis including junctionopathies such as a CMS, botulism, tick paralysis, and numerous drugs or toxins including organophosphates and snake envenomation; myopathies including centronuclear myopathy, myotubular myopathy, muscular dystrophy, immune mediated polymyositis, glycogen storage myopathy, lipid/mitochondrial myopathies, hyperadrenocorticoid myopathy, hypoadrenocorticoid myopathy, hypothyroid myopathy, hyperthyroid myopathy, hypokalemic myopathy,

hyperkalemic myopathy, hypoglycemic myopathy; motor or mixed polyneuropathies including acute idiopathic polyradiculoneuritis, degenerative polyneuropathies, diabetic poluneuropathy, hyperadrenocorticoid polyneuropathy, hyperchylomicronemia, hyperoxaluria, hypothyroid neuropathy, paraneoplastic neuropathies, chronic inflammatory demyelinating/relapsing polyneuropathy, protozoal polyradiculoneuritis, toxic polyneuropathies (Bailey 2012, Penderis and Martin-Vaquero 2016, Platt and Shelton 2013). As for CMSs, correlation with the signalment and history of the patient is required in order to prioritise conditions in the differential diagnoses list (Bailey 2012, Penderis and Martin-Vaquero 2016, Platt and Shelton 2013).

3.3.2. Minimum database

A minimum database consisting of a serum biochemistry, haematology profile, and urinalysis enables evaluation of metabolic causes of neuromuscular weakness (Shelton 2010). Additionally, the minimum database permits identification of abnormalities that require intervention, particularly in animals experiencing dysphagia or megaoesophagus who might have compromised fluid or nutritional intake (Penderis and Martin-Vaquero 2016). Furthermore, the results of the minimum database can dictate whether further endocrinological testing is required (Penderis and Martin-Vaquero 2016). Additionally, an electrocardiogram allows evaluation of the presence of third-degree atrioventricular block (Hackett et al. 1995).

3.3.3. Thoracic imaging

Thoracic imaging in the form of thoracic radiographs or computed tomography enables evaluation of the presence of a CMM and/or megaoesophagus (Shelton 1997, Shelton 2000).

To evaluate the latter using radiography, such radiographs should ideally be obtained consciously as sedation and general anaesthesia might cause iatrogenic megaoesophagus (Penderis and Martin-Vaquero 2016). Should thoracic radiographs be normal despite historical suggestion of megaoesophagus, then oesophagography with liquid barium and fluoroscopy could be performed although there is a risk of causing/exacerbating aspiration pneumonia (Pollard 2012). In those circumstances, oesophageal scintigraphy appears to be a safer alternative (Pollard 2012). Thoracic imaging can also identify features consistent with aspiration pneumonia which might have resulted from megaoesophagus (Pollard 2012).

3.3.4. Pharmacological testing

Similar to CMSs, pharmacological testing using a short-acting AChE-I such as edrophonium chloride or neostigmine methylsulphate can rapidly provide a provisional diagnosis of MG (Cridge et al. 2020, Dewey et al. 1997). The underlying mechanism of action is the same as in post-synaptic CMSs where the acetylcholinesterase enzyme is inhibited from hydrolysing ACh thereby lengthening its residence time in the neuromuscular cleft, hence enhancing binding of ACh to the functional AChRs, resulting in an improvement in neuromuscular transmission and ultimately muscle strength (Pascuzzi 2003, Webb 1971). The test is performed in the same manner described for CMSs and a dramatic improvement in skeletal muscle weakness and fatigability following pharmacological testing supports the diagnosis of MG (Shelton 2002). Both edrophonium chloride and neostigmine methylsulphate can be used, however edrophonium production by the sole producer was discontinued in December 2017 (Dickinson et al. 2005, Cridge et al. 2020). Again, pharmacological testing lacks both specificity and sensitivity and does not provide a definitive diagnosis (Cridge et al. 2020, Dewey et al. 1997). Indeed, CMSs and other neuromuscular disorders such as polymyositis

can yield a positive response, while a positive response is not always seen in MG (Cridge et al. 2020, Dickinson et al. 2005). False negative responses in MG are thought to be most common in the fulminant form due to a lack of enough function AChRs to elicit a positive response (Khorzad et al. 2011). There is often little to no improvement observed with this test in the focal form of the disease, with the exception of the palpebral reflex, hence it is usually not performed (Dewey 1997). As when performed for the presumptive diagnosis of a CMS, there is a risk of eliciting a cholinergic crisis and placement of an intravenous cannula and pre-emptive premedication with an antimuscarinic agent such as atropine at a dosage of 0.02-0.04 mg/kg SC, IM, IV or glycopyrrolate at a dosage of 0.01-0.03 subcutaneous, intramuscular, intravenous is recommended (Newark 2007, Penderis and Martin-Vaquero 2016, Rusyniak et al. 2004).

3.3.5. Electrophysiology

As for CMSs, electromyography is usually normal in MG, but in severe chronic cases, abnormal spontaneous activity in the form of fibrillation potentials and positive sharp waves can be seen (Cuddon 2002). Motor nerve conduction studies are normal (Cuddon 2002). Supramaximal repetitive nerve stimulation usually reveals a 10% or more decremental response and is more likely to be seen in the generalised MG compared to the focal form (Cuddon 2002). Again, electrodiagnostic findings are non-specific given that they can also occur with post-synaptic CMSs and other neuromuscular disorders (Cuddon 2002, Shelton et al. 2002). Although electrophysiology does not permit a definitive diagnosis to be made, similar to pharmacological testing, it allows for a rapid presumptive diagnosis to be established, so that treatment can be started promptly while serological measurement of AChR antibody concentration is pending (Khorzad et al. 2011). In human MG, single fibre

electromyography is also performed due to its high sensitivity for the disease (Cuddon 2002). Single fibre electromyography involves recording evoked action potentials from a single muscle fibre using a needle electrode with a very small recording surface (Cuddon 2002). From this, jitter, defined as the degree of variation of the onset latency of the action potentials of a given muscle fibre between stimulations can be determined (Cuddon 2002). Jitter serves as a measure of the neuromuscular safety factor (Cuddon 2002). Under normal circumstances, neuromuscular transmission is practically constant between stimulations for a given muscle fibre, however junctionopathy results in increased variability of neuromuscular transmission and therefore an increase in jitter as is seen in myasthenia gravis in humans (Cuddon 2002). A method for performing single fibre electromyography has been described in the dog (Añor et al. 2003). Again, general anaesthesia is required to perform these tests which can present a significant risk in animals with MG (Khorzad et al. 2011).

3.3.6. Demonstration of immunoglobulin localised to the endplate region

Another test which supports the diagnosis is demonstration of immune complexes at the level of the NMJ (Pflugfelder et al. 1981, Shelton et al. 1990). Two methods are described, immunocytochemistry of a fresh muscle biopsy or by incubating serum with stored normal canine muscle samples (Pflugfelder et al. 1981, Shelton et al. 1990). Common to both methods is the use of staphylococcal protein A conjugated with horseradish peroxidase where the staphylococcal protein A is used as a secondary antibody which binds to the fragment crystallizable region of the primary antibody which is the anti-AChR antibody (Dubois-Dalcq et al. 1977). Conjugation of the staphylococcal protein A with horseradish peroxidase enables its visual identification through fluorescence (Dubois-Dalcq et al. 1977). The staphylococcal protein A-horseradish peroxidase conjugate is not specific for antibodies directed against the

AChR and this test is therefore not specific for MG (Pflugfelder et al. 1981). Despite this, this test has good screening properties given that a negative result rules out the possibility of the disease (Shelton et al. 1990).

3.3.7. Serum acetylcholine receptor antibody concentration measurement

The "gold standard" and only definitive test for the diagnosis of MG involves demonstration of serum antibodies directed against the AChR using immunoprecipitation radioimmunoassay (Shelton 2010). For optimal results, use of species-specific assay is recommended (Shelton 2010). Sensitivity of this test is 98% for the generalised form of the disease and false positives are very rare (Shelton 2010). An AChR antibody concentration >0.6 nmol/l in dogs, and >0.3 nmol/l in cats is diagnostic for the disease (Shelton 2010). There is however a long turnaround time associated with this assay (Shelton 2010). In order to avoid false negatives, submitted serum should be collected before immunosuppressive therapy (Shelton 2010).

3.4. Treatment

Treatment is divided between supportive care, symptomatic treatment aiming at improving the clinical signs, and immunomodulation aiming to address the underlying immune process (Khorzad et al. 2011, Shelton 2002).

3.4.1. Supportive care

3.4.1.1. Aspiration pneumonia

Given the high prevalence of megaoesophagus with MG, prevention and/or treatment of aspiration pneumonia is essential due to the increased morbidity and mortality associated with this complication (Shelton 2002). Prevention involves adopting modified feeding

practices aimed at reducing regurgitation (Shelton 2002). Treatment involves implementing appropriate antimicrobial therapy ideally based on culture and sensitivity, along with nebulisation and coupage, and regularly alternating recumbency in order to prevent hypostatic lung oedema (Shelton 2002).

3.4.1.2. Intravenous fluid therapy

Many dogs and cats with megaoesophagus become dehydrated because of regurgitation. In such cases, intravenous fluid therapy is indicated to maintain hydration (Shelton 2002).

3.4.1.3. Nutritional support

In the presence of megaoesophagus, modified feeding practices are required to reduce regurgitation. Elevated feeding involves feeding the patient in an upright position both during and after feeding for 15 minutes (Penderis and Martin-Vaquero 2016, Shelton 2002). This aims to keep the oesophagus in a vertical position to promote movement of food into the stomach (Penderis and Martin-Vaquero 2016, Shelton 2002). A specifically designed chair called “Bailey chair” can be used to facilitate this labour-intensive feeding method (Penderis and Martin-Vaquero 2016, Shelton 2002). Additionally, each individual patients will have an optimal food consistency which they tolerate best, and which the clinician must determine, usually through trial and error (Penderis and Martin-Vaquero 2016, Shelton 2002). If regurgitation cannot be managed using elevated feeding, a nasogastric, oesophageal, or ideally gastrostomy tube should be placed (Penderis and Martin-Vaquero 2016, Shelton 2002). The benefit of a gastrostomy tube is that head elevation is no longer required and that the risk of aspiration pneumonia is reduced compared to nasogastric and oesophageal tubes (Penderis and Martin-Vaquero 2016, Shelton 2002). The disadvantages of gastrostomy tubes

are the requirement for general anaesthesia, and that only liquid food can be administered (Penderis and Martin-Vaquero 2016, Shelton 2002).

Figure 3: Dog with myasthenia gravis being fed using a Bailey chair

3.4.1.4: Respiratory support

In patients with severe weakness affecting the respiratory muscles, respiratory support though intermittent positive pressure ventilation is indicated (Khorzad et al. 2011, Shelton 2002).

3.4.2. Symptomatic therapy

Symptomatic therapy aims to resolve the clinical signs using AChE-Is (Khorzad et al. 2011, Shelton 2002). Acetylcholinesterase inhibitors prolongs the residence time of ACh in the synaptic cleft, thereby allowing more ACh to bind to the AChR, thereby improving neuromuscular transmission (Pascuzzi 2003, Webb 1971). It is usually administered in the form of pyridostigmine bromide at a dosage of 0.5-3 mg/kg orally in dogs, and 0.25 mg/kg orally in cats, every 8-12 hours (Khorzad et al. 2011, Shelton 2002). Treatment should be started at the lower end of the reference range and slowly increased until the clinical signs have resolved whilst minimising adverse effects (Khorzad et al. 2011, Shelton 2002). Cats are reported to be more sensitive to the adverse effects of AChE-Is than dogs, and it has been suggested that they respond better to immunosuppression (Khorzad et al. 2011, Shelton 2002). Adverse effects are due to overstimulation of the muscarinic cholinergic receptors resulting in hypersalivation, vomiting, diarrhoea, bronchoconstriction, and bradycardia, as well as overstimulation of the nicotinic cholinergic receptors resulting in muscle fasciculation and exacerbation of muscle weakness (Khorzad et al. 2011, Shelton 2002). If treatment by mouth is not possible, then pyridostigmine bromide can be administered through the gastrostomy tube if present (Khorzad et al. 2011, Shelton 2002). Additionally, intravenous neostigmine bromide can also be administered at a dosage of 0.04 mg/kg every 6 hours, or as

a constant rate infusion at a dosage of 0.01-0.03 mg/kg/hr (Khorzad et al. 2011, Shelton 2002). There is a degree of variability in the clinical response to AChE-Is however the reason for this is unknown (Khorzad et al. 2011, Shelton 2002). Furthermore, it appears that the clinical response of the oesophageal skeletal muscle is not as pronounced as that usually seen in the appendicular skeletal muscles (Shelton et al. 1990).

3.4.3. Immunosuppression

Should cases fail to adequately respond to AChE-Is, immunosuppression is then considered, and aims to address the underlying auto-immune aetiology (Khorzad et al. 2011, Shelton 2002). This potential benefit however needs to be carefully weighed against the risk of suppressing the immune system in animals with or at risk of aspiration pneumonia (Khorzad et al. 2011, Shelton 2002).

3.4.3.1. Prednisolone

Glucocorticosteroids are steroid hormones that suppress and prevent inflammation (Singh et al. 2004). Glucocorticosteroids exert both genomic and non-genomic effects (Singh et al. 2004). The genomic effects modulate the expression of genes responsible for inflammation and leukocyte function, whereas the non-genomic effects inhibit cellular metabolism and energy production of immune cells (Singh et al. 2004). Glucocorticoids are the most commonly used immunosuppressant medication in dogs and cats with MG (Shelton 2002). An initial dosage of 0.5 mg/kg orally every 12 hours is recommended in dogs, and 1-2 mg/kg orally every 12 hours in cats, which can then be slowly increased to effect as needed (Shelton 2002). A disadvantage of glucocorticosteroids however is that they can exacerbate skeletal muscle weakness, especially in dogs, whereas cats appear more resistant to the adverse

effects (Dickinson and LeCouteur 2004, Shelton 2002). Other common adverse effects include polyphagia, polyuria, polydipsia, panting, restless behaviour, gastrointestinal upset, opportunistic infections, skeletal muscle breakdown, pot-bellied appearance, bilateral flank alopecia, skin thinning (Elkholly et al. 2020).

3.4.3.2. Azathioprine

Azathioprine is a cytotoxic antimetabolite that interferes with deoxyribonucleic acid synthesis, targeting cell mediated immunity, particularly lymphocytes with a preference for T lymphocytes (Aarbakke et al. 1997, Elion 1993). Azathioprine has successfully been used in the treatment of MG in a case series of dogs in which clinical remission was achieved in three out of five dogs and in which AChR antibody concentration decreased significantly over time (Dewey et al. 1999). The recommended dosage is 1-2 mg/kg orally every 24-48 hours (Coates and Jeffery 2014). Adverse effects include gastrointestinal upset, myelosuppression, hepatotoxicity, and pancreatitis (Viviano 2013).

3.4.3.3. Cyclosporine

Cylcosporine is a lipophilic polypeptide (Stahelin 1986). Cyclosporine inhibits calcineurin thereby preventing T-cell activation, and decreasing production of interleukin IL-2, IL-3, IL-4, tumour necrosis factor alpha, and interferon gamma (Halloran 1996). Cyclosporine has been successfully used in the treatment of MG in two dogs (Bexfield et al. 2006). Clinical remission was achieved in the first case, and an improvement of clinical signs occurred in the second (Bexfield et al. 2006). The recommended dose is 3-6 mg/kg orally every 12 hours (Coates and Jeffery 2014). The specificity of cyclosporine for lymphocytes is an attractive feature given the concerns for aspiration pneumonia (Viviano 2013). Adverse effects are

usually transient and dose dependant and include mild gastrointestinal upset, gingival hyperplasia, opportunistic infections, hepatotoxity, allergic reactions, and lymphoproliferative disorders (Viviano 2013).

3.4.3.4. Mycophenolate mofetil

Mycophenolate mofetil is a prodrug of mycophenolic acid and is a purine synthesis inhibitor which reduces T and B cell proliferation (Villaroel et al 2009). Mycophenolate mofetil has been used in the treatment of MG in dogs, however there is contradictory evidence as to its beneficial effects (Abelson et al. 2009, Dewey et al. 2007, Dewey et al. 2010). In a case series of three dogs and a case report of one dog, oral administration of mycophenolate mofetil resulted in rapid resolution of clinical signs, as well as immune remission in the dog in the case report (Abelson et al 2009, Dewey et al. 2007). However in a retrospective study of 27 dogs with MG, no benefit was shown using mycophenolate mofetil alongside pyridostigmine bromide over pyridostigmine bromide on its own (Dewey et al 2010). The recommended dose is 7-20 mg/kg orally every 12 hours, and the IV dose is 15-20 mg/kg diluted in 500 mls of 0.45% sodium chloride and 2.5% dextrose administered over 4 hours (Penderis and Martin-Vaquero 2016). Like cyclosporine, mycophenolate mofetil's specificity for lymphocytes and neutrophil sparing property is thought to be beneficial given the concern for aspiration pneumonia (Viviano 2013). Adverse effects predominantly involve gastrointestinal upset (Viviano 2013).

3.4.4. Plasmapheresis and intravenous immunoglobulins

Both plasmapheresis and intravenous immunoglobulins are used for the treatment of MG in humans, especially in patients with severe or worsening clinical signs (Gilhus 2016).

Plasmapharesis has been reported with success as an adjunct therapy in the treatment of MG in both dogs and cats (Bartges et al 1990, Dörfelt et al 2021, Vitalo et al 2021). In plasmapheresis the immunoglobulins are filtered and removed from the plasma before being returned to the patient (Dörfelt et al 2021, Fernandez-Zarzoso et al 2019). In MG, this process effectively removes the pathogenic AChR antibodies (Dörfelt et al 2021).

Intravenous immunoglobulins has also been reported to be successful as an adjunct therapy in the treatment of MG in dogs (Penderis and Martin-Vaquero 2016). The dosage used was 0.5 g/kg (Penderis and Martin-Vaquero 2016). Intravenous immunoglobulins bind to the self-antigen recognition site, thereby preventing binding of the pathological autoantibodies to the self-antigens (Viviano 2013). They also increase the catabolism of autoantibodies by preventing their recycling, and cause a reduction of various cytokines (Viviano 2013).

3.5. Monitoring the course of the disease

Although definitive for the diagnosis of myasthenia gravis, the serum AChR antibody concentration value correlates poorly with the severity of the disease (Shelton 2002). However serial measurement of serum AChR antibody concentration over time is a good indication of disease status in an individual (Shelton 2002). It is also known in dogs without neoplasia that the natural course of the disease involves spontaneous remission in the absence of immunosuppression (Shelton and Lindstrom 2001). The natural course of MG in cats remains unknown.

3.6. Prognosis

The prognosis for MG is guarded to fair as the 1-year mortality rate for MG is 40-60% in dogs and 15% in cats (Dewey et al 1997, Ducote et al. 1999, Khorzad et al. 2011, Shelton

1997). Aspiration pneumonia and respiratory failure are the most frequent causes of death from MG in dogs and cats (Dewey et al 1997, Ducoté et al. 1999).

4. Current state of knowledge

Despite CMSs and MG being well characterised conditions, there are several aspects which warrant further investigation.

There are limited reports on the long-term outcome of cats with MG with only 2 retrospective studies published to date (Ducoté et al 1999, Hague et al 2015). In the first study, a low mortality rate of 15% was documented, however in the second and more recent study, 58% of affected cats underwent euthanasia (Ducoté et al 1999, Hague et al 2015). Hence long-term outcome of MG in cats remains unclear with conflicting results published to date (Ducoté et al 1999, Hague et al 2015). Furthermore, the natural course of MG in cats has not been investigated to date (Ducoté et al 1999, Hague et al 2015, Mayousse et al 2015, Meeking et al 2008, Nagata et al 2017).

