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CANINE DEGENERATIVE MYELOPATHY: LATEST UPDATES AND PRELIMINARY RESULTS OF MESENCHYMAL STEM CELLS TREATMENT

MIELOPATIA DEGENERATIVA DEL CANE: ULTIMI AGGIORNAMENTI E RISULTATI PRELIMINARI DEL TRATTAMENTO CON CELLULE STAMINALI MESENCHIMALI

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ABSTRACT

Canine Degenerative Myelopathy (DM) is a neurodegenerative disorder affecting dogs in late adulthood. The clinical picture typically starts as a slowly progressive and unpainful T3 to L3 myelopathy. Then, the clinical spectrum progresses to a Lower Motor Neuron flaccid paraplegia and it gradually ascends to affect the thoracic limbs causing tetraplegia. In the last stage bulbar signs occur and, if euthanasia is not performed, DM-affected dogs die from respiratory failure. The prevalence for DM among the population is 0.19%. Large breeds are more represented than small breeds (Coates and Wininger, 2010). According to the literature, the main risk factors for developing the disease are the breed and homozygosity for SOD1:c.118G > A. Since the discovery of the SOD1 mutation in the majority of DM affected dogs, DM has been considered a natural occurring model for Amyotrophic Lateral Sclerosis (ALS) (Awano et al., 2009). Similarities between these two disorders include genetic basis, clinical hallmarks and histopathological features. Unfortunately, they also share the lack of an effective therapy. Therefore, their prognosis remains poor. Over the last years, an increasing number of studies on both SOD1 transgenic mice and humans has evaluated that Mesenchymal Stem Cells (MSC) therapy is a promising treatment for ALS based on its neuroprotective effects. In addition, biomarkers for DM are still not available in clinical practice, though they could be an instrument to obtain an earlier diagnosis and to objectively monitor the disease. This thesis has three objectives. In the first place, it evaluates the signalment and history of the 12 DM-affected dogs which were referred to the veterinary teaching hospital of Parma University (OVUD) between February 2019 and March 2022. Among these dogs, weight over 25 kg was found to be predominant in DM affected dogs and it was also correlated to earlier onset of symptoms. Further investigation with a larger sample is needed to assess whether weight could be a risk factor for developing DM. In the second place, the thesis describes the preparation of MSCs from adipose tissue and the intravenous and intrathecal administration for the therapy of DM in two dogs which were referred to OVUD between 1st May 2021 and 31st March 2022. Finally, samples of Cerebrospinal Fluid (CSF) and serum has been collected to evaluate phosphorylated neurofilament heavy (pNF-H) levels as a

potential marker of DM. As pNF-H values will be available, they will be compared to other sample from dogs without neurological disorders.

1. ANATOMY OF NERVOUS SYSTEM

The nervous system can be classified into two systems: the Central Nervous system (CNS) and the Peripheral Nervous system (PNS).

The CNS consists of the brain and spinal cord, which are centrally located and are the center of neural functions. The PNS includes all the nervous tissue that is not part of the brain and spinal cord. Hence, it comprehends the nerves, ganglia, and sensory receptors. The PNS transports the sensory signals coming from the external and internal environment to the CNS. It also transports motor signals from the CNS to the peripheral effectors, which are skeletal muscles, cardiac muscle, smooth muscles, secretory glands (Uemura, 2015).

1.1 Cells of the Nervous System

The two categories of cells that constitute the nervous system are the neurons and the neuroglia. Neurons have peculiar cell shapes with a membrane capable of generating electrical impulses. These travel from one neuron to the next via synapses which form a complex neuronal network designed for information processing. The neuronal cell body is also known as the soma or perikaryon. Axons frequently branch at great distance from the cell body, where they can either synapse with other neurons or connect with target organs. The other neuronal processes are the dendrites. Dendrites and perikarya are the receptive sites of impulses from other neurons (Uemura, 2015).

Neuroglia are the most abundant cells in nervous system, constituting over 90% of tissue. They fill the space in the nervous system not occupied by neurons and blood vessels and their role is to provide structural, metabolic, and protective support for neurons. Generally, unlike neurons, neuroglia continue to divide also in adult life. Neuroglia are different in the CNS comparing to the PNS. More specifically, in the CNS cell types include microglia, ependymal cells, oligodendrocytes, and astrocytes. By contrast, Schwann cells are the only neuroglia that is found in the PNS (Uemura, 2015).

1.2 Anatomy of the Central Nervous System

The Central Nervous System is composed by the Brain and the Spinal Cord. It is a tubular structure which originates in the embryo from a proliferation of ectodermal epithelial cells referred to as the neurectoderm (de Lahunta et al., 2021). The brain

is protected by the skull bones and includes the cerebrum, the brainstem and the cerebellum. The cerebrum consists of two hemispheres that are divided into lobes. It is connected to the brainstem which can be divided in the midbrain, the pons and the medulla oblungata. The brainstem is dorsally connected to the cerebellum via the cerebellar peduncles, whilst caudally it is connected to the spinal cord (de Lahunta et al., 2021).

1.3 The spinal cord

In medium-sized and large dogs, the spinal cord extends from the foramen magnum to the level of the sixth or seventh lumbar vertebra. Instead, in small dogs it extends slightly further. The spinal cord is surrounded by the vertebrae, meninges, and CSF which protects it from injuries. Its roles are to mediate messages between the brain and peripheral nerves and also to serve as a center for spinal reflexes (Uemura, 2015). Therefore, the spinal cord is responsible for most of the sensation and motility of the body. Particularly, after entering the spinal cord, sensory nerve fibers synapse with various neurons which can be interneurons of the local spinal circuitry or projection neurons that constitute the ascending tracts, or even motor neurons that innervate the muscle. The role of ascending sensory tracts is to convoy sensory information to the brain stem, cerebellum, and cerebral cortex. The motor tracts originate from the cerebral cortex and the brain stem and then descend in the spinal cord. Their function is to regulate motor neurons via interneurons (Uemura, 2015).

The functions of the spinal cord can be summarized in the following three points:

- mediation of motor signals that descends from the cerebrum and brain stem.
- mediation of sensory information, which ascends the spinal cord.
- acts as a center for spinal reflexes. This means that the spinal cord has a local circuit to carry out reflexes, which requires both sensory fibers of the dorsal roots and motor fibers of the ventral roots. More specifically, motor axons exit the spinal cord through the ventral root to innervate skeletal muscles, smooth muscles, and glands. Sensory information is carried by neurons which have their soma in the dorsal root ganglion, while their axons enter the spinal cord through the dorsal roots (Uemura, 2015).

If a transverse section of the spinal cord is obtained, two structurally and functionally distinct areas are revealed. Those are the peripheral white matter and the central gray matter. Their names are due to the fact that in fresh specimens the peripheral part appears glistening white, while the “H” shaped part is grey. In fact, white matter consists mostly of longitudinally oriented myelinated axons, but also some nonmyelinated axons are present. The reason of the white color of this area is the presence of myelin. By contrast, the gray matter appears less white because it contains the neuronal cell bodies (Uemura, 2015).

The gray matter is located in the central part of the spinal cord, where its shape resembles the letter “H.” The dorsal and ventral arms of this “H” are known as the dorsal horn and ventral horn. The gray matter around the central canal and between the dorsal and ventral horns is referred to as the intermediate substance. The intermediolateral nucleus of the sympathetic division is located in the lateral edge of the intermediolateral substance at the level of the thoracic (T1–T13) and cranial lumbar (L1–L3) cord segments. The protuberance formed by this nucleus is known as the lateral horn. The gray matter is formed by neuronal cell bodies, their dendrites and axons, surrounded by glia cells (Uemura, 2015).

The white matter of each half of the spinal cord can be distinguished into dorsal, lateral, and ventral funiculi. The term “funiculus” means “a little cord” in Latin. The white matter between the dorsolateral fasciculus and the dorsomedian septum is the dorsal funiculus. The dorsolateral fasciculus is made of lightly myelinated and nonmyelinated fibers and is located in the narrow area of white matter that extends from the dorsolateral sulcus to the dorsal end of the dorsal horn. The white matter situated between the ventral median fissure and the ventrolateral sulcus is the ventral funiculus which is the site where the ventral rootlets emerge. The lateral funiculus is the remaining white matter located between the dorsal and ventral funiculi. Moreover, the so-called white commissure is the white matter located ventral to the central intermediate substance, that connects the right and left ventral funiculi medially. In the cervical (C1, C8) and upper thoracic segments (T1, T2), the dorsal funiculus can be distinguished into a medial fasciculus gracilis and a lateral fasciculus cuneatus. Their names come from Latin, in fact “fasciculus” means “a little boundle”, while “gracilis” refers to the slender appearance of this ascending sensory tract. By contrast, “cuneatus” comes from the fact that this

ascending sensory tract is wedge-shaped. Generally, the lateral and ventral funiculi are formed by several ascending sensory and descending motor tracts that have specific roles and anatomical connections (Uemura, 2015). The ascending tracts include the over-mentioned fasciculus gracilis and cuneatus but also the dorsal spinocerebellar tract, the ventral spinocerebellar tract, the spinothalamic tract, the nucleus of the dorsal spinocerebellar tract. The descending upper motor neuron pathways include the lateral corticospinal tract, the rubrospinal tract, the medullary reticulospinal tract, the vestibulospinal tract, the pontine reticulospinal tract (Coates and Wininger, 2010). Moreover, axons of local spinal circuits connect adjacent segments of the spinal cord. These intersegmental axons are a part of the circuits for spinal reflexes and ascend or descend in the so-called fasciculus proprius, which is the white matter immediately adjacent to the gray matter. Thus, the fasciculus proprius mediates intersegmental reflexes that can be tested with the Neurological Examination such as the scratch reflex and cutaneous trunci reflex (Uemura, 2015).

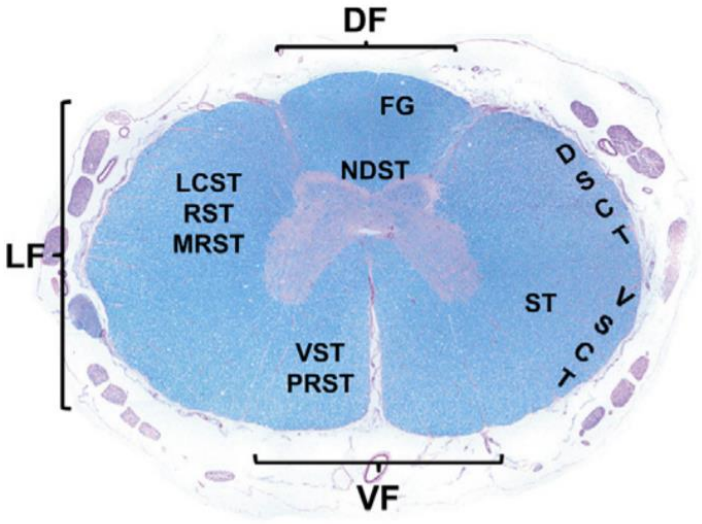

Figure 1: Luxol fast blue stain of a transverse section of a normal mid-thoracic spinal cord labeled for the white matter regions (DF, dorsal funiculus; LF, lateral funiculus; VF, ventral funiculus), containing the ascending (FG, fasciculus gracilis; DSCT, dorsal spinocerebellar tract; VSCT, ventral spinocerebellar tract; ST, spinothalamic tract; NDST, nucleus of the dorsal spinocerebellar tract) fibers and descending (LCST, lateral corticospinal tract; RST, rubrospinal tract; MRST, medullary reticulospinal tract; VST, vestibulospinal tract; PRST, pontine reticulospinal tract) UMN pathways (Coates and Wininger, 2010).

1.4 Upper Motor Neuron and Lower Motor Neuron

Neurologic examination includes evaluation of gait. Gait generation results from the interaction of Upper Motor Neuron (UMN) and Lower Motor Neuron (LMN) (Garosi and Lowrie, 2014; Platt and Olby, 2014).

The UMN system includes any efferent neuron originating within the cerebral cortex, basal nuclei or brainstem that synapses through an interneuron with a LMN to modify its activity. UMNs are responsible for the initiation and maintenance of normal movements and for the maintenance of tone in the extensor muscles to support the body against gravity. UMNs also inhibits myotatic reflexes. Lesions of the UMN system result in loss of motor function and release of the inhibitory effect that the UMN system has on LMNs located caudal to the level of the injury. This disinhibition is usually more evident in the extensor muscles (Garosi and Lowrie, 2014; Platt and Olby, 2014).

The UMN system can be divided into pyramidal and extrapyramidal systems. This classification is more relevant in primates than in domestic animals because their pyramidal system is more anatomically and functionally developed (de Lahunta et al., 2021).

The soma of neurons of the pyramidal system are located predominantly in the motor area of the cerebral cortex. From there, their axons descend towards the spinal cord through the white matter of the cerebral hemisphere and brainstem, passing by the pyramid which is an anatomical structure located on the ventral surface of the medulla. The term “pyramid” derives from its triangular shape. It contains only the projection pathway of the neurons of the pyramidal system. In conclusion, the pyramidal system constitutes a monosynaptic pathway which starts in the cerebrum and descends to the spinal cord by way of the pyramids of the medulla (de Lahunta et al., 2021).

By contrast, soma of neurons of the extrapyramidal system are located in the cerebral cortex, including the motor area. Their axons descend to the brainstem directly or passing through basal (subcortical) nuclei, hence they synapse with neurons in the basal nuclei and brainstem nuclei. Then, axons descend to the spinal cord without entering in the pyramids of the medulla. Thus, the

extrapyramidal system is a multineuronal, multisynaptic corticospinal pathway (de Lahunta et al., 2021).

Nevertheless, the pyramidal and extrapyramidal systems overlap anatomically and function together. It is worth highlighting that extrapyramidal system is more important in domestic animals, whereas the pyramidal system is more developed and functionally relevant in primates (de Lahunta et al., 2021).

The LMN system connects the CNS with the muscles of an effector organ. The cell body of a LMN is located in the ventral horn of the spinal cord grey matter or within the cranial nerve nucleus of the brainstem. The axon of a LMN leaves the CNS as a ventral nerve root, becomes a spinal nerve and then a peripheral nerve. It synapses with the effector organ that can be either a muscle or a gland. The LMN is the last neuron in a chain of neurons that is responsible for the muscular contraction needed to maintain posture, support bodyweight and provide gait (Garosi and Lowrie, 2014; Platt and Olby, 2014).

2. DEGENERATIVE MYELOPATHY

2.1 Definition and name

Canine Degenerative Myelopathy (DM) is a fatal neurodegenerative disorder beginning in late adulthood.

It was first named and described by Averill in 1973 as a proprioceptive ataxia and upper motor neuron (UMN) paresis of the hind limbs not related to disc protrusions and spondylotic reactions as argued before by several authors (Averill, 1973). Since most of the earlier studies were focused on German Shepherds, it has been referred to as German Shepherd Dog Myelopathy (Braund and Vandevelde 1978), further evidence has shown that it can affect several breeds leading to the disuse of this name. In 1975 Griffiths and Duncan reported a series of cases with a higher degree of dorsal root involvement than in previous works, claiming that the name of the disease should have been “chronic degenerative radiculomyelopathy”, but with no luck (Griffiths and Duncan, 1975). More recently the clinical spectrum has been broadened to involve both the UMN and the lower motor neuron (LMN) systems (Coates and Wininger, 2010). Among all the names that have been proposed over the years “Degenerative Myelopathy” is the most indicated because it doesn’t limit the clinical spectrum and the signalment into too narrow borders.

2.2 Signalment

Over the years, DM has been histologically confirmed in several breeds: German Shepherd Dog (Averill, 1973), Siberian Husky (Bichsel et al. 1983), Miniature Poodle (Matthews, 1985), Boxer (Miller et al. 2009), Pembroke Welsh Corgi (Coates et al. 2007), Chesapeake Bay Retrievers, Rhodesian Ridgeback (Awano et al. 2009), Bernese Mountain Dog, Standard Poodle, Kerry Blue Terrier, Cardigan Welsh Corgi, Golden Retriever, Wire Fox Terrier, American Eskimo dog, Soft-coated Wheaten Terrier, Pug (Coates and Wininger, 2010) and mixed breed (Averill, 1973). The disorder has been reported without histopathologic confirmation in Irish Terrier, Kerry Blue Terrier (Griffiths et Duncan, 1975), Labrador Retriever, Bernese Mountain Dog, Hovawart, Kuvasz, Collie, Belgian Sheperd, Giant Schnauzer, Soft-coated Wheathen Terrier, Mastiff, Borzoi (Kathmann et al. 2006) and Great Dane (Polizopoulou et al. 2008).

Coates and colleagues reported breed-specific prevalence rates for DM among 432.467 dogs presented to veterinary teaching hospitals between January 1, 1990, and December 31, 1999. Results can be seen in the following table (Coates et al., 2007).

Breed	**Prevalence of DM Dogs (%)**
All dogs	0.19
German Shepherd Dog	2.01
Welsh Corgi, Cardigan	1.51
Chesapeake Bay Retriever	0.83
Rhodesian Ridgeback	0.74
Irish Setter	0.68
Boxer	0.59
Welsh Corgi, Pembroke	0.58
Fox Terrier, Wire	0.52
Collie	0.38
Old English Sheepdog	0.38
Mixed Breed	0.15

Table 1: breed-specific prevalence rates for DM (Coates et al., 2007)

The prevalence for DM in all dogs is 0.19%. The highest prevalence found among breeds is 2.01% in German Shepherd Dog. Large breeds are more represented than small breeds. Among smaller dogs, Welsh Corgi, Cardigan are the most DM-affected with a prevalence of 1.51 (Coates et al. 2007).

Regarding sex prevalence, there appears to be a female predominance (1.6:1) in Corgis (Coates et al. 2007) but not in other breeds (Kathmann et al. 2006). In the study that evaluated DM in Corgis, the female prevalence could be linked to breeders being more aware of the study than owners and likely to keep more females into old age (Coates et al. 2007).

The mean age of onset in large breed dogs is 9 years, whereas in Pembroke Welsh Corgi the onset disease is, on average, 10.9 years (Coates and Wininger, 2010). Further investigations are needed to evaluate the mean onset age of other small breed dogs.

Age of death ranges from 10.5 to 16 years for Pembroke Welsh Corgi, (median, 12,6 years) (Coates et al. 2007).

The duration of clinical signs before death in Pembroke Welsh Corgi ranges from 10 to 37 months, the mean duration is 19 months (Coates, et al., 2007). A study reports that, if euthanasia is not performed, DM-affected dogs die from respiratory failure, approximately three years or more after disease onset (Oyake et al., 2016).

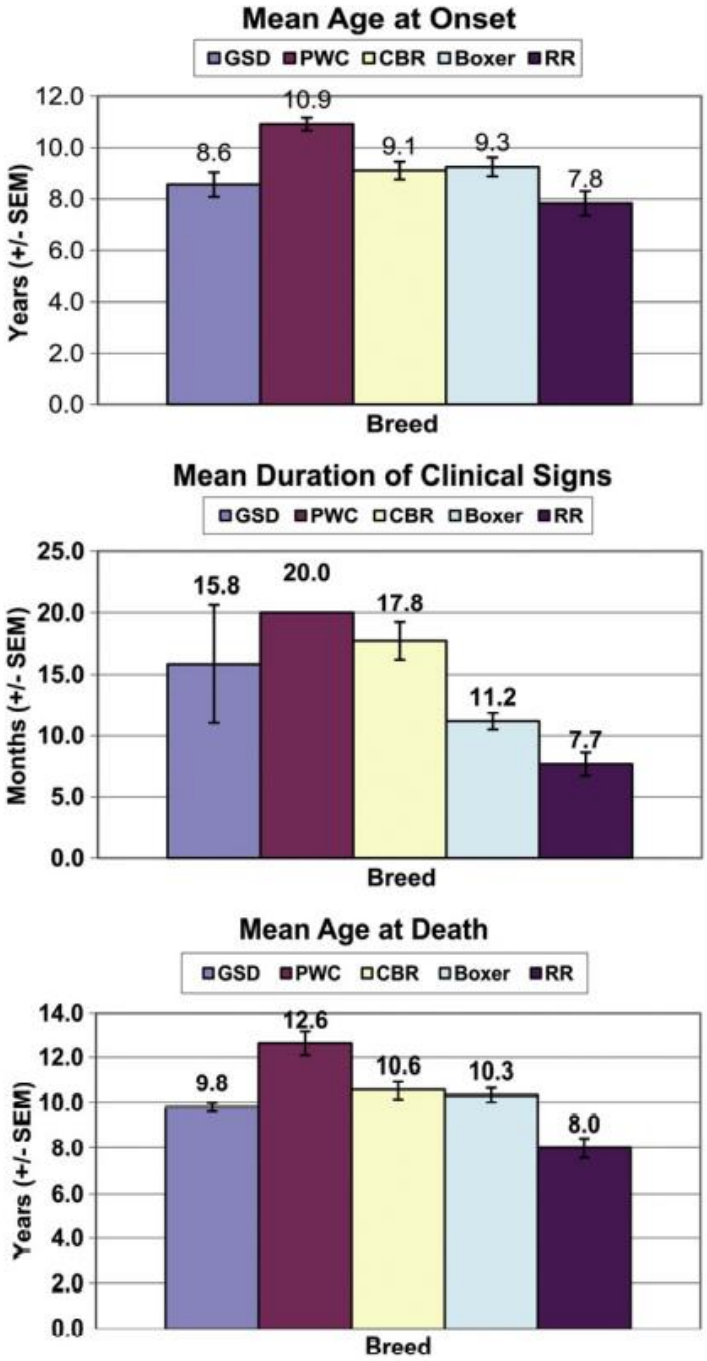

Figure 2: mean age at disease onset, mean disease duration and mean age at death of affected German Shepherd Dog (GSD), Pembroke Welsh Corgi (PWC), Chesapeake Bay Retriever (CBR) and Rhodesian Ridgeback (RR) (Coates and Wininger 2010).

In one article, is reported that DM was diagnosed in a 6-years-old cat. The patient developed ataxia, paraparesis of hind limbs, loss of conscious proprioception over a period of 8 months and was euthanized. A histological examination of the spinal cord was performed and showed lesions that are compatible with DM diagnosis. Particularly, a diffuse degeneration of myelin was more evident in the thoracolumbar segment. The aetiology of the deterioration wasn't clear (Mesfin et al., 1980) Since 1980, no other suspected case of DM in cats has been reported in literature.

2.3 Signs and symptoms

The clinical description of DM given by early and latest studies differs substantially. In fact, signs of DM in the first reports were limited to UMN spastic paraparesis and hind limb proprioceptive ataxia with a T3 to L3 localization. All the dogs examined in these reports were German Shepherd Dogs or large-breed dogs and euthanasia was performed early in the course of the disease not allowing researchers to fully delineate the progression of the disease. (Averill, 1973). More recently, as authors were able to report cases with longer disease duration, the clinical spectrum has been significantly broadened. To better understand the course of the disorder, it is useful to distinguish four stages. The first two stages are considered "early stages" and the last two "late stages".

1 Early 6-12 months from the onset	UMN paraparesis and proprioceptive ataxia	• Progressive general proprioceptive ataxia • Asymmetric and spastic paraparesis • Postural reaction deficits in pelvic limbs • Intact spinal reflexes or decreased patellar reflex • Lack of paraspinal hyperesthesia
2	LMN paraparesis to	• Mild to moderate loss of muscle

Early 9-18 months	paraplegia	mass in pelvic limbs • Reduced to absent spinal reflexes in pelvic limbs • Non ambulatory paraparesis to paraplegia • +/- urinary and faecal incontinence
3 Late 14-24 months	LMN paraplegia to thoracic limbs weakness	• Signs of thoracic limbs weakness • Flaccid paraplegia • Absence of spinal reflexes in pelvic limbs • Severe loss of muscle mass in pelvic limbs • Urinary and faecal incontinence
4 Late >36 months	LMN tetraplegia and brain stem signs	• Flaccid tetraplegia • Difficulty with swallowing and tongue movements • Absence of spinal reflexes in all limbs • Reduced to absent cutaneous trunci reflex • Generalized and severe loss of muscle mass • Urinary and faecal incontinence

Table 2: stages of DM (Coates and Wininger, 2010).

During the early stage, the clinical picture typically consists of a slowly progressive and unpainful T3 to L3 myelopathy. At this stage the signs of DM are loss of pelvic limbs proprioceptive ability leading to asymmetric lameness and postural reaction deficits in hind limbs. Spastic paraparesis is indicative of UMN dysfunction. At the

exordium of symptoms, spinal reflexes can still be intact, though patellar reflex may be exaggerated, normal or decreased. Although hyporeflexia of patellar reflex is reported as normal in older dogs (Levine et al., 2002), some authors have suggested that, in DM, the involvement of the dorsal roots of the femoral nerve may inhibit sensory impulses from stretch receptors located in the quadriceps muscle. Flexor reflexes can be normal or show crossed extension that is suggestive of UMN dysfunction. At physical examination, the nails can be worn because of toe dragging. While most large breed dogs progress to hind limbs paraparesis within 6 to 9 months from onset of clinical signs, the progression is slower in Corgis (Coates and Wininger, 2010).