Myasthenia gravis is currently classified in dogs and cats according to generalisation, progression, and severity of skeletal muscle weakness and fatigability based on a previous classification system used for humans which has not been updated in over twenty-five years (Dewey et al 1997, Dickinson and Le Couteur 2004, Khorzad et al. 2011, Shelton 2002). However, factors such as the presence or absence of a thymoma, or administration of thiourylene medication in cats, can influence treatment, outcome, or both, frequently leading to the division of affected dogs and cats into separate disease groups based on these factors (Bell et al 2012, Dickinson and Le Couteur 2004, Ellis et al 2019, Hague et al 2015, Khorzad

et al. 2011, Lainesse et al 1996, Shelton et al 1997, Shelton et al 2000, Shelton et al 2001, Shelton 2002).

Historically, CMSs were termed congenital MG which was thought to solely be the result of a postsynaptic deficiency of AChRs in the absence of antibodies directed against them (Dickinson and Le Couteur 2004, Indieri et al. 1983, Lennon et al 1980). However, it is now clear a congenital post-synaptic AChR deficiency is only one of several clinically heterogeneous congenital syndromes affecting the NMJ, resulting in skeletal muscle weakness and fatigability (Abitbol et al. 2015, Blakey et al. 2017, Dickinson et al. 2005, Gandolfi et al. 2015, Malik et al. 1993, Proschwsky et al 2007, Rinz et al. 2015, Shelton 2016, Shelton 2017, Tsai et al. 2019). These syndromes are collectively referred to as CMSs and considered as a separate disease entity from MG (Shelton 2016, Shelton 2017). There is currently no established classification system for CMSs in dogs and cats.

A significantly higher incidence of MG associated megaoesophagus is reported in dogs (88%) compared to cats (40%), or even humans in which there are only a few reported cases (Desuter et al. 2015, Ducoté et al. 1999, Shelton et al. 1997). This has been attributed to the tunica muscularis of the canine oesophagus being described as solely composed of skeletal muscle as opposed to that of cats or humans in which the distal third and two thirds are respectively reported to contain smooth muscle (Hague et al. 2015, Meyer et al. 1986). This description of the tunica muscularis of the oesophagus of dogs as being solely composed of skeletal muscle is based on a population of 9 crossbreed dogs in the United States of America and 22 dogs in Germany (Mann and Shorter 1964, Bush 1980). Recently, however, the distal third of the tunica muscularis was reported to be composed of smooth muscle in a population of 8 Iraqi dogs (Dawood et al. 2022). This would be consistent with the tunica muscularis

described in most omnivores in which there a mixture of skeletal and smooth muscle, as opposed to that of ruminants which is solely composed of skeletal muscle (Majewski et al. 2003, Sukon et al. 2009).

5. Aims of the thesis

The aims of the present thesis are the following:

- Evaluate the long-term outcome of cats with MG without evidence of a CMM.
- Evaluate the natural course of the disease in cats with MG without evidence of a CMM.
- Update the classification system used for MG in dogs and cats based on comparison with published classification systems in human beings.
- Establish a classification system for CMSs in dogs and cats.
- Evaluate the tunica muscularis composition of the canine oesophagus.

6. Hypotheses

A set of hypotheses were formulated:

- The long-term outcome of cats with myasthenia gravis without evidence of a cranial mediastinal mass is often favourable.
- The natural course of the disease can involve spontaneous remission in cats with MG without evidence of a CMM.
- The tunica muscularis of the canine oesophagus is solely composed of skeletal muscle.

Chapter II: Long-term outcome of cats with myasthenia gravis without evidence of a cranial mediastinal mass

1. Introduction

There are limited reports on the long-term outcome of cats with MG with only 2 retrospective studies published to date (Ducoté et al 1999, Hague et al 2015). In the first study, a low mortality rate of 15% was documented, however in the second and more recent study, 58% of affected cats underwent euthanasia (Ducoté et al 1999, Hague et al 2015). Hence long-term outcome of MG in cats remains unclear with conflicting results published to date (Ducoté et al 1999, Hague et al 2015). Furthermore, the natural course of MG in cats has not been investigated to date (Ducoté et al 1999, Hague et al 2015, Mayousse et al 2015, Meeking et al 2008, Nagata et al 2017).

2. Materials and methods

Ethical approval was sought and gained from the University of Nottingham School of Veterinary Medicine and Science's ethics committee for this study (Ethical approval number 3238 201001). The medical records of all cats diagnosed with MG, between 2005 and 2018 from two veterinary referral hospitals were reviewed for inclusion into the present study. Criteria used for inclusion consisted of a diagnosis of MG, thoracic imaging, serum biochemistry including measurement of creatine kinase (CK) activity, and a haematology profile. The diagnosis of MG was based on clinical signs compatible with the disease and an abnormal serum AChR antibody concentration. Serum AChR antibody concentration was measured by immunoprecipitation radioimmunoassay using a feline-specific antigen with a diagnostic value of 0.3 nmol/L (Shelton et al 2000). All imaging studies of the thorax were interpreted by a board-certified veterinary radiologist. Criteria used for exclusion were the presence of an identifiable CMM on thoracic imaging or administration of methimazole or carbimazole. The wheelbarrow exercise stress test (WEST) was employed during the

neurological examination of some cats to evaluate their degree of skeletal muscle strength and tolerance to exercise. This test involved elevating and supporting the caudal half of the body while moving the cat forward thereby promoting ambulation through the thoracic limbs. The medical records were reviewed for signalment, history, reasons for referral, general and neurological examination findings, diagnostic tests performed, treatment, short-term follow-up, long-term outcome and repeated measurement of serum AChR antibody concentration if performed. When undertaken, electromyography was conducted under general anaesthesia in normothermic cats by a board-certified veterinary neurologist, at least seven days after the onset of clinical signs, using a disposable bipolar concentric needle electrode. Skeletal muscles evaluated as part of the electromyographic study consisted of the appendicular and epaxial muscles, as well as those of the head. Motor nerve conduction studies and repetitive nerve stimulation studies were not routinely performed as part of a neuromuscular workup in these two veterinary referral hospitals during the inclusion period. Throughout this study, the term immune remission was used to define the absence of clinical signs of MG along a with a normal serum AChR antibody concentration after discontinuation of treatment, whereas spontaneous remission referred to the resolution of clinical signs of MG alongside normalisation of serum AChR antibody concentration in the absence of any treatment. Short-term follow-up information was obtained approximately two weeks after initial presentation in all cats upon informing their owner of the diagnosis. Long-term outcome was evaluated through an interview with the owner six months after diagnosis alongside a repeated general and neurological examination of their cat if alive, as well as a final interview at the time of writing of this study if their cat was still alive six months after confirmation of the diagnosis.

3. Results

3.1. Population

Eight cats met the inclusion criteria. The population comprised six pure-bred cats; Siamese (n = 2), British Shorthair (n = 2), Bengal (n = 1), Norwegian Forest (n = 1), as well as two Domestic Shorthair cats. There was an equal number of male and female cats, all of which were entire aside from one male and one female which were neutered. Age of the cats at the time of presentation varied from one to eleven years (median age of six years).

3.2. Clinical presentation

Duration of clinical signs before presentation ranged from three days to eleven months, and the evolution included deterioration, waxing and waning, plateauing, but also improvement. Reasons for referral included tetraparesis (n = 4), a plantigrade stance (n = 3), paraparesis (n = 2), reluctance to walk (n = 2), cervical ventroflexion (n = 2), appendicular skeletal muscle tremors (n = 2), inability to jump (n = 1), generalised skeletal muscle atrophy (n = 1), dysphagia (n = 1), and lethargy (n = 1). General examination did not reveal any abnormality in any of the cats. Neurological examination abnormalities included a decreased withdrawal reflex in all limbs (n = 5), a plantigrade stance (n = 5), paraparesis (n = 4), a decreased hopping response in all limbs (n = 4),appendicular skeletal muscle tremors (n = 3), a decreased extensor postural thrust (n = 3), tetraparesis (n = 2), a decreased hopping response restricted to the pelvic limbs (n = 2), cervical ventroflexion (n = 2), bilateral weakness and fatigability of the facial skeletal muscles involved in the menace response and palpebral reflex (n = 2), generalised skeletal muscle atrophy (n = 1), and a decreased withdrawal reflex restricted to the pelvic limbs (n = 1). The WEST was performed on six cats, five of which

demonstrated skeletal muscle weakness and fatigability, progressing to exhaustion within one minute of this activity and were not able to resume normal ambulation until a short period of rest. The neurological examination did not reveal any abnormality in one cat.

3.3. Diagnostic test results

Serum biochemistry and haematology profile did not reveal any abnormality aside from serum creatine kinase activity which was increased in two cats (295 and 1201 U/L). Thoracic imaging consisted of plain radiographs in all cats, none of which revealed any abnormality. Two cats underwent an abdominal ultrasound, neither of which revealed any abnormality. Magnetic resonance imaging was performed in two cats. The brain and cervical spinal cord region were evaluated in one, whereas the thoracolumbar spinal cord region was assessed in the other cat. No abnormality was reported in either magnetic resonance imaging studies. Cerebrospinal fluid collected from the cistern of the conus medullaris in the lumbosacral region (n = 2), or from both the cistern of the conus medullaris in the lumbosacral region and the cerebellomedullary cistern (n = 2), was submitted for analysis in four cats. Cerebrospinal fluid analysis revealed albumino-cytological dissociation in 1 cat on both the cisternal and lumbar samples (45 mg/dL on the cisternal sample, 65 mg/dL on the lumbar sample) and did not reveal any abnormality in the remaining three cats. A single dose of neostigmine methylsulphate was administered intravenous to one cat as part of a neostigmine challenge test, resulting in transient complete resolution of the previously observed bilateral weakness and fatigability of the facial skeletal muscles involved in the menace response and palpebral reflex. Electromyography was performed in five cats, two of which had prolonged insertional activity as well as fibrillation potentials in multiple skeletal muscles across all limbs. Electromyography did not reveal any abnormality in the remaining three cats, including the

two cats with an increased serum creatine kinase activity. Gastrocnemius (n = 2), cranial tibial (n = 1), and bicep femoris (n = 1) muscle biopsies were collected from the two cats in which electromyography was abnormal. Muscle biopsies did not reveal any abnormality in either cat. A sciatic-tibial nerve biopsy was obtained from one cat, which did not reveal any abnormality. Four cats underwent serological testing for feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV), which were negative. Serology for *Toxoplasma gondii* in four cats demonstrated evidence of previous exposure but not of active infection in two cats, whereas the remaining two cats were naive to the protozoa. Polymerase chain reaction was performed on cerebrospinal fluid for *Toxoplasma gondii* (n = 3), Coronavirus (n = 3), and FeLV (n = 1). Polymerase chains reactions were performed on blood for *Toxoplasma gondii* (n = 2), Coronavirus (n = 2), and FIV (n = 1). All polymerase chain reaction results were negative. Serum AChR antibody concentration was abnormal in all cats, ranging from 0.59 to 8.4 nmol/L (median value of 4.55 nmol/L).

3.4. Treatment

The decision to initiate treatment as well as the protocol used was clinician dependent. In the four cats in which no treatment was given, the clinician elected not to administer any treatment given that the owners had reported that their cat was already improving by the time of initial presentation, diagnosis, or both. Treatment consisted of an immunosuppressive course of prednisolone at a dosage of 1 mg/kg PO every 12 hours for one month in four cats. This dosage of prednisolone was then slowly tapered, before being discontinued within six months after having been started. The remaining four cats did not receive any treatment. None of the cats received any supportive treatment or therapy with anticholinesterase agents.

3.5. Outcome

All cats were alive and had improved upon short-term follow-up, although they had not fully recovered. Outcome six months after diagnosis was excellent in all cats as their repeated general and neurological examinations performed at least three weeks after any treatment had been discontinued did not reveal any abnormality, and as their owners reported complete resolution of the previously observed clinical signs. Serum AChR antibody concentration measurement was repeated in all cats six months after diagnosis and at least three weeks after any treatment had been discontinued. At such time, serum AChR antibody concentrations had normalised in all cats, indicating that immune remission was attained in all cats, including the four cats which did not receive any treatment, and whose natural course of disease therefore involved spontaneous remission. Serum AChR antibody concentration was also measured three months after diagnosis in one cat undergoing treatment with prednisolone, at which point the concentration had decreased, but immune remission had not occurred yet. At the time of writing of this study, five cats had died or were euthanised at an old age due to unrelated disease but were not reported to have experienced any reoccurrence of the clinical signs of MG at any point during their life, whereas the remaining three cats were still alive and were also not reported to have experienced any reoccurrence of the clinical signs of MG at least four years after diagnosis.

4. Discussion

We report a population of eight cats diagnosed with MG without evidence of a CMM in which long-term outcome was excellent and immune remission was systematically achieved. There was spontaneous remission in all four cats that did not receive any treatment. Clinical presentation was heterogeneous, as the duration and evolution of clinical signs before presentation as well as their severity at the time of examination varied considerably between cats. Lastly, skeletal muscle weakness and fatigability either induced or exacerbated by the WEST were the most consistent neurological examination abnormalities associated with MG in cats in this study.

Clinical presentation was heterogeneous despite the fact that all cats were diagnosed with the generalised form of MG, and that megaoesophagus was not detected in any cat. Not only did the duration of clinical signs vary significantly from acute to chronic presentations as previously described, but the evolution and severity of clinical signs was also eclectic (Dickinson and LeCouteur 2004). Some were deteriorating or plateauing while others waxed and waned or improved with time, and neurological examination abnormalities at the time of initial examination ranged from absent to severe skeletal muscle weakness and fatigability. It is reasonable to consider that the immune-mediated basis of MG in cats is a continuous spectrum of disease, ranging from severe and acute fulminating skeletal muscle weakness and fatigability through to potentially subclinical and chronic skeletal muscle weakness and fatigability. This spectrum, alongside spontaneous remission, could explain the heterogeneous presentation of cats with MG.

Due to the sedentary nature of cats, it is often challenging to evaluate skeletal muscle strength and tolerance to exercise in the consultation room. Additionally, skeletal muscle weakness and fatigability can be subtle in cats with MG. An advantage of the WEST is the ability to generate physical activity in order to evaluate skeletal muscle strength and tolerance to exercise. The WEST was employed in an attempt to induce or exacerbate any skeletal muscle weakness and fatigability, particularly in cats with vague clinical signs. In the present study, 5 of the 6 cats evaluated demonstrated skeletal muscle weakness and fatigability progressing to exhaustion within 1 minute of this activity and were not able to resume normal ambulation until a short period of rest. This included one cat in which neuromuscular disease was uncertain from the neurological examination as the only abnormality was intermittent appendicular skeletal muscle tremors. Upon performing the WEST, it became apparent that the cat was intolerant to exercise and that the appendicular skeletal muscle tremors might have represented appendicular skeletal muscle weakness. The only cat in which the WEST did not reveal evidence of skeletal muscle weakness nor fatigability had a normal neurological examination and had been described to have already improved by the owners at the time of initial presentation. Skeletal muscle weakness and fatigability induced or exacerbated by the WEST were the most consistent neurological examination abnormalities associated with MG in this study.

Cerebrospinal fluid analysis revealed albuminocytological dissociation in one cat, which has not been reported in cats with MG. An increased cerebrospinal fluid total protein concentration was documented in some of the patients included in a study evaluating cerebrospinal fluid in human MG, in which the presence of cerebrospinal fluid anti-AChR antibodies was demonstrated, and a significant correlation between cerebrospinal fluid total

protein concentration and cerebrospinal fluid anti-AChR antibody concentration was observed (Müller et al 1987). Another study analysing cerebrospinal fluid in human MG also reported an increase in total protein in some patients, which was either caused by blood contamination or by concurrent diseases (Thorlacius et al 1985). Given that magnetic resonance imaging was unremarkable in the cat with albuminocytological dissociation, the increased total protein concentration might have resulted from blood contamination, or it could otherwise be hypothesized that it might be linked to the putative presence of anti-AChR antibodies in the cerebrospinal fluid.

The decision to initiate treatment as well as the protocol used was clinician dependent. In the four cats in which no treatment was given, the clinician elected not to administer any treatment given that the owners had reported that their cat was already improving by the time of initial presentation, diagnosis, or both. None of the cats required supportive therapy as there were no specific issues to address such as dehydration, regurgitation/dysphagia, aspiration pneumonia, or respiratory difficulties due to weakness and fatigue of the respiratory skeletal muscles. None of the cats received treatment with anticholinesterase agents. Anticholinesterase agents have been successfully reported in the treatment of MG in cats (Dickinson and LeCouteur 2004, Shelton 2002). Prednisolone used at an immunosuppressive dose has also been successful in the treatment of MG in cats and has been suggested to be more beneficial than anticholinesterase agents (Dickinson and LeCouteur 2004, Mayousse et al 2017, Shelton 2002). Not only do corticosteroids address the underlying aetiology, they can also increase neuromuscular transmission, and cats appear tolerant to the adverse effects of prednisolone, even at high doses (Berrih-Aknin and Le Panse 2014, Dickinson and LeCouteur 2004, Hall et al. 1977, Mayousse et al 2017, Shelton

2002). In agreement with the latter, none of our cats experienced adverse effects including deterioration of their degree of skeletal muscle weakness after administration of an immunosuppressive course of prednisolone. From our results, it is not possible to draw conclusions as to the optimal therapy for cats with MG without evidence of a CMM. However, given the natural history of the disease includes spontaneous remission in cats diagnosed with MG without evidence of a CMM, treatment is therefore not always necessary in this disease population.

In our population, the six-month survival rate was 100% and long-term outcome was excellent in all cats. A major difference between our population and that of previous studies in which outcome was generally unfavourable is that cats with evidence of a CMM were excluded from the present study (Ducoté et al 1999, Hague et al 2015, Mayousse et al 2017,). Although it is not possible to comment as to the outcome of cats diagnosed with MG and a CMM, it is intuitive that their outcome would relate in part to the prognosis associated with their CMM. Given that the long-term outcome of feline MG is favourable when there is no evidence of a CMM, attempting to rule out the presence of a CMM by performing thoracic imaging therefore refines prognosis.

Immune remission from MG was more frequent in our population (100%) than in a previous study (9.2%), and we report the first incidences of spontaneous remission as defined as the resolution of clinical signs of MG along with normalisation of serum AChR antibody concentration in the absence of any treatment (Hague et al 2015). The reasons for this might be threefold.

Our population was restricted to cats diagnosed with MG without evidence of a CMM. This might be of clinical relevance given that the majority of cats reported to have achieved immune remission do not have evidence of a CMM (Hague et al 2015, Mayousse et al 2017, Meeking et al 2008, Nagata et al 2017). Perhaps, the ability to achieve immune remission might be affected by the presence of a CMM. This would appear plausible given that some CMMs, such as thymomas, are hypothesized to induce MG as part of a paraneoplastic syndrome in humans (Lindstrom 2000, Berrih-Aknin and Le Panse 2014).

Another factor might be that the previous study was not able to evaluate serum anti-AChR antibody concentration over a sufficient amount of time for immune remission to occur (Hague et al 2015). In this previous study, serial measurement of serum anti-AChR antibody concentration was performed in nineteen cats affected by MG without evidence of a CMM, of which the majority (88%) had a decrease in serum AChR antibody concentration despite some experiencing an initial increase (Hague et al 2015). Because the timescale over which serum AChR antibody concentration evaluation occurred is unknown in this previous study, perhaps some of these concentrations might have normalised if given more time (Hague et al 2015). It is worthy to note that immune remission can take up to eighteen months to occur in dogs with MG (Shelton and Lindstrom 2001).