If euthanasia is not performed, clinical sign progress to LMN flaccid paraplegia and gradually ascend to affect the thoracic limbs causing tetraplegia. As the dog is becoming non-ambulatory, LMN signs such as hyporeflexia of the patellar and withdrawal reflexes, flaccid paralysis and severe loss of appendicular muscle mass occur. Signs typically begin in the pelvic limbs and progress to affect the thoracic limbs. In the late stage of DM, loss of muscle mass is described. Although early reports have attributed muscle atrophy to disuse, recent studies suggest that the flaccidity can be secondary to denervation (Awano et al., 2009). In dogs with advanced disease, cranial nerves are also involved, and the patient can consequently experience swallowing difficulties and inability to bark. Urinary and faecal incontinence occur in the late stage when paraplegia is already established (Coates, 2014; Oyake et al., 2016).

For large breed dogs, pet owners usually choose to perform euthanasia when the patient no longer supports weight in pelvic limbs and needs walking assistance. Small dog breeds are often reported to be cared by the owner over longer time because the management is easier (Coates et al., 2007).

In the late stage of DM, dysfunctional changes occur in the intercostal muscles resulting in respiratory dysfunction. Hypoventilation provokes hypoxemia in the later stages of DM. If euthanasia is delayed, DM-affected dogs die from respiratory failure, approximately three years or more after disease onset (Oyake et al., 2016).

2.4 Aetiology and Pathogenesis

Several studies have considered various hypothesis on DM aetiology, but a lot is yet to discovery to fully understand the underlying etiopathology leading to the disorder. The recent breakthrough of SOD1 mutations as the major risk factor for DM has been a milestone in the research on DM. Since then, various studies on the role of SOD1 in DM aetiology have been performed. The most dominant hypothesis is currently that DM occurs by a “gain of toxic function” of the mutant SOD1 protein (Nakata et al., 2021). Nevertheless, more on pathogenetic mechanisms is still to clarify.

Over the years, many hypotheses have been made and rejected. For example, nutritional, metabolic, oxidative stress, excitotoxic mechanisms have been proposed to explain DM etiopathology (Coates and Wininger, 2010).

Historically, DM was associated to the presence of multiple osseus plaques in the dura mater that were believed to compress nerve roots causing paraparesis (Morgan, 1969). This have been claimed to be wrong because the prevalence and anatomical distribution of dural plaques is not consistent with epidemiology and clinical features of DM (Averill, 1973). Averill discussed the possibility that vascular insufficiency of the spinal cord could be implied in DM pathogenesis, but no evidence was found. In fact, his study didn’t demonstrate the presence of ischemic lesions and, additionally, the onset of symptom of an ischemic disease is acute (Averill, 1973).

Averill also suggested an association with vitamin B12 deficiency basing on the similarities of DM with human Sub-acute Combined Degeneration (SCD), a disease affecting human spinal cord that has been linked with B12 hypovitaminosis, but the lesions of this pathology are consistently different from DM pathological features. In 1984, a study measured serum concentration of B12 vitamin and found hypovitaminosis in 3 out of 6 DM-affected dogs. Hypovitaminosis B12 was found to be correlated with the occurrence of small intestinal disorders and the authors speculated that enteropathy and the consequent lack of nutrients could lead to DM lesions (Williams et al. 1984).

Also, Vitamin E deficiency has been considered. In fact, low levels of vitamin E are implied in aetiology of Ataxia with Vitamin E Deficiency (AVED), a human disorder

that has been associated with a mutation of α-tocopherol transfer protein (α-TTP), that is implied in the metabolism of α-tocopherol. This mutation damages α-TTP function, provoking the loss of α-tocopherol from dietary vitamin E. Low α-tocopherol levels in blood cause the accumulation of free radical oxygen, ultimately leading to the development of lesions in the nervous system. (Williams et al., 1985) E hypovitaminosis have been also associated with degenerative myeloencephalopathy and motor neuron disease in horses (Coates and Wininger, 2010). In 1985 serum levels of vitamin E of a group of DM-affected German Shepherd dogs were compared to a control group. The Vitamin E serum levels were found to be slightly lower in DM-affected dogs (Williams et al., 1985). Nevertheless, another study demonstrated higher levels of vitamin E in DM-affected Shepherd dogs compared to a control group composed by other breeds. If compared to unaffected German Shepherd dogs, the serum levels of vitamin E have not been found to be significantly higher (Johnston et al. 2001). Furthermore, expression levels of the a-tocopherol transfer protein mRNA are not significant (Fechner et al., 2003). Moreover, supplementation with vitamin E doesn't influence progression of DM in affected dogs (Johnston, 2001).

Griffith and Duncan (1975) addressed DM as a "dying-back" disease or distal axonopathy. Nevertheless, the following studies argued that distribution of DM lesions doesn't resemble typical findings of a "dying-back" disease that are symmetrical and start in the distal axonal portion to spread towards the proximal parts of the axons (Braund and Vandevelde, 1978).

The possibility of an immune-mediated aetiology has been firstly proposed in 1980 by Waxmann and colleagues. The data from their study show lower responses to thymus-dependent mitogens by peripheral blood leukocytes obtained from dogs with DM. The degree of the depression of the proliferative response corresponded with the clinical status of patients. Researchers didn't find a relation between the disease process and the restricted peripheral blood leukocyte deficiency (Waxmann et al., 1980). Later, the same research group suggested that immune-mediated events lead to chronic demyelinating disorders as happens in human Multiple Sclerosis. Neuroinflammation in the pathogenesis of DM needs further exploration because of its role in ALS and other neurodegenerative diseases (Coates and Wininger, 2010).

In DM, as in human ALS, the possibility of an excitotoxic aetiology involving Glutamate has been investigated. Glutamate is the predominant excitatory amino acid in the CNS. Excitatory amino acid transporters (EAATs) on the cell membrane have the role to reuptake extracellular glutamate into the cell, modulating homeostasis of synaptic transmission. Among these transporters, astrocytic glutamate transporter 1 (GLT-1 or EAAT2) and glutamate/aspartate transporter (GLAST or EAAT1) are responsible for most of the glutamate transport. Moreover, glutamine synthetase (GS), that catalyses the production of glutamine from glutamate and ammonia, is found in astrocytes and oligodendrocytes and may be involved in the maintenance of the glutamate concentration. Excessive glutamate exposure is toxic to neurons because of massive Ca2+ entry into the cell. Since also motor neurons are vulnerable to excitotoxicity, it is believed that excitotoxicity and oxidative stress play a major role in motor neuron loss in patients with ALS. It has also been reported that the downregulation of GLT-1 is involved in the disease progression in human patients with ALS and in some types of SOD1-transgenic ALS model mice (Ogawa et al., 2013). In 2013, a study examined 5 DM-affected Pembroke Welsh Corgi with a SOD1 mutation, 5 non-DM dogs of the same breed, and 5 Beagles without neurologic signs to assess the neuronal changes and the expression levels of 2 glial excitatory amino acid transporters (GLT-1 and GLAST). The number of neurons in the spinal ventral horns of the DM dogs was significantly decreased, whereas no change was observed in the cell size. Chromatolysis, lipofuscin-laden neurons, and marked synapse loss were also reported. GLT-1 expression was highly decreased in DM dogs, whereas GLAST expression showed no significant change. The results indicate that excitotoxicity related to the reduced expression of GLT-1, but not GLAST, may be involved in neuron loss in DM, as in human ALS, though intraneuronal events may differ between the two diseases (Ogawa et al., 2013).

The role of genetics in DM will be discussed in the following paragraphs.

2.4.1 Genetics

An inherited basis for DM has been suggested by epidemiology because of the uniformity of clinical signs, age, histopathology, and breed predilections. An obstacle to the study of DM genetics has been represented by the late onset of disease since it makes difficult collecting data from parents and siblings to show

evidence to this theory. (Coates and Wininger, 2010) Until now, familiar DM has been reported in the Siberian Husky (Bichsel et al., 1983), Pembroke Welsh Corgi (Coates et al., 2007), Chesapeake Bay Retriever (Long et al., 2009) the Rhodesian Ridgeback and Boxer (Coates and Wininger, 2010).

Over the years, multiple studies on the role of genetics in DM have been conducted. The following paragraphs' aim is to delineate the history of research on this topic that has led to consider genetic as the main risk factor for DM (Nakata et al., 2021).

Braund and Vendevelde in 1978 were the first to suggest the possible implication of genetics in DM considering the strong breed predisposition of German Shepherd dogs but without providing scientific support (Braund and Vendevelde, 1978).

The genetic theory was proposed again in 2006, when a study found a point mutation in hypervariable region 2 of DLA-DRB1 in DM-affected German Shepherd dogs. They also suggested that DM could have a genetic basis resembling primary progressive multiple sclerosis in human beings. The allele was named **1101J* and has been reported to be homozygous in DM-affected German Shepherd dogs and heterozygous in healthy dogs of the same breed (Clemmons et al., 2006). A DNA test to detect this allele was proposed by the University of Florida. Few years later, another study argued that, according to their findings, alleles of DLA-DRB1 are not unique in German Shepherd dogs having DM. Hence, it was concluded that the detection of allele *01101J cannot be used to diagnose or predict DM (Clark et al., 2008).

In 2007, a study on twenty-one Pembroke Welsh Corgi suggested a familial disease basing on Pedigree analysis results. The advanced age of onset of signs is an obstacle that has been made segregation analysis difficult. The following picture shows family relationships from a family of Pembroke Welsh Corgi dogs comprehending 27 affected individuals (Coates et al., 2007).

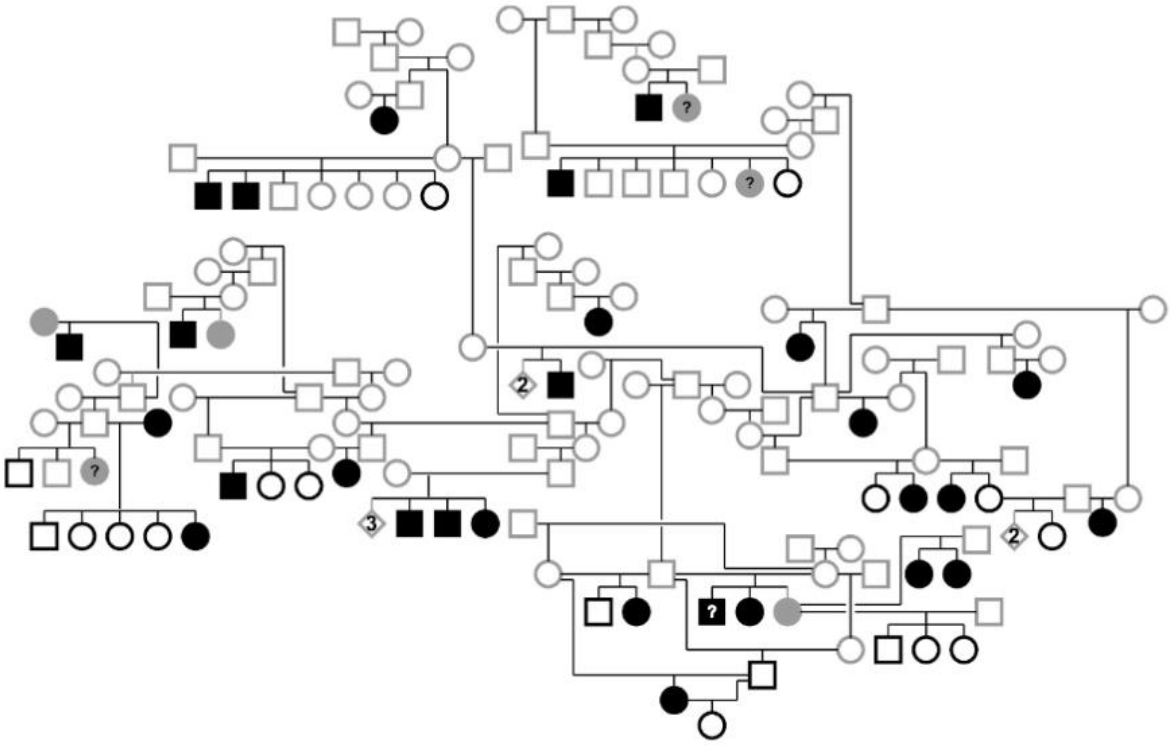

Figure 3: Pedigree showing family relationships from a family of Pembroke Welsh Corgi dogs that contains 27 affected individuals with available DNA. Squares are males, and circles are female; diamonds are numbers of siblings of unknown sex. Solid symbol indicates familial dm-affected dogs. Open symbol represents clinically normal dogs. Solid symbols with question marks represent dogs with clinical signs of familiar dm but without a histopathologic confirmation of the diagnosis. Black symbols represent dogs with available DNA samples. Gray symbols represent dogs with no DNA samples (Coates et al., 2007).

A huge step forward to understand the aetiology of DM was made in 2009, when Awano and colleagues found a mutation in Superoxide Dismutase (SOD1) in DM-affected dog. This mutation resembles the SOD1 mutations in human Amyotrophic Lateral Sclerosis (ALS), an adult-onset neurodegenerative disease involving both upper and lower motor neuron that shares several clinical similarities with DM. Therefore, DM is now considered the first natural occurring animal model for ALS (Awano et al., 2009).

In Awano and colleagues' study, DNA samples from 38 DM-affected Pembroke Welsh corgi and 17 related clinically normal controls were collected and used to perform genome-wide association mapping, an approach used in genetics research to link a disease to its genetic variation. The single nucleotide polymorphisms with strongest association with DM were clustered in a region of chromosome 31, called CFA31. This region contains 3 genes: *SOD1*, *TIAM1*,

and *SFRS15*. *SOD1* was considered a good candidate because mutations in human *SOD1* can cause ALS. *SOD1* was re-sequenced in both normal and DM-affected dogs, and a G to A transition was found in exon 2, which corresponds to nucleotide 118 of the cDNA, and predicted a glutamic acid to lysine missense mutation at amino acid 40. Homozygosity for the A allele (A/A) was associated with DM in all the five dog breeds taken in examination by this study: Pembroke Welsh corgi, German Shepherd dog, Boxer, Rhodesian ridgeback, and Chesapeake Bay retriever. However, some dogs were homozygous for the mutation but free of clinical signs, suggesting age-related incomplete penetrance. Findings suggests that the disease is probably inherited in an autosomal recessive manner. The histopathologic examination of DM-affected patients showed myelin and axon loss in the lateral white matter of spinal cord. Additionally, cytoplasmic inclusions that bind anti-superoxide dismutase 1 antibodies were found in neurons from spinal cord of DM-affected dogs. This finding is particularly interesting because these inclusions resemble those seen in spinal cord from familiar ALS-affected humans with *SOD1* mutations. Hence, canine DM is considered a spontaneously occurring animal model for ALS (Awano et al., 2009).

The discovery of the SOD1:c.118G>A missense mutation has made DM the first spontaneously occurring SOD1-linked ALS in animals. Another pathology of veterinary interest that has been claimed to share several similarities with human ALS is Equine Motor Neuron Disease (EMDN) (Coates and Wininger, 2010). EMDN is a horse spontaneous adult-onset neurologic disorder caused by the degeneration of motor neurons in the spinal cord and brain stem. Its symptoms and signs, pathological lesions, and epidemiologic distribution resemble those of human motor neuron disease (MND), a term that includes several human motor neuron progressive diseases including ALS. The aetiology of both MND and EMDN is unknown, although several hypotheses have been made (Mohammed et al., 2007). No evidence for SOD1 mutations in EMDN has been found (Coates and Wininger, 2010).

In human familial ALS, multiple genes have been associated with the disorder and more than 145 *SOD1* mutations have been described. Hence, it is reasonable to assume that many more mutations could be implied in the aetiology of DM. In 2011, Wininger and colleagues reported a case of DM in a Bernese Mountain Dog

with a novel *SOD1* missense mutation. The age at onset and the progression of the disease were indicative of DM, although the neurologic signs progressed slower than expected. The DNA test for the SOD1:c.118G > A mutation commonly associated with DM was normal (homozygous for the G allele). It was found a SOD1:c.52A 4 T missense mutation that causes the substitution of a serine for threonine at position 18 in the amino acid sequence of SOD1. This amino acid substitution could be a neutral sequence variant unrelated to DM, but researchers stated that it could be linked to DM because further analysis showed the presence of cytoplasmic aggregates that bound anti-SOD1 antibodies in motor neurons from the patient spinal cord. These aggregates resemble those found both in other DM-affected dogs that were homozygote of the SOD1:c.118A allele, and in human familial ALS cases associated with several SOD1 mutations, and even in transgenic murine ALS models expressing mutant human SOD1. The aggregates might form because amino acid substitutions could make conformation of SOD1 unstable. However, it wasn't still clear if the aggregates are the cause to neurodegeneration or contribute to it or even if they are produced by neurodegenerative processes. Additionally, as stated by researchers, the definitive evaluation about the implication of SOD1:c.52 T allele in DM aetiology would have required clinical and pathological evaluations of many more canine SOD1:c.52 T homozygotes (Wininger et al., 2011). Their theory was made stronger by another study conducted in 2014 that is discussed in the next paragraph.

A successive study was designed to evaluate the distribution of the 2 mutant SOD1 alleles (SOD1:c.118A and SOD1:c.52T) across 222 breeds. The results show that SOD1:c.118A allele is widely distributed in the overall canine population. In fact, it was found in 124 different canine breeds and in mixed-breed dogs, that were 56% of the breeds represented in the study. The worldwide presence of the mutation in the other 98 breeds can't be excluded because the mean number of genotyped dogs from each of those breeds was only 23.4 and in a half of them less than 10 dogs were genotyped. Thus, further investigation is needed to clarify the prevalence of the allele in those breeds. The widespread diffusion of the SOD1:c.118A allele suggests that it originated before the establishment of individual breeds. The natural selective pressure couldn't lower the presence of the mutation because the disorder clinically manifest in adult or old dogs. Among

similar breeds, great variations in frequency of *SOD1:c.118A* allele were evidenced. For example, all the tested Greater Swiss Mountain Dogs were homozygous for the ancestral *SOD1:c.118G* allele, whereas among Bernese Mountain Dogs, a closely related breed, the frequency of *SOD1:c.118A* allele was 38% (Zeng et al., 2014).

As stated by the authors, it should be noted that the breed-specific allele frequencies determined by this study are unlikely to reflect the real overall allele frequencies for each breed because dogs weren't selected randomly but for a variety of reasons (Zeng et al., 2014).

Interestingly, the SOD1:c.52T allele was found to be limited to Bernese Mountain Dogs. The *SOD1:c.52T* allele frequency among the 912 genotyped Bernese Mountain Dogs was 3.5%, much lower if compared to the 38% *SOD1:c.118A* allele frequency in the same breed. The development of signs of DM in one out of two (the second was still young when the study was published) of *SOD1:c.52T* homozygous, and in 4 out of 24 heterozygotes at both *SOD1:c.52* and *SOD1:c.118* supports the assertion that the *SOD1:c.52T* allele can cause or contribute to the development of DM. However, it is also reported that some *SOD1:c.118* heterozygous dogs with no other *SOD1* missense mutations have developed DM. Researchers concluded that further studies that analyses cases of DM in *SOD1:c.52T* homozygotes would provide more definitive evidence that the *SOD1:c.52T* allele is effectively implicated in the aetiology of DM (Zeng et al., 2014).

The same study also included histopathologic examination of 249 spinal cords from 38 different breeds and mixed breed. 213 were from dogs that had received a presumptive DM diagnosis while still alive, others were included in a control group. In 45 cases the presumptive diagnosis was not confirmed by histopathology, hence they were included in the control group. Out of the 168 spinal cord from DM confirmed cases:

- 157 dogs were *SOD1:118A* homozygous,
- 9 dogs (2 Bernese Mountain Dogs, 2 Chesapeake Bay Retrievers, 2 German Shepherd Dogs, 2 Rhodesian Ridgebacks, and 1 Alaskan Husky) were *SOD1:c.118A*/*G* heterozygotes. One of the

2 *SOD1:118A*/*G* heterozygous Bernese Mountain Dogs was a *c.118A*/*G* plus c.52A/T compound heterozygote. They had no other sequence variants in their *SOD1* amino acid coding regions.

- 2 dogs were *SOD1:c.118G* homozygotes. One of them was the Bernese Mountain dog descripted by Wininger and colleagues in 2011 that was homozygous for the T allele at *SOD1:c.52*. The other one was a 11-year-old German Shepherd Dog in which the disorder was characterized by a slightly slower rate of progression and a more pronounced sensorimotor neuropathy than DM-average. No significant *SOD1* sequence variants were found in DNA from this patient (Zeng et al., 2014).

115 spinal cords with confirmed DM were examined by immunohistochemistry with anti-SOD1 antibodies and compared to 58 controls. Cytoplasmic aggregates containing *SOD1* antigen were found in spinal cord neurons in all but 1 of the examined dogs with histopathological confirmed DM. The exception was a German Shepherd Dog without any sequence variants in *SOD1* coding regions. Assessed that in human familial ALS multiple mutation in several genes are implied in aetiology of the disorder, we can suppose that the DM in the German Shepherd Dog without SOD1 aggregates derives from another genetic or acquired cause that has not been yet identified. Interestingly, among the 58 control spinal cords, 6 out of 18 spinal cords from patients that were homozygous for the *SOD1:c.118A* allele had SOD1 aggregates and 4 of 25 spinal cords from dogs that were A/G heterozygotes at *SOD1:c.118* had SOD1 aggregates. These aggregates were not found in any of the 15 spinal cords from control dogs that were homozygous for the *SOD1:c.118G* allele. The researchers supposed that some or all the control dogs with cytoplasmic SOD1 aggregates were in a preclinical stage of DM when euthanized (Zeng et al., 2014). This was supported by studies of murine models overexpressing mutant human *SOD1* transgenes in which SOD1-containing aggregates developed before their motor neuron deficits become apparent (Stieber et al., 2000).

To estimate the relative risks for developing DM for dogs with different *SOD1:c.118* genotypes (*A*/*A* versus *A*/*G* versus *G*/*G*) avoiding biases, an analysis was performed only to the 137 dogs that fulfilled some requirements. Data shows that in this cohort 60% of the 30 *SOD1:c.118A* homozygous dogs

developed clinical signs typical of DM whereas only 4% of the 55 *SOD1:c.118A*/*G* heterozygotes and only 6% of the 53 *SOD1:c.118G* homozygotes developed clinical signs of DM. Therefore, the risk of developing DM for *SOD1:c.118A*/*G* heterozygotes is similar to that of *G*/*G* homozygotes and significantly lower than that for *SOD1:c.118A* homozygotes. Hence, the strategy adopted by some breeders of avoiding the production of *SOD1:c.118A* homozygous puppies is rational, especially for breeds with high *SOD1:c.118A* allele frequencies in which stricter mating strategies could provoke the loss of other desirable traits (Zeng et al., 2014).

In the following years, several other studies have been performed to assess the frequency of SOD1 alleles in various populations of dogs. For example, one research investigated the frequency of missense mutation SOD1:c.118G > A among dogs in Mexico (Ayala-Valdovinos et al., 2018), another one examinated the prevalence within a referral population of German Shepherd dogs from the UK (Holder et al., 2014), another provided epidemiological data on the frequency of this allele in German Shepherds in Brazil (Santos et al., 2011), one study considered Czechoslovakian Wolfdogs breed from Slovakia (Jakabová et al., 2016), and also Belgian Malinois dogs in Greece have been investigated (Mataragka et al., 2021). These studies show that missense *SOD1* mutation is widespread among different dog populations of all over the world adding further evidence that genetic testing in breeding programs is a rational tool.

2.4.2 Role of SOD1

Mutations in *SOD1* gene is the main risk factor for developing DM, although underlying mechanisms that lead to neural degeneration are still elusive (Nakata et al., 2021). The *SOD1* protein is one of the most abundant proteins in the CNS and is 153 amino acids in length. It functions as a free radical scavenger. The first ALS causative genetic mutations in *SOD1* were discovered in 1973 by Rosen and colleagues. Since then, more than 140 different *SOD1* mutations have been found in ALS patients. Mutations in *SOD1* gene of ALS patients are distributed in all 5 exons and provoke alterations of amino acids throughout the *SOD1* protein (Coates and Wininger, 2010). In veterinary medicine, *SOD1* from DM-affected dogs often has a c.118G >A missense mutation in exon 2 that predicts an E40K substitution (Awano et al., 2009). Another missense mutation (c.52A > T) has been

identified in the *SOD1* gene in Bernese Mountain Dogs, which predicts a T18S substitution (Wininger et al., 2011). Hence, DM is a spontaneous animal model of ALS. Crisp and colleagues in 2013 investigated the biochemical properties of canine mutant SOD1 and confirmed a strong link between SOD1-mediated diseases in humans and dogs (Crisp et al., 2013). The mutant SOD1 protein is prone to form aggregates in cultured cells, and aggregates has been found also in spinal neurons and astrocytes of DM affected dogs. Consequently, it is believed that these aggregates could be closely associated with the pathogenesis of DM. (Nakamae et al., 2015). Aggregation probably occurs because the mutations reduce the net negative charge of SOD-1. The SOD-1 isoforms with reduced net negative charge may be prone to aggregation because of reduced repulsive Coulombic forces or because of increased interaction with anionic membrane surfaces (Coates and Wininger, 2010). Nevertheless, the mutant SOD1 proteins (canine E40K and T18S) preserve full enzymatic activity (Crisp et al., 2013). Hence, the most dominant hypothesis is currently that DM occurs by a "gain of toxic function" of the mutant SOD1 protein. (Crisp et al., 2013; Nakata et al., 2021) This theory is supported also by the presence of subclinical white matter degeneration in the spinal cords of asymptomatic dogs with homozygotes of mutant SOD1 and heterozygotes (Kobatake et al., 2017).