Thirdly, the lack of occurrence of immune remission among cats without evidence of a CMM in the previous study might have related to failure to identify neoplastic disease at the time of presentation (Hague et al 2015). Neoplasia has been identified in dogs in which immune remission was not achieved as long as three years after diagnosis of AMG (Shelton and Lindstrom 2001). The authors however note that MG in cats has not been associated with neoplasia located outside the mediastinum to date.

Immune-mediated polymyositis was definitively ruled out in two cats and was suspected not to be present in the remaining six cats as their serum creatine kinase activity was either within normal limits or because electromyography did not reveal any abnormality when their serum creatine kinase activity was increased. The outcome of cats affected by MG concurrently with immune mediated polymyositis is relatively unknown as there are only two reported cases (Mayousse et al 2017). Of these two cases, one was treated with anticholinesterase agents alone and died, whereas the other also received prednisolone and achieved immune remission (Mayousse et al 2017). If some of our cats were concurrently affected by undiagnosed immune mediated polymyositis, it is possible that this comorbidity might not have affected their outcome or their ability to achieve immune remission. Outcome of immune mediated polymyositis is generally favourable, and treatment of immune mediated polymyositis also involves immunosuppression using prednisolone or might not be necessary given that spontaneous remissions have also been reported for this condition (Dickinson and Le Couteur 2004).

There are limitations to our study. Our sample size is small and is therefore perhaps not truly representative of the population of cats affected by MG without evidence of a CMM. Short-term follow-up and long-term outcome were respectively evaluated solely or in part using information provided by the owners of the included cats; hence, they may be subjective and prone to the caregiver placebo effect. Lastly, given the superiority of computed tomography compared to plain thoracic radiography for the evaluation of mediastinal masses in humans, plain thoracic radiographs could have failed to identify the presence of a CMM in our population (Rebner et al 1987).

5. Conclusions

Cats diagnosed with MG without evidence of a CMM have a favourable outcome and frequently achieve immune remission. Moreover, the natural history of feline MG includes spontaneous remission when there is no evidence of a CMM. Attempting to rule out the presence of a CMM therefore refines prognosis, and treatment is not always necessary in this disease population. Additionally, clinical presentation was heterogeneous, comprising cats that were improving or with a normal neurological examination at the time of presentation, and skeletal muscle weakness and fatigability induced or exacerbated by the WEST were the most consistent neurological examination abnormalities associated with MG in cats in this study.

Chapter III: Classification of myasthenia gravis and congenital myasthenic syndromes in dogs and cats

1. Introduction

Myasthenia gravis is currently classified in dogs and cats according to generalisation, progression, and severity of skeletal muscle weakness and fatigability based on a previous classification system used for humans which has not been updated in over twenty-five years (Dewey et al 1997, Dickinson and Le Couteur 2004, Khorzad et al. 2011, Shelton 2002). However, factors such as the presence or absence of a thymoma, or administration of thiourylene medication in cats, can influence treatment, outcome, or both, frequently leading to the division of affected dogs and cats into separate disease groups based on these factors (Bell et al 2012, Dickinson and Le Couteur 2004, Ellis et al 2019, Hague et al 2015, Khorzad et al. 2011, Lainesse et al 1996, Shelton et al 1997, Shelton et al 2000, Shelton et al 2001, Shelton 2002).

Historically, CMSs were termed congenital MG which was thought to solely be the result of a postsynaptic deficiency of AChRs in the absence of antibodies directed against them (Dickinson and Le Couteur 2004, Indieri et al. 1983, Lennon et al 1980). However, it is now clear a congenital post-synaptic AChR deficiency is only one of several clinically heterogeneous congenital syndromes affecting the NMJ, resulting in skeletal muscle weakness and fatigability (Abitbol et al. 2015, Blakey et al. 2017, Dickinson et al. 2005, Gandolfi et al. 2015, Malik et al. 1993, Proschwsky et al 2007, Rinz et al. 2015, Shelton 2016, Shelton 2017, Tsai et al. 2019). These syndromes are collectively referred to as congenital myasthenic syndromes (CMSs) and considered as a separate disease entity from MG (Shelton 2016, Shelton 2017). There is currently no established classification system for CMSs in dogs and cats.

2. Materials and methods

The aim of the literature search was to identify all published classification systems for MG and CMSs in humans, dogs, and cats. Search strategies included use of electronic search engines for publication databases, searching through the reference list of publications and proceedings of relevant scientific conferences. Electronic databases used were Pub Med (www.ncbi.nlm.nih.gov/PubMed), CAB Abstracts (www.cabdirect.org) and Google Scholar (www.scholar-google.com). The search terms used in the search engines were as follows: (human OR people OR blank) or (dog OR dogs OR canine) or (cat OR cats OR feline) and (classification OR review) and (myasthenia gravis OR acquired myasthenia gravis OR congenital myasthenic syndromes OR congenital myasthenic syndrome OR congenital myasthenia gravis). Manual searches for articles from the reference list of publications and major veterinary neurology conference meeting proceedings and relevant textbook chapters was also carried out. Conference proceedings were searched for the annual symposium of the European Society and College of Veterinary Neurology (ESVN/ECVN) and the American College of Veterinary Internal Medicine (ACVIM).

3. Results

3.1. Congenital myasthenic syndromes

3.1.1. Terminology and definition used in humans

In humans, the term CMSs is used to refer to a clinically heterogeneous group of genetic disorders causing aberrant neuromuscular transmission (Engel et al 2015, Engel et al 2018, Farmakidis et al 2018, Vanhaesebrouck and Beeson 2019).

3.1.2. Classification used in humans

Although CMSs share the common features of skeletal muscle weakness and fatigability associated with inadequate neuromuscular transmission, their clinical presentation (ie, age of onset, presenting signs, distribution of skeletal muscle weakness and fatigability, and response to treatment) varies according to the mutation (Engel et al 2015, Engel et al 2018, Farmakidis et al 2018, Vanhaesebrouck and Beeson 2019, Lee et al 2018, Finsterer 2019). Over 30 mutations have been identified in humans, and affect proteins involved in NMJ structure, function, or repair (Engel et al 2015, Engel et al 2018, Farmakidis et al 2018, Vanhaesebrouck et al 2019). Numerous criteria can be used to classify these CMSs although the most frequently used classification system is based on the affected NMJ component or components, thereby organizing them into presynaptic, synaptic, postsynaptic, and concurrent presynaptic- and postsynaptic CMSs (Table 2) (Engel et al 2015, Farmakidis et al 2018, Vanhaesebrouck and Beeson 2019). These CMSs are then further categorised according to the mechanism of the defect of neuromuscular transmission, the affected protein, and ultimately the mutated gene (Table 2) (Engel et al 2015, Farmakidis et al 2018, Vanhaesebrouck and Beeson 2019). For some mutations, however, the location of the

affected protein or proteins is unknown, meaning that these CMSs cannot presently be classified, and are referred to as "other" (Engel et al 2015, Farmakidis et al 2018).

Table 2: Classification of congenital myasthenic syndromes in humans as described by Vanhaesebrouck and Beeson (2019)

Site of defect	**Mechanism**	**Gene**	**Protein**
Presynaptic	Defect in ACh recycling	*SLC5A7* (solute carrier family 5 member 7)	ChT (choline transporter)
	Defect in ACh synthesis	*CHAT*	ChAT (choline acetyltransferase)
	Defects in loading of ACh in synaptic vesicles	*SLC18A3* (solute carrier family 18 member A3)	VAchT (vesicular acetylcholine transporter)
	Defect in synaptic vesicle docking, priming, fusing and exocytosis	*SNAP25B* (synaptosomal nerve-associated protein 25)	Soluble N-ethylmeleimide-sensitive factor-attachment protein receptor 25
		UNC13A (unc-13 Homolog A)	Munc 13 (mammalian uncoordinated-13)
		SYB1 (Synaptobrevin-1)/VAMP1 (vesicle associated membrane Protein 1)	SYB1/VAMP1
		SYT2 (synaptotagmin 2)	SYT2
		PREPL (prolyl-endopeptidase Like)	PREPL
	Defect in axonal transport of proteins	*MYO9A* (myosin IXA)	MYO9A
Synaptic	Acetylcholinesterase deficiency	*COLQ*	COLQ

	Synaptic basement membrane defects	*COL13A1* (collagen type 13 α1)	COL13A1
		LAMA5 (laminin α5)	LAMA5
		LAMB2 (laminin β2)	LAMB2
	Defects in AChR clustering pathway	*AGRN* (agrin)	AGRN
Postsynaptic	Reduced numbers of AChR (AChR deficiency)	*CHRNE*, *CHRNA1* (cholinergic receptor nicotinic α1 subunit), *CHRNB1* (cholinergic receptor nicotinic β1 subunit), *CHRND* (cholinergic receptor nicotinic δ subunit)	AChR subunits
	Kinetic changes in AChR function (slow channel syndromes)	*CHRNA1, CHRNB1, CHRND, CHRNE*	AChR subunits
	Kinetic changes in AChR function (fast channel syndromes)	*CHRNA1, CHRND, CHRNB1, CHRNE*	AChR subunits
	Defect in AChR clustering pathway	*LRP4*	LRP4
		MUSK	MUSK
		DOK7 (downstream of kinase 7)	DOK7
		RAPSN (rapsyn)	RAPSN
	Defect in skeletal muscle voltage-gated sodium channel	*SCN4A* (sodium voltage gated channel α4)	SCN4A
	Plectin deficiency	*PLEC* (plectin)	PLEC
Pre + post synaptic	Defective glycosylation	*ALG2* (A-1,3,Mannosyltranferase)	ALG2
		ALG14 (ALG14 UDP-N-Acetylglucosaminyltransferase Subunit)	ALG14
		DPAGT1 (dolichyl-phosphate N-acetyl-	DPAGT1

		glucosaminephosphotransferase 1)	
		GFPT1 (glutamine-fructose-6-phosphate transaminase 1)	GFPT1
		GMPPB (GDP-mannose-pyrophosphorylase)	GMPPB

3.1.3. Terminology and definition in dogs and cats

Previous terminology has frequently referred to these syndromes as congenital MG, but more recent publications follow the terminology used in humans and use the term CMS because it more appropriately characterises the genetic and clinical heterogeneity of these syndromes (Shelton 2016, Rinz et al 2014, Shelton 2017). Similar to humans, CMSs also are defined in dogs and cats as a clinically heterogeneous group of genetic disorders causing aberrant neuromuscular transmission (Shelton 2016, Shelton 2017).

3.1.4. Classification in dogs and cats

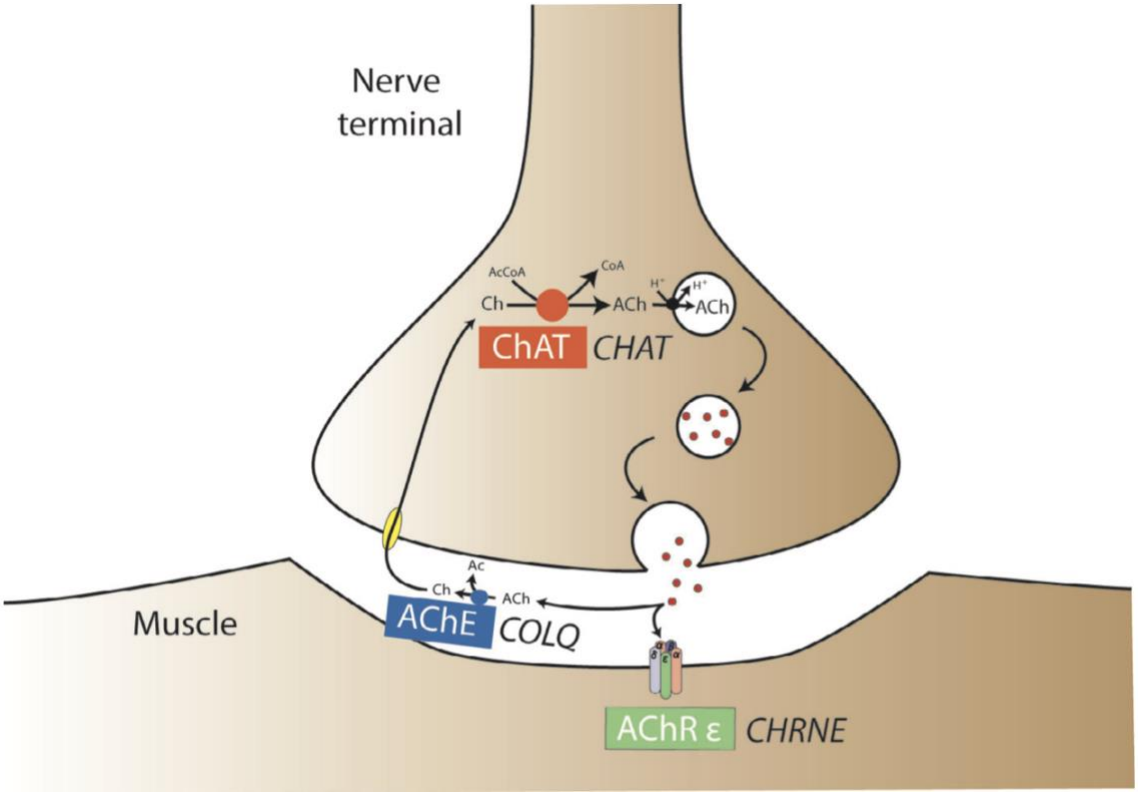
Given the shared genetic basis of CMSs in humans, dogs, and cats, a similar classification system of CMSs in dogs and cats to that used in humans appears appropriate. Following the classification system used in humans, we propose to classify CMSs in dogs and cats according to the affected NMJ component, the mechanism of the defect of neuromuscular transmission, the affected protein, and ultimately the mutated gene (Table 3) (Vanhaesebrouck and Beeson 2019). A concurrent presynaptic and postsynaptic category is not included in this classification of CMSs in dogs and cats because at the present time such CMSs are not reported in these species (Shelton 2016, Tsai et al. 2019). This situation might change as new CMSs are discovered. Despite efforts to classify CMSs in dogs and cats, many

suspected CMSs presently cannot be classified because of a lack of identification of an underlying genetic mutation (Indieri et al 1983, Dickinson et al. 2005, Blakey et al. 2017, Johnson et al 1975, Jenkins et al 1976, Miller et al 1983, Joseph et al 1988, Van Ham 1992). Among these suspected CMSs, many are suspected to be postsynaptic because of a lack of postsynaptic AChRs, (Indieri et al 1983, Dickinson et al. 2005, Blakey et al 2017, Miller et al 1983), but these suspected CMSs still cannot be classified, because concurrent presynaptic involvement cannot be excluded because of a lack of knowledge of the suspected underlying genetic mutation, (Indieri et al 1983, Dickinson et al. 2005, Blakey et al 2017, Miller et al 1983) and as such a deficiency although marginal also is reported in some synaptic CMSs in dogs (Rinz et al 2014, Tsai et al. 2019).

Table 3: Classification of congenital myasthenic syndromes in dogs and cats

Affected neuromuscular junction component	**Mechanism of the defect of neuromuscular transmission**	**Protein**	**Gene**	**Species, breed**
Presynaptic	Defect in ACh synthesis	ChAT (choline acetyltransferase)	*CHAT*	Dogs: Old Danish Pointing dog
Synaptic	ACh deficiency	COLQ	*COLQ*	Dogs: Labrador Retriever, Golden Retriever Cats: Sphynx, Devon Rex
Postsynaptic	AChR deficiency	AChR ε subunit	*CHRNE*	Dogs: Jack Russell Terrier, Heideterrier

Figure 4: Illustration of congenital myasthenic syndromes reported in dogs and cats as published by Mignan et al. (2020)



3.1.4.1. Presynaptic congenital myasthenic syndromes

A presynaptic CMS is reported in Old Danish Pointing dogs. (Prochowsky et al 2007, Flagstad 1982, Flagstad et al 1989, Trojaborg and Flagstad 1982). It involves a missense mutation in exon 6 of the choline acetyltransferase (*CHAT*) gene the normal function of which is the synthesis of ACh, and is inherited in an autosomal recessive manner.

(Prochowsky et al 2007). Reported onset is twelve to sixteen weeks of age, and a period of exercise is required before observation of skeletal muscle weakness and fatigability, at which time palpation of the affected skeletal muscles can identify hypertonicity (Proschowsky et al 2007, Flagstad et al 1989, Trojaborg and Flagstad 1982). A decremental response is observed upon repetitive nerve stimulation, but a preceding high frequency conditioning train is required, and postsynaptic AChR concentration is normal (Proschowsky et al 2007, Flagstad et al 1989, Trojaborg and Flagstad 1982). In humans with CHAT-associated CMS, conditioning with 10 Hz repetitive nerve stimulation is often required to elicit a decremental response and this is thought to be due to the nerve terminal containing sufficient ACh stores (Nicolau and Milone 2019). Neuromuscular junction autoantibody testing for the AChR is negative (Flagstad et al 1989, Trojaborg and Flagstad 1982). Administration of AChE-Is has no clinical or electrophysiologic effect, although guanidine can result in transient electrophysiologic improvement (Proschowsky et al 2007, Trojaborg and Flagstad 1982). Genetic testing for this mutation is available for this breed (Trojaborg and Flagstad 19820. In the *CHAT*-associated CMS of Old Danish Pointing dogs, administration of AChE-Is had no clinical effect (Proschowsky et al 2007, Trojaborg and Flagstad 1982). Treatment for *CHAT*-associated CMSs in humans includes medications that increase the amount of ACh in the synaptic cleft, such as AChE-Is or 3,4-diaminopyridine (3,4-DAP) (Lee et al 2018, Finsterer et al 2019).

3.1.4.2. Synaptic congenital myasthenic syndromes

A synaptic CMS is identified in Labrador Retrievers, Golden Retrievers, Sphynxes and Devon Rexes (Rinz et al 2014, Tsai et al. 2019, Abitbol et al. 2015, Gandolfi et al. 2015). These CMSs involve a mutation in the collagen-like tail subunit of asymmetric

acetylcholinesterase (*COLQ*) gene, which anchors acetylcholinesterase (AChE) to the basal lamina of the NMJ (Rinz et al 2014, Tsai et al. 2019, Abitbol et al. 2015, Gandolfi et al. 2015). In Labrador Retrievers, it is caused by a nonsynonymous mutation in exon 14, and is inherited in an autosomal recessive manner (Rinz et al 2014). Reported onset is two to three weeks of age, electromyography is normal, and a decremental response is observed upon repetitive nerve stimulation (Rinz et al 2014). Skeletal muscle and nerve biopsy results are normal, but postsynaptic AChR concentration is marginally decreased (Rinz et al 2014). Neuromuscular junction autoantibody testing for the AChR is negative (Rinz et al 2014). Administration of pyridostigmine bromide results in worsening of skeletal muscle weakness and fatigability (Rinz et al 2014). Genetic testing for this mutation is available for this breed (Rinz et al 2014). In Golden Retrievers, it is caused by a nonconserved missense mutation in exon 13 that is inherited in an autosomal recessive manner (Tsai et al. 2019). Reported onset is six to eight weeks of age, electromyography is normal, but motor nerve conduction studies can identify a mild decrease in the amplitude of the M wave, and repetitive nerve stimulation identifies a decremental response (Tsai et al. 2019). Skeletal muscle and nerve biopsy results are normal, but postsynaptic AChR concentration also is marginally decreased (Tsai et al. 2019). Neuromuscular junction autoantibody testing for the AChR is negative (Tsai et al. 2019). Administration of edrophonium chloride either results in worsening of skeletal muscle weakness and fatigability or has no effect, whereas administration of albuterol results in temporary improvement of skeletal muscle weakness and fatigability (Tsai et al. 2019). Genetic testing for this mutation is available for this breed (Tsai et al. 2019). In Sphynx and Devon Rex cats, the mutation is shared, and is a missense mutation in exon 15, which is inherited in an autosomal recessive manner (Abitbol et al. 2015, Gandolfi et al. 2015). Reported onset is three to twenty-three weeks of age, affected

cats often have decreased skeletal muscle mass and dorsal protrusion of the scapulae (Abitbol et al. 2015, Gandolfi et al. 2015, Malik et al 1993). Electromyography can disclose positive sharp waves and complex repetitive discharges (Abitbol et al. 2015, Gandolfi et al. 2015). A decremental response can be observed upon repetitive nerve stimulation, but in some cats, repetitive nerve stimulation is within normal limits (Abitbol et al. 2015, Gandolfi et al. 2015). Skeletal muscle biopsy samples frequently have dystrophic changes whereas nerve biopsy results are normal, and postsynaptic AChR concentration is normal (Abitbol et al. 2015, Gandolfi et al. 2015, Malik et al 1993). Neuromuscular junction autoantibody testing for the AChR is negative (Abitbol et al. 2015, Gandolfi et al. 2015). Administration of edrophonium chloride can result in exacerbation of skeletal muscle weakness and fatigability (Gandolfi et al. 2015). Genetic testing for this mutation is available for these breeds (Abitbol et al. 2015, Gandolfi et al. 2015). Administration of AChE-Is either has no effect or results in exacerbation of clinical signs in the *COLQ*-associated CMSs reported in dogs and cats (Rinz et al 2014, Tsai et al. 2019, Gandolfi et al. 2015). Administration of albuterol, however, can result in a temporary improvement of clinical signs in the *COLQ*-associated CMS reported in Golden Retrievers (Tsai et al. 2019). Similarly, *COLQ*-associated CMSs in humans respond positively to β2-adrenergic receptor agonists such as albuterol and ephedrine, which are thought to stabilize the NMJ and decrease dispersion of AChRs (Lee et al 2018, Finsterer et al 2019). Acetylcholinesterase inhibitors or 3,4-DAP however are contraindicated in *COLQ*-associated CMSs in humans because their administration results in exacerbation of clinical signs (Lee et al 2018, Finsterer et al 2019). This is because the excess synaptic ACh resulting from the reduction in ACh breakdown from the COLQ mutation is exacerbated by acetylcholinesterase inhibitors and 3,4-DAP (Lee et al 2018, Finsterer et al 2019).

3.1.4.3. Postsynaptic congenital myasthenic syndromes

A postsynaptic CMS is described in Jack Russell Terriers and in a Heideterrier (Rinz et al. 2015, Herder et al 2017). Both of these CMSs are associated with a mutation in the cholinergic receptor nicotinic ε subunit (*CHRNE*) gene, which codes for the ε subunit of the AChR (Rinz et al. 2015, Herder et al 2017). In Jack Russell Terriers it is caused by a deletion mutation in exon 7, and is inherited in an autosomal recessive manner (Rinz et al. 2015). Reported onset is usually six to eight weeks of age (Rinz et al. 2015). Electromyography is normal, but a decremental response is observed upon repetitive nerve stimulation (Rinz et al. 2015). Skeletal muscle and nerve biopsy results are normal, but postsynaptic nicotinic AChR concentration is markedly decreased (Rinz et al. 2015). Neuromuscular junction autoantibody testing for the AChR is negative (Rinz et al. 2015). Administration of AChE-Is results in electrophysiologic as well as temporary clinical improvement (Rinz et al. 2015). Genetic testing for this mutation is available for this breed (Rinz et al. 2015). Jack Russell terriers with *CHRNE*-associated CMS generally benefit from treatment with AChE-Is, but the response can be transient because drug resistance occurs (Rinz et al. 2015). Similarly, *CHRNE*-associated CMSs in humans benefit from AChE-Is, but also from 3,4-DAP (Lee et al 2018, Finsterer et al 2019). Additionally, drug resistance to AChE-Is also is observed in *CHRNE*-associated CMSs in humans, and addition of a β2-adrenergic receptor agonist to their treatment is recommended because it counteracts the adverse effects of long-term AChE-I treatment on the NMJ (Vanhaesebrouck et al 2019). In the Heideterrier, a nonsynonymous mutation in exon 31 is reported in 1 dog with skeletal muscle weakness and fatigability (Herder et al 2017). Reported onset was <1 week of age,

and skeletal muscle weakness and fatigability were described to initially affect the thoracic limbs (Herder et al 2017).

3.2. Myasthenia gravis

3.2.1. Terminology and definition used in humans

Myasthenia gravis is defined in humans as skeletal muscle weakness and fatigability caused by antibodies against components of the NMJ of skeletal muscle (Berrih-Aknin et al 2014, Gilhus and Verschuuren 2015, Gilhus 2016). This definition is used because antibodies are not limited to the AChR, but instead can be directed against muscle specific kinase (MUSK), or low-density lipoprotein receptor-related protein 4 (LRP4) (Berrih-Aknin et al 2014, Gilhus and Verschuuren 2015, Gilhus 2016).

3.2.2. Classification used in humans

Because clinical presentation, diagnosis, optimal treatment, and outcome vary among human MG patients, subgrouping is necessary (Berrih-Aknin et al 2014, Gilhus and Verschuuren 2015, Gilhus 2016). It is performed according to the autoimmune disease mechanism, protein or proteins targeted, status of the thymus gland, genetic characteristics, response to treatment, and whether skeletal muscle involvement is focal or generalised (Berrih-Aknin et al 2014, Gilhus and Verschuuren 2015, Gilhus 2016). Subgroups include AChR antibody positive ocular, early onset, late onset, or thymoma-associated MG, AChR antibody negative MUSK or LRP4 autoantibody positive MG, and seronegative MG (Table 4) (Berrih-Aknin et al 2014, Gilhus and Verschuuren 2015, Gilhus 2016).

Table 4: Classification of myasthenia gravis subgroups in humans as described by Gilhus and Verschuuren (2015)

MG subgroups	**Autoantibody target**	**Age of onset**	**Sex**	**Haplotype associations**	**Thymic status**
Early onset	AChR	<50 years	Female predisposition	Various	Hyperplasia common
Late onset	AChR (titin, ryanodine receptor)	>50 years	Male predisposition	Various	Atrophy common
Thymoma	AChR (titin, ryanodine receptor)	Any age	N/A	N/A	Lymphoepithelioma
MUSK	MUSK	Any age	Female predisposition		Normal
LRP4	LRP4	Any age	N/A	N/A	Normal
Ocular	Variable	Any age	N/A	N/A	Normal
Seronegative	Unknown	Any age	N/A	N/A	Normal or hyperplasia

3.2.3. Terminology and definition in dogs and cats

Myasthenia gravis classification historically has included both acquired and congenital forms of the disease (Shelton 2002, Dickinson and Le Couteur 2004). In recent years, the term CMS has replaced the term congenital MG in dogs and cats, and the term MG now refers only to autoimmunity against the NMJ (Shelton 2016, Shelton 2017).

3.2.4. Classification in dogs and cats

Current classification of MG includes focal, generalised and acute fulminating presentations based on a previous classification system used in humans (Dewey et al 1997, Ducoté et al 1999). Focal MG is defined as weakness in ≥1 focal skeletal muscle group that does not involve the appendicular skeletal muscles (Shelton 2002, Dickinson and Le Couteur 2004, Dewey et al 1997, Ducoté et al 1999, Shelton et al 1990). These focal skeletal muscle groups are the facial, oesophageal, pharyngeal, and laryngeal skeletal muscles (Shelton 2002, Dickinson and Le Couteur 2004, Dewey et al 1997, Ducoté et al 1999, Shelton et al 1990). Generalised MG is defined as appendicular skeletal muscle weakness, which can range from mild to severe, with or without facial, oesophageal, pharyngeal, or laryngeal skeletal muscle involvement (Shelton 2002, Dickinson and Le Couteur 2004, Khorzad et al. 2011, Dewey et al 1997, Ducoté et al 1999). Acute fulminant MG is defined as an acute, rapidly progressive and very severe form of generalised MG frequently but not necessarily causing respiratory failure and death (Shelton 2002, Dickinson and Le Couteur 2004, Khorzad et al. 2011, Dewey et al 1997, Ducoté et al 1999, King and Vite 1998, Richardson 2011). This classification is widely accepted and will therefore be retained. However, we believe that differentiation between generalised and fulminant presentations can be difficult because of lack of objective criteria to differentiate a fulminant presentation from a severe generalised presentation, and that they might represent a continuous spectrum of disease. A separately classified acute fulminant MG form no longer exists in people, rather it is accepted that the disease spectrum of generalised MG ranges from mild to extreme weakness with some patients initially presenting in a myasthenic crisis, which is objectively defined as onset

or exacerbation of skeletal muscle weakness to the point that intubation and mechanical ventilation are required (Berrih-Aknin et al 2014, Gilhus and Verschuuren 2015, Gilhus 2016, Wendell and Levine 2011). In people, a myasthenic crisis can occur at any time point in the disease including as the initial presentation of the disease (Wendell and Levine 2011). As in humans, the autoimmune disease mechanism can affect treatment, outcome, or both, and relates to the presence or absence of a thymoma, or administration of thiourylene medication in cats (Shelton 2002, Dickinson and Le Couteur 2004, Khorzad et al. 2011, Shelton et al 2000, Hague et al 2015, Bell et al 2012, Ellis and Tappin 2019, Lainesse et al 1996, Shelton and Linstrom 2001). This classification therefore introduces subgroup classification according to the autoimmune disease mechanism. Lastly, as in humans, NMJ autoantibody testing can be negative in some dogs with generalised MG (Shelton 2002, Shelton 2010). Such cases must fulfil specific criteria to be referred to as seronegative, including serum AChR antibody testing to have been negative at least twice (Shelton 2002, Shelton 2010). A seronegative subgroup was not included in the previous classification of MG in dogs because of a lack of defining criteria (Dewey et al 1997). Such criteria are now available, and classification based on seronegativity is therefore possible in dogs (Shelton 2002, Shelton 2010). A summary of this study's proposed classification of MG in dogs and cats is presented in Table 5.

Table 5: Classification of myasthenia gravis in dogs and cats

Focal myasthenia gravis	Nonthymoma associated subgroup
	Thymoma associated subgroup
Generalised myasthenia gravis	Nonthymoma associated subgroup
	Thymoma associated subgroup
	Thiourylene medication associated subgroup (cats only)
	Seronegative subgroup (dogs only)
Acute fulminant myasthenia gravis	Nonthymoma associated subgroup
	Thymoma associated subgroup

Additional characteristics of the disease that are used in humans to further classify patients, such as protein or proteins targeted, thymus gland status, and genetic characteristics cannot be used at this time for classification of MG in dogs and cats. The underlying reasons are discussed below. Regardless of the subgroup, certain dogs and cats can present with associated coexisting diseases (Shelton 2002, Ducoté et al 1999, Shelton 1998, Penderis and Martin-Vaquero 2015, Dewey et al 1995, Hackett et al 1995, Levine et al 2005, Mayousse et al 2017, Hill et al 2013, Singh et al 2010). The implications of the presence of these coexisting diseases on patients with MG are currently unknown (Shelton 2002). Although such affected dogs and cats are classified into specific subgroups, we recognize that treatment, outcome, or both might differ from the remainder of their respective subgroup depending on the concurrent condition or conditions. However the decision to include such cases into a specific subgroup appears reasonable given that all other aspects of their MG are consistent with the subgroup in which they are categorised.

3.2.4.1. Non–thymoma-associated myasthenia gravis

Dogs and cats in the focal, generalised, and acute fulminating categories that do not have a thymoma are referred to by this designation. The targeted protein is the AChR, although striational autoantibodies against titin and the ryanodine receptor are reported in some dogs (Shelton 2002, Dickinson and Le Couteur 2004, Khorzad et al. 2011, Hague et al 2015, Shelton et al 2001). These striational autoantibodies are typically accompanied by AChR autoantibodies when clinical signs of skeletal muscle weakness and fatigability are present, and are thought not to be pathogenic, hence they are not classified separately, although they might serve as markers of disease severity (Gilhus and Verschuuren 2015, Gilhus 2016, Shelton et al 2001). Attempts at categorising some dogs and cats into early or late onset non–thymoma-associated MG subgroups, as in humans, are reported (Mayousse et al 2017, Shelton et al 2001, Wolf et al 2017). These range from being based on severity of clinical signs alone, to using the bimodal age of onset alongside either demonstration of an association with the *DLA-DQB1* haplotype, itself frequently observed in humans with early onset MG, or by identification of striational antibodies, which are predominantly but not exclusively reported in late onset MG in humans (Mayousse et al 2017, Shelton et al 2001, Wolf et al 2017, Gilhus 2016). However, the frequency of thymic hyperplasia or atrophy is unknown in dog and cats, as is the benefit of removing the thymus in those cases (Dewey et al 1997, Hague et al 2015, Day 1997). The presence of an age-related sex predisposition as seen in humans is not clear in dogs and cats, and despite a bimodal age of onset, no cut-off value permits separation into early and late onset MG subgroups (Dewey et al 1997, Ducoté et al 1999). Because of this lack of necessary information, it is prudent not to subgroup dogs and cats into early and late onset non–thymoma-associated MG subgroups at this time.

3.2.4.2. Thymoma-associated myasthenia gravis

Dogs and cats in the focal, generalised, and acute fulminating categories that have a thymoma are referred to by this designation. In these dogs and cats, MG is the result of a paraneoplastic syndrome (Shelton 2002, Dickinson and Le Couteur 2004, Khorzad et al. 2011, Dewey et al 1997, Hague et al 2015). The targeted protein is the AChR, and striational autoantibodies against titin and the ryanodine receptor also are reported in some dogs (Shelton 2002, Dickinson and Le Couteur 2004, Khorzad et al. 2011, Dewey et al 1997, Hague et al 2015, Shelton et al 2001). In creating a thymoma-associated MG subgroup, the authors recognize that classification of dogs or cats with any concurrent neoplasia, but specifically those with cranial mediastinal neoplasia, might be difficult because it could be argued that such tumours also might induce MG as part of a paraneoplastic syndrome (Shelton 2002). However, aside from individual reports, no neoplasia is repeatedly identified in conjunction with MG in dogs, cats, or even humans, suggesting that the presence of concurrent neoplasia that is not a thymoma might be coincidental (Shelton 2002, Krotje et al 1990, Moore et al 1990, Ridyard et al 2000, Hague et al 2015, Gilhus 2016).

3.2.4.3. Thiourylene medication-associated myasthenia gravis

Myasthenia gravis occurs secondarily to the administration of thiourylene medication in some cats as a consequence of a reversible break in tolerance to self-AChRs (Shelton 2002, Dickinson and Le Couteur 2004, Shelton et al 2000, Bell et al 2012, Ellis and Tappin 2019). Myasthenia gravis appears drug-induced and reversible in this subgroup because clinical signs often resolve after discontinuation of the medication (Dickinson and Le Couteur 2004). This subgroup is only present in the generalised category of MG, because all cats with thiourylene medication-associated MG to date have been reported to have generalised clinical

signs (Shelton 2002, Dickinson and Le Couteur 2004, Shelton et al 2000, Bell et al 2012, Ellis and Tappin 2019).

3.2.4.4. Seronegative myasthenia gravis

Acetylcholine receptor autoantibody testing by radioimmunoassay is negative in approximately 2% of dogs with generalised MG and they are referred to as seronegative (Shelton 2002). To be classified as such, dogs are required to have a general and neurological examination, pharmacologic, and electrophysiologic findings consistent with MG, as well as normalisation of clinical signs after AChE-I treatment and for serum AChR autoantibody testing by radioimmunoassay to have been negative at least twice (Shelton 2002). Because AChR autoantibody testing can be negative early in the course of the disease and because many patients subsequently can seroconvert, measurement of serum AChR should be repeated 1 to 2 months after a negative AChR test (Shelton 2002). Prior treatment with immunosuppressive medication can result in a negative result, and should therefore be ruled out. Possible explanations as to why AChR autoantibody radioimmunoassay testing can be negative in dogs with MG include damage to the antigenic epitopes during the process of solubilization thereby preventing recognition of autoreactive AChR autoantibodies, the majority of autoantibodies being bound in the skeletal muscle thereby causing circulating AChR autoantibody concentration to be within normal limits, or autoantibodies being directed against the toxin binding site (Shelton et al. 1990). Additionally, autoantibodies can be directed against other components of the postsynaptic NMJ (Shelton 2010). This has been demonstrated in 1 dog seronegative for the AChR in which autoantibodies were detected against MUSK (Shelton 2010). Availability of MUSK autoantibody testing is limited and no established reference range is available in dogs (Shelton 2010). The percentage of dogs with

focal MG, and cats with focal or generalised MG, that are seronegative is unknown (Shelton 2002). Such cases might exist, however because they have not been reported and no criteria are available to define them, such subgroups are not included in this classification.

4. Discussion

This review of the literature has provided a classification system of MG and CMSs in dogs and cats to aid recognition of the disease groups for both conditions, as well as guide treatment, refine prognosis, and provide a framework for additional studies of these conditions.

Given the degree of heterogeneity of the various congenital myasthenic syndromes in dogs and cats highlighted in this novel classification system, it has become evident that diagnostic test results must be interpreted in light of the specific CMS being investigated. For instance, administration of edrophonium chloride as part of a challenge test can result in transient marked improvement of skeletal muscle weakness and fatigability, but can otherwise result in exacerbation of skeletal muscle weakness and fatigability, or might have no effect depending on the CMS (Shelton 2002, Rinz et al 2014, Rinz et al. 2015, Tsai et al. 2019, Prochowsky et al 2007, Gandolfi et al. 2015). Similarly, although a decremental response is frequently observed upon repetitive nerve stimulation in most CMSs, there is a CMS in cats in which RNS often can be normal, and a CMS in dogs in which a preceding high frequency conditioning train usually is required before observation of a decremental response (Rinz et al 2014, Rinz et al. 2015, Tsai et al. 2019, Prochowsky and Flagstad 2007, Abitbol et al. 2015, Gandolfi et al. 2015). Ultimately though, genetic testing is required to achieve a definitive diagnosis, and when negative, whole genome sequencing analysis should be considered and

might permit discovery of previously unknown mutations (Tsai et al. 2019, Blakey et al. 2017).

With the recent identification of the genetic mutations responsible for some CMSs in dogs and cats, it is now clear that the causative gene plays a pivotal role as to which symptomatic treatment benefits a given CMS (Rinz et al 2014, Rinz et al. 2015, Tsai et al. 2019, Prochowsky et al 2007, Gandolfi et al. 2015, Trojaborg and Flagstad 1982). Efforts must therefore be made to obtain a genetic diagnosis, permitting institution of appropriate symptomatic treatment because inappropriate symptomatic treatment can result in exacerbation of clinical signs (Rinz et al 2014, Tsai et al. 2019, Gandolfi et al. 2015).

Given the shared pathogenesis, and similarities in the response to a given symptomatic treatment among CMSs in humans, dogs, and cats with shared causative mutations, it is logical to consider similar treatments in dogs and cats as those used in humans for a given CMS, which emphasises the importance of correctly classifying CMSs in dogs and cats.

Although this classification system has been purposefully designed to accommodate possible novel disease groups, revisions eventually will be needed.

5. Conclusion

Updated terminology adopted from human medicine defines MG as an autoimmune disorder that impairs neuromuscular transmission by the production of autoantibodies against the NMJ of skeletal muscle. Congenital myasthenic syndromes are a clinically heterogeneous group of genetic disorders causing aberrant neuromuscular transmission. Both conditions encompass disease groups, the recognition of which is important with regard to treatment, outcome, or both. This review of the literature has provided a classification system of MG and CMSs in

dogs and cats to aid recognition of the disease groups for both conditions, as well as guide treatment, refine prognosis, and provide a framework for additional studies of these conditions. This classification system has been purposefully designed to accommodate possible novel disease groups, but revisions eventually will be needed.