2.4.3 Role of microRNAs

Lately, microRNA's role in DM pathogenesis has been investigated. MicroRNAs (miRNAs) are small, approximately 18–25 nucleotides, non-coding RNAs involved in the negative regulation of gene expression and in all cellular processes, such as development, differentiation, cell proliferation and apoptosis. One miRNA can regulate multiple mRNAs, while one mRNA can be regulated by more than one miRNA. Their dysregulation is implied in various disorders and the evaluation of different profiles of miRNA is useful to investigate underlying mechanisms of various diseases. In fact, by means of bioinformatics approaches it is possible to identify genes and miRNAs possibly linked to a disease of interest (Nakata et al., 2021). In human medicine there is an increasing interest in the research upon miRNAs in neurogenerative disorders, especially specific miRNAs are involved in ALS pathogenesis and have been proposed as markers of ALS (Rinchetti et al., 2017). Lately, regarding veterinary medicine, plasma miR-26b has been claimed to

be a potential diagnostic biomarker of DM (Nakata et al., 2019). The involvement of miRNA in DM has been further investigated by Nakata and colleagues in 2021. The comparation of the spinal miRNA expression profiles of DM-affected dogs with those of control dogs allowed to discover that in DM-affected patients three miRNAs are up-regulated and 18 miRNAs are down-regulated. This anomaly in the spinal miRNA expression profiles could indicate a failure in the regulation of the RNA metabolism in DM patients. Mechanisms of target clusters of miRNAs were investigated through bioinformatic tools, particularly GO (Gene Ontology) analysis was performed through the DAVID online database. Down-regulation miRNAs target clusters are associated to tissue development of bone, skeletal muscle, and the circulatory system. Authors suggest that these clusters may be induced as a compensation for muscle atrophy because of the widespread loss of muscle mass occurring in the late stage of DM. Up-regulated miRNAs target clusters (miR-23a, miR-142 and miR-221) are related with protein metabolism, including RNA transcription or protein ubiquitination, and cellular response, including telomerase activity, intercellular signal transduction or response to drugs. Since the main hypothesis on DM is currently that it is caused by “gain of toxic function” of mutant SOD1 protein, authors focused their analysis on the mechanism of protein ubiquitination, a process that has been linked to several degenerative diseases. Ubiquitin is a small protein highly conserved through the evolution scale. It is covalently attached to other proteins in the form of polyubiquitination as tag for proteasome-mediated degradation. Dysfunction of the ubiquitin-proteasome system is suspected be a consequence of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and ALS. Ubiquitinated and SOD1-immunopositive inclusions are neuropathological typical findings of human familial ALS and the expression of mutant SOD1 are suspected to be involved into proteasomal inhibition and eventually motor neuronal death. Concerning DM, immunohistochemistry of the spinal cords of DM-affected patients evidences that mutant SOD1 aggregations are not ubiquitin immune-positive. The miRNAs up-regulated in the spinal cords of DM-affected dogs has been proved to significantly increase the proportion of cells with mutant SOD1 aggregations in vitro. Hence, these findings suggests that dysregulated miRNAs in DM inhibit ubiquitination of misfolded SOD1 proteins, resulting in the accumulation of mutant SOD1 aggregations (Nakata et al., 2021).

3. DIAGNOSIS

A definitive diagnosis of DM is determined post-mortem by histopathologic examination of the spinal cord. A presumptive clinical diagnosis is currently based on the following criteria:

- compatible history and clinical signs,
- presence of *SOD1* mutation (SOD1:c.118A and/or SOD1: c.52T)
- exclusion of orthopaedic disorders, other spinal cord and peripheral nerve diseases that can mimic DM (Toedebusch et al., 2017).

Typically, a dog that has DM is taken to the veterinary when the owner is concerned by its lameness. The steps that the clinician follows to reach a diagnosis are: signalment, history, general physical examination, orthopaedic examination, neurologic examination, list of differential diagnosis and collateral analysis to exclude or confirm the diagnosis.

Firstly, the clinician must evaluate:

- Signalment: specie, breed, age, sex, other distinguishing characteristics.
- History: environment, diet, medical history, reproductive history, vaccination status and current and past medications.

Concerning DM, targeted patients are adult or old dogs. Large-size dogs are the most frequently affected by DM. Among different breeds, the highest prevalence is in German Shepherds dog (2,01%), Cardigan Welsh Corgi (1,51%). Prevalence in canine population is 0.19% (Coates et al., 2007).

Then, general physical examination must follow. System approach or Head to Toe exam can be used. System approach comprehends the evaluation of:

1. General appearance and skeletal development
2. State of nutrition, muscle trophism
3. Mentation and level of consciousness
4. Attitude and particular clinical signs
5. Skin and subcutis
6. Eyes, buccal, genital mucosa
7. Explorable lymph nodes
8. Temperature

9. Pulse
10. Respiratory rate and pattern
11. Organic functions

Signs linked to DM that can be collected during a general physical examination are various. The general practitioner can observe ataxia and abnormalities in gait. Nails can be worn as a consequence of toe dragging. In DM late stage, loss of muscle mass, urinary and faecal incontinency can occur.

Neurologic examination is needed to make a presumptive diagnosis or a list of differentials, suggest further investigations, and stage the disease.

Orthopaedic examination should be performed to exclude various orthopaedic diseases that can either mimic or coexist with DM such as hip dysplasia, cranial cruciate ligament rupture (Braund, 1987) and arthrosis.

Complete blood count, serum biochemistry and urinalysis in DM-affected dogs are normal (Coates and Wininger, 2010).

3.1 Neurological Examination

The aims of the neurological examination are to evaluate if the signs observed are a consequence of a nervous system lesion, to understand where the lesion is located and to make a list of differential diagnosis. Then, diagnostic investigations are carried out to confirm or exclude the diagnosis. The first step is taking an accurate and complete history and signalment as previously descripted in this thesis. Then, the clinician must encourage the owner to give a clear description of the concern trying to avoid any ambiguity that could lead to misdiagnosis. The onset of the symptoms must be investigated through carefully questioning to assess if it is acute (onset over minutes to hours), subacute (onset over days), chronic (onset over several days, weeks or months), episodic (animal returns to normal between episodes). The evolution can be progressive, static, improving or waxing and wining. These elements can help the clinician in defining a proper list of differentials because specific causes are associated to a specific type of onset and evolution as showed in the graph below (Garosi and Lowrie, 2014; Platt and Olby, 2014). If the dog is DM-affected, the onset is defined as chronic, and the evolution of the condition is recognised as progressive (Coates and Wininger, 2010).

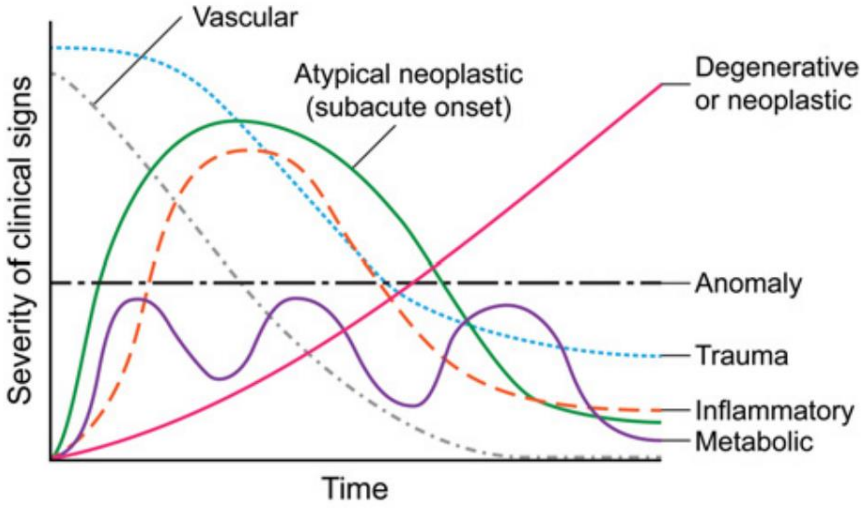

Figure 4: sign-time graph of neurologic diseases. This applies to the majority of cases but there are exceptions in all categories (Dewey and da Costa, 2016).

Before neurologic examination is extremely important to assess if the dog has been treated with any drug and when the therapy has been suspended, particularly for corticosteroids that can be an obstacle in the assessment of neurologic clinical signs. Sometimes, it happens that the owner of a suspected DM-affected dog refers that the patient was previously treated with corticosteroids, but with no improvement. In other DM mimics like Intervertebral disc disease, the symptoms can be mitigated by such therapy, and this can help the clinician in defining a list of differentials. Before neurological examination, a general physical examination must be carried out as previously described in this thesis. Then the neurologic examination can be performed. A general overview is presented in the following table (Garosi and Lowrie, 2014; Platt and Olby, 2014).

Part I: hands-off examination	• Mental status and behaviour • Posture and body position at rest • Evaluation of gait • Identification of abnormal involuntary movements
Part II: hands-on	• Cranial nerve assessment

examination	• Postural reaction testing • Spinal reflexes; muscle tone and size • Sensory evaluation

Table 2: general overview of a neurological examination (Garosi and Lowrie, 2014; Platt and Olby, 2014).

3.1.1 Part 1: hands-off examination

Mental status and behaviour

The state of consciousness depends on two anatomical structures: the ascending reticular activating system (ARAS) within the brainstem and the cerebral cortex. All sensory modalities (except muscle and joint proprioception) send input to the ARAS that projects diffusely to all areas of the cerebral cortex through the thalamus. Mental status mustn't be confused with behaviour, that is associated with the limbic system, consisting of portions of the cerebrum and diencephalon (Garosi and Lowrie, 2014; Platt and Olby, 2014). State of consciousness can be classified as:

- Normal.
- Depressed: drowsiness and loss of responsiveness to environmental stimuli.
- Stuporous: state of unconsciousness that can be roused by painful stimulus.
- Comatose: state of unconsciousness that can't be roused by painful stimulus.

Changes in awareness and behaviour include disorientation, delirium, aggression, compulsive walking, loss of learned behaviour, vocalizing, head pressing (Garosi and Lowrie, 2014; Platt and Olby, 2014).

In DM-affected dogs, mental status, awareness, and behaviour are normal (Coates and Wininger, 2010).

Posture and body position at rest

Common abnormalities in posture and body position at rest are head tilt, head turn, ventroflexion of the neck, spinal curvature (scoliosis, lordosis, kyphosis),

derecebrate rigidity, decerebellate rigidity, Schiff-Sherrington posture (Garosi and Lowrie, 2014; Platt and Olby, 2014). A DM-affected dog doesn't have any of the signs mentioned but at first stage shows an abnormal pelvic limb position (knuckling, abducted, adducted or crossed over) due to UMN dysfunction. Then, when the disease begins to involve the LMN, the dog shows difficulty in bearing its own weight initially on posterior limbs and in late stage also on the anterior limbs (Coates and Wininger, 2010).

Evaluation of gait

Examination of gait should be conducted in a place where the patient can move freely over a non-slip surface. If the animal can't walk, body support should be provided in order to detect any voluntary movement. Normal gait requires intact function of multiple anatomical structures such as brainstem, cerebellum, spinal cord and sensory and motor peripheral nerves, neuromuscular junctions and muscles. Gait generation results from the interaction of UMN and LMN. When evaluating gait, the clinician should assess whether the dog has ataxia, paresis, paralisis, circling (Garosi and Lowrie, 2014; Platt and Olby, 2014).

Ataxia is an abnormality of coordination. There are different types of ataxia that are described in the table below.

Type of ataxia	**Neurolocalization**	**Clinical signs**
Proprioceptive	General proprioceptive pathways: • Peripheral nerve • Dorsal root • Spinal cord • Brainstem • Cerebral cortex	Abnormal postural reactions with limb paresis.
Vestibular	Vestibular apparatus: • Vestibular nuclei (central)	Head tilt, leaning, falling or rolling to one side, abnormal nystagmus, strabismus, normal (peripheral) or

	• Vestibular portion of CN VIII or vestibular receptors (peripheral)	abnormal (central) postural reactions. Crouched posture, reluctance to move and wide head excursion in case of bilateral dysfunction.
Cerebellar	Cerebellum	Wide-based stance, intention tremors of the head, loss of balance and truncal sway, dysmetric gait, pendular nystagmus, delayed onset and dysmetric hopping reactions, ipsilateral menace deficit with normal vision, absence of limb paresis and proprioception placement deficits, and normal mentation.

Table 3: Classification of ataxia (Garosi and Lowrie, 2014; Platt and Olby, 2014).

Paresis is an abnormality in the strength of the voluntary movement. Paresis means that some voluntary movements are still present, while paralysis is the total loss of voluntary movement. The clinician can distinguish between paresis given by a lesion of UMN or LMN because their clinical manifestation is different. In fact, UMN lesions typically result in the loss of the inhibitory effect that the UMN system has on LMNs located caudal to the level of the injury. This disinhibition is usually more apparent in extensor muscles and results clinically in spastic paresis or paralysis (Garosi and Lowrie, 2014; Platt and Olby, 2014). UMN paresis and general proprioceptive ataxia occur with lesions affecting the brainstem or spinal cord. By contrast, a LMN lesion causes a flaccid paresis, difficulty in weight bearing and a short-strided gait. When standing, the affected limbs may exhibit a tremor in the muscles. LMN paresis visible in gait can be a consequence of lesions

affecting the peripheral nerves, neuromuscular junction and muscles (Garosi and Lowrie, 2014; Platt and Olby, 2014).

Lameness can affect one or multiple limbs. Lameness of one limb can be either orthopaedic or can be associated with nervous systems diseases such as lateralized disc extrusion or nerve root tumour and can be referred to as “nerve root signature”. It also can be associated with other pathologies and affect more than one limb (Garosi and Lowrie, 2014; Platt and Olby, 2014).

In a dog with early-stage DM, clinical signs of DM are general proprioceptive ataxia, spastic paresis (that indicates UMN disease), lameness, and weakness. The onset is often asymmetric. Then, LMN flaccid paraparesis occurs. Next, the disease progresses to paraplegia and ultimately to tetraplegia (Coates and Wininger, 2010).

Identification of abnormal involuntary movements

Abnormal involuntary movements include seizure, tremors, myotonia, head bobbing (Garosi and Lowrie, 2014; Platt and Olby, 2014). The clinical spectrum of DM doesn’t include these signs (Coates and Wininger, 2010).

3.1.2 Part 2: hand-on examination

Cranial nerve assesment

To assess cranial nerve (CN) functions, the clinician performs various tests that are summarized in the table below.

Test	**Afferent cranial nerve**	**Intermediate brain region**	**Efferent cranial nerve**	**Principal effect**
Palpebral reflex	CN V – trigeminal (ophthalmic or maxillary)	Brainstem	CN VII – facial	Blink elicited by touching the medial or lateral canthus of the eye

Corneal sensation	CN V – trigeminal (ophthalmic)	Brainstem	CN VII – facial CN VI – abducent	Blink and globe retraction elicited by touching the cornea
Vestibulo-ocular reflex	CN VIII – vestibulocochlea	Brainstem	CN III – oculomotor CN IV – trochlear CN VI – abducent	Nystagmus induced by moving the head
Menace response	CN II – optic	Forebrain; cerebellum; brainstem	CN VII – facial	Blink elicited by a menacing gesture
Response to stimulation of nasal mucosa	CN V – trigeminal (ophthalmic)	Forebrain; brainstem	None	Withdrawal of the head elicited by touching the nasal mucosa
Pupillary light reflex	CN II – optic	Brainstem	CN III – oculomotor	Pupillary constriction elicited by shining a

				light in the eye
Gag reflex	CN IX – glossopharyngeal CN X – vagus	Brainstem	CN IX – glossopharyngeal CN X – vagus	Contraction of the pharynx elicited by its palpation

Table 4: main cranial nerve tests (Garosi and Lowrie, 2014; Platt and Olby, 2014).

In MD latest stage of disease, brain stem signs occur. Cranial nerve signs include swallowing difficulties and an inability to bark (Coates and Wininger, 2010).

Postural reaction testing

The awareness of the position and movements of the body is called sense of kinaesthesia and depends on proprioreceptors located in joints, tendons and muscles (general proprioception) or in the inner ear (special proprioception). Those receptors project to the cerebral cortex where they are consciously perceived (Garosi and Lowrie, 2014; Platt and Olby, 2014).

Postural reactions teste complex pathways that generally are composed by an afferent arc (a proprioceptive receptor, a peripheral sensory nerve, the spinothalamic ascending pathways and the contralateral somatic sensory area of the cerebral cortex) and an efferent arc (the contralateral motor cortex, the descending motor pathways within the brainstem and spinal \cord, the peripheral motor nerve and the skeletal effector muscles). As postural reactions involve many anatomical structures, they do not provide specific information on the localization of the lesion but testing them is a valid tool for detecting subtle disfunction and asymmetry (Garosi and Lowrie, 2014; Platt and Olby, 2014).

The sense of kinaesthesia is tested through the assessment of the postural reactions listed below:

- Proprioceptive placing (paw position or ‘knuckling’ response): this test evaluates the conscious awareness of limb position and movement in

space. Proprioceptive placing is performed by turning over the paw so the dorsal surface is in contact with the ground and determining how quickly the animal corrects the paw position. When performing this test, the animal should be standing squarely on all four limbs and the majority of the animal's bodyweight should be supported.

- Hopping reactions: the hopping reaction is tested by holding the patient and placing most of its bodyweight on one limb while the animal is moved laterally. The normal reaction is hopping on the tested limb to accommodate a new body position following the centre of gravity. This test highlights subtle ataxia or weakness of each limb.
- Wheelbarrowing: it is specific for the thoracic limbs. The pelvic limbs are lifted off the ground by supporting the animal under the abdomen and forcing it to walk forwards. With this test subtle thoracic limb weakness and ataxia can be easily detected.
- Extensor postural thrusting: it is specific for the pelvic limbs. The animal is supported by the chest caudal to the thoracic limbs and the pelvic limbs are lowered to the floor, forcing the animal to walk backwards. This test highlights pelvic limb weakness and ataxia
- Hemi-walking: This test evaluates the ability to move using the thoracic and pelvic limbs of one side while holding the contralateral limbs. When the animal is moved laterally, the clinician can assess the coordination of the movements.
- Visual or tactile placing response: Tactile placing is performed with the eyes of the dog covered. The patient is lifted and the distal part of the thoracic limb is moved towards the edge of a table. When the dorsal surface of the paw touches the edge of the table, the dog should immediately place its foot on the table. Visual placing is performed by allowing the animal to see the table, consequently the dog should reach for the surface before the paw touches it (Garosi and Lowrie, 2014; Platt and Olby, 2014).

In a DM-affected dog, postural reaction testing become abnormal firstly in one pelvic limb, then the signs become symmetric and in the late stage begin to affect also thoracic limbs (Coates and Wininger, 2010).

Spinal reflexes

Spinal reflex evaluation includes and evaluation of the thoracic limbs, the pelvic limbs and examination of the tail and anus.

The evaluation of the thoracic limbs consists of:

- Withdrawal (flexor) reflex: the clinician pinches a digit of the patient with fingers or a haemostat in order to obtain a noxious stimulus that provoke a reflex contraction of the flexor muscles and withdrawal of the tested limb. This reflex evaluates the integrity of spinal cord segments C6–T2, its associated nerve roots, brachial plexus and peripheral nerves (axillary, musculocutaneous, median and ulnar nerves). Additionally, the contralateral limb should be observed for extension (crossed-extensor reflex), indicating an UMN lesion cranial to the C6 spinal cord segment.
- Extensor carpi radialis reflex: the clinician strikes the extensor carpi radialis muscle belly with a reflex hammer at the proximal region of the antebrachium keeping the carpus slightly flexed. If spinal cord segments C7–T2, their associated nerve roots and the radial nerve are not lesioned, there is a slight extension of the carpus.
- Biceps brachii and triceps reflexes: these reflexes are less reliable than the withdrawal and extensor carpi radialis reflexes and are not always present in the normal animal. They evaluate the integrity of spinal cord segments C6–C8 (biceps), C7–T1 (triceps), their associated nerve roots and the musculocutaneous (biceps) and radial (triceps) nerves (Garosi and Lowrie, 2014; Platt and Olby, 2014).

The evaluation of the pelvic limbs includes:

- Withdrawal (flexor) reflex: a normal reflex consists in flexion of the hock, stifle and hip. It evaluates the integrity of spinal cord segments L4–S2, their associated nerve roots and the femoral and sciatic nerves. Moreover, a crossed-extensor reflex indicates an UMN lesion cranial to the L4 spinal cord segment.
- Patellar reflex: this test is performed with the dog in lateral recumbency, the stifle slightly flexed and the limb is supported by placing a hand under the tight. Striking the patellar tendon with a reflex hammer elicitates the reflex

contraction of the quadriceps femoris muscle. If the reflex is absent or weak, there is a lesion of the L4–L6 spinal cord segments or the femoral nerve. Occasionally a weak reflex can be due to old age or previous stifle disease. In absence of others neurological signs, an exaggerated patellar reflex can be due to the dog being nervous or excited. If there is a lesion cranial to the L4 spinal cord segment, the reflex can be normal or exaggerated. Hyperreflexia can also indicate a lesion that involves the sciatic nerve or the L6–S2 spinal cord segment. This pseudo-hyperreflexia results from the decreased tone in the muscles that flex the stifle and normally counteract stifle extension during the patellar reflex.

- Cranial tibial and gastrocnemius reflexes: these reflexes are less reliable than the patellar reflex. They evaluate the integrity of spinal cord segments L6–S1 (cranial tibial) and L7–S1 (gastrocnemius), their associated nerve roots and the peroneal (cranial tibial) and tibial (gastrocnemius) peripheral nerves (Garosi and Lowrie, 2014; Platt and Olby, 2014).

Then, the clinician evaluates the tail and anus through the perineal reflex. This reflex is tested through a stimulation of the perineum with a haemostat that results in contraction of the anal sphincter and flexion of the tail. The anatomical structured involved in the reflex are the caudal nerves of the tail, the pudendal nerve and spinal cord segments S1–Cd5 (Garosi and Lowrie, 2014; Platt and Olby, 2014).

Regarding DM, at the onset of disease spinal reflex abnormalities are consistent with UMN paresis localized to the T3–L3 spinal cord segments. Patellar reflexes may be normal or exaggerated; however, hyporeflexia of the patellar reflex has also been described (Griffiths and Duncan, 1975). Flexor reflexes may also be normal or show crossed extension (suggestive of chronic UMN dysfunction) (Coates and Wininger, 2010). Hyporeflexia of the patellar and withdrawal reflexes, flaccid paralysis and widespread muscle atrophy, beginning in the pelvic limbs (LMN signs) manifest as the dog becomes non-ambulatory. In the last stage of DM, perineal reflex abnormality and faecal and urinary incontinency can occur (Coates and Wininger, 2010).

Muscle tone

Resting muscle tone is normal or increased in case of UMN paresis and decreased to absent in case of LMN paresis (Garosi and Lowrie, 2014; Platt and Olby, 2014). In a DM affected dog, the first stage of the disease is characterized by UMN paresis of pelvic limbs and the muscle tone is increased. Then, when LMN paresis occur, the muscles tone become decreased to absent. In addition, in the late stage of the disease, muscle atrophy occurs initially confined to pelvic limbs and ultimately involving all appendicular muscles (Coates and Wininger, 2010). Most reports have attributed the loss of muscle mass to disuse (Averill, 1973; Griffiths and Duncan, 1975; Coates et al., 2007), but flaccidity in dogs with protracted disease suggests denervation (Awano et al., 2009).

Sensory evaluation

Evaluation of the sensory system largely depends on tests for nociception because touch, pressure and temperature sensation are extremely difficult to assess objectively in dogs. The aim of this evaluation is to detect and map any area of sensory abnormalities. Anaesthesia, hypoaesthesia, hyperaesthesia refer to a loss, impairment, increased sensitivity to a normal level of stimulation, respectively. Analgesia, hypoalgesia and hyperalgesia refer to loss, impairment and increased sensitivity to pain, respectively (Garosi and Lowrie, 2014; Platt and Olby, 2014).

The tests that the clinician performs for sensory evaluation are:

- Cutaneous sensory testing: Cutaneous sensation is evaluated by pinching the skin with a haemostat. The normal responses are a withdrawal reflex and a behavioural response (e.g. turning the head or vocalization). A normal withdrawal reflex indicates that the cutaneous nerve tested, the spinal cord segments and the efferent motor neuron of the withdrawal reflex are functional. The behavioural response implies the conscious perception of the stimulus and consequently indicates that the cutaneous nerve being tested, the afferent nociceptive pathways within the spinal cord and brain, and the appropriate portions of the cerebral cortex are functional (Garosi and Lowrie, 2014; Platt and Olby, 2014).

- Evaluation of the cutaneous trunci (panniculus) reflex: the clinician performs this test pinching the skin on the dorsal trunk between T2 and L4–L5 that leads to the contraction of the cutaneous trunci muscles bilaterally. The sensory nerve from the skin enters the corresponding segment of the spinal cord. Then, afferent sensory information ascends the spinal cord and synapses bilaterally at the C8-T1 spinal cord segments with the motor neurons of the lateral thoracic nerve, which courses through the brachial plexus and innervates the cutaneous trunci muscle. This test is performed from caudal to cranial on each side of the spine, starting at the level of the wings of ilium. If the reflex is present at this level the entire pathway is intact and further testing is not necessary. With spinal cord lesions, this reflex is lost caudal to the spinal cord segment affected (Garosi and Lowrie, 2014; Platt and Olby, 2014). In DM affected dogs, the cutaneous trunci reflex is initially preserved, but it become reduced or absent in the end stage, as tetraplegia occurs. (Coates and Wininger, 2010)
- Nociception testing: For consciously perceiving pain, the sensory component of the peripheral nerves and the associated spinal cord segments, the spinal cord and brainstem and the related thalamocortical system must all be intact and functional. Since the nociceptive fibres are located deep in the spinal cord white matter and project to both sides of the spinal cord, only a severe bilateral spinal cord lesion impairs nociception. The nociception testing is performed at the same time of the withdrawal test. If the gentle squeeze applied to the digits to elicit the withdrawal reflex is not followed by any behavioural response, heavier pressure is applied (Garosi and Lowrie, 2014; Platt and Olby, 2014). In DM affected dogs, nociception is preserved. (Coates and Wininger, 2010)
- Palpation: the clinician palpates head, spine and limbs of the dog to detect any deformity, focus of pain and restricted movement. Palpation of the spine is started by applying gentle downward pressure on the spinous process and then along the transverse processes. The degree of pressure applied should be increased progressively. Simultaneous palpation of the abdomen can help to detect the focus of the pain (Garosi and Lowrie, 2014; Platt and Olby, 2014). Particularly, in a suspected case of DM in a dog, the palpation of the spine is relevant because a DM-affected dog shows no pain

whilst in other mimics such as IVDD there is a hyperalgesia of the affected tract of the spine (Coates and Wininger, 2010).

3.2 Differential Diagnosis

A presumptive ante-mortem diagnosis is based on the recognition of the progression of clinical signs and the exclusion of other diseases that cause progressive myelopathy. To make it even more challenging, it is not uncommon for old dogs to have neurological and orthopaedic diseases that can either mimic or coexist with DM. Neurological disorders includes:

- Intervertebral disc disease (IVDD)
- Degenerative lumbosacral stenosis
- Spinal cord neoplasia
- Inflammatory disease

Orthopaedic disorders to consider are:

- Hip dysplasia
- Cranial cruciate ligament rupture (Braund, 1987)

If a careful examination is performed, these disorders can be distinguished from DM because neurological findings are different. For example, paw replacement (proprioceptive positioning) is useful to differentiate between orthopaedic and neurologic disease because dogs with orthopaedic disorder will have normal paw replacement. Other spinal cord disorders differ in anatomic distribution, clinical signs, and age of onset (Coates et Wininger, 2010). For example, DM affected dog shows no pain, while IVDD is painful. Moreover, signalment can indicates a breed predisposition, for example chondrodystrophic breeds, as Pembroke Welsh Corgi, are prone to Hansen type I intervertebral disc disease, whilst older large breed dogs are prone to Hansen type II intervertebral disc disease (Smolders et al., 2013).

Further explanations on the most relevant differentials can be found in the following paragraphs.

3.2.1 Intervertebral disc disease

As showed in the following figure, type I intervertebral disc disease implicates complete rupture of the dorsomedial or dorsolateral annulus fibrosus and dorsal

longitudinal ligament with extrusion of degenerated nucleus pulposus material. Type II intervertebral disc disease implicates partial ruptures and disorganization of the annulus fibrosus, and bulging of nucleus pulposus, annulus fibrosus and dorsal longitudinal ligament towards the dorsomedial or dorsolateral side (Smolders et al., 2013).

IVDD can be ruled out in differential diagnosis thanks to neurologic examination, particularly the presence of pain, acute or subacute symptoms onset and neuroimaging. Many imaging techniques have been described to be useful for diagnosis of IVDD. Radiographs are helpful to identify calcification and intervertebral disc degeneration but there are notable limitations in its sensibility. Myelography is more useful than radiographs but has been largely supplanted by cross-sectional imaging. Computed tomography (CT) with or without myelography, and magnetic resonance imaging (MRI) are currently the most sensible methods to analyse the anatomy of spinal cord and, consequently, to evaluate the presence or absence of IVDD. (Da Costa, et al., 2020)

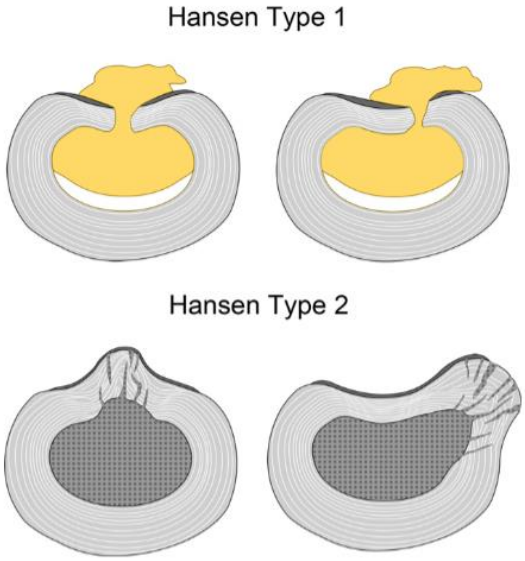

Figure 5: the picture shows the annulus fibrosus (grey), the dorsal longitudinal ligament (dark grey), the extrusion of degenerated nucleus pulposus material (yellow) (Smolders et al., 2013).

3.2.2 Degenerative lumbosacral stenosis

Degenerative lumbosacral stenosis (DLSS) is a common neurological disease typically affecting large breed dogs, including German Shepherd dog, Boxer and Rhodesian Ridgeback. Over the past 40 years, DLSS has been called in many other ways: cauda equina syndrome, cauda equina compression, lumbosacral

stenosis or disease and lumbosacral instability. DLSS, as the name suggests, is a multifactorial degenerative disorder that causes stenosis of the vertebral canal and compression of the cauda equina or its blood supply (Meij, et al., 2010). Several pathologies are implied in its pathogenesis. The most relevant causes of DLLS are:

- degeneration of the disc that can prelude to Hansen type II (or less frequently type I) IVD herniation
- ventral subluxation of S1 (lumbosacral instability) and misalignment of the facet joints
- congenital vertebral abnormalities like symmetric or asymmetric transitional or extra vertebrae
- proliferation of the soft tissues surrounding the cauda equina such as hypertrophy of the interarcuate ligament, the joint capsule, and epidural fibrosis
- sacral osteochondrosis
- vascular compromission of the blood supply to the spinal nerves (Meij, et al., 2010).

Owners often report a history of pelvic limb lameness, difficulty with rising, sitting, or lying down, reluctance to jump or climb, and/or getting into the car, dragging of toes, a low carriage of the tail, and urinary or faecal incontinence. Clinical findings during neurological examination are associated to the compression of cauda equina and are caudal lumbar or lumbosacral pain, hyperesthesia of the lumbosacral region, unilateral or bilateral pelvic limb lameness, and posterior paresis. The most immediate clinical finding that allows to discriminate between DM and DLSS is pain evoked by pressure applied over the lumbosacral region. Lumbosacral pain needs to be differentiated from pain evoked by hyperextension of the hip joints. In some patients are reported non-weight bearing pelvic limb lameness and nerve root signature (pain apparent on palpation or traction of the limb), both caused by unilateral compression of the L7 and/or S1 nerves (Meij, et al., 2010). Other neurologic signs in DLSS include LMN signs of the pelvic limbs such as paresis, atrophy of muscles innervated by the sciatic nerve (L6, L7, S1), hyporeflexia of the withdrawal reflex and cranial tibial reflex (Meij, et al., 2010). Moreover, pseudo-hyperreflexia of the patellar reflex is sometimes reported. The

patellar reflex may appear exaggerated due to hypotonia of the flexor muscles of the stifle that normally antagonize the quadriceps muscles (Worth et al., 2019). In critically affected dogs, urinary and faecal incontinence can occur. When proprioceptive ataxia and proprioceptive deficits occur, main differential diagnosis that needs to be considered in addition to DLSS are degenerative myelopathy, thoracolumbar IVD herniation, or neoplasia.

The presumptive diagnosis of DLSS in dogs is based on anamnesis, clinical signs, orthopaedic and neurologic examinations. Imaging techniques are required to make a definitive diagnosis of DLSS or to rule it out of our list of differentials (Meij, et al., 2010).

3.2.3 Spinal cord neoplasia

Generally, spinal cord neoplasia is more common in old dogs but can appear at any age. Neurological signs are mostly chronic and progressive, although an acute setting of symptoms can occur when neoplasia is associated with spontaneous haemorrhage, impairment of vascular supply or loss of a compensatory mechanism to the surrounding tissue. Main factors influencing the clinical picture are lesion size, collocation, histological nature, growth rate and associated inflammatory response. Neurological sings can be lateralized or symmetrical, and typically suggest a focal lesion. In addition, paraneoplastic neurological syndromes can occur (Garosi, 2014; Platt and Olby, 2014).

Spinal cord neoplasia can be classified as extradural, intradural-extramedullary, and intramedullary depending on their location relative to the spinal cord and the dura mater. Examples of each category are:

- extradural: metastasis, vertebral tumours (sarcomas, plasma cell tumours), lymphoma.
- intradural-extramedullary: meningiomas, nerve sheath tumours, metastasis.
- intramedullary: ependymomas, gliomas, metastasis, round cell tumours (Coates, 2014; Platt and Olby, 2014).

After an accurate anamnesis, clinical and neurologic examination, the clinical pathway must include imaging techniques to discriminate between neoplasia and DM (Coates, 2014; Platt and Olby, 2014).

3.2.4 Inflammatory disease

Inflammatory diseases can be sterile or infectious. Their onset can be acute, subacute, or insidious, depending on the cause. Clinical signs typically progress if no treatment is given, although sometimes they may wax and wane early after onset. Neurological signs can indicate a focal or multifocal lesion that can be asymmetrical or symmetrical (Garosi, 2014; Platt and Olby, 2014). Examples of sterile or infectious inflammatory disease are reported below:

- Infectious: meningitis/myelitis (aetiology can be viral, fungal, bacterial, protozoal, rickettsial, agal, spinal empyema), discospondylitis (aetiology is mostly bacterial, fungal), vertebral physitis.
- Non-infectious: granulomatous meningoencephalomyelitis, steroid-responsive meningitis–arteritis, vasculitis (Coates, 2014; Platt and Olby, 2014).

Diagnostic pathway to reach a definitive diagnosis must be evaluated case to case.

3.3 Diagnostic instruments

Diagnostic methods useful to evaluate spinal cord disorders and to presumptively diagnose MD include radiography, myelography, computed tomography, magnetic resonance imaging (MRI), DNA testing, cerebrospinal fluid (CSF) analysis and electrodiagnostic testing (Coates, et al., 2007; Platt and Olby, 2014).

Spinal imaging (radiography, myelography, MRI, CT) is routinely performed to exclude degenerative lumbosacral syndrome, intervertebral disc disease and spinal cord neoplasia. (Coates and Wininger, 2010) The gold standard for spinal cord is MRI (Coates, 2014; Platt and Olby, 2014). Imaging sometimes shows disc protrusions that can confound the diagnosis of DM. Therefore, the clinician must evaluate if the lesions showed by imaging can explain the symptoms or are just concomitant to DM based on his own experience (Coates and Wininger, 2010). Recently, Diffusion Tensor Imaging (DTI) has been proposed as a tool to detect spinal cord lesions correlated to DM in patients while still alive (Johnson et al., 2021).

A DNA test is commercially available and routinely used by clinicians. It is based on a mutation in the SOD1 gene and dogs that are homozygous for this mutation

are at risk for developing the disorder. Despite of that, some dogs homozygous for the mutation remain asymptomatic because the mutations are incompletely penetrant (Dewey and da Costa, 2016). SOD1 gene is also associated with human ALS, therefore DM is considered a naturally occurring animal model for ALS (Awano et al., 2009).

CSF analysis can help rule out meningitis, in fact in DM-affected dogs it shows no cytologic or protein abnormalities (Coates et Wininger, 2010).

Additionally, over the last years, innovative biomarkers have been proposed both for the diagnosis and the monitoring of DM. Particularly, a study shows that phosphorylated neurofilament heavy (pNF-H) is a promising biomarker for diagnosis of DM, though further studies are needed to examine pNF-H on larger cohort of DM mimics, especially central and peripheral axonopathies (Toedebusch et al., 2017). In 2019, plasma miR-26b has been proposed as a potential diagnostic biomarker of DM (Nakata et al., 2019). Other biomarkers that have been studied are myelin basic protein (MBP) (Oji, et al., 2007) and 8-isoprostane (Coates, et al., 2007).

Electrodiagnostic testing has been performed on DM patients. Electromyography (EMG) and study of nerve conduction velocities have shown different electrical activity depending on the stage of the disease (Awano et al., 2009).

DTI, electrodiagnostic testing, DNA testing and biomarkers are described in the following sections.

3.3.1 Diffusion Tensor Imaging

Considering DM-correlated lesions, standard MRI can only show that affected dogs have smaller spinal cords than healthy dogs (Jones et al., 2005), but DM also causes white matter spinal cord lesions that are undetectable on traditional neuroimaging. To spot them, some authors have recently proposed diffusion tensor imaging (DTI), an advanced MRI technique that's gaining popularity among clinicians and researchers as a promising tool for studying white matter in living humans and animals, both in healthy and pathological conditions (Johnson et al., 2021).

DTI is a variant of diffusion-weighted imaging (DWI), an MRI method that is based on water diffusion rate within a tissue. DTI doesn't need contrast and can be used

on almost all modern MR scanners with relatively fast scan times (Ranzenberger et al., 2021). In human medicine DTI has been used to study a wide range of neurological disorders including:

- deformation (deviation, infiltration, destruction) of white matter caused by tumours
- neurodegenerative disorders such as multiple sclerosis and Alzheimer's
- epilepsy
- neuropsychiatric disorders including schizophrenia
- development disorders like dyslexia, autism, and attention deficit hyperactivity disorder
- movement disorders as Parkinson's disease
- delineation of the anatomy of immature brains and changes in the microstructure of white matter during aging (Soares et al., 2013)
- ALS (Sage et al., 2007)

In Veterinary Medicine, in the neurological field, DTI has been used to study the anatomy of white matter tracts in the dog brain (Jacqmot et al., 2013), anatomy of the spinal cord (Hobert et al., 2013) and to evaluate several pathologies. Especially, multiple research projects have successfully studied canine spinal cord injury (SCI) with different approaches that focus on clinically relevant aspects. For example, one study compared different DTI findings in acute and chronic SCI (Wang-Leandro et al., 2017). One other focalized on acute SCI in dogs in order to find a possible imaging biomarker regarding the correlation between duration of injury and clinical severity (Lewis et al., 2020). So far, DTI has been used to study DM in two research projects (Lewis et al., 2021; Johnson et al., 2021). It follows some technical explanation on DTI workflow and basic concepts along with an analysis of these two works.

DTI workflow is complex and implicates multiple skills such as detecting imaging artifacts, the application of a specific protocol and the understanding of neuroanatomical complexity. In fact, as showed by the following picture, once understood the application fields of DTI, researchers must choose an appropriate acquisition protocol. Data requires quality control, pre-processing, format conversion, distortions and motion correction and skull stripping. Then, tensors are

estimated (Soares et al., 2013). A tensor is a voxel that contains scalar values constituting a vector, those values depend on water molecules diffusion and orientation within a voxel and, in the final analysis, within tissues (Ranzenberger et al., 2021). The resulting data can be visualized through scalar indices (e.g. fractional anisotropy and mean diffusivity) or through tractography. Quantitative analysis methods that can be performed on data include ROI (region of interest) based analysis, voxel-based analysis, histogram analysis and tractography/fibre tracking. Each method is useful for a specific purpose, for example Region of Interest (ROI) analysis allows to focus on a region or subset of voxels. The results can be incorporated into functional MRI or structural MRI. Eventually, results interpretation can be carefully made (Soares et al., 2013).

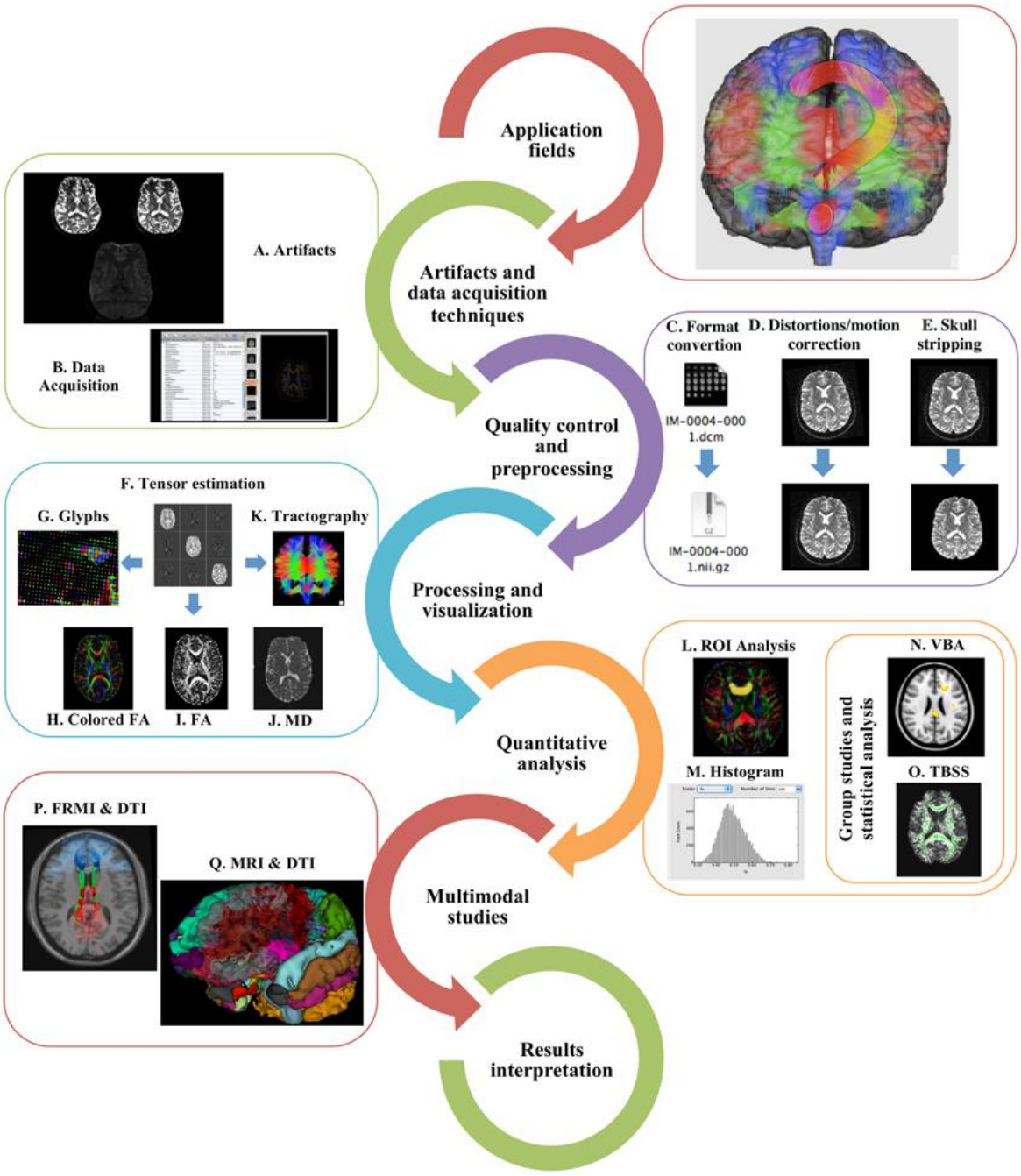

Figure 6 : DTI workflow (Soares et al., 2013)

DTI is based on the measurement and quantification of water diffusion within different tissues. Water diffusion varies depending on the cytoarchitecture of a tissue, its integrity and presence of barriers. In white matter, since axons are organized in parallel bundles, the diffusion of water molecules mainly occurs along one direction. This preferentially oriented diffusion is known as anisotropic diffusion. In the grey matter, diffusion is less anisotropic because of its cytoarchitecture, whereas in the CSF fluid water molecules move randomly and equally in all directions, and water diffusion is therefore described as isotropic

(Soares et al., 2013). A voxel is the three-dimensional volume of space representing the smallest unit that composes an MRI image. The parameter that expresses the degree of diffusion anisotropy in a voxel is called fractional anisotropy (FA). It ranges from 0 to 1, and tissues with high anisotropy, such as spinal cord white matter, have a value close to 1. FA decreases when white matter is lesioned, thus it is considered a highly sensitive but nonspecific biomarker of neuropathology. Another commonly used parameter for spinal cord DTI is mean diffusivity (MD, also called the apparent diffusion coefficient or ADC) that represents the mathematical average diffusion of water molecules within a voxel. Spinal cord white matter has moderate MD values, that may increase or decrease depending on the histopathological progression of a lesion (Vedantam et al., 2014). For example, an increase in tissue water and inflammation can result in MD increases, whereas cell proliferation can cause MD decreases (Alexander et al., 2007).

Recently, a study has tested both these parameters on dogs with DM and has shown that affected dogs had significant decreases in FA within the regions of the spinal cord that had high expected lesion load. Decreases in FA were most significant in dogs with severe forms of DM and correlated with neurological grade. Findings suggest that DTI is useful as a non-invasive marker of microstructural change within the spinal cord, and, particularly, FA has the potential to be a biomarker for lesion development in DM and could play a role in diagnosis and monitoring of the disease. By contrast, MD did not show alterations corresponding to expected lesion load and only showed statistically significant alterations in the caudal lumbar region (Johnson et al., 2021).

Another study on the same topic evaluated fractional anisotropy, mean diffusivity, axial diffusivity, and radial diffusivity on specific regions of interest. In this study, DTI indices didn’t differentiate DM-affected from control dogs, detect longitudinal changes or differentiate disease severity. Hence, these results didn’t support brain DTI as an imaging biomarker for the diagnosis and monitoring of DM (Lewis et al., 2021).

Both studies were conducted on small number of dogs, hence further investigation is needed to assess the role that DTI could have in DM research and clinical practice (Lewis et al., 2021; Johnson et al., 2021).

3.3.2 Electrodiagnostic testing

Electrophysiological tests allow to study the electrical activity of muscles, neural tissue, and neuromuscular junction as a function of time. Electrical activity that these studies records, can be spontaneous as in electroencephalography (EEG) and electromyography (EMG), or it can be the consequence of stimulation as in nerve conduction velocity (NCV) measurements and evoked potential studies. These studies are minimally invasive although they require anaesthesia. The main obstacles to their diffusion in clinical practice are the cost of the equipment and the experience needed for conducing and interpreting them correctly (Poncelet and Poma, 2014; Platt and Olby, 2014).

Electrodiagnostic testing has been conducted on DM patients. Particularly, EMG and study of nerve conduction velocities show variations of electrical activity depending on the stage of the disease (Awano et al., 2009).

Generally, EMG is used in clinical practice because it can identify denervated muscles and differentiate myopathies. Technically, EMG is conducted using a concentric electrode that measures the electrical activity in a portion of muscle small as its tip. A normal relaxed muscle is electrically silent. An exception is the end plate noise that is recorded when the electrode is close to an endplate region because miniature endplate potentials and endplate spikes occur (Poncelet and Poma, 2014; Platt and Olby, 2014).