Chapter IV: Composition of the tunica muscularis of the oesophagus in dogs

1. Introduction

A significantly higher incidence of MG associated megaoesophagus is reported in dogs (88%) compared to cats (40%), or even humans in which there are only a few reported cases (Desuter et al. 2015, Ducoté et al. 1999, Shelton et al. 1997). This has been attributed to the tunica muscularis of the canine oesophagus being described as solely composed of skeletal muscle as opposed to that of cats or humans in which the distal third and two thirds are respectively reported to contain smooth muscle (Hague et al. 2015, Meyer et al. 1986). This description of the tunica muscularis of the oesophagus of dogs as being solely composed of skeletal muscle is based on a population of 9 crossbreed dogs in the United States of America and 22 dogs in Germany (Mann and Shorter 1964, Bush 1980). Recently, however, the distal third of the tunica muscularis was reported to be composed of smooth muscle in a population of 8 Iraqi dogs (Dawood et al. 2022). This would be consistent with the tunica muscularis described in most omnivores in which there a mixture of skeletal and smooth muscle, as opposed to that of ruminants which is solely composed of skeletal muscle (Majewski et al. 2003, Sukon et al. 2009).

2. Materials and methods

2.1. Study design

Dogs submitted for post-mortem investigation to the University of Nottingham's veterinary pathology service between June 2022 and February 2023 were prospectively recruited. Inclusion criteria were (1) the absence of any clinical history of oesophageal disease as well as (2) the absence of any oesophageal abnormalities/disease on post-mortem investigation performed by a board-certified veterinary pathologist. Consent for inclusion into the study

was gained through the veterinary pathology submission form (Supplement 1). Population sample size was determined using an online sample size calculator (Sample Size Calculator, Maple Tech International LLC, 2203 Timberlock PI, Suite 252, The Woodlands, Texas 77380, https://www.calculator.net/sample-size-calculator.html) with a confidence level set at 95% and a population proportion set at 98%. The resulting recommended required sample size was thirty dogs. The sample group was split equally into n=10 small breed dogs (<10 kg of body weight), n=10 medium breed dogs (>10 kg - < 30 kg of body weight), and n= 10 large breed dogs (> 30 kg of body weight).

2.2. Ethical consideration

Ethical approval was sought and gained from the University of Nottingham School of Veterinary Medicine and Science's ethics committee for this study (Ethical approval number 3238 201001).

2.3. Tissue trimming

The oesophagus was removed in its entirety, then folded over its middle point. Entire transverse sections of the most proximal five centimetres, most distal five centimetres, and middle five centimetres were then collected. When the length of the oesophagus was less than fifteen centimetres, entire transverse sections of the most proximal third, most distal third, and middle third were collected.

2.4. Tissue fixation

Samples were trimmed into cassettes and fixed in 10% neutral buffered formalin (10% Neutral Buffered Formalin, CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom) for a minimum of 48 hours.

2.5. Tissue processing

Tissue processing was carried out using a tissue processor (Epredia™, Excelsior™ AS Tissue Processor, Shandon Diagnostics Ltd, Tudor Road, Manor Park, Runcorn, Chechire, WA7 1TA, United Kingdom) using the following protocol. Tissues were placed in 10% neutral buffered formalin (10% Neutral Buffered Formalin, CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom) for one hour, followed by immersion in six consecutive alcohol solutions of increasing purity each for one hour (Industrial Denatured Alcohol 99%, CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom), followed by immersion in three consecutive Xylene solutions of increasing purity each for 90 minutes (Xylene, CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom), followed by 3 consecutive melted paraffin bathes set at 62 degrees Celsius for 90 minutes each (Cellwax plus (S), CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom).

2.6. Tissue embedding

Tissue was embedded using an embedding station (Leica EG-1160, Leica Microsystems Ltd, Larch House, Woodlands Business Park, Breckland, Linford Wood, Milton Keynes, MK14 6FG, United Kingdom) using the following protocol. Cassettes were placed into a cassette bath consisting of melted paraffin set at 61 degrees Celsius (Cellwax plus (S), CellPath Ltd,

80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom). A suitably sized stainless-steel mould (Stainless Steel Base Mould, CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom) was then placed onto the embedding station's heated work surface set at 60 degrees Celsius. Cassettes were then removed from the melted paraffin bath and the cassette lid was removed using heated forceps set at 62 degrees Celsius (Cellceps Plus 2 mm serrated heated forceps, CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom). Melted paraffin (Cellwax plus (S), CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom) from the melted paraffin dispensing chamber set at 61 degrees Celsius was then added to the mould so to cover the bottom of the mould sufficiently. The tissue was then placed and orientated into the mould using heated forceps set at 62 degrees Celsius (Cellceps Plus 2 mm serrated heated forceps, CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom). The mould and tissue were then placed onto the embedding station's cold plate, set at -0.5 degrees Celsius so to allow the tissue to be held into position by the cooling paraffin. Care was given not to cool the tissue and paraffin for too long so to avoid creating separate layers of paraffin which might fracture during trimming. The cassette was then pushed down on top of the mould so to form a seal before being topped up with more melted paraffin (Cellwax plus (S), CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom) so to fill the cassette to result in the cassette being 75% full. The cassette and mould were then moved back to the cold plate. Once set, the paraffin block was lifted out of the mould and any excess wax was trimmed from the edges using a heated block trimmer set at 90 degrees Celsius (Block Trimmer Plus, CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom). All paraffin blocks were visually inspected for cracks and/or air bubbles. If any cracks or air was

identified, the block was then placed back into a cassette bath and the embedding process was repeated as previously described.

2.7. Tissue trimming

Paraffin blocks were trimmed using a sectioning microtome (Leica RM2235, Leica Microsystems Ltd, Larch House, Woodlands Business Park, Breckland, Linford Wood, Milton Keynes, MK14 6FG, United Kingdom) along with a microtome blade (Infinity microtome blade DT31N50, C. L. Sturkey Inc, 824 Cumberland Street, Lebanon, Pennsylvania, 17042, United States of America) with the clearance angle set at five degrees. Ten micrometre thick transverse sections were trimmed off of the paraffin blocks until the tissue surface was exposed. Once the paraffin blocks were satisfactorily trimmed, they were placed face down onto an ice block ahead of tissue cutting.

2.8. Tissue cutting

Tissue was cut using a sectioning microtome (Leica RM2235, Leica Microsystems Ltd, Larch House, Woodlands Business Park, Breckland, Linford Wood, Milton Keynes, MK14 6FG, United Kingdom) with the clearance angle set at five degrees along with a microtome blade (Infinity microtome blade DT31N50, C. L. Sturkey Inc, 824 Cumberland Street, Lebanon, Pennsylvania, 17042, United States of America). Five micrometre transverse sections were cut for slides which were to undergo haematoxylin and eosin or phosphotungstic acid-haematoxylin staining, whereas 3 micrometre transverse sections were cut for the slides that were to undergo immunohistochemistry for smooth muscle actin staining. Transverse sections were cut until they formed a ribbon of four sections. The ribbons were then delicately placed over a distilled water bath (Purified/De-ionised Water, CellPath Ltd, 80

Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom) set at 45 degrees Celsius, allowing any wrinkles to disappear. Care was also taken to release/remove any air bubbles from the distilled water bath beforehand. Sections were then gently separated using forceps and the best sections were picked up using a slide submerged in the water. Slides that were to undergo haematoxylin and eosin (Knittel StarFrost, Waldemar Knittel Glasbearbeitungs GmbH, Varrentrappstr. 5, D-38114 Braunschweig, Deutschland), or phosphotungstic acid-haematoxylin (Knittel StarFrost Adhesive, Waldemar Knittel Glasbearbeitungs GmbH, Varrentrappstr. 5, D-38114 Braunschweig, Deutschland) staining were then labelled with the block identification number using a heat and chemical resistant marker pen (CellMark Marker Black, CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom). Slides (TOMO IHC Adhesive Glass Slide, Matsunami Glass Ind Ltd, 2-1-10 Yasaka-cho, Kishiwada City, Osaka 596-0049, Japan) that were to undergo smooth muscle actin immunohistochemistry were labelled using a compatible sticker label (Leica BOND Universal Slide Labels, Leica Microsystems Ltd, Larch House, Woodlands Business Park, Breckland, Linford Wood, Milton Keynes, MK14 6FG, United Kingdom). Slides were then left to drain by being placed in a leaning position against the side of the water bath on top of a piece of paper for fifteen minutes. Slides were then placed on a hotplate set at 60 degrees Celsius for 60 minutes to allow the sections to adhere to the slide. Following this, slides were placed in a slide rack (Slide Rack AGH112, Agar Scientific Ltd, Unit 7, M11 Business Link, Personage Lane, Stansted, Essex, CM24 8GF, United Kingdom) ahead of staining.

2.9. Haematoxylin and eosin staining

Slides were dewaxed by being immersed in xylene (Xylene, CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom) for five minutes, followed by alcohol for five minutes (Alcohol 99%, CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom), before being rinsed under tap water for thirty seconds. The slides were then immersed in a haematoxylin solution (VFM Harris Haematoxylin Stain, CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom) for five minutes, before being rinsed under tap water for thirty seconds. The slides were then differentiated in acid alcohol (Acid Alcohol 3%, Atom Scientific Ltd, Unit 2A, East Tame Business Park, Rexcine Way, Hyde, Cheshire, SK14 4GX, United Kingdom) over five slow dips or until the background was clear. The slides were then rinsed under tap water for thirty seconds. The slides were then immersed in Scott's tap water bluing reagent (Scott's Tap Water, CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom) for 10 seconds. The slides were then rinsed under tap water for 30 seconds. The slides were then dipped in an eosin solution (Eosin Y 1% Aqueous, CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom) for 5 minutes. The slides were then rinsed under tap water for 30 seconds. The slide were then dehydrated in an alcohol solution (Industrial Denatured Alcohol 99%, CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom), cleared in xylene (Xylene, CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom), and mounted using pertex (Pertex Mounting Medium, Histolab Products AB, Södra Långebergsgatan 36, Västra Frölunda, 36, 421 32, Sweeden), before

being covered with a cover slide (ClariTex Coverslip, CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom).

2.10. Phosphotungstic acid-haematoxylin staining

Slides were dewaxed by being immersed in xylene (Xylene, CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom) for five minutes, followed by alcohol for five minutes (Alcohol 99%, CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom), before being rinsed under tap water for thirty seconds. The slides were then immersed in a coplin jar (Coplin Jar, Agar Scientific Ltd, Unit 7, M11 Business Link, Personage Lane, Stansted, Essex, CM24 8GF, United Kingdom) containing 50 millilitres of potassium permanganate solution (Potassium permanganate 0.5%, Atom Scientific Ltd, Unit 2A, East Tame Business Park, Rexcine Way, Hyde, Cheshire, SK14 4GX, United Kingdom) together with 2.5 millilitres of sulphuric acid (Sulphuric Acid <3%, Atom Scientific Ltd, Unit 2A, East Tame Business Park, Rexcine Way, Hyde, Cheshire, SK14 4GX, United Kingdom) for five minutes. The slides were then bleached by pipetting a sufficient amount of oxalic acid (Oxalic Acid 5% Solution, Atom Scientific Ltd, Unit 2A, East Tame Business Park, Rexcine Way, Hyde, Cheshire, SK14 4GX, United Kingdom) onto the slide for one minute. The slides were then rinsed in tap water. The slides were then immersed inside another coplin jar containing haematoxylin in phosphotungstic acid-haematoxylin solution (Haematoxylin solution in PTAH, Atom Scientific Ltd, Unit 2A, East Tame Business Park, Rexcine Way, Hyde, Cheshire, SK14 4GX, United Kingdom) for 24 hours. The slides were then rinsed in tap water. The slide were then dehydrated in an alcohol solution (Industrial Denatured Alcohol 99%, CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom), cleared in xylene (Xylene, CellPath

Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom), and mounted using pertex (Pertex Mounting Medium, Histolab Products AB, Södra Långebergsgatan 36, Våstra Frölunda, 36, 421 32, Sweeden), before being covered with a cover slide (ClariTex Coverslip, CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom).

2.11. Smooth muscle acting immunohistochemistry

Immunohistochemistry was carried out using an automated immunohistochemistry staining machine (Leica Bond III Fully Automated IHC and ISH Staining System, Leica Microsystems Ltd, Larch House, Woodlands Business Park, Breckland, Linford Wood, Milton Keynes, MK14 6FG, United Kingdom) using the following protocol. Slides were placed on the machine specific rack (Leica Bond III Fully Automated IHC and ISH Staining System, Leica Microsystems Ltd, Larch House, Woodlands Business Park, Breckland, Linford Wood, Milton Keynes, MK14 6FG, United Kingdom) and were covered with a machine compatible cover slide (Leica BOND Universal Covertiles, Leica Microsystems Ltd, Larch House, Woodlands Business Park, Breckland, Linford Wood, Milton Keynes, MK14 6FG, United Kingdom). Slides were dewaxed inside the automated immunohistochemistry staining machine (Leica Bond III Fully Automated IHC and ISH Staining System, Leica Microsystems Ltd, Larch House, Woodlands Business Park, Breckland, Linford Wood, Milton Keynes, MK14 6FG, United Kingdom) using a dewaxing solution (Dewax-Solution, Leica Microsystems Ltd, Larch House, Woodlands Business Park, Breckland, Linford Wood, Milton Keynes, MK14 6FG, United Kingdom). Following this, slides underwent epitope retrieval by being exposed to 150 microliters of a citrate solution (BOND Epitope Retrieval

Solution 1, Leica Microsystems Ltd, Larch House, Woodlands Business Park, Breckland, Linford Wood, Milton Keynes, MK14 6FG, United Kingdom) for 20 minutes.

Slides then underwent an endogenous peroxidase block by being exposed to 150 microliters of a peroxidase blocking solution (Peroxidase-Block, Leica Microsystems Ltd, Larch House, Woodlands Business Park, Breckland, Linford Wood, Milton Keynes, MK14 6FG, United Kingdom) for five minutes. Slides then underwent three consecutive bond washes, each time using 150 microliters of a bond wash solution (BOND Wash Solution 10X Concentrate, Leica Microsystems Ltd, Larch House, Woodlands Business Park, Breckland, Linford Wood, Milton Keynes, MK14 6FG, United Kingdom) that was diluted 1:10 with distilled water (Purified/De-ionised Water, CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom) per slide. The slides were then exposed for fifteen minutes to 150 microliters of a rabbit polyclonal to alpha smooth muscle actin primary antibody (Anti-alpha smooth muscle actin antibody ab5694, Abcam Plc, 152 Grove Street, Waltham, Massachusetts, 02453, United States of America) which was diluted 1:800 with an antibody diluent (Leica Bond Primary Diluent AR9352, Leica Microsystems Ltd, Larch House, Woodlands Business Park, Breckland, Linford Wood, Milton Keynes, MK14 6FG, United Kingdom). Slides then underwent another three consecutive bond washes, each time using 150 microliters of a bond wash solution (BOND Wash Solution 10X Concentrate, Leica Microsystems Ltd, Larch House, Woodlands Business Park, Breckland, Linford Wood, Milton Keynes, MK14 6FG, United Kingdom) that was diluted 1:10 with distilled water (Purified/De-ionised Water, CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom) was used. Slides were then exposed to 150 microliters of a post primary antibody solution (Post Primary Block, Leica Microsystems Ltd, Larch House,

Woodlands Business Park, Breckland, Linford Wood, Milton Keynes, MK14 6FG, United Kingdom). Slides then underwent another three consecutive bond washes, each time using 150 microliters of a bond wash solution (BOND Wash Solution 10X Concentrate, Leica Microsystems Ltd, Larch House, Woodlands Business Park, Breckland, Linford Wood, Milton Keynes, MK14 6FG, United Kingdom) that was diluted 1:10 with distilled water (Purified/De-ionised Water, CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom) was used. The slides were then exposed to a 150 microliters of a polymer-based detection solution (Polymer, Leica Microsystems Ltd, Larch House, Woodlands Business Park, Breckland, Linford Wood, Milton Keynes, MK14 6FG, United Kingdom) for 8 minutes. Slides then underwent another 2 consecutive bond washes, each time using 150 microliters of a bond wash solution (BOND Wash Solution 10X Concentrate, Leica Microsystems Ltd, Larch House, Woodlands Business Park, Breckland, Linford Wood, Milton Keynes, MK14 6FG, United Kingdom) that was diluted 1:10 with distilled water (Purified/De-ionised Water, CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom) was used. Slides were then exposed to 150 microliters of a 3,3′-diaminobenzidine tetrahydrochloride hydrate solution mixed with a hydrogen peroxide stain enhancing solution (DAB enhancer, Leica Microsystems Ltd, Larch House, Woodlands Business Park, Breckland, Linford Wood, Milton Keynes, MK14 6FG, United Kingdom) for 10 minutes. Slides were then rinsed with distilled water (Purified/De-ionised Water, Leica Microsystems Ltd, Larch House, Woodlands Business Park, Breckland, Linford Wood, Milton Keynes, MK14 6FG, United Kingdom). Slides were then exposed to 150 microliters of a haematoxylin solution (Haematoxylin, Leica Microsystems Ltd, Larch House, Woodlands Business Park, Breckland, Linford Wood, Milton Keynes, MK14 6FG, United Kingdom) for five minutes. Following this, slides were removed from the

immunohistochemistry machine and were transferred to a slide rack (Slide Rack AGH112, Agar Scientific Ltd, Unit 7, M11 Business Link, Personage Lane, Stansted, Essex, CM24 8GF, United Kingdom) before being rinsed in tap water. The slide were then dehydrated in an alcohol solution (Industrial Denatured Alcohol 99%, CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom), cleared in xylene (Xylene, CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom), and mounted using pertex (Pertex Mounting Medium, Histolab Products AB, Södra Långebergsgatan 36, Våstra Frölunda, 36, 421 32, Sweeden), before being covered with a cover slide (ClariTex Coverslip, CellPath Ltd, 80 Mochdre Enterprise Park, Newtown, Powys, SY16 4LE, United Kingdom).

2.12. Tissue analysis

Slides were manually evaluated by a single observer (TM) under the supervision of a board-certified veterinary pathologist (WW), with a binocular light microscope (Leica DM750, Leica Microsystems Ltd, Larch House, Woodlands Business Park, Breckland, Linford Wood, Milton Keynes, MK14 6FG, United Kingdom). Haematoxylin and eosin was used for identification of the muscoa, submucosa, tunica muscularis and serosa layers. Identification of skeletal muscle was based on observation of the characteristic striated pattern created by the sarcomeres along with eccentric position of the nuclei all of which were highlighted by the strong positive phosphotungstic acid-haematoxylin staining giving the tissue a blue to purple haematoxylin-based colour (Valentine 2017). Positive brown/yellow staining in the tunica muscularis on smooth muscle actin immunohistochemistry was used to identify smooth muscle. Positive and negative controls were used for both the PTAH staining and SMA immunohistochemistry protocols. A transverse section of duodenum small intestine and

a section of bicep femoris muscle were respectively used as positive controls for the PTAH staining and SMA immunohistochemistry protocols while a section of liver was used as a negative control for both the PTAH staining and SMA immunohistochemistry protocols.

2.13. Statistical analysis

Statistical analysis of the muscular composition of the oesophagus was performed through determination of exact confidence intervals using an online exact confidence interval calculator (Kohn and Senyak 2021).

3. Results:

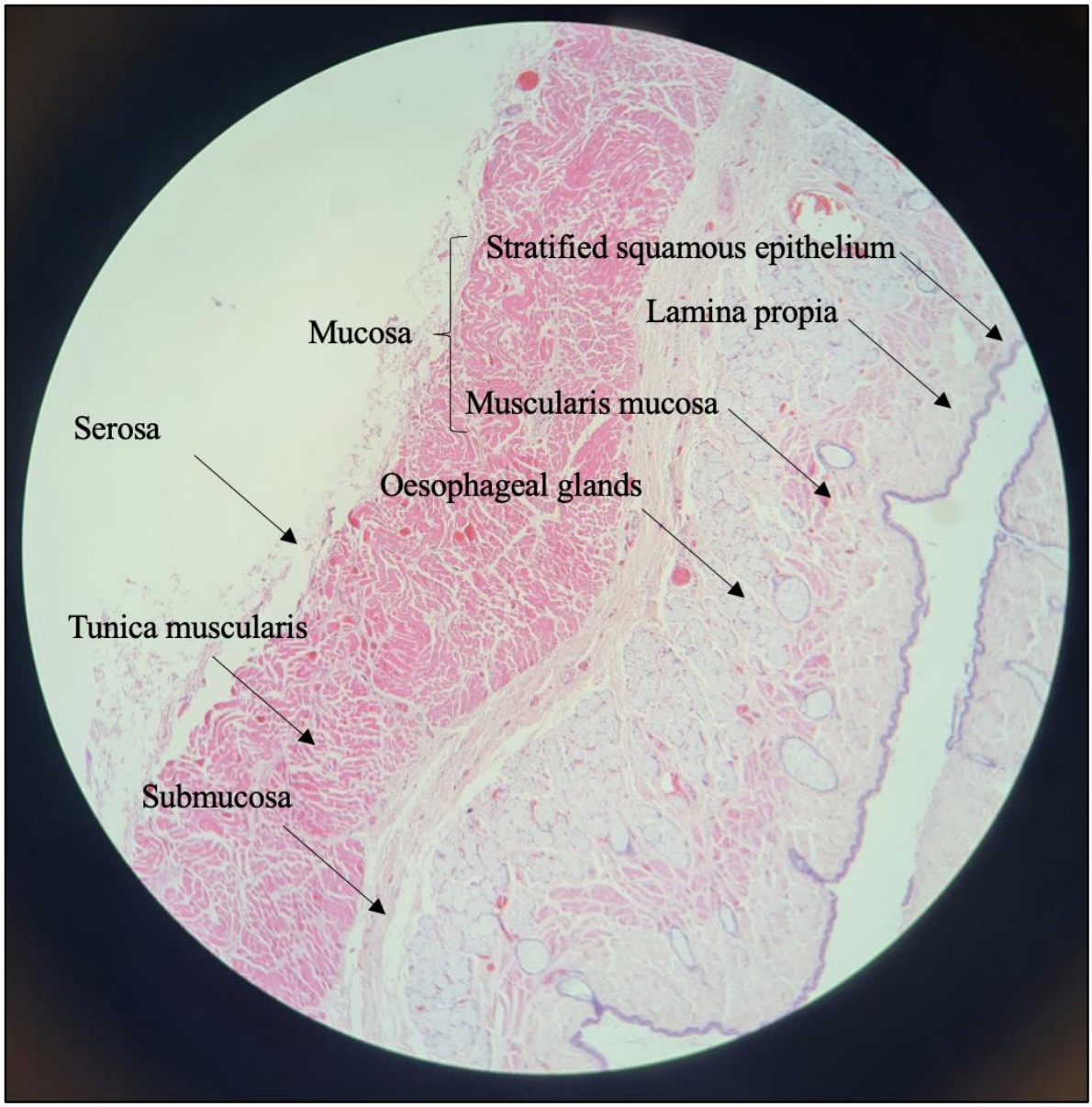
3.1. Population

Thirty dogs were recruited as per the inclusion criteria. The small dog population recruited (n=10) consisted of Shih-tzu (n=3), Boston terrier (n=2), Jack Russel Terrier (n=2), Miniature Pinscher (n=1), Toy Poodle (n=1), and crossbreed (n=1). The medium dog population recruited (n=10) consisted of Springer Spaniel (n=3), Border Collie (n=2), crossbreed (n=1), Beagle (n=1), Airedale terrier (n=1), Pembroke Welsh Corgi (n=1), and French Bulldog (n=1). The large dog population recruited (n=10) consisted of Labrador Retrievers (n=3), Golden Retrievers (n=2), Rottweiler (n=1), Siberian Husky (n=1), Doberman Pinscher (n=1), Rhodesian Ridgeback (n=1), Newfoundland (n=1). Eighteen dogs were male, and twelve dogs were female. Twenty-four dogs were neutered while six dogs were entire. Age at the time of inclusion into the study ranged from eleven months of age to thirteen years of age (median age of eight years).

3.2. Haematoxylin and eosin staining

Haematoxylin and eosin staining was consistent across all thirty dogs, throughout the cervical, thoracic, and abdominal regions of the oesophagus, and revealed four histological layers. The mucosal layer was composed from inward to outward of a stratified squamous epithelium layer, loose connective tissue consistent with lamina propria, and a small muscular layer consistent with muscularis mucosa respectively. The mucosa was surrounded by a dense irregular layer of connective tissue and glands consistent with submucosa, itself surrounded by a muscular layer consistent with tunica muscularis, and finally an external fibrous layer consistent with serosa.

Figure 5: Microscope image of the canine oesophagus stained with haematoxylin and eosin

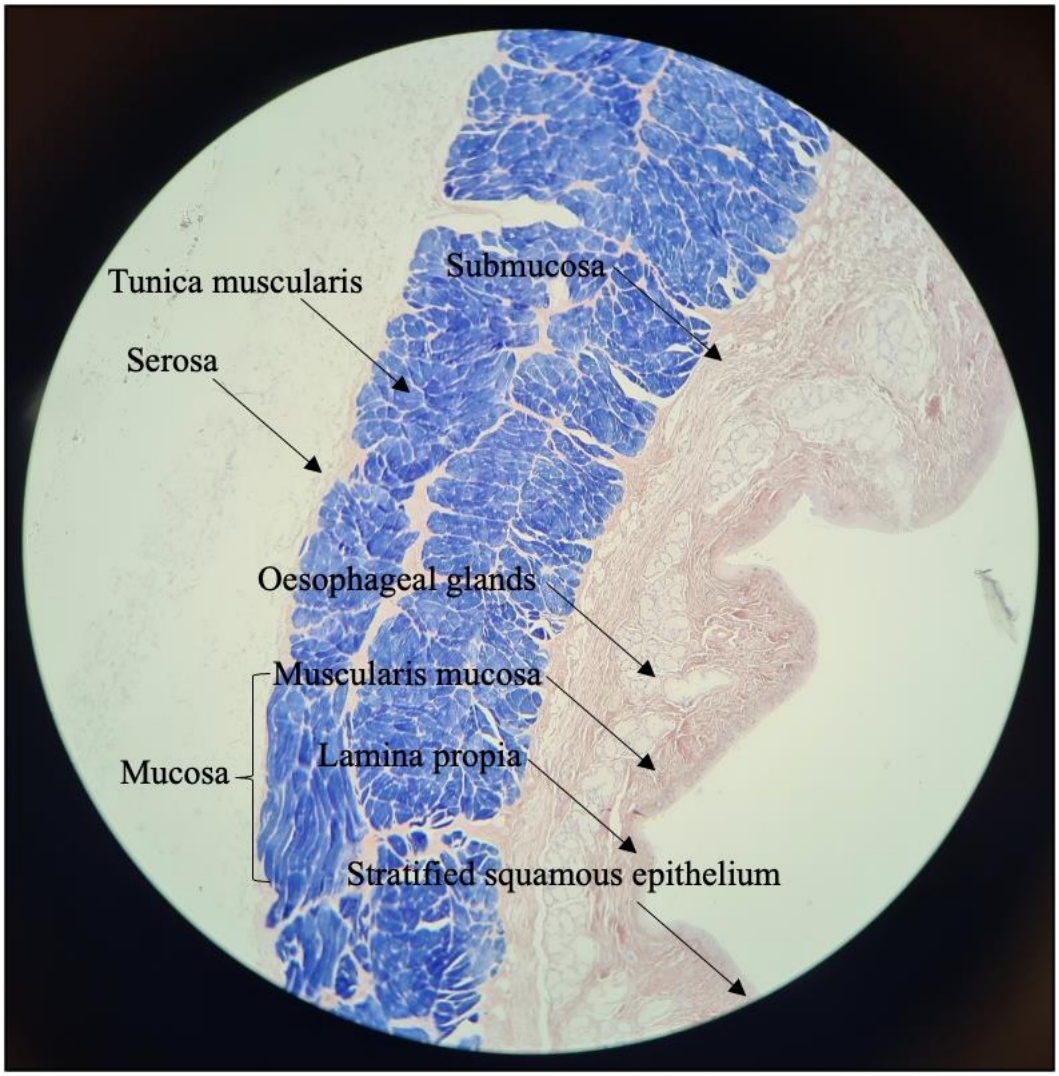


3.3. Phosphotungstic acid haematoxylin staining

Phosphotungstic acid haematoxylin staining was consistent across all thirty dogs, throughout the cervical, thoracic, and abdominal regions of the oesophagus, revealing strong positive staining of the entire tunica muscularis layer, revealing cross-striations consistent with two

layers of skeletal muscle. There was no positive staining observed in the remaining layers of the oesophagus except for some positive staining inside blood vessels in the mucosa, and submucosa layers, most consistent with fibrin.

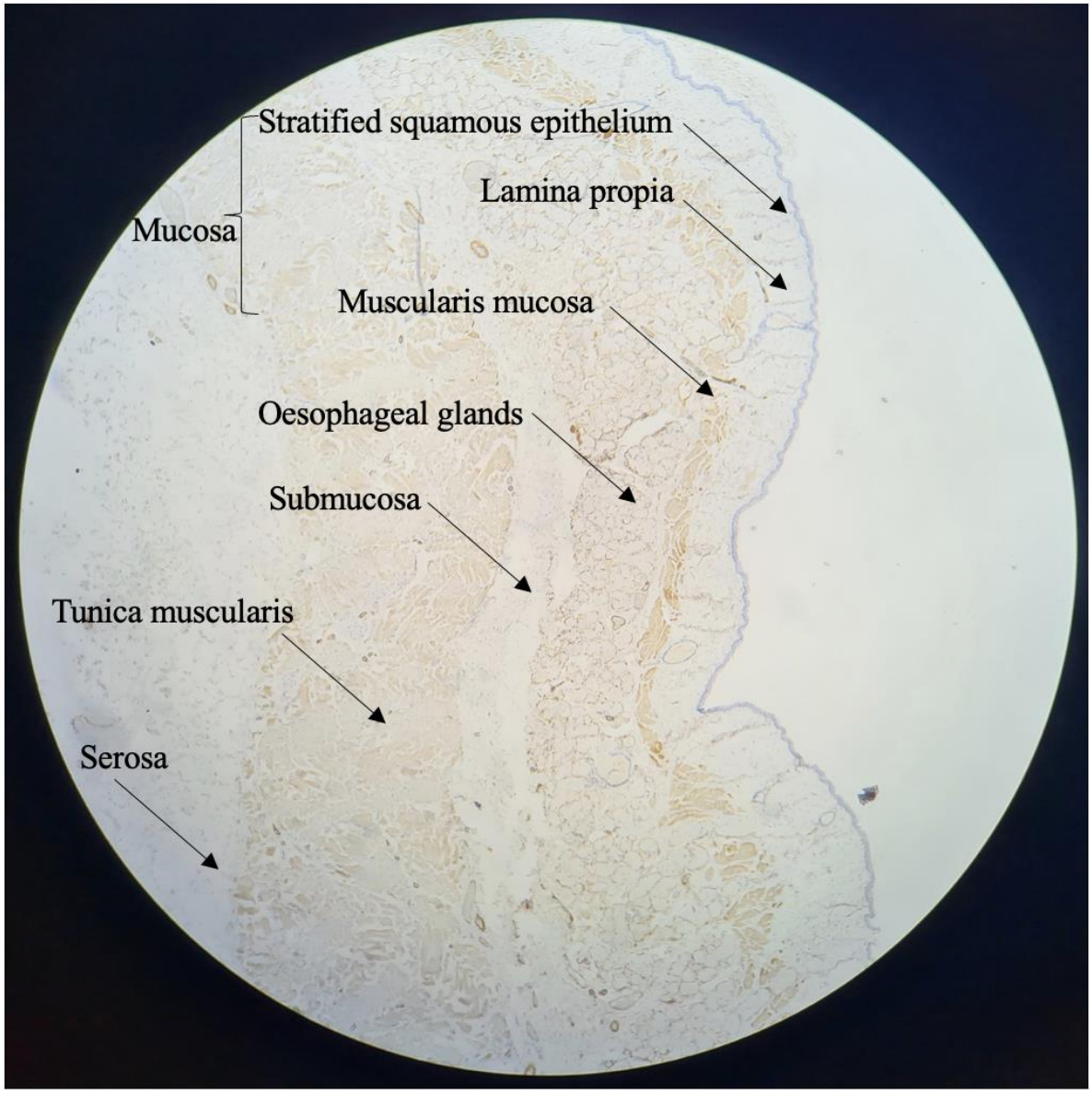
Figure 6: Microscope image of the canine oesophagus stained with phosphotungstic acid haematoxylin



3.4. Smooth muscle actin immunohistochemistry

Smooth muscle actin staining was consistent across all thirty dogs, throughout the cervical, thoracic, and abdominal regions of the oesophagus, revealing strong positive staining in the mucosal layer consistent with the smooth muscle layer that is the muscularis mucosa. No positive staining was observed in the tunica muscularis, or in any of the other remaining layers of the oesophagus.

Figure 7: Microscope image of the canine oesophagus stained with smooth muscle actin immunohistochemistry



3.5. Statistical analysis

Using a 95% confidence level, the exact confidence interval was calculated as 0.0% (0.0-11.6%) given that the oesophagus was solely composed of skeletal muscle and did not contain any smooth muscle in any of the thirty dogs.

4. Discussion

We report the largest study to date to evaluate the composition of the tunica muscularis of the canine oesophagus. Our results have demonstrated that the tunica muscularis of the canine oesophagus is solely composed of skeletal muscle throughout, and that the smooth muscle muscularis mucosa is present throughout all regions of the oesophagus. These results are consistent with those reported by Mann and Shorter (1964) and Bush (1980), however differ from those published by Dawood et al (2022) which report that the tunica muscularis in the abdominal region of the canine oesophagus is solely composed of smooth muscle, and that there is no muscularis mucosa in the cervical region of the canine oesophagus. These conflicting results could be explained by the lack of use of phosphotungstic acid haematoxylin and smooth muscle actin staining in the study by Dawood et al (2022) (Kösemehmetoğlu and Johnson 2023, Pernick 2023). Another possible explanation would be population based anatomical differences between our United Kingdom based population and that of the study by Dawood et al (2022) which comprised an Iraqi based population.

A significantly higher incidence of MG associated megaoesophagus (88%) is reported in dogs compared to cats (40%), or even humans in which there are only a few reported cases (Desuter et al. 2015, Ducoté et al. 1999, Shelton et al. 1997). Within a species, it has been postulated that the incidence of MG associated megaoesophagus is negatively correlated with

the amount of smooth muscle in the tunica muscularis layer of the oesophagus (Hague et al. 2015). Our results have demonstrated that the tunica muscularis of the oesophagus of the dog does not contain any smooth muscle, and is therefore entirely composed of skeletal muscle, whereas in cats, the distal third of the tunica muscularis is reported to be solely composed of smooth muscle whilst the proximal two thirds of the tunica muscularis of the oesophagus is described as solely composed of skeletal muscle (Bremner et al 1970). Hence our findings support the theory that MG associated megaoesophagus incidence is negatively correlated with the amount of smooth muscle in the tunica muscularis layer of the oesophagus.

Knowledge of the composition of the tunica muscularis of the canine oesophagus is also important with regards to management of megaoesophagus. Medications aimed at improving oesophageal peristalsis by exerting an effect on the smooth muscle of the tunica muscularis of the oesophagus are recommended in the management of megaoesophagus in dogs (Penderis-Vaquero 2016). Our results suggest that these medications are unlikely to be beneficial in megaoesophagus in dogs given the absence of any smooth muscle in the tunica muscularis of the canine oesophagus. Furthermore, these medications are also unlikely to have a beneficial effect on the smooth muscle of the muscularis mucosa given that it does not significantly contribute to oesophageal peristalsis (Kook 2014). From this, medications targeting skeletal muscle such as AChE-Is, would be considered the most beneficial for MG associated megaoesophagus. However despite this, there is still anecdotal evidence that the effect of AChE-Is on oesophageal skeletal muscle is lesser than that on the facial and appendicular skeletal muscles (Shelton et al. 1990).

Exact confidence interval statistical analysis was used to correlate this study's results to the wider United Kingdom dog population. Using a 95% confidence level, the exact confidence

interval was calculated as 0.0% (0.0-11.6%) given that the oesophagus was solely composed of skeletal muscle and did not contain any smooth muscle in any of the thirty dogs. Therefore, although our results strongly suggest that the tunica muscularis of the oesophagus is solely composed of skeletal muscle in the majority of dogs, there is a possibility that up to 11.6% of the dog population could in fact have some degree of smooth muscle in the tunica muscularis layer of the oesophagus.

There are limitations to our study. All samples were evaluated by a single observer (TM) without use of whole slide imaging software which might have reduced human error, as well as enabled measurements to be taken, and permitted objective quantitative analysis of the muscular components of the oesophagus to be performed. This limitation was mitigated by the fact that the observer (TM) was supervised by a board-certified veterinary pathologist (WW) who provided training and supervision on reviewing the slides, from the cervical, thoracic, and abdominal regions of the oesophagus of the small, medium, and large dog groups. Furthermore, naked eye analysis was deemed acceptable given that the tunica muscularis was solely composed of skeletal muscle, and that the muscularis mucosa was solely composed of smooth muscle. Additionally, our samples of the abdominal region of the oesophagus did not include the lower oesophageal sphincter which prevented analysis of the muscular composition of the latter. This is a limitation given that the lower oesophageal sphincter has been described as containing a significant smooth muscle component whose inability to relax has been proposed as a possible cause of MG induced megaoesophagus. Finally, the observer (TM), and supervisor (WW) were not blinded to the location origin in the oesophagus of the samples, hence there is a possibility of confirmation bias.

5. Conclusions

Throughout the cervical, thoracic, and abdominal regions of the canine oesophagus, the tunica muscularis is solely composed of skeletal muscle, whereas the muscularis mucosa is solely composed of skeletal muscle. These findings support the theory that MG associated megaoesophagus incidence is negatively correlated with the amount of smooth muscle in the tunica muscularis layer of the oesophagus. Given the lack of smooth muscle in the tunica muscularis, medications targeting the skeletal muscle should be prioritised in management of MG associated megaoesophagus.

Chapter V: Conclusions

1. Conclusions

This aim of this thesis was to investigate treatment and outcome in myasthenia gravis in dogs and cats as well as a provide a framework for future studies. This entailed performing three heterogenous studies.

Firstly, the long-term outcome of cats with myasthenia gravis without evidence of a cranial mediastinal mass was retrospectively evaluated. This retrospective study was the first to investigate the natural course of the disease in cats. This study demonstrated that cats diagnosed with MG without evidence of a CMM often have a favourable outcome and frequently achieve immune remission. Moreover, the natural history of feline MG includes spontaneous remission when there is no evidence of a CMM. Therefore, attempting to rule out the presence of a CMM therefore refines prognosis, and treatment is not always necessary in this disease population.

Secondly, a review of the literature on myasthenia gravis and congenital myasthenia gravis was performed to respectively update and provide classification of myasthenia gravis and congenital myasthenic syndromes in dogs and cats. Updated classification of MG in dogs and cats retains differentiation based on focal, generalised or fulminant presentation, but also takes into account the autoimmune disease mechanism or seronegativity. Congenital myasthenic syndromes are classified according to the affected NMJ component, the mechanism of the defect of neuromuscular transmission, the affected protein, and ultimately the mutated gene responsible. These classification systems of MG and CMSs in dogs and cats aid recognition of the disease groups for both conditions, as well as guide treatment, refine prognosis, and provide a framework for additional studies of these conditions.