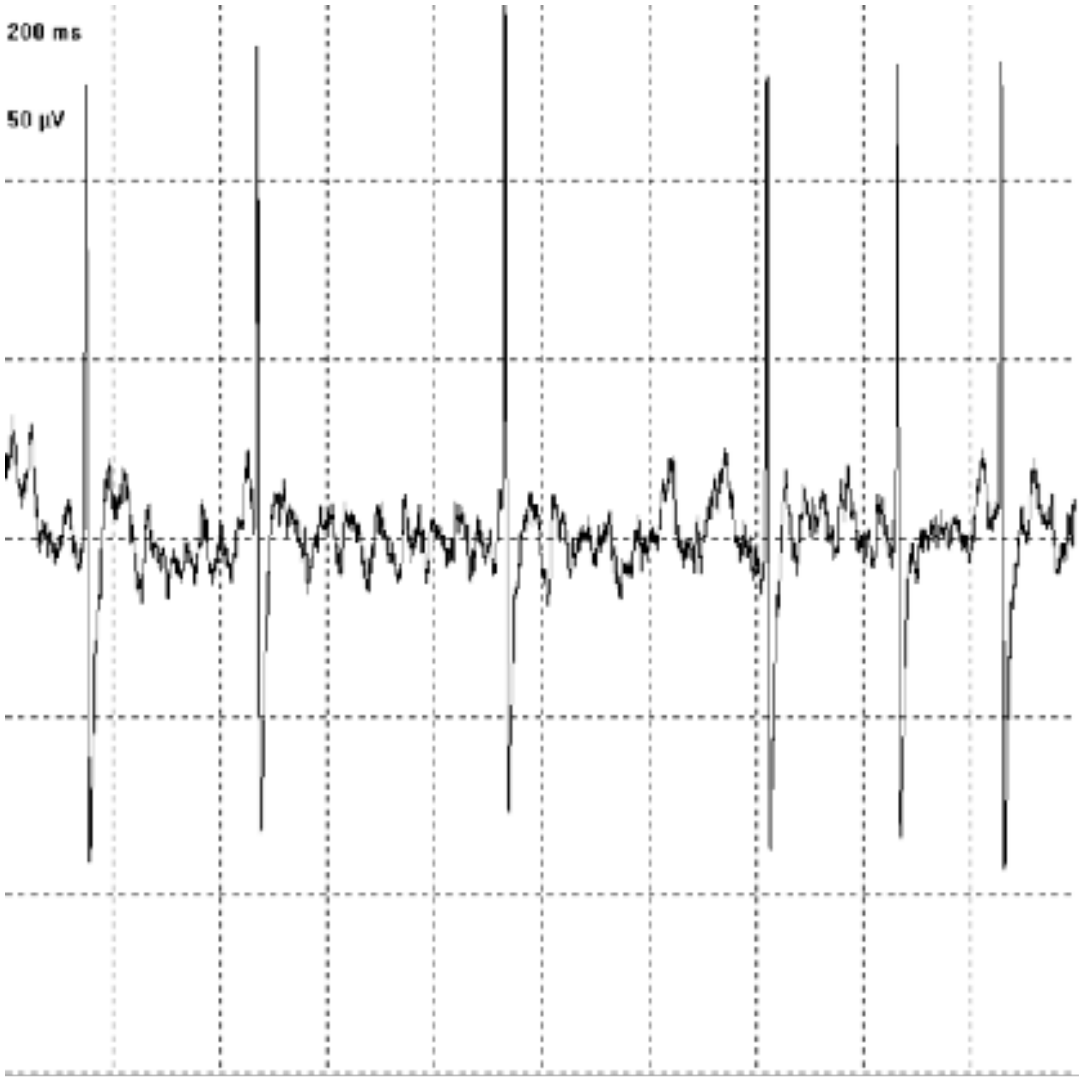

Figure 7: Miniature end-plate potentials and end-plate spikes are recorded in the endplate region.

Another normal finding is insertion potentials, which follow concentric needle electrode movements. In fact, mechanical triggering of muscle fibre potentials results in random neurotransmitter release at the level of the neuromuscular junction and mechanical stimulation of the muscle fibres. Regarding pathological findings, denervated muscle fibres show spontaneous depolarization, that is mostly recorded in the form of fibrillation potentials and positive sharp waves (Poncelet and Poma, 2014; Platt and Olby, 2014).

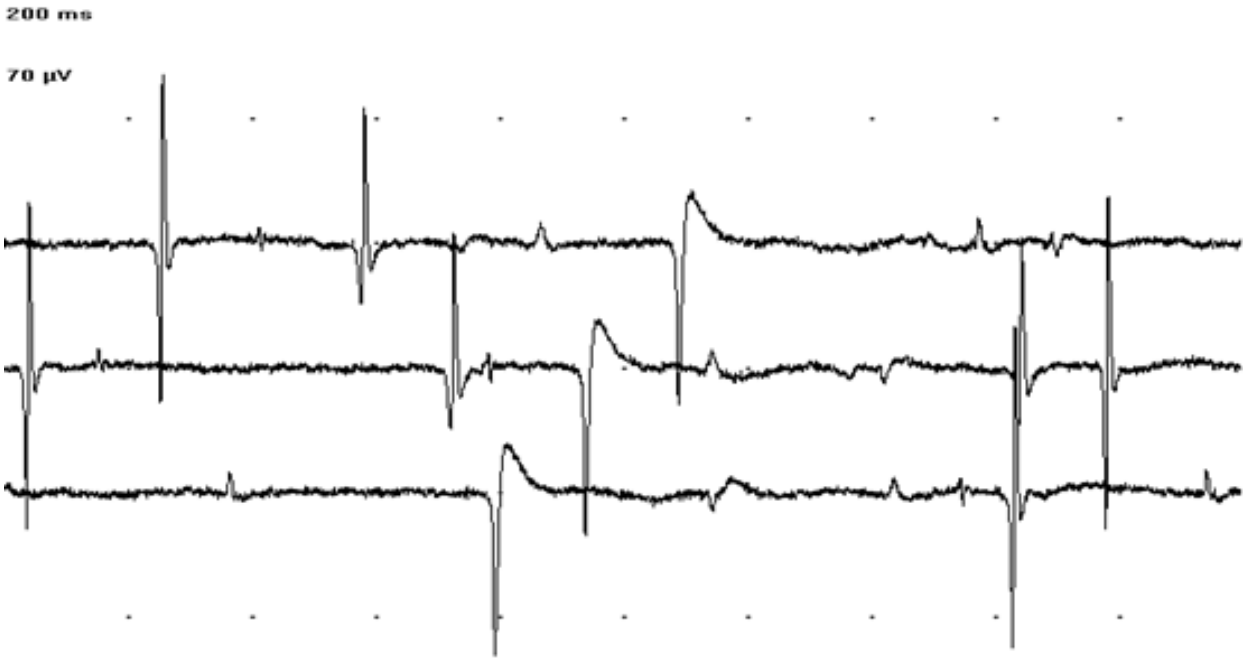

Figure 8: in a denervated muscle, the clinician can record spontaneous electrical activity, such as fibrillation potentials) and positive sharp waves.

The study of maximum motor nerve conduction velocity (NCV) is used to investigate neuropathies. To obtain the motor NCV in one of the targeted muscles, a clinician must stimulate a motor nerve at a minimum of two sites and record the evoked electrical activity, that is the compound action potential, commonly referred to as CMAP. Sites of placement of the electrodes are shown in the following picture (Poncelet and Poma, 2014; Platt and Olby, 2014).

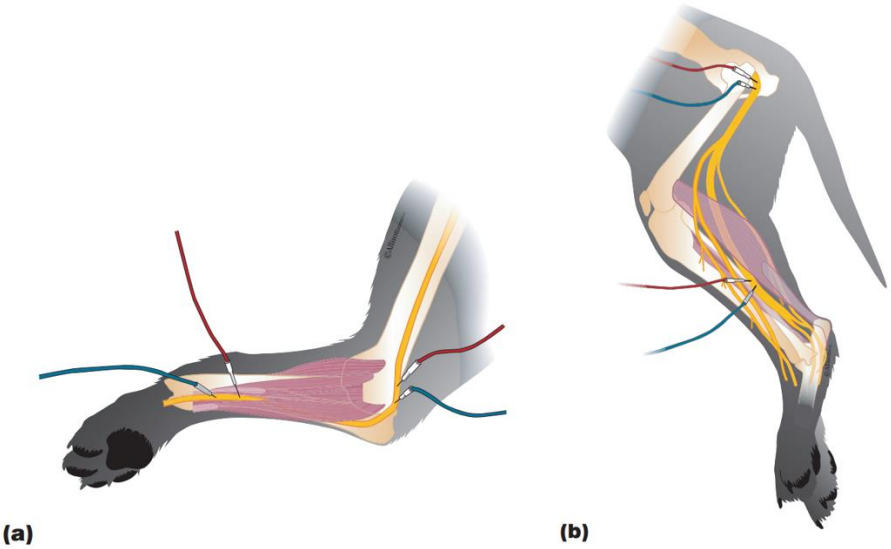

Figure 9: two sites of placement for the stimulating electrodes for maximum motor NCV measurement at proximal and distal locations along (a) the ulnar nerve and (b) the sciatic–tibial nerve (Poncelet and Poma, 2014; Platt and Olby, 2014).

Regarding the applications of electrodiagnostic on the study of DM, there is evidence that in the early stage of DM, EMG doesn't detect any spontaneous activity, and nerve conduction velocities are within normal ranges (Awano et al., 2009).

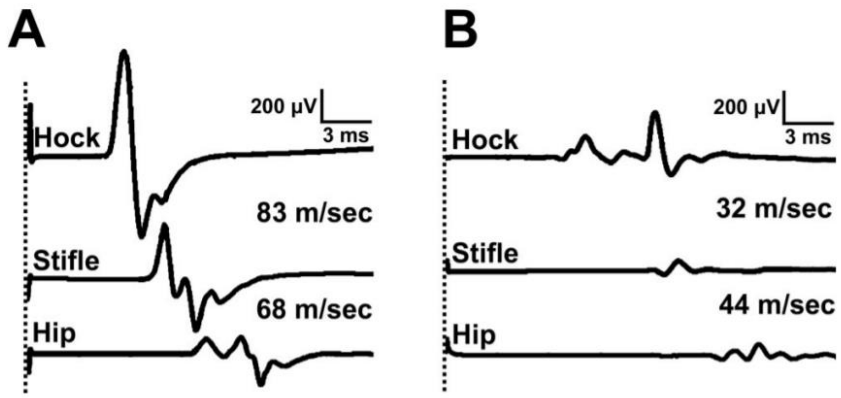

Figure 10: ischiatic/tibial M wave (CMAP) recordings obtained after stimulation at the hock, stifle, and hip. The patient was a 10-year-old boxer affected by DM. The picture compares early and late findings. (a) in early stage, the motor nerve conduction velocities between proximal and distal stimulation sites were within or above the normal mean values for the tibial nerve (66.9 2.4 m/s), though the m wave amplitudes (6.0, 3.1 and 0.8 mv) were below the normal range of 22.2 +/- 2.6 mv. (b) in the late stage, m wave recordings detected further decreases in amplitude (1.2, 0.6, and 0.4 mv) with marked temporal dispersion. Also, the proximal and distal motor nerve conduction velocities were decreased when compared with the normal reference range. These findings provide evidence of motor axonopathy and demyelination in the late stage of DM (Awano et al., 2009).

In the late stage of DM, EMG shows multifocal spontaneous activity in the distal appendicular musculature. The most common waveforms recorded are fibrillation potentials and sharp waves. Recordings of M waves (compound muscle action potentials, CMAP) from stimulation of the tibial and ulnar nerves reveals temporal dispersion and decreases in amplitudes. The proximal and distal motor nerve conduction velocities are decreased comparing to the normal reference range. Hence, electrodiagnostic testing can show motor axonopathy and demyelination in the late stage of DM (Awano et al., 2009).

3.3.3 DNA testing

Since the earlier studies, a genetic component has been highly suspected to be relevant in DM aetiology basing on the epidemiology of the disorder (Braund and Vandevelde, 1978). A recent study confirmed this hypothesis using a genome wide mapping association that detected a point mutation in exon two of the canine Sod1 gene, predicting G to A nucleotide transition at 118th nucleotide.

Homozygosity for SOD1:c.118G > A allele has been proved to be a risk factor for the development of DM in dogs (Awano et al., 2009).

Research on SOD1 mutation has led to a commercially available test able to detect homozygosity or heterozygosity for SOD1:c.118G > A. If associated with clinical investigations on suspected DM cases, this DNA test is useful because it can support a presumptive diagnosis (Awano et al. 2009). Although the SOD1 mutation is strongly associated with DM, there are a part of the asymptomatic population that is homozygous or heterozygous for the mutation without showing any symptoms throughout all their lives (Awano et al. 2009).

The DNA test can be used by dog breeders as a tool to avoid the production of homozygous puppies. In fact, dogs homozygous with the mutation will contribute one chromosome with the mutant allele to all their offspring, while the heterozygous parent could pass the mutant allele to half of its offspring. Therefore, the SOD1 DNA test can be implemented in mating strategies to reduce the prevalence of DM, especially in breeds with high SOD1:c.118A allele frequencies. Nevertheless, overly aggressive breeding programs could create a “bottleneck” effect leading to the loss of desirable traits and possibly selecting for other diseases (Coates and Wininger, 2010).

DNA test for DM can detect the presence or absence of disease-linked alleles but it cannot rule out a diagnosis because other mutations either in the same gene or in another gene could provoke similar phenotype. In addition, if the clinician suspects DM in a Bernese Mountain Dog, also SOD1:c.52T allele needs to be considered (Zeng et al., 2014).

3.3.4 Biomarkers

Since a proportion of dogs homozygous for the SOD1 mutation do not develop DM, the identification of the SOD1 mutation in patients can’t be considered specifically diagnostic for the disease. Hence, there is a clinical need for the development of specific biomarkers for DM to support diagnosis.

In recent times, several studies were conducted to analyse possible biomarkers for the diagnosis and the monitoring of DM. A good biomarker should be both sensible and specific to the disease and should differentiate between DM and its mimics in a quick and simple way.

Biomarkers that have been investigated include myelin basic protein (MBP) (Oji, et al., 2007), 8-isoprostane (Coates, et al., 2007), chaperone protein clusterin (Shafie et al., 2014) phosphorylated neurofilament heavy (pNF-H) (Toedebusch et al., 2017), plasma microRNA miR-26b (Nakata et al., 2019).

Myelin basic protein (MBP)

In 2007 Oji and colleagues evaluated MBP concentration in the CSF of 8 DM-affected german shepherd dogs and compared them to 8 not affected mixed breed dogs. A commercially available ELISA made for detection of human MBP was used. MBP is a protein only found in the nervous system that composes 30% of the total protein in the myelin sheath and it is encoded by a single gene expressed by oligodendrocytes. The presence of MBP in CSF has been previously demonstrated in humans with demyelinating disease and demyelinating lesions in the CNS. In humans, increased concentration of MBP in CSF is not disease specific but is a marker of demyelination (Oji, et al., 2007).

In dogs with DM mean MBP concentration in CSF from the lumbar cistern was significantly higher compared to control dogs, that suggests the presence of a demyelinating lesion. Mean MBP concentration in CSF samples from the cisterna magna of dogs with DM was slightly but not significantly higher than that in the cisterna magna of normal dogs. Dogs with other types of neurologic diseases were not tested. The study shows that MBP can be a marker for demyelinating lesions but there is no data on MBP in DM mimics. Hence, as it happens in humans, MBP doesn't seems to have the basis to be specific for DM (Oji, et al., 2007).

8-Isoprostane

8-iso-prostaglandin F2α (8-isoprostane) is a vasoactive prostanoid that can be measured in CSF. It is considered a reliable and stable biomarker of free radicals of oxygen generation in vivo. Additionally, it was suspected that, since it is a vasoconstrictor, it could have been involved in vascular changes during progression of DM. DM-affected dogs and control group had the same concentration of 8-isoprostane. Authors claim that the concentration could be underestimated because the samples were collected from cerebellomedullary cistern and suggest collection from both cerebellomedullary cistern and

lumbosacral cistern for next studies on DM biomarkers (Coates, et al., 2007). Hence, 8-isoprostane is not to be considered a biomarker of DM.

Chaperone protein clusterin

Chaperone protein clusterin (apolipoprotein J) is a protein that is protective against endoplasmic reticulum stress-mediated apoptosis and oxidative stress. It also serves as an extracellular chaperone influencing protein aggregation. A comparison of clusterin CSF levels in several neurological conditions showed that clusterin was elevated in both DM and chronic IVDD. These findings indicate that clusterin may potentially serve as a marker for chronic spinal cord disease in dog, but it does not differentiate DM from chronic IVDD (Shafie et al., 2014).

Phosphorylated neurofilament heavy (pNF-H)

Phosphorylated neurofilament heavy (pNF-H) is an abundant structural protein of myelinated motor axons that has been proposed as a biomarker of both canine and human nervous system damage (Toedebusch et al., 2017). Neurofilaments are only found in neurons, therefore their detection unambiguously indicates neuronal damage. They consist of three subunits called NF-L, NF-M, and NF-H. NF-H contains unusual tandemly repeated peptides centred on the sequence lysine-serine-proline (KSP). In axonal neurofilaments almost all the serine residues are phosphorylated (Shaw et al., 2005). Phosphorylated KSP repeats within the carboxy terminus of pNF-H are immunogenic and increase the stability of the NF-H protein (Toedebusch et al., 2017). Therefore, pNF-H is more resistant to proteases than either NF-L or NF-M. When axonal injury occurs, pNF-H is released into serum and CSF in significant amounts and, because its resistance to degradation, it can be readily detected with appropriate ELISA or other assays (Shaw et al., 2005).

In veterinary medicine, pNF-H has been investigated as a biomarker in dogs with acute SCI, in paraplegic dogs with intervertebral disc herniation (Nishida et al., 2014; Murthy et al., 2021) and in DM-affected dogs (Toedebusch et al., 2017). Regarding IVDH, the disease is classified in five grades considering its severity. pNF-H concentration in serum was significantly higher in grade 5 than grade 4 dogs. All the dogs in the study that had high pNF-H levels 1–3 days after injury did not regain the ability to walk after surgery. Furthermore, dogs with clinical signs of

progressive myelomalacia after surgery, also had high levels of pNF-H between 1 and 3 days after injury. Hence, serum pNF-H is a promising biomarker for SCI and myelomalacia, although further investigation was recommended by authors (Nishida et al., 2014).

In 2021, another study evaluated the time course of serum pNF-H concentration in dogs affected by IVDH. Data shows that dogs that developed progressive myelomalacia had significantly higher serum pNF-H concentrations after surgery compared to all other cohorts at 24 hours. A time of peak after SCI was not detected, but generally progressively increased serum pNF-H concentrations are observed in the first 14 days after SCI. However, dogs that do not recover had a greater increase in serum pNF-H concentration between postoperative days 3 and 14. Authors suggested that a similar study should be performed on a larger cohort of dogs (Murthy et al., 2021).

Olby and colleagues, in 2019 published a study on possible serum biomarkers to predict recovery from SCI secondary to thoracolumbar intervertebral disc extrusion (IVDE). Serum concentrations of glial fibrillary acidic protein (GFAP), phosphorylated neurofilament heavy chain (pNFH) and S100β were evaluated using an ELISA test. GFAP and S100β are markers of astrocytic injury whereas pNFH is a marker of axonal injury. GFAP and S100β concentrations rose for the first 1 to 3 days and then they were undetectable by 14 and 28 days, respectively. This suggests that there was early release by cells around the time of injury, but this phase ended within a few days as astrocytes either recovered cellular membrane integrity or died and were removed. pNFH concentrations peaked at 14 days and it were detectable at 56 days, likely reflecting ongoing Wallerian degeneration in white matter. Data showed that the presence of serum GFAP in the first 3 days after onset of paralysis can predict motor recovery in patients with paralysis due to acute IVDE with good accuracy. The introduction in clinical practice of a bedside test that measures serum concentrations of both GFAP and possibly pNFH would be useful. Authors claim that the study should be replicated in a larger cohort, in fact one limit of this work was the small sample size (Olby et al., 2019).

In human medicine, increased pNF-H concentrations in both blood and CSF have shown high diagnostic value and are associated with disease progression in

patients diagnosed with ALS. In 2017, Toedebusch and colleagues investigated CSF and serum pNF-H concentrations in DM-affected dogs using a commercially available ELISA. The study involved 53 DM-affected, 27 neurologically normal (including young and old dogs), 7 asymptomatic at-risk (based on the presence of genetic mutation), and 12 DM-mimic dogs. The pNF-H level in CSF was not significantly different between young and old control dogs. The variance in concentrations of pNF-H in CSF was higher among the old than the young dogs. Comparing DM-affected dogs to old control dogs, median pNF-H concentration in CSF was significantly increased in all stages of DM. No difference was found in median pNF-H concentration in CSF between asymptomatic, at-risk dogs and DM mimics, whereas median CSF pNF-H concentration was increased in the early stage of DM. In addition, serum pNF-H concentration was measured, but no difference was found between old control dogs and DM-affected at any stage. pNF-H cut-off concentration above 20.25 ng/mL in CSF yielded the optimal discrimination between DM stage 1, asymptomatic at-risk and DM-mimics dogs. No significant differences in median CSF pNF-H concentration were observed between DM stages. The lack of significant correlation between DM disease stage and CSF pNF-H concentration was expected since also human ALS shows a wide variability in CSF pNF-H concentrations, which are weakly associated with the ALS function rating scale. This aspect can be explained by disease-related alterations in the regulation of NF-H. In fact, as happens in ALS patients, DM-affected dogs have early and progressive ectopic phosphorylation of NF-H within neuronal perikarya, without a significant decline in total pNF-H protein in the lumbar tract of the spinal cord. Although the underlying mechanisms are not fully understood, disease-specific alterations in NF synthesis, turnover, and axonal transport have been found to be involved in NF accumulation and likely to influence detectable CSF levels throughout disease (Toedebusch et al., 2017).

In conclusion, phosphorylated neurofilament heavy (pNF-H) is a promising biomarker for diagnosis of DM, though further studies are needed to examine pNF-H on larger cohort of DM mimics, especially central and peripheral axonopathies. Longitudinal studies that examine individual dogs throughout the course of the disease should be performed. Moreover, future studies should investigate pNF-H serum concentration on a larger sample population because the

fact that no difference was found between DM and control dogs was unexpected. In fact, pNF-H is readily detectable in the serum of dogs with acute, severe spinal injury and ALS patients. Authors suggest that the lack of detectable difference could be caused by the variability of serum pNF-H within all groups. Furthermore, unidentified concurrent axonopathy in a subset of these control dogs cannot be excluded, in fact no electrodiagnostic tests were performed to exclude this diagnosis and axons are highly sensitive to metabolic and mechanical stress (Toedebusch et al., 2017).

Plasma microRNA miR-26b

In 2019, researchers investigated plasma microRNA (miRNA) profiles of DM-affected Pembroke Welsh Corgis to identify novel biomarkers. MicroRNAs are small (18–25 nucleotides) non-coding RNAs that have regulatory functions by targeting messenger RNAs (mRNAs) for cleavage or translational repression. They have a regulatory role in various cellular processes such as cellular growth, differentiation, cellular proliferation, and apoptosis. Hence, miRNAs are key regulators of various biological functions in the nervous system, such as neuronal differentiation, synaptic plasticity, and neuroinflammation. Because the expression of some miRNAs is specific to tissues or biological stages, changes in specific miRNA concentrations in the CNS are involved in the progression of neurodegenerative disorders. Some miRNAs are detectable in biological fluids like blood, urine, and CSF and are in a relatively stable form because they are encapsulated in microvesicles. MicroRNAs have potential as future diagnostic biomarkers of neurodegenerative diseases in both human and veterinary medicine. (Nakata et al., 2019) In human medicine there are studies that evaluate specific miRNAs as markers of ALS and their role in ALS pathogenesis (Rinchetti et al., 2017).

Plasma levels of 277 miRNAs were quantified using an RT-qPCR array that identified 11 up-regulated miRNAs and 7 down-regulated miRNAs in DM-affected dogs. Authors identified 3 miRNAs (miR-26b, miR-181a, and miR-196a) that are likely to regulate several genes associated with SOD1. Among those miRNAs, miR-26b had the best diagnostic accuracy for distinguishing DM dogs from not affected-dogs. The plasma level of miR-26b was significantly higher in the DM group than in the healthy control group. A positive correlation was observed

between increases in the plasma level of miR-26b and disease progression. Hence, findings suggest that plasma miR-26b is a potential diagnostic biomarker of DM (Nakata et al., 2019).

4 HISTOPATHOLOGY

DM is correlated to various histopathological lesions in different anatomical structures including the spinal cord, nerves, and muscles. To date, histopathological examination of the spinal cord is required to make a definitive post-mortem diagnosis of DM, since there aren't biomarkers, diagnostic tool or pathognomonic signs which allow us to make a definitive ante-mortem diagnosis in everyday medical practice. However, histopathology is not only useful in clinical practice to confirm the suspect of DM, but it is also a fundamental tool to study underlying mechanisms of the disorder. The purpose of the following paragraphs is to delineate the most relevant histopathological findings and to deepen some aspects such as the role of glial cells in DM which is gaining increasing interest among researchers.

4.1 Spinal cord pathology

The histopathological hallmark of DM is progressive axonal loss and demyelination of the spinal cord, that predominantly occurs in the caudal thoracic area. These lesions are detectable in all funiculi and involve the somatic sensory, general proprioceptive sensory, and motor tracts. However, the most severely affected anatomical structure is the dorsolateral part of the lateral funiculus (March et al., 2009). The pathology of the gray matter remains largely unknown. Nevertheless, neuronal cell body degeneration and loss were observed in the spinal cord of DM cases (Ogawa et al., 2014; Nakamae et al., 2021).