Finally, the composition of the tunica muscularis of the oesophagus in dogs was prospectively evaluated in small, medium, and large breed dogs using haematoxylin and eosin, phosphotungstic acid haematoxylin, and smooth muscle actin staining. This study confirmed that the tunica muscularis of the canine oesophagus is solely composed of skeletal muscle throughout the cervical, thoracic, and abdominal regions and supports the theory that MG associated megaoesophagus incidence is negatively correlated with the amount of smooth muscle in the tunica muscularis layer of the oesophagus.

1.1. Future directions

Together, the results of this thesis highlight future research avenues in MG and CMSs as well as provide a framework for such studies. The retrospective study of the long-term outcome of cats with myasthenia gravis without evidence of a cranial mediastinal mass revealed that treatment isn't always necessary, and that outcome was generally favourable in this disease population even including spontaneous immune remission. Furthermore, novel classification of MG in dogs and cats accounts for the presence or absence of a thymoma. However, there are no guidelines as to when to start treatment in cats with MG without evidence of a cranial mediastinal mass nor an optimal treatment approach of MG in dogs and cats (Shelton et al. 2002). Additionally, temporal evolution of serum AChR in dogs and cats with thymoma-associated myasthenia gravis remains unclear, as is the benefit of thymectomy in these cases (Nagata et al. 2017, Robat et al 2013, Rusbridge et al 1996, Shelton 2002). Evaluation of the short and long-term effects of thymectomy or lack of in dogs and cats with thymoma-associated myasthenia gravis could help improve treatment and outcome. Novel classification of MG in dogs and cats questioned the possibility that some dogs with MG seronegative for antibodies against the AChR could in fact have antibodies against MUSK, or LRP4 as is the

case in people (Gilhus and Verschuuren 2015). Future studies should investigate the presence of MUSK or LRP4 antibodies in AChR seronegative MG cases as well as investigate the seronegative MG incidence in cats. The classification of CMSs provides a framework for treatment trial in dogs and cats based on medications used in humans for a given shared genetically confirmed CMS. The results of the prospective study of the composition of the tunica muscularis of the canine oesophagus support the theory that MG associated megaoesophagus incidence is negatively correlated with the amount of smooth muscle in the tunica muscularis layer of the oesophagus. The mechanisms underpinning selective involvement of the oesophageal, facial, pharyngeal, or laryngeal muscles in focal MG however remain unknown and warrant further investigation. Postulated theories include safety margin differences for neuromuscular transmission between different muscle groups as well as antigenic differences in the AChR itself (Engel 1988, Oda and Shibasaki 1988).

Supplementary material

Supplement 1

University of Nottingham
UK | CHINA | MALAYSIA

School of Veterinary Medicine and Science
Pathology Service
Phone: 07813 537686
Fax: 01159 516550
Email: veterinary-pathology@nottingham.ac.uk

For internal use only Accession Number:
Date received: Date of PM:

Request form for post mortem examination and disposal For clinical associates

Please tick one:
□ **Teaching case** (report of macroscopical findings)
□ **Full work-up** (formal report required)

Submitting Veterinary Practice:
Clinician:

Address:

Phone:
Fax:
Email:

Report to be sent by Email ☐ or Fax ☐

Owner's details:
Owner's name: .....
Animal's name/ID: .....
Passport/microchip: .....
Reference No (if any): .....

DETAILS OF SUBMISSION
Species: .....
Breed: .....
Colour/markings: .....
Sex: ..... Age:..... Weight: .....
Date of Death: ..... Euthanized? Yes ☐ No ☐

Travel outside the UK? No ☐ Yes ☐
If yes: date and place travelled to:
.....
.....

HISTORY/CLINICAL FEATURES

I confirm that the owner has given consent for the post mortem examination and *disposal of the above animal and we are aware that, as part of this procedure, material may be used for educational and/or research purposes.

Signature of submitting Vet: ..... Date: .....
NECROPSIES WILL NOT BE PERFORMED WITHOUT THE SIGNATURE OF THE REFERRING VETERINARIAN

*Carcases will be incinerated; carcases can be released to a pet cremation service ***by prior arrangement*** with the referring Veterinary Practice. Arrangements must be clearly indicated below.

Individual cremation requested Yes ☐ No ☐
If yes, please state your usual cremation service provider for us to arrange collection
.....

In addition to the necropsy and histology, other diagnostic testing (microbiology, toxicology etc.) may be available on request at an additional cost.

References

Aarbakke J, Janka-Schaub G, Elion GB. Thiopurine biology and pharmacology. Trends in Pharmacological Sciences. 1997;18(1):3-7.

Abelson A, Shelton GD, Whelan MF, Cornejo L, Shaw S, O'Toole TE. Use of mycophenolate mofetil as a rescue agent in the treatment of severe generalised myasthenia gravis in three dogs. *Journal of Veterinary Emergency and Critical Care.* 2009;19(4):369-74.

Abitbol M, Hitte C, Bossé P, Blanchard-Gutton N, Thomas A, Martignat L, Blot S, Tiret L. A *COLQ* missense mutation in Sphynx and Devon Rex cats with congenital myasthenic syndrome. *Plos One*. 2015;10(9):e0137019.

Adrian R, Costantin L, Peachey L. Radial spread and contraction in frog muscle fibres. *Journal of Physiology*. 1969;204:231-257.

Añor S, Lipsitz D, Williams DC, Tripp L, Willits N, Maselli R, LeCouteur RA. Evaluation of jitter by stimulated single-fiber electromyography in normal dogs. *Journal of Veterinary Internal Medicine.* 2003;17(4):545-550.

Bailey KS. Myasthenia gravis. In: *Small animal neurological emergencies.* 1st Edition. London. Taylor & Francis Group;2012:433-446.

Baker RW, Hughson FM. Chaperoning SNARE assembly and disassembly. *Nature Reviews Molecular Cell Biology*. 2016;17:1465-1487.

Bartges JW, Klausner JS, Bostwick EF, Hakala JE, Lennon VA. Clinical remission following plasmapheresis and corticosteroid treatment in a dog with acquired myasthenia gravis. *Journal of the American Veterinary Medical Association*. 1990;196(8):1276-1278.

Bell ET, Mansfield CS, James FE. Immune-mediated myasthenia gravis in a methimazole-treated cat. *Journal of Small Animal Practice*. 2012;53(11):661-663.

Berrih-Aknin S, Le Panse R. Myasthenia gravis: a comprehensive review of immune dysregulation and etiological mechanisms. *Journal of Autoimmunity.* 2014;52:90-100.

Berrih-Aknin S, Frenkian-Cuvelier M, Eymard B. Diagnostic and clinical classification of autoimmune myasthenia gravis. *Journal of Autoimmunity*. 2014;48-49:143-148.

Bexfield NH, Watson PJ, Herrtage ME. Management of myasthenia gravis using cyclosporine in 2 dogs. *Journal of Veterinary Internal Medicine.* 2006;20(6):1487-1490.

Blakey TJ, Michaels JR, Guo LT, Hodshon AJ, Shelton GD. Congenital myasthenic syndrome in a mixed breed dog. *Frontiers of Veterinary Science.* 2017;(4):173. 10.3389/fvets.2017.00173

Bremner CG, Shorter R, Ellis Jr FH. Anatomy of feline esophagus with special reference to its muscular wall and phrenooesophageal membrane. *Journal of Surgical Research.* 1970;10(7):327-331.

Bush C. Structure of the esophagus of dogs. *Acta Anatomica (Basel)*. 1980;107(30):339-360.

Calderon J, Bolanos P, Caputo C. The excitation-contraction coupling mechanism in skeletal muscle. *Biophysical Reviews*. 2014;6(1):133-160.

Cetin H, Beeson D, Vincent A, Webster R. The structure, function, and physiology of the fetal and adult acetylcholine receptor in muscle. *Frontiers of Molecular Neuroscience*. 2020. https://doi.org/10.3389/fnmol.2020.581097.

Coates JR, Jeffery ND. Perspectives on meningoencephalitis of unknown origin. *Veterinary Clinics of North America: Small animal practice.* 2014;44(6):1157-85.

Cridge H, Little A, José-López, Pancotto T, Michaels J, Menchetti M, Suñol A, Lipsitz D, Beasley MJ. The clinical utility of neostigmine administration in the diagnosis of acquired myasthenia gravis. *Journal of Veterinary Emergency and Critical Care.* 2021;31(5):647-655.

Cuddon PA. Acquired immune-mediated myasthenia gravis in a cat. *Journal of Small Animal Practice.* 1989;30(9):511-516.

Cuddon PA. Electrophysiology in neuromuscular disease. *Veterinary Clinics of North America: Small animal practice.* 2002;32(1):31-62.

Dawood MS, Abood DA, Hameza AY. The histological and histochemical features of the esophagus in local breed dogs (Canis familiaris). *The Iraqi Journal of Veterinary Sciences.* 2022;36(4):1069-1074.

Dawson JR. Myasthenia gravis in a cat. *Veterinary Record.* 1970;86(19):562-563.

Day MJ. Review of thymic pathology in 30 cats and 36 dogs. *Journal of Small Animal Practice*. 1997;38(9):393-403.

De Harven E, Coers C. Electron microscope study of the human neuromuscular junction. *Journal of Biophysical and Biochemical Cytology*. 1959;6(1):7-10.

De Lahunta A, Glass E, Kent M. Lower motor neuron: spinal nerve, general somatic efferent system. In: *De Lahunta's Veterinary neuroanatomy and clinical neurology.* 5th Edition. St Louis MO. Saunders;2020:106-167.

Desaki J, Uehara Y. The overall morphology of neuromuscular junctions as revealed by scanning electron microscopy. *Journal of Neurocytology.* 1981;10(1):101-110.

Desuter G, Van Parijs V, Gardiner Q. Megaesophagus associated with myasthenia gravis: a rare condition in humans. *B-ENT.* 2015;11(1):63-66.

Dewey CW, Shelton GD, Bailey CS, et al. Neuromuscular dysfunction in five dogs with acquired myasthenia gravis and presumptive hypothyroidism. *Progress in Veterinary Neurology.* 1995;6:117-123.

Dewey CW, Bailey CS, Shelton GD, Kass PH, GH Cardinet 3rd. Clinical forms of acquired myasthenia gravis in dogs: 25 cases (1988-1995). *Journal of Veterinary Internal Medicine.* 1997;11(2):50-57.

Dewey CW. Acquired myasthenia gravis in dogs part 1. *Compendium for Continued Education for the Practicing Veterinarian.* 1997;19(12):1340-1353.

Dewey CW, Coates JR, Ducoté JM, Meeks JC, Fradkin JM. Azathioprine therapy for acquired myasthenia gravis in five dogs. *Journal of the American Animal Hospital Association.* 1999;35(5):396-402.

Dewey CW, Boothe DM, Rinn KL, Coates JR, Burkholder WJ. Treatment of a myasthenic dog with mycophenolate mofetil. *Journal of Veterinary Emergency and Critical Care.* 2007;10(3):177-187.

Dewey CW, Cerda-Gonzalez S, Fletcher DJ, Harb-Hauser MF, Levine JM, Badgley BL, Olby NJ, Shelton GD. Mycophenolate mofetil treatment in dogs with serologically diagnosed acquired myasthenia gravis: 27 cases (1999-2008). *Journal of the American Veterinary Medical Association.* 2010;236(6):664-668.

Dickinson PJ, LeCouteur RA. Feline neuromuscular disorders. *Veterinary Clinics of North America: Small animal practice.* 2004;34(6):1307-1359.

Dickinson PJ, Sturges BK, Shelton GD, LeCouteur RA. Congenital myasthenia gravis in Smooth-Haired Miniature Dachshund dogs. *Journal of Veterinary Internal Medicine.* 2005;19(6):920-3.

Dörfelt S, Fischer A, Meyer-Lindenberg A, Dörfelt R. Feline acquired thymoma-associated myasthenia gravis managed with surgery and therapeutic plasma exchange. *Veterinary Record Case Reports.* 2021;9(4).e211. https://doi.org/10.1002/vrc2.211

Drachman DB, Angus DW, Adams RN, Michelson JD, Hoffman GJ. Myasthenia antibodies cross-link acetylcholine receptors to accelerate degradation. *New England Journal of Medicine.* 1978;298:1116-1122.

Drachman DB, Adams RN, Josifek LF, Self SG. Functional activities of autoantibodies to acetylcholine receptors and the clinical severity of myasthenia gravis. *New England Journal of Medicine.* 1982;307:769-775.

Dubois-Dalcq M, McFarland H, McFarlin D. Protein A-Peroxidase: A valuable tool for the localization of antigens. *The Journal of Histochemistry and Cytochemistry*. 1977;25(11):1201-1206.

Ducoté JM, Dewey CW, Coates JR. Clinical forms of acquired myasthenia gravis in cats. *Compendium for Continued Education of the Practicing Veterinarian*. 1999;21(5):440-448.

Ebashi S. Regulatory mechanism of muscle contraction with special reference to Ca-troponin-tropomyosin system. *Essays in Biochemistry*. 1974;10:1-36.

Elkholly DA, Brobelt DC, Church DB, Pelligand L, Mwacalimba K, Wright AK, O'Neill DG. Side effects to systemic glucocorticoid therapy in dogs under primary veterinary care in the UK. *Frontiers of Veterinary Science*. 2020. 10.3389/fvets.2020.00515

Elion GB. The pharmacology of azathioprine. *Annals of the New York Academy of Science*. 1993;685:400-407.

Ellis J, Tappin S. Management and resolution of acquired myasthenia gravis in a carbimazole-treated hyperthyroid domestic shorthair cat. *Veterinary Record Case Reports*. 2019;7(1):e000806.

Engel AG. Myasthenia gravis and myasthenia syndromes. *Annals of Neurology*. 1984;16:519-534.

Engel AG. Congenital myasthenic syndromes. *Journal of Child Neurology*. 1988;3:233-246.

Engel AG, Arahate K. The membrane attack complex of complement at the endplate in myasthenia gravis. *Annals of the New York Academy of Sciences.* 1987;505:326-332.

Engel AG, Shen XM, Selcen D, Sine SM. Congenital myasthenic syndromes: pathogenesis, diagnosis, and treatment. *Lancet Neurology*. 2015;14(5):420-434.

Engel AG. Congenital myasthenic syndromes in 2018. *Current Neurology and Neuroscience Reports*. 2018;18(8):46.

Farmakidis C, Pasnoor M, Barohn RJ, Dimachkie MM. Congenital myasthenic syndromes: a clinical and treatment approach. *Current Treatment Options in Neurology*. 2018;20(9):36.

Feng Z, Ko CP. Schwann cells promote synaptogenesis at the neuromuscular junction via transforming growth factor beta 1. *Journal of Neuroscience*. 2008;28(39):9599-9609.

Fernández-Zarzoso M, Gómez-Segui I, de la Rubia J. Therapeutic plasma exchange: Review of current indications. *Transfusion and Apheresis Science.* 2019;58(3):247-253.

Fill and Coronado. Ryanodine receptor channel of sarcoplasmic reticulum. *Trends in Neurosciences*. 1988;11(10):453-457.

Finsterer J. Congenital myasthenic syndrome. *Orphanet Journal of Rare Disorders*. 2019;14(1):57.

Flagstad A. A new hereditary neuromuscular disease in the dog breed "Gammel Dansk Honsehund". Genetic investigations. *Hereditas*. 1982;96(2):211-214.

Flagstad A, Trojaborg W, Gammeltoft S. Congenital myasthenic syndrome in the dog breed Gammel Dansk Hønsehund: clinical, electrophysiological, pharmacological and immunological comparison with acquired myasthenia gravis. *Acta Veterinaria Scandinavica.* 1989;30(1)89-102.

Flucher, BE, Daniels MP. Distribution of Na^+ channels and ankyrin in neuromuscular junctions is complementary to that of acetylcholine receptors and 43 kd protein. *Neuron.* 1989;3:163-175.

Forgash JT, Chang Y, Mittelman NS, Petesch S, Benedicenti L, Galban E, Hammond JJ, Glass EN, Barker GJ, Shelton GD, Luo J, Garden OA. *Journal of Veterinary Internal Medicine.* 2021;35(5):2315:2326.

Franzini-Armstrong C, Porter K. Sarcolemmal invaginations constituting the T system in fish muscle fibres. *Journal of Cellular Biology*. 1964;22:675–696.

Gandolfi B, Grahn RA, Creighton EK, et al. A *COLQ* variant associated with Devon Rex and Sphynx feline hereditary myopathy. *Animal Genetics*. 2015;46:711-715.

Gilhus NE, Verschuuren JJ. Myasthenia gravis: subgroup classification and therapeutic strategies. *Lancet Neurology*. 2015;14(10):1023-1036.

Gilhus NE. Myasthenia gravis. *New England Journal of Medicine.* 2016;375(26):2570-81.

Hackett TB, Van Pelt DR, Willard MD, Martin LG, Shelton GD, Wingfield WE. Third degree atrioventricular block and acquired myasthenia gravis in four dogs. *Journal of the American Veterinary Medical Association.* 1995;206(8):1173-1176.

Hague DW, Humphries HD, Mitchell MA, Shelton GD. Risk factors and outcome in cats with acquired myasthenia gravis (2001-2012). *Journal of Veterinary Internal Medicine.* 2015;29(5):1307-1312.

Hall ED, Riker WF, Baker T. Glucocorticoid effect on the edrophonium responsiveness of normal and degenerating mammalian motor nerve terminals. *Annals of Neurology.* 1977;2(5):404-408.

Hall JE, Hall ME. Membrane physiology, nerve, and muscle. In: *Guyton and Hall: Textbook of medical physiology.* 13th Edition. Philadelphia, Pennsylvania. Elsevier;2016:47-105.

Halloran PF. Molecular mechanisms of new immunosuppressants. *Clinical Transplant*. 1996;10:118-123.

Hasselbach W. Relaxing factors and the relaxation of muscle. *Progress in Biophysics and Molecular Biology*. 1964;14:167-222.

Herder V, Ciurkiewicz M, Baumgärtner M, Jagnnathan V, Leeb T. Frame-shift variant in the *CHRNE* gene in a juvenile dog with suspected myasthenia gravis-like disease. *Animal Genetics*. 2017;48(5):625.

Hill PB, Collins D, Fearnside S, Olivry T. Putative paraneoplastic pemphigus and myasthenia gravis in a cat with a lymphocytic thymoma. *Veterinary Dermatology*. 2013;24(6):646-649.

Hong IHK, Etherington SJ. Neuromuscular junction. *Encyclopedia of Life Sciences.* 2011. https://doi.org/10.1002/9780470015902.a0023202.

Hughes T. The early history of myasthenia gravis. *Neuromuscular disorders.* 2015;15:878-886.

Huxley H. Evidence for continuity between the central elements of the triads and extracellular space in frog sartorius muscle. *Nature*. 1964; 202:1067.

Huxley H. The mechanism of muscular contraction. *Science*. 1969;164:1356-1366.

Indieri RJ, Creighton SR, Lambert EH, Lennon VA. Myasthenia gravis in two cats. *Journal of the American Veterinary Medical Association.* 1983;182(1):57-60.

Jeffery ND. Corticosteroid use in small animal neurology. *Veterinary Clinics of North America: Small animal practice.* 2014;44(6):1059-74.

Jenkins WL, Van Dyk E, McDonald CB. Myasthenia gravis in a Fox Terrier litter. *Journal of South African Veterinary Association.* 1976;47:59-62.

Johnson RP, Watson ADJ, Smith J, Cooper BJ. Myasthenia in Springer Spaniel littermates. *Journal of Small Animal Practice.* 1975;16:641-647.