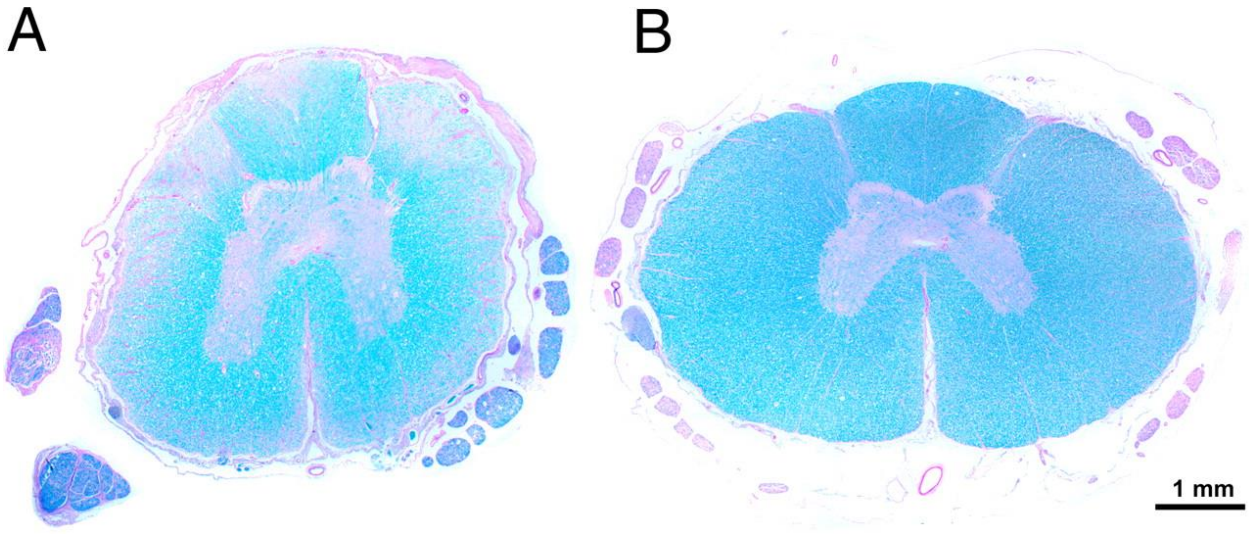

Figure 11: Spinal cord histopathology. (A) Luxol fast blue-periodic acid Schiff staining of a thoracic spinal cord cross-section from a DM-affected 13-year-old Pembroke Welsh Corgi. The white matter

degeneration is depicted by regions of pallor where there has been loss of nerve fibers. (B) A similarly stained spinal cord cross section from an unaffected 13-year-old Labrador Retriever. Note there is no evidence of nerve fiber loss. The bar in the lower right of the photomicrograph indicates the magnification (Awano et al., 2009).

Immunostaining using an antibody against SOD1 protein shows the presence of SOD1 cytoplasmic inclusion bodies, characterised as well-defined dark clumps (Awano et al. 2009). This finding is similar to those found in ALS patients and transgenic models expressing mutant human SOD1 (Awano et al. 2009).

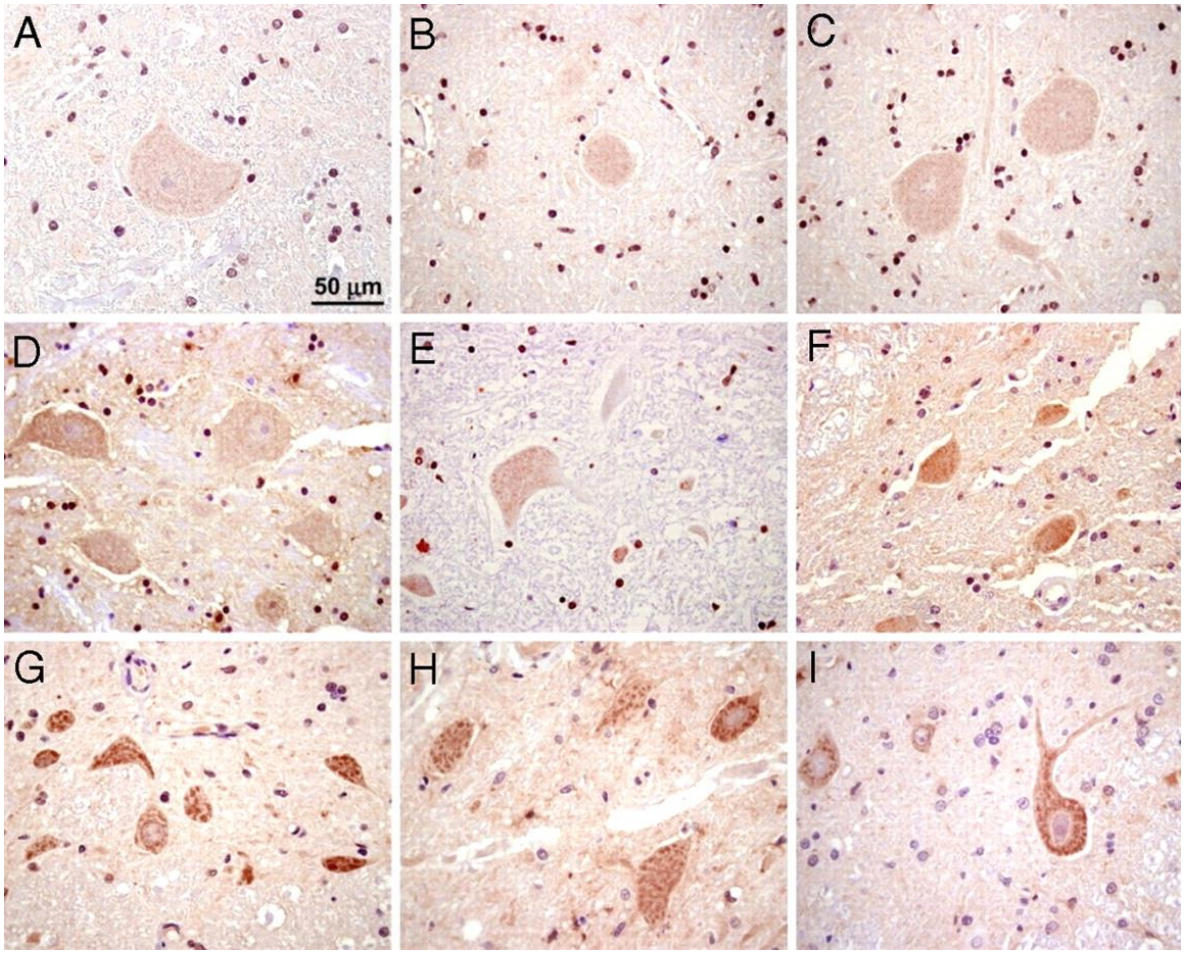

Figure 12: immunohistochemical staining with anti-SOD1 antibody in representative sections from the spinal cords from 3 G/G homozygous asymptomatic control dogs (A–C), 3 A/G heterozygous asymptomatic control dogs (D–F), and 3 A/A homozygous dogs with a confirmed diagnosis of DM (G–I). The samples were from a 13-year-old Rhodesian Ridgeback (A), an 8-year-old Labrador Retriever (B), a 13-year-old Labrador Retriever (C), an 8-year-old Australian Shepherd (D), a 13-year-old Tibetan Terrier (E), an 8-year-old German Shepherd Dog (F), an 8-year-old Rhodesian Ridgeback (G), a 13-year-old Pembroke Welsh Corgi (H), and a 10-year-old Boxer (I). The bar in A indicates the magnification for all spinal cord cross-sections (Awano et al., 2009).

Over the last years, researchers have focused on the key role that glial cells play in the pathogenesis of neurodegenerative diseases such as ALS. Specifically, proinflammatory cytokines and chemokines are overproduced by increased numbers of activated glial cells such as astrocytes and microglia and released in the parenchyma provoking neuroinflammation of the spinal cord (Hashimoto et al., 2021). Activated microglia are commonly observed in the spinal cords of ALS patients, and they express surface molecules such as major histocompatibility complex class II (MHC II) molecules. Activated microglia produce proinflammatory cytokines including interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and chemokines such as C-single-bond-C-motif-chemokine-ligand 2 (CCL2) causing infiltration of peripheral leucocytes into spinal cords, which leads to neuron injury (Hashimoto et al., 2021). Therefore, it is believed that attenuation of neuroinflammation is a potential therapeutic strategy for ALS. In DM, different studies have reported increased glial cells and inflammatory molecules (Lovett et al., 2014; Hashimoto et al., 2021). Specifically, Lovett and colleagues through immunohistochemistry found that expression of hsp70 was significantly increased in ependymal cells lining the spinal cord central canal of DM-affected dogs. This was associated with enhanced CD18 positivity in the gray matter of DM-affected dogs. CD18+ cells had mostly a macrophage/microglial morphology. Hence, their findings indicate a possible pro-inflammatory state (Lovett et al., 2014). Hashimoto and colleagues investigated DM spinal cords through immunohistochemistry and quantitative real-time RT-PCR. Their results suggest a proinflammatory state of the microenvironment in the DM spinal cord in which activated microglia and astrocytes play important roles by secreting a set of cytokines, chemokines, and expressing adhesion molecules. Specifically, they found evidence of a significantly enhanced transcriptions of IL-1β, TNF-α, CCL2 and vascular cell adhesion molecule−1 mRNA in the spinal cords of DM dogs. Moreover, immunohistochemistry for the class II major histocompatibility complex molecules HLA-DR and CCL2 indicated that the immunopositive areas of activated macrophages/microglia and CCL2 protein were significantly increased in DM, and CCL2 protein was mainly overproduced by astrocytes (Hashimoto et al., 2021).

4.2 Brain pathology

In literature, there are only a few studies that have examined the brain pathology of DM-affected dogs. Johnston and colleagues described abnormalities in the red nucleus (origin of the rubrospinal tract) and lateral vestibular nucleus of the brainstem, and in the lateral (dentate) and fastigial nucleus of the cerebellum. Specifically, their findings included chromatolysis, occasional neuronophagia and gliosis (Johnston et al. 2000). Nevertheless, other researchers who examined brains from DM-affected dogs by light microscopy did not find lesions in the brain (Averill, 1973; Braun and Vandevelde; Coates and Wininger, 2010). In 2009 March and colleagues described brain sections as within normal limits, except for areas of mild astrogliosis in gray matter of the caudal medulla (March et al., 2009).

4.3 Peripheral nerve and muscle pathology

Intercostal and pelvic limb muscles from late stage DM-affected dogs show pathological changes consistent with denervation atrophy (Shelton et al., 2012; Morgan et al., 2013). Specifically, myofibers from dogs with advanced DM exhibit variability in size and shape with hypertrophy and atrophy fibers. This is typical of denervation. Additionally, in the end stage of the disease, fibrosis occurs (Awano et al., 2009).

Peripheral nerve specimens from DM affected dogs show nerve fiber loss as indicated by axonal degeneration, endoneurial fibrosis, numerous inappropriately thinly myelinated fibers, and secondary demyelination (Awano et al., 2009). Additionally, thoracic motoneurons that innervate the intercostal muscles were found to contain cytoplasmic aggregates which stain with an anti-SOD1 antibody (Awano et al., 2009; Morgan et al., 2013).

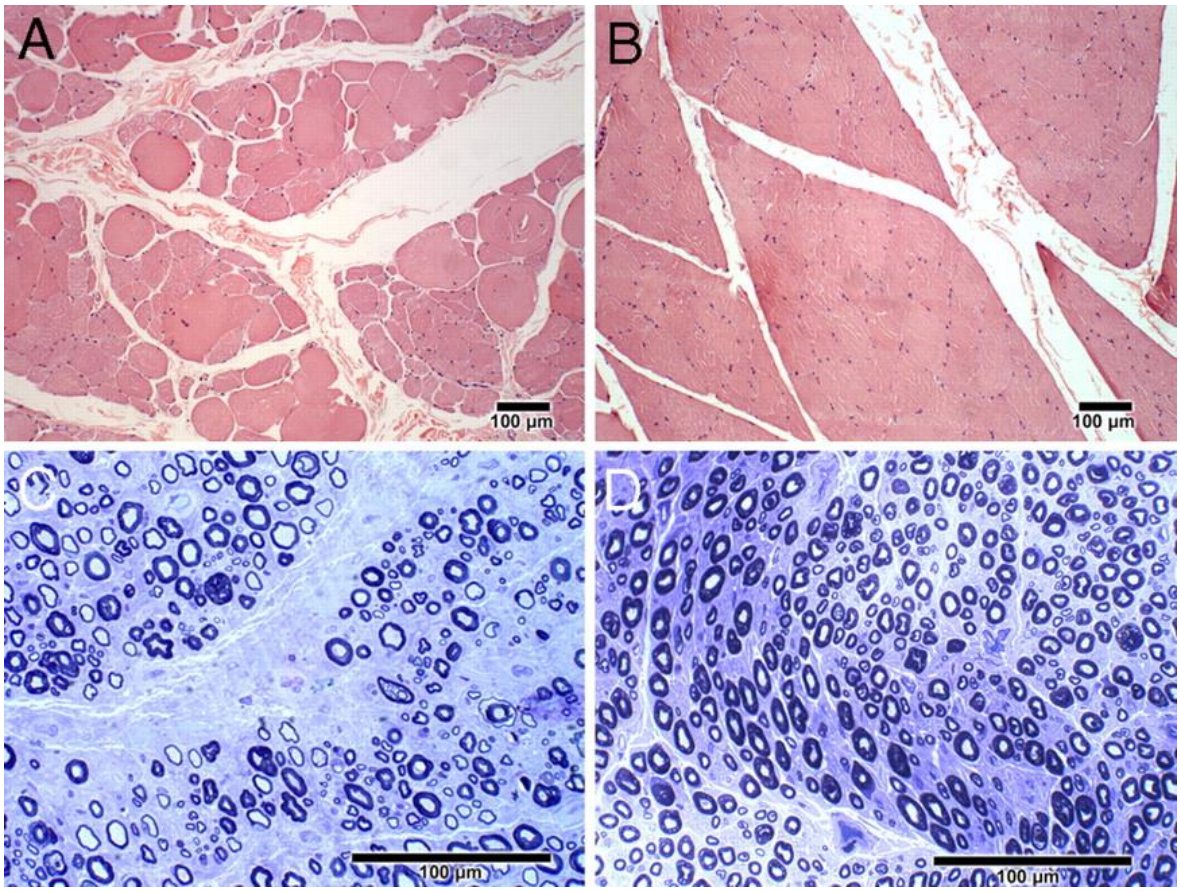

Figure 13: skeletal muscle and peripheral nerve histopathology in advanced DM. (A) H&E stained paraffin sections of the gastrocnemius muscle from a 13-year-old DM-affected Pembroke Welsh Corgi showed excessive variability in myofiber size with large and small groups of atrophic fibers consistent with denervation. (B) For comparison, a similarly stained gastrocnemius muscle from an age-matched control dog. (C) Toluidine blue stained resin embedded sections of the peroneal nerve from the same Pembroke Welsh Corgi showed substantial myelinated fiber loss, endoneurial fibrosis and secondary demyelination. (D) For comparison, a similarly stained peroneal nerve from an age-matched control dog. Bars in the lower right of all figures indicate the magnification (Awano et al., 2009).

5 INNOVATIVE THERAPEUTIC APPROACHES IN AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic lateral sclerosis (ALS) is a progressive adult-onset neurodegenerative disease that invariably leads to death. “Amyotrophy” refers to atrophy of muscle fibers that are denervated because of motor neuron degeneration. “Lateral sclerosis” refers to the degeneration of the posterior and lateral tracts of spinal cord. ALS was first described in 1869 by the French neurologist Jean-Martin Charcot, therefore it is also called Charcot’s disease. It became widely known to the public when it was diagnosed to one of baseball’s most beloved players, Lou Gehrig. Thus, it is also known as Lou Gehrig’s disease (Coates and Wininger, 2010).

5.1 Epidemiology, pathogenesis and classification of ALS

ALS is the most common adult motor neuron disease in humans. It mostly affects people in their fourth to sixth decade of life. The overall survival is 3 to 5 years for more than 50% of ALS patients, (Coates and Wininger, 2010) although survival is very variable, with some people living more than 50 years. The factors influencing the heterogeneity in survival might be related to environmental or genetic differences in susceptibility or could be stochastic (Shatunov and Al-Chalabi, 2021). Approximately 5% to 10% of ALS cases are familial (familial ALS, abbreviated in fALS), whilst the others are sporadic ALS (Coates and Wininger, 2010). It is worth noting that about 10% of people with sporadic ALS have mutations in genes that are known to cause familial ALS (van den Berg et al., 2021). Mutations in the SOD1 gene account for 20% of familial ALS cases. The mode of inheritance for fALS is mostly autosomal dominant, though autosomal recessive ALS has also been reported (Shatunov and Al-Chalabi, 2021).

ALS is a heterogeneous disease in its clinical presentation, onset of clinical signs, and survival (Coates and Wininger, 2010). It primarily affects upper and lower motor neurons, but also frontotemporal and other regions of the brain. The extent to which each neuronal population is affected varies between individuals (Al-Chalabi et al., 2016). This variability requires a classification system which takes into consideration multiple aspects to provide an indication of treatment and prognosis and to enable analysis in clinical trials of homogeneous groups for a more effective approach to therapy. El-Escorial criteria for ALS are currently the

standard method for diagnosing ALS, but among researchers the classification of ALS is a widely discussed topic (Al-Chalabi et al., 2016).

Generally, the hallmark signs of ALS include progressive UMN and LMN systems degeneration affecting initially a region and then spreading to other regions (Coates and Wininger, 2010). Some ALS patients exhibit cognitive abnormalities that may precede or occur after the onset of symptoms. At disease onset, an ALS patient usually presents with only upper or LMN signs that involve mainly muscles innervated by bulbar (brainstem) neurons or limb muscles. These different forms of ALS are known as UMN onset, LMN onset, or bulbar onset (Coates and Wininger, 2010). About two-thirds of patients with typical ALS have a spinal form of the disease related to focal muscle weakness that may start distally or proximally in the upper or lower limbs. Most of these patients go on to develop bulbar and respiratory symptoms. Canine DM is similar in its clinical spectrum to UMN-onset ALS (Coates and Wininger, 2010).

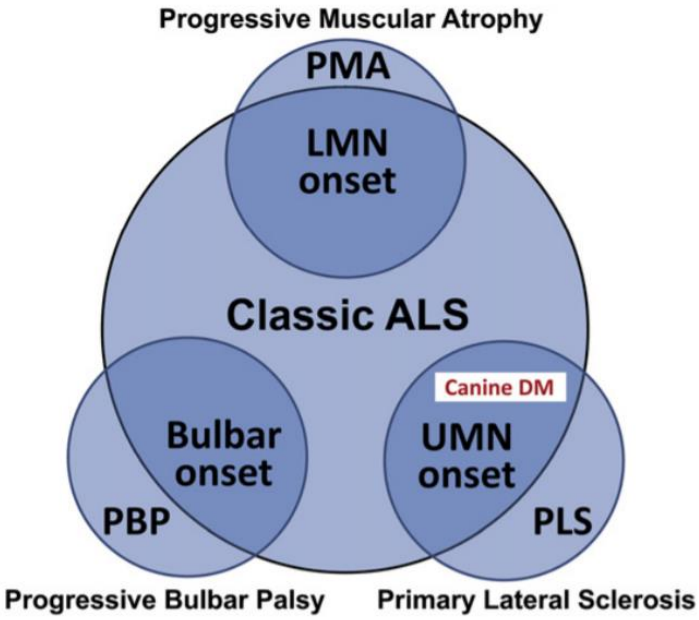

Figure 14: clinical spectrum of amyotrophic lateral sclerosis (ALS) in humans. Clinical signs of classic ALS manifest generalized UMN and LMN involvement. At onset, ALS may present only with UMN, LMN, or brainstem (bulbar) signs. Thus, these forms of ALS are termed UMN onset, LMN onset, or bulbar onset. The clinical spectrum of ALS can overlap with separate disease subtypes: progressive muscle atrophy (PMA), progressive bulbar palsy (PBP), and primary lateral sclerosis (PLS). Canine degenerative myelopathy has similarities in its clinical spectrum to UMN-onset ALS (Coates and Wininger, 2010).

The ALS pathogenetic mechanisms are still unclear. To date, multiple studies have demonstrated the involvement of several altered signaling pathways, such as mitochondrial dysfunction, glutamate excitotoxicity, oxidative stress and neuroinflammation (Bonafede and Mariotti, 2017).

Genetics has definitely a role in the pathogenesis of fALS but it may play a role also in sporadic ALS. To date, over 30 different genes have been discovered in familial ALS. In approximately 60-80% of fALS patients a gene mutation can be identified, of which C9orf72 (40%), SOD1 (20%), FUS 1-5% and TARBDP (1-5%) are the most common in Europe. (van den Berg et al., 2021) Mutations in the SOD1 gene were the first to be identified in ALS and, to date, more than 150 mutations have been found (Bonafede and Mariotti, 2017). The prevalence of mutations differs among different ethnic groups, for example the most common ALS gene in Asia is SOD1, followed by FUS, C9orf72, and TARDBP (Zou et al., 2017). Approximately 10% of sporadic ALS cases have mutations in known FALS-genes and first-degree relatives of sporadic patients are eight-fold higher risk for developing the disease. Rigid dichotomizing ALS into familial and sporadic disease is considered an oversimplification because there are multiple similarities in genetic architectures between familial and sporadic disease (van den Berg et al., 2021).

As in DM, there is no definitive diagnostic test or biomarkers to distinguish between ALS and its mimics. Hence, confirmation of diagnosis is based on clinical findings, electromyography results, and exclusion of mimics (Al-Chalabi et al., 2016).

Histopathological similarities between DM and ALS include myelinated axon loss and gliosis within the spinal cord, myelinated axon loss in peripheral nerves, and muscle atrophy. In addition, accumulations of cytoplasmic aggregates containing SOD1 within motor neurons occur in DM and some forms of ALS. Although ALS is generally considered primarily a disease of the motor system, growing evidence indicates sensory involvement in some cases. Sensory deficits are also characteristic features of DM (Morgan et al., 2014).

5.2 Pharmacological Therapy

Starting from 1994, when the first rodent model overexpressing SOD-1 was obtained, many studies have developed novel therapeutic agents for ALS using rodent models (Lutz, 2018). However, they have had poor therapeutic success when translated to human patients with ALS. It is believed that the poor translation of the therapies was due to these ALS transgenic animals overexpressing specific human gene mutations (Alexander et al., 2004). The identification of E40K Sod1 mutation in DM has established a genetic link between DM and ALS, therefore DM has become the first spontaneously occurring animal model of ALS (Awano et al. 2009). Compared with the SOD1 rodent models, dogs with DM are more similar to ALS patients in terms of size, structure, complexity of their nervous systems and duration of the disease. For these reasons, dogs with DM can be considered a good disease model for the evaluation of potential therapeutic interventions for ALS (Nardone et al., 2016; Kobatake et al., 2021).

Currently, no effective treatment to cure ALS or to significantly alleviate symptoms is available. Regarding pharmacological therapy, the first drug approved by US Food and Drug Administration (FDA) has been Riluzole (2-amino-6-trifluoromethoxy benzothiazole, also known as Rilutek). It acts as an inhibitor of glutamate release from the presynaptic terminals by blocking voltage-gated sodium channels, thus limiting glutamate excitotoxicity. Other neuroprotective pharmacological actions of Riluzole include the modulation of the NMDA ionotropic receptors, the inactivation of voltage-dependent sodium channels and the inhibition of the uptake of the inhibitory neurotransmitter γ–aminobutyric acid. The clinical use of Riluzole prolongs life of patients by 3 months, although the results obtained in clinical trials are often controversial (Bonafede and Mariotti, 2017).

Edaravone (Radicava) has been recently approved by FDA. It is a free radical scavenger that relieves the effects of oxidative stress. Evidence for efficacy is controversial and limited to short-term beneficial effects (Witzel et al., 2022).

The use of these pharmacological treatments has a minimal impact on the disease course because they are directed against one or a few pathological mechanisms involved in ALS. For better therapeutic approach, it could be helpful to counteract different pathogenetic mechanisms at the same time. Thus, since the transplantation of stem cells and gene therapy can act via multiple mechanisms, in

the last years an increasing interest has been addressed to these therapeutic approaches (Bonafede and Mariotti, 2017).

5.3 Gene Therapy

Since ALS can be caused by mutations in SOD1 and the complete absence of the SOD1 gene does not cause the disease in mice, researchers have tested the effect of the SOD1 gene silencing in laboratory animals. In particular, a lentivirus encoding for a RNA silencing (siRNA) that catalyzes the selective degradation of SOD1-mRNA was injected in the muscle or in the spinal cord of transgenic mouse models. The expression of SOD1 was reduced, and consequently the neurodegeneration appeared delayed. Nevertheless, data about disease progression and survival are controversial (Bonafede and Mariotti, 2017).

Other studies have investigated the effect of an antisense oligonucleotide directed to SOD1-mRNA. It decreased the concentration of SOD1 mRNA and related protein in the spinal cord, that lead to a longer survival in the SOD1(G93A) rat model. A clinical trial was performed in phase I and showed the safety and tolerability of intrathecal administration of antisense oligonucleotide. Unfortunately, this treatment requires a constant infusion of antisense oligonucleotide (Bonafede and Mariotti, 2017).

To conclude, gene therapy requires to be optimized for a successful approach to neurodegenerative diseases (Bonafede and Mariotti, 2017).

5.4 Stem Cell Therapy

Mesenchymal stem cells (MSCs) can differentiate into cells of mesodermal endodermal and ectodermal lineages, including neurons and glial cells. For this reason, MSCs have been investigated as a possible therapy for neurodegenerative diseases for which pharmacological current approaches can only partially slow down diseases, such as Alzheimer's Disease, Amyotrophic Lateral Sclerosis, Huntington's Disease, and Parkinson's Disease. Promising results obtained in vitro and in animal models have prompted several clinical studies that have assessed safety of the procedures and improvements in the progression of some disorders. Although some mechanisms of MSCs are still to clarify, some known effects of the administration of stem cells are neurogenesis and angiogenesis stimulation, antiapoptotic, immunomodulatory, and anti-

inflammatory actions. Most effects derive from their paracrine expression of neurotrophic factors and cytokines which are mainly delivered at damaged regions because of the propensity of MSCs to home to injured sites (Lo Furno et al., 2018).

In adult life, stem cells have been identified in several tissues. In fact, stem cells contribute throughout the entire life to the replacement of dead cells in many organs such as the blood, skin, or gastrointestinal tract. Regarding the central nervous system (CNS), for a long time it was believed that only microglia, astrocytes, and oligodendrocytes proliferate in the adult organism, whereas neurons were considered unable to divide. This is not entirely true because neural stem cells (NSCs) can continuously generate new functional neurons in at least two areas of the brain, the dentate gyrus of the hippocampus and the subventricular zone adjacent to the lateral ventricles (Lo Furno et al.; 2018).

Different lines of cells that have been explored to assess a possible therapeutic activity in neurodegenerative disorders are:

- Embryonic stem cells (ESC): they have pluripotency (can give rise to any cellin the body) and very high proliferative potential. However, they raise ethical/religious issues and involve the risk of easily producing tumors (Lo Furno et al., 2018).
- Fetal Stem Cells (FSC): multipotent cells that can give rise to any cell of a certain germ layer. They raise ethical/religious issues (Forostyak et al., 2017).
- Induced pluripotent stem cells (iPSCs): autologous somatic cells that are reprogrammed and could differentiate to neural cells. Their safe use has not yet been properly assessed (Lo Furno et al.; 2018).
- Mesenchymal Stem Cells: MSCs are somatic adult derived cells that can be either multi or oligopotent (Forostyak et al., 2017). They are available in hight amount. Moreover, they have low immunogenicity and can differentiate into multiple cell lineages. These include neurons and glial cells when cultured under specific conditions. Moreover, they don’t raise ethical or religious issues. MSCs can be extracted from several tissues, but the most studied MSCs are those from umbilical cord (UC-MSCs), bone marrow (BMSCs) and adipose tissue (ASCs) (Lo Furno et al.; 2018).

- Neural Stem Cells (NSCs): They have been found in dentate gyrus of the hippocampus and the subventricular zone adjacent to the lateral ventricles. NSCs can differentiate to neurons, astrocytes, and oligodendrocytes. Unfortunately, since they are extracted from the nervous tissue, autologous NSCs are hardly available in sufficient amounts without harm for the patient. In addition, data on NSC transplantation are controversial, since when injected in vivo, they may remain in the undifferentiated form. They are also susceptible to immune responses upon allogeneic transplantation (Lo Furno et al.; 2018).

Over the last years, MSCs administration as a possible therapeutical approach to ALS patients has been widely investigated and has become an intensely debated field of study. The purpose of the following paragraphs is to give a focus on the use of MSCs in ALS highlighting its possible therapeutical mechanisms and considering practical aspects such as type of cells, frequency, quantity and route of administration (Forostyak et al., 2017).

MSCs have a broad therapeutic potential that still hides many unknown features. It is thought that their action is due to multiple mechanisms, including paracrine activity and various actions on damaged cells. Particularly, MSCs secrete a cocktail of several growth factors (GFs), cytokines and exosomes. GFs that are known to be involved in ALS and were successfully tested *in vivo* to assess their role in neuroprotection or disease modification are: glia cell-line derived neurotrophic factor (GDNF), insulin growth factor type-1 (IGF-1), brain-derived neurotrophic factor (BDNF), neural growth factor (NGF), VEGF and others that seem to play less significant role in ALS pathology (Forostyak et al., 2017). Moreover, it has been reported that after an intrathecal or combined application of MSC, the level of apoptosis and inflammation decreases leading a better survival of motor neurons (Forostyak et al., 2017). The potential properties of MSCs are represented in the following figure.

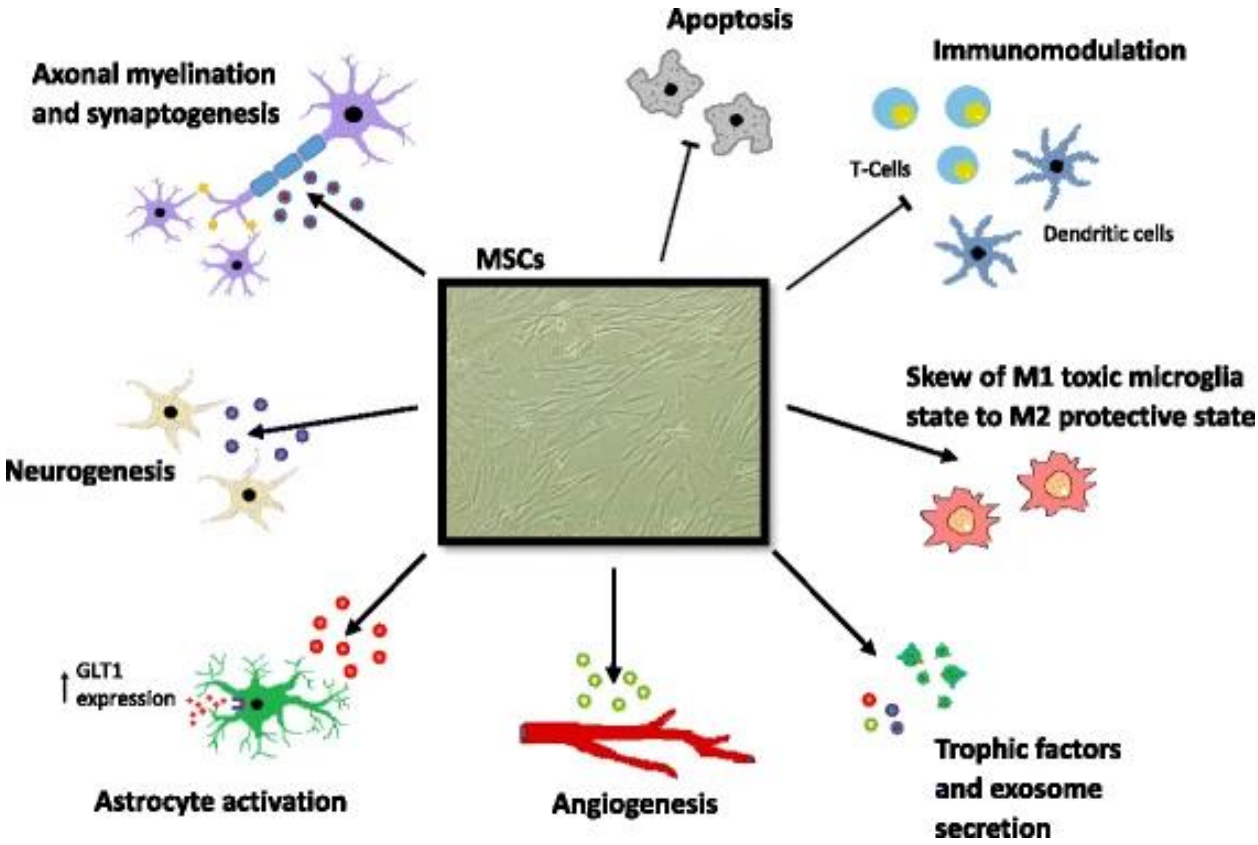

Figure 15: Possible mechanisms of MSCs efficacy in neurodegeneration. MSCs may provoke therapeutic responses because of paracrine effects and cell-to-cell contacts. The ability of MSCs to secrete cytokines, growth factors and exosomes could induce and support regeneration processes, such as angiogenesis, synaptogenesis, axonal re-myelination and neurogenesis. Because of immunomodulatory properties, MSCs could limit inflammatory responses in the CNS by inhibiting maturation and migration of dendritic cells, suppression of lymphocyte activation and proliferation, and by reducing gliosis. In addition, MSCs have anti-apoptotic actions, and could attenuate excitotoxicity by modulating astrocyte functions (Ciervo et al., 2017).

In the last two decades, increasing number of studies have been conducted on animal models such as $SOD1^{G93A}$ transgenic mice to explore underlying mechanisms and to assess therapeutical activity of MSCs. Researchers have tested the effect of stem cells derived from different tissues and administrated through different routes and different frequencies (Forostyak et al., 2017). Studies on rodents demonstrated positive effects of MSC on motor activity and survival after being delivered via various routes (mostly intrathecal, but also intravenous, intraspinal, intramuscular, or combined). MSCs effects are dose- and passage-dependent, and it has been showed that MSC from earlier passages (up to the fifth) are more suitable therapeutic application due to their stability, anti-inflammatory and neuroprotective effects (Forostyak et al., 2017).

These encouraging results have been followed in the last years by several phase 1/2 clinical trials on human patients. Most of these has employed MSCs of different origin, mostly autologous bone marrow or adipose tissue. This wide use of bone marrow and adipose tissue derived cells is explained by the easiness of derivation and manipulation with autologous cells from patients, legal issues and long history of clinical application. Most of these studies are conducted on a small number of patients and the follow up performed in most cases was not longer than 24 months. Several studies reported that stem cells improved tested parameters in ALS patients (Forostyak et al., 2017). For example, a clinical trial completed in 2016 in Czech Republic evaluated the safety and the efficacy of autologous multipotent MSC in patients with ALS (Sykova et al., 2017). The trial involved 26 patients with sporadic ALS, who received a single intrathecal administration of autologous MSCs. Authors reported that 80% of patients preserved forced vital capacity values (a parameter that is measured to assess the progression of ALS in patients) above 60% for 12 months. In addition, 14 patients with a remarkable pretreatment decline in functional scales, had significant reduction/stabilization in their total functional score decline at 3 months after application, which was less pronounced at 6 and 9 months (Sykova et al., 2017). Another phase 1/2 clinical trial took place in 2010 in Israel in 15 patients with multiple sclerosis (MS) and 19 ALS patients. The researchers administrated 60 million autologous MSCs intrathecally in combination with 20 million MSCs intravenously. In addition, in some patients MSCs were marked with ferumoxides to track them in vivo using an MRI (Karussis et al., 2010; Ciervo et al., 2017). No severe adverse effects occurred, and disease stabilization was obtained in some patients. MRI screening 24 h, 48 h, 1 and 3 months after the infusion of cells, revealed the presence of ferumoxides in nerve roots, meninges and the parenchyma of the spinal cord. Nevertheless, the contrast agent could also be ingested by phagocytes which had migrated to inflammatory lesions. In addition, an analysis of peripheral blood from ALS patients obtained 4 h and 24 h after infusion of MSCs, showed a remarkable increase of CD4+ CD25+ regulatory T cells and a reduction in activated dendritic cells and lymphocyte proliferation. These findings were suggestive of the immunomodulatory functions of MSCs (Karussis et al., 2010; Ciervo et al., 2017).

Despite an increasing number of clinical trials proving the safety of the procedure there is a great need for bigger multicentre trials. In addition, these should include placebo group of patients. Moreover, a unification of design regarding routes of application, different type of cells, and ways of clinical evaluation is needed to obtain more homogenous data between the trials in the future (Forostyak et al., 2017). Finally, markers that detect the disease at early stage are relevant for the success of cell-based therapy because at the beginning of neurodegeneration stem cells might bring more benefits in rescuing neurones from inevitable death, if compared with the therapy at the terminal-stage of ALS. Furthermore, markers would permit to objectively monitor the progression of the disease (Forostyak et al., 2017).

6 TREATMENT OF DEGENERATIVE MYELOPATHY

Although DM has been studied for fifty years, there is no specific therapy that has been proven to be significantly successful.

The first attempt to treat DM had been the administration of immunosuppressive drugs such as glucocorticoids, cyclophosphamide and azathioprine, basing on the hypothesis that MD was an immune-mediated neurodegenerative disorder. Unfortunately, none of these drugs had a positive effect on disease progression (Clemmons, 1992). Another protocol included the use of epsilon-aminocaproic acid, N-acetylcysteine in combination with vitamins B, C and E and daily exercise. Prednisolone was given for the first two weeks and upon worsening of neurological signs. These medications did not show any better results than physiotherapy alone (Polizopoulou et al., 2008).

In 2006 Kathmann and colleagues evaluated the effect of daily intensive physiotherapy on progression of DM in 22 affected dogs. The 9 dogs that received intensive physiotherapy had longer survival time (mean 255 days) than the 6 dogs with moderate (mean 130 days) and the 7 with no (mean 55 days) physiotherapy. Moreover, affected dogs which received physiotherapy remained ambulatory longer than animals that did not receive physical treatment. Intensive daily physiotherapy was defined in the study as gait exercise at least three to five times daily, with either massage and passive joint movement three times a day or daily hydrotherapy, whilst moderate physiotherapy included gait exercise maximally three times a day and hydrotherapy or massage once a week. More information about the prescribed physiotherapy is found in the table below (Kathmann et al., 2006). Since the time of this publication, physiotherapy for dogs with DM has been widely recommended to improve the patient's quality of life (Miller et al., 2020).

Type of activity	**Instructions**	**Duration and frequency**
Active exercise	• Slow walking. • If needed, knuckling is prevented by a sling around the paw and pulling the	5–10 minutes at least 5 times a day.

	dog's limb by each step with it. • Frequent exercises preferred to long exercises. • Exercise must be adapted to animal's condition. • Dog sits and gets up several times. • Assistance with a sling if needed. • Attention is paid to correct placement of the paws. • If the weight shifts while standing, the owner should make the dog bear his weight once on the left, then on the right side by pushing him gently at the level of the hip. • Changing of ground (grass, asphalt, sand). • Stair climbing, walking uphill.	
Passive exercise	• Gentle, slow extension and flexion of each joint of both hind limbs (starting distally, manipulating of each joint performed separately). • Maintaining the range of physiologic motion of each joint. • The limb is always fixed proximally to the joint and the distal partis moved	3 times/d, 10 times in each articulation.
Massage	• Massage is started and finished with stroking. • Gentle massage (kneading) of the entire paravertebral muscles and the	3 times/d.

	limbs, starting from distal to proximal.	
Hydrotherapy	• If available, walking on underwater treadmill; otherwise, swimming or walking in water, depending on dog's ability. • Adaptation to animal's condition is important. • Assistance with a sling as needed. • Weight shifting while standing in water: making the dog bear its weight once on the left, then on the right by pushing him gently at the level of the hip	At least once a week, 5–20 minutes.
Paw protection	• With a bandage or socks and shoes.	While walking.

Table 6: prescribed physiotherapy for dogs with DM (Kathmann et al., 2006).

In 2020, Miller and colleagues examined the impact that adding photobiomodulation therapy (PBMt) to rehabilitation therapy had on the progression of DM. Before discussing Miller's research, some information on PBMt is needed. Light or PhotoBioModulation therapy (PBMt) is an intervention currently used as an integral part of many rehabilitation protocols and to treat a variety of conditions in veterinary medicine (Miller et al., 2020). Although the exact biochemical mechanism is not precisely known, various studies have assessed that laser therapy can alter the inflammatory response. Increases in reactive oxygen species (ROS), adenosine triphosphate (ATP), and nitric oxide (NO) are thought to be the main factors that underlie laser's therapeutic effects. Some explanations on these mechanisms follow. ROS increases because there is a higher rate of oxidative phosphorylation which releases electrons that are accepted by oxygen to produce ROS. With appropriate doses of laser therapy, a

slight increase of ROS concentrations is thought to activate the beneficial antioxidant enzymes (such as superoxide dismutase and catalase) (Hochman, 2018). Moreover, PBMt provokes a surplus of ATP that could be used for tissue repair. Specifically, the photo-stimulation at certain wavelength of enzyme cytochrome c oxidase in the mitochondrial respiratory chain results in a higher rate of electron transfer, increased proton transport, and a subsequent increase in mitochondrial membrane potential, ATP synthase, and eventually ATP. The cell can use this additional energy for tissue repair (Hochman, 2018). In addition, applying PBMt, the amount of NO bound to the heme-copper active site of cytochrome c oxidase can be photo-dissociated. Finally, the release of NO into surrounding tissues promotes angiogenesis, mediates on vasodilatation, and modulates the inflammatory and immune response. Over the last years, laser therapy therapeutic potential has been investigated for many diseases. Areas of particular interest include pain, wounds, musculoskeletal conditions, and some neurologic disorders. In veterinary neurology PBMt has been studied as a possible therapy to mitigate symptoms of spinal cord injury and peripheral nerve damage. For spinal cord injury, the results of various studies conducted on dogs are controversial (Hochman, 2018). Regarding peripheral nerve damage, some studies have documented beneficial use of PBMt for peripheral nerve, for example in 2007 a pilot study assessed that in patients with long-term peripheral nerve injury non-invasive 780-nm laser phototherapy can progressively improve nerve function, which leads to significant functional recovery (Rochkind et al., 2007). Concerning PBMt in general, it is worth noting that laser parameters including wavelength, fluence, wattage, and overall dose needs to be in specific ranges to have a therapeutic effect (Hochman, 2018). In 2020, Miller and colleagues evaluated the effect of PBMt on DM progression. Two different protocols of PBMt with physiotherapy (Protocol A and Protocol B) were established and then compared to historical data expectations (Miller et al., 2020). These were given by previously published study in which information on progression of ambulation status and/or survival data is reported for larger groups of dogs with DM: Kathmann et al., Polizopoulou et al., and Kanazono et al. (Kanazono et al., 2013). In-clinic and at-home exercises were the same for both laser-treated groups. PBMt was always performed through the dog's coat without shaving it. The laser probes were in direct contact with the dog's coat and skin over the spinal column, as well

as 5–7 cm lateral to the right and left sides of the spinal column in the paraspinal musculature, from approximately the T3 vertebral body to the lumbosacral junction. Protocol A was given to 6 dogs, whilst Protocol B was performed on 14 dogs. The table below shows the light parameters (wavelength, radiant power, irradiance, fluence) and the details about the treatment (protocol, area, time) (Miller et al., 2020).

	Protocol A group	**Protocol B group**
Wavelength (nm)	904	980
Radiant power (W)	0,5	6-12 Depending on patient's size, larger patients treated at higher power; irradiance increased with increase in power.
Irradiance (W/cm^2) at skin surface	0,5	1,2-2,4 Depending on patient's size, larger patients treated at higher power; irradiance increased with increase in power.
Fluence (J/cm^2)	8 (per “point”)	14–21 (average over treated area)
Treatment protocol	Point-to-point “grid method” technique at a total of 20 points spread	Continuously moving grid pattern over the entire treatment area at a speed

	throughout the treatment area according to manufacturer's instructions. (Respond Model 2400XL Laser, Respond Systems, Inc., Branford, CT.)	of 1–3 in/sec according to manufacturer's recommendations. (Companion Therapy Laser CTC-15, LiteCure, LLC, DE.)
Treatment area (cm^2)	650-1000 Treatment area increased with larger patient size.	650-1000 Treatment area increased with larger patient size.
Treatment time	~5 min, 20 sec	Between 25–26 min, 15 sec Depending on patient's size, larger patients treated at higher power; irradiance increased with increase in power.

Table 7: Protocol A and Protocol B groups Photobiomodulation Theraphy Treatment Parameters. (Miller et al., 2020)

Dogs in the PTCL-B group had longer survival from the onset of symptoms (38.2 ± 14.67 months) than those in the PTCL-A group (11.09 ± 2.68 months). Similarly, nonambulatory paresis or paralysis occurred later in the PTCL-B group (31.76 ± 12.53 months from onset of symptoms), than the PTCL-A group (8.79 ± 1.60 months from onset of symptoms) and the historical data group. Currently, authors are planning a trial with a larger sample size, tighter inclusion criteria and placebo-controlled treatment (Miller et al., 2020).

In 2021 Kobatake and colleagues evaluated DM progression in 8 Pembroke Welsh Corgis that had been administered an oral supplement containing curcumin

(Neuroact, Veterinarian Medical Development Company Limited, Saitama, Japan). Their outcomes were compared to the disease progression of 32 cases with no curcumin supplementation. Their choice to administrate curcumin was based upon a study which concluded that it might improve the probability of survival as an add-on treatment to Riluzole in human patients with ALS, although further studies with larger sample sizes and of longer duration were needed to confirm these findings (Kobatake et al., 2021). Curcumin is a polyphenolic compound that has neuroprotective effects such as antioxidant and anti-inflammatory activities. Moreover, a study conducted in vitro showed that curcumin molecules bound strongly to the aggregation-prone regions of the mutant SOD1 proteins and blocked the exposed aggregation site, leading to the inhibition of the formation of SOD1 unstructured aggregates. In addition, curcumin is thought to have beneficial effects on muscles. The 8 dogs treated with curcumin had a significantly longer survival time than those who had not received treatment. The timing of the onset of thoracic limb paresis was not significantly different between the curcumin and control groups; however, non-weight bearing of the hind/thoracic limbs was prolonged in the curcumin-treated dogs when compared with the control dogs. The timing of respiratory disfunction onset between the two groups wasn't significantly different. As stated by authors, dog owners who choose to administer curcumin to their dogs, despite the unproven effectiveness of curcumin in DM, may be more engaged in conservative care such as physical therapy. Therefore, extended survival may be because of the dog owner's approach to care and not because of the therapeutic effects of curcumin. Improvement in motor function may be attributed to the anti-inflammatory and antioxidant effects of curcumin on joints rather than to the pathology of DM itself. Additional studies with more uniform conditions especially concerning physiotherapy are required to assess the effectiveness of curcumin (Kobatake et al., 2021).

7 EXPERIMENTAL CONTRIBUTE

This work has three purposes. Firstly, it evaluates the signalment and history of DM-affected dogs which were referred to the veterinary teaching hospital of Parma University (Ospedale Veterinario Universitario Didattico di Parma, OVUD) between February 2019 and March 2022.

The second purpose is to describe the preparation and administration of MSCs from adipose tissue for the therapy of DM in two dogs which were referred to OVUD between 1st May 2021 and 31st March 2022.

Moreover, samples of CSF and serum has been collected to evaluate pNF-H levels as a potential marker of DM. The values obtained will be compared to other sample from dogs without neurological disorders.

The clinical records were analysed retrospectively. All the procedures were approved by the ethical committee of University of Parma.

8 MATERIALS AND METHODS

12 dogs with suspected DM were referred to the veterinary teaching hospital of Parma University (Ospedale Veterinario Universitario Didattico di Parma, OVUD) between February 2019 and March 2022. Their breed, sex, age at onset of symptoms, weight, age at euthanasia is showed in the following table.

Dog	**Breed**	**Sex**	**Age at onset of symptoms (years-month)**	**Weight (kg)**	**Age at euthanasia (years-month)**
Kira	Czechoslovakian Wolfdog	F	11-8	>25	
Shiro	Hovawart	M	9-5	>25	
Killa	Czechoslovakian Wolfdog	F	8-1	>25	
Balù	Mixed Breed	M	9-3	30	Not known
Laika	Mixed Breed	F	9-5	56	10
Nana	Mixed Breed	F	10-2	16	10-10
Mollypolly	Mixed Breed	F	9-10	15	
Sofia	Mixed Breed	F	9-5	16	
Lulù	German Shepherd	F	8-3	35	
Lupo	German Shepherd	M	-	35	9-3
Olly	Mixed Breed	F	10-6	26	
Zeus	Czechoslovakian Wolfdog	M	5-9	35	

The diagnostic workup included complete blood count, serum biochemistry, radiography, neurologic examination, MRI or TC and genetic test for SOD1:c.118G > A. The results are reported in the table below. Complete blood count, serum biochemistry, radiography and RMI or TC showed no alteration. Neurologic examination assessed the presence of progressive general proprioceptive ataxia, paraparesis with an asymmetric exordium, postural reaction deficits in pelvic limbs, lack of paraspinal hyperesthesia. Based on these findings, the dogs received a presumptive diagnosis of DM. Physiotherapy was prescribed to them.