Joseph RJ, Carrillo JM, Lennon VA. Myasthenia gravis in the cat. *Journal of Veterinary Internal Medicine.* 1988;2(2):75-79.

Kamenskaya MA, Elmqvist D, Thesleff S. Guanidine and neuromuscular transmission. II. Effect on transmitter release in response to repetitive nerve stimulation. *Archives of Neurology*. 1975;32:510–518.

Kent M, Glass EN, Acierno M, Shelton GD. Adult onset acquired myasthenia gravis in three Great Dane littermates. *Journal of Small Animal Practice.* 2008;49(12):647-650.

King LG, Vite CH. Acute fulminating myasthenia gravis in five dogs. *Journal of the American Veterinary Medical Association*. 1998;212(6):830-834.

Khorzad R, Whelan M, Sisson A, Shelton GD. Myasthenia gravis in dogs with an emphasis on treatment and critical care management. *Journal of Veterinary Emergency and Critical Care.* 2011;21(3):193-208.

Kohn MA, Senyak J. Sample size calculators (Website). UCSF CTSI. 20 December 2021. Available at http://www.sample-size.net/. Accessed 11 March 2023.

Kook PH. Oesophageal motility in small animals. In: 24th Annual ECVIM-CA Congress, Mainz, Germany, 4 September 2014 - 6 September 2014.

Kösemehmetoğlu K, Johnson G. Actin, alpha smooth muscle type. PathologyOutlines.com website. Accessed 08 June 2023. https://www.pathologyoutlines.com/topic/stainsalphasmoothmuscleactin.html

Krotje LJ, Fix AS, Potthoff AD. Acquired myasthenia gravis and chollangiocellular carcinoma in a dog. *Journal of the American Veterinary Medical Association.* 1990;197(4):488-490.

Kuffler SW, Yoshikami D. The number of transmitter molecules in a quantum: an estimate from iontophoretic application of acetylcholine at the neuromuscular synapse. Journal of Physiology. 1975;251(2):465-482.

Lainesse MF, Taylor SM, Myers SL, Haines D, Fowler JD. Focal myasthenia gravis as a paraneoplastic syndrome of canine thymoma: improvement following thymectomy. *Journal of the American Animal Hospital Association*. 1996;32(2):111-117.

Lee M, Beeson D, Palace J. Therapeutic strategies for congenital myasthenic syndromes. *Annals of the New York Academy of Sciences*. 2018;1412(1):129-136.

Lennon VA, Lambert EH, Palmer AC, Cunningham JG, Christie TR. Acquired and congenital myasthenia gravis in dogs: a study of 20 cases. In: *Myasthenia Gravis-Pathogenesis and Treatment*. Tokyo, Japan. Tokyo University Press;1980:41-54.

Levine JM, Bergman RL, Coates JR, Shelton GD. Myasthenia gravis and hypothyroidism in a dog with meningomyelitis. *Journal of the American Animal Hospital Association*. 2005;41(4):247-251.

Lindstrom JM. Acetylcholine receptors and myasthenia. *Muscle and Nerve.* 2000;23(4):453-477.

Lipsitz D, Berry JL, Shelton GD. Inherited predisposition to myasthenia gravis in Newfoundlands. *Journal of the American Veterinary Medical Association.* 1999;215:946-58.

Malik R, Mepstead K, Yang F, Harper C. Hereditary myopathy of Devon Rex cats. *Journal of Small Animal Practice*. 1993;34:539545.

Mann CV, Shorter RG. Structure of the canine esophagus and its sphincters. *Journal of Surgical Research.* 1964;4(4):160-163.

Martin AR. Quantal nature of synaptic transmission. *Physiology Review*. 1965;46:51–66.

Martin, AR. Amplification of neuromuscular transmission by postjunctional folds. *Proceedings of the Royal Society of London*. 1994;258:321-326.

Mason KV. A case of myasthenia gravis in a cat. *Journal of Small Animal Practice.* 1976;17(7):467-472.

Mayousse V, Jeandel A, Blanchard-Gutton N, Escriou C, Gnirs K, Shelton GD, Blot S. Evaluation of coexisting polymyositis in feline myasthenia gravis: A case series. *Neuromuscular disorders.* 2017;27(9):804-15.

Meeking SA, Prittie J, Barton L. Myasthenia gravis associated with thymic neoplasia in a cat. *Journal of Veterinary Emergency and Critical Care.* 2008;18(2):177-183.

Meyer GW, Austin RM, Brady CE 3rd, Castell DO. Muscle anatomy of the human esophagus. *Journal of clinical gastroenterology.* 1986;8(2):131-134.

Mignan T, Targett M, Lowrie M. Classification of myasthenia gravis and congenital myasthenic syndromes. *Journal of Veterinary Internal Medicine.* 2020;34(5):1707-1717.

Miller LM, Lennon VA, Lambert EH, Reed SM, Hegreberg GA, Miller JB, Ott RL. Congenital myasthenia gravis in 13 smooth fox terriers. *Journal of the American Veterinary Medical Association.* 1983;182:694-697.

Miller LM, Hegreberg GA, Prieur DJ, Hamilton MMJ. Inheritance of congenital myasthenia gravis in smooth fox terrier dogs. *Journal of Heredity*. 1984;75(3):163-166.

Miyazawa A, Fujiyoshi Y, Unwin N. Structure and gating mechanism of the acetylcholine receptor pore. *Nature*. 2003;423(6943):949-955.

Moore AS, Madewell BR, Cardinet 3rd GH, Shelton GD. Osteogenic sarcoma and myasthenia gravis in a dog. *Journal of the American Veterinary Medical Association.* 1990;197(2):226-7.

Müller KMI, Taskinen E, Lefvert AK, Pirskanen R, Livanainen M. Immunoactivation in the central nervous system in myasthenia gravis. *Journal of Neurological Sciences.* 1987;80(1):13-23.

Nachmansohn D, Machado AL. The formation of acetylcholine. A new enzyme: "Choline acetylase". *Journal of neurophysiology.* 1943;6(5):397-403.

Nachmansohn D, Wilson IB. The enzymic hydrolysis and synthesis of acetylcholine. *Advances in Enzymology and Related Areas of Molecular Biology*. 1951;12:259-339.

Nagata N, Miyoshi T, Otake Y, Suzuki H, Kagawa Y, Yamagami T, Irie M. Temporal deterioration of neurological symptoms and increase of serum acetylcholine receptor antibody levels after thymectomy: a case report of a cat with myasthenia gravis. *Journal of Veterinary Medical Science.* 2017;78(12):1893-1896.

Newark J. Nerve agents. *Neurologist.* 2007;13(1):20-32.

Nicolau S, Milone M. The electrophysiology of presynaptic congenital myasthenic syndromes with and without facilitation: From electrodiagnostic findings to molecular mechanisms. *Frontiers of Neurology.* 2019;10. 10.3389/fneur.2019.00257

Oda K, Shibasaki H. Antigenic difference of acetylcholine receptor between single and multiple form end-plates of human extraocular muscle. *Brain Research.* 1988;449:337-340.

O'Dair HA, Holt PE, Pearson GR, Gruffydd-Jones TJ. Acquired immune-mediated myasthenia gravis in a cat associated with a cystic thymus. *Journal of Small Animal Practice.* 1991;32(4):198-202.

Palmer AC, Barker J. Myasthenia in the dog. *Veterinary Record.* 1974;95(20):452-454.

Palmer AC, Goodyear JV. Congenital myasthenia in the Jack Russell Terrier. *Veterinary Record.* 1978;103(19):433-434.

Palmer AC, Lennon VA, Beadle C, goodyear JV. Autoimmune form of myasthenia gravis in a juvenile Yorkshire Terrier x Jack Russell Terrier hybrid contrasted with congenital (non-autoimmune) myasthenia gravis of the Jack Russell. *Journal of Small Animal Practice.* 1980;21:359-364.

Pascuzzi RM. The edrophonium test. *Seminars in Neurology.* 2003;23(1):83-88.

Penderis J, Martin-Vaquero P. Junctionopathies: disorders of the neuromuscular junction. In: *A practical guide to canine and feline neurology.* 3rd Edition. Ames, Iowa: Wiley-Blackwell;2016:521-557.

Pernick N. Trichrome. Phosphotungstic acid haematoxylin. PathologyOutlines.com website. Accessed 08 June 2023. https://www.pathologyoutlines.com/topic/stainstrichrome.html

Pflugfelder CM, Cardinet 3rd GH, Lutz H, Holliday TA, Hansen RJ. Acquired canine myasthenia gravis: Immunocytochemical localization of immune complexes at neuromuscular junctions. Muscle & Nerve. 1981;4(4):289-295.

Platt S, Shelton GD. Exercise intolerance and collapse. In: BSAVA Manual of canine and feline neurology. 4th Edition. Gloucestershire. British Small Animal Association;2013:342-367.

Pollard RE. Imaging evaluation of dogs and cats with dysphagia. *International Scholarly Research Notices*. 2012. https://doi.org/10.5402/2012/238505

Proschowsky HT, Flagstad A, Cirera S, Joergensen CB, Fredholm M. Identification of a mutation in the CHAT gene of Old Danish Pointing dogs affected with congenital myasthenic syndrome. *Journal of Heredity*. 2007;98:539-543.

Rebner M, Gross BH, Robertson JM, Pennes DR, Spizarny DL, Glazer GM. CT evaluation of mediastinal masses. *Computerized Radiology.* 1987;11(3):103-110.

Richardson D. Acquired myasthenia gravis in a poodle. *Canadian Veterinary journal.* 2011;1(1):16-22.

Ridyard AE, Rhind SM, French AT, et al. Myasthenia gravis associated with cutaneous lymphoma in a dog. *Journal of Small Animal Practice.* 2000;41(8):348-351. 10.1111/j.1748-5827.2000.tb03216.x

Rinz CJ, Levine J, Minor KM, et al. A *COLQ* missense mutation in Labrador Retrievers having congenital myasthenic syndrome. *Plos One*. 2014;9(8):e106425.

Rinz CJ, Lennon VA, James F, et al. A *CHRNE* frameshift mutation causes congenital myasthenic syndrome in young Jack Russell terriers. *Neuromuscular Disorders*. 2015;25:921-927.

Robat CS, Cesario L, Gaeta R, Miller M, Schrempp D, Chun R. Clinical features, treatment options and outcome in dogs with thymoma: 116 cases (1999-2010). *Journal of the American Veterinary Medical Association*. 2013;243(10):1448-1454.

Rodríguez Cruz PMR, Cossins J, Beeson D, Vincent A. The neuromuscular junction in health and disease: Molecular mechanisms governing synaptic formation and homeostasis. *Frontiers in Molecular Neuroscience.* 2020;3(13):610964. 10.3389/fnmol.2020.610964

Roghani A, Feldman J, Kohran SA, Shirzadi A, Gundersen CB, Nicholas B, Edwards RH. Molecular cloning of a putative vesicular transporter for acetylcholine. *Proceedings of the National Academy of Sciences of the United States of America.* 1994;91(22):10620-624.

Rusyniak DE, Nanagas KA. Organophosphate poisoning. *Seminars in Neurology.* 2004;24(2):197-204.

Sandow A. Excitation-Contraction coupling in muscular response. *Yale Journal of Biology and Medicine*. 1952;25(3):176-201.

Sanes JR, Lichtman JW. Induction, assembly, maturation and maintenance of a postsynaptic apparatus. *Nature Reviews Neuroscience.* 2001;2(11):791-805.

Sanes JR. The basement membrane/basal lamina of skeletal muscle. *Journal of Biological Chemistry*. 2003;278(15):12601-12604.

Scott-Moncrieff JC, Cook JR, Lantz GC. Acquired myasthenia gravis in a cat with thymoma. *Journal of the American Veterinary Medical Association.* 1990;196(8):1291-1293.

Serfilippi LM, Quance JL. Pathology in practice. *Journal of the American Veterinary Medical Association*. 2018;253(2):173-176.

Singh A, Boston S, Poma R. Thymoma-associated exfoliative dermatitis with post-thymectomy myasthenia gravis in a cat. *Canadian Veterinary Journal*. 2010;51(7):757-760.

Singh N, Rieder M, Tucker MJ. Mechanisms of glucocorticoid-mediated anti-inflammatory and immunosuppressive action. *Paediatric and Perinatal Drug Therapy*. 2004;6(2):107-115.

Shelton GD, Cardinet GH, Lindstrom JM. Canine and human myasthenia gravis autoantibodies recognize similar regions on the acetylcholine receptor. *Neurology.* 1988;38(9):1417-1423.

Shelton GD, Willard MD, Cardinet 3rd GH, Lindstrom J. Acquired myasthenia gravis: selective involvement of oesophageal, and facial muscles. *Journal of Veterinary Internal Medicine.* 1990;4:281-284.

Shelton GD, Joseph R, Richter K, et al. Acquired myasthenia gravis in hyperthyroid cats on Tapazole therapy [Abstract]. Journal of veterinary internal medicine. 1997;11:120.

Shelton GD, Schule A, Kass PH. Risk factors for acquired myasthenia gravis in dogs: 1154 cases (1991-1995). *Journal of American veterinary medical association.* 1997;211(1):1428-1431.

Shelton GD. Myasthenia gravis: lessons from the past 10 years. *Journal of Small Animal Practice.* 1998;39:368-372. 10.1111/j.1748-5827.1998.tb03734.x

Shelton GD, Ho M, Kass PH. Risk factors for acquired myasthenia gravis in cats: 105 cases (1986-1998). *Journal of the American Veterinary Medical Association.* 2000;216(1):55-57.

Shelton GD, Lindstrom JM. Spontaneous remission in canine myasthenia gravis: Implications for assessing human Myasthenia gravis therapies. *Neurology.* 2001;57(11):2139-2141.

Shelton GD, Skeie GO, Kass PH, Aarli JA. Titin and ryanodine receptor autoantibodies in dogs with thymoma and late-onset myasthenia gravis. *Veterinary Immunology and Immunopathology*. 2001;78(1):97-105.

Shelton GD. Myasthenia gravis and disorders of neuromuscular transmission. *Veterinary clinics of North America: Small animal practice.* 2002;32(1):189-206.

Shelton GD. Routine and specialized laboratory testing for the diagnosis of neuromuscular disease in dogs and cats. *Veterinary Clinical Pathology.* 2010;39(3):278-295. 10.1111/j.1939-165X.2010.00244.x

Shelton GD. Myasthenia gravis and congenital myasthenic syndromes in dogs and cats: a history and mini-review. *Neuromuscular Disorders*. 2016;26(6):331-334.

Shelton GD. Muscular disorders. In:. *Textbook of Veterinary Internal Medicine: Diseases of the Dog and the Cat*. Vol 2. 8th Edition. St. Louis, Missouri: Elsevier;2017:2149.

Stenner VJ, Parry BW, Holloway SA. Acquired myasthenia gravis associated with a non-invasive thymic carcinoma in a dog. *Australian Veterinary Journal.* 2003;81(9):543-546. 10.1111/j.1751-0813.2003.tb12883.x

Stepaniuk K, Legendre L, Watson S. Acquired myasthenia gravis associated with oral sarcoma in a dog. *Journal of Veterinary Dentistry.* 2011;28(4):242-249. 10.1177/089875641102800405

Shivamurthy P, Parker MW. Cardiac manifestation of myasthenia gravis: A systematic review. *International Journal of Cardiology Metabolic and Endocrine.* 2014;5:3-6

Stahelin H. Cyclosporin A. A historical background. *Progress in Allergy*. 1986;38:19-27.

Sudhof TC. The presynaptic active zone. *Neuron*. 2012;75(1):11-25.

Sukon P, Timm KI, Valentine BA. Oesophageal anatomy of the Llama (Lama glama). *International Journal of Morphology*. 2009;27(3):811-817.

Tanabe T, Beam KG, Adams BA, Tetsuhiro N, Numa S. Regions of the skeletal muscle dihydropyridine receptor critical for excitation-contraction coupling. *Nature*. 1990;346:567-569.

Thorlacius S, Aarli JA. The cerebrospinal fluid in myasthenia gravis. *Acta Neurologica Scandinavica*. 1985;72(4):432-436.

Trojaborg W, Flagstad A. A hereditary neuromuscular disorder in dogs. *Muscle and Nerve.* 1982;5(9):30-38.

Tsai KL, Vernau KM, Winger K, Zwueste DM, Sturges BK, Knipe M, Williams DC, Anderson KJ, Evans JM, Guo LT, Clark LA, Shelton GD. Congenital myasthenic syndrome in Golden retrievers is associated with a novel *COLQ* mutation. *Journal of Veterinary Internal Medicine*. 2019;34(1):258-265.

Uemura EE. Structure and function of neurons and neuroglia. In: *Fundamentals of canine neuroanatomy and neurophysiology.* 1st Edition. Ames, Iowa. Wiley-Blackwell;2015:11-37.

Valentine BA. Skeletal muscle. In: Pathologic Basis of Veterinary Disease. 6th Edition. Mosby;2017:908-953.

Van Ham LML. A myasthenic-like syndrome in young related mongrel dogs. *Progress in Veterinary Neurology.* 1992;3:135-139.

Vanhaesebrouck AE, Beeson D. The congenital myasthenic syndromes: expanding genetic and phenotypic spectrums and refining treatment strategies. *Current Opinion in Neurology*. 2019;32(5):696-703.

Vanhaesebrouck AE, Webster R, Maxwell S, et al. β2-Adrenergic receptor agonists ameliorate the adverse effect of long-term pyridostigmine on neuromuscular junction structure. *Brain*. 2019;142(12):3713-3727.

Villaroel MC, Hidalgo M, Jimeno A. Mycophenolate mofetil: An update. *Drugs Today*. 2009;45(7):521-532.

Vitalo A, Buckley G, Londoño L. Therapeutic plasma exchange as adjunct therapy in 3 dogs with myasthenia gravis and myasthenia-like syndrome. *Journal of Veterinary Emergency and Critical Care.* 2021;31(1):106-111.

Viviano KR. Update on immunosuppressive therapies for dogs and cats. *Veterinary Clinics of North America: Small animal practice.* 2013;43(5):1149-70.

Wallace ME, Palmer AC. Recessive mode of inheritance in myasthenia gravis in the Jack Russell Terrier. *Veterinary Record.* 1984;114(14):350.

Webb SN. Mechanism of action of neostigmine at the neuromuscular junction. *Journal of Pharmacy and Pharmacology.* 1971;23(1):227-228.

Wendell LC, Levine JM. Myasthenic crisis. *Neurohospitalist*. 2011;1(1):16-22.

Wismer T, Means C. Toxicology of newer insecticides in small animals. *Veterinary Clinics of North America: Small Animal Practice*. 2012;42(2):335-347.

Wolf Z, Vernau K, Safra N, et al. Association of early onset myasthenia gravis in Newfoundland dogs with the canine major histocompatibility complex I. *Neuromuscular Disorders*. 2017;27(5):409-416.

Wood SJ, Slater CR. Action potential generation in rat slow- and fast-twitch muscles. *Journal of Physiology.* 1995;486:401-410.

Wood SJ, Slater CR. The contribution of postsynaptic folds to the safety factor for neuromuscular transmission in rat fast- and slow-twitch muscles. *Journal of Physiology*. 1997;500;165-176.

Wood SJ, Slater CR. Beta-spectrin is co-localized with both voltage-gated sodium channels and ankyrin G at the rat neuromuscular junction. *Journal of Cellular Biology*. 1998;140:675-684.

Wood SJ, Slater CR. Safety factor at the neuromuscular junction. *Progress in Neurobiology*. 2001;64:393-429.

Wu M, Majewski M, Wojtkiewicz J, Vanderwinden JM, Adriaensen D, Timmermans JP. Anatomical and neurochemical features of the extrinsic and intrinsic innversation of the striated muscle in the porcine esophagus: evidence for regional and species differences. Cell and Tissue Research. 2003;311(3):289-297.