Name	**TC or MRI**	**Genetic test for SOD1:c.118G > A**
Kira	TC	Heterozygous
Shiro	TC	Homozygous
Killa	MRI	Not performed
Balù	TC	Homozygous
Laika	MRI	Homozygous
Nana	TC	Homozygous
Mollypolly	MRI	Homozygous
Sofia	TC	Homozygous
Lulù	MRI	Homozygous
Lupo	MRI, TC	Homozygous
Olly	TC	Homozygous
Zeus	TC	Homozygous

Table 8: complete blood count, serum biochemistry, imaging, genetic testing of dogs that were diagnosed with DM between February 2019 and March 2022 at the veterinary teaching hospital of Parma University.

Among these 12 dogs, 2 dogs diagnosed with DM were referred to the veterinary teaching hospital of Parma University (Ospedale Veterinario Universitario Didattico di Parma, OVUD) between 1st May 2021 and 31st March 2022. Olly was a female, spayed, 11 years old mixed breed; she was 10 years and 6 months old at onset of symptoms. Zeus was a 6-year-old male Czechoslovakian Wolfdog; he was 5 years and 9 months old at onset of symptoms. Both had not previously been affected by any other disease during their life. The diagnostic work up was previously

discussed. The genetic test detected homozygosity for SOD1:c.118G > A for both dogs.

For both cases physiotherapy was recommended. In addition, an experimental therapy was proposed. This consisted of an administration of autologous Mesenchymal Stem Cells (MSCs) derived from adipose tissue every two months for three times. The amount of the administration was 2 million MSCs for intrathecal infusion and 2 million/kg MSCs for intravenous infusion. To objectively monitor the progression of the disease during the treatment, the examination of pNF-H levels in CSF and serum was performed before each MSCs administration. A commercially available ELISA registered for humans was used.

The following table shows the dates of the biopsies of adipose tissue and of the administrations of MSCs.

Name	**Biopsy of adipose tissue**	**First administration of MSCs**	**Second administration of MSCs**	**Third administration of MSCs**
Olly	30/06/2021	02/09/2021	04/11/2021	03/03/2022
Zeus	21/07/2021	07/10/2021	02/12/2021	03/03/2022

Table 9: dates of the biopsies of adipose tissue and of the administrations of MSCs.

The next paragraphs provide a description of the general process followed to obtain autologous MSCs from abdominal adipose tissue, the administration of MSCs and the measurement of pNF-H levels in CSF and serum.

8.1 Biopsy of adipose tissue and transport to the laboratory

General anaesthesia was performed. The skin overlying the biopsy site underwent routine aseptic preparation. The skin was incised with a scalpel and carefully dissected to facilitate the excision of 4 grams of adipose tissue from the abdomen. The sample was placed in a sterile tube that had been previously filled with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with Penicillin (100 UI/ml) and Streptomycin (100 µg/ml). Then, it was immediately transported to the laboratory.

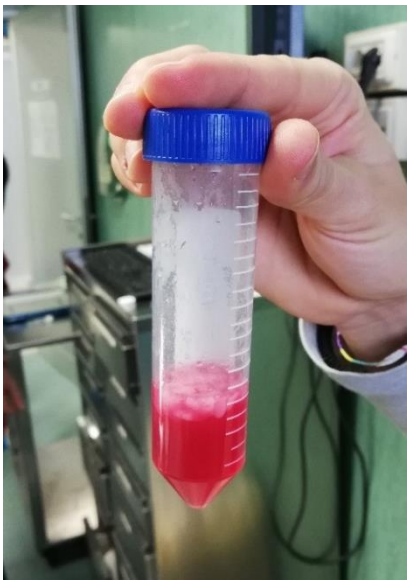

Figure 16: adipose tissue in the medium containing DMEM, Penicillin (100 UI/ml) and Streptomycin (100 µg/ml).

8.2 Isolation and conservation of MSCs

The sample of adipose tissue was washed in Petri dish with Ethanol 70% and then with Phosphate Buffered Saline (PBS) for three times. In sterile conditions, it was cut into smaller pieces using a scalpel and a forceps and weighted. After that, the sample was immersed in a solution containing Collagenase Type I (0,1%) and DMEM in a proportion of 5 ml of solution for each gram of tissue. Successively, it was incubated in an orbital shaker at 37 °C for 1 hour for enzymatic digestion. After this time, it was centrifuged for 10 minutes at 200 xg to obtain the separation of adipocytes, supernatant and pellet.

Next, the supernatant was removed with a Pasteur pipette, and the pellet was resuspended in 2 ml of DMEM previously supplemented with 10% of FBS (Fetal Bovine Serum). After that, cells were seeded in a culture flask of 25 cm² (Cellstar®) containing 5 ml of DMEM supplemented with 10% FBS, Penicillin (100 UI/ml), Streptomycin (100 µg/ml) and Polymyxin B (2,5 µg/ml). Cells were maintained there until 80% confluence and were subsequently detached with Trypsin 1% in EDTA and expanded until passage 3 (P3). The volume was divided into 6 micro vials that were immersed in liquid nitrogen for conservation.

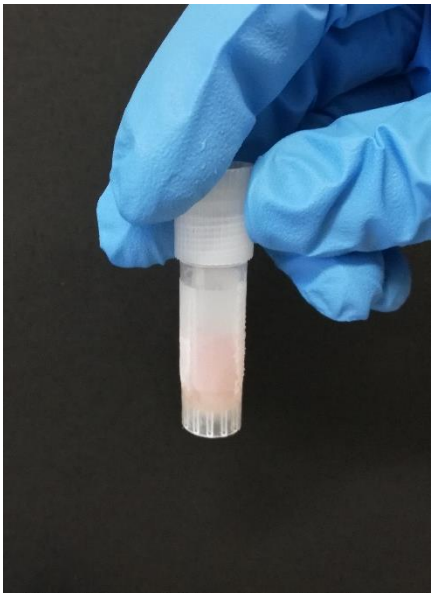

Figure 17: micro vial containing MSCs in the medium.

8.3 Thawing of MSCs

The micro vials were thawed at 37°C. Then, the solution contained in each micro vial was transferred into a sterile tube (Clearline®) and diluted with sterile Ringer's lactate. The tubes were centrifuged at 180xg for 10 minutes.

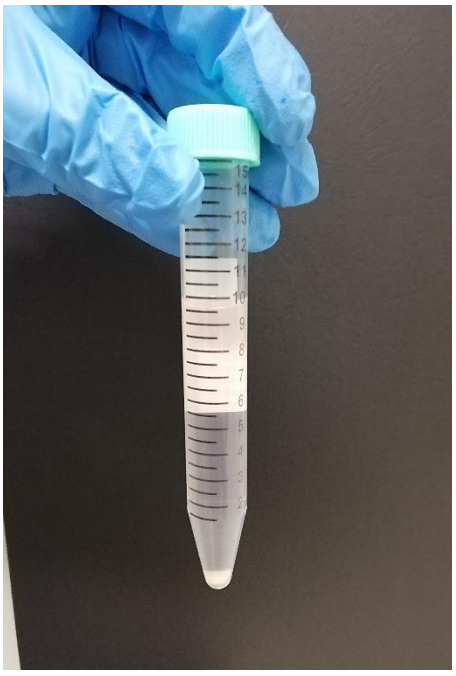

Figure 18: The supernatant and the pellet are visible in the tube after centrifugation.

After that, the supernatants were eliminated, and the pellets of each tube was resuspended in 2 ml of Ringer's lactate. This volume was further diluted to reach the concentration of 2 million for each kilo of weight of the patient in a total volume

Figure 19: The syringes are filled with the two volumes obtained at the end of the process, ready for the administration.

of 15 ml for the intravenous infusion. In addition, 2 million of cells were diluted to reach a volume of 0,8 ml for the intrathecal administration. Two syringes were filled with the two volumes obtained.

8.4 Transport to the operating room and administration

For the transport to the operating room, the two syringes were placed into an insulating box. The syringes were gently and continuously stirred before and during the administration. General anaesthesia was performed. For the intravenous administration the 15 ml syringe was connected to a three-way stopcock valve, while for the intrathecal administration the 0,8 ml syringe was attached to a lumbar puncture needle. The rate of infusion was 1 ml/min for intravenous administration whilst for the intrathecal administration it was 0.10 ml/min.

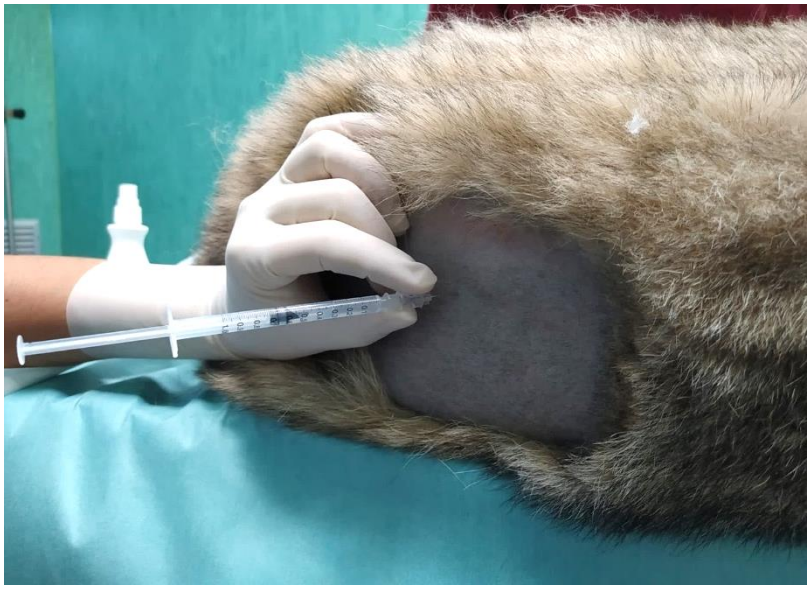

Figure 20: intrathecal administration of MSCs.

Figure 21: intravenous administration of MSCs.

8.5 Measurement of pNF-H levels in CSF and serum

CSF and serum samples were collected before each administration of MSCs and were stored at -20 °C. A commercially available ELISA registered for humans was

used. CSF was not always collectable due to technical reasons. The data on pNF-H levels in both CSF and serum are still not available.

9 RESULTS AND DISCUSSION

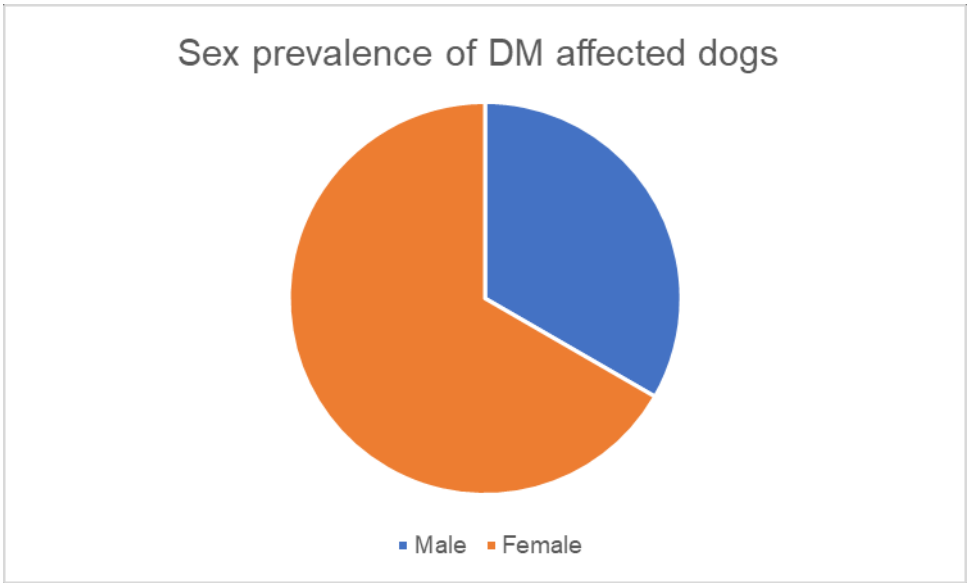

Figure 22: Pie chart of sex prevalence in DM-affected dogs.

Regarding sex prevalence, females were 2/3 of all dogs. This was not expected since in literature no sex predominance is reported (Kathmann et al. 2006). Nevertheless, female predominance was reported in Corgi in one paper, but it was probably a consequence of breeders being more aware of the study than owners and likely to keep more females into old age. (Coates et al. 2007) In this thesis, female predominance was probably caused by the small number of patients.

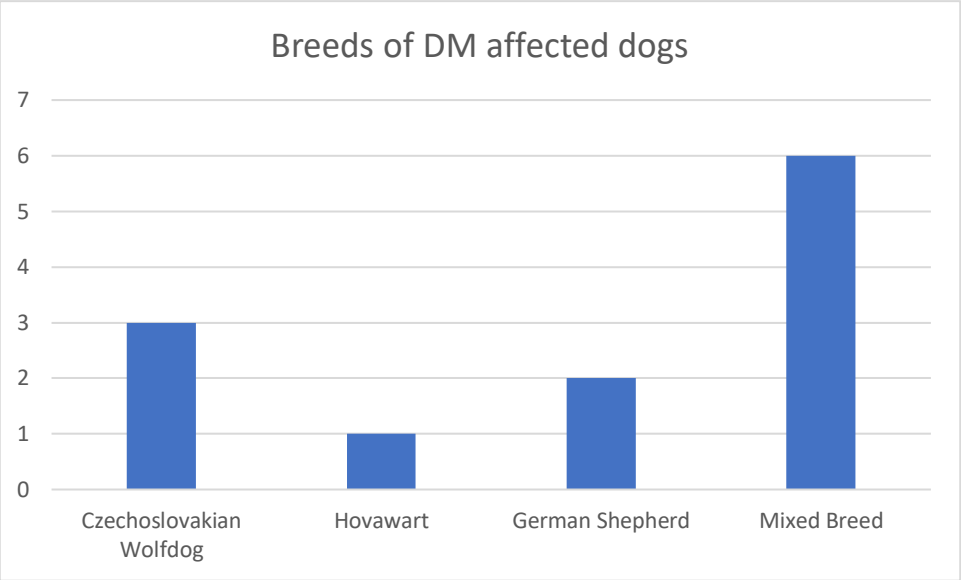

Figure 23: This histogram shows the breeds of DM-affected dogs.

Mixed breed dogs were the most represented with 50% of the sample; 25% were Czechoslovakian Wolfdog, German Shepherd dogs were 16,7%, and Hovawart were 8,3%. In the literature these breeds are also represented as predisposed to DM. (Coates and Wininger, 2010) Nevertheless, 50% of mixed breed was not expected since among all the population of mixed breed dogs, prevalence of DM-affected dogs is only 0,15% as shown in Coates and Wininger, 2010. This might be due to the small number of patients and to the high number of mixed breed dogs in the canine population.

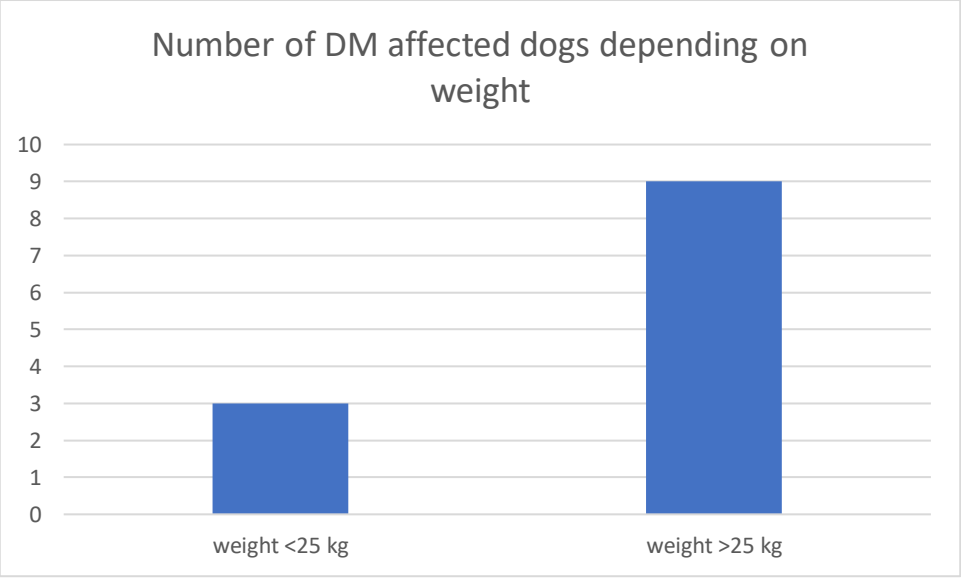

Figure 24: This histogram shows the number of DM-affected dogs depending on weight.

The 75% of dogs were >25 kg of weight, whilst only 25% were <25 kg.

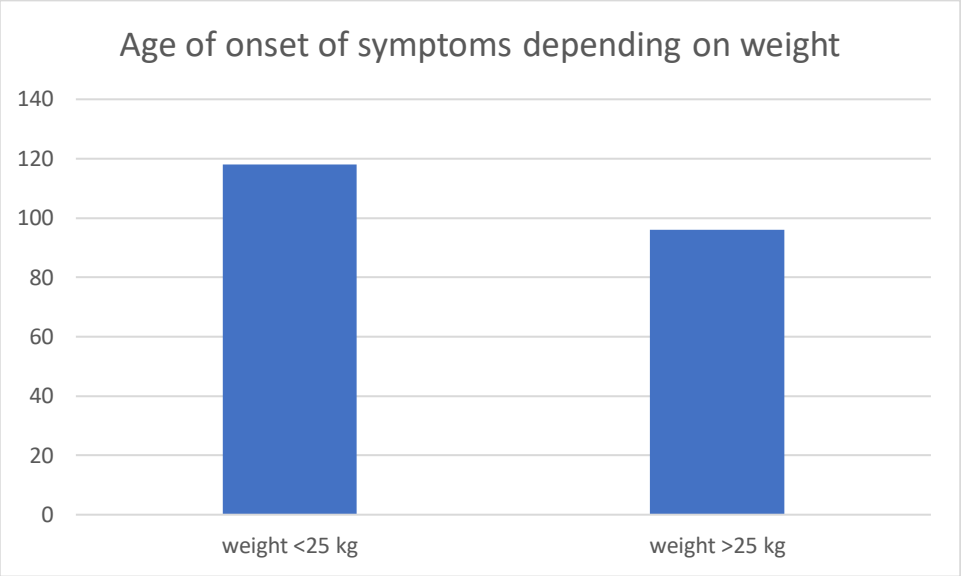

Figure 25: This histogram shows the age of onset of symptoms in months of DM-affected dogs depending on weight.

The mean age of onset of symptoms was 111 months (9 years and 3months), with a minimum age of 5 years and 9 months in a Czechoslovakian Wolfdog, whilst the oldest dog was a 11 years and 8 months old heterozygous Czechoslovakian Wolfdog. The mode was 113 months (9 years and 5 months). In the literature the mean age of onset is reported to be 9 years in large breed dogs, whereas in Pembroke Welsh Corgi the onset disease is, on average, 10.9 years (Coates and Wininger, 2010).

Dogs that weighted less than 25 kg were 25% of the sample, while 75% weighted more than 25 kg. Moreover, dogs that weighted less than 25 kg had an age of onset of 118 months (9 years and 10 months). By contrast, age of onset of patients over 25 kg is 96 months (8 years). As a result, we could assume that weight higher than 25 kg is a risk factor for developing DM.

Genetic test was performed on 11 dogs out of 12. Homozygous dogs were 10/11. Only one patient was heterozygous for SOD1:c.118G > A. He was a Czechoslovakian Wolfdog and had an age of onset of 11 years and 8 months, considerably far above the average. The duration of clinical signs was more than 4 years, considerably longer that the average reported in the literature (Coates and Wininger, 2010). This could be a consequence of heterozygosity, and to the dedication of the owner that provided the best management and was willing to

keep alive the patient as long as he could. Zeng and colleagues in 2014 reported that only 4% SOD1:c.118A/G heterozygotes dogs developed clinical signs of DM. A potential risk factor could be that the dog weighted more than 25 kg. It would be interesting to analyse data from Zeng and colleagues (2014) to evaluate the weight of their sample of 55 heterozygous dogs in correlation with the development of symptoms and the age of onset of symptoms. Moreover, in future studies, it would be interesting to evaluate the progression of DM in heterozygous dogs compared to progression in homozygous dogs, particularly using pNFH to obtain more objectivity in comparison to clinical evaluation and anamnesis. In fact, the data on onset of symptoms are influenced by the human factor since some owners can notice very mild symptoms while others notice symptoms later. In addition, also data on duration of symptoms are influenced by the decision of the owner to perform euthanasia because they depend not only by the disease progression, but also on the human factor.

Data on the measurements of pNFH are not avaiable yet. When avaiable, they could be a tool to obtain an early diagnosis, and to evaluate the progression of the disease for both clinical and research purposes.

Regarding the preparation of MSCs, DMSO was used for cryoprotection. After thawing, centrifugation is needed to remove DMSO which is toxic for cells. Needles used to fill the syringes with MSCs suspended in Ringer's lactate were 18G to minimize the damage due to manipulation. Thermic shock was avoided by keeping the syringes in an insulated box while transported to the operating room. Cells were maintained into plastic syringes and tubes for the shortest time to avoid cells' adherence to plastic. For the same purpose, the syringes were gently and continuously stirred before and during the administration.

Future studies, especially for the evaluation of administration of MSCs should include a higher number of patients, a control group, and more data regarding physiotherapy. In addition, the measurement of pNFH could lead to more objectivity in monitoring of the disease progression. Moreover, it would be interesting to also measure miRNAs for several reasons. Firstly, miR-26b was evaluated as a potential biomarker by Nakata and colleagues (2019) but their sample was composed only by Pembroke Welsh Corgis. Hence, it should be tested also on other breeds and on a larger sample. In addition, miR-26b and

pNFH should be compared on the same sample of dogs to evaluate which one has the best diagnostic accuracy for distinguishing DM-affected patients from not affected-dogs. Moreover, plasma levels of miRNAs could be quantified using an RT-qPCR array to assess how MSCs therapy impact on miRNA profile. In fact, miRNAs are thought to have a key role in the anti-inflammatory and immunomodulatory activity of MSCs (Giunti et al., 2021). These data could be compared to those from Nakata and colleagues (2019) that identified 11 up-regulated miRNAs and 7 down-regulated miRNAs in DM-affected Pembroke Welsh Corgis out of 277 miRNA considered.

10 CONCLUSION

Although DM has been studied for many years, there is no specific therapy that has been proven to be significantly successful. Hence, prognosis for DM-affected dogs remains poor.

Since the discovery of the SOD1 mutations in the majority of DM affected dogs, DM has been considered a natural occurring model for ALS. These neurodegenerative disorders, besides having a similar genetic basis, share multiple similarities regarding their underlying pathological mechanisms, histopathological features and clinical hallmarks. Unfortunately, both disorders lack an effective therapeutical approach (Awano et al., 2009). According to the literature, the main risk factors in DM are the breed and homozygosity for SOD1:c.118G > A. Among DM-affected dogs which were referred to the veterinary teaching hospital of Parma University (Ospedale Veterinario Universitario Didattico di Parma, OVUD) between February 2019 and March 2022, weight over 25 kg was found to be predominant in DM affected dogs and it was also correlated to earlier onset of symptoms. Further investigation with a larger sample are needed.

Over the last years, the safety of administration of MSCs through different routes has been demonstrated by several studies conducted either on laboratory animal and humans. In addition, results obtained mostly on SOD1 transgenic mice, but also in some human clinical trials, are encouraging towards the therapeutical effect of MSCs on ALS (Ciervo et al., 2017). To the author's knowledge, no studies regarding the administration of MSCs in DM-affected dogs are available, although this would be relevant for both DM and ALS research. This thesis describes the preparation and administration of MSCs from adipose tissue for the therapy of DM in two dogs which were referred to OVUD between 1st May 2021 and 31st March 2022. Moreover, samples of CSF and serum has been collected to evaluate pNF-H levels as a potential marker of DM. The values obtained are not avaiable yet. They will be compared to other sample from dogs without neurological disorders and with DM-mimics. Indeed, another important aspect in clinical practice of both DM and ALS is the lack of biomarkers which could offer the opportunity to obtain an early diagnosis and to monitor the disease in an objective manner. The availability of biomarkers would also permit to objectively test new (and old) therapeutical approaches avoiding biases linked to the human factor. Recently

pNFH have been proposed for those purposes for both ALS and DM. Compared to other biomarkers, pNFH appears to be sufficiently sensible and specific and can be measured with an ELISA. Nevertheless, further investigation about their measurement in other mimics and in both CSF and blood are still required.

Moreover, in future studies, measurement of miRNAs should be considered for several reasons. Particularly, miR-26b is considered a potential biomarker of DM, although further investigation is still required (Nakata et al., 2019). In addition, the quantification of other miRNAs before and after MSCs therapy should be carried out to elucidate the mechanisms of MSCs action (Giunti et al., 2021).

